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JOURNAL OF AOAC INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence

AOAC INTERNATIONAL

JAINEE 75(6)949-1160 (1992)
ISSN 1060-3271

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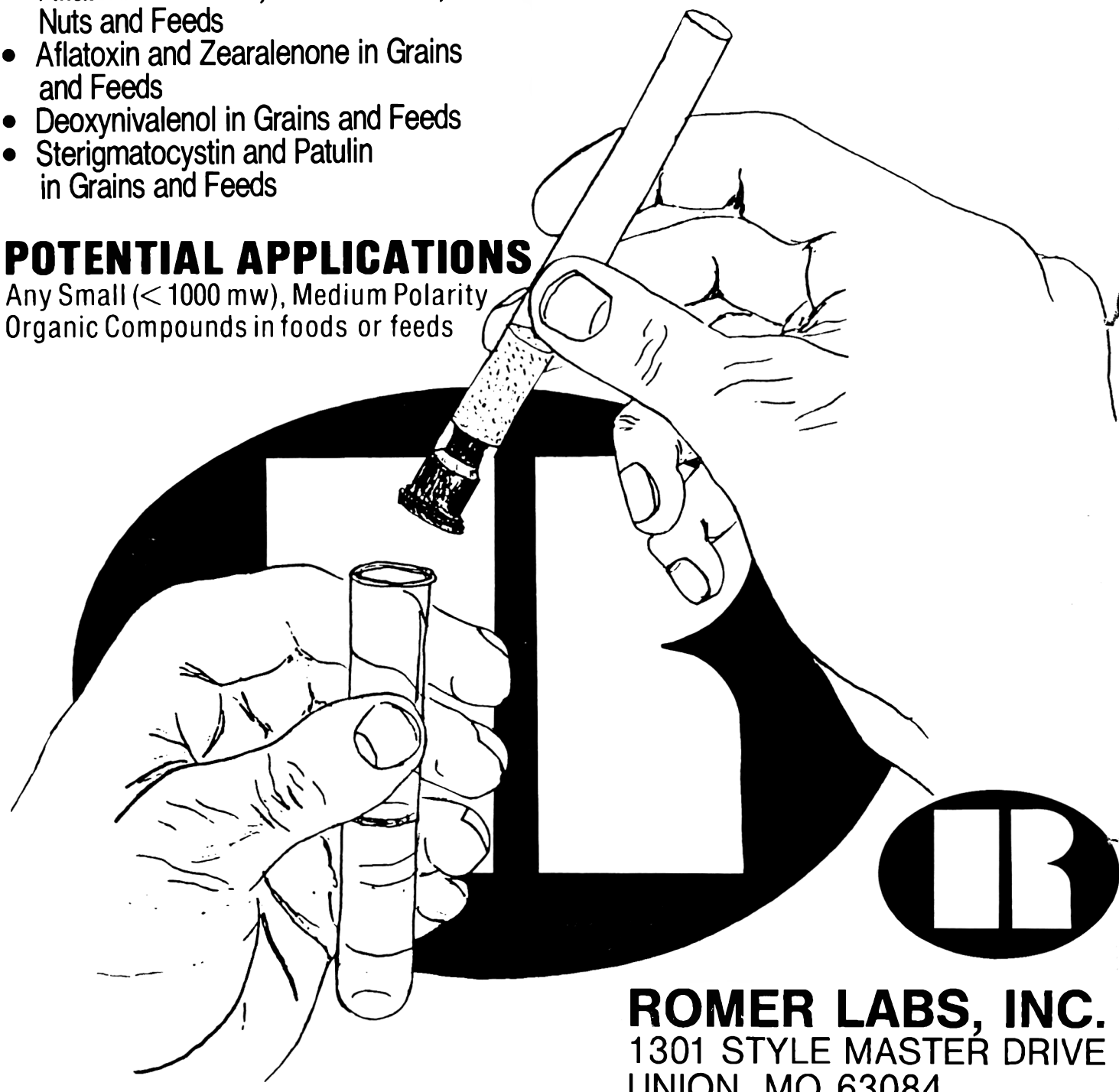

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JOURNAL OF AOAC INTERNATIONAL

A Peer-Reviewed Journal Publishing Basic and Applied Research in the Analytical Sciences Related to Foods, Drugs, Agriculture, and the Environment

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The *Journal of AOAC International* (ISSN 1060–3271) is published bimonthly by AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 75 (1992) are: Members, $110.00 in U.S., $145.00 outside U.S.; Nonmembers, $160.00 in U.S., $190.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.
POSTMASTER: Send address changes to AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA.

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Discharge Ionization Detector Assembly

The Model 24–600 temperature regulated discharge ionization detector (DID) is engineered for gas analysis in the 1–10 ppm range; it has a dynamic linear range of 5 ppb to <1%. The unit will integrate with most existing gas chromatographs, can be designed into new GC process systems, or used as a replacement detector to upgrade an older instrument. The assembly consists of 2 components: a DID detector in a helium purged housing and a control unit that includes an electrometer, high voltage power supply, digital display for dc voltage and discharge current (mA), and a detector temperature control with digital readout. GOW-MAC Instruments, Inc.
Circle No. 365 on reader service card.

Microprocessor Based Smart Universal Actuator

An inexpensive, microprocessor based, smart universal actuator for accurate positional control of rotary valves adapts to different types of valves from various manufacturers, such as Rheodyne, Valco, Whitey, Autoclave, etc. An encoder provides high accuracy and a microprocessor enables the user to modify the angle of rotation instantly. The actuator can be controlled through a manual display, TTL logic, or RS-232C. Applications include chromatographic valves, flow switching valves, high pressure valves, stream selection valves, and sample injection valves in any area where control of gas or liquid flow is required. Thar Designs, Inc.
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LC Carbamate Analysis

For analysis of carbamates in compliance with EPA methods 531.1 and 5, the EC5100 post-column analysis system can be connected to an LC system as simple as a binary pump, sample injector, and fluorescence detector. It comes complete with the method, post-column derivitization instrument, 2 columns, OPA reagent chemicals, 1-year warranty, and unlimited applications and troubleshooting assistance. The instrument includes low-pulsation reagent pumps, 500 µL reactor, column heater, TTL-compatible communications, and eluent pre-heater. Oxygen barriers for OPA preservation and anti-backflow safety devices protect the columns, reagents, and instrument, ensuring reproducibility and ppb sensitivity. Pickering Laboratories, Inc.
Circle No. 367 on reader service card.

150 PSI Glass Reactor

A 1 L glass reactor capable of withstanding pressures to 150 psi at 300°F permits researchers to observe, photograph, or video catalytic reactions. This observation proves valuable in studying reaction variables, such as mixing, color and viscosities, as well as allowing researchers to witness the reaction under process conditions. The glass reactor features a jacketed vessel for optimum heat transfer, and uses a magnetically coupled, packless rotary impeller system for effective mixing without leakage or contamination. The glass reactor assembly is pedestal mounted and incorporates a swing-away protective shield. The glass reactor is designed for applications in research laboratories of the pharmaceutical, chemical, and petrochemical industries. Autoclave Engineers.
Circle No. 368 on reader service card.

Model 3550-UV Microplate Reader

The Model 3550-UV microplate reader offers the broad range of functions required for the wide variety of assays now being routinely performed in the laboratory. In addition to enzyme immunosorbant assays, almost any test system that produces a chromogenic or light scattering result can be analyzed on the new reader. Protein determination, cell viability, DNA detection, NAD/NADH assay, kinetic ELISA, and kinetic enzyme analysis are just a few examples of the growing list of routine assays being transferred from the cuvette to the microplate format. The flexibility and multifunctional nature of the microplate reader makes it ideal for laboratories with diverse microplate analysis needs. Bio-Rad Laboratories.
Circle No. 369 on reader service card.

Folding Safety Scissors

A compact, high-quality folding scissor, Slip-N-Snip safety scissors are fabricated with stainless surgical steel blades and chrome-plated metal handles. Their sturdy construction makes them an excellent scissor for the medical and scientific professions. When not in use, Slip-N-Snip scissors can be easily folded up with the blades completely shielded, and can be carried with complete safety, unlike standard scissors. B-J Scientific Products, Inc.
Circle No. 370 on reader service card.

Programmable Multi-Step Filament Pyrolysis System

A multi-step filament pyrolysis system prepares solid samples such as polymers, composites, and fibers for analysis using gas chromatography, mass spectroscopy, or FT-IR. The Pyroprobe 2000 allows up to 5 steps per method to be programmed and 9 methods to be stored for easy recall. The platinum filament and the heated interface are independently programmable in steps containing initial temperature, heating rate, and final temperature. CDS Analytical, Inc.
Circle No. 371 on reader service card.

Flat Bottom Cylindrical Jars with Lids

Sturdy, uniform walled, flat bottom cylindrical jars, with glass lids as an option, are for applications requiring wall strength and clarity. Suitable for use as chromatography tanks, these cylindrical jars are precision ground and exceptionally clear. The evenness of the hand blown walls provides excellent strength, because thickness increases proportionally with the diameter of the product. Woods' Glass.
Circle No. 372 on reader service card.

Compact High Pressure Washer

The HDS 600C and HDS 700C cold-hot-steam pressure washers are designed to save time and money. They feature infinitely variable operating pressure, water volume, and temperature control, and precise chemical metering. Each unit offers a +90% fuel efficient burner system for maximum fuel savings; complete machine shut-off at the trigger gun for safe and convenient operation; direct drive motor; removable corrosion resistant detergent tank; and a durable, impact and corrosion resistant cover for maximum protection. Alfred A. Kärcher, Inc.
Circle No. 373 on reader service card.

Continuous Water Analyzer

The Vista VOC continuous water analyzer, an advanced, full automated system for trace analysis of volatile organic compounds (VOC) in water at concentrations in the low ppb range, can be used at any plant location to monitor plant process influent and effluent, noncontact cooling water, clean water used in product production, as well as municipal water treatment. Use of specific EPA methods (500 and 600 series) helps ensure that regulated Maximum Contaminant Levels are not exceeded. ABB Process Analytics, Inc.
Circle No. 374 on reader service card.

Titrate by PC II System

Titrate by PC software provides a menu driven series of steps to string modules together to perform rapid titrations to a known endpoint, to titrate for multiple endpoints, and to maintain a stat-level of

a known constituent. Once a titration procedure is developed, it is stored in a library for recall and easy duplication of results. Operating from DOS, the software provides the probe reading, the change in probe reading, the titrant added, cumulative titrant as well as first and second differentials. The user programs the best suited variable for identifying the equivalence point, and the software calculates, prints, and saves the final results. The titrate by PC system operates from the same hardware platform as the company's pH by PC and the ISE by PC systems. Computer Chemistry Corp.
Circle No. 375 on reader service card.

Eppendorf Disposable Pipet Tips

Eppendorf brand disposable pipette tips are manufactured from virgin polypropylene according to rigid quality standards. Flex-Tips are available in yellow and metal-free versions for Eppendorf pipette sizes up to 100 µL, and in blue for Eppendorf pipettes 101 to 1000 µL. Clear tips are useful for atomic absorption procedures. Eppendorf pipette tips are available bulk packed. The yellow and blue tips are also available tray mounted, or in sterile packaging. Sterile tips are indivudally wrapped and radiation sterilized. Bio-Rad Laboratories.
Circle No. 376 on reader service card.

Microsoft Windows Chemistry Programs

Over 60 Microsoft Windows programs for the chemist are highlighted in a new catalog. Environmental programs include Electronic EPA Methods, CLP Methods, Air Toxic Methods, MSDS Report Manager, CLP Forms Templates, and NPDES Report Templates. New programs for calculating alkalinity, hardness, molecular formulae from mass spectra data, and corrosion rates are described. The catalog also includes new programs for standards additions calibration, standards dilution modeling, a new chemical dictionary, QA/QC control charting program, instrument maintenance and inventory system, molecular drawing, chemical conversions, water indices, chromatography equations, a neural network program, periodic table database, and many more. WindowChem Software, Inc.
Circle No. 377 on reader service card.

High Dynamic Range Imaging System

The heart of the HiDRIS (High Dynamic Range Imaging) system is the most sensitive CCD camera commercially available. These cameras, which are available in visible or UV sensitive versions, feature high sensitivity (512 ×512-element camera requires only 10 photons for an A/D count), high gain, low noise, and extraordinary quantum efficiency. Efficient thermoelectric or cryogenic cooling of the sensor virtually eliminates detector dark current, allowing many hours of exposure time for the detection of low photon rates. TE-cooled detectors can operate at –80°C without the need of nitrogen purge. The HiDRIS software is intuitive, requiring only a few minutes of instruction. Context-sensitive, on-line help assists in manual-free operation. The HiDRIS software is operated entirely from a single virtual instrumental panel. It is almost totally mouse driven, using up/down arrows, check boxes, slide bars, cross hairs, and a zoom box. Setting critical parameters, executing experiments, and controlling file operations is a simple matter of pointing and clicking. HiDRIS applications include spectroscopy, microscopy, material characterization, and low-light level image acquisition, and astronomy.
Circle No. 378 on reader service card.

For Your Information

Meetings

November 17–18, 1992: AOAC Eastern Ontario/Quebec Section Meeting, Longueuil, Quebec, Canada. Contact: Michele Bono, Health Protection Bldg, 1001 St. Laurent, Longueuil, PQ, J4K 1C7, Canada, telephone 514/646-1353.

November 18–19, 1992: AOAC Central Section Meeting, Kalamazoo, MI. Contact: Sungsoo Lee, Kellogg Co., 235 Porter St, PO Box 3423, Battle Creek, MI 49016-3423, telephone 616/961-2823.

December 1, 1992: AOAC New York/New Jersey Section Meeting, Piscataway, NJ. Contact: Alex MacDonald, Hoffmann-La Roche, Inc., 340 Kingsland St, Nutley, NJ 07110, telephone 201/235-4641.

December 1–3, 1992: AOAC Board of Directors Meeting, Arlington, VA. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/ 522-3032.

January 21–22, 1993: AOAC Pacific Southwest Section Meeting, Napa, CA. Contact: Paul Bolin, DFA of California, PO Box 86, 1855 S. Van Ness, Fresno, CA 93721, telephone 209/233-7249.

January 31–February 2, 1993: AOAC Southwest USA Section Meeting, Little Rock, AR. Contact: Thomas Wilson, Colonial Sugars, Inc., PO Box 3360, Gramercy, LA 70052-3360, telephone 504/869-5521.

February 1–4, 1993: AOAC Southeast USA Section Meeting, Atlanta, GA. Contact: Jan Hobson, Griffith Corp., Rocky Ford Rd, PO Box 1847, Valdosta, GA 31603-8635, telephone 912/242-8635.

March 29–30, 1993: AOAC Europe Section Meeting, Barcelona, Spain. Contact: J. Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres 6, 08029 Barcelona, Spain, telephone 343-32288 06.

March 1993: AOAC MidAtlantic USA Section Meeting. Contact: David B. MacLean, 6422 Alloway Ct, Springfield, VA 22152, telephone 703/451-1578.

May 10–12, 1993: AOAC Northeast Section Meeting, Guelph, Ontario, Canada. Contact: Cathy Burchat, Ontario Ministry of Agriculture and Foods, Bldg No. 43, McGilvray St, University of Guelph, Guelph, ON, N1G 2W1, Canada, telephone 519/823-8800.

June 7–9, 1993: AOAC Midwest Section Meeting, W. Des Moines, IA. Contact: P. Frank Ross, USDA National Vet Serv Lab Toxicol Lab, PO Box 844, 1800 Dayton Rd, Ames, IA 50010, telephone 515/239-8542.

July 25–29, 1993: 107th AOAC International Annual Meeting and Exposition, Washington, DC. Contact: Margaret Ridgell, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

Conacher Elected 1992–1993 AOAC President

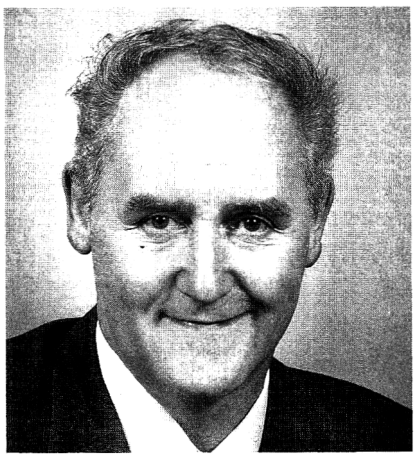
Henry (Harry) B.S. Conacher, director of the Bureau of Chemical Safety, Health Protection Branch, Ottawa, Ontario, Canada, became President of AOAC International for 1992–1993 following the business meeting at the AOAC International Annual Meeting in Cincinnati, OH, on September 3, 1992.

Conacher has been actively involved with AOAC for more than 20 years, serving as a member (1981–1983) and chairman (1983–1987) of Committee C (Methods Committee on Foods I) and as an Associate Referee for Brominated Oils (1970–1980) and Fats and Fatty Acids by GLC (1973–1980). He was instrumental as a member of the Official Methods Board between 1983 and 1987 and also was chairman to the Governance Council (1988–1989) and the Task Force on Validation of Methods in Crisis Situations (1987–1988), and served as a member on the Wiley Award Committee. A member of the Board of Directors since 1988, Conacher has served as a Director for 4 years. In recognition of at least 10 years of meritorious service, Conacher was named Fellow of the AOAC in 1984 at the 98th AOAC International Annual Meeting.

As director of the Bureau of Chemical Safety, Conacher is responsible for the direction of research, evaluation, and standard setting activities of some 150 scientific personnel involved with agricultural chemicals, food additives, food packaging materials, incidental additives, and chemical contaminants in the food supply. He has been with the Health Protection Branch, Canada, since 1968, when he was initially involved as a research scientist in the Food and Nutrition Research Divisions. Since that time, he has been head of the Food Additives and Contaminants Section (1975–1979) and chief of the Food Research Division (1980–1992). In the later position, he planned, established, and managed research programs and handled the administration of the division's some 40 scientists and technicians. He also coordinated the activities of the division with other bureau divisions and with outside organizations, and provided advice, information, and recommendations for development of enforcement of food protection legislation relating to chemicals in foods.

In a brief speech following receipt of the gavel at the business meeting in Cincinnati, Conacher indicated there were 2 issues that he wished to emphasize during his term as President. These were transparency in operation and the development of a plan "to provide direction to the Association over the next 5 years or so."

By transparency, Conacher indicated he meant communication — before, during, and after decision making — together with the involvement of as many of the Association members as was feasible. He did, however, indicate the challenge was to find the appropriate



balance between transparency and the timeliness of decisions.

He went on to say that it is vitally important for an organization to know "why it is there," and "where it is going." Conacher said that the former has been partially addressed for the Association by the recent development of the new mission statement; however, a future course remained to be developed. This he promised to initiate during the upcoming year.

Conacher has long been involved in other professional activities. He was chairman to the National Academy of Sciences Committee on Environmental Monitoring and Chemical Analysis, the Four-Nation International Committee on Chemistry of Antioxidants, the Technical Advisory Committee (4th session) of the FAO/WHO Committee on Food Monitoring in Geneva, Switzerland, and the IUPAC Food Chemistry Commission Working Group on Halogenated Hydrocarbons Environmental Contaminants, and served as vice chairman on the IUPAC Food Chemistry Commission. He is currently serving as a member to the Advisory Committee of the National Research Council Marine Program, and this year chaired the CIFST National Conference in Ottawa.

The author of nearly 100 scientific papers, reviews, and book chapters, Conacher graduated from St. Andrews University in Scotland, with an honors B.S. in Chemistry in 1961, and received his Ph.D. in Lipid Chemistry in 1968, also from this university.

AOAC looks forward to Conacher's term as President of the Association.

AOAC Board Slate Elected, Propositions and Methods Approved

A new slate of officers, 2 propositions to amend the AOAC International Bylaws, and 69 methods recommended for adoption as final action are among the items recently approved by a vote of AOAC membership.

The following Board slate, which the Nominating Committee proposed, was elected for the 1992–1993 year:

President-Elect: Arvid Munson, Phoenix Regulatory Associates, Inc., Sterling, VA

Secretary-Treasurer: Alan R. Hanks, Office of the Indiana State Chemist, West Lafayette, IN

Directors:

- Paul R. Beljaars, Inspectorate of Health Protection Directorate, The Netherlands
- George H. Boone, Food and Drug Administration, Brooklyn, NY
- Eugene J. Klesta, Chemical Waste Management, Inc., Alsip, IL
- Raymond Matulis, Kraft General Foods, Glenview, IL
- Albert E. Pohland, Food and Drug Administration, Washington, DC
- P. Frank Ross, U.S. Department of Agriculture, Animal Health Inspection Service, Ames, IA

Henry B.S. Conacher, having been previous elected, will serve on the 1992–1993 Board as President as will Edgar R. Elkins who will serve as Immediate Past President. Beljaars, Boone, Klesta, and Matulis are new Board members.

As amended in 1991, the Bylaws now specify that 6 Directors be elected (to increase the size of the Board from 9 to 10 members) to staggered 3-year terms. The 1991 proposition provided that the 6 Directors would be elected to initial 1-, 2-, and 3-year terms to begin the staggered term system. This was accomplished by proposing Pohland and Ross for 1-year terms, Boone and Klesta for 2-year terms, and Beljaars and Matulis for 3-year terms.

This Board slate was approved, together with the propositions and methods, by mail ballots sent to all AOAC members this past June. From the 693 valid ballots received, the 2 propositions amended the AOAC Bylaws as follows:

- The Board was granted the authority to grant waivers of dues to unemployed members upon written request.
- The word "regional" was deleted in all references to Regional Sections in the Bylaws to give AOAC sections more latitude in choosing their names and to eliminate the various international geographical ambiguities associated with the word "regional."

This year, for the first time, the ballot also included a section for member adoption of final action methods. The 69 methods recommended for adoption as final action were approved.

1992 Fellows of the AOAC

In recognition of at least 10 years of meritorious service to the Association, the following individuals received honors as 1992 Fellows of the AOAC at the 106th AOAC International Annual Meeting in Cincinnati, OH:

Jack L. Boese, FDA.—Associate Referee (10 methods adopted): 1979–1987, mites in stored foods; 1982–present, performance materials in foods and drugs, 1988–present, rodent gnawing of packaging materials and foods, salivary analyze test, 1979–1982, automated filth analysis, 1979–1981, soluble insect and other animal filth, 1979–1988, filth in dried mushrooms. General Referee: 1986–present, filth and extraneous ma-

terials in foods and drugs. Member, 1990–present, Methods Committee F. Short course instructor, 1987, extraneous materials workshop. 1992 winner of General Referee Award.

Michael H. Brodsky, Ontario Ministry of Health.—Member, 1986–1991, Laboratory Quality Assurance Committee. Member, 1986–1992, Meetings, Symposia, and Educational Programs Committee. Member, 1989–1991, Interlaboratory Studies Committee. Member, 1986–1988, Chairman, 1990–1992, Official Methods Board. Member 1983–1988, Methods Committee F.

Richard Ellis, USDA.—Member, 1985–1990, Chairman, 1987–1990, Long Range Planning Committee. Member, 1985–1990, Guidelines for Collaborative Studies Committee. Member, Nitrosamine Committee. General Referee (9 methods approved): 1979–1986, meat, poultry, and meat and poultry products. Member, 1986–1987, 1989–1990, Methods Committee C. Member, 1988–1989, Official Methods Board.

Erika Hargesheimer, City of Calgary.—Member, 1982–1991, Chairman, 1986–1990, Methods Committee H. Member, 1986–1990, Official Methods Board. General Referee (3 methods approved): cooperative studies. 1990 winner of General Referee Award.

Gayle A. Lancette, FDA.—Member, 1991–present, Official Methods Board. Member, 1985–1987, Methods Committee G. Member, 1990–1991, Chairman, 1991–present, Methods Committee F. Associate Referee (3 methods adopted): 1977–1989, *Bacillus cereus*, 1982–1987, *Staphylococcus*. 1989 winner of Associate Referee Award.

Donald A. Mastrorocco, Jr, Hershey Chocolate, USA.—Member, 1982–1991, Chairman, 1988–1991, Methods Committee F. Member, 1988–1991, Official Methods Board. Chairman, 1985–1986, Ad Hoc Committee on Biological and Pass/Fail Methods. Co–chairman, 1988–1989, Ad Hoc Task Force on Test Kits. Associate Referee (1 method adopted): 1978–1981, extraneous materials in chocolate liquor.

David L. Osheim, NVSL.—Member, 1985–1987, Chairman, 1988–1990, Regional Sections Committee. Member, 1988–1989, Governance Council. Member, Meetings, Symposia, and Educational Program Committee. Chair-

man, 1991–present, Membership Committee. Associate Referee (2 methods adopted): 1981–1991, copper in animal tissues, 1984–present, fluoride in animal tissues. 1987 winner of Associate Referee Award.

P. Frank Ross, USDA.—Member, 1989–present, Board of Directors. Member, 1985–1986, Fellows Committee. Member, 1984–1987, Meetings, Symposia, and Educational Programs Committee. Member, 1987–1988, Chairman, 1988–1989, Constitution Committee. General Referee (6 methods approved): veterinary analytical toxicology. 1987 winner of General Referee Award. 1992 winner of Harvey W. Wiley Award.

Stephen Slahck, Mobay.—Member, 1986–present, Methods Committee A. Associate Referee (8 methods adopted): 1982–1987, propoxur, 1982–1987, methiocarb, 1982–1987, aminocarb, 1984–1990, oxthioquinox, 1988–present, cyfluthrin, 1984–present, oxydemeton–methyl, 1984–1990, anilazine, 1984–1990, triademefon, 1984– present, azinphos–methyl. 1986 winner of Associate Referee Award.

H. Mike Stahr, Iowa State University.—Member, 1991–present, Regional Section Committee. Member, Archives Committee. Member, University Participation. Associate Referee: 1971–present, sodium monofluoroacetate, 1985–present, pesticides in toxicological samples.

Nominations for these awards were made by AOAC members, were reviewed and recommended by the Committee on Fellows, and finally were approved by the Board of Directors.

Methods Committee Associate Referee Awards

Created in 1986, the Methods Committee Associate Referee Awards recognize the best Associate Referee in a committee for a given year. Those named for 1992 are:

■ **Joseph G. Sebranek**, Iowa State University, Department of Animal Science, Ames, IA 50011, Associate Referee for Crude Protein in Meat and Meat Products, Combustion Method, nominated by the Methods Committee on Foods I

■ **James T. Tanner**, U.S. Food and Drug Administration, Division of Nutrition, Washington, DC 20204, Associate Referee for Analysis of Milk-Based Infant Formula, Phase V, nominated by the Methods Committee on Foods II

■ **Brian Worobey**, Health and Welfare Canada, Bureau of Chemical Safety, Tunney's Pasture, Ottawa, ON, K1A OL2, Canada, Associate Referee for Diquat and Paraquat Residues in Potatoes, Liquid Chromatographic Method, nominated by the Methods Committee on Residues

■ **Russell S. Flowers**, Silliker Laboratories, Inc., Chicago Heights, IL 60411, Associate Referee for *Salmonella* in Foods, Alternative (Two-Step) Immunodiffusion Method, nominated by the Methods Committee on Microbiology and Extraneous Materials

■ **James E. Longbottom**, U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH 45268, Associate Referee for Trace Elements in Water, Inductively Coupled Plasma Mass Spectrometric Method, nominated by the Methods Committee on Environmental Quality.

General Referee Award to Boese

The 1992 winner of the General Referee Award, granted by the Official Methods Board in recognition of outstanding leadership and substantial contribution to method development, is Jack L. Boese, General Referee for Filth and Extraneous Materials in Foods and Drugs.

In that capacity, Boese oversees method development in some 20 topic areas. Under his leadership, 11 methods have been adopted.

Boese is an entomologist employed at the U.S. Food and Drug Administration in Washington, DC. He has been actively involved with AOAC for many years, serving as a member of Committee F (Methods Committee for Filth and Extraneous Materials in Foods and Drugs) since 1979. He was also named a Fellow in 1992.

Other nominees for the General Referee of the Year were Margaret Clarke (Methods Committee on Foods II) and Kenneth P. Stoub (Methods Committee on Environmental Quality).

Collaborative Study of the Year Award — 1992

The Official Methods Board named Joseph G. Sebranek the winner of the Collaborative Study of the Year Award for 1992 for "Crude Protein in Meat and Meat Products, Combustion Method." Sebranek who works for Iowa State University in Ames, IA, had his study nominated by the Methods Committee on Foods I.

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.

Nominations were received from 2 other Methods Committees: the Methods Committee on Foods II nominated "Analysis of Milk-Based Infant Formula, Phase V (Folic Acid, Pantothenic Acid, Vitamin E, and Vitamin A)" by James T. Tanner; and "Insoluble Dietary Fiber in Food and Food Products, Enzymatic–Gravimetric Method (Phosphate Buffer)" by Leon Prosky; and the Methods Committee on Microbiology and Extraneous Materials nominated "*Listeria* sp., Biochemical Identification Method (Micro-ID Listeria)" by Donald L. Higgins and Barbara J. Robison.

Michael J. McCall Named 1992 AOAC Scholarship Winner

Michael J. McCall, a 21-year-old junior at Illinois State University, has been selected by the AOAC International Harvey W. Wiley Scholarship Committee as the 1992 scholarship award winner. He will receive $1,000 toward his senior year expenses.

McCall has been involved for 3 semesters in undergraduate research, working on the development or modification of analytical methods for environmental sampling. He recently presented the results of his research at an undergraduate research symposium at Illinois State University.

Currently, McCall's research project involves nitrate/nitrite/ammonia analysis in aquaculture tank effluents.

Highlights of AOAC International Annual Meeting

Change was in the air in Cincinnati last month when AOAC International held its 106th annual meeting! And President Edward R. Elkins reminded attendees that this was the first meeting for the Association with its new name and logo. More than just a name change — an emphasis on international was evident everywhere, from the technical program to the entertainment.

On Sunday evening, the Opening Reception featured foods from every continent with its "AOAC Around the World" theme. At the 7 food stations representing the 7 continents of the world, attendees and guests sampled global cuisine ranging from Australian steak and kidney pie to African samosas. Competition was keen in the AOAC International Global Guessing Game, which challenged everyone to compete for prizes by estimating the number of AOAC members on each continent. After comparing hundreds of answer sheets to the actual numbers furnished by AOAC Membership Coordinator Carrie Glavin, prizes were awarded: **Robert L. Bradley**, University of Wisconsin Department of Food Science; **Anant V. Jain**, University of Georgia Diagnostic Laboratories; **Jon E. McNeal**, U.S. Department of Agriculture; **Jerry Ngeh-Ngwainbi**, Kellogg Co.; **Henry Ostapenko**, Heinz USA; **Harry Reid**, *Food Chemical News*; **Erika Smith**,

Cornell University; **Jennifer Wo**, Nutrition International; and **Yiu Kwa Wo**, Nutrition International.

On Tuesday evening, more than 250 attendees and guests cruised the Ohio River aboard the Funliner for the AOAC International Dixieland Riverboat Cruise. Newly-elected Board Member Paul Beljaars of the Inspectorate of the Health Protection Directorate in Maastricht, The Netherlands, sat in on drums with the dixieland band on board and entertained guests and crew. Many members were amazed to discover this "hidden talent," but many already knew that Beljaars is a talented musician who tours annually with his band. Even the entertainment had an international flair!

The 1992 AOAC International Annual Meeting in Cincinnati last month also inaugurated several changes that were very popular with meeting attendees and exhibitors. Beginning with the streamlined registration process, attendees enjoyed improved services and scheduling to enhance the meeting experience. Badges and tickets were mailed to all attendees who registered by the August 3 advance registration deadline. After a brief pause in the Express Registration lines to pick up badge holder, program, and other meeting materials, they were on their way.

On Monday morning, the Opening Session featured the Keynote Address, "International Perspectives in Certification and Accreditation," by Antonio S. Mendes, Official for Internal Market and Industrial Affairs at the Directorate General Commission of the European Communities, Brussels, Belgium. Mendes gave an excellent overview of the activities of the Commission of the European Communities in certification. Potential problem areas in mutual recognition of certification programs and approaches to their solutions were examined. His address provided considerable insight into the technical difficulties encountered in establishing free trade and the importance of mutually recognized analytical methodologies.

During his year-end address at the Opening Session, President Elkins recounted for the audience the numerous accomplishments of the Association

during the past year that reflect long-range Association policies, including an increased commitment to making AOAC a truly international organization through participation in international activities and interaction with international organizations. He also described the implementation of the test kit verification program, the new peer-verified methods program, the creation of the new Mid-Atlantic AOAC section, and the expansion of the AOAC's publishing activities.

Many of these important initiatives can be credited back to the member surveys conducted in 1989 on long-range planning questions, as well as the 1990 in-depth focus groups surveys, according to Elkins. "For an association to meet the needs of its members and other constituents, it needs to ask its members what their needs are. AOAC has been doing just that," said Elkins. He concluded that another membership survey was undertaken jointly this spring with many of the other associations belonging to the Council of Engineering and Scientific Society Executives, and "we fully expect to implement measures that will respond to the needs brought to light by the survey."

Following the speeches, presentation were made of the 1992 Harvey W. Wiley Award, Fellow of the AOAC Awards, General Referee of the Year Award, Collaborative Study of the Year Award, Committee Associate Referee of the Year Awards, and Employee Service Awards. (The Wiley Award Address, formerly presented at the Opening Session, was the featured presentation of the Wiley Award Symposium.)

The abbreviated Opening Session ended by 10:00 a.m., giving attendees a chance to visit the 95 exhibiting companies without missing any of the technical program. Samuel Page, Chairman of the AOAC Committee on Meetings, Symposia, and Educational Programs, reported: "Most people really liked the revised format of the Opening Session. In fact, I heard few, if any, complaints about anything. Everyone had nothing but praise for the meeting, the facilities, and the city."

For the first time this year, the Exhibit Hall featured a Harvey Wiley Award winner's booth, giving attendees a chance to personally talk to and congratulate P. Frank Ross, U.S. Department of Agriculture, Animal Health Inspection Service, this year's award winner. The new Statistics Committee Booth, which offered hands-on experience with the AOAC statistics software, was another popular feature of the Exhibit Hall.

Innovations in the technical program included the addition of the Harvey W. Wiley Symposium featuring Ross's presentation entitled "What Are We Going to Do With This Dead Horse?" to a standing-room-only audience, and the expansion of the Regulatory Roundtable to a full day. John Richard and Glenn Bennett of USDA's Northern Regional Research Center in Peoria, IL, organized the Wiley Symposium, "Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action." The Regulatory Roundtable focused on international trade, with a morning session on the North American Trade Zone Regulations, organized by Bobbi Dresser, U.S. Food and Drug Administration. Alex Williams, Government Chemist of England (retired), organized the afternoon session on EC Regulations.

Five additional symposia included: Microbiology Update; Milk: Antibiotics and Other Contaminants; Nutrition Labeling of Lipids; Process Sensors and Control; and Forensic Methods and Product Tampering. Ten poster sessions were organized around the AOAC Methods Committee topics.

Expanding the popular Open Forum and dividing it into 2 sessions provided an opportunity for feedback from attendees on all aspects of the meeting, as well as other governance and technical issues.

FDA *Bacteriological Analytical Manual*

A new seventh edition of the FDA *Bacteriological Analytical Manual* (BAM) became available from AOAC in October 1992. This new edition contains methods preferred by the U.S. Food and Drug Administration for the microbiological analysis of foods. New topics include an overview of rapid methods and tests, and new chapters include "*Listeria monocytogenes*," "Serodiagnosis of *Listeria monocytogenes*," and "Identification of Foodborne Bacterial Pathogens by Gene Probe." Most other chapters have been revised and expanded, and all have been brought up to date.

Although procedures in this 529-page manual have not achieved official AOAC status through collaborative testing, they represent all the methodology currently in use in FDA laboratories. They have been found most effective for the detection of microorganisms and certain of their metabolic products.

The BAM's loose-leaf format facilitates the updating of procedures; some methods presented in earlier BAM editions are no longer in general use and have been eliminated.

Contents

The contents include: Food Sampling and Preparation of Sample Homogenate; Microscopic Examination of Foods; Aerobic Plate Count; *Escherichia coli* and the Coliform Bacteria; *Salmonella*; *Shigella*; *Campylobacter*; *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*; *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and Other *Vibrio* spp. *Listeria monocytogenes*; Serodiagnosis of *Listeria monocytogenes*; *Staphylococcus aureus*; Staphylococcal Enterotoxins; *Bacillus cereus*; Diarrheagenic Enterotoxin; Clostridium perfringens; *Clostriduum botulinum*; Yeasts, Molds, and Mycotoxins; Parasitic Animals in Foods; In-

hibitory Substances in Milk; Rapid HPLC Determination of Sulfamethazine in Milk; Examination of Canned Foods; Modification of Headspace Gas Analysis Methodology, using the SP4270 Integrator; Examination of Containers for Integrity; Microbiological Methods for Cosmetics; Identification of Foodborne Bacterial Pathogens by Gene Probes; and Investigation of Food Implicated in Illness. Appendixes include: Rapid Methods for Detecting Foodborne Pathogens; Most Probable Number Determination; and Media and Reagents.

This new 529-page manual can be ordered from AOAC International by sending the following: purchaser's name and address, a check (U.S. funds on U.S. banks only) or MasterCard, VISA, or Diners credit card information (name of card, card number, and expiration date) and card holder's signature to AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, Va 22201. Price is $89.00 in the U.S., $109.80 outside the U.S. for AOAC members, $99.00 in the U.S., $122.00 outside the U.S. for non-members. Credit card orders may be placed by mail, telephone 703/522-3032, or fax 703/522-5468.

Methods Adopted First Action

As directed by the Board of Directors, the Official Methods Board is responsible for consideration of methods for first action approval. The following methods were adopted first action at the Official Methods Board meeting August 29, 1992, in conjunction with the 106th AOAC International Annual Meeting in Cincinnati, OH, and became official at that time. These methods will be published in the fourth supplement (1993) to the 15th edition (1990) of *Official Methods of Analysis*.

■ *Drugs and Related Topics*: Sulfamethazine Residues in Milk, Liquid Chromatographic Method

■ *Foods I*: Iodine (as Iodide) in Pasteurized Liquid Milk and Skim Milk Powder, Liquid Chromatographic Method

■ *Foods II*: Crude Protein in Cereal Grains and Oilseeds, Generic Combustion Method

Analysis of Milk-Based Infant Formula, Phase IV: Iodide in Ready-To-Feed Milk-Based Infant Formula, Ion-Selective Electrode Method; Linoleic Acid in Ready-To-Feed Milk-Based Infant Formula, Gas Chromatographic Method; Vitamin D_3 (Cholecalciferol) in Ready-To-Feed Milk-Based Infant Formula, Liquid Chromatographic Method; and Trans-Vitamin K_1 (Phylloquinone) in Ready-To-Feed Milk-Based Infant Formula, Liquid Chromatographic Method

(1→3)(1→4)-Beta-D-Glucan in Grains & Cereals, Enzymatic- Spectrophotometric Method

Alcohol and Original Gravity Content In Beer, SCABA Method

■ *Microbiology and Extraneous Materials*: Total Coliforms and ***E. coli*** in Foods, Confirmed Detection by Coli-Complete® Substrate Supporting Disc Method

■ *Feeds, Fertilizers, and Related Topics*: Sampling of Liquid Fertilizers, Texas Liquid Sampler Method

■ *Environmental Quality*: Ethylene Thiourea (ETU) Residue in Finished Drinking Waters, Gas Chromatographic Method with Nitrogen-Phosphorus Detection

Chlorinated Acidic Residues in Finished Drinking Waters, Gas Chromatographic Method with Electron Capture Detection.

Instructions to Authors

Scope of Articles and Review Process

The *Journal of AOAC International* 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 desktop publisher; *(4)* desktop publisher sends page proofs to author for final approval.

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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.*

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2. To Managing Editor, AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, 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, acknowledgements, references); 4.Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.

3. Suggest in a covering letter the names of at least 4 qualified reviewers, i.e., individuals engaged in or versed in research of the type reported.

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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

(1) 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

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1990) 15th 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.

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Journal Information

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AOAC *Official Methods* are methods that have been validated by an AOAC-approved collaborative study, recommended by the appropriate AOAC General Referee, Methods Committee, and the Official Methods Board, and adopted and published according to the Bylaws of the Association. Published papers that include such methods are distinguished by the words Collaborative Study in the title and by footnotes that indicate Association actions.

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

European Representatives. For information about AOAC and its publication, persons outside the U.S. may also contact the following: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 31-8389-18725; Derek C. Abbott, 33 Agates Lane, Ashtead, Surrey, KT21 2ND, UK, telephone 44-3722-74856.

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Books in Brief

Instrumentation in Analytical Chemistry, 1988—1991. Edited by Louise Voress. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 350 pp. Price: U.S. and Export: $44.95. ISBN 0-8412-2191-X.

An anthology of 48 articles that first appeared in *Analytical Chemistry*, this volume provides an excellent overview of the entire field of analytical instrumentation and features state-of-the-art developments. This fourth volume in the series focuses on robotics, computers, and laboratory data management; atomic and molecular spectroscopy, electroanalytical chemistry and chemical sensors; separations; mass spectroscopy; and surface analysis. Also included is an introduction by Royce Murray, editor of *Analytical Chemistry*.

Chemical Safety Sheets Book. Published by Samson Chemical Publishing USA, 610 S. Albert St, Mount Prospect, IL 60056, 1992. 1050 pp. Price: 124.00.

Designed specifically for use by hazardous material response teams, chemical transporters, safety managers, and other first responders to chemical emergencies, the *Chemical Safety Sheets Book* includes vital information on chemical hazards, exposure symptoms, preventative measures, fire extinguishing, first aid, and other critical safety information on over 900 common chemicals. The 1050-page book also includes key NFPA and DOT/UN labeling data as well as current U.S. and European exposure standards. For simple use in emergency situations, the book is alphabetized by chemical name and also indexed by both chemical (trade) name and DOT/UN identification number. All relevant information on each individual chemical is contained on a single, easy-to-follow page.

Methods for the Determination of Metals in Environmental Samples. By C.K. Smoley. Published by Lewis Publishers, 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 328 pp. Price: U.S. $59.95/Outside U.S. $72.00. ISBN 0-87371-831-3.

Methods for the Determination of Metals in Environmental Samples presents a detailed description of 13 analytical methods covering 35 analytes that may be present in a variety of sample types. The methods involve a wide range of analytical instrumentation including inductively coupled plasma (ICP)/atomic emission spectroscopy (AES), ICP/mass spectroscopy (MS), atomic absorption spectroscopy (AA), ion chromatography (IC), and liquid chromatography (LC). The application of these techniques to such a diverse group of sample types is a unique feature of this book. Sample types include waters ranging from drinking water to marine water, in addition to industrial and municipal wastewater, groundwater, and landfill leachate. The book also includes methods that will accommodate biological tissues, sediments, and soils. Methods in this book can be used in several regulatory programs because of their applicability to many samples types. For example, ICP/AES, ICP/MS, and AA methods can be used in drinking water and permit programs. Methods applicable to marine and estuarine waters can be used for the EPA's National Estuary Program. Terminology is consistent throughout the book, an important feature especially for the quality control sections where standardized terminology is not yet available.

Capillary Electrophoresis: Principles, Practice, and Applications. By S.F.Y. Li. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 570pp. Price: U.S.$225.50/Dfl.395.00. ISBN0-444-89433-0.

Capillary electrophoresis (CE) has had a very significant impact on the field of analytical chemistry in recent years as the technique is capable of very high resolution separations, requiring only small amounts of samples and reagents. Furthermore, it can be readily adapted to automatic sample handling and real time data processing. Many new methodologies based on CE have been reported. Rapid, reproducible separations of extremely small amounts of chemicals and biochemicals, including peptides, proteins, nucleotides, DNA, enantiomers, carbohydrates, vitamins, inorganic ions, pharmaceuticals, and environmental pollutants have been demonstrated. A wide range of applications have been developed in greatly diverse fields, such as chemical, biotechnological, environmental, and pharmaceutical analysis.

Biosensor Design and Application. Edited by Paul R. Mathewson and John W. Finley. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 230 pp. Price: U.S. and Export: $54.95. ISBN 0-8412-2494-3.

This book presents a contemporary discussion of currently available and potential applications of biosensors, and examines diverse approaches to the basic problems of microsizing analytical instrumentation, including the use of intact crustacea antenna, various forms of redox reactions, use of antibody-medicated reactions, and optical- and spectrophotometric-based methods. It describes requirements for using biosensors in experiments conducted in space as well as an interesting approach to the development of more efficient procedures for commercialization of biosensor technologies.

The JOURNAL OF AOAC INTERNATIONAL

EDITORIAL PROFILE

The *Journal of AOAC International* is the official bimonthly journal of AOAC International. Published for analytical scientists who develop and use analytical methodology, it is used by its subscribers in their day-to-day work in the fields of food composition and contamination, feeds, pharmaceuticals, cosmetics, agricultural and household chemicals, water analysis, and environmental control. Users include analytical chemists, biologists, microbiologists, biochemists, toxicologists, spectroscopists, statisticians, forensic and other scientists in laboratory, administrative, and top management positions in industry, government, and universities in the United States and more than 90 other countries.

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Harvey W. Wiley Award for the Development of Analytical Methods

A $3,000 annual award presented to an outstanding scientist or scientific team for analytical contributions in an area of interest to AOAC International.

December 1 is the closing date for nominations for the current year's award. Nominees, however, continue to be eligible for three additional years without renomination and their eligibility may be extended an additional four years by written request of the nominator.

AOAC International Fellow Award

Any member who has given at least 10 years of meritorious service to AOAC may be nominated. Awards are based on accumulated service. Members may send letters in support of eligible candidates to AOAC International.

February 15 is the deadline for submitting nominations for the current year's award.

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AOAC INTERNATIONAL

For more information or application forms, contact:
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CHEMICAL CONTAMINANTS MONITORING

- Food and Drug Administration Survey of Menthyl Mercury in Canned Tuna—N. Yess

FEEDS

- Assay of Oxytetracycline in Animal Feed by Liquid Chromatography and Microbiological Plate Assay—M.L. Hasselberger

TRANSACTIONS OF THE 106TH AOAC INTERNATIONAL ANNUAL MEETING AND EXPOSITION

KEYNOTE ADDRESS

- International Perspectives in Certification and Accreditation—A.S. Mendes

PRESIDENT'S ADDRESS

- AOAC International — 1991–1992: The Year in Review—E. Elkins

CHEMICAL CONTAMINANTS MONITORING

Survey of Bottled Drinking Water Sold in Canada. Part 1. Lead, Cadmium, Arsenic, Aluminum, and Fluoride

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Samples of bottled water (n = 172) offered for sale in Canada were analyzed for lead, cadmium, arsenic, aluminum, and fluoride: means and ranges (μg/g) found were, respectively, 0.0026 (<0.0010–0.074), 0.00018 (<0.0001–0.0004), 0.0030 (<0.001–0.048), 0.027 (<0.010–0.568), and 0.543 (<0.050–5.85). Comparison of levels among mineral waters (n = 64), spring waters (n = 77), and miscellaneous waters (n = 31) indicated appreciable differences only in the case of fluoride. For fluoride, the means and medians (μg/g) for mineral, spring, and miscellaneous waters were 1.179 and 0.455, 0.152 and 0.090, and 0.201 and <0.050, respectively. No samples were found in violation of the tolerances in the Canadian Food and Drug Regulations; however, 1 sample (in a lead-soldered can) contained lead and 15 samples contained fluoride at levels above the limits recommended by the Guidelines for Canadian Drinking Water (tap-water) Quality.

In Canada, consumable water is generally classified into 3 categories: drinking water (i.e., tap water), bottled mineral or spring water, and other bottled waters (e.g., distilled waters and flavored waters). These last 2 classes fall under the prepackaged designation in accordance with the Canadian Food and Drug Regulations (1).

No regulatory standards have been established at the federal level for tap water, because responsibility in this area rests mainly with the provinces and municipalities. However, a subcommittee of the Federal–Provincial Advisory Committee on Environmental and Occupational Health has established guidelines for tap-water quality (2).

On the other hand, all prepackaged water is classified as a food in Canada, and consequently, the sale of such products is subject to all the provisions of the Canadian Food and Drugs Act and Regulations (1).

In recent years, consumption and use of bottled waters in Canada have increased considerably (personal communication, M. Lavallée, Water Resources Division, Department of the Environment, Government of Quebec, Canada, 1986). Furthermore, there is a public perception that bottled water is in some manner safer than tap water (personal communication, N. McEwen, Chemical Evaluation Division, Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Canada, 1991). Because of this, the Canadian regulations (1) pertaining to bottled water are currently under review. As part of this review, a survey of a variety of selected chemicals in bottled waters (both imported and domestic) on the Canadian market was undertaken. This report discusses the levels of lead, cadmium, arsenic, aluminum, and fluoride found. Part 2 of the survey deals with volatile organic compounds (VOCs) (3).

Experimental

During March and April 1990, representative samples of many different brands of bottled water were collected from across Canada. Samples consisted of 5 containers, with a minimum volume of 750 mL/container. If only smaller containers were available, sufficient containers were taken to provide a sample size of total volume greater than 3.5 L. From each sample, 3 containers of any size were taken for analysis of VOCs (3), and all remaining containers from each sample were taken for trace element analysis. In all, 172 different brands and varieties were sampled for trace elements.

For classification purposes, samples were divided by type (spring, mineral, or others that included deionized, distilled, and community tap water) and by origin (bottled in Canada or imported) according to the bottle label.

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Table 1. Graphite-furnace atomic absorption spectrometric instrumental conditions

Variable	Lead and cadmium	Arsenic	Aluminum
Lamp	Hollow cathode	EDL	Hollow cathode
Wavelength, nm	283.3 (lead) 228.8 (cadmium)	193.7	309.3
Aliquot volume			
Sample, µL	10	10	10
Modifier, µL			2
Temperature program[a]			
Drying temperature, °C	70 (5, 0) 140 (25, 20)	120 (0.2, 10) 225 (2, 35)	70 (5, 0) 120 (15, 20)
Ashing, °C	650 (5, 20)	900 (5, 6)	1000 (3, 20)
Atomization, °C[b]	2000 (1, 3)	2650 (0.9, 2)	2700 (1, 3)
Cleanout, °C	2000 (0, 3) 2700 (1, 3)	2650 (0, 1)	2700 (0, 1)
Cooldown, °C	40 (15, 4)	40 (13, 5)	40 (15, 3)

[a] Temperature program steps expressed in °C followed by the ramp and hold times in seconds (in parentheses).
[b] Argon gas flow stopped 1 s before and during the atomization step.

Sample Pretreatment for Trace Element Determinations

Waters poured from smaller, original bottles were analyzed directly for all elements. Large containers (20–50 L) were subsampled into acid-washed polyethylene bottles before shipping to the Atlantic Region, Health Protection Branch Laboratory, Halifax, for analysis. All samples were acidified with 1% nitric acid (w/v) before analysis for lead, cadmium, and arsenic. The pH was not measured. Unacidified portions were used for aluminum and fluoride determinations.

All bottles of water were shaken well just before analytical subsampling.

Analytical Instrumentation

A Varian Model 875 spectrometer with GTA-95 furnace and pyrolytic graphite L'vov platform was used to determine lead, cadmium, arsenic, and aluminum. Instrumental conditions are summarized in Table 1.

For fluoride determinations, a Corning Model 10 pH/mV meter was used in conjunction with a fluoride-specific electrode (Orion Model 94-09) and single-junction reference electrode (Orion Model 90-01).

Reagents

Deionized water was used throughout. All reagents were analytical reagent grade unless otherwise specified.

Lead and Cadmium Method

A 2 mL aliquot of modification solution containing 25% (v/v) nitric acid and 0.5% (w/v) ammonium dihydrogen phosphate was added to 8 mL water sample in a polystyrene centrifuge tube (Falcon No. 2095). Solutions were left overnight and analyzed by graphite-furnace atomic absorption spectrometry using standards with the same modifier.

Arsenic Method

Arsenic was analyzed by using a matrix modifier developed specifically for arsenic coprecipitated from food matrixes containing varying amounts of iron and copper (4). A 2.5 mL aliquot of modifier containing 80 µg/mL Ni(II), 1000 µg/mL Fe(III), and 800 µg/mL Cu(II) in 40% (v/v) nitric acid was added to 7 mL water sample in a polystyrene centrifuge tube. After the solution was allowed to stand overnight, 0.5 mL 15 000 µg/mL barium(II) (as nitrate) was added. Solutions were left standing a minimum of 1 h then analyzed instrumentally.

Aluminum Method

In a polystyrene centrifuge tube containing 9 mL water sample, 0.5 mL nitric acid (Baker Instra-Analyzed Grade) and 0.5 mL 20% citric acid (Baker Analyzed) were added. Samples were analyzed in the presence of 2 µL 100 µg/mL palladium (prepared by diluting BDH Assured palladium 1000 µg/mL AA standard) added by the autosampler.

Fluoride Method

Water samples were degassed in an ultrasonic bath (some of the samples were carbonated). A 5 mL aliquot of sample was added to a Teflon-FEP beaker along with 5 mL total ionic strength adjustment buffer with 1,2-cyclohexylene dinitrilotetraacetic acid (Fisher Grade, Orion No. 94-09-09A). While the mixture was stirred over an insulated magnetic stirrer, elec-

Table 2. Quality control results for trace elements

Parameter	Lead	Cadmium	Arsenic	Aluminum	Fluoride
			Rec. studies		
n	29	30	31	28	12
Av. rec., %	98	95	100	115	105
Rec. SD, %	9	7	7	14	6
Minimum rec., %	78	82	88	82	97
Maximum rec., %	123	122	113	147	113
Spike added, μg/mL	0.05	0.005	0.02	0.05	5
			Control sample 1[a]		
n	7	8	15	11	17
Prepared level, μg/mL	0.030	0.0030	0.020	0.020	0.70
Av. found, μg/mL	0.029	0.0028	0.018	0.038	0.69
SD	0.002	0.0001	0.001	0.009	0.03
Minimum found, μg/mL	0.026	0.0026	0.016	0.026	0.63
Maximum found, μg/mL	0.032	0.0030	0.020	0.057	0.75
			Control sample 2[a]		
n	7	7	15	9	
Prepared level, μg/mL	0.070	0.0070	0.080	0.080	
Av. found, μg/mL	0.069	0.0067	0.077	0.104	
SD	0.004	0.0002	0.003	0.008	
Minimum found, μg/mL	0.066	0.0065	0.074	0.095	
Maximum found, μg/mL	0.077	0.0070	0.081	0.120	

[a] Prepared by spiking demineralized water.

trode measurements were taken after stable readings were obtained (3–4 min). Standards were treated in the same manner as the samples. Fifteen standards were run before and after samples, and after every 6–7 samples, 3 of the standards were run to check calibration.

Standards

A minimum of 5 nonzero working standards (prepared from BDH Assured AA standards and from Alpha Ventron sodium fluoride) were used for each trace element. Working standards were prepared by diluting stock standards with the same modifiers as samples. Nonlinear algorithms were used for calibration.

Quality Assurance

Quality assurance included preparation of laboratory reference waters as well as recovery of spikes added to water samples. Spiked water samples and one of the laboratory reference waters were analyzed within most analytical batches. The number of batches taken to analyze all samples varied with the element determined. Results are presented in Table 2 and appear satisfactory except for the high average recovery (190%) obtained for aluminum in control sample 1, which appears to have been contaminated.

Statistical Operations

For calculation of statistical parameters, when the concentration fell below the detection limit of the method, the detection limit was used.

For all elements except fluoride, the detection limit was 3 times the standard deviation in mass units of multiple reagent blanks (a minimum of 2), included with each analytical batch, divided by the sample weight. Thus, the detection limit for each element could vary from batch to batch.

For fluoride, the lowest standard concentration excluding the reagent blank was 0.05 μg/mL. Samples with potentials corresponding to concentrations below this standard were arbitrarily given the concentration of <0.05 μg/g.

Results and Discussion

Table 3 summarizes the concentrations of elements found in the water samples.

The mean, median, and range of aluminum concentrations were 0.027, 0.012, and <0.010–0.57 μg/g, respectively. The difference between the means for Canadian spring water (0.016 μg/g) and mineral water (0.029 μg/g) was statistically significant ($P = 0.013$), but there was no difference between

Table 3. Trace-element levels (μg/g) in bottled water samples

Product		Aluminum	Fluoride	Lead	Cadmium	Arsenic
			All samples			
$n = 172$	Average	0.027	0.543	0.0026	0.00018	0.0030
	Median	0.012	0.135	<0.0010	<0.00020	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.568	5.850	0.0740	0.00040	0.0480
			Mineral water			
All samples ($n = 64$)	Average	0.029	1.179	0.0021	0.00016	0.0035
	Median	0.016	0.455	<0.0010	<0.00010	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.217	5.850	0.0140	0.00030	0.0480
Canadian ($n = 20$)	Average	0.029	0.968	0.0032	0.00019	0.0015
	Median	0.018	0.365	<0.0020	<0.00020	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.075	4.200	0.0140	0.00030	0.0030
Imported ($n = 44$)	Average	0.028	1.275	0.0017	0.00015	0.0045
	Median	0.014	0.555	<0.0010	<0.00010	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.217	5.850	0.0040	0.00030	0.0480
			Spring water			
All samples ($n = 77$)	Average	0.016	0.152	0.0017	0.00018	0.0026
	Median	0.010	0.090	<0.0010	<0.00010	<0.0020
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.208	0.960	0.0040	0.00030	0.0290
Canadian ($n = 61$)	Average	0.016	0.163	0.0018	0.00019	0.0019
	Median	0.010	0.090	<0.0010	<0.00020	<0.0020
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.208	0.960	0.0040	0.00030	0.0030
Imported ($n = 16$)	Average	0.016	0.107	0.0013	0.00014	0.0054
	Median	0.011	0.080	<0.0010	<0.00010	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.071	0.230	0.0020	0.00030	0.0290
			Miscellaneous waters			
All samples ($n = 31$)[a]	Average	0.049	0.201	0.0058	0.00021	0.0030
	Median	0.013	0.050	<0.0020	<0.00020	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.568	1.120	0.0740	0.00040	0.0340
Canadian ($n = 28$)	Average	0.052	0.218	0.0057	0.00021	0.0032
	Median	0.013	0.050	<0.0020	<0.00020	<0.0010
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	0.568	1.120	0.0740	0.00040	0.0340
Imported ($n = 2$)	Average	0.010	0.050	0.0025	0.00020	0.0015
	Median	<0.010	<0.050	0.0025	0.00020	<0.0015
	Minimum	<0.010	<0.050	<0.0010	<0.00010	<0.0010
	Maximum	<0.010	<0.050	0.0040	0.00030	<0.0020

[a] Number includes 1 sample of miscellaneous water that could not be classified by origin.

Table 4. Statistically significant differences in concentrations by using a 2-tailed *t*-test[a]

Element	Data set 1	Mean	Data set 2	Mean	Significance
Aluminum	Canadian mineral water	0.029	Canadian spring water	0.016	P = 0.013
Fluoride	Canadian mineral water	0.968	Canadian spring water	0.163	P = 0.013
	Imported mineral water	1.28	Imported spring water	0.107	P = 0.0001

[a] Heteroscedasticity (unequal variances between groups) is assumed.

the domestic and imported products (Table 4). The distinction between spring water and mineral water is based on solids content, with many regulatory guidelines defining mineral water as that from a natural source with more than 500 μg/mL total dissolved solids. Thus, with the high level (8.1%) of aluminum in the earth's crust, the greater concentration of aluminum in mineral waters is predictable.

The mean, median, and range of fluoride concentrations were 0.543, 0.135, and <0.050–5.85 μg/g, respectively (Table 3). Spring water contained 0.152 μg/g fluoride, which is statistically lower ($P < 0.0001$) than mineral waters, which have a mean of 1.179 μg/g fluoride. Significant differences were found both for imported and Canadian products (Table 4).

The mean, median, and range of lead concentrations were 0.0026, <0.0010, and <0.0010–0.074 μg/g, respectively (Table 3). No statistically significant differences relating to source or type of water were found.

The mean, median, and range of cadmium concentrations were 0.00018, <0.00020, and <0.0001–0.0004 μg/g, respectively (Table 3). Concentrations did not depend on the source or type of water.

The mean, median, and range of arsenic concentrations in all samples were 0.003, <0.001, and <0.001–0.048 μg/g, respectively (Table 3). No statistically significant differences relating to source or type of water were found.

No samples were found in violation of the tolerances for lead and arsenic in Table 1, Division 15 of the Food and Drug Regulations (1). One sample of domestic purified drinking water in a lead-soldered can contained 0.074 μg/g lead, more than the maximum concentration of 0.05 μg/mL recommended in the Canadian Drinking Water Guidelines (2). Fifteen samples contained more fluoride than the maximum recommended concentration of 1.5 μg/mL (2).

References

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(2) *NH&W 1989 Guidelines for Canadian Drinking Water Quality* (1989) Health and Welfare Canada, Ottawa, ON, Canada

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CHEMICAL CONTAMINANTS MONITORING

Graphite-Furnace Atomic Absorption Spectrometric Determination and Survey of Total Aluminum, Copper, Manganese, Molybdenum, and Tin in Infant Formulas and Evaporated Milks

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A graphite-furnace atomic absorption spectrometric (GFAAS) method using a single simple digestion was developed to determine aluminum, copper, manganese, molybdenum, and tin in infant formulas and evaporated (evap.) milks. Citric acid (0.5 mL 20%) and 3 mL HNO_3 were added to 2 g formula (ready-to-use basis) in unwashed polystyrene test tubes. Tubes were capped, and solutions were digested 6–18 h at 55°C. Digests were diluted to 10 mL with water and analyzed. Standards were prepared in a matrix of 30% HNO_3 and 1% citric acid without digestion. Palladium (1 μg) was used as matrix modifier for aluminum and tin determinations. The mean detection limits of the method were 0.021, 0.043, 0.006, 0.063, and 0.073 μg/g for aluminum, copper, manganese, molybdenum, and tin, respectively. The elements were determined in 191 Canadian infant formulas and evap. milk samples. Means, medians, and ranges (μg/g) of metals in soy-based ready-to-use formulas were as follows: 1.62, 0.54, and 0.23–5.2 for aluminum; 0.87, 0.88, and 0.71–1.14 for copper; 0.63, 0.22, and 0.042–2.4 for manganese; 0.068, <0.049, and <0.048–0.14 for molybdenum; and 0.20, 0.15, and <0.074–0.54 for tin. Means, medians, and ranges (μg/g) of metals in milk-based ready-to-use formulas were as follows: 0.18, 0.13, and <0.011–0.66 for aluminum; 0.61, 0.58, and 0.44–0.93 for copper; 0.097, 0.094, and 0.053–0.16 for manganese; 0.033, <0.042, and <0.012–0.067 for molybdenum; and 0.23, 0.18, and 0.067–0.91 for tin. Respective levels in evap. milks (as sold) were as follows: 0.08, 0.051, and <0.017–0.27 for aluminum; 0.103, 0.087, and 0.045–0.31 for copper; 0.071, 0.062, and 0.048–0.16 for manganese; 0.14, <0.17, and <0.07–<0.19 for molybdenum; and 2.84, 0.46, and 0.032–48 for tin.

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Physiological effects (1) and complex metabolic interactions of toxic and nutritional elements (2, 3), coupled with the economic need for more efficient and rapid methods of analysis, pointed to the need for development of a simple graphite-furnace atomic absorption spectrometric (GFAAS) method applicable to aluminum, copper, manganese, molybdenum, and tin in infant formulas and evaporated (evap.) milks.

Although many GFAAS methods for the determination of individual or multiple elements have been published (4–6), multielement methods are less common, and analytical considerations in their application to routine low-level analyses have been all but ignored. Such considerations include cross contamination and instrumental base line and sensitivity drift. Furthermore, many of the multielement methods have failed to include tin and aluminum, in part because of the greater difficulty in their analysis. Specifically, analyses for aluminum, present at 8% in the earth's crust and less than 10 ng/g in some milk samples, are subject to contamination; HNO_3 digests of tin in the absence of organic matter (blanks) result in tin loss as metastannic acid. Therefore, the GFAAS method described here was developed, and its digestion procedure allows for inclusion of tin.

Experimental

Apparatus

(a) *Graphite-furnace atomic absorption spectrometer.*—Model 875-ABQ with GTA-95 graphite furnace (Varian Associates of Canada Ltd, Ottawa, ON, Canada), equipped with deuterium-continuum simultaneous background corrector; graphite-furnace atomizer; pyrolytically coated plateau and partitioned graphite tubes; L'vov platforms; digital recorder for integrated absorbance readings; programmable autosampler capable of picking up and pipetting blank, sample, or standard, and modifier from different sample cups.

(b) *Water bath.*—Capable of holding test tube rack and maintaining temperature at 55 ±2°C (Grant Instruments Ltd, Cambridge, UK). Fill bath with deionized water, and arrange

tube holder so that water does not touch the upper third of the tubes. Do not cover water bath to prevent evaporation.

(c) *Centrifuge tubes.*—15 mL polystyrene, graduated, with screw caps (Falcon Corp., Lincoln Park, NY 07035, Cat. No. 2095), or equivalent. Test 10 tubes from each lot for contamination by pipetting 1 mL 30% HNO_3 into tubes, capping, shaking, and analyzing for each element directly by GFAAS. The standard deviation for aluminum, the element most indicative of contamination, should be <3 ng/mL. (Ultimately, the detection limit is governed by the standard deviation of the blank, and this test assures that contribution of the tubes to the blank standard deviation is minimal.)

(**Notes**: Samples are extremely susceptible to contamination during workup and analysis. This is particularly true for aluminum. Wash hands thoroughly before handling labware, avoid talc [in gloves], body powders, dust, powdered samples, and drafts. Never touch the lip or any other part of a tube that may come in contact with solutions. Wash each pipet just before use with concentrated HNO_3, and rinse directly in a stream of deionized water. Avoid touching the pipet tip to any surface other than the solutions being pipetted. Avoid using glassware of any type other than that used to prepare the stock standards: acid-washed polypropylene or polystyrene labware is recommended.)

Although digestion tubes are capped during digestion, keep external area between cap and tube free of water and dust.

(d) *Demineralizer.*—Milli-Q (Millipore Corp., Bedford, MA 01730; Cat. No. ZD20,115 84).

(e) *Atomizer.*—HGA-400 (Perkin-Elmer Corp., Norwalk, CT 06859).

Reagents

(a) *Water.*—ASTM Type III. Prepare in demineralizer. Aliquots of water pipetted directly into graphite furnace must give no aluminum signal.

(b) *HNO_3 and HCl.*—High purity. Prepare from reagent grade acids using a quartz sub-boiling still, and use wherever these acids are specified. Follow standard safety precautions to avoid burns and breathing vapors.

(c) *Metals for preparation of standards.*—High-purity copper (Ventron Corp., Danvers, MA 01923, Cat. No. 00098), high-purity manganese (Ventron, Cat. No. 400022), and 99.999% palladium powder (Aldrich Chemical Co., Milwaukee, WI 53201, Cat. No. 20,393-9) for modifier.

(d) *Copper and manganese stock solutions.*—1000 µg/mL. Dissolve 1.000 g metal in ca 110 mL 50% HNO_3, and dilute to 1 L with water. For stock solutions containing 10 µg/mL copper in 5% HNO_3 and 1 µg/mL manganese in 2% HNO_3, dilute individual 1000 µg/mL standards with appropriate matrix solutions.

(e) *Molybdenum stock solution.*—1000 µg/mL. Dissolve 1.840 g ammonium molybdate, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Fisher Scientific, Pittsburgh, PA 15219, Cat. No. A674) in 100 mL 50% (v/v) ammonia solution with heating, cool to room temperature, and dilute to 1 L with water. For stock solutions containing 10 µg/mL molybdenum in 2.5% ammonia solution, dilute 1000 µg/mL standard with matrix solution.

(f) *Tin and aluminum stock solutions.*—1000 µg/mL, commercial preparations, tin (J.T. Baker, Inc., Phillipsburg, PA 08865, No. 1-6943) and aluminum (Mallinckrodt Corp., St. Louis, MO 63134, Cat. No. H500-500NY). For stock solutions containing 10 µg/mL tin in 5% (v/v) HCl acid, and 10 µg/mL aluminum in 5% HNO_3, individually dilute the 1000 µg/mL standards with appropriate matrix solutions.

(h) *Citric acid.*—20% (w/v) in water. Dissolve 20 g citric acid monohydrate, "Baker Analyzed" (J.T. Baker, Cat. No. 0110-1) in water, and dilute to 100 mL.

(h) *Palladium modifier.*—500 µg/mL. Heat 0.25 g palladium in 50 mL 50% (v/v) solution of HNO_3 and HCl. Dilute to 500 mL with water. Store in acid-washed polyethylene or polypropylene bottle.

Preparation of Standards

(a) *Contamination control.*—Calibrate 10, 20, 50, and 100 µL pipets or adjustable pipets (with plastic tips) to accurately deliver solutions by weighing water. Wash plastic pipet tips by first drawing up HNO_3, and then water. Prepare empty 15 mL polystyrene centrifuge tubes by checking for cracks. Using GFAAS, test 10 µg/mL copper stock standard for molybdenum and manganese contamination, 10 µg/mL molybdenum stock standard for copper and manganese contamination, and 1 µg/mL stock manganese standard for copper and molybdenum contamination.

(b) *Aluminum working standards.*—Containing 0, 20, 40, 60, 80, and 100 ng/mL. Add 6 mL 50% HNO_3 and 0.5 mL 20% citric acid to each of 6 centrifuge tubes. Pipet 0, 20, 40, 60, 80, and 100 µL 10 µg/mL aluminum stock standard into the respective tubes. Dilute to 10 mL mark with water.

(c) *Tin working standards.*—Containing 0, 50, 100, 150, and 200 ng/mL. Add 6 mL 50% HNO_3 and 0.5 mL 20% citric acid to each of 5 centrifuge tubes. Pipet 0, 50, 100, 150, and 200 µL 10 µg/mL tin stock standard into the respective tubes. Dilute to 10 mL with water.

(d) *Combined copper, manganese, and molybdenum working standards.*—Containing 0, 50, 100, 150, and 200 ng/mL copper and molybdenum, and 0, 5, 10, 15, and 20 ng/mL manganese. Add 6 mL 50% HNO_3 and 0.5 mL 20% citric acid to each of 5 centrifuge tubes. Pipet 0, 50, 100, 150, and 200 µL of each of the 10 µg/mL copper, 1 µg/mL manganese, and 10 µg/mL molybdenum stock standards. Dilute to 10 mL with water.

Sample Collection and Storage

Canadian infant formulas and evap. milks were collected in 1987 by the Field Operations Directorate of Health and Welfare Canada (7), transferred to clean polystyrene centrifuge tubes, and stored frozen. One determination was made on each of 191 different lots of infant formulas and evap. milks.

Sample Digestion

Accurately weigh 2 g ready-to-use formula, 1 g concentrated liquid formula or evap. milk, or 0.3 g formula powder into centrifuge tubes. Add 1.7 mL water to any tubes containing formula powders and 2 mL water to the 3 blanks run with each

Table 1. Graphite-furnace atomic absorption spectrometric parameters for aluminum, copper, manganese, molybdenum, and tin

Parameter	Al	Cu	Mn	Mo	Sn
Aliquot volumes per atomization, μL:					
Blank (2% HNO_3)	2	2	2	1	2
Palladium modifier	2	—	—	—	2
Sample/standard	10	10	10	5[a]	10
Ashing temp, °C	1000	700	700	1000	1000
Ashing time, s	10	10	10	10	10
Ashing ramp, s	10	1	1	10	10
Cool-down temp, °C	40	—	—	—	40
Cool-down time, s	15.0	—	—	—	15.0
Atomization temp, °C	2600	2100	2100	2650	2600
Ramp time, s	1.5	1.1	1.1	1	1.5
Hold time, s	4.5	3.5	3.5	4.0	3.5
Gas, L/min	0	0	0	0	0
Cleanout temp, °C	2700	2600	2600	2850	2700
Hold time, s	1	1	1	3	1
Gas, L/min	3	3	3	3	3
Tube (1-partitioned, 2-plateau)	2	1	1	1	2
L'vov platform	yes	no	no	no	yes
Wavelength, nm	309.3	327.4	279.5	313.3	235.5
Slit width, mm	0.5	0.5	0.5	0.5	0.5

[a] Because of the low molybdenum concentrations in formulas and milks, a more appropriate sample aliquot volume would be 10 or 15 μL.

series of samples. Into each tube, pipet 0.5 mL 20% citric acid and 3 mL HNO_3. Cap tubes snugly, and heat in water bath at 55°C for 6 h or overnight. Cool to room temperature, and wipe water from tube, moving downward from the cap, using a clean cellulose wipe. Wearing protective polyethylene gloves containing no talc, cover cap with lint-free cellulose wipe and release pressure. Wipe tube of any droplets near the cap, and carefully remove cap without touching the edge of the tube or the cap. Place cap, top down, on a clean surface. Dilute contents to 10 mL mark with water, cap, shake, and let stand until analysis.

Quality Control Samples

(a) *Sample spikes.*—Containing 500 ng aluminum, 500 ng copper, 50 ng manganese, 500 ng molybdenum, and 500 ng tin. Choose a typical sample for the run, and weigh portions into 8 tubes. Spike 2 tubes with 50 μL 10 μg/mL aluminum stock solution, 2 tubes with 50 μL 10 μg/mL tin stock solution, and 2 tubes with 50 μL each of 10 μg/mL molybdenum, 10 μg/mL copper, and 1 μg/mL manganese stock solutions. The remaining 2 tubes contain the unspiked sample.

(b) *Reference sample.*—Choose a typical sample for the survey, and accurately weigh aliquots into a sufficient number of tubes so that 2 tubes are included with each analytical series. Include extra tubes for samples requiring repeat analyses. Analyze 2 tubes with each analytical series and refrigerate remaining tubes.

Instrumental Determination

Set up spectrometer and furnace according to manufacturer's instructions, using spectrometric parameters in Table 1. Different furnaces may require different conditions, and some specific comments are offered to allow for this.

The aliquot volumes indicated in Table 1 may be adjusted; however, the appropriateness of any chosen aliquot volume can be evaluated by running standards and samples using the volume chosen and comparing it with a smaller volume on the same group of samples and standards. If the resulting sample concentrations differ for the 2 aliquot volumes, additional investigation of conditions is required. Also, as a general guideline, if the precision or sensitivity of standards or samples changes with changes in the drying temperature, then the aliquot volume is too large.

Of the 5 elements, aluminum and tin are subject to the most interferences, and 2 measures are taken to minimize this potential problem. First, 1 μg palladium (2 μL 500 μg/mL palladium matrix modifier solution) is added to the furnace with the sample. This necessitates a cleanout step after atomization to avoid gradual accumulation of palladium in the graphite tube. Second, it is important to use a L'vov platform and stabilized-temperature platform atomization (STPF) conditions. This involves a cool-down step (40°C for 15 s) before atomization, an atomization ramp time that is as fast as the furnace power supply can provide, a somewhat stable temperature during the full

atomization period, and integrated absorbance measurements for as long as a positive absorbance is observed.

Integrated absorbance measurements are also made for copper, manganese, and molybdenum; however, a L'vov platform is not used because these elements are less susceptible to interferences and because the longer atomization period required for platform atomization leads to more rapid tube deterioration. For molybdenum, the integration period covered only about 80% of the total peak because of rapid tube deterioration if the atomization time was extended further. The cleanout procedures following molybdenum atomization should be carefully evaluated to eliminate the possibility of carryover from one atomization to the next.

For some models of commercial atomizers, 2 or 3 (6 s) cleanout steps (with argon flow on) at 2800°C may be needed to effectively remove residual molybdenum.

Arrange routine analytical sequence to determine standards first, followed by 3 reagent sample blanks, 2 reference samples, 2 duplicates of a sample characteristic for the run, 2 duplicates of the same sample spiked, 20 other samples (1 analysis each), and the standards again. If use of conventional automation of the available instrumentation is more convenient, recalibrate on all standards after every 5–15 sample or blank solutions, depending on the accuracy desired and the expected sensitivity change of the element during the run. In this case, the 3 sample blanks should be evenly spread during the run: one at the beginning of the analysis, one midway through the analysis, and one at the end (see *Results and Discussion*).

Pour fresh solutions into the autosampler cups, including the appropriate standards and spikes 3 times: once for aluminum, once for tin, and once for the remaining 3 elements.

Calculations

Our own computer program was used to perform calculations automatically. Steps followed by the program are outlined below.

Calculate the detection limit as 3 times the pooled standard deviation of the standard deviations of the 2 sets of blanks (standards and samples). Ignore baseline drift, which would affect the standard deviation of the standard blank run before and after the samples. The above-mentioned detection limit, expressed in nanograms absolute, is characteristic of the analytical run. For each individual sample, the detection limit is expressed as the above-mentioned value divided by the actual sample weight.

Assess baseline drift using absorbances of the standard blank run before and after the samples. If baseline drift is greater than 3% of the highest standard absorbance and, from observation of the strip-chart recording, the base line drifted uniformly, linearly adjust all absorbances to compensate for the drift.

Calibrate on the first group of standards, and calculate the blank-corrected nonzero concentrations of the samples and the second standard group. If all standard concentrations in the second group are higher or lower than the nominal concentrations, linearly adjust, beginning with the first solution run following the first standard group, all concentrations for the percentage change in sensitivity. If the sensitivity change is not unidirectional for all standards, calibrate on the average absorbances of the 2 sets of standards.

Alternatively, calculate concentrations using the automation available on commercial GFAAS systems.

Statistical Treatment of Results

Elemental concentrations in formulas and evap. milks that were less than the detection limit of the methodology are reported as being at the detection limit.

When multiple analytical series are run, evaluate the concentrations obtained for the reference sample. If the concentration in one or more of the series is a statistical outlier, reanalyze all samples in the series.

Contribution of Formulas to Dietary Intake by Infants

Formula intakes from a Nutrition Canada 24 h recall survey were used to estimate contribution of the formulas and evap. milks to the dietary intake of the infants (7). These intakes were 423 g/day (106 g/kg/day) for infants 0–1 month old and 637 g/day (110 g/kg/day) for infants 1–3 months old. Estimates assume that the only source of the element is the formula or milk fed to the infant.

Results and Discussion

Method Development

Methodology is a modification of that developed for aluminum in formulas and evap. milks (7). The major difference was the addition of citric acid before digestion to prevent precipitation of stannic acid in the absence of organic matter (reagent blanks). Improvement in recovery through use of citric acid was about 10% using the polystyrene centrifuge tubes and about 60% using glassware.

The choice of palladium as a matrix modifier for aluminum and tin was based on its previous application, in combination with ammonium citrate, to tin (5). Its suitability as a modifier for aluminum was confirmed in this study by use of the HGA-400 atomizer. Four modifier combinations, with and without citric acid, were tested: (*1*) no modifier, (*2*) 8% hydrogen in the inert gas, (*3*) 1 μg palladium, and (*4*) 1 μg palladium with 8% hydrogen in the inert gas. The palladium modifier with 1% citric acid present and no hydrogen in the inert gas gave best peak shape and peak height agreement between the standard and sample (Figure 1). (Peak heights and shapes are more sensitive indicators of the potential for interferences than peak areas.) Palladium with hydrogen, which has been proposed as a modifier (8), gave poorer agreement between samples and standards.

In multielement methods, the use of multielement standards should be avoided, unless each stock standard is checked for traces of each of the other elements. In this work, aluminum was a contaminant in all stock standards of the other elements; therefore, separate aluminum standards were prepared. The presence of tin contamination in the copper stock standard required the preparation of separate tin working standards.

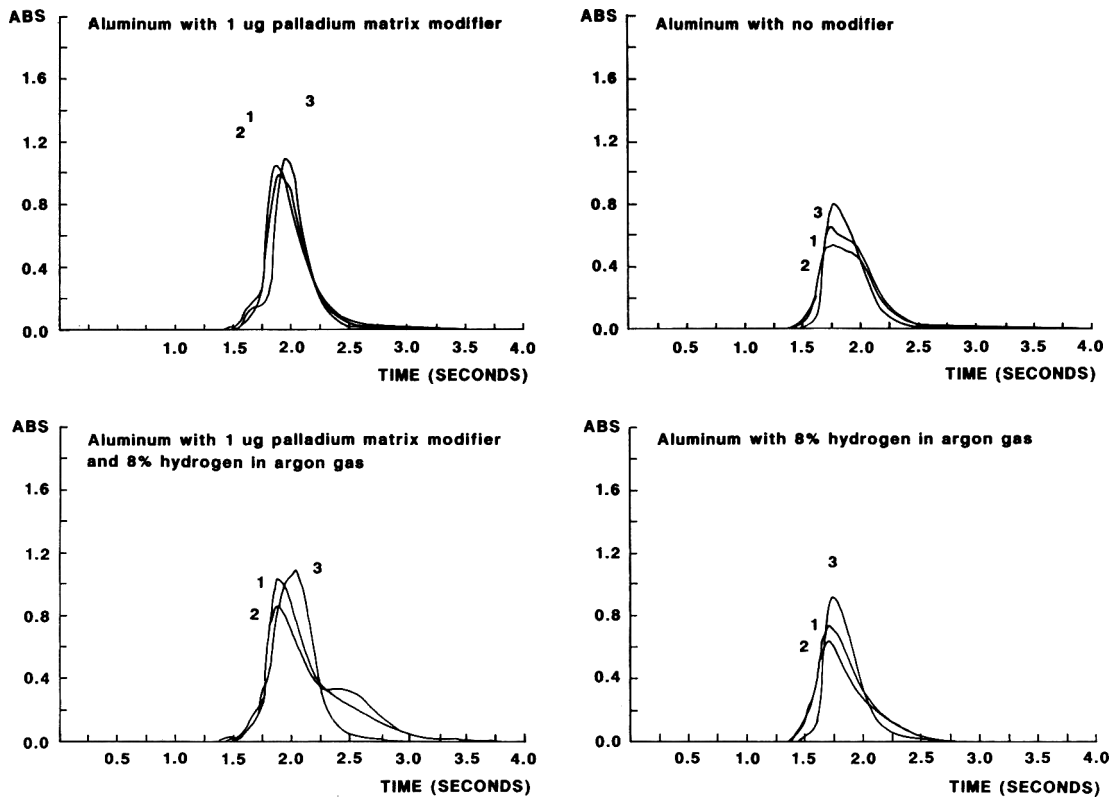

Figure 1. Effect of different modifier combinations on aluminum absorbance signals for standard with (*1*) 1% citric acid, (*2*) standard with no citric acid, and (*3*) infant formula digested according to the method (citric acid added before digestion).

The technique of using microliter pipets to prepare working standards is proposed to minimize the need for standard blank compensation. In routine analytical practice, as exemplified in reputable analytical manuals (9), it is usually assumed that the stock standards used to prepare working standards have a negligible blank, and no separate blank for each stock standard is needed. This assumption cannot be made for aluminum. For example, the aluminum blank for the working standards was occasionally as high as 10 or 20 ng/mL, and the same or greater magnitude of blank can be expected for the stock standards, because Pyrex-type pipets were used for their preparation. Had an aluminum stock standard of 1 μg/mL been used to prepare the working standards, a 1–2% bias in standard concentrations would have resulted. This bias can be overcome either by preparing a separate standard blank and adding it to each of the working standards or by ensuring that the stock standard blank is negligible when compared with the concentration of the stock standard. In the case of aluminum, this can only be ignored when the stock standard is 10 μg/mL or greater.

The method is not amenable to fully automated multielement instrumental determinations for several reasons. Separate standards are needed for some of the elements. Tin and aluminum are determined with a platform in plateau-type tubes, whereas the others are determined by use of partitioned tubes without a platform. Furthermore, despite the cleanout step, palladium traces remaining in the graphite tube after aluminum and tin determinations severely depressed the analytical signal for the other elements and necessitated the insertion of a palladium-free tube. Finally, the high atomization temperature needed for molybdenum would cause rapid deterioration of the graphite tube if a platform were present.

For routine GFAAS determinations, periodic standard sensitivity changes and changes in base line are common. Molybdenum sensitivity gradually decreased during the analytical run for this method. This appeared to be caused by the rigorous atomization conditions and gradual enlargement of the injection hole of the graphite tube. Sophisticated instruments deal with problems of this nature automatically by recalibrating after running a specified number of samples. The problem with this approach is that calibration is based on the standards run before samples, and those samples at the end of the run will have the greatest error associated with them. Our own system assumes linear drift during the analytical run and deals with it by running standards both before and after samples and by incrementally adjusting the sample concentrations for standard and baseline drift. Table 2 illustrates the effects of adjustment on the calculated concentrations of molybdenum standards run before and after samples. The sensitivities of the first group of

Table 2. Effect of sensitivity and baseline adjustments on calculated concentrations (ng/mL) of molybdenum standards run before and after samples

Nominal concn	Uncorrected (averaged)[a]	Baseline drift corrected[b]	Sensitivity corrected[c]	Both[d]
Before samples				
0	4.1	1.4	0.0	0.0
25	29.7	27.2	25.0	25.0
50	59.4	56.8	50.0	50.0
100	118.7	115.7	100.0	100.0
150	171.3	168.0	150.0	150.0
After samples				
0	−3.2	−0.4	−3.1	−0.3
25	20.9	23.6	25.1	26.8
50	41.7	44.2	49.6	49.4
100	85.9	88.5	101.4	98.0
150	131.3	134.3	155.1	148.8

[a] Normal calibration averaging absorbances for standards run before and after samples.
[b] Calibration as in footnote a after incrementally adjusting all absorbances for a shift in base line as determined by the reagent blanks for the standards.
[c] Calibration on standards run before samples, calculation of concentrations of second standards based on this calibration, and then incremental adjustment of all concentrations to correct for a decrease in standard sensitivity.
[d] Incremental adjustment of absorbances for a shift in base line followed by calibration and standard sensitivity adjustment, as in footnote *c*.

Table 3. Accuracy of GFAAS determination based on standard reference materials

Material	Element	*N*[a]	Certified, μg/g	Found, μg/g
Wheat flour (NBS 1567)	Cu	4	2.0 ± 0.3	2.0 ± 0.1
	Mn	4	8.5 ± 0.5	8.45 ± 0.09
	Mo	4	(0.4)[b]	0.25 ± 0.09
Milk powder (NBS 1549)	Al	4	(2)[b]	0.26 ± 0.07
	Cu	5	0.7 ± 0.1	0.56 ± 0.04
	Mn	5	0.26 ± 0.06	0.33 ± 0.03
	Mo	5	(0.34)[b]	0.41 ± 0.12
	Sn	4	(<0.5)[b]	<0.02
Rice flour (NBS 1568)	Cu	5	2.20 ± 0.3	2.17 ± 0.07
	Mn	5	20.1 ± 0.4	20.2 ± 0.20
	Mo	5	(1.6)[b]	1.43 ± 0.07

[a] No. of samples.
[b] Uncertified levels.

Table 4. Analytical figures of merit over 10 batches of samples run under routine analytical conditions

Parameter	Al	Cu	Mn	Mo	Sn
Mean (range) for laboratory reference material, μg/g	0.102 (0.077–0.121)	1.20 (1.08–1.28)	0.224 (0.18–0.26)	0.072 (0.025–0.13)	0.255 (0.19–0.31)
Repeatability SD, μg/g	0.0063	0.030	0.016	0.076	0.014
Repeatability CV, %	6.2	2.5	6.9	106.0	5.4
Day-to-day reproducibility SD, μg/g	0.013	0.048	0.021	0.032	0.036
Day-to-day reproducibility CV, %	12.6	3.9	9.4	44.2	14.2
Mean (range) detection limit, μg absolute	0.021 (0.006–0.057)	0.043 (0.012–0.115)	0.006 (0.001–0.016)	0.063 (0.005–0.17)	0.073 (0.018–0.26)
Mean (range) sample spike rec., %	104 (80–135)	98 (93–103)	103 (86–133)	114 (97–124)	96 (75–112)

Table 5. Levels (μg/g) of elements in infant formulas and evap. milks

	Aluminum				Copper			
Sample	N[a]	Mean	Median	Range	N[a]	Mean	Median	Range
Formula								
Ready-to-use	51	0.60	0.23	<0.011–5.2	52	0.69	0.68	0.44–1.14
Milk-based	36	0.18	0.13	<0.011–0.66	36	0.61	0.58	0.44–0.93
Soy-based	15	1.62	0.54	0.23–5.2	16	0.87	0.88	0.71–1.14
Concd liquid	53	0.60	0.39	0.042–2.24	53	1.26	1.26	0.72–1.8
Milk-based	37	0.27	0.23	0.042–0.71	37	1.12	1.14	0.72–1.4
Soy-based	16	1.36	1.47	0.70–2.24	16	1.59	1.62	1.27–1.8
Powdered	64	3.02	1.48	<0.10–8.5	64	4.1	3.9	0.031–8.2
Milk-based	36	1.37	0.77	<0.10–5.5	36	3.9	3.8	0.031–6.6
Soy-based	28	5.15	5.40	0.13–8.5	28	4.3	4.5	0.052–8.2
Evap. milk	22	0.08	0.051	<0.017–0.27	22	0.103	0.087	0.045–0.31

	Manganese				Molybdenum			
Sample	N[a]	Mean	Median	Range	N[a]	Mean	Median	Range
Formula								
Ready-to-use	52	0.26	0.095	0.042–2.4	52	0.044	<0.043	<0.012–0.14
Milk-based	36	0.097	0.094	0.053–0.16	36	0.033	<0.042	<0.012–0.067
Soy-based	16	0.63	0.22	0.042–2.4	16	0.068	<0.049	<0.048–0.14
Concd liquid	53	0.41	0.21	0.065–3.1	53	0.058	<0.046	<0.012–0.27
Milk-based	37	0.21	0.21	0.065–0.44	37	0.041	<0.045	<0.012–<0.06
Soy-based	16	0.87	0.37	0.096–3.1	16	0.097	<0.052	<0.024–0.27
Powdered	63	2.8	2.5	0.47–11.7	64	0.30	<0.32	<0.030–0.86
Milk-based	35	1.94	1.02	0.47–7.3	36	0.27	<0.31	<0.034–<0.51
Soy-based	28	3.9	3.0	1.3–11.7	28	0.34	<0.33	<0.030–0.86
Evap. milk	22	0.071	0.062	0.048–0.16	22	0.14	<0.17	<0.07–<0.19

	Tin			
Sample	N[a]	Mean	Median	Range
Formula				
Ready-to-use	52	0.22	0.17	0.067–0.91
Milk-based	36	0.23	0.18	0.067–0.91
Soy-based	16	0.20	0.15	<0.074–0.54
Concd liquid	53	0.47	0.26	<0.026–2.6
Milk-based	37	0.57	0.35	<0.026–2.6
Soy-based	16	0.25	0.13	<0.080–1.18
Powdered	64	0.33	<0.28	<0.03–<0.78
Milk-based	36	0.34	<0.28	<0.03–<0.76
Soy-based	28	0.33	<0.26	<0.19–<0.78
Evap. milk	21	2.84	0.46	0.032–48

[a] No. of samples.

Table 6. Comparison of current with previous aluminum results on the same samples

Sample	Current level, μg/g[a]		Previous level, μg/g[a]	
	Mean	Median	Mean	Median
Formula				
Ready-to-use				
Milk-based	0.18	0.13	0.13	0.091
Soy-based	1.62	0.54	1.98	0.84
Concd liquid				
Milk-based	0.27	0.23	0.22	0.18
Soy-based	1.36	1.47	1.41	1.21
Powdered				
Milk-based	1.37	0.77	0.72	0.50
Soy-based	5.1	5.4	9.4	8.3
Evap. milk	0.080	0.051	0.093	0.052

[a] Fewer samples were analyzed in this study than were analyzed previously (7), which contributes to some of the differences.

standards were about 30% higher than those of the last. Adjustment for sensitivity change greatly improved agreement with the nominal concentrations.

Method Performance

Accuracy of the method was confirmed by analysis of reference materials with certified levels of the elements (Table 3). Agreement with certified levels was excellent, except for copper in milk powder.

Analytical figures of merit for the method are presented in Table 4. That these figures were obtained under routine analytical conditions must be stressed, because better results can be obtained if only a few samples are analyzed in a run.

Detection limits were estimated as 3 times the pooled standard deviations for the standard blanks and sample blanks.

The reason for this approach was that during some of the runs, a change in base line, as represented by the standard blank run before and after the samples, occurred. Thus, it was possible for the standard deviation of the standard blank to be greater than the standard deviation of the sample blanks (true reagent blanks). Because the detection limit is a function of both sets of blanks, the standard deviations of both sets were used for its estimation.

A portion of the detection limit for aluminum was due to contamination from the unwashed disposable centrifuge tubes. This was measured only after completion of the study. In 24 h, 1 mL 10% HNO_3 in each tube dissolved 3.6 ±2.7 ng in Falcon 2095 polystyrene tubes, 24 ±1.4 ng in Corning 25310 polystyrene tubes, and 26 ±7.8 ng in Evergreen 222-2469-G80 polypropylene tubes (10 tubes of each type). The corresponding contributions to detection limits for the formulas and evap. milks, based on 2 mL ready-to-use formula, were 4.1, 2.1, and 11.7 ng/g.

Future Methodological Considerations

The analytical conditions chosen for molybdenum were inadequate for optimal determination. In retrospect, molybdenum standard concentrations should have been in the range of 0–50 ng/mL, and the aliquot volume pipetted into the furnace should be increased to a minimum of 10 μL.

Although this survey involved only the 5 elements discussed above, the same procedure can be applied to other elements in infant formulas and evap. milks. Specifically, preliminary investigations showed that lead in formulas or milks stored in lead-soldered cans can be determined using ammonium dihydrogen phosphate matrix modifier and STPF conditions. Lead levels in formula in lead-free cans are too low for accurate determination by this method. Nickel was not tested, but its level in some infant formulas is high enough to be determined. GFAAS conditions similar to those used for copper are probably appropriate.

Aluminum in Formulas and Milks

Table 5 shows means, medians, and ranges of aluminum and other elements found in the various infant formulas and evap. milks (as sold).

Except in powdered soy-based formulas, aluminum levels were in good agreement with those reported previously (7) for the same samples (Table 6). For example, of the ready-to-use formulas, the mean level in milk-based formulas (0.18 μg/g) was one-ninth of that found in the soy-based formulas (1.6 μg/g). Powdered soy-based formulas were lower in the present study. Repeat analyses of the samples, however, confirmed the lower levels, and reasons for this discrepancy were not found.

The contribution of the formulas and evap. milks to the dietary intakes of aluminum by infants 0–3 months old (Table 7) ranged from 4 to 179 μg/kg/day, depending on the age of the infant and the type of formula or milk fed to the infant. These are well below the Food and Agriculture Organization/World Health Organization (FAO/WHO) provisional tolerable weekly intake (PTWI) of 7 mg/kg (10).

A comparison of these aluminum levels and intakes with other published values has been made previously (7).

Table 7. Contribution of infant formulas or evap. milk only to the dietary intake of elements by infants 0–3 months old

	Intake of elements, μg/day									
	0–1 month olds					1–3 month olds				
Sample	Al	Cu	Mn	Mo	Sn	Al	Cu	Mn	Mo	Sn
Formula										
Ready-to-use										
Milk-based	76	257	41	14	100	114	387	62	21	150
Soy-based	686	369	266	29	84	1031	555	401	43	127
Concd liquid										
Milk-based	58	236	45	9	121	88	355	68	13	182
Soy-based	288	337	183	21	53	434	506	276	31	79
Powdered										
Milk-based	89	253	126	17	22	134	379	190	26	33
Soy-based	335	278	256	22	21	505	419	386	33	32
Evap. milk	17	22	15	29	600	26	33	23	44	903
	Intake of elements, μg/kg/day									
	0–1 month olds					1–3 month olds				
Sample	Al	Cu	Mn	Mo	Sn	Al	Cu	Mn	Mo	Sn
Formula										
Ready-to-use										
Milk-based	19	65	10	4	25	20	67	11	4	26
Soy-based	173	93	67	7	21	179	97	70	8	22
Concd liquid										
Milk-based	15	60	11	2	31	15	62	12	2	32
Soy-based	73	85	46	5	13	75	88	48	5	14
Powdered										
Milk-based	23	64	32	4	6	23	66	33	5	6
Soy-based	85	70	65	6	5	88	73	67	6	6
Evap. milk	4	6	4	7	152	4	6	4	8	157

Copper in Formulas and Milks

The average copper level in ready-to-use formulas was 0.69 μg/g, about 7 times the level of 0.103 μg/g in evap. milk (Table 5). Copper levels in the soy- and milk-based formulas were almost the same. These results are comparable with 0.46 μg/g copper found previously in human milk and 0.052 μg/g found in milk-based formulas without added copper (11).

The contribution of the formulas to the dietary intake of copper by infants varied according to formula type from 60 to 97 μg/kg/day (Table 7), a range that appears nutritionally adequate (12). The contribution of evap. milk to the copper intake by infants 0–3 months old would be about one-tenth the above value.

Manganese in Formulas and Milk

The average level of 0.26 μg/g in ready-to-use formulas (Table 5) is slightly lower than the 0.38 μg/g found previously in Canadian formulas (13). Average manganese levels were about 4–6 times higher in soy-based formulas than in milk-based ones. On an as-consumed basis, manganese levels in milk-based formulas were about 3 times higher than those in evap. milks, which contained an average of 0.036 μg/g as consumed. Literature values for manganese in cow's milk range from 0.016 to 0.190 μg/g (6).

Contributions of the formulas and milks to the dietary intakes of manganese by infants 0–3 months old ranged from 15 μg/day (4 μg/kg/day) for evap. milk to 401 μg/day (70 μg/kg/day) for soy-based ready-to-use formulas (Table 7).

This range agrees with a previous estimation of 30 μg/kg/day for infants 1–6 months old (14) but is higher than an estimate of 0.5–0.9 μg/kg/day for exclusively breast-fed infants 1–3 months old (12).

Molybdenum in Formulas and Milk

Molybdenum levels were generally low, with slightly higher levels in soy-based formulas compared with milk-based ones. Evap. milk contained 0.07 μg/g molybdenum on a ready-to-use basis. This falls within a literature range of 0.024 to 0.075 μg/g reported for cow's milk in different countries (6).

The contribution of the formulas and evap. milks to the dietary intake of molybdenum by infants 0–3 months old varied from 9 to 43 μg/day, depending on age and the type of formula fed (Table 7). For formula-fed infants, a dietary intake of 6 μg/kg/day was reported by von Stockhausen (14), a value that agreed with the range of 2–8 μg/kg/day found in this study.

Tin in Formulas and Milk

Tin levels were generally less than 1 μg/g on a ready-to-use basis, and there was no apparent difference between soy-based and milk-based formulas (Table 5). For evap. milks, a difference was observed between milks stored in soldered cans and milks stored in solder-free cans. In 8 lots of milks stored in solder-free cans, the average and median tin levels were 0.55 and 0.46 (range, 0.35–0.79) μg/g. In 12 lots of milk stored in cans sealed with tin–lead solder, the average and median tin levels were 15 and 0.94 (range, 0.47–64) μg/g, respectively. Of the evap. milk in soldered cans, those from one lot of one manufacturer averaged 56 (range, 48–64) μg/g, while those from the remaining 3 manufacturers contained low levels: mean, 1.06 (range, 0.47–2.0) μg/g.

The contribution of formulas to the dietary intake of tin was generally low: 5–32 μg/kg/day. The dietary intake of tin by infants could have reached 157 μg/kg/day if they had been fed evap. milk instead of infant formula. All of these intakes are well below the FAO/WHO PTWI of 14 mg/kg (2 mg/kg/day).

Additional investigations are required on evap. milks to establish whether the higher tin levels (56 μg/g) from one of the manufacturers have been reduced after conversion to solder-free cans, or whether the source of the tin was the can lining.

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CHEMICAL CONTAMINANTS MONITORING

Survey of Butyltin, Cyclohexyltin, and Phenyltin Compounds in Canadian Wines

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Butyltin, cyclohexyltin, and phenyltin compounds were extracted with 0.05% tropolone in pentane from enzymatically hydrolyzed wine samples. Methyl derivatives made by Grignard reaction were quantitated by gas chromatography (GC)/atomic absorption spectrometry (AAS). Wine samples contained <0.1–138 ng dibutyltin/mL. Lower levels of monobutyl- and tributyltin were also detected by GC/AAS. Poly(vinyl chloride) (PVC) liner material used in some commercial transport tanks contained extractable butyltins. Imported wines sampled from these containers and Canadian wines blended with wine imported in PVC-lined tanks had considerably higher butyltin levels than did wines shipped in stainless steel. Analysis by GC/mass spectrometry confirmed the presence of butyltins in wine and PVC liner samples and, in addition, detected monophenyl- and monocyclohexyltin compounds at levels below the GC/AAS method detection limits of 0.04–0.05 ng Sn/mL.

Organotin compounds have diverse uses, including wood preservation, poly(vinyl chloride) (PVC) plastic stabilization, catalysis, agricultural chemicals, and as antifouling agents in marine paint (1, 2). Global production of organotins has been estimated to be at least 35 000 metric tons (1).

Dialkyltin compounds such as dioctyl- or dibutyltin diisooctylthioglycolate, dioctyl- or dibutyltin maleate, and dioctyl- or dibutyltin dilaurate are used in PVC stabilization. Monoalkyltin compounds are blended with dialkyltins to produce a synergistic effect (2). Octyltin compounds are permitted in food-contact PVC (2) because of their low mammalian toxicity (3).

Tributyltin compounds are typically used in some antifoulant marine paint and wood preservation products (4). However, concerns over the effects on nontarget organisms, particularly molluscs (5, 6), have resulted in restrictions on the use of alkyltin-based marine paints in recent years.

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Agricultural application of organotins is primarily limited to the use of triphenyltin on vegetable crops as a fungicide (2). Tricyclohexyltin hydroxide (Plictran) was used for many years as an acaricide on fruit crops. In 1987, it was withdrawn from the market worldwide by the manufacturer after additional studies revealed teratogenic effects in test animals at dosage levels that precluded adequate margins of safety, particularly in the context of human occupational exposure (7). However, cyclohexyltin residues have been found recently in mollusc samples, a fact suggesting its continued presence in the food supply (8).

A recent study of Canadian and foreign beers and wines found butyltin levels exceeding 100 ppb in several Canadian wine samples, with most other Canadian and foreign wines containing much lower or undetectable levels. Very low levels of phenyl- and cyclohexyltin were also present (9). Possible sources of butyltin residues include contaminated irrigation water or use of nonfood-grade PVC products in storage, transportation, or production facilities.

Therefore, the purpose of this study was to determine the extent and levels of butyltin contaminants in Canadian wines and to examine possible source(s) of the contamination.

Experimental

Reagents and Standards

ACS reagent chemicals and distilled-in-glass grade solvents (Caledon Laboratories Ltd, Georgetown, ON, Canada) were used. Pepsin (1:10 000) was purchased from Sigma Chemical Co. (St. Louis, MO). Methylmagnesium chloride was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Tropolone was purchased from Fluka Chemical Corp. (Ronkonkoma, NY).

Butyltin trichloride ($BuSnCl_3$), purchased from Research Organic/Inorganic Chemical Corp. (Sun Valley, CA), was vacuum distilled; no other tin compounds were found in the distillate. Details concerning the commercial source, cleanup, or synthesis of all other alkyltin salts and methylated derivatives used in this study have been described (9, 10).

Instrumentation

Details of the gas chromatographic (GC)/atomic absorption spectrometric (AAS) system have been reported elsewhere

(10–12). GC conditions were changed as follows: glass column (1.8 m × 6 mm od, 2 mm id), packed with 3% 100/120-mesh OV-7 on Chromosorb W-HP (Chromatographic Specialties Ltd, Brockville, ON, Canada); carrier gas, helium, 20 mL/min; transfer line, 1.2 m × 0.25 mm id deactivated fused silica; transfer line temperature, 275°C; injector temperature, 225°C; temperature program, 75°C (0.5 min hold) to 130°C (no hold) at 25°C/min, then to 155°C (no hold) at 15°C/min, followed by a linear increase of 25°C/min to 310°C (0.5 min hold). AAS operating conditions were as follows: Hamamatsu tin hollow cathode lamp (Hamamatsu Photonics K.K., Hamamatsu, Japan); current, 15 mA; wavelength, 224.6 nm; band pass, 0.5 nm; quartz T-tube furnace temperature, 800°C; furnace support gas flow rates, H_2 No. 1 at 30 mL/min, H_2 No. 2 at 15 mL/min, air at 45 mL/min.

A VG Analytical 7070EQ mass spectrometer (EBQQ configuration) coupled to a Varian VISTA 6000 gas chromatograph was used for confirmation. The system (using the conventional magnetic sector only) was operated in the electron impact mode (70 eV) at a mass resolution of 1000. GC operating parameters were as follows: DB-5 capillary column (30 m × 0.25 mm id; J&W Scientific, Folsom, CA); carrier gas, He (18 psi); on-column injector program, 80°C (0.5 min hold) to 250°C at a linear increase of 80°C/min; column program, 70°C (1.5 min hold) followed by a linear increase of 5°C/min to 85°C (no hold) and then to 300°C at a linear increase of 15°C/min.

Selected monitored ion masses were *m/z* 205 and 207 for monobutyltrimethyltin ($BuMe_3Sn$), 205 and 207 for dibutyldimethyltin (Bu_2Me_2Sn), 247 and 249 for tributylmethyltin (Bu_3MeSn), 225 and 227 for monophenyltrimethyltin ($PhMe_3Sn$), 231 and 233 for monocyclohexyltrimethyltin ($CyMe_3Sn$), 314 and 316 for dicyclohexyldimethyltin (Cy_2Me_2Sn), 299 and 301 for tricyclohexylmethyltin (Cy_3MeSn), and 349 and 351 for triphenylmethyltin (Ph_3MeSn).

Sample Preparation

Wine samples from 3 provinces were purchased at the retail level by Health and Welfare Canada inspectors or obtained from provincial liquor control board warehouses. All samples were stored at room temperature until analysis. Champagne samples were cooled before being opened and were degassed by sonification.

The investigation of possible butyltin contamination from PVC products involved collection of wine samples that had been in contact with either PVC-lined or stainless-steel transport containers. Samples for this transport container study were obtained directly from wineries. PVC liner samples from the transport containers (used to import foreign wines for blending purposes) were also provided by one of the provincial liquor control boards. These samples were either cut into 1 mm × 1 mm sections (methylene chloride extraction) or as a larger (ca 25 mm × 10 mm) single section (xylene extraction).

Analytical Methods

Details of the wine extraction method appear elsewhere (9). Briefly, wine samples (30 mL) with 0.5 g ascorbic acid added were hydrolyzed enzymatically overnight at pH 2 with pepsin. The pH was then lowered to 1 with HCl, and 2 extractions were made with 0.05% tropolone in pentane. After the pooled organic extract was concentrated to 1 mL under a nitrogen stream, 1 mL tetrahydrofuran and 0.5 mL methylmagnesium chloride were added, and the sample was tumbled 10 min. Prechilled nitric acid (0.5M) was slowly added, followed by 0.9 mL isooctane. Samples were then tumbled briefly, centrifuged, and the aqueous layer discarded. An additional wash with 18 MΩ/cm deionized water followed. The organic layer was adjusted to 2.0 mL and then dried over anhydrous sodium sulfate. One modification was made to the procedure: The amount of ascorbic acid was lowered from 1.5 g to 0.5 g. Organotin levels in the wine samples were quantitated by comparison with external standards. Thirty wine samples were analyzed in duplicate to test reproducibility of the procedure. The method detection limits (Table 1) ranged from 0.04 to 0.05 ng Sn/mL sample.

Two extraction methods were used with the PVC liner samples: (*1*) 30 min extraction by rotary tumbling with 5 mL methylene chloride in screw-capped 10 mL centrifuge tubes and (*2*)

Table 1. Method detection limit (MDL)

Analyte	Mean $N_{p\text{-}p}$ (mV)[a]	N_{SD} (mV)[b]	Response factor[c]	LOD[d]	MDL[e]	
					ng Sn/mL	ng $R_xSn^{(4-x)+}$/mL[f]
$BuMe_3Sn$	0.01004	0.00364	318.4	6.7	0.04	0.1
Bu_2Me_2Sn	0.01004	0.00331	357.4	7.1	0.05	0.1
Bu_3MeSn	0.01059	0.00329	387.9	7.9	0.05	0.1
Cy_2Me_2Sn	0.00937	0.00324	364.2	7.0	0.05	0.1
Cy_3MeSn	0.01041	0.00245	441.0	7.8	0.05	0.2
Ph_3MeSn	0.01007	0.00276	403.2	7.4	0.05	0.1

[a] Mean peak-to-peak baseline noise of 20 measurements.
[b] Standard deviation of $N_{p\text{-}p}$.
[c] Inverse of slope from linear regression (pg Sn/mV).
[d] Limit of detection, (mean $N_{p\text{-}p}$ + $3N_{SD}$) × response factor (pg Sn).
[e] Method detection limit, (LOD/inj. vol.) × (extract vol./sample vol.) × 10^{-3}.
[f] R = butyl, cyclohexyl, or phenyl.

120 min extraction with boiling xylene heated in 150 mm × 20 mm test tubes. A subsample of each extract was derivatized with methylmagnesium chloride, as described previously.

Recovery Experiments

Samples of red and white wine were spiked at 2 levels (0.9–2.8 or 8.9–14.5 ng/mL) with a mixture containing tributyltin bromide, dibutyltin dibromide, butyltin trichloride, dicyclohexyltin dibromide, tricyclohexyltin bromide, and triphenyltin chloride before hydrolysis. The percent recovery of each compound was determined by dividing the mean peak area of the compound recovered from the spiked samples with the mean peak area of the compound in a blank hydrolysate extract spiked just before derivatization.

Results and Discussion

Extraction Methods

Recoveries of the 6 organotins from white and red wine averaged 92% (83–100%) and 93% (82–114%), respectively (Table 2), with the method reported in this paper. Coextractives from the wine samples had no effect on derivatization yields. The wine sample duplicates (Table 3) generally show very good agreement over a wide range of reported values.

The amount of ascorbic acid was modified, because preliminary work indicated that monobutyltin recoveries were improved by lowering the added quantity to 0.5 g. Earlier recovery studies (9) did not include monobutyltin, because a purified standard was not available at that time. Enzymatic hydrolysis remained necessary to prevent emulsion formation during extraction.

Because organotins are extractable from PVC products (13), 3 PVC liners were examined to determine the presence or absence of extractable alkyltins, using the outlined methylene chloride and xylene extraction procedures.

Wine Sample Analysis

A GC/AAS chromatogram of a sample containing mono-, di-, and tributyltin is shown in Figure 1. Dibutyltin was the predominant organotin compound present, with 23.2% of all tested wine samples containing 1.1–138.1 ng/mL (Table 3, Figure 2a). Monobutyltin was found less frequently, with 10.8% of the samples containing levels of 1.1–21.4 ng/mL. Tributyltin levels were much lower than either dibutyl- or monobutyltin, with levels of <0.1–1.9 ng/mL.

Butyltin contamination varied by province. Wines from province A had a low range of dibutyltin levels (Figure 2b), with none of the samples having levels exceeding 10 ng/mL (Table 3). Wines collected from province B (Figure 2c) had a greater percentage of occurrences (45.4%) in the 0.1–1.0 ng dibutyltin/mL range than samples from province A; one sample from province B contained 70.6 ng dibutyltin/mL. Wine samples from province C contained the highest levels of butyltins (Figure 2d), with values ranging up to 138.1 ng dibutyltin/mL.

GC/MS analysis of 20 wine samples confirmed the presence of monobutyltin as $BuMe_3Sn$ (*m/z* 207 $[M\text{-}CH_3]^+$), dibutyltin as Bu_2Me_2Sn (*m/z* 207 $[M\text{-}C_4H_9]^+$), and tributyltin as Bu_3MeSn (*m/z* 249 $[M\text{-}C_4H_9]^+$). GC/MS also detected monophenyltin as $PhMe_3Sn$ (*m/z* 227 $[M\text{-}CH_3]^+$) and monocyclohexyltin as $CyMe_3Sn$ (*m/z* 233 $[M\text{-}CH_3]^+$) at levels below the GC/AAS detection limits (Figure 3a).

Table 2. Mean recoveries of organotin compounds from wine

Analyte	Wine type	Spiking level, ng/mL	Mean rec., % ± SD[a]
$BuSnCl_3$	White	8.9	86 ± 2
		0.9	91 ± 8
	Red	8.9	82 ± 2
		0.9	82 ± 6
Bu_2SnBr_2	White	8.9	91 ± 1
		0.9	94 ± 11
	Red	8.9	94 ± 8
		0.9	100 ± 10
Bu_3SnBr	White	14.5	91 ± 3
		1.5	90 ± 4
	Red	14.5	95 ± 2
		1.5	114 ± 13
Cy_2SnBr_2	White	13.6	95 ± 2
		2.7	96 ± 5
	Red	13.6	91 ± 1
		2.7	98 ± 7
Cy_3SnBr	White	13.8	83 ± 5
		2.8	92 ± 8
	Red	13.8	85 ± 2
		2.8	89 ± 4
Ph_3SnCl	White	13.8	91 ± 1
		2.8	100 ± 5
	Red	13.8	91 ± 2
		2.8	99 ± 4

[a] $N = 5$.

Transport Container Study

Wines associated with stainless steel transport containers had butyltin levels ranging from <0.1 to 4.0 ng/mL. However, wines transported in PVC-lined containers had consistently higher butyltin levels (0.3–64 ng/mL) (Table 4), which suggests that the PVC transport tanks could be a butyltin source.

PVC Liner Analysis Results

Of the 3 PVC transport tank liner materials examined, 2 contained extractable butyltins (Table 5). Monobutyl-, dibutyl-, and tributyltin were found in the grey/black laminate. The grey/white laminate (Table 5) contained lower levels of dibutyl- and tributyltin (0.1–2.6 μg/g). No detectable levels of

Table 3. Alkyltin levels in wine

Province	Winery No.	Wine type	Analyte concn, ng/mL		
			$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
A	1	White	<0.1	<0.1	<0.1
A	1	White	<0.1	<0.1	<0.1
A	1	Champagne	<0.1	<0.1	<0.1
A	2	Sparkling	<0.1	<0.1	<0.1
A	3	Champagne	<0.1	<0.1	<0.1
A	3	White	<0.1	<0.1	<0.1
A	3	White	<0.1	<0.1	<0.1
A	3	Red	<0.1	<0.1	<0.1
A	3	Red	<0.1	<0.1	<0.1
A	4	Sparkling	<0.1	<0.1	<0.1
A	4	White	<0.1	<0.1	<0.1
A	4	Red	<0.1	<0.1	<0.1
A	4	Red	<0.1	<0.1	<0.1
A	4	White	<0.1	<0.1	<0.1
A	5	White	<0.1	<0.1	<0.1
A	5	Red	0.2	0.6	<0.1
A	6	White	<0.1	<0.1	<0.1
A	7	White	<0.1	<0.1	<0.1
A	7	Red	<0.1	<0.1	<0.1
A	7	White	<0.1	<0.1	<0.1
A	7	White	<0.1	<0.1	<0.1
A	8	Red	<0.1	<0.1	<0.1
A	8	White	<0.1	<0.1	<0.1
A	8	White	<0.1	<0.1	<0.1
A	9	White	0.2	<0.1	<0.1
A	9	White	<0.1	<0.1	<0.1
A	9	Red	0.1	<0.1	<0.1
A	9	White	<0.1	<0.1	<0.1
A	10	Red	0.6	4.1	<0.1
A	10	White	0.2	1.5	0.1
A	10	White	0.1	1.6	<0.1
A	10	White	0.2	1.4	<0.1
A	11	White	<0.1	0.1	<0.1
A	11	White	<0.1	<0.1	<0.1
A	11	White	<0.1	0.2	<0.1
A	11	Red	<0.1	<0.1	<0.1
A	12	White	<0.1	<0.1	<0.1
A	12	White	<0.1	<0.1	<0.1
A	12	White	<0.1	<0.1	<0.1
A	12	White	<0.1	<0.1	<0.1
A	13	Red	<0.1	<0.1	<0.1
A	13	Red	<0.1	<0.1	<0.1
A	13	White	<0.1	<0.1	<0.1
A	13	White	<0.1	<0.1	<0.1
A	14	Red	0.2	4.8	<0.1
A	15	Red	<0.1	<0.1	<0.1
A	15	Red	<0.1	<0.1	<0.1
A	15	White	<0.1	<0.1	<0.1
A	15	White	<0.1	<0.1	<0.1
A	16	White	<0.1	<0.1	<0.1
A	16	Red	<0.1	0.1	<0.1
A	16	Red	<0.1	<0.1	<0.1
A	16	White	<0.1	<0.1	<0.1
A	17	Red	<0.1	<0.1	<0.1

Table 3. ***Continued***

Province	Winery No.	Wine type	Analyte concn, ng/mL		
			$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
A	17	Red	<0.1	<0.1	<0.1
A	17	White	<0.1	<0.1	<0.1
A	17	White	<0.1	<0.1	<0.1
B	18	White	<0.1	<0.1	<0.1
B	18	White	<0.1	<0.1	<0.1
B	18	Red	<0.1	<0.1	<0.1
B	19	White	0.3	0.7	<0.1
B	19	Red	0.2	0.3	<0.1
B	19	Red	0.3	0.6	<0.1
B	19	White	8.3	70.6	1.0
B	19	Blush	<0.1	0.1	<0.1
B	20	White	<0.1	0.2	<0.1
B	20	White	<0.1	<0.1	<0.1
B	21	Red	<0.1	<0.1	<0.1
C	1	Champagne	<0.1	<0.1	<0.1
C	1	White	<0.1	<0.1	<0.1
C	1	Red	<0.1	<0.1	<0.1
C	1	Sparkling	<0.1	<0.1	<0.1
C	1	White	<0.1	<0.1	<0.1
C	2	Burgundy	<0.1	<0.1	<0.1
C	2	White	<0.1	<0.1	<0.1
C	2	Red	<0.1	<0.1	<0.1
C	2	White	<0.1	<0.1	<0.1
C	3	White	4.5	66.6	0.6
C	3	Red	0.3[a]	1.6[a]	<0.1
			(0.3–0.4)	(1.6–1.7)	—
C	3	Red	16.3	138.1	0.7
C	3	Champagne	<0.1	<0.1	<0.1
C	3	White	<0.1	0.1	<0.1
C	3	Red	2.4[a]	14.4[a]	0.1[a]
			(2.3–2.6)	(14.3–14.4)	(<0.1–0.1)
C	3	Red	15.0	84.5	0.5
C	3	White	<0.1	0.1	<0.1
C	3	White	1.3[a]	15.4[a]	0.3[a]
			(1.0–1.7)	(15.2–15.5)	(0.1–0.5)
C	3	White	5.2	56.0	0.6
C	3	White	<0.1	0.3	<0.1
C	3	White	0.1	0.3	<0.1
C	3	White	0.2[a]	0.2[a]	<0.1
			(0.2–0.3)	(0.1–0.2)	—
C	3	Red	18.7	108.1	0.7
C	3	White	<0.1	<0.1	<0.1
C	22	Champagne	<0.1	<0.1	<0.1
C	23	Red	<0.1	0.5[a]	<0.1
			—	(0.5–0.5)	—
C	23	White	<0.1	0.7[a]	<0.1
			—	(0.6–0.7)	—
C	23	Red	<0.1[a]	0.9[a]	<0.1
			(<0.1–0.1)	(0.8–1.0)	—
C	23	White	0.1[a]	1.2[a]	<0.1
			(0.1–0.2)	(1.2–1.2)	—
C	24	Red	<0.1	0.3[a]	<0.1
			—	(0.3–0.3)	—

Table 3. ***Continued***

Province	Winery No.	Wine type	Analyte concn, ng/mL		
			$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
C	24	White	0.2[a]	0.3[a]	<0.1
			(0.2–0.2)	(0.3–0.3)	—
C	24	Sparkling	<0.1	0.3[a]	—
			—	(0.3–0.3)	—
C	24	Red	4.4[a]	27.6[a]	0.1[a]
			(4.4–4.4)	(27.1–28.1)	(0.1–0.1)
C	24	White	<0.1	0.2[a]	<0.1
			—	(0.2–0.2)	—
C	25	Sparkling	<0.1	<0.1	<0.1
C	26	Sparkling	0.2	2.4[a]	<0.1
			—	(2.3–2.5)	—
C	26	Champagne	9.3[a]	107.6[a]	0.7[a]
			(8.4–10.2)	(101.9–113.3)	(0.7–0.7)
C	26	Red	3.6[a]	61.8[a]	0.7[a]
			(3.1–4.1)	(61.6–61.9)	(0.7–0.7)
C	26	White	<0.1	<0.1	<0.1
C	26	Red	<0.1	<0.1	<0.1
C	26	White	<0.1	0.7[a]	<0.1
			—	(0.7–0.7)	—
C	27	Champagne	<0.1	<0.1	<0.1
C	27	Red	<0.1	<0.1	<0.1
C	27	White	0.7	6.5	<0.1
C	27	White	<0.1	0.4	<0.1
C	28	White	<0.1	0.2[a]	<0.1
			—	(0.2–0.2)	—
C	28	Red	<0.1	<0.1	<0.1
C	29	Red	<0.1	<0.1	<0.1
C	29	Red	<0.1	<0.1	<0.1
C	29	White	<0.1	<0.1	<0.1
C	29	White	<0.1	<0.1	<0.1
C	30	Sparkling	0.5[a]	1.8[a]	<0.1
			(0.5–0.5)	(1.7–1.8)	—
C	30	Red	<0.1	0.4[a]	<0.1
			—	(0.4–0.4)	—
C	30	White	0.1	<0.1	<0.1
C	30	Red	<0.1	<0.1	<0.1
C	30	White	<0.1	<0.1	<0.1
C	31	White	0.8	4.8	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	Red	<0.1	<0.1	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	<0.1	0.1	<0.1
C	31	White	0.2	2.0	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	0.2	1.3	<0.1
C	31	White	<0.1	0.4	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	Red	<0.1	<0.1	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	0.2	<0.1	<0.1
C	31	White	0.1	0.8	<0.1
C	31	Red	<0.1	<0.1	<0.1
C	31	White	0.1	2.8	<0.1

Table 3. ***Continued***

Province	Winery No.	Wine type	Analyte concn, ng/mL		
			$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
C	31	White	<0.1	<0.1	<0.1
C	31	Red	0.1	0.3	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	<0.1	<0.1	<0.1
C	31	White	0.2	1.9	<0.1
C	31	White	<0.1	<0.1	<0.1
C	32	White	<0.1	0.2[a]	<0.1
			—	(0.2–0.2)	—
C	32	Red	15.2[a]	90.6[a]	0.8[a]
			(14.7–15.7)	(89.9–91.2)	(0.8–0.9)
C	32	White	<0.1	<0.1	<0.1
C	33	White	<0.1	<0.1	<0.1
C	33	Red	<0.1	<0.1	<0.1
C	33	White	<0.1	<0.1	<0.1
C	34	White	<0.1	<0.1	<0.1
C	34	Red	<0.1	<0.1	<0.1
C	34	Sparkling	<0.1	<0.1	<0.1
C	34	White	<0.1	<0.1	<0.1
C	34	Red	0.4	2.5	<0.1
C	34	Red	<0.1	<0.1	<0.1
C	35	White	6.8[a]	103.0[a]	1.0[a]
			(6.5–7.1)	(102.7–103.3)	(1.0–1.0)
C	35	Red	<0.1	<0.1	<0.1
C	35	Champagne	<0.1	0.1[a]	<0.1
			—	(0.1–0.1)	—
C	35	White	0.9[a]	11.5[a]	0.2[a]
			(0.8–0.9)	(10.9–12.1)	(0.1–0.2)
C	36	White	<0.1	0.2	<0.1
C	36	White	4.8	32.2	0.3
C	36	White	0.3	1.3	<0.1
C	36	White	0.3	5.9	<0.1
C	36	White	0.2	2.9	<0.1
C	36	White	2.1	20.2	0.1
C	36	Red	1.1	12.7	<0.1
C	36	White	3.5	37.9	0.5
C	36	Red	9.5	109.7	1.9
C	36	White	0.5	3.3	<0.1
C	36	White	1.0	6.0	<0.1
C	36	Red	0.2	1.8	<0.1
C	36	White	5.6	45.2	0.2
C	36	Red	21.4	121.2	1.3
C	37	White	<0.1	<0.1	<0.1
C	37	White	<0.1	<0.1	<0.1
C	38	Sparkling	<0.1	<0.1	<0.1
C	39	Red	<0.1	<0.1	<0.1
C	39	White	<0.1	0.5[a]	<0.1
			—	(0.5–0.5)	—
C	39	White	<0.1	0.5[a]	<0.1
			—	(0.5–0.6)	—
C	40	White	<0.1	<0.1	<0.1
C	40	White	<0.1	0.3	0.4

[a] Sample duplicates, mean value (range in parentheses).

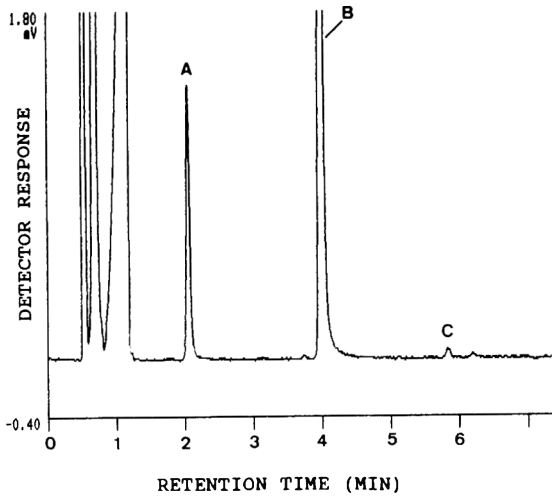

Figure 1. GC/AAS chromatogram of Canadian wine sample containing (A) $BuSn^{3+}$ (as $BuMe_3Sn$), (B) Bu_2Sn^{2+} (as Bu_2Me_2Sn), and (C) Bu_3Sn^{+} (as Bu_3MeSn).

butyltins were extracted from the yellow/white laminate (Table 5). Dibutyltin levels predominated (<0.1–202.9 μg/g), with lower amounts of monobutyltin (<0.1–79.3 μg/g) and tributyltin (<0.1–3.3 μg/g) also present. Xylene extracted all 3 butyltins, whereas methylene chloride appeared to extract more dibutyl- and tributyltin. (Table 5).

GC/MS analysis confirmed the presence of all 3 butyltins (Figure 3b). These values are qualitative only, because the recovery efficiency of the extraction methods was not determined. However, the PVC used in 2 of the tested liners (grey/white laminate and grey/black laminate) was obviously formulated with butyltin stabilizers.

Conclusions

Although the majority of wines tested contained low or undetectable levels of organotins, some products contained levels well above background. Canadian wines blended with imported wine (which had been transported in PVC-lined tanks) and imported wines sampled directly from PVC-lined tanks were found to have considerably higher butyltin levels than products transported in stainless steel tanks. Of 3 PVC liners tested, 2 contained extractable butyltins, indicating a butyltin stabilizer formulation. Therefore, the use of nonfood-grade PVC in transport tanks appears to have been the source of the butyltin contamination of wines bottled in Canada.

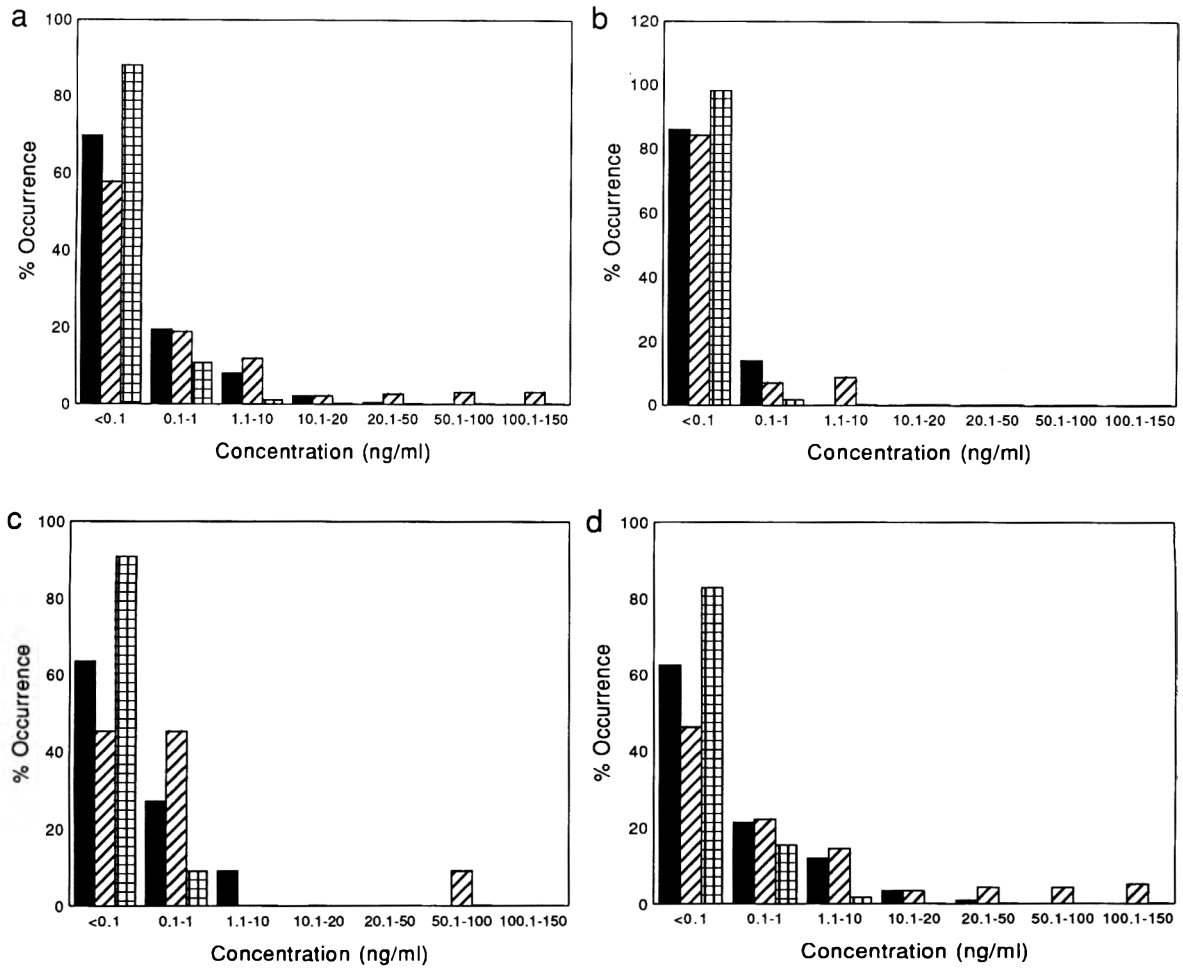

Figure 2. Frequency histogram of (■) monobutyltin, (▨) dibutyltin, and (▦) tributyltin levels in wines from (a) all tested provinces, (b) province A, (c) province B, and (d) province C.

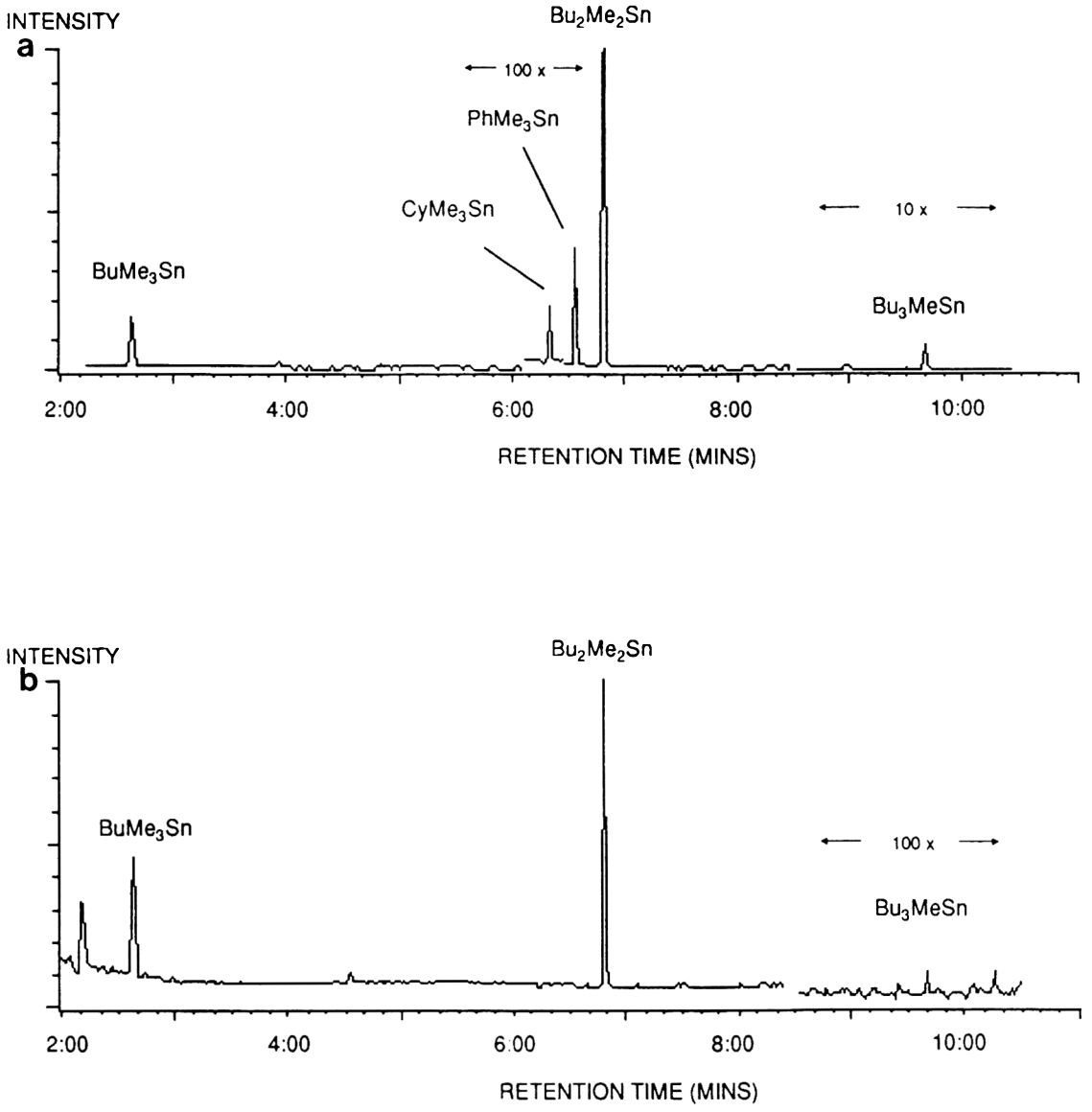

Figure 3. GC/MS confirmation of (a) wine and (b) PVC liner sample containing monobutyltin as $BuMe_3Sn$ (*m/z* 207 $[M\text{-}CH_3]^+$), dibutyltin as Bu_2Me_2Sn (*m/z* 207 $[M\text{-}C_4H_9]^+$), tributyltin as Bu_3MeSn (*m/z* 249 $[M\text{-}C_4H_9]^+$), monophenyltin as $PhMe_3Sn$ (*m/z* 227 $[M\text{-}CH_3]^+$), and monocyclohexyltin as $CyMe_3Sn$ (*m/z* 233 $[M\text{-}CH_3]^+$).

Acknowledgments

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Table 4. Butyltin levels in wines exposed to poly(vinyl chloride) (PVC) or stainless steel (SS) transport containers

Sample			Analyte concn, ng/mL		
Winery No.	Wine type	Container type	$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
3	White	SS	<0.1	0.4	<0.1
3	Red	SS	3.5	4.0	<0.1
3	Red	PVC	38.1	64.0	1.2
3	White	SS	<0.1	<0.1	<0.1
31	White	PVC	7.4	49.6	0.8
31	White	PVC	6.8	46.7	0.7
31	White	PVC	7.1	47.0	0.8
36	White	PVC	5.6	31.3	0.3

Table 5. Alkyltin levels in poly(vinyl chloride) (PVC) transport liners

		Analyte concn, µg/g		
Liner sample	Extraction method[a]	$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^{+}
Grey/white PVC laminate	1	ND[b]	2.6	0.1
	2	ND	0.8	ND
Yellow/white PVC laminate	1	ND	ND	ND
	2	ND	ND	ND
Grey/black nitrile–PVC laminate with PVF[c] barrier	1	ND	202.9	3.3
	2	79.3	145.9	2.5

[a] 1, methylene chloride, 30 min; 2, hot xylene, 120 min.
[b] Not detected.
[c] Poly(vinyl fluoride).

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DRUG RESIDUES IN ANIMAL TISSUES

Effect of Cold-Temperature Storage on Stability of Benzylpenicillin Residues in Plasma and Tissues of Food-Producing Animals

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A recently developed liquid chromatographic method is used to determine benzylpenicillin residues in incurred tissue and plasma samples from experimental animals stored at freezer temperatures of –20°C and –76°C. It was observed that penicillin-incurred plasma samples stored at –20°C for 2 months were relatively stable, showing a gradual loss of only 10–20% of the residues present at the beginning of the storage period. Loss of penicillin residues from bovine kidney and liver stored at –20°C occurred at a rate that was more rapid than from plasma. Losses in benzylpenicillin concentrations of up to 20% were observed in kidney and liver samples stored at –20°C after as little as 10 days of storage. Gluteal muscle samples stored at the same temperature showed almost 50% reduction in benzylpenicillin concentration after only 10 days of storage. However, incurred tissue samples stored at –76°C remained stable. The depletion of benzylpenicillin residues from edible tissues and biological fluids follows a first-order rate law.

Meat samples received for residue analysis in regulatory laboratories must be stored for various periods of time in a cooled state, before analyses. The choice of storage temperature, which has traditionally been –20° to –22°C, has usually been dictated by some knowledge of the effect of temperature on the stability of the analyte in question. Honikel et al. (1) studied the effect of storage temperature (–22°C) and processing on tetracycline residues in meat and bones and observed no losses in concentration of tetracyclines with cold storage. O'Brien et al. (2) investigated the effect of cooking and cold storage temperature (–20°C) on biologically active antibiotic residues in meat and found that chloramphenicol, oxytetracycline, streptomycin, and sulfadimidine showed no measurable losses in concentration with storage and cooking. In the same investigation, however, ampicillin concentrations decreased with storage and cooking. Recently, MacNeil et al. (3) also reported that they had observed no losses in the residue levels of oxytetracycline-incurred tissues stored at –20°C for 80 days.

In Canada, tissue samples collected for residue testing as part of the federal meat inspection program are frozen at the abattoir, packed with freezer bags in an insulated container, and shipped by air express to the Health of Animals Laboratory, Saskatoon, Saskatchewan. Such samples are normally received at the laboratory in a frozen or semithawed state, where they are catalogued and stored in a freezer at –20° to –22°C until analysis, usually within 24 h of sample receipt.

In a recent study conducted in our laboratory on the depletion of benzylpenicillin (penicillin G) residues from tissues of steers administered with extra-label doses of procaine penicillin G (unpublished data), we observed marked depletion in levels of the drug in penicillin-incurred tissues held in a walk-in cooler at ca 4°C for about 2 weeks after first being analyzed by liquid chromatography (4). Tissue samples stored in a –20°C freezer over the same period also were found to contain lower penicillin levels than were present initially.

In the literature, ampicillin is the only β-lactam antibiotic for which the effect of storage at –20°C on stability in tissue has been investigated (2). For penicillin G, DePaolis et al. (5) studied the decomposition products produced during the storage of penicillin G–incurred beef and chicken samples at –2°C, but there is no report in the literature on the effect of storage temperatures lower than –2°C on the stability of benzylpenicillin residues in edible tissues. Recently, Wiese and Martin (6) studied the degradation of penicillin G in fortified bovine plasma samples that were stored at room temperature, –20°C, and –70°C. They observed that degradation of penicillin G was so rapid that even plasma samples stored at –70°C must be analyzed within a month of storage to guarantee a sample with no more than a small percentage loss of the analyte.

Because meat samples received from federally inspected abattoirs in Canada and plasma samples from our own experimental animals are usually stored at –20° to –22°C prior to analysis, it was felt prudent to examine the effect, if any, that

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storage temperatures lower than −2°C might have on the stability of benzylpenicillin residues.

This paper describes the results of experimental work conducted to examine the effect of storage at −20°C for plasma, and −20°C and −76°C for tissues, on the stability of benzylpenicillin residues in food-producing animals.

Experimental

Apparatus

(a) *Filter papers.*—GF/B, 5.5 cm (Whatman Chemical Separations, Inc., Clifton, NJ 07014).

(b) *Funnel.*—5.8 cm Buchner.

(c) *Solid-phase extraction cartridges.*—6 mL (500 mg) BondElut C_{18} (Varian Sample Preparation Products, Harbor City, CA 90710) and 1 mL (100 mg) C_{18} Baker cartridges (John's Scientific Inc., Toronto, Ontario, Canada).

(d) *Polytron homogenizer.*—With 20 mm probe (Brinkmann Instruments, Ltd, Rexdale, Ontario, Canada).

(e) *Mechanical shaker.*—Eberbach flat bed.

(f) *Liquid chromatograph.*—Waters 501 pump, 712 autosampler, and Nova-Pak C_{18} column (4 μm; 3.9 mm × 150 mm), 994 photodiode array detector (Waters Chromatography Division, Mississauga, Ontario, Canada). Data acquisition, operation of pump, and injector were controlled by Waters 820 chromatography workstation. Mobile phase flow through column was set at 1 mL/min; analytes were detected at 325 nm at sensitivity setting of 0.005 AUFS.

(g) *Blender.*—Sunbeam, Oskar Jr. (Sunbeam Corp., Ltd, Toronto, Ontario, Canada).

Reagents

(a) *Ethacilin.*—300 000 IU/mL of procaine penicillin G (Rogar/STB Inc., London, Ontario, Canada).

(b) *Penicillin V potassium salt and penicillin G sodium salt.*—Sigma Chemical Co., St. Louis, MO 63178.

(c) *1,2,4-Triazole.*—Aldrich Chemical Co., Inc., Milwaukee, WI 53201.

(d) *Mercuric chloride.*—BDH, Toronto, Ontario, Canada.

(e) *Sodium tungstate.*—Fisher Scientific, Nepean, Ontario, Canada.

(f) *Sodium thiosulfate.*—Anachemia Science, Winnipeg, Manitoba, Canada.

(g) *Water.*—Barnstead RO/Nanopure ultrafiltration unit.

All other reagents were of reagent grade.

Preparation of Derivatizing Reagent, Mobile Phase, Standard, and Elution Solutions

Preparation of derivatizing reagent (2M 1,2,4-triazole containing 0.01M mercuric chloride), mobile phase (75% 0.1M phosphate buffer, pH = 6.5, containing 0.0157M thiosulfate, and 25% acetonitrile), and elution solution (60% acetonitrile, 5% 0.2M phosphate buffer, and 35% water) are all described in detail elsewhere (4). Stock standard solutions of 1000 μg/mL of each of penicillin G and penicillin V (internal standard) were prepared in water. These solutions were further diluted with water to prepare 5 μg/mL working standard solutions.

Animal Experiment

(a) *Storage temperature study on incurred tissues from slaughtered animals.*—Three healthy crossbred finished beef steers that had not been previously treated with any antibiotics were held at a University of Saskatchewan feedlot and fed ration of silage, ground hay, barley grain, mineral supplement, and water. Steers were injected intramuscularly with procaine penicillin G once daily for 5 consecutive days. One steer (No. 1), weighing 430 kg, was injected with a dose of 24 000 IU (36.4 mL) per kg body weight. Two other steers (No. 2 and 3), weighing 466 and 460 kg, respectively, were each injected with a dose of 66 000 IU (3 equal volume injections of 32.3 mL) per kg body weight. Animals were withdrawn from the drug for 2 days after the last injection and slaughtered at a local abbatoir. About 800–1000 g each of gluteal muscle (contralateral to injection site), kidney, and liver were collected from each animal and transported to the Health of Animals Laboratory. Tissue samples from each slaughtered animal were sorted into 9 groups, each containing ca 40 g of each of gluteal muscle, kidney, and liver. One group of tissues from each animal was analyzed immediately (zero storage). Five sets of tissues were stored in −20°C freezer, and the remaining set of 3 was stored in −76°C freezer until analysis.

(b) *Storage temperature study on plasma from live animal.*—A fourth steer (No. 4), weighing 460 kg, also with no known antibiotic treatment history and held at same feedlot as animals No. 1–3, was injected intramuscularly with a dose of 66 000 IU/kg body weight once daily for 5 consecutive days. On the fifth day, blood samples were collected into heparinized tubes from the left jugular vein at 0, 15, 30, 60, and 120 min following injection. Blood samples were centrifuged 10 min at 4500 *g*, and plasma was harvested, aliquoted into 5 mL portions, and stored at −20°C until being assayed.

Extraction and Cleanup of Penicillin G from Tissues and Plasma

About 40 g of each thawed tissue sample was homogenized in a blender, and 5.0 g samples were accurately weighed into 50 mL polypropylene centrifuge tubes. To each tube, 300 μL of 5 μg/mL penicillin V standard solution (internal standard) was added, and samples were extracted with water and a mixture of 0.17M sulfuric acid and 5% tungstate solution.

Frozen penicillin-incurred plasma samples were allowed to thaw at room temperature, and 2 mL samples were pipeted into 50 mL polypropylene centrifuge tubes. To this, 120 μL of 5 μg/mL penicillin V working standard solution was added, and penicillins in plasma were extracted with 30 mL water, 2 mL 0.17M sulfuric acid, and 2 mL 5% sodium tungstate solution.

Plasma and tissue extracts were loaded onto conditioned C_{18} cartridges (500 mg capacity for tissues and 100 mg capacity for plasma) and eluted with 1.0 mL and 0.5 mL elution solutions for tissue (4) and plasma samples (unpublished data), respectively. Equal volume of derivatizing reagent was added

Table 1. Effect of −20°C storage temperature on stability of penicillin G in bovine plasma

	Mean concn (± SD[a]) of penicillin G, ng/mL				
	Days of storage				
Sampling time, min	0	5	12	62	95
15	1091	1050 ± 105	995 ± 75	943 ± 47	841 ± 95
30	1221	1194 ± 74	1164 ± 180	1002 ± 88	903 ± 65
60	1357	1325 ± 91	1280 ± 108	1187 ± 140	993 ± 98
120	1313	1308 ± 110	1298 ± 68	1253 ± 34	1038 ± 77

[a] Three replicate analyses of plasma from steer No. 4 were conducted for each sample. Single determinations were conducted for each sample at zero time.

to each eluant and allowed to react in 65°C water bath for 30 min. Between 50 and 100 µL aliquots of filtered, derivatized samples were injected into the liquid chromatograph for analysis. Response ratios (UV response of penicillin G to UV response of penicillin V as internal standard) calculated from chromatograms of incurred plasma and tissues were used to calculate concentrations of penicillin G from calibration curve of response ratio vs concentration of penicillin G.

Results and Discussion

Table 1 shows the concentrations of penicillin G detected in bovine plasma collected from steer No. 4 at defined times after the injection on the fifth day, stored at −20°C, and analyzed periodically over a period of 95 days. According to these data, penicillin residues in plasma stored at −20°C for a period of 2 months remain relatively stable, showing a gradual loss of only 10–20% of the residue present at the beginning of the storage period. More substantial losses (>20%) of penicillin residues occurred with prolonged storage at −20°C, as noted with the findings for 95 days of storage.

In a previous experiment conducted at our laboratory (unpublished data) to define the minimum sample size of tissue (liver, kidney, and gluteal) to be taken from an incurred bulk animal tissue to provide a homogeneous representative sample (repeatable analytical data with CV ≤ 15%), we found that the coefficients of variation (CVs) obtained from the analysis of 8 individual 5 g samples subsampled from homogenized 40 g incurred tissues taken from various portions of the bulk tissue were ≤ 15%. Application of the Student's *t*-test to the calculated means of the 8 analytical results from each 40 g lot showed no statistically significant difference ($P = 0.05$) from the means of multiple analysis from other 40 g lots. Similar observations were made when sample sizes of 100 g were homogenized and eight 5-g samples were subsampled and analyzed by liquid chromatography. However, when sample sizes ≤20 g were sampled from the bulk material and subsampled for analysis, means from quadruplicate analyses were found to be statistically different from lot to lot, and CVs ranging from 20 to 60% were obtained. In our laboratory, therefore, we routinely use ca 40 g sample sizes from the bulk material for homogenization, before subsampling 5 g amounts for analysis. In addition, the 40 g sample lots were stored unblended, and were blended only

Table 2. Effect of −20°C storage temperature on stability of penicillin G residues in tissues from 3 steers[a]

		Mean concn ± SD, ng/g[b]			
		Days of storage			
Matrix	Steer No.	0	10	33	99
Liver	1	836 ± 7	700 ± 10	632 ± 50	404 ± 13
	2	1397 ± 208	1230 ± 30	1131 ± 76	730 ± 62
	3	2140 ± 263	1819 ± 64	1611 ± 44	1072 ± 131
Kidney	1	528 ± 60	448 ± 24	413 ± 31	328 ± 23
	2	374 ± 5	314 ± 26	269 ± 49	172 ± 23
	3	1182 ± 57	938 ± 75	879 ± 19	408 ± 18
Gluteal muscle	1	23 ± 1	13 ± 3	NA[c]	ND[d]
	2	29 ± 6	13 ± 2	NA	ND
	3	26 ± 1	11 ± 2	NA	ND

[a] Steers were injected intramuscularly with procaine penicillin G and slaughtered after 2 days withdrawal.
[b] Average of 3 analyses.
[c] NA—Samples were not analyzed.
[d] ND—Samples were analyzed but were found to contain penicillin G levels below the detection limit of 5 ng/g for the assay.

Table 3. Effect of −76°C storage temperature on stability of penicillin G residues in tissues from 3 steers[a]

Matrix	Steer No.	Mean concn ± SD, ng/g[b]		
		Days of storage		
		0	99	189
Liver	1	836 ± 7	799 ± 10	760 ± 40
	2	1397 ± 208	1290 ± 111	1205 ± 85
	3	2140 ± 263	2133 ± 38	2013 ± 136
Kidney	1	528 ± 60	501 ± 6	475 ± 25
	2	374 ± 5	355 ± 17	336 ± 15
	3	1182 ± 57	1123 ± 36	1061 ± 157
Gluteal muscle	1	23 ± 1	21 ± 5	22 ± 5
	2	29 ± 6	25 ± 4	19 ± 6
	3	26 ± 1	21 ± 5	23 ± 4

[a] Steers were injected intramuscularly with procaine penicillin G and slaughtered after 2 days withdrawal.
[b] Average of 3 analyses.

on the day of the analysis after they had been allowed to thaw at room temperature. This was done to prevent the suspected accelerated losses of penicillin residues (personal communication, Craig D. Salisbury, Gary Korsrud, and Valerie Martz, Agriculture Canada, Health of Animals Laboratory, Saskatoon, 1991) from animal tissues blended prior to storage.

Tables 1 and 2 show that penicillin residues in tissues stored at −20°C decreased more rapidly than penicillin residues in plasma. Gluteal muscles showed losses of nearly 50% of the initial residue present within 10 days of storage at −20°C. Losses of penicillin residues from kidney and liver were not as rapid as from gluteal muscle. Using the Student's *t*-test, the mean penicillin G concentrations of kidney samples No. 1, 2, and 3, and liver sample No. 1 were statistically different ($P = 0.05$) from the initial concentrations after only 10 days of storage at −20°C; however, liver samples No. 2 and No. 3, stored at the same temperature, did not show any significant difference in concentration until after 33 days of storage. With the exception of liver sample No. 1 (Table 3), all tissue samples stored at −76°C showed no statistically significant difference ($P = 0.05$) in concentration from the initially measured values, even after 189 days of storage.

The loss of penicillin G from plasma stored at −20°C followed a first-order rate law (7). The average rate constant and half-life calculated for the depletion of penicillin G from blood samples collected from steer No. 4 and stored at −20°C are presented in Table 4. A typical graph showing first-order kinetics for the depletion of penicillin G in plasma is shown in Figure 1. According to Wiese and Martin (6), plasma samples fortified with penicillin G at concentrations ranging from 0.1 to 100 μg/mL and stored at −20°C would have had losses up to 50% of their initial concentrations by 75 days after storage. In our study, the predicted half-life for penicillin G residues in plasma obtained from a steer treated with an extra-label dose of penicillin G and stored at −20°C was 276 days, a slower rate of degradation than predicted for fortified plasma samples by Wiese and Martin (6). The difference in these 2 results may be due to differences in the samples used for the experiments, i.e., fortified vs incurred, where factors such as binding constants of penicillin G to plasma albumin may be different for incurred and fortified samples. The rates of loss of penicillin residues from kidney and liver also followed a first-order reaction rate law. However, there were insufficient data points to reliably evaluate the kinetics of the depletion of penicillin G in gluteal muscle samples. Table 4 also shows a summary of the first-order kinetic parameters calculated for the depletion of penicillin G from plasma stored at −20°C, and livers and kidneys stored at −20°C and −76°C, using the data presented in Tables 1–3. The average rate of decrease in penicillin residues from plasma stored at −20°C is approximately one-half the rate

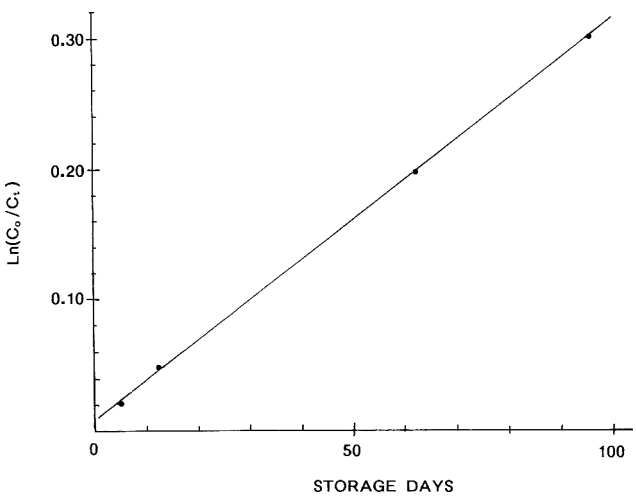

Figure 1. Plot of the logarithm of the initial concentration, C_0, to the concentration at a given time (t), C_t, of penicillin G in plasma against time, using the 30 min plasma data from steer No. 4 (Table 1). The first-order rate constant is obtained from the slope of the line.

Table 4. Average first-order kinetic values for degradation of penicillin G in penicillin-incurred plasma and tissue samples

Matrix	Storage temperature, °C	Mean ± SD rate constant, $days^{-1}$	Mean ± SD half-life, days
Plasma	−20	0.00256 ± 0.00040	276 ± 44
Liver	−20	0.00611 ± 0.00017	114 ± 3
	−76	0.000652 ± 0.000101	1080 ± 167
Kidney	−20	0.00670 ± 0.00321	123 ± 67
	−76	0.000611 ± 0.000019	1134 ± 37

in livers and kidneys stored at the same temperature. At these rates, it is predicted that it would take 276 ± 44, 114 ± 3, and 123 ± 67 days for penicillin G concentrations in plasma, liver, and kidney stored at −20°C, respectively, to depreciate by 50%. The average rates of decrease of penicillin G residues from liver and kidney samples stored at −76°C, however, were one-tenth the rates measured for the depletion at −20°C. At this rate of decrease, it would take about 2 years for the concentration of penicillin G residues in penicillin-incurred kidney and liver samples stored at −76°C to fall to 50% of their initial concentration. For laboratories contemplating the storage of penicillin-incurred samples for short periods before analysis, the kinetic data summarized in Table 4 predict that penicillin concentrations in liver and kidney samples stored at −20°C for 1, 5, and 10 days would have decreased to 99, 97, and 94%, respectively, of their concentrations at the time of storage.

Conclusions

The results of these experiments have demonstrated that significant losses of penicillin G residues in gluteal muscle stored at −20°C occur in as little as 10 days of storage. Such samples lose about 50% of their initial concentration of penicillin G after only 10 days of storage. Losses are less, but still significant, for penicillin-incurred plasma samples stored at −20°C for more than 2 months prior to analysis. However, penicillin G residues in tissue samples stored at −76°C are very stable. We, therefore, recommend that analytical laboratories receiving samples for penicillin analysis note the extent to which penicillin G decreases in tissues and biological fluids stored in conventional freezers and that they modify their analytical and sample storage protocols accordingly. Otherwise, quantitative analyses conducted on such stored samples will reflect residue concentrations at the time of sample analysis, but may not adequately reflect concentrations at time of sample submission.

Acknowledgments

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Confirmatory Method for Five Sulfonamides in Salmon Muscle Tissue by Matrix Solid-Phase Dispersion

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A reversed-phase (C_{18}) liquid chromatographic (LC) method was developed for the analysis of 5 sulfonamides (sulfadiazine [SDZ], sulfamerazine [SMRZ], sulfamethazine [SMTZ], sulfadimethoxine [SDMX], and sulfapyridine [SP]) in salmon muscle tissue. Spiked tissue samples were extracted by matrix solid-phase dispersion. The LC mobile phase gradient consisted of acetonitrile–aqueous 0.01M ammonium acetate (pH 5.5). The potentiators trimethoprim and ormetoprim were also resolved with this chromatographic system. The method detection limits at the 99% confidence level were 48, 66, 228, and 150 ppb for SP, SMRZ, SMTZ, and SDMX, respectively, and approximately 100 ppb for SDZ. The average percent recoveries of analytes from salmon muscle tissue were 66, 66, 71, 82, and 75% for SDZ, SP, SMRZ, SMTZ, and SDMX, respectively.

The use of sulfonamides for the control of fish diseases on fish farms has raised public concern for the need to monitor these compounds. For this reason, we previously developed a thin-layer chromatographic screening method for the 5 sulfonamides sulfadiazine (SDZ), sulfamerazine (SMRZ), sulfamethazine (SMTZ), sulfadimethoxine (SDMX), and sulfapyridine (SP) (1). The present paper describes a confirmatory method by liquid chromatography (LC).

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 600E pump, Model 990 photodiode array detector (250–290 nm monitored), WISP 712 autosampler (20 µL injection volume) (Waters Chromatography Div., Mississauga, ON), Supelcosil LC18-DB (5 µm) 25 cm × 4.6 mm id column (Supelco, Inc., Bellefonte, PA 16823), Waters 990 data-processing system (Waters Chromatography Div.).

(b) *Mortar and pestle.*—4 oz (118 mL) or 8 oz (237 mL), glass (Canlab, Vancouver, BC).

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(c) *Column.*—Brinkman cartridge, plastic column with screen mesh filter at bottom (Brinkman, Rexdale, ON).

(d) *Vacuum manifold.*—Absorbex SPU (BDH Chemicals, Vancouver, BC).

(e) *Centrifuge tube.*—Plastic, 15 mL (Sarstedt Canada Inc., St. Laurent, PQ); plastic, 1.5 mL (Canlab).

(f) *pH meter.*—Corning Glass Works, Science Products, Corning, NY 14831.

(g) *Centrifuge.*—Silencer H-103N (Western Scientific Services Ltd).

Reagents and Materials

(a) *Solvents and chemicals.*—All solvents were LC grade and obtained from BDH Chemicals. Sulfonamides and potentiators were obtained from Sigma Chemical Co. (St. Louis, MO 63178) and Burroughs Wellcome (Kirkland, PQ).

(b) *Sample dispersant.*—C_{18}-functionalized silica gel (40 µM) (Analytichem International, Inc., Harbor City, CA 90710). Before use, material was washed twice with hexane, dichloromethane, and methanol. After last methanol wash, material was partially dried so that it was "clumpy" but not dripping with methanol and not free-flowing powder. This material was placed in foil-covered beaker and used immediately.

(c) *Mobile phase.*—Acetonitrile–aqueous 0.01M ammonium acetate (pH 5.5). Percent acetonitrile was varied as follows (linear gradients): 18% at 0 min, to 30% at 24 min, to 80% at 26–55 min, to 18% at 57 min.

(d) *Standards.*—Stock (2000 ppm) solutions of SMTZ, SDMX, and SP were made in 50 mL methanol. Stock solutions (50 mL) of SDZ and SMRZ contained 3 drops of concentrated ammonia and 1 mL water, respectively, to aid dissolution. Spiking standard mixtures were prepared by diluting stock solutions with methanol.

(e) *Salmon.*—Farmed coho salmon were purchased at local market.

Sample Processing and LC Analysis

Salmon muscle tissue (0.50 ± 0.08 g) was placed into glass mortar. Spiking solution (10 µL) was placed on tissue (methanol on tissue blanks). One "scoop" (ca 5 mL, 2 g dry weight) methanol-damp C_{18} material was added. Sample was ground ca 1 min with pestle until homogeneous mixture was obtained.

Contents of mortar were transferred to Brinkman cartridge (10 mL plastic syringe barrel with filter paper disk has also been used [2]) and tightly compacted with glass rod (4 mm diameter).

Column was placed on vacuum manifold, washed with 8 mL 10% toluene in hexane (ca 1 drop/s; discard), and aspirated dry. Glass test tube, containing folded Whatman No. 1 filter paper (4.25 cm) inserted ca 2 cm below top of tube, was placed under column, and analytes were eluted with 8 mL dichloromethane (ca 1 drop/s). Filter paper caught any C_{18} material from column. (In-line syringe filters were found to clog when dichloromethane was added, presumably because of swelling of Teflon filter membrane.) Dichloromethane eluate was transferred to plastic centrifuge tube (15 mL), centrifuged 15 min at 3900 rpm, and decanted to another plastic centrifuge tube. This solution was evaporated to dryness under nitrogen in 40°C water bath. Wall of tube was washed down twice with 1 mL methanol; each wash was evaporated to dryness.

Methanol (200 µL) was added to sample residue. Tube contents were vortex-mixed 20 s, and 20 µL was injected into LC system.

Method Detection Limit

Method detection limits (MDLs) were determined from the following equation:

$$\text{MDL} = S\,(t_{0.99,\,N-1})$$

where $t_{0.99,N-1}$ = Student's t value appropriate for the number of observations and 99% confidence level, and S = standard deviation of the peak areas of a group of samples spiked at a recommended level of 1–5 times the method detection limit (3).

Eight salmon muscle tissue samples were spiked with the 5 sulfonamides at 400 ppb and analyzed along with 8 nonspiked (blank) samples. The analyte peak areas were corrected for any interference found in blank samples by subtracting the average (n = 8) peak area of the interference in blanks from the analyte peak areas in spiked samples.

For each analyte, standard deviations of the 8 interference-corrected peak areas were calculated, and this number served as the value of S in the above equation, where $t_{0.99,\,N-1} = 2.896$. The unit of the resulting value of MDL was peak area. This was converted to concentration (ppb) by using single-point calibration method.

Method Linear Range

Salmon muscle tissue samples were spiked with all analytes at 7 levels in duplicate: 60, 100, 200, 300, 400, 1000, and 5000 ppb. Linear regression parameters were calculated from the peak area results.

Results and Discussion

LC Analysis

Of the reported mobile phases examined (2, 4–9), acetonitrile–0.15M $NH_4H_2PO_4$, pH 4.85 (12.6 + 87.4) (6) gave the best resolution of the group of analytes under investigation.

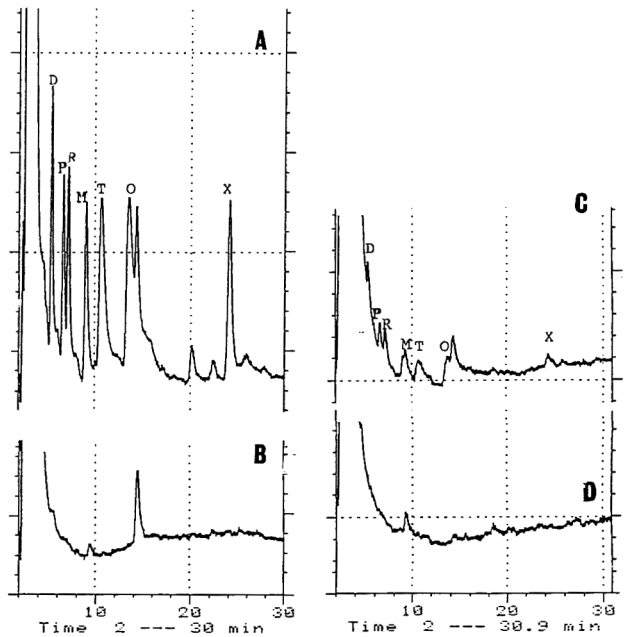

Figure 1. Liquid chromatograms of salmon muscle extracts: (A) spike level = 400 ppb, (B) spike level = 0 ppb (analyzed concurrently with "A"), (C) spike level = 60 ppb, and (D) spike level = 0 ppb (analyzed concurrently with "C"); peak labeling: SDZ, SMRZ, SMTZ, SP, TMP, OMP, SDMX; UV detector wavelength = 270 nm.

This mobile phase was further optimized by varying the percent acetonitrile and the pH of the aqueous phase. Further improvement was achieved by replacing monobasic ammonium phosphate with ammonium acetate. The optimum mobile phase composition was found to be acetonitrile–0.01M aqueous ammonium acetate, pH 5.5 (18 + 82). A mobile phase gradient was required for the analysis of tissue samples because of the presence of late-eluting endogenous compounds (see *Reagents and Materials*). LC resolution of the 5 sulfonamides is shown in Figure 1. The potentiators trimethoprim (TMP) and ormetoprim (OMP) were also separated with this LC system.

Method Detection Limit

The calculated method detection limits (99% confidence level) for SDZ, SP, SMRZ, and SDMX ranged from 33 to 150 ppb (Table 1). The method detection limit for SMTZ was 228 ppb. This relatively high value was, in part, due to the presence of a small interfering peak for this analyte (Figures 1a and b), the area of which was quite variable (%RSD = 25). The magnitude of this unidentified interference was similar to the SMTZ response at a spiking level of 60 ppb, as shown in Figures 1c and d.

These calculated method detection limits were checked by analyzing samples spiked with all analytes at 60 ppb. At this spiking level, all analytes were detected (Figure 1c), with the exception of SMTZ, which was obscured by the interfering peak noted previously (Figure 1d). SDZ was partially obscured by the solvent peak (Figure 1c). This interference rendered the calculated MDL of 33 ppb incorrect, and an MDL of 100 ppb was estimated for this analyte.

Table 1. Method parameters

Analyte	MDL, ppb[a]	RSD, %[b]	Av. Rec., %[c]	RSD, %[b]	Linear regression parameters		
					r^2	Slope	y-intercept
SDZ	~100[d]	2	66	3	0.990	1.46	-90.18
SP	48	4	66	1	0.986	1.13	-89.97
SMRZ	66	5	71	14	0.999	1.34	-36.40
SMTZ	228	19	82	6	0.999	1.27	33.89
SDMX	150	12	75	5	0.999	0.37	-224.34

[a] Method detection limit.
[b] $n = 8$.
[c] Over 3 spiking levels; data in Figure 2.
[d] Calculated value = 33 ppb.

The difference observed in the blank tissue samples (Figures 1b and 1d) may be because these samples were from different fish.

Intra-assay Reproducibility

The intra-assay variability of the method, as indicated by the relative standard deviation (RSD) of the peak areas of 8 replicate spiked (400 ppb) tissue samples, was generally less than 13% (Table 1). Excluding SMTZ, the average RSD was 7%.

Recovery

Recoveries of the analytes from salmon muscle were found not to be related to spiking level in the 400–5000 ppb range (Figure 2). Average recoveries ranged from 66 to 82% (Table 1). Low recoveries with a solvent extraction method have been reported (8) for SDMX (55%) in spiked salmon muscle tissue. Recoveries of the 5 sulfonamides from trout (a salmonid) muscle tissue were found to be 10–20% higher than from salmon tissue in one experiment. Lower recoveries from salmon may be related to its relatively higher cholesterol (8) and fat/oil contents (10).

It was important to process the tissue samples within 0.5 h of weighing to prevent reduced recoveries because of tissue dehydration (data not shown).

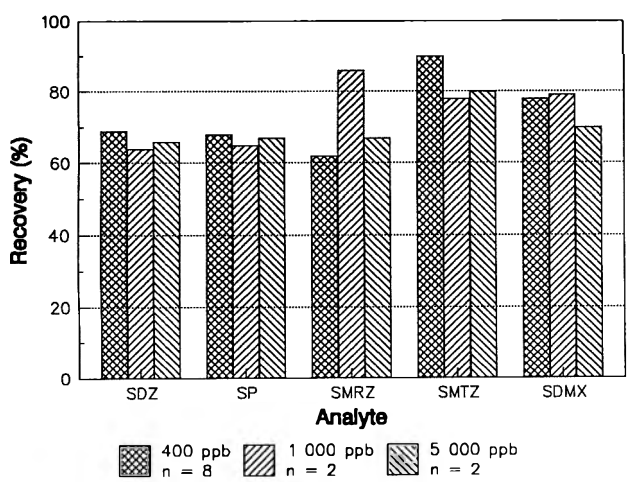

Figure 2. Average recoveries of sulfonamides from salmon muscle tissue spiked at 3 levels. Overall average recoveries are given in Table 1.

The above recoveries were not confirmed for samples containing "bioincurred" analytes.

Method Linear Range

Linear detector response was observed for all analytes in the 60–5000 ppb spiking range. Correlation coefficients (Table 1) ranged from 0.986 to 0.999 (average = 0.994).

Salmon Liver and Kidney Tissue

Preliminary investigation showed that the method may be applicable to salmon liver tissue but not to kidney tissue because of excessive tissue interferences (data not shown).

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Gas Chromatographic/Mass Spectrometric Determination of Oxolinic, Nalidixic, and Piromidic Acid in Fish

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A gas chromatographic/mass spectrometric (GC/MS) method using selected ion monitoring (SIM) mode has been developed for determination of oxolinic, nalidixic, and piromidic acid in fish. Drugs are extracted with ethyl acetate from a sample homogenized with phosphate buffer (pH 6.0). The extract is partitioned into 3% sodium bicarbonate solution, and the aqueous phase is re-extracted with ethyl acetate after neutralization. After evaporation, the residue is dissolved in methanol and reduced with sodium tetrahydroborate. The reaction mixture is treated with acid, extracted with ethyl acetate, and re-extracted with ethyl acetate after alkalinization. The combined ethyl acetate extract is passed through an alumina cartridge column, concentrated, and analyzed by GC/MS in SIM mode. Percent recoveries and coefficients of variation (CV%) from silver salmon fortified at the 0.1 ppm level were 92.2 (4.4) for oxolinic, 100.6 (12.8) for nalidixic, and 92.1 (3.8) for piromidic; at the 0.01 ppm level, they were 80.5 (6.2) for oxolinic, 81.5 (8.2) for nalidixic, and 69.6 (8.5) for piromidic. The detection limit is 0.003 ppm.

In recent years, fish for use as food in Japan have been artificially cultivated on a large scale in both fresh water and sea water, and drugs are used to prevent and to treat diseases of fish. Oxolinic acid (1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxo-3-quinolinecarboxylic acid) (Figure 1a), nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) (Figure 1b), and piromidic acid (8-ethyl-5,8-dihydro-5-oxo-2-[1-pyrrolidinyl]pyrido[2,3-d]pyrimidine-6-carboxylic acid) (Figure 1c) are antimicrobial agents that are widely used in the cultivation of fish such as salmon, rainbow trout, sweetfish, carp, eel, and yellowtail.

Drug residues in cultivated fish may endanger human safety. In Japan, the Food Safety Law established a zero residue level for all antimicrobial agents in foods in 1971. Hence, routine screening for oxolinic, nalidixic, and piromidic acid residues in cultivated fish is necessary, but the available methods of analysis are limited. Endo et al. used the microbial assay for oxolinic acid in animal tissues (1), but that method lacks sensitivity and specificity. Fluorometry (2) and thin-layer chromatography (3) were used to determine nalidixic acid. Liquid chromatographic (LC) methods were developed for the assay of pharmaceutical preparations, plasma, and urine samples (4–7) and, recently, for the analysis of residues in fish samples (8–11). The Japanese official method also uses an LC determination; however, its detection limit is not sufficient, and identification that relies solely on retention data is not specific enough to support regulatory action.

Gas chromatography/mass spectrometry (GC/MS) is the most reliable and sensitive technique for residue analysis. Coworkers and I have been developing GC/MS methods for drug residue analysis in which selected ion monitoring (SIM) method was used exclusively.

However, a limited number of GC methods for oxolinic, nalidixic, and piromidic acid were reported, and only nalidixic and piromidic acid in tablets and plasma were analyzed by GC after esterification (12–14). As I reported earlier (15), esters of oxolinic acid were very polar and not suitable for GC determination at residue levels; therefore, I developed a GC/MS SIM method for the determination of oxolinic acid residues in fish. In this method, oxolinic acid is reduced with sodium tetrahydroborate to give a compound (Figure 1d) that is sufficiently volatile to be suitable for GC. This reduction could be applied to nalidixic and piromidic acids with slight modification to give the products (e) and (f) in Figure 1. The compounds (d), (e), and (f) have good GC characteristics and give sharp, symmetrical peaks on gas chromatograms at moderate column temperatures. Therefore, I developed a new GC/MS method using these reduction products for the simultaneous determination of oxolinic, nalidixic, and piromidic acid in fish at residue levels. This method is sensitive and specific enough to support regulatory action; therefore, it is useful for daily screening and for identification of detected drug residues.

METHOD

Apparatus

(a) *Homogenizer.*—Biotron Model BT 10 20 350D (Biotrona Co. Ltd, Kussnacht, Switzerland).

(b) *Wrist-action shaker.*—Model 8-1-W (Yayoi Co. Ltd, Tokyo, Japan).

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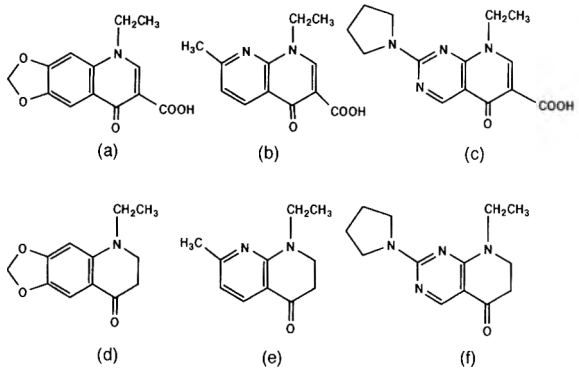

Figure 1. Structures of oxolinic (a), nalidixic (b), and piromidic (c) acids and reduction products of oxolinic (d), nalidixic (e), and piromidic (f) acids.

(**c**) *Rotary evaporator.*—Model N-1 (Tokyo Rika Kikai, Tokyo, Japan).

(**d**) *Pasteur pipet.*—Disposable, 7095B-9 (Corning Glass Works, New York, NY).

(**e**) *GC column.*—DB-5 fused silica open tubular column (15 m × 0.25 mm; film thickness, 0.25 μm) (J&W Scientific, Folsom, CA).

(**f**) *Gas chromatograph/mass spectrometer.*—Model 5890 Series II GC system with a split/splitless injector port, Model 5970B mass selective detector (quadrapole mass spectrometer). The system is operated with 59970 MS Chemistation computer software (Hewlett-Packard, Engelwood, CO). The capillary column (DB-5) is directly interfaced to the mass spectrometer ion source. Operating conditions: injection port temperature, 270°C; column temperature, initial at 100°C, hold for 2 min, program at 30°C/min to 190°C, and then at 20°C/min to 270°C, hold for 11 min; transfer line temperature, 270°C. Carrier gas (helium) column head pressure, 5 psi (flow, 0.8 mL/min); positive ion mode; ionization voltage, 70 eV (fixed); electron multiplier, 2400 V; SIM detection ions for oxolinic acid, *m/z* 219, 204, 176; for nalidixic acid, *m/z* 190, 176, 175; and for piromidic acid, *m/z* 246, 218, 217.

Reagents

(**a**) *Ethyl acetate, methanol, anhydrous sodium sulfate.*—Pesticide grade (Wako Chemicals, Osaka, Japan).

(**b**) *Sodium dihydrogen phosphate dihydrate, sodium bicarbonate, sodium tetrahydroborate, hydrochloric acid, sodium hydroxide.*—Reagent grade (Wako Chemicals).

(**c**) *Cartridge aluminum column.*—SepPak Alumina N (Waters Division of Millipore, Milford, MA).

(**d**) *Phosphate buffer (pH 6.0).*—Dissolve 31.2 g sodium dihydrogen phosphate dihydrate in 600 mL water, adjust to pH 6.0 by adding 1M sodium hydroxide solution (22–23 mL), and dilute to 1 L with water.

(**e**) *Saturated sodium bicarbonate solution.*—Add 40 g sodium bicarbonate to 500 mL water and mix well. Let stand until layers separate, and use supernatant solution.

(**f**) *Sodium bicarbonate solution, 3%.*—Dissolve 30 g sodium bicarbonate in 1 L water.

(**g**) *Hydrochloric acid, 5N.*—Add 417 mL hydrochloric acid to 500 mL water, mix well, and dilute to 1 L with water.

(**h**) *Hydrochloric acid, 0.1N.*—Dilute 2 mL 5N hydrochloric acid to 100 mL with water.

(**i**) *pH indicator paper.*—Type TC (Cat. No. 2611628) (Whatman International Ltd, Maidstone, UK).

(**j**) *Drugs.*—Oxolinic acid (No. O-0877), nalidixic acid (No. N-8878), piromidic acid (P-6542) (Sigma Chemical Co., St. Louis, MO).

(**k**) *Standard stock solutions and working solutions.*—Accurately weigh 10.0 mg of each drug, dissolve each in methanol, and dilute to 100 mL (100 ppm stock solution). Combine and dilute stock solutions to 1.0, 0.5, and 0.1 ppm with methanol.

Extraction and Cleanup

Cut sample to ca 0.5 cm pieces, and weigh 10.0 g into 100 mL centrifuge tube. Add 20 mL phosphate buffer solution (pH 6.0) and homogenize 5 min. Wash homogenizer shaft with 40 mL ethyl acetate, and add this wash to above centrifuge tube. Shake 10 min, and centrifuge 10 min at 2000 rpm (1000 × *g*). Pipet upper ethyl acetate layer to 100 mL separatory funnel. Add 20 mL ethyl acetate to centrifuge tube, break up residual lumps with spatula, and shake 10 min. Centrifuge 10 min at 2000 rpm (1000 × *g*), and pipet ethyl acetate into above separatory funnel. Shake ethyl acetate extract with 10, 10, and 5 mL portions of 3% sodium bicarbonate solution, and transfer aqueous layer to another 100 mL separatory funnel. Add 1.5 mL 5N hydrochloric acid, mix well, and add 0.1N hydrochloric acid to adjust solution to pH 6 (check with pH indicator paper). Extract twice with 30 mL and then 20 mL ethyl acetate. Dry ethyl acetate solution over anhydrous sodium sulfate for 5 min, filter into 100 mL pear-shape flask, and evaporate solvent in rotary evaporator at 45°C. Transfer residue quantitatively to 10 mL test tube with 1.0 and 0.5 mL methanol.

Sodium Tetrahydroborate Reduction

Cool methanol solution with ice, add ca 4 mg sodium tetrahydroborate, and let solution react 30 min at 0°C with occasional swirling. Add 4 mL 0.1N hydrochloric acid, mix well, and check that pH = 2 with pH indicator paper. If more basic, adjust to pH 2 with 0.1N hydrochloric acid. Warm solution at 40°C for 5 min on water bath. Cool solution with ice, and extract twice with 2 mL ethyl acetate. Wash organic extract with 0.3 mL phosphate buffer (pH 6.0), and dry over anhydrous sodium sulfate. To aqueous phase, add saturated sodium bicarbonate solution from Pasteur pipet to make pH 9.0 (ca 20 drops), and extract twice with 2 mL ethyl acetate. Dry organic extract over anhydrous sodium sulfate. Combine 2 ethyl acetate solutions.

Wash SepPak Alumina N cartridge column with 2 mL ethyl acetate, and discard this wash. Pass ethyl acetate solution through this column, followed by 1 mL ethyl acetate. Collect all of the eluate, evaporate solvent, and dissolve residue in 1 mL acetone, this solution is ready for GC/MS determination. Store under nitrogen atmosphere in refrigerator or immediately proceed to GC/MS determination. Perform GC/MS analysis within 3 days after reduction.

Preparation of Standard Solution for Calibration Graph

Pipet 1 mL of each working solution, 0.1, 0.5, and 1.0 ppm, to 10 mL test tube and cool with ice-water. Add ca 4 mg sodium tetrahydroborate, and let solution react 30 min at 0°C with occasional swirling. Add 4 mL 0.1N hydrochloric acid, mix well, and check pH with pH indicator paper. If more basic than pH 2, adjust to pH 2 with 0.1N hydrochloric acid. Warm solution 5 min at 40°C on water bath. Cool reaction mixture, and extract twice with 2 mL ethyl acetate. Wash organic extract with 0.3 mL phosphate buffer (pH 6.0), and dry over anhydrous sodium sulfate. To aqueous phase, add saturated sodium bicarbonate solution from Pasteur pipet to pH 9.0 (ca 20 drops), and extract twice with 2 mL ethyl acetate. Dry organic extract over anhydrous sodium sulfate. Combine 2 ethyl acetate solutions. Wash SepPak Alumina N cartridge column with 2 mL ethyl acetate, and discard this wash. Pass ethyl acetate solution through column, followed by 1 mL ethyl acetate. Collect all eluate and evaporate solvent. Dissolve residue in 1 mL acetone. This solution is ready for GC/MS determination. Store under nitrogen atmosphere in refrigerator or immediately proceed to GC/MS determination. Prepare fresh every 3 days.

GC/MS SIM Determination

Set GC/MS conditions described in *Apparatus* (**f**). The detection ions of SIM are *m/z* 219, 204, 176, 190, 176, 175, 246, 218, and 217. Inject 2 µL standard solutions, and obtain peak areas from SIM chromatograms of *m/z* 204, 175, and 217. Construct calibration graph by plotting peak areas vs amounts injected (ng). Inject 2 µL sample solution, and identify peaks of reduction products of oxolinic, nalidixic, and piromidic acid by retention time in SIM. Calculate amounts injected (ng) by comparing peak areas of *m/z* 204, 175, and 217 with calibration graph. Calculate drug residue concentration in sample by the following:

$$Y = x/2W$$

where x = amount detected in 2 µL sample solution injected (ng); W = sample weight (g); and Y = drug residue concentration in sample (ppm).

Confirm drugs by comparing ratios of peak areas of 3 SIM chromatograms of sample solution with peak area ratios of standard solution. Ratio must agree within ±20% range.

Results and Discussion

As reported earlier (15), methyl or *n*-butyl oxolinate was not suitable for GC determination at the residue level. Therefore, I tried other possible derivatives and found that sodium tetrahydroborate reduction of oxolinic acid proceeded smoothly to give a fluorescent product. The structure of this product (Figure 1d) was confirmed by the mass spectrum (Figure 2d) (M^+, *m/z* 219; M^+-CH_3, *m/z* 204; M^+-CH_3-CO, *m/z* 176). This compound (d) is considered to be formed by decarboxylation of an intermediate product (Figure 3g), which is produced by Michael-type addition of hydride ion (H^-) to oxolinic acid. Compound (d) is smoothly eluted from GC columns and gives a sharp, symmetrical peak at moderate temperatures; thus, it is well suited for GC analysis.

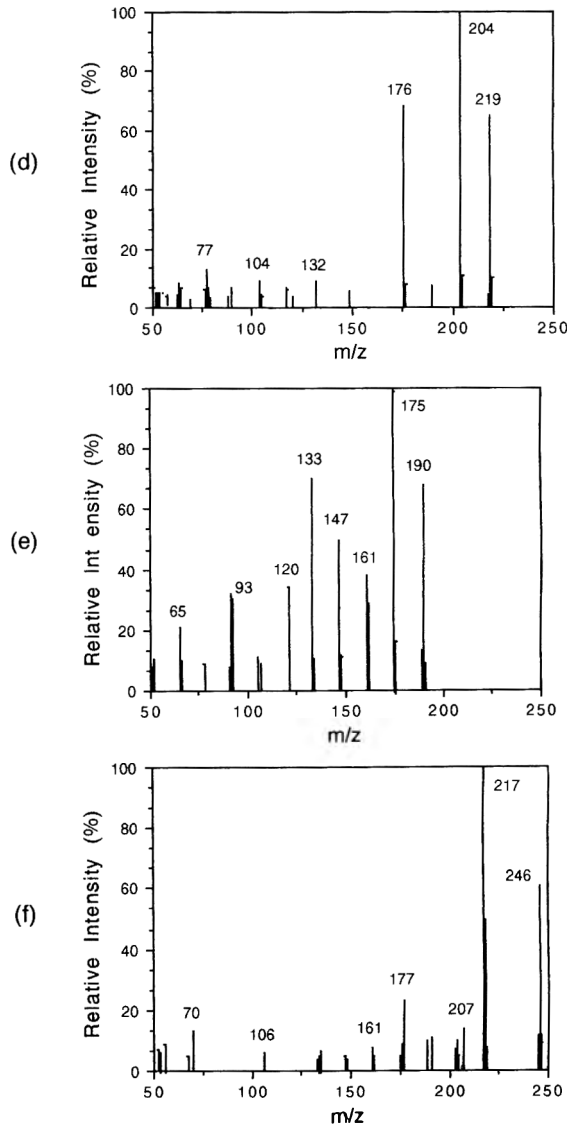

Figure 2. Mass spectra of reduction products of oxolinic (d), nalidixic (e), and piromidic (f) acids (20 ng each injected).

Because nalidixic and piromidic acids (Figures 1b and 1c) have similar structural features, I tried reducing these acids with sodium tetrahydroborate. I found that reduction proceeded smoothly and gave 2 fluorescent products, (e) and (f) in Figure 1. These products are considered to be formed by the same reaction scheme shown in Figure 3, and the structures were confirmed by their mass spectra (Figures 2e and 2f). Compounds (e) and (f) have good GC characteristics and give sharp, symmetrical peaks on gas chromatograms. Because nalidixic and piromidic acid are also used in cultivation of fish as antimicrobial agents, residues of the 3 drugs may be simultaneously determined in cultivated fish. Hence, I developed a GC/MS method for determining residue of these 3 drugs in cultivated fish, by using the above tetrahydroborate reduction products.

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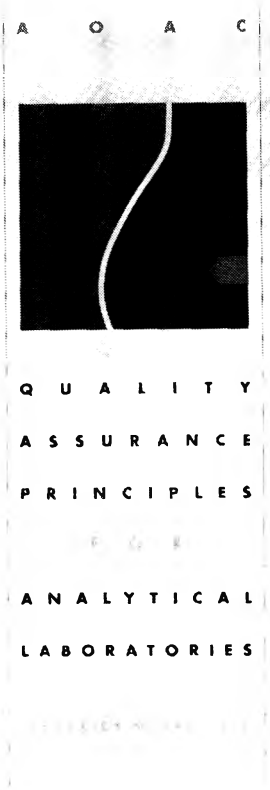

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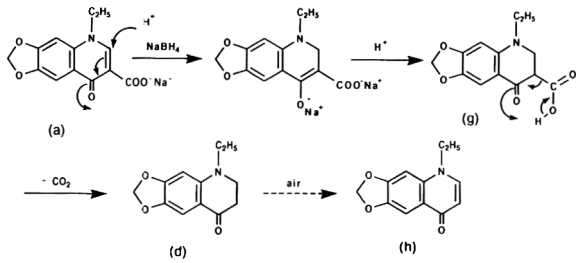

Figure 3. Reaction scheme for reduction of oxolinic acid with sodium tetrahydroborate.

The optimum reaction conditions of sodium tetrahydroborate reduction for the 3 drugs are slightly different from the conditions for oxolinic acid only (15), because of the possibility of reducing the carbon–nitrogen double bonds in nalidixic and piromidic acids. Reduction at 20°C for 5 min did not give maximum yields of products for nalidixic and piromidic acids. Reduction at 0°C (ice-cooled) produced yields that were more constant and high, but a longer reaction time was necessary compared with reaction at 20°C. A reaction time of 30 min gave the best results in these experiments.

After reduction, the reaction mixture must be treated with hydrochloric acid to destroy excess tetrahydroborate and to promote decarboxylation. When the amount of hydrochloric acid was not sufficient to make the solution acidic, the yields of the reduction products decreased considerably. The pH of the reaction mixture after hydrochloric acid addition is critical and should be carefully established with pH indicator paper to be pH 2 in each reduction. Addition of 0.1N hydrochloric acid to the reduction mixture to adjust to pH 2 and warming this solution at 40°C for 5 min are the optimum conditions for the 3 drugs.

Pure crystalline reduction products (d), (e), and (f) were prepared by preparative scale reduction (50 mg each) followed by purification using preparative thin-layer chromatography on silica gel. The absolute yields of the reduction products were then obtained on the bases of the pure reduction products. The yields from 1.0 μg of each starting material were as follows: (d) oxolinic acid. 51.2%; (e) nalidixic acid, 59.6%; and (f) piromidic acid, 85.5%.

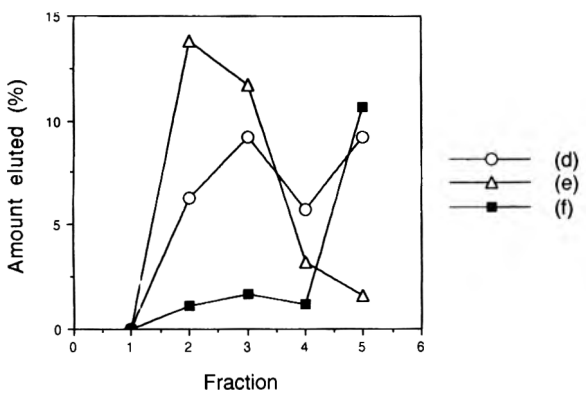

Figure 4. Elution of reduction products of oxolinic (d), nalidixic (e), and piromidic (f) acids from SepPak Alumina N. Eluant (4 mL): 1, CH_2Cl_2; 2, CH_2Cl_2–diethyl ether (1 + 1); 3, CH_2Cl_2–diethyl ether (1 + 2); 4, diethyl ether; and 5, ethyl acetate.

Table 1. Recoveries (%) of oxolinic, nalidixic, and piromidic acids from silver salmon muscle

Parameter	Oxolinic	Nalidixic	Piromidic
0.1 ppm fortification	93.8	94.2	91.0
	95.9	115.7	87.7
	91.1	82.4	94.9
	85.7	108.3	90.7
	94.4	102.4	96.4
Mean rec. %	92.2	100.6	92.1
CV, %	4.4	12.8	3.8
0.01 ppm fortification	82.9	79.1	71.8
	83.8	76.3	62.9
	74.8	89.0	74.1
Mean rec., %	80.5	81.5	69.6
CV, %	6.2	8.2	8.5

Compound (d) slowly changed to a dehydrogenated compound (Figure 3h) when the diethyl ether solution was exposed to air at room temperature (15). Compound (h) eluted after compound (d), and the structure was confirmed by the mass spectrum (M^+, *m/z* 217; M^+-CH_3, *m/z* 202; M^+-CH_3-CO, *m/z* 174). However, this oxidation was not realized when diethyl ether was not used as solvent. Compound (f) is fairly unstable and decomposed rapidly on standing in air (2 days) without solvent. Therefore, the reduction products must be determined by GC/MS as quickly as possible, and the solutions must be stored under nitrogen atmosphere in a refrigerator. Still, I recommend GC/MS determination within 3 days after reduction,

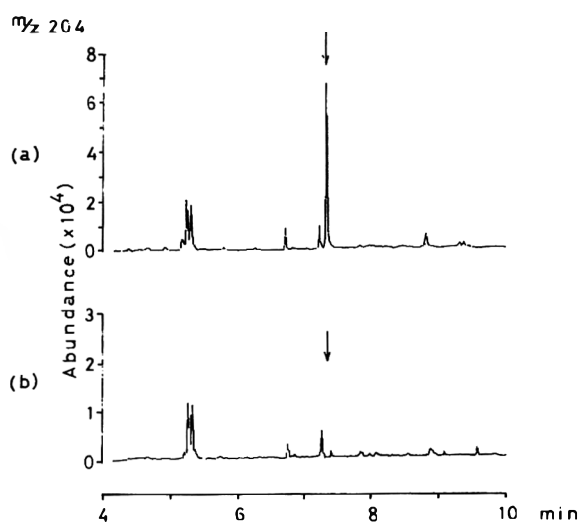

Figure 5. SIM chromatograms (*m/z* 204) of extract from (a) silver salmon fortified with 0.1 ppm oxolinic acid and (b) blank silver salmon. Arrows indicate retention time of reduction product of oxolinic acid.

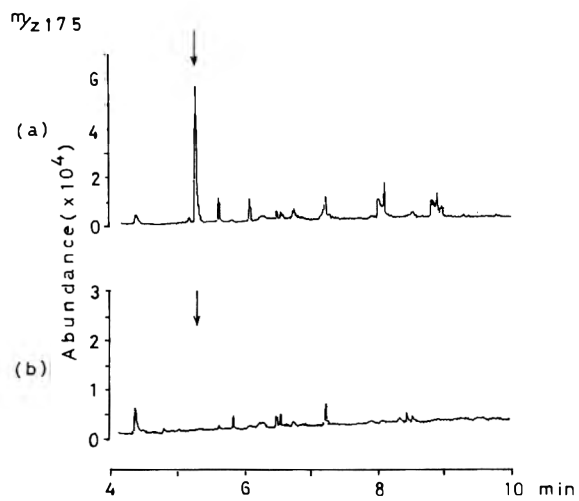

Figure 6. SIM chromatograms (*m/z* 175) of extract from (a) silver salmon fortified with 0.1 ppm nalidixic acid and (b) blank silver salmon. Arrows indicate retention time of reduction product of nalidixic acid.

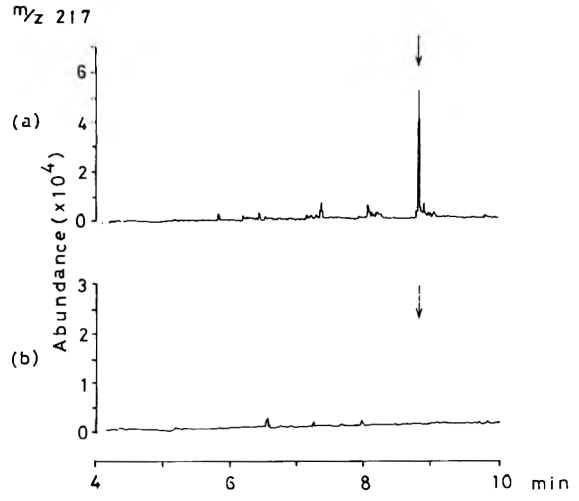

Figure 7. SIM chromatograms (*m/z* 217) of extract from (a) silver salmon fortified with 0.1 ppm piromidic acid and (b) blank silver salmon. Arrows indicate retention time of reduction product of piromidic acid.

because the reduction product of piromidic acid (f) slowly decomposed after 1 week even under these conditions.

Sphon (16) pointed out that 3 ions are necessary for the qualitative identification of an endogenous drug residue by use of low-resolution MS. Therefore, 3 distinct ions, including the base ion, are selected from the mass spectrum of each reduction product for SIM detection ions. Quantitation is effected by comparing the peak areas on the *m/z* 204, 175, and 217 SIM chromatograms (the base ion of each compound) with a calibration graph. SIM using these ions is most sensitive. Almost straight lines were obtained in the range of 0.2–20 ng standard injected, and the calibration graph for quantitation was constructed in the range of 0.2–2.0 ng of injection amounts. Qualitative identification of drug residues in each case is indicated by the presence of a peak at the same retention time on the 3 SIM chromatograms, and their peak ratios are similar (±20%) to those of the standards. In experiments with silver salmon at fortification levels of 0.1 and 0.01 ppm, the ratios of the drug residues agreed within ±20% of the expected range obtained from standards.

I used the extraction method reported by Browning and Pratt (2) for the determination of nalidixic acid. Because oxolinic, nalidixic, and piromidic acid are amphoteric, the sample must be extracted with ethyl acetate after homogenization with phosphate buffer (pH 6.0). Kasuga et al. (9) also used this extraction method for the LC determination of oxolinic, nalidixic, and piromidic acids in fish and obtained good extraction efficiencies. As a cleanup procedure, partition of ethyl acetate extract into aqueous sodium bicarbonate solution and re-extraction of the aqueous phase after neutralization effectively removed oily substances and interfering peaks to give almost satisfactory results. After sodium tetrahydroborate reduction, the sample extract is passed through a chromatographic column to completely remove high-boiling polar compounds for protection of the open-tubular column. A silica gel cartridge column is used for oxolinic acid determination (15) because of its simplicity, small amount of necessary solvent, and constant activity of adsorbent. However, the reduction products of nalidixic and piromidic acid were not completely eluted from silica and Florisil cartridge columns with ethyl acetate as eluant. An alumina column is more suitable for chromatography of basic compounds, and Figure 4 shows the elution pattern of 3 reduction products from SepPak Alumina N. Ethyl acetate is necessary and sufficient for eluting the 3 reduction products.

Recoveries were determined by adding the 3 drugs at 0.1 and 0.01 ppm to blank silver salmon muscle tissue, which was previously analyzed by this method to confirm that no drug was detected. Table 1 shows the results. Figures 5–7 show the SIM chromatograms of extracts from silver salmon fortified with each drug at 0.1 ppm (a) and from blank silver salmon (b). The detection limit, designated as 3 times the background level, is 0.003 ppm in muscle tissues (also instrument-dependent).

An experienced analyst can prepare and analyze 10 samples in 3 days (8 h/day), and confirmation of the residue drug is also possible. This method is useful for both daily screening and qualitative identification of residues of oxolinic, nalidixic, and piromidic acids in cultivated fish. Also, this method is, to the best of my knowledge, the first to determine oxolinic, nalidixic, and piromidic acids by GC/MS.

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DRUG RESIDUES IN ANIMAL TISSUES

HPLC Receptorgram: A Method for Confirmation and Identification of Antimicrobial Drugs by Using Liquid Chromatography with Microbial Receptor Assay. I. Sulfonamides in Milk

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A determinative method was developed for confirmation, identification, and quantitation of 12 sulfonamide residues and *p*-aminobenzoic acid (PABA) in milk. This method, termed "HPLC receptorgram," uses liquid chromatography in conjunction with the microbial receptor assay (MRA) to monitor sulfonamides. The MRA for sulfonamides (commercialized as the Charm II test) uses a microcrobial receptor and [^{3}H]sulfamethazine tracer. Sulfonamides in milk bind to the receptor and inhibit the binding of the tracer to the receptor. Milk samples spiked at 10 ppb for each sulfonamide result in at least a 50% decrease in binding on the HPLC receptorgram, with the exception of sulfanilamide and sulfacetamide, which give approximately a 25% decrease. Additionally, milk samples spiked at the minimum detection level of the MRA assay can be confirmed, identified, and quantitated with coefficients of variation (%) of 7.6–24. Analysis of market milk samples found positive by the MRA showed 9 samples containing sulfamethazine, 4 samples containing sulfadimethoxine, 2 samples containing sulfadoxine, 2 samples containing sulfadiazine or sulfathiazole, and 1 sample each containing sulfisoxazole or sulfapyridine. Three samples contained multiple sulfonamide residues. Ten sulfonamides are determined with an isocratic buffer elution system containing 22% acetonitrile mobile phase. *p*-Aminobenzoic acid (PABA), an interfering sulfonamide analog, sulfanilamide, and sulfacetamide are eluted by using a 10% acetonitrile mobile phase.

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Sulfonamides are an important family of antimicrobial drugs that are widely used in the dairy industry for the treatment and prevention of disease. For these reasons, sulfonamides and other families of antibiotics have provided enormous benefits to the dairy and meat industry. However, the misuse of antibiotics can result in the presence of antibiotic residues in milk and meat, which may pose a health threat to the consumer and are in violation of the Code of Federal Regulations (1–5). Thus, the monitoring of incurred residues of antibiotics in market milk or meat is essential for ensuring the safety and adequacy of food as well as providing an indication of the proper usage of antibiotics at the producer level.

The U.S. Food and Drug Administration (FDA) has established safe levels for all sulfonamides at 10 ppb (5). Of the few screening tests available commercially, most are immunoassays specific to a single sulfonamide. However, the microbial receptor assay (MRA), which has a group capability, was adopted final action by AOAC as part of a 7-family test (6). In several surveys of market milk in North America, the MRA was used to detect sulfonamides as well as other antimicrobial drugs (7–9).

For regulatory purposes, the identification of the specific contaminant in food samples is mandatory. The few methods for determination of sulfonamides in milk and meat that have been published include thin-layer chromatography (10, 11), liquid chromatography (LC) (12, 13), and gas chromatography/mass spectrometry (GC/MS) (14–16). LC is the method of choice for regulatory purposes, because GC/MS is a complicated and cumbersome procedure and in most cases lacks the sensitivity required for quantitation at the established safe levels. The monitoring systems for LC are UV absorbance/diode array detection, fluorescence, and electrochemistry. A particular residue is identified by comparing its retention time with that of a known standard. Peak heights or areas are used to quantitate contaminants in milk samples. Because the composition of market milk samples will vary as a reflection of differences in diet and treatment of individual dairy cows or herds, liquid chromatograms of market milk samples will not have a consistent background. In analyses of a large drug family such as sulfonamides, separation of all sulfonamide peaks from interferences is difficult, and both false positives and errors in quantitation may result (17). Thus, the existing LC technique is not sufficient to confirm, identify, and quantitate positive samples in the 1–10 ppb range.

Our goals in the development of the "HPLC receptorgram" for sulfonamides were to simplify the extraction procedure so that it would recover all sulfonamides, to reach quantitation levels below the FDA established safe level, and to eliminate false results. These goals could be reached by combining LC separation capability with the high specificity of the microbial receptor assay. The method requires a simple extraction, and the partially purified and concentrated extract is directly applied to LC. The eluate is collected, and the individual fractions are assayed for sulfonamide activity by the MRA. Analytes are tentatively identified and quantitated by comparing fraction(s) positive for sulfonamide activity with known sulfonamide standards. A preliminary outline of the HPLC receptorgram method has been discussed within group E47 of the International Dairy Federation (IDF) and was published in IDF Bulletin No. 258 under the general heading "Tentative Confirmation and Identification Tests" (18).

METHOD

Apparatus

(**a**) *Liquid chromatograph.*—LKB 2150 LC pump (Pharmacia LKB, Piscataway, NJ), Waters 990 system with photodiode array detector, and NEC Powermate/Multisync II computer (Waters Chromatography Div., Milford, MA); St. John electrical injection valve with Rheodyne 7010 sample injection valve and 200 µL loop (St. John Association, Beltsville, MD); Foxy 200 fraction collector (Isco, Lincoln, NE).

(**b**) *LC column.*—Lichrosorb RP-8 column (10 µm, 250 × 4.6 mm) (Alltech, Deerfield, IL) with Waters precolumn filter.

(**c**) *Filters.*—Acrodisc 0.2 µm LC 13 PVDF filters (Gelman Sciences, Ann Arbor, MI), Nylon-66 0.2 µm filters (Rainin, Woburn, MA), 0.8 µm filters (Millipore Corp., Bedford, MA).

(**d**) *Filter holder.*—For 0.8 µm filter (Millipore), 47 mm glass filter holder for vacuum filtration (Rainin, Woburn, MA).

(**e**) *Syringes.*—1 and 20 mL syringes (Becton-Dickinson, Rutherford, NJ).

(**f**) *Drying system.*—Vacuum oven (NAPCO, Tualatin, OR) or Speed-Vac (Savant, Hickesville, NY) connected to refrigerated condenser trap (Savant) and vacuum pump.

(**g**) *Extraction columns.*—Bond Elut C_8 octyl 500 mg columns (Varian, Harbor City, CA); Bond Elut empty 4 mL capacity reservoirs with 20 µm frits (Varian) filled with 500 mg analytical grade macroporous cation resin AG MP-50 (Bio-Rad Laboratories, Richmond, CA).

(**h**) *Extraction system.*—Automated multisyringe pump extraction system (Charm Sciences, Malden, MA).

(**i**) *Centrifuge tubes.*—Disposable polypropylene 50 mL nonsterile centrifuge tubes and 1.5 mL flat-top microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA).

(**j**) *Centrifuges.*—IEC clinical centrifuge (International Equipment Co., Needham Heights, MA); Multifuge (American Scientific Products, McGaw Park, IL).

(**k**) *Water bath.*—Water bath at 60°C (Brinkmann, Westbury, NY).

(**l**) *Assay system.*—Charm II system for assay of sulfonamides (Charm Sciences, Malden, MA).

Reagents

(**a**) *Solvents.*—LC grade acetonitrile, methanol, and water (J.T. Baker, Phillipsburg, PA); double-distilled water obtained from Barnstead Fi-stream II system (Sybron Barnstead, Boston, MA).

(**b**) *Mobile phase.*—Isocratic buffer containing 22% acetonitrile in 10mM ammonium acetate buffer, pH 4.6. Add 1.54 g ammonium acetate (J.T. Baker) to 1 L LC water, and adjust to pH 4.6 with glacial acetic acid (J.T. Baker). Add LC water to 2 L, and filter through 47 mm (0.2 µm) filter under vacuum. Add 565 mL acetonitrile. Prepare isocratic buffer containing 10% acetonitrile by adding 100 mL acetonitrile to 900 mL 10mM ammonium acetate buffer, pH 4.6.

(**c**) *McIlvaine/EDTA buffer.*—Mix 272.5 mL 0.1M citric acid (Sigma Chemical Co., St. Louis, MO) with 227.5 mL 0.2M dibasic sodium phosphate (Sigma). Add 37.2 g ethylenediaminetetraacetic acid (Sigma), and adjust to pH 4.5 with ammonium hydroxide (J.T. Baker) or phosphoric acid (J.T. Baker). Add water to make 1 L buffer.

(**d**) *Oxalate buffer.*—Mix 4.26 g ammonium oxalate (Sigma) with 3.6 g oxalic acid (Sigma), and add 180 mL water. Adjust pH to 3.8 with ammonium hydroxide, and dilute to 200 mL with water.

(**e**) *Sulfonamide standards.*—Prepare stock solutions of sulfadiazine (SDZ), sulfathiazole (STHZ), sulfapyridine (SPY), sulfamerazine (SMR), sulfamethazine (SMZ), sulfachloropyridazine (SCP), sulfadoxine (SDX), sulfisoxazole (SSX), sulfamethoxazole (SMXZ), sulfadimethoxine (SDM), sulfanilamide (SNA), and sulfacetamide (SAA) (U.S. Pharmacopeial Convention, Rockville, MD) at 1 mg/mL in metha-

Table 1. Microbial receptor assay (MRA) of 10 sulfonamides for establishing a minimal detection level

Drug species	Bs/Bo value (at 3 spiked concn, ppb)[a]			MDL, ppb[b]	BsBo for sample spiked at MDL[a]	CV, %
	2	5	10			
SDZ	0.68	0.35	0.29	3	0.58	3.4
STHZ	0.68	0.43	0.29	4	0.57	6.9
SPY	0.85	0.62	0.47	5	0.65	4.6
SMR	0.54	0.47	0.34	2	0.71	4.8
SMZ	0.81	0.61	0.44	5	0.66	7.7
SCP	0.64	0.32	0.26	2	0.58	4.4
SDX	0.70	0.55	0.46	5	0.65	6.8
SSX	0.54	0.29	0.19	2	0.65	14.0
SMXZ	0.46	0.28	0.23	1	0.65	4.1
SDM	0.57	0.40	0.35	2	0.64	8.0

[a] Negative raw milk was spiked with 10 sulfonamides and analyzed on the MRA using the competitive procedure (Charm II Test, Kit SMTBL010). Results are expressed as spiked sample result divided by average zero negative control (Bs/Bo). All assays were performed in triplicate.

[b] Minimal detection level (MDL) is defined as the lowest concentration that can be detected with accuracy. For this study, this level was established at 3 SD from zero (Bs/Bo = 0.65). Standard deviation was calculated from raw milk obtained from a control herd.

nol and store at −20°C; PABA (Sigma). Prepare diluted stock standards for LC and milk samples fortified with sulfonamides fresh daily. Use tritium-labeled SMZ and SDM (Charm Sciences) as internal standards for retention time calibration and recovery determinations.

Sample Preparation

To 50 mL centrifuge tube, add 20 mL milk sample followed by 20 mL McIlvaine/EDTA buffer. Heat 15 min at 60°C or until milk curdles. Centrifuge 5 min at high speed in clinical centrifuge, and filter extract through 0.8 μm filter. If resistance becomes excessive, use new filter. Load 28 mL extract onto activated C_8 Bond Elut column, followed by 5 mL water wash. Elute sample with 2.5 mL 100% methanol into 13 × 100 mm test tube. Dry extracted sample in heated Speed-Vac. Dissolve sample with 100 μL LC buffer, and filter through 0.2 μm Acrodisc filter into microcentrifuge tube. Wash test tube with another 100 μL LC buffer, and filter through same filter. Purge filter with air to obtain maximum recovery. Sample is now ready for LC analysis.

For determination of PABA, SNA, and SAA, use different sample preparation procedure. To 20 mL milk, add 5 mL 30% (w/v) trichloroacetic acid (Sigma). Heat 15 min at 50°C or until milk curdles. Centrifuge 5 min at high speed in clinical centrifuge, and filter extract through 0.8 μm filter into fresh 50 mL centrifuge tube. Add 2.5 mL 0.35M oxalate buffer, pH 3.8, and adjust pH to 2.0 with 0.75 mL 10% ammonium hydroxide. Centrifuge as above for 5 min. Load sample onto activated cation exchange column (Bio-Rad AG MP-50 resin). Activate cation exchange column by washing twice with 3 mL methanol, followed by washing twice with 3 mL water. Wash with 2 mL methanol, and elute with 2 mL 0.2M phosphate buffer (pH 8.0)–methanol (1 + 1) into test tube. Do not allow column to dry out during procedure. Dry eluate in heated Speed-Vac, and prepare as above for LC analysis.

Liquid Chromatography

Elute sulfonamides with isocratic gradient containing 22% acetonitrile in 10mM ammonium acetate buffer, pH 4.6, at 1 mL/min flow rate. Separate SDZ, STHZ, and SPY with isocratic gradient of 19.5% methanol in 10mM ammonium phosphate buffer, pH 5.5, at 1.0 mL/min flow rate. Elute PABA with isocratic gradient of 10% acetonitrile in 10mM ammonium acetate buffer, pH 4.6, at 1 mL/min flow rate. Inject standard solution of sulfonamides daily to calibrate column and time windows for collection of sulfonamide peaks. If necessary, adjust composition of LC elution buffer to obtain optimal separations. Perform blank injection, and analyze chromatogram to ensure base line with no contamination by standards. For LC analysis of extracted samples, load all of sample (ca 170 μL) onto 200 μL loop. Wash microcentrifuge tube containing sample with minimal amount of water (ca 30 μL), and load onto loop.

Calibration of Time Windows

To correlate retention times with time window collection of peaks of interest, use tritium-labeled sulfamethazine and sulfadimethoxine. Compare retention time of sulfamethazine and sulfadimethoxine peaks with peak for radioactivity. Adjust time window collection for peaks of interest for this system with known delay time. Calibrate time windows for each sulfonamide accordingly from sulfonamide standard injection.

Microbial Receptor Assay of LC Fractions

Dry fractions from LC analysis in Speed-Vac or vacuum oven at 60°C. Reconstitute fractions in 4 mL antibiotic-free skim milk. Perform MRA for sulfonamides according to manufacturer's instructions (Charm Sciences) or as described in detail by Charm and Chi (6), except assay 2 mL milk rather than 5 mL. Repeat assay of positive fractions with remaining 2 mL milk, or dilute as appropriate to fit standard curve for

Table 2. Calibration of HPLC receptorgram and set up of time windows for collection of 12 sulfonamides and PABA

Peak	Sulfa drug	Retention time, min	Time windows, min	
			Left time	Right time
	Mobile phase: 22% acetonitrile			
1	SDZ	7.28	6.8	7.6
1	STHZ	7.35	6.8	7.6
2	SPY	7.9	7.61	8.3
3	SMR	9.10	8.5	9.5
4	SMZ	10.4	9.9	10.9
5	SCP	15.5	14.9	16.2
6	SDX	17.5	16.8	18.05
7	SSX	18.75	18.1	19.3
8	SMXZ	20.05	19.35	20.85
9	SDM	34.9	33.7	36.0
	Mobile phase: 10% acetonitrile			
1	SNA	7.15	6.5	8.4
2	PABA	11.8	11.2	12.4
3	SAA	12.9	12.41	13.7
	Mobile phase: 19.5% methanol			
1	SDZ	9.05	8.36	9.65
2	STHZ	15.23	14.60	16.10
3	SPY	16.83	16.15	17.90

quantitation. For PABA, reconstitute SNA and SAA fractions in 2 mL milk, and assay as above.

Sulfonamide Standard Curves

Prepare standard curves for each sulfonamide by using MRA. Fortify antibiotic-free milk with each sulfonamide, and dilute to achieve appropriate concentrations.

Fortification of Milk Samples

Prepare fortified milk samples for each sulfonamide at 10 ppb and at concentration for each sulfonamide that gives 35% decrease in binding (limit of determination reported by manufacturer).

Results and Discussion

FDA has set the safe level for individual or total sulfonamides in milk at 10 ppb. The MRA can readily screen for sulfonamides at this level. MRA (commercialized as the Charm II test) for sulfonamides uses a microbial receptor and [^{3}H]sulfamethazine as a tracer. When the receptor is incubated with milk, contaminating sulfa drugs will bind to the receptor and inhibit the binding of [^{3}H]sulfamethazine to the receptor. The amount of tritium bound to the receptor is measured, and a

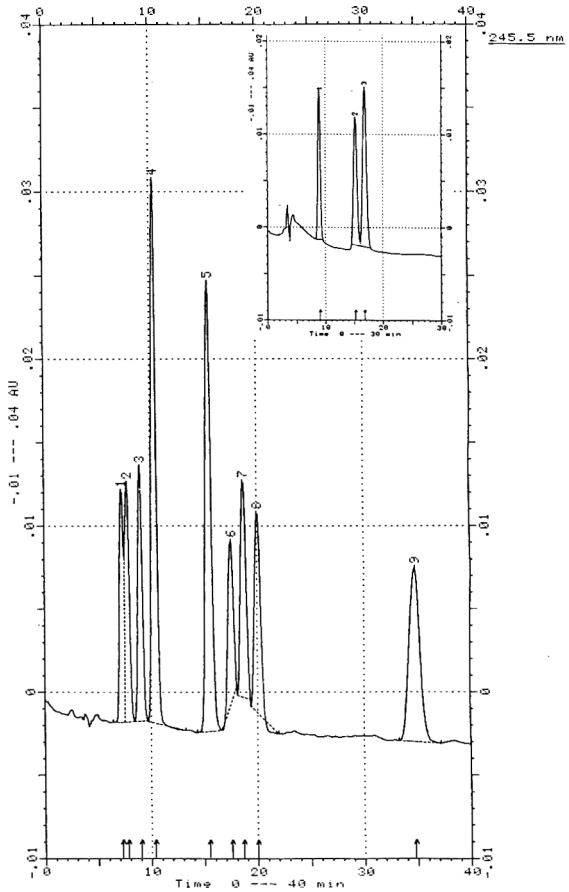

Figure 1. LC chromatogram of 10 sulfonamide standards. Isocratic gradient containing 22% acetonitrile in 10mM ammonium acetate buffer, pH 4.6; flow rate, 1 mL/min. Peaks: 1, SDZ (70 ng), STHZ (110 ng); 2, SPY (250 ng); 3, SMR (250 ng); 4, SMZ (600 ng); 5, SCP (600 ng); 6, SDX (300 ng); 7, SSX (300 ng); 8, SMXZ (200 ng); and 9, SDM (1200 ng). Inset: LC chromatogram of SDZ (peak 1, 125 ng), STHZ (peak 2, 225 ng), and SPY (peak 3, 300 ng). Elution: isocratic gradient of 19.5% methanol in 10mM ammonium phosphate buffer, pH 5.5, at 1.0 mL/min flow rate.

Bs/Bo value is determined, where Bs is the counts per minute (cpm) of the sample and Bo is the cpm of the zero control.

At the minimum detection level (MDL) for the MRA, sulfonamides can be detected at 1–5 ppb (Table 1). MDL values for SNA, SAA, and PABA are 10, 10, and 1 ppb, respectively. Although the MDL for the receptor assay in a single assay of market milk is set at 35% decrease in binding (3 standard deviations from zero), the screening level for the FDA-established safe level by this assay is approximately 50% binding with 10 ppb sulfamethazine as the standard. MRA values for the 10 sulfonamides at 10 ppb are shown in Table 1. Sulfonamides with high specificity for the receptor reach the Bs/Bo value of 50% at levels below 10 ppb. Therefore, LC methodology is needed to identify and confirm sulfonamides at 2.5-fold lower levels than the FDA established safe level.

The HPLC receptorgram assay uses LC in conjunction with MRA for confirmation and identification of sulfonamides in

Table 3. HPLC receptorgram for preliminary study of milk samples spiked at 10 ppb[a]

Sample	Peak	Bf/Bo[b]
SDZ	1	0.27
STHZ	1	0.41
SPY	2	0.46
SMR	3	0.27
SMZ	4	0.38
SCP	5	0.41
SDX	6	0.44
SSX	7	0.17
SMXZ	8	0.22
SDM	9	0.20

[a] n = 1 with fractions assayed in duplicate. Each milk sample was run separately.
[b] Bf/Bo is the cpm of the fraction divided by the cpm of zero control milk.

milk samples. The sulfonamides are identified by using the MRA to assay collected LC fractions for sulfonamide activity and correlating the positive fractions with retention times of known sulfonamide standards (Table 2; Figure 1). Twelve sulfonamides and PABA were selected for this study. The chromatogram of sulfonamide standards obtained with 22% acetonitrile in the mobile phase shows the separation of 9 peaks, with sulfathiazole and sulfadiazine coeluting in peak 1 (Figure 1). To achieve an acceptable separation and identification of SDZ, STHZ, and SPY by the HPLC receptorgram, an isocratic gradient of 19.5% methanol in 10mM ammonium phosphate buffer, pH 5.5, can be used (Figure 1, inset). For each peak of interest, fractions are collected in defined time windows, as shown in Table 2. Table 2 also shows the retention times and time windows for SNA, PABA, and SAA obtained with the 10% acetonitrile mobile phase.

Because FDA has established 10 ppb as the safe level for sulfonamides, a preliminary study was performed by spiking milk samples with sulfonamides at 10 ppb. HPLC receptorgram results from this study are shown in Table 3. At the 10 ppb level, all the sulfonamides assayed by this method showed >50% decrease in binding, which indicates that the method can adequately detect all 10 sulfonamides at the 10 ppb level. Market milk samples can be quantitated by comparing results with HPLC receptorgrams of spiked standards. If necessary, the reconstituted LC fraction can be diluted to fit the linear portion of the standard curve. Radiolabeled sulfamethazine or sulfadimethoxine is used to monitor the recovery and check the retention times of sulfonamides throughout the procedure.

Because market milk samples may contain more than one sulfonamide or active metabolite from a parent compound, a more detailed HPLC receptorgram study was conducted at the MDL of the MRA for each sulfonamide. In this study, milk was spiked with 10 sulfonamides at concentrations ranging from 1 to 5 ppb. The spiked milk samples were assayed by MRA to confirm that the initial concentrations of sulfonamide in these samples were near their established MDLs (Table 1). Three HPLC receptorgrams were performed for each sulfonamide, and fractions were assayed in duplicate for every run. Results from this study are presented in Table 4. Fractions not containing the sulfonamide of interest were negative. At the MDL, each sulfonamide is positive with at least a 25% decrease in binding on the HPLC receptorgram, and the final concentration of the sulfonamide for each run is derived from the observed decrease in binding and recovery factor. Because fractions are reconstituted in the same antibiotic-free milk, the coefficient of variation (CV) among negative fractions is less than in market milk samples; therefore, the MDL (3 standard deviations from zero) can be established at 20% binding. Repeatability of the HPLC receptorgram at the MDL has a CV% ranging from 8.0 to 24.0.

To demonstrate the effectiveness of the HPLC receptorgram method, 16 milk samples from 1991 that screened positive for sulfonamides by MRA were analyzed. Results of the analysis in terms of Bf/Bo (Bf is the cpm of the LC fraction, and Bo is the cpm of the zero control on MRA) for each fraction are shown in Table 5. Eight samples (1, 3, 5, 7, 8, 11, 12, and 14) are considered at or above the FDA safe level by LC. The re-

Table 4. HPLC receptorgram results of milk samples spiked at MDL for 10 sulfa drugs

Sulfonamide	Spiked level, ppb	Run 1[a]	Run 2	Run 3	Av.	CV, %[b]
SDZ	3.0	2.3	3.3	3.1	2.9	16.0
STHZ	4.0	3.4	2.8	2.1	2.8	24.0
SPY	5.0	5.5	5.6	4.8	5.3	17.5
SMR	2.0	1.5	1.3	1.3	1.4	10.3
SMZ	5.0	2.5	4.5	4.9	4.0	23.6
SCP	2.0	1.6	1.9	1.9	1.8	9.0
SDX	5.0	3.0	3.0	2.1	2.7	17.5
SSX	2.0	1.2	1.1	1.2	1.2	7.6
SMXZ	1.0	1.1	1.3	1.2	1.2	10.0
SDM	2.0	1.5	1.7	1.6	1.6	8.0

[a] Run values (ppb) represent the average of active fractions assayed in duplicate; calculations include recovery factor from radioactive experiments.
[b] n = 6.

Table 5a. HPLC receptorgrams for 16 milk samples in 1991 that were screened as positive on the MRA[a]

	Bs/Bo value at 9 sulfa peaks[b]								
Sample	1	2	3	4	5	6	7	8	9
1	1.05	1.07	0.99	0.76	1.03	1.06	0.19	0.28	1.05
2	1.05	0.96	0.96	1.02	0.97	0.97	1.06	1.01	0.41
3	0.38	0.42	1.04	1.05	0.97	0.80	1.01	0.92	1.02
4	1.11	1.05	1.19	1.15	1.18	1.12	1.13	1.18	0.45
5	1.01	0.95	1.04	0.68	1.10	0.43	1.01	0.97	0.92
6	1.00	1.04	1.07	0.52	0.94	1.00	1.03	0.99	0.93
7	0.99	1.10	1.01	1.01	0.95	0.50	1.00	0.95	0.75
8	0.9	0.94	0.95	0.50	1.04	0.99	1.06	1.11	1.00
9	0.91	1.10	0.96	0.68	1.03	1.01	1.05	0.94	1.00
10	0.96	0.98	0.99	0.61	1.03	0.99	0.98	0.99	1.07
11	1.06	1.00	0.94	0.52	1.02	1.07	1.02	0.95	0.95
12	0.85	1.01	1.03	0.30	1.10	1.02	0.93	1.02	1.04
13	0.93	1.03	1.02	0.97	1.01	0.99	1.10	0.95	0.5
14	1.12	1.11	1.08	0.37	1.07	1.13	0.93	1.01	1.14
15	0.59	0.87	1.03	0.99	0.97	1.01	1.06	1.05	1.01
16	1.06	0.62	1.04	0.97	1.03	0.96	1.03	0.91	1.00

[a] Results reported as Bf/Bo, cpm of the fraction divided by the cpm of the zero control.
[b] Sulfa peak numbers refer to LC fractions collected in the defined time windows shown in Table 2.

maining 8 samples were confirmed as positive for sulfonamides but at levels below 10 ppb by LC. However, if the initial screening values are used, 11 samples are at or above 10 ppb. SMZ was found in 9 samples, SDM in 4 samples, SDX in 2 samples, STHZ/SDZ in 2 samples, and SSX and SPY each in 1 sample.

Samples 1, 5, and 7 contained multiple sulfonamides. In sample 3, sulfonamide activity overlapped between the SDZ/STHZ and SPY fractions. Injection of standards after the run indicated that the retention times were slightly late (about 20 s). This sample, therefore, contains SDZ/STHZ, although the presence of SPY cannot be ruled out. To resolve such a problem, HPLC receptorgram analysis of the sample should be repeated with 19.5% methanol in 10mM ammonium phosphate, pH 5.5, as shown in Figure 1 (inset), to separate SDZ, STHZ, and SPY.

Table 5b. Summary of milk sample HPLC receptorgram results and comparison with MRA screening values

			Total Sulfonamides in sample	
Sample	MRA initial screening SMZ equiv., ppb[a]	Identified sulfa drug	MRA initial corrected, ppb[b]	MRA quantitation from LC fraction, ppb
1	≥20	SSX, SMZ	≥10	≥18
2	11.5	SDM	7	6
3	≥20	SDZ/STHZ	≥12	≥16
4	≥20	SDM	≥14	6[c]
5	14	SDX, SMZ	12	14
6	5	SMZ	5	6
7	≥20	SDX, SDM	10	10
8	11	SMZ	11	8
9	10	SMZ	10	5
10	10	SMZ	10	6.5
11	11	SMZ	11	8.0
12	12	SMZ	12	11
13	≥20	SDM	6	5
14	13	SMZ	13	10
15	10	SDZ/STHZ	4	4
16	8	SPY	9	6

[a] SMZ equivalents are the concentration of sulfonamides in terms of SMZ in samples as calculated from the initial MRA screening.
[b] MRA result corrected for total sulfa drug(s) identified by the HPLC receptorgram.
[c] An equal activity was found in a fraction eluting at 3.6–5.6 min.

In a recent study, Smedley and Weber used 2 isocratic conditions at 12 and 30% methanol to separate 10 sulfonamides (12). Under these conditions, STHZ and SDZ were also sufficiently separated. MRA will determine only active metabolites. As indicated in Table 5, the producer samples and market milk samples mostly show contamination by a single sulfonamide. Thus, no active metabolites or breakdown products could be detected by this method. Radiolabeled sulfonamides are needed to verify the existence of the parent compound and its active metabolites and breakdown products in an incurred study. Smedley and Weber (12), although pointing to multiple extraneous peaks in an incurred study, provide no clear confirmation that these are all true metabolites of sulfonamides.

Sulfonamides and PABA are analogs, and at the 1 ppb level, PABA will cause a positive result on MRA. Although milk does not contain PABA and PABA has not been found in any commercial milk samples, identification of PABA is important. A different extraction procedure was developed for PABA, because PABA does not bind sufficiently to the C_8 extraction column. This procedure is also suitable for determination of SAA and SNA. With an isocratic mobile phase of 10% acetonitrile in 10mM ammonium acetate, pH 4.6, PABA elutes at 11.8 min, SNA at 7.15 min, and SAA at 12.9 min. Time windows are collected for SNA, PABA, and SAA, as indicated in Table 2. The Bf/Bo result for PABA is 61.3 ± 3.5% (n = 3), indicating that PABA can be readily detected by the HPLC receptorgram method at 1 ppb. Similarly, SNA and SAA could be detected at 10 ppb, with Bf/Bo at 71 ± 7.0% (n = 2) and 75 ± 1.0% (n = 2), respectively.

Ten milk samples can be prepared simultaneously and are ready for LC analysis in about 1 h. Each run takes about 40 min to complete. LC fractions dried in the Speed-Vac are ready for assay in 2–3 h. A large number of samples can be dried overnight in the vacuum oven and the fractions assayed the next day. Assay of up to 12 fractions takes only 15 min.

A significant advantage of the HPLC receptorgram method over traditional detection methods is that analyte bioactivity is used to monitor LC fractions to confirm and identify sulfonamides. MRA, which is commonly used to screen milk samples for positives, is also used to confirm positives after separation by LC. Because the assay limit of detection is 2- to 5-fold below the 10 ppb safe level, milk samples containing more than 1 sulfonamide but totaling 10 ppb can be characterized. Confidence is high that a sample can be confirmed and that the individual sulfonamide can be identified and quantitated. The high specificity of the assay (coefficient of inhibition is $0.1–1.0\times10^{-10}$M) ensures a negative base line regardless of the milk origin, because interferences that may be present in the initial screening test are removed by the HPLC receptorgram procedure. In our laboratory, this method was used to evaluate several hundred milk samples from 1988 to 1991, and no false negatives were observed. A few unknown milk samples, as well as a few standard spiked samples, gave positive results in a fraction correlating with 3.6–5.6 min window. Therefore, this fraction may contain an active metabolite or active breakdown product from a parent sulfa drug.

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DRUG FORMULATIONS

Spectrophotometric Method for Determination of Cephalexin in Its Dosage Forms

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A simple, rapid, and specific method was developed for determination of cephalexin and its dosage forms. The method is based on the reaction of cephalexin with acetylacetone–formaldehyde reagent to give a yellow chromophore measurable at 400 nm. The color is stable for 3 h. Beer's law is valid within a concentration range of 10–100 μg/mL for cephalexin. All variables were studied to optimize the reaction conditions. The method is specific for amino β-lactam antibiotics. Non-amino β-lactam antibiotics do not interfere. No interference was observed in the presence of common pharmaceutical adjuvants. The validity of the method was tested by analyzing cephalexin tablets, capsules, and oral suspension. Good recoveries were obtained for these preparations. The results were comparable with those obtained by the official procedure.

Pharmaceutical preparations containing cephalexin have been available for many years. Methods for their analysis as pure substance and in dosage forms include titrimetry (1, 2), polarography (3, 4), fluorometry (5, 6), colorimetry (7–9), and liquid chromatography (LC) (10). The compendia (11, 12) recommend the volumetric or UV-spectrophotometric method. The former is not satisfactory for microquantities; the latter suffers from absorbance interferences. In the present work, the applicability of the buffered acetylacetone–formaldehyde reagent (pH 4.3) for the quantitative determination of amino β-lactam antibiotics has been investigated; the result is a simple, rapid, accurate, and selective colorimetric method for the quantitative determination of cephalexin in pure form and in pharmaceutical formulations.

Experimental

Apparatus and Reagents

(a) *Ultraviolet/visible spectrophotometer.*—Beckman Model 25.

(b) *pH meter.*—Systronic digital Model 355.

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(c) *Chemicals.*—Pharmaceutical grade cephalexin and probenecid were obtained as gifts from various manufacturers and were used as working standards without further treatment. All other reagents were analytical grade; water was double-distilled.

(d) *Dosage forms.*—Various commercial preparations were purchased from local markets.

(e) *Reagents solution.*—Prepare by mixing 16.0 mL 0.2M sodium acetate solution and 34.0 mL 0.2M acetic acid with 7.8 mL freshly distilled acetylacetone and 15.0 mL formaldehyde. Keep 5 min in boiling water bath, cool, and adjust pH to 4.3. Dilute with water to 100 mL.

Preparation of Standard Solution

Dissolve accurately weighed amount of cephalexin in water, and dilute quantitatively with water to obtain 0.005M solution of cephalexin.

Preparation of Samples

Tablets.—Weigh and powder 20 tablets, transfer accurately weighed quantity of powder equivalent to 183 mg cephalexin to 100 mL volumetric flask, and dissolve in and dilute to volume with water. Shake well and filter through Whatman No. 1 paper. Discard first portion of filtrate. Use clear solution obtained as stock solution (0.005M).

Cephalexin–probenecid tablets.—Treat similarly.

Capsules.—Thoroughly mix contents of 20 capsules, weigh, and proceed as described under *Tablets*.

Oral suspension.—Accurately weigh quantity of powder equivalent to 183 mg cephalexin, and treat as described under *Tablets*.

Determination

Transfer 1 mL of either standard or sample solution to 25 mL volumetric flask, add 4 mL reagent solution, let stand for 30 min at 35°C, and dilute to volume with water. Measure absorbance of yellow solution at 400 nm against blank prepared similarly, but take 1 mL water instead of standard or sample solution.

Results and Discussion

A characteristic yellow color with an absorption maximum at 400 nm was developed when cephalexin was reacted with

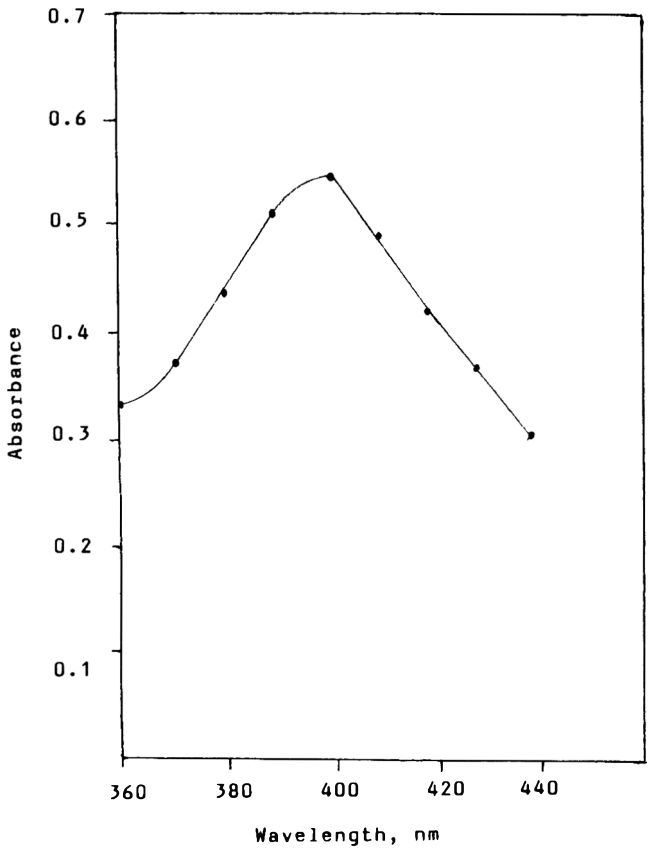

Figure 1. Absorption spectrum of reaction product of cephalexin with acetylacetone–formaldehyde reagent.

acetylacetone–formaldehyde reagent in acetate buffer (pH 4.3) (Figure 1).

On the basis of the literature background and our findings, the scheme for the formation of chromogen due to the reaction of acetylacetone and formaldehyde with cephalexin (RNH_2) could be obtained, as shown in Figure 2.

In the present work, a linear correlation was obtained between absorbance and concentration of cephalexin over the range of 10–100 μg/mL, with good correlation coefficient and small intercept (Table 1). The apparent molar absorptivity and Sandell's sensitivity for cephalexin were 2.675×10^3 L

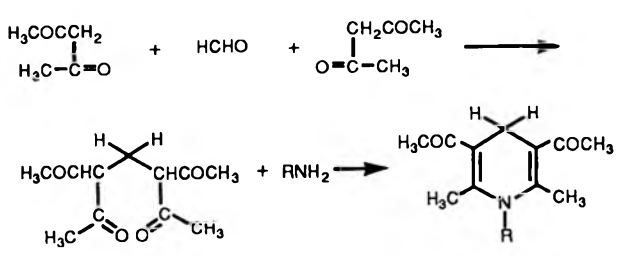

Figure 2. Scheme for the formation of chromogen due to the reaction of acetylacetone and formaldehyde with cephalexin (RNH_2).

Table 1. Numerical results for calibration curve of cephalexin

Standard cephalexin concn, μg/mL	Absorbance at 400 nm[a]
7.32	0.053
14.64	0.090
21.96	0.164
29.28	0.206
36.60	0.275
43.92	0.333
51.24	0.388
58.56	0.424
65.88	0.486
73.20	0.535
80.52	0.555
87.84	0.617
95.16	0.670
102.48	0.731
109.80	0.783

$r = 0.9895$
Intercept = –0.0051
Slope = 0.5463
$n = 15$

[a] Average of 5 determinations.

Table 2. Effect of reagent concentration on cephalexin absorbance

Reagent, mL	Cephalexin absorbance at 400 nm[a]
0.5	0.316
1.0	0.466
1.5	0.500
2.0	0.510
3.0	0.535
4.0	0.541
5.0	0.540
6.0	0.541

[a] Average of 3 determinations.

Table 3. Effect of reaction time on cephalexin absorbance

Time, min	Cephalexin absorbance at 400 nm[a]
5	0.258
10	0.400
15	0.471
20	0.516
30	0.540
40	0.541
60	0.545
120	0.546
180	0.545

[a] Average of 3 determinations.

Table 4. Effect of pH on color intensity of cephalexin

pH	Cephalexin absorbance at 400 nm[a]
3.5	0.380
4.0	0.469
4.1	0.538
4.3	0.541
4.5	0.543
4.8	0.506
5.0	0.422
5.3	0.180
5.6	0.080

[a] Average of 3 determinations.

Table 5. Effect of dilution by different solvents on absorption intensity of developed color

Solvent	Cephalexin absorbance at 400 nm[a]
Water	0.535
Ethanol	0.532
Methanol	0.527
Isopropanol	0.525
Dioxane	0.466
Acetonitrile	0.490
Dimethylformamide	0.508
Dimethyl sulfoxide	0.521

[a] Average of 3 determinations.

Table 6. Determination of cephalexin in presence of cephaloridine by proposed method[a]

Cephaloridine, mg	Cephalexin, %[b]
50	100.19
100	100.14
250	100.06
500	100.05
1000	100.01

[a] Cephalexin, 500 mg.
[b] Average of 3 determinations.

Table 7. Determination of cephalexin in presence of probenecid by proposed method[a]

Probenecid, mg	Cephalexin, %[b]
250	100.06
500	100.01
1000	99.90
1500	98.91
2000	98.89

[a] Cephalexin, 500 mg.
[b] Average of 3 determinations.

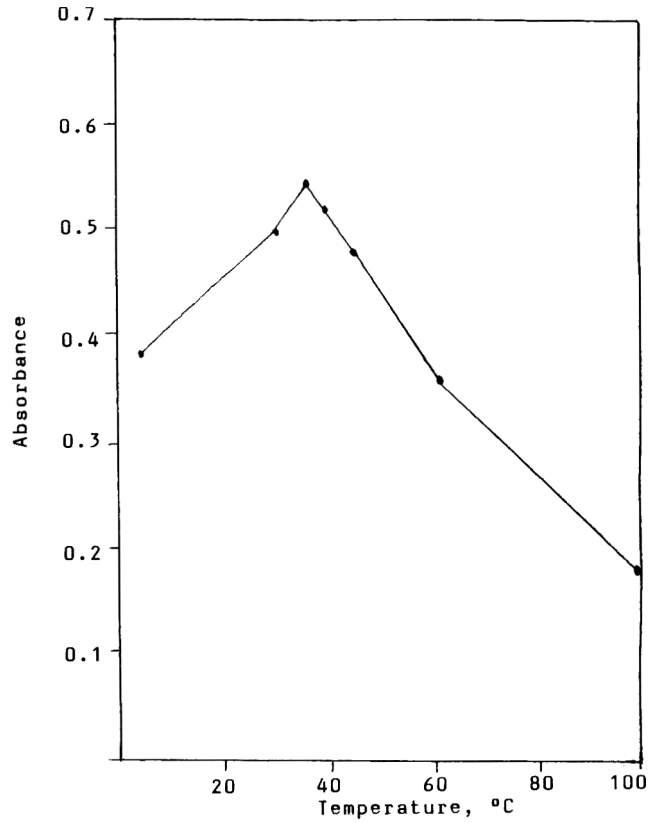

Figure 3. Effect of temperature on reaction.

$mol^{-1}cm^{-1}$ and 0.146 μg cm^{-2}/0.001A, respectively. The colored species was stable for 3 h at 35°C.

Optimum Reaction Conditions

Effect of reagent concentration.—The amount of reagent solution was varied to determine the volume required for optimum color intensity (Table 2). Results showed that 4 mL was optimum. On further increase in the volume of reagent solution, the absorbance remained constant. Therefore, 4 mL of reagent solution was used throughout this work.

Effect of reaction time.—Maximum color intensity was achieved by reacting cephalexin with acetylacetone–formalde-

Table 8. Determination of cephalexin in presence of 1000 mg each of excipients in synthetic mixtures by proposed method[a]

Excipient	Cephalexin, %[b]
Starch	99.95
Dicalcium phosphate	99.86
Talc	100.01
Magnesium stearate	99.68
Lactose	99.56

[a] Cephalexin, 500 mg.
[b] Average of 3 determinations.

Table 9. Assay of cephalexin in commercial preparations and bulk drug

Sample	Drug	Claimed, mg	Found, mg[a]		Rec., % ± SD
			Pharmacopeial method (11)	Proposed method	
Bulk drug	Cephalexin	—	99.84	100.03	100.03 ± 0.20
	Cephalexin	—	100.70	100.83	100.83 ± 0.17
Tablet	Cephalexin	125	126.52	126.00	100.80 ± 0.41
	Cephalexin	250	250.60	249.80	99.92 ± 0.36
	Cephalexin + PR[b]	250	252.15	251.93	100.77 ± 0.47
Capsule	Cephalexin	250	255.27	255.00	102.00 ± 0.29
	Cephalexin	500	502.00	501.80	100.36 ± 0.33
Oral suspension	Cephalexin	125	127.00	126.85	101.48 ± 0.56

[a] Average of 5 determinations.
[b] PR = Probenecid, 250 mg.

hyde reagent for 30 min at 35°C. Further standing, up to 180 min, caused no significant change in absorbance (Table 3).

Effect of pH.—Aqueous medium seems to be optimum for this reaction. Decrease in color intensity was observed by carrying out the reaction at lower pH (2.5, 3.0, and 3.5) or higher pH (5.0, 6.0, and 7.0) without any shift of λ_{max}. Optimum color intensity was obtained in the pH range 4.1–4.5. Therefore, pH 4.3 was selected for use throughout this work (Table 4).

Effect of temperature on color intensity.—The reaction of cephalexin with the reagent solution was carried out at elevated temperature. When the temperature was increased, the absorbance decreased. Therefore, this reaction was carried out at 35 ± 1°C (Figure 3).

Effect of dilution with different solvents.—Dilution of the colored products by solvents like ethanol, methanol, dioxane, dimethylfermamide, dimethyl sulfoxide, and water showed no effect on λ_{max}, but the intensity of absorption was influenced slightly. Water was used in this work (Table 5).

Selectivity of Reaction

Selectivity of the method was checked by examining the effect of acetylacetone–formaldehyde reagent on non-amino β-lactam cephalosporins. No color was produced with any of these compounds. This indicates the high selectivity of the method for β-lactam antibiotics with the primary amino group in the side chain.

The proposed procedure can detect impurities or distinguish cross-contamination from non-amino β-lactam cephalosporins (Table 6). The procedure would not be stability-indicating, because it cannot detect the cephalexin precursor, 7-amino-desacetoxy cephalosporanic acid.

Determination of Cephalexin in Presence of Probenecid

The proposed method could be used for the determination of cephalexin in the presence of different concentrations of probenecid up to the ratio 1:4, as shown in Table 7.

Interference

The proposed method is selective for the determination of cephalexin. Common excipients such as starch, talc, magnesium stearate, and dicalcium phosphate did not affect the development, intensity, or stability of the color (Table 8).

Table 10. Recovery of cephalexin in pharmaceutical formulations by official and proposed methods

Sample	Label claim	Cephalexin added, mg	Found, mg[a]		Rec., % ± SD
			Official method (11)	Proposed method	
Tablet	125 mg/tab	25	148.60	149.00	119.20 ± 0.49
	125 mg/tab	50	174.85	175.62	140.50 ± 0.54
	250 mg/tab	50	299.15	301.00	120.40 ± 0.51
	250 mg/tab	100	350.09	350.75	140.30 ± 0.60
Capsule	250 mg/cap	50	298.80	300.25	120.10 ± 0.39
	250 mg/cap	100	349.50	350.86	140.40 ± 0.47
	500 mg/cap	50	549.80	550.45	110.10 ± 0.42
	500 mg/cap	100	599.00	601.68	120.30 ± 0.49
Oral suspension	125 mg/5 mL	25	148.37	149.59	119.70 ± 0.60
	125 mg/5 mL	50	174.75	175.09	140.10 ± 0.65

[a] Average of 5 determinations.

Analysis of Pharmaceutical Preparations

The proposed method was applied successfully to the determination of cephalexin in tablets (either singly or in combination with probenecid), capsules, and oral suspension. Commonly encountered excipients did not interfere. The results obtained compared favorably with those by the official method (11) (Table 9).

Reliability and suitability of the proposed method were confirmed by adding known quantities of cephalexin to various preanalyzed cephalexin formulations and by analyzing the mixtures by the proposed method.

The recoveries of cephalexin are shown in Table 10.

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ENVIRONMENTAL QUALITY

Comparison of Analytical Methods Used to Determine Metal Concentrations in Environmental Water Samples

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Al, Cd, Cu, Fe, Pb, Mn, and Zn were determined in water samples collected from Clear Creek and North Clear Creek, CO, that had been impacted by acid mine drainage. These samples contained a wide range of total suspended solid concentrations. The samples were prepared and digested by dissolved, acid-soluble, total recoverable, U.S. Environmental Protection Agency (EPA total), and Contract Laboratory Program (CLP) total procedures. The CLP method resulted in the highest reported metal concentrations for 67% of the analyses (primarily Al, Cd, Cu, and Fe), the EPA total method for 11% of the analyses (mainly Mn and Zn), and the acid-soluble method for 9% of the analyses. For the remainder (13%), 2 or more methods resulted in similar metal concentrations. The same analyses using the Three Kids Mine standard reference material demonstrated that the EPA total and total recoverable methods underestimate the maximum metal concentrations in sediment-bearing water samples. In addition, use of acid-soluble or total methods is inappropriate to determine compliance with aquatic water quality standards that were originally promulgated on the basis of dissolved concentrations.

Sampling of anthropogenically impacted surface and groundwaters has proliferated since passage of the 1976 Resource Conservation Recovery Act (RCRA) and the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA). However, despite efforts to ensure analytical reproducibility and applicability, a number of different methods are used to measure total aqueous metal concentrations and to evaluate conformity of stream and groundwaters to aquatic water quality criteria (AWQC).

The most common assays used in environmental monitoring programs consist of total and dissolved methods, operationally defined as an unfiltered sample and a sample passed through a 0.45 μm filter, respectively. Total metals in an environmental aqueous sample are commonly measured using either an EPA total (1), an EPA total recoverable (1), or a contract laboratory program (CLP) total (2) method, all of which invoke similar analytical techniques but use different sample preparation and digestion procedures (Table 1). In addition to discrepancies in total metals analysis, the appropriate methodology for use in evaluating compliance with AWQC remains uncertain.

Originally, standards (3) were promulgated on the basis of bioassays using the dissolved protocol (1) to report concentrations to which target organisms responded. However, this technique is not used unilaterally in aqueous sample analysis across the United States to establish conformity with the applicable standards. For example, the State of Montana uses the acid-soluble method (4), deemed analogous to a "bioavailable fraction," to determine compliance with AWQC standards. In addition, Federal AWQC for As, Cd, Cu, and Pb are based on acid-soluble concentrations, whereas the same criteria for Ag, Cr, Ni, and Zn are based on total recoverable concentrations.

It is also possible that the efficacy of analytical methods may vary depending on the refractory nature of any sediment entrained in the water column upon sample collection. For example, sediment in which precipitated amorphous iron hydroxide predominates is liable to release more metal per gram during sample digestion than the same mass of quartz hosting the same absolute metal concentration. In the case of waters containing low concentrations of total suspended solids (TSS), the total and dissolved concentrations should be similar and independent of the digestion procedure. However, samples with high TSS concentrations should show increasing metal concentrations as the particulate fraction in the sample is subjected to an increasingly vigorous digestion.

At least 3 designated total methods are commonly used to digest and analyze aqueous samples, all of which involve slightly different analytical protocols that can result in inconsistencies in the reported metal concentrations. For example, apart from the different strengths of the acids used in the digestion in the EPA total method (step a), there is no defined sample volume requirement, whereas in step e, an undetermined mass of nitric acid may be added (Table 1). Consequently, different metal recoveries may be reported by different laboratories using the same method, depending on the refractory nature of the material and the different methods of sample treatment during the digestion phase. The objective of this paper is to com-

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Table 1. Summary of analytical protocols used in study

Step	CLP total	EPA total	Total recoverable	Acid soluble	Dissolved
Field filtering	None	None	None	None	Yes (0.45 μm)
Field preservation	HNO_3	HNO_3	HNO_3	HNO_3	HNO_3
	pH < 2	pH < 2	pH < 2	pH = 1.75–2.0	pH < 2
Holding time	Usually <6 mo for metals	Usually <6 mo for metals	Usually <6 mo for metals	3 days	Usually <6 mo for metals
Laboratory filtering	After digestion	After digestion	After digestion	Yes (0.45 μm after 16 h)	None
Laboratory digestion	ICP/flame AA:				Depends on specification: can directly analyze or any of the total preparations may be followed.
	a. 100 mL samples	a. 50–100 mL sample	a. 100 mL sample	a. Let stand for 16 h at pH = 1.75 ± 0.1	
	b. 2 mL 1:1 HNO_3 10 mL 1:1 HCl	b. 3 mL concd HNO_3	b. 5 mL 1:1 HCl	b. Filter through 0.45 μm filter	
	c. Digest to 25–50 mL	c. Digest to near dryness	c. Digest to 15–20 mL		
	d. Filter	d. 3 mL concd HNO_3	d. Filter		
		e. Add more HNO_3 and digest to near dryness	*Note:*		
	Furnace:	f. 5 mL 1:1 HCl or HNO_3	HCl is omitted for furnace analysis		
	a. 100 mL	g. Digest			
	b. 1 mL H_2O_2 (30%)	h. Filter			
	c. Digest to 25–50 mL				
	d. Filter				
Reference	(2)	(1)	(1)	(4)	(2)

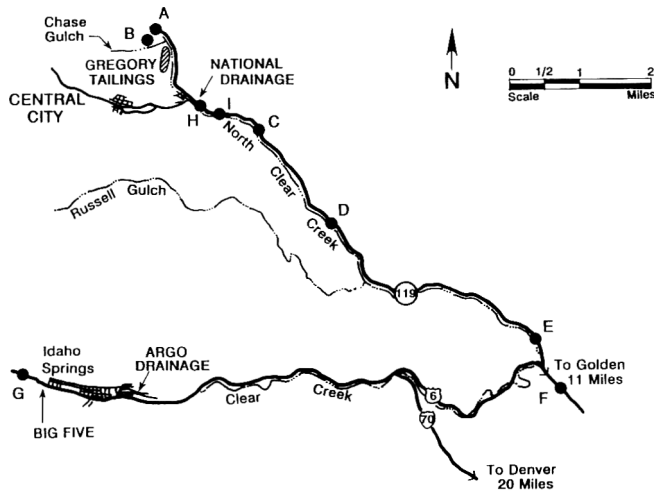

Figure 1. Clear Creek study area, showing sample sites.

pare the analytical methods commonly used to determine metal concentrations in environmental aqueous samples from the perspective of method compatibility and applicability to water quality criteria.

Methodology

The study was conducted at the Clear Creek/Central City Superfund site, located approximately 30 miles west of Denver, CO, in the northeast portion of the Colorado Mineral Belt. The site consists of the streams and tributaries of Clear Creek and North Clear Creek, and the adjacent tailings and waste rock piles (Figure 1). The study area contains approximately 800 abandoned mines, 25 active mining operations, and at least 4 perennial tunnel adit discharges that have historically contributed acidic, metalliferous waters to Clear Creek.

Samples A–G (27–564 mg/L TSS) were collected across the 3 m wide creek channel in clean plastic buckets (Alconox rinse, followed by 1% HNO_3 and deionized water rinse); 2 additional samples, H and I, were collected in the vicinity of the National Drainage by agitating the bed sediment to provide higher TSS solutions and then sampling the overlying water column downstream. Sample H (TSS = 2480 mg/L) was composed principally of sand in the solid fraction; sample I (TSS = 1600 mg/L) contained iron oxide, which had encrusted the stream bed.

At each site, one subsample, representing the dissolved metal fraction, was filtered through a 0.45 μm nitrocellulose filter and immediately acidified to pH 2.0. A second bulk sample, the acid-soluble fraction, was acidified to pH 2.0 with HNO_3 and then filtered through a 0.45 μm nitrocellulose filter by the analytical laboratory. Three additional subsamples remained unfiltered but were immediately acidified with HNO_3 to pH 2.0 for later digestion and analysis by EPA total, CLP total, and EPA total recoverable methods.

Sample J was prepared by mixing 6.7 g of Three Kids Mine (TKM) waste rock standard reference material (SRM) obtained from the Environmental Monitoring Support Laboratory (EMSL), Las Vegas, NV, in 4.5 L deionized water (TSS = 1489 mg/L). The slurry was stirred continuously, and samples were

collected and treated in a manner analogous to those collected from Clear Creek and North Clear Creek.

The TKM SRM has been analyzed by the CLP total method a number of times by different laboratories (5). Consequently, the metal content, as reported according to this protocol, is well known. The TKM sample was included to compare the digestion procedures used in this study and the true total value reported by EPA for the SRM. Although the TKM SRM is a solid, its use in solution is appropriate because it simulates the dissolution of waste rock that erodes into adjacent creeks in many mineralized areas of the western United States.

On the basis of the known total concentration of the standard sample as reported by EPA, the equivalent theoretical total metal recovery may be calculated as follows:

$$\text{Calculated recovery (g/L)} = \frac{\text{mass of standard (g)} \times \text{true metal concentration (g/g)}}{\text{Volume of fluid (L)}} \quad (1)$$

Using Pb as an example:

$$\mu\text{g Pb/L} = \frac{6.9\text{ g} \times 5830\ \mu\text{g/g}}{4.5\text{ L}} = 8939\ \mu\text{g/L} \quad (2)$$

This value represents the Pb concentration present in the solution based on the reported solid concentrations, assuming that particulate material was distributed homogeneously throughout the parent solution. The EPA-sanctioned concentrations (5) may then be compared with the concentrations reported for each metal determined in this study to calculate the digestion efficiency of the various techniques.

To ensure that the data were valid and representative, a comprehensive field and laboratory quality assurance/quality control (QA/QC) program was incorporated into the sampling and analytical design. All samples were analyzed within 2 weeks of sample collection, well within the 6-month holding time established for metals by EPA (6). Comparison between bottle blanks and field equipment blanks demonstrated that decontamination procedures were effective (all analyses were below method detection limits) except for low concentrations (14 µg/L) of Zn carried over in the field blank. This is not problematic, because for all samples except the TKM SRM, it represents <5% of the lowest recorded Zn concentration in any sample collected in the field. The TKM SRM contained a lower Zn level (30 µg/L) in the dissolved fraction concentrations; however, this sample was prepared in the laboratory after careful cleaning of all filtering equipment.

With the exception of cadmium, all analyses were conducted by inductively coupled plasma (ICP) techniques at ENSECO Laboratory, Arvada, CO, according to EPA–CLP-prescribed QA/QC protocols (7). Cadmium was determined by graphite furnace atomic absorption (GFAA), following the same protocols (7). In addition, sample duplicates (splits of 1 sample) and blind standard analyses were performed through both routine analytical service (RAS) and special analytical services (SAS) requests to the EPA and CLP.

The laboratory QA/QC samples run with each batch of 20 samples included matrix spike, matrix spike duplicate, serial dilution, laboratory control samples, and preparation blanks. Each set of analyses was performed on a separate batch of samples; therefore, each sample set had its own QA/QC. Typical results are provided in Table 2. The relative percent difference for EPA total metal concentrations based on duplicate samples for all analytes were <17%, within the criteria of 20% established by EPA for water samples. Analytical matrix spike recoveries using standards traceable to NBS standards (e.g., Metals Control WP 284-2), at concentrations that were approximately 10% of the reported value, were between 90 and 110%, with the exception of Pb in the total recoverable analysis (114%); therefore, these analyses are accurate within 10% of the actual concentration (Table 2), and the ICP and GFAA signal responses were due to the analyte. Overall, the QA/QC program demonstrated that these data (Table 3) are accurate, precise, reproducible, and acceptable without qualification.

Results

The metals considered in this study were Al, Cd, Cu, Fe, Mn, Pb, and Zn. The concentration of other metals (Ag, As, Ba, Be, Cr, Co, Hg, Ni, Sb, and V) were determined but either were below the detection limit or failed to meet QA/QC criteria. Generally, reported metal concentrations increased in the order of digestion rigor, i.e., dissolved < acid-soluble < EPA total recoverable < EPA total < CLP total (Table 3), corresponding to the increasing strength of the acidic solution used in the digestion routine. Assuming that the method resulting in the highest metal concentration is most representative of the actual total in the sample, the CLP method resulted in the reported maximum total for the 7 metals 47 of 70 times; the EPA total method, 8 times; the acid-soluble method, 6 times; and the EPA total recoverable method, 2 times. The highest concentrations were reported by the acid-soluble and CLP methods 5 times; by both EPA and CLP total methods 1 time; and by EPA, CLP, and acid-soluble methods 1 time.

Maximum Mn concentrations were reported by 4 different methods. Generally, the acid-soluble method resulted in the highest concentration at locations that were representative of background conditions (sample A) or that were a mile or more downstream from a source of tailings and waste rock, namely, the low-TSS samples C, D, E, F, and G. This suggests that the extraction efficiency of the acid-soluble procedure is as effective as any of the total methods for waters containing low suspended solids (<200 mg/L). In contrast, sample I was a high-TSS water (1600 mg/L) for which the CLP method was most efficient at solubilizing Mn. With the exception of sample F, the CLP total procedure resulted in the highest Al concentrations and generally provided the highest reported concentration for Cd, Cu, and Pb.

Iron in the Clear Creek system precipitates from aqueous solution as an amorphous solid (8) that can adsorb trace metals under neutral pH values (9), and desorb these metals under more acidic conditions (10). In the Clear Creek environment,

Table 2. Representative quality assurance/quality control data

Analyte	Bottle blank	Equip. decont. blank (mg/L)[a]	Clear Creek sample A duplicate recoveries (mg/L)		Relative percent difference	Matrix spike recoveries (sample E)									
						Dissolved		Acid-soluble		Total recoverable		EPA total[b]		CLP total	
						Sample	Spike, %	Sample	Spike, %	Sample	Spike, %	Sample	Spike, %	Sample	Spike, %
Al	<20	<20	526	495	6	60	101	400	109	838	102	640	90–110	1000	102
Cd	<0.1	<0.1	1.7	1.6	6	3.5	100	3.4	97	2.9	95	3.9	90–110	4.0	94
Cu	<4	<4	48	49	2	14	105	103	102	93	106	83	90–110	108	105
Fe	<26	<26	1770	1980	11	<26	96	3160	101	3910	97	3300	90–110	4480	97
Mn	<4	<4	1000	1060	6	1260	98	1360	103	1190	100	1100	90–110	1290	98
Pb	<5	<5	5.8	6.9	17	<5	100	12	106	16	114	21	90–110	20	96
Zn	<2	14	531	457	15	766	100	1060	108	899	95	880	90–110	1000	94

[a] Equipment decontamination blank.
[b] Spike determination for routine analytical service (RAS) requests were all within the contract prescribed limits.

amorphous ferric hydroxide was observed to have precipitated at stations B, H, and I in the bed sediment. Maximum Fe concentrations generally resulted from the most vigorous digestion procedure.

Some of the metals (e.g., Cd, Cu, and Zn) appear to correlate with Fe in the dissolved and acid-soluble treatments (Table 4), indicating that these metals are sorbed to, or co-precipitated with, the easily dissolved amorphous ferric hydroxide present in the entrained and bed sediment fractions. As the digestion procedure became more vigorous, Fe no longer correlated with Cd, Cu, and Zn. These data demonstrate that the amorphous ferric hydroxide matrix is more refractory and is not dissolved until a laboratory digestion step more vigorous than the acid-soluble treatment is included in the sample preparation.

At the highest TSS values (1600 mg/L for sample I and 2480 mg/L for sample H), the total metal concentrations are substantially higher than the dissolved concentrations, suggesting that much of the sediment in the sample is susceptible to attack by acid digestion. A comparison of the total digestion procedures indicates that the CLP method resulted in the highest metal concentrations for samples consisting principally of amorphous ferric hydroxide (samples H and I).

The acid-soluble fraction resulted in the highest reported Zn concentrations for 5 of the 9 samples (A, D, E, F, and G); however, all were within 10% of the CLP value. These 5 samples were collected from sites representing background conditions (A and G) or several miles from a source area (Samples D, E, and F). Samples collected from areas immediately downstream from tailings and waste rock piles (e.g., H) all had the highest reported metal concentrations in the CLP fraction. This observation suggests that the distance from a metal source and the type of mineral matrix are also important factors in assessing the efficacy of the recommended digestion procedure.

For the TKM SRM, the highest reported metal concentrations, with the exception of Cd, were obtained by the CLP total method, whereas the total recoverable method results in the lowest concentrations (except for Pb). The CLP and total recoverable methods generally bracket the reported value obtained by the EPA total procedure. Except for Cd and Pb, which were at the lower end of the reported range, the CLP total method resulted in values for the SRM that were within 10% of the upper end of the reported range (Table 3). These data demonstrate that the use of the TKM SRM in the slurry form provides metal concentrations consistent with the range reported by EPA (5).

Conclusions

This study shows that differences exist in the maximum concentrations of Al, Cd, Cu, Fe, Mn, and Zn determined by 3 different techniques designed to determine the total metal concentration. Of the 3 methods, the CLP total method is more representative of the true total metal concentration for Al, Cu, Fe, Mn, and Zn. Consequently, for environmental samples, the total recoverable and EPA total methods may underestimate total metal concentrations in solution. In fact, if the objective is

Table 3. Analytical data (μg/L) by site and method

Sample (TSS, mg/L)	Preparation method	Al	Cd	Cu	Fe	Mn	Pb	Zn
A (36)	Dissolved	86	1.8	14	45	3.5×10^2	6.1	3.3×10^2
	Acid-soluble	1.8×10^2	1.6[a]	23	3.9×10^2	4.4×10^2	9.9	$3.8^a \times 10^2$
	Total recoverable	7.8×10^2	1.2	17	1.5×10^3	3.4×10^2	17	2.9×10^2
	EPA total	5.9×10^2	1.5	20	1.3×10^3	4.1×10^2	18[a]	3.6×10^2
	CLP total	$9.1^a \times 10^2$	1.6[a]	24[a]	$1.7^a \times 10^3$	$4.4^a \times 10^2$	17	$3.8^a \times 10^2$
B (564)	Dissolved	89	5.0	15	1.5×10^2	8.2×10^2	10	8.3×10^2
	Acid-soluble	1.5×10^3	9.5	98	2.5×10^3	1.3×10^3	2.7×10^2	1.9×10^3
	Total recoverable	9.4×10^3	9.0	2.3×10^2	4.3×10^4	1.3×10^3	7.1×10^2	2.0×10^3
	EPA total	9.8×10^3	1.3	2.4×10^2	4.5×10^4	1.4×10^3	1.0×10^2	2.1×10^3
	CLP total	$1.2^a \times 10^4$	11[a]	$2.7^a \times 10^2$	$5.1^a \times 10^4$	$1.5^a \times 10^3$	$2.0^a \times 10^3$	$2.2^a \times 10^3$
C (158)	Dissolved	56	4.8	16	3.8×10^3	2.2×10^3	<5.0	1.1×10^3
	Acid-soluble	9.1×10^2	5.0	1.9×10^2	1.0×10^4	$2.4^a \times 10^3$	28	1.3×10^3
	Total recoverable	1.8×10^3	3.9	1.7×10^2	1.2×10^4	1.8×10^3	1.0×10^2	1.0×10^3
	EPA total	1.7×10^3	5.5[a]	1.8×10^2	1.3×10^4	2.0×10^3	64	1.2×10^3
	CLP total	$3.3^a \times 10^3$	5.5[a]	$2.6^a \times 10^2$	$1.9^a \times 10^4$	$2.4^a \times 10^3$	87[a]	$1.5^a \times 10^3$
D (124)	Dissolved	75	4.0	13	2.6×10^2	2.2×10^3	<5.0	9.3×10^2
	Acid-soluble	1.3×10^3	5.5[a]	2.5×10^2	1.3×10^4	$2.3^a \times 10^3$	34	$1.4^a \times 10^3$
	Total recoverable	2.1×10^3	4.4	2.4×10^2	1.6×10^4	2.1×10^3	91	1.3×10^3
	EPA total	2.2×10^3	5.5[a]	2.3×10^2	1.6×10^4	1.9×10^3	64	1.2×10^3
	CLP total	$3.0^a \times 10^3$	5.5[a]	$2.7^a \times 10^2$	$1.9^a \times 10^4$	$2.2^a \times 10^3$	$1.0^a \times 10^2$	$1.4^a \times 10^3$
E (27)	Dissolved	60	3.5	14	<26	1.3×10^3	<5.0	7.7×10^2
	Acid-soluble	4.0×10^2	3.4	1.0×10^2	3.2×10^3	$1.4^a \times 10^3$	12	1.1×10^3
	Total recoverable	8.4×10^2	2.9	93	3.9×10^3	1.2×10^3	16	9.0×10^2
	EPA total	6.4×10^2	3.9	83	3.3×10^3	1.1×10^3	21[a]	8.8×10^2
	CLP	$1.0^a \times 10^3$	4.0[a]	$1.1^a \times 10^2$	$4.5^a \times 10^3$	1.3×10^3	20	$1.0^a \times 10^3$
F (36)	Dissolved	86	2.3	15	<26	1.7×10^3	<5.0	2.9×10^2
	Acid-soluble	5.8×10^2	3.0	92	3.5×10^3	$1.9^a \times 10^3$	9.1	$8.3^a \times 10^2$
	Total recoverable	6.3×10^2	2.4	86	3.5×10^3	1.7×10^3	18	7.2×10^2
	EPA total	$9.6^a \times 10^2$	2.9	90	$4.6^a \times 10^3$	1.7×10^3	25[a]	7.7×10^2
	CLP total	9.2×10^2	3.5[a]	$1.1^a \times 10^2$	4.4×10^3	1.8×10^3	15	7.9×10^2
G (188)	Dissolved	4.2×10^3	28	9.7×10^2	5.6×10^3	1.8×10^4	6.4	8.5×10^3
	Acid-soluble	4.8×10^3	28	$1.0^a \times 10^3$	3.1×10^4	$1.9^a \times 10^4$	42	$9.1^a \times 10^3$
	Total recoverable	6.1×10^3	22	8.6×10^2	2.9×10^4	1.6×10^4	35	7.2×10^3
	EPA total	6.0×10^3	26	9.1×10^2	3.0×10^4	1.8×10^4	24	8.3×10^3
	CLP total	$7.2^a \times 10^3$	32[a]	$1.0^a \times 10^3$	$3.4^a \times 10^4$	1.8×10^4	51[a]	8.4×10^3
H (2480)	Dissolved	58	4.2	14	1.9×10^3	1.8×10^3	<5.0	9.5×10^2
	Acid-soluble	1.8×10^3	7.5	5.6×10^2	1.2×10^4	2.4×10^3	1.4×10^2	1.6×10^3
	Total recoverable	7.6×10^3	11	8.8×10^2	2.6×10^4	3.0×10^3	$4.7^a \times 10^2$	2.2×10^3
	EPA total	9.3×10^3	12[a]	$1.1^a \times 10^3$	3.4×10^4	$3.5^a \times 10^3$	2.7×10^2	2.7×10^3
	CLP total	$1.3^a \times 10^4$	11	9.6×10^2	$6.0^a \times 10^4$	3.1×10^3	2.9×10^2	$3.4^a \times 10^3$
I (1600)	Dissolved	81	5.5	11	3.6×10^3	2.9×10^3	<5.0	1.4×10^3
	Acid-soluble	1.3×10^3	7.0	3.6×10^2	3.7×10^4	3.3×10^3	1.9×10^2	1.8×10^3

Table 3. ***Continued***

Sample (TSS, mg/L)	Preparation method	Al	Cd	Cu	Fe	Mn	Pb	Zn
	Total recoverable	1.6×10^4	8.0	6.6×10^2	2.1×10^5	3.2×10^3	2.2×10^2	2.7×10^3
	EPA total	1.2×10^4	8.5	5.5×10^2	1.8×10^5	2.8×10^3	2.4×10^2	2.2×10^3
	CLP	$2.0^a \times 10^4$	11^a	$8.1^a \times 10^2$	$2.5^a \times 10^5$	$3.8^a \times 10^3$	$6.1^a \times 10^2$	$3.3^a \times 10^3$
J (1489)	Dissolved	52	2.0×10^{-1}	7.4	<20	61	<5.0	30
	Acid-soluble	1.1×10^3	$5.0^a \times 10^{-1}$	1.4×10^2	97	1.2×10^4	<5.0	1.4×10^2
	Total recoverable	2.1×10^4	3.0×10^{-1}	3.2×10^2	1.2×10^4	6.2×10^4	3.7×10^2	5.0×10^2
	EPA total	2.6×10^4	3.0×10^{-1}	4.0×10^2	1.5×10^4	1.3×10^5	3.2×10^3	6.6×10^2
	CLP total	$3.2^a \times 10^4$	2.0×10^{-1}	$5.2^a \times 10^2$	$2.4^a \times 10^4$	$1.9^a \times 10^5$	$6.9^a \times 10^3$	$8.5^a \times 10^2$
Av. SRM value[b]		1.5×10^4	<1.0	2.7×10^2	1.1×10^4	9.2×10^4	5.8×10^3	4.3×10^3
Calculated rec.	Low	1.2×10^4	<1.0	3.4×10^2	9.1×10^3	1.1×10^5	6.6×10^3	4.9×10^2
	Av.	2.3×10^4	<1.5	4.1×10^2	1.7×10^4	1.4×10^5	8.9×10^3	6.5×10^2
	High	3.5×10^4	12	4.8×10^2	2.5×10^4	1.8×10^5	1.1×10^4	8.2×10^2
Difference[c]		6	n.c.[d]	8	4	7	4	3

[a] Values represent highest reported metal concentrations by method for each sample (see Figure 1 for site location).
[b] Ref. 5, mg/kg.
[c] Percent difference between Sample J CLP total data and the closer of the low or high end of the range calculated on the basis of EPA reported CLP solid concentration (5) recalculated using Eq.1.
[d] Noncalculable.

Table 4. Pearson correlation matrix by treatment

Treatment	Metal	Cd	Cu	Fe	Pb
Dissolved[a]	Cu	0.98	—	—	—
	Fe	0.77	0.69	—	—
	Pb	0.19	0.15	−0.10	—
	Zn	0.99	0.99	0.77	0.14
Acid-soluble[a]	Cu	0.90	—	—	—
	Fe	0.63	0.72	—	—
	Pb	0.20	0.09	0.30	—
	Zn	0.99	0.99	0.62	0.06
EPA total recoverable[a]	Cu	0.73	—	—	—
	Fe	0.20	0.41	—	—
	Pb	0.13	0.31	0.19	—
	Zn	0.96	0.72	0.24	−0.02
EPA total[a]	Cu	0.77	—	—	—
	Fe	0.18	0.33	—	—
	Pb	−0.28	0.09	−0.06	—
	Zn	0.95	0.71	0.17	−0.21
CLP total[a]	Cu	0.75	—	—	—
	Fe	0.20	0.54	—	—
	Pb	−0.28	−0.07	−0.07	—
	Zn	0.99	0.83	0.28	−0.25

[a] The 5% level of significance for n = 10 is 0.58 (12).

to determine the maximum total metal concentration, it is necessary to understand both the sample mineralogy and the source of the material before an analytical method is selected. For example, the CLP method resulted in the highest metal concentration when the solid fraction entrained in the water column originated from the tailings and waste rock, whereas farther downstream, other methods resulted in higher concentrations, presumably because of the type of suspended solid (11). This is an important distinction, because it presupposes that a sampling program will be designed for a specific purpose, with a comprehension of how data from samples analyzed for total metals are to be used.

Finally, the analytical procedures used to promulgate AWQC should be carefully reviewed. Because the CLP total method appears to be a more vigorous procedure in terms of these weak acid digestions, consideration should be given to using this method rather than the EPA total method if the objective is to determine the maximum metal concentration in aqueous environmental samples. However, if the objective is to evaluate compliance with regulatory standards, the dissolved method, rather than the acid-soluble or total recoverable method, should be selected on the basis of the criteria used to establish the aqueous metal concentrations in the original toxicological investigation (3).

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ENVIRONMENTAL QUALITY

Extractive Spectrophotometric Determination of Trace Amounts of Sulfur Dioxide in Air

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A sensitive spectrophotometric method was developed for the determination of trace amounts of sulfur dioxide (SO_2) in air after SO_2 has been fixed in a buffered formaldehyde solution. The reaction of iodate with the fixed SO_2 in the presence of an acid and an excess of chloride leads to the formation of ICl_2^- ions. The resulting ICl_2^- species forms an ion-pair with pararosaniline cation; the product is extracted into isopentyl alcohol and measured spectrophotometrically at 560 nm. The color system obeys Beer's law over the range 0–40 μg SO_2. The color is stable for 72 h from the time of extraction. The molar absorption coefficient of the color system is $4.5 \times 10^3 Lmol^{-1}cm^{-1}$. The coefficient of variation is 3.6% for 10 determinations at 20 μg SO_2. The effect of interfering gases on the determination is discussed. The method was applied to the determination of SO_2 at low concentrations, and the results obtained were compared with the widely used West and Gaeke method. The method can be used to determine as low as 2 μg SO_2.

Air pollution due to sulfur dioxide (SO_2) has mainly been a consequence of the widespread use of sulfur and its compounds in manufacturing and industrial processes and the increased use of fossil fuels as a source of energy. Because the presence of SO_2 in ambient air is known to be a health hazard, the development of analytical methods for its determination has attracted considerable attention.

The West and Gaeke method (1), because of its simplicity, sensitivity, and specificity, has been widely used for the colorimetric determination of SO_2 in the atmosphere after the SO_2 has been absorbed in 0.1M tetrachloromercurate (TCM) solution. However, the analysis must be carried out carefully with close attention to temperature and pH (2). Further, the TCM absorber has some disadvantages, such as the use of highly toxic mercury(II) chloride and the instability of the fixed SO_2 complex (3). To overcome these disadvantages, Dasgupta et al. (4) advocated the use of a buffered formaldehyde solution for trapping SO_2. The trapped SO_2 is stable for 30 days without appreciable loss of the fixed SO_2, and the absorbent permits a sampling rate of ≤0.3 L/min for quantitative collection of atmospheric SO_2. Later, Dasgupta (5) modified his trapping solution by incorporating 0.1 mM disodium 1,2-cyclohexylenedinitrilotetraacetic acid into the buffered formaldehyde solution to mask the unusual interference due to Mn(II), and recommended a sampling rate ≤0.4 L/min for quantitative collection of atmospheric SO_2. Recently, we reported the use of a modified buffered formaldehyde solution (6) for the quantitative collection of SO_2 from the atmosphere at a sampling rate of 0.5 L/min for 6 h.

Many sensitive methods have been reported for the determination of SO_2 based on its reducing property (7–9). Recently, 2 methods have been reported, both of which are based on the interaction of the fixed SO_2 with iodate in a chloride-containing acidic medium. The method reported by Balasubramanian and Kumar (6) is based on the reaction of the fixed SO_2 with iodate in an acidic medium and excess chloride to form ICl, which is stabilized as ICl_2^- ion. The ICl formed is used to iodinate 2′,7′-dichlorofluorescein to form 2′,7′-dichloro-4′,5′-diiodofluorescein. The iodinated product is extracted into a solvent mixture of 15% isopentyl acetate in isopentyl alcohol and measured spectrophotometrically at 535 nm. The color system is stable for 90 min after extraction. Selvapathy et al. (10) reported a sensitive method based on the ion-pair formation between the anionic chloro complex of iodine and the pyronine G cation. The anionic chloro complex of iodine is generated by the interaction of fixed SO_2 with iodate in a chloride-containing acidic medium. The ion pair is extracted into benzene, and the absorbance is measured at 535 nm. Even though the method is highly sensitive ($\varepsilon = 4.5 \times 10^4 Lmol^{-1}cm^{-1}$), it has the disadvantages of high blank absorbance (0.16A) and poor color stability (absorbance measurements must be completed within 10 min after extraction).

This paper describes the study and evaluation of the variables that govern the formation of an ion pair between ICl_2^-, generated by the reaction between iodate and the fixed SO_2, and pararosaniline cation, to provide the basis for a sensitive spectrophotometric method for the determination of SO_2.

Materials and Methods

Apparatus

The absorbance was measured by using a Carl Zeiss PMQ II spectrophotometer with 10 mm quartz cells. Fritted glass

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bubblers with suitable suction devices were used for trapping SO_2 from air. The flow rate of the air was measured by a rotameter.

Reagents

All chemicals used were analytical reagent grade, and distilled water was used for preparing the reagent solutions.

(a) *Sulfur dioxide standard solution.*—5 μg/mL. Dissolve 0.2 g anhydrous sodium sulfite in 500 mL water, and standardize iodimetrically (11). Dilute suitable volume of this solution with 7mM buffered formaldehyde solution to give a 5 μg/mL solution of SO_2. This solution remains stable for at least a month.

(b) *Pararosaniline hydrochloride solution.*— 0.001%. Dissolve 0.025 g pararosaniline hydrochloride (BDH, England) in 125 mL 5M sulfuric acid, and dilute to 250 mL with water in calibrated flask. Dilute 25 mL of this solution to 250 mL with 2.5M sulfuric acid in calibrated flask. Solution is stable for 1 month if stored in brown bottle.

(c) *Sulfuric acid.*—5.5M. Dilute 306 mL sulfuric acid (specific gravity, 1.84) to 1 L with water.

(d) *Buffered formaldehyde trapping solution.*—Dilute 530 μL formaldehyde solution (37%), 1.36 g sodium acetate trihydrate, and 600 μL acetic acid to 1 L with water. Solution is 7mM in formaldehyde and 10mM in sodium acetate and acetic acid; pH 4.76 at 25°C.

(e) *Potassium iodate solution.*—0.4% m/v.

(f) *Sodium chloride solution.*—6% m/v.

(g) *Sulfamic acid solution.*—5% m/v.

(h) *Sodium hydroxide solution.*—4.5M.

(i) *Isopentyl alcohol.*—Solvent for extraction.

Sampling

Air samples were collected by drawing 20–200 L air through fritted glass bubblers containing 20 mL buffered formaldehyde trapping solution for 40–400 min at 0.5 L/min. Solution was diluted to 50 mL with trapping solution before determination.

Determination

Into 50 mL calibrated flask, 5 mL 0.4% potassium iodate, 2 mL 5.5M sulfuric acid, and 1 mL 6% sodium chloride solution were added. A 15 mL aliquot of buffered formaldehyde trapping solution containing ≤40 μg SO_2 (fixed as formaldehyde–hydrogen sulfite addition compound) was treated with 1 mL 4.5M sodium hydroxide to decompose the adduct. Solution was then introduced into 50 mL calibrated flask through long-stemmed funnel with tip kept well immersed into reagent solution to avoid SO_2 loss. Solution was mixed, and 2.5 mL 0.001% pararosaniline (PRA) solution was added. Solution was diluted with water to 50 mL final volume and allowed to stand for 5 min. It was then transferred into 125 mL separatory funnel and extracted with 5 mL isopentyl alcohol for 1 min. Organic layer was separated, transferred into test tube, and treated with ca 1 g anhydrous sodium sulfate to remove trace amounts of water. Absorbance of organic extract was measured at 560 nm in 10 mm cells against reagent blank run through entire procedure. SO_2 concentration was established by reference to calibration graph prepared by treating 0–8 mL standard SO_2 solution (0–40 μg SO_2) with 15 mL buffered formaldehyde trapping solution and proceeding as described previously.

Results and Discussion

In this study, SO_2 was fixed in the modified trapping solution that we developed (6). This trapping solution, consisting of 7mM formaldehyde buffered at pH 4.76 with 10mM sodium acetate–acetic acid buffer, permitted a sampling rate of 0.5 L/min for a period of 6 h when 15 mL of the solution was used for sampling. The sampling period can be increased to 8 h when 20 mL trapping solution is used instead of 15 mL. Preliminary studies were carried out with a 5 mL solution of 0.4% potassium iodate, 2 mL 5.5M sulfuric acid, 1 mL 6% sodium chloride, and 2.5 mL 0.001% PRA solution. The resulting solution, which contained 20 μg SO_2 (fixed as the hydrogen sulfite addition compound) in 15 mL buffered formaldehyde trapping solution after treatment with 1 mL 4.5M sodium hydroxide to decompose the adduct, was diluted to 50 mL with water. No difference in color between the blank and sample was observed. The solution was then transferred into a 125 mL separatory funnel and extracted 1 min with 5 mL isopentyl alcohol. The results indicated that there was a difference between the color of blank and sample and that the extraction of the ion pair into the solvent was selective, because the absorbance of the blank at 560 nm was quite low (0.024A) and that of the sample was high (0.285A).

The optimum acidity for the extraction of ion pair was first established. A constant and maximum absorbance was obtained when the acidity of the reaction mixture was in the 0.255–0.355M range for sulfuric acid. Above 0.355M, the sample absorbance decreased; below 0.255M, the blank absorbance increased. Hence, a solution maintained at 0.305M in sulfuric acid was selected as the optimum acidity for the reaction.

Similar studies revealed that 3 mL 0.4% potassium iodate, 2 mL 0.001% PRA solution, and 0.5 mL 6% sodium chloride solution were sufficient to provide a constant and maximum absorbance. In the present study, however, we recommend using 5 mL 0.4% potassium iodate solution, 2.5 mL 0.001% PRA solution, and 1 mL 6% sodium chloride solution.

The formation of ion pair was almost instantaneous, and mixing the phases for about 30 s was sufficient for its quantitative extraction into the isopentyl alcohol solvent. The color system after extraction was stable for 72 h at room temperature.

The extraction behavior of various solvents (5 mL) was tested for the extraction of the ion pair. No color was observed when benzene and *n*-hexane were used as extraction solvents. The extraction behavior of other solvents is given in Table 1. These data show that only isopentyl alcohol was optimal, because the extraction of the ion pair was maximum and the blank was very low with this solvent. The absorption spectra of the ion pair formed from different concentrations of SO_2 was recorded and is shown in Figure 1. Curve A shows the absorption spectrum of the reagent blank (2.5 mL 0.001% PRA). Curve F

Table 1. Extractabiiity of ion pair into various solvents (SO_2 = 20 μg)

No.	Solvent	Absorbance		
		Blank	Sample	Sample vs blank
1	Ethyl acetate[a]	0.010	0.080	0.070
2	CCl_4	0.002	0.004	0.002
3	1-Butanol[a]	0.080	0.280	0.200
4	Isobutyl alcohol[a]	0.070	0.240	0.170
5	IBMK	0.020	0.135	0.115
6	Isopentyl alcohol	0.025	0.310	0.285

[a] 10 mL of solvent was used for extraction instead of 5 mL.

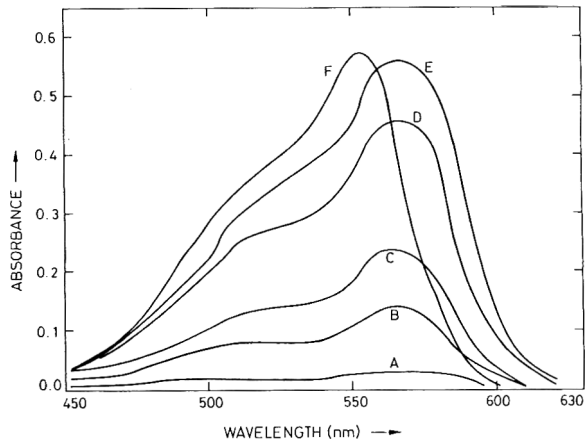

Figure 1. Absorption spectra measured against solvent blank: (A) reagent blank; (B) SO_2, 7.5 μg; (C) SO_2, 15 μg; (D) SO_2, 30 μg; (E) SO_2, 40 μg; and (F) pararosaniline (1.54×10^{-5}M) in isopentyl alcohol solvent.

shows the spectrum of identical concentrations of PRA prepared in isopentyl alcohol. Comparison of curves A and F indicates that free PRA is not extracted into isopentyl alcohol under the experimental conditions. Curves B, C, D, and E show the spectra resulting from the extraction of the ion pair between ICl_2^- and PRA. The calibration graph was rectilinear over the range 0–40 μg standard SO_2. The molar absorption coefficient was $4.5 \times 10^3 Lmol^{-1}cm^{-1}$. The precision of the method was evaluated by establishing the concentration of 10 samples containing 20 μg standard SO_2. The mean recovery was 19.5 μg, with a relative standard deviation of 3.6%.

Nature of Extractable Species

In the present study, an attempt was made to form an ion pair between ICl_2^-, generated by the reaction of SO_2 with iodate in an acidic medium containing excess of chloride ions, and PRA, rather than between ICl_2^- and other cationic dyes. As already reported, the methods based on the formation of an ion pair between rhodamine 6 G (9) or pyronine G (10) with anionic chloro complex of iodine suffer from high blank absorbance values (0.10A for the rhodamine 6 G method and 0.16A for the pyronine G method) and poor color stability (absorbance measurements must be completed within 10 min after extraction by the pyronine G method).

Recently, we have established the formation of ICl_2^- species (6) from the reaction between SO_2 and iodate in an acidic medium containing excess chloride ions (in accordance with equation 1) and used it for the iodination of 2′,7′-dichlorofluorescein.

$$IO_3^- + 2SO_2 + 2H^+ + 2Cl^- + H_2O \rightarrow ICl_2^- + 2H_2SO_4 \quad (1)$$

In the present study, when 2′,7′-dichlorofluorescein was replaced by PRA, an absorbance value of 0.285A for 20 μg SO_2 was observed on extraction with isopentyl alcohol. This observed absorbance may be due to the extraction of iodinated PRA or the ion pair formed between ICl_2^- and PRA cation. The possibility of the other competing direct reaction of SO_2 with PRA and formaldehyde to form Schiff's base type of colored product (1, 4) under the present experimental conditions is ruled out because of high acidity (0.305M in H_2SO_4) and the presence of the oxidizing agent, potassium iodate. This conclusion was also confirmed by the observation that there was no color difference between the blank and the sample in both aqueous medium and the organic (isopentyl alcohol) layer when an experiment was carried out in the absence of potassium iodate.

Experiments were carried out to establish the nature of the species responsible for the observed absorbance. Using potassium iodate, PRA, and 20 μg SO_2 solutions, the color was developed and extracted into isopentyl alcohol. The corresponding reagent blank was also prepared and extracted into isopentyl alcohol. The organic layers from sample and blank were equilibrated with 5 mL neutral aqueous 5% potassium iodide solution. This operation resulted in the formation of a yellow color in the aqueous layer for the sample which, on treatment with starch solution, gave a blue color indicating the presence of iodine. No such blue color was observed with the blank. The formation of iodine with the sample can be explained as a result of the interaction of iodide with the ICl_2^- anion (in accordance with equation 2), which was present as an ion pair with PRA cation in the organic layer.

$$ICl_2^- + I^- \rightarrow I_2 + 2Cl^- \quad (2)$$

If free iodate was extracted as an ion pair with PRA, this can also interact with iodide to generate iodine and give a blue color with starch solution. This possibility of iodine generation was ruled out by the observation that no blue color was observed with the blank. Furthermore, when experiments were carried out with twice the optimum concentration of iodate, no blue color was observed with the blank. This result clearly indicates that no free iodate is extracted as an ion pair with PRA.

Instead of the ion pair being extracted into the organic layer, in the case of an iodinated PRA, it cannot generate iodine on treatment with aqueous neutral potassium iodide. Hence, the color observed in the organic layer is due to the ion pair of

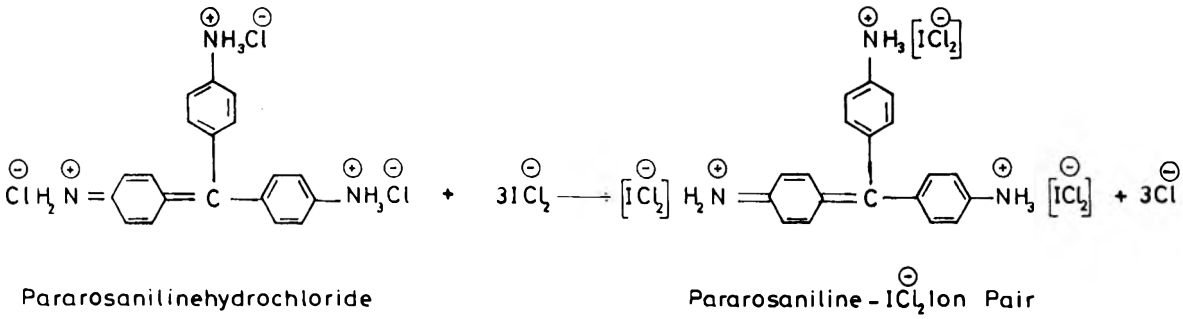

Figure 2. Species responsible for color.

ICl_2^- and PRA cation. In addition, the composition of the ion pair was also established by the mole-ratio method. The result indicated that the PRA:ICl_2^- mole ratio was 1:3 (Figure 2). The extracted ion pair of ICl_2^- and PRA was stable for 72 h from the time of extraction, and the color system had a low blank absorbance (0.024A), unlike the earlier reported ion pair systems of cationic dyes with anionic chloro complex of iodine (9, 10).

Effect of Interfering Species

The effect of common air pollutants on the determination of 20 μg SO_2 was studied by introducing the gas under examination into the trapping solution in the form of anions together with SO_2. Nitrogen dioxide, when present at all levels, caused a low recovery of SO_2. The negative interference of up to 1000 μg nitrogen dioxide was eliminated by the addition of 2 mL 5% (m/v) solution of sulfamic acid to the trapping solution after sampling. Hydrogen sulfide interfered seriously at all levels, causing positive errors. This is due to a similar reaction of H_2S with iodate, generating ICl_2^- ions. The interference of as much as 15μg H_2S was overcome by adding 2 mL 5% sulfamic acid (to provide the acidity) and 5 mL 0.4% potassium iodate solution to the trapping solution after sampling. The H_2S fixed in the trapping solution (due to its solubility) reacts with potassium iodate in the presence of sulfamic acid to liberate iodine, which is extracted into benzene. Under these conditions, SO_2, which gets fixed as the hydrogen sulfite addition compound of formaldehyde, is stable and does not react with potassium iodate. After removal of the iodine generated by H_2S with 5 mL benzene, the aqueous layer containing the SO_2 as hydrogen sulfite addition compound was treated with 1 mL 4.5M sodium hydroxide solution to decompose the addition compound, and the color was developed according to the recommended procedure.

Application of Method

The proposed method was used for the determination of low concentrations of SO_2 generated from 2 permeation devices (type H and M) developed by Balasubramanian et al. (12). These simple permeation devices were made with poly(tetrafluoroethylene) as the permeation medium for sulfur dioxide; they generate 214 ± 5 and 125 ± 4 ppb SO_2, respectively, when dry air is passed through the system at a flow rate of 0.26 ± 0.01 L/min. The SO_2 concentrations of the permeation devices were established by using a Seres Model SF 30 pulsed UV fluorescence monitor capable of measuring 1–10 000 ppb SO_2 with an accuracy of ±1%.

The dry air containing SO_2 that came out of the permeation devices (H and M) was mixed with fresh air and was then collected in 20 mL buffered formaldehyde trapping solution at a flow rate of 0.5 L/min for 8 h. The volume of the trapping solution after sampling was diluted to 50 mL with the buffered

Table 2. Determination of SO_2 generated from permeation devices[a]

Permeation device	West and Gaeke method			Proposed method			Estimated concn of SO_2, ppb[b]
	SO_2 μg/ 15 mL	SO_2 μg/ 50 mL	SO_2, ppb	SO_2 μg/ 15 mL	SO_2 μg/ 50 mL	SO_2, ppb	
M	12.50	41.67	127.5	12.60	42.00	128.6	125 ± 4
	12.30	41.03	125.6	12.50	41.67	127.5	
	12.10	40.33	123.4	12.20	40.67	124.5	
	12.30	41.03	125.6	12.70	42.33	129.5	
H	21.50	71.67	219.4	21.70	72.33	221.4	214 ± 5
	21.00	70.00	214.3	21.00	70.00	214.3	
	21.10	70.33	215.3	21.30	71.00	217.3	
	21.00	70.00	214.3	21.30	71.00	217.3	

[a] Sampling rate = 0.5 L/min, sampling volume = 20 mL, and sampling time = 8 h. Volume of sample taken for analysis was 15 mL in all instances: 20 mL sampled solution was made up to 50 mL with buffered formaldehyde, before analysis.

[b] Determined by using a Seres SF 30 pulsed ultraviolet fluorecence monitor.

formaldehyde solution. A 15 mL aliquot of this solution was analyzed by the proposed procedure and the standard West and Gaeke method (1). Although the sampling rate was maintained at 0.5 L/min, the concentration of SO_2 in ppb was calculated by taking the volume of air passing through the permeation devices (H and M), containing 214 and 125 ppb SO_2, respectively, at a flow rate of 0.26 L/min. The results in Table 2 show that the concentrations of SO_2 obtained by both methods are comparable.

Conclusion

Sulfur dioxide at levels as low as 2 μg can be precisely determined by the proposed procedure. The calibration graph is rectilinear in the 0–40 μg SO_2 range. The relative standard deviation is 3.6% for 10 determinations of 20 μg SO_2. The color system is stable for 72 h after extraction into isopentyl alcohol. The application of the proposed method to the determination of trace amounts of SO_2 from 2 permeation devices demonstrated the usefulness of the method.

Acknowledgment

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MEAT AND MEAT PRODUCTS

Determination of Total Fat in Meat and Meat Products by Solvent Extraction after Hydrochloric Acid Hydrolysis: NMKL[1] Interlaboratory Study

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A gravimetric method including acid hydrolysis and solvent extraction according to SBR (Schmid, Bondzynski, and Ratzlaff) for the determination of total fat in meat and meat products was collaboratively studied in 12 laboratories. The study aimed at investigating whether an older SBR method for the determination of fat in meat and meat products, published by the Nordic Committee on Food Analysis in 1974, could be simplified without significant losses in precision. The study samples consisted of 12 materials in the form of 6 matched pairs of meat types: ham, beef trimmings, pork loin, black pudding, sausage, and liver paté. The fat contents of the samples varied between 2.6 and 44.4 g fat per 100 g. The participating laboratories were asked to analyze the samples by both the old and the new methods. The results indicated that there was no significant difference between the results obtained by the 2 methods. The precision of the revised method in most respects meets the U.S. Department of Agriculture, Food Safety and Inspection Service requirements on methods for the analysis of meat and meat products. The relative standard deviation for repeatability of the method varied from 0.93% for the 44.5 g/100 g level of fat to 4.5% for the 2.5 g/100 g level. The relative standard deviation for reproducibility varied correspondingly between 1.2 and 6.1%. For samples with a fat content <5 g/100 g, the revised method gave somewhat lower fat concentrations than the older method. However, the mean differences were small and did not exceed the precision (repeatability and reproducibility) of the method. Ten of the 12 participating laboratories found the revised method easier to work with than the original method.

For labeling purposes and for calculation of energy content, food laboratories need reliable and preferably simple methods of determining the fat contents of foods. A gravimetric NMKL method including acid hydrolysis and solvent extraction according to SBR (Schmid, Bondzynski, and Ratzlaff) has been used in the Nordic countries for a long time. It was felt that this method could be simplified. It was also recognized that the method needed to be revised in view of laboratory safety. In the original method, samples are first heated over a flame with concentrated hydrochloric acid in a beaker and, after addition of alcohol, transferred to another vessel for extraction with a mixture of diethyl ether and petroleum ether. The extract is then evaporated on a water bath. In the revised, simplified version, acid treatment and extraction take place in the same vessel. Dilute acid is used instead of concentrated acid. The heat treatment takes place in a boiling water bath, so that the boiling does not need to be watched as closely as boiling over a flame. The ether is distilled off in a Soxhlet apparatus, which is preferable from a safety point of view and also allows reuse of the solvent in subsequent extractions of the same sample. Performing the acid treatment of samples and their extraction with ether in the same vessel saves time and eliminates the difficulties associated with transferring ether solutions containing fat from one vessel to another.

Both methods were subjected to full collaborative studies in which 12 Nordic laboratories determined the fat contents of 12 materials present as split-level pairs, i.e., 2 nearly identical materials that differ only slightly in analyte (fat) concentration.

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This method was accepted as an official NMKL method at the 43rd NMKL Annual Meeting, Stykkisholmcer, Iceland, 1989.

The results of the study of the revised method are presented in this paper.

Interlaboratory Study

Six materials were selected for the study: ham, beef trimmings, pork loin, sausage, black pudding, and liver paté. All sample materials were raw. Because the sample material is completely disintegrated during the acid hydrolysis, the changes occurring in the sample matrix during cooking are considered not to have any impact on the recovery of the fat in the subsequent solvent extraction. Thus, the results of this study are applicable to cooked products as well.

Two samples of each material with rather similar fat concentrations were prepared in a meat products plant. The samples were packaged in plastic film in 100 g batches. Thus, the study materials consisted of 12 samples in the form of 6 matched pairs. The samples were randomly numbered. The homogeneity of the samples was tested by analyzing 6 batches of each material. The samples were considered to be homogeneous, because the variations between batches (variance) were not greater than the variations within batches (Fisher's F test [1]). Heat treatment of the samples could not be used as a means of preservation, and the samples were stored at a temperature of −20°C.

The samples were packed with carbon dioxide ice. The 12 samples were mailed to each of the 12 participants by express air mail. All laboratories reported that the samples were still frozen on arrival. The laboratories were requested to store the samples at ≤−18°C before performing single determinations of the fat content according to the methods under comparison. The instructions had been sent to the laboratories well in advance to allow them to practice the methods. The laboratories were also requested to first familiarize themselves with the method by analyzing 2 practice samples of known fat content that were provided and then to perform a single determination of each sample material provided. Only in obvious cases of test failure were the analyses to be repeated. The same analyst was to analyze all samples by both the old NMKL method and the revised, simplified method under study.

Revised Method

Principle

The sample is treated with 8M hydrochloric acid, and ethanol is added; the liberated fat is extracted with a mixture of diethyl ether and petroleum ether. The solvent is then evaporated, and the fat is weighed.

Apparatus and Reagents

All chemicals must be analytical grade. A blank run with reagents should give a value not exceeding 0.5 mg. Try other brands of solvents (**g**)–(**i**) (1) if blank values exceed 0.5 mg.

(**a**) *Meat chopper.*—With perforated plate and openings 2 and 3 mm.

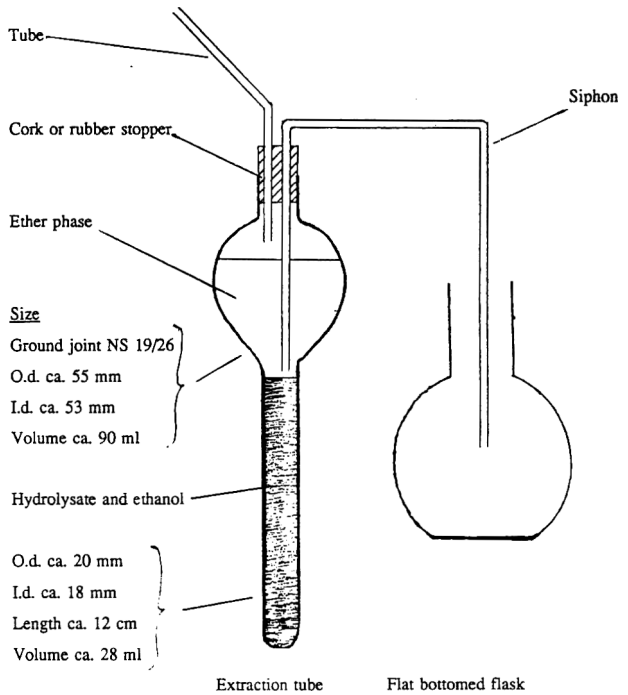

Figure 1. Apparatus (extraction tube equipped with siphon) for transfer of fat-containing ether phases from extraction tube to flask after completed fat extraction.

(**b**) *Food processor.*—1500–3000 rpm.

(**c**) *Extraction tube.*—Eichloff-Barthel (see Figure 1), with ground glass stopper and equipped with siphon.

(**d**) *Water bath.*—Explosion-proof, 80–90°C, with equipment for Soxhlet extraction (Soxhlet extraction and condenser, NS 29); alternatively, explosion-proof rotatory evaporator suitable for ether and petroleum ether evaporations.

(**e**) *Drying oven.*—102–105°C, or vacuum oven, 60°C, 7–13 kPa.

(**f**) *Thin aluminum foil.*—0.01 mm.

(**g**) *Hydrochloric acid.*—8 mol/L. Dilute 670 mL hydrochloric acid (12 mol/L; sp. gr., 1.18 g/cm^3) to 1 L with deionized or distilled water.

(**h**) *Pumice.*—Ignited and granulated.

(**i**) *Diethyl ether.*—Free from peroxides. Ether containing stabilizing agent is recommended.

(**j**) *Petroleum ether.*—b.p. 40–60°C.

Storage of Samples

Store sample in plastic bag, airtight container, or similar airtight wrapping. Sample may be stored up to 3 days at ≤5°C. Store for longer periods at ≤−18°C.

Sample Preparation

Use representative sample of ≥200 g. Remove sample quantitatively from its wrapping. Include gravy, jelly, fat, and anything else that has separated from product in package. Thaw frozen material in refrigerator. Chop sample immediately after removal from refrigerator; cut into small pieces, and pass twice through chopper (**a**). For very fatty samples, use 3 mm plate

Table 1. Interlaboratory study results (g/100 g) for determination of fat in meat and meat products

Collaborator	Ham		Beef trimmings		Black pudding		Sausage		Liver paté		Pork loin	
1	2.66	2.99	4.96	5.28	14.6	15.4	20.5	25.8	31.2	32.9	43.6	44.1
2	2.45	2.75	4.95	5.00	15.7	14.8	20.5	27.1	32.7	33.7	44.8	45.1
3	2.84	2.92	5.02	5.22	14.0	15.5	19.6	26.9	32.4	33.4	43.4	45.2
4	2.21	2.67	4.36	4.72	14.0	14.9	20.3	26.2	31.9	33.1	45.0	44.8
5	2.33	2.64	4.43	5.01	14.1	15.0	19.1	25.0	31.6	32.8	43.7	44.5
6	2.53	2.55	4.39	5.46	13.2	14.7	20.0	26.8	31.5	32.7	43.8	44.9
7	2.55	2.56	4.77	4.89	14.3	15.7	19.1	25.5	31.8	33.0	43.9	43.6
8	2.77	2.83	4.86	5.43	14.7	15.1	21.7	27.7	32.7	33.1	43.4	43.6
9	2.65	2.59	4.68	5.03	13.8	14.6	19.4	25.9	31.8	32.9	43.8	43.9
10	2.48	2.61	4.74	5.26	15.8	16.2	20.2	26.4	32.6	32.8	43.9	44.4
11	2.70	2.70	5.03	5.33	14.0	15.3	19.8	26.0	31.9	32.9	44.2	44.3
12	2.45	2.70	4.55	5.07	14.4	15.2	19.9	26.3	32.2	32.0	44.7	44.9

openings. Mix thoroughly after each grinding. Alternatively, homogenize in food processor (**b**). For samples with very loose consistency, i.e., food items in which all ingredients are finely comminutated, e.g., emulsion sausages, mash and mix well with fork. To control homogeneity of (duplicate) minced sample, add spoonful of charcoal powder to material before homogenization, and afterward, check color of material, which should be homogeneous dispersal of grey to black, depending on color of sample. Store prepared sample in airtight container at ≤5°C, or ≤–18°C if sample is to be stored >3 days.

Determination

Before weighing, carefully mix any condensed water or meat juice separated from sample during storage with remaining contents.

For samples containing < ca 25 g fat/100 g, weigh 4.5–5 g to nearest milligram onto piece of aluminum foil. For samples containing > ca 25 g fat/100 g, weigh ca 3 g. Fold foil around sample, and place sample in extraction tube (**c**).

Add 10 mL hydrochloric acid, 8 mol/L (**g**), and let aluminum foil disintegrate. When hydrogen formation has stopped, place tube in boiling water bath. When solution has warmed up, stopper tube with glass stopper and let stand for total time of 1 h from immersion. Carefully mix contents of tube after 15, 30, and 45 min. Do not let pieces of sample stick to glass above liquid surface.

Remove tube from water bath, and let cool to ≤30°C. Add 10 mL 95% ethanol and mix. Add 25 mL diethyl ether (**i**), and stopper tube. Turn tube upside down several times. To release pressure, ease stopper between turnings. Turn tube upside down and shake vigorously for 30 s. Leave tube for ≥2 min; add 25 mL petroleum ether (**j**), and mix by inverting tube several times. Ease stopper between inversions. To allow phases to separate, let tube stand overnight.

Add few small pieces of pumice (**h**) to flat-bottom flask to prevent bumping. Dry flask in oven (**e**) ≥1 h. Cool in desiccator, and weigh to nearest milligram.

Using siphon (**c**), transfer ether phase to flask as follows: Adjust shorter leg of siphon so that end is 5–10 mm above border between 2 phases. Take care not to transfer any part of lower phase or any emulsion between phases to flask. Force liquid into siphon by blowing gently into tube (see Figure 1). After siphoning, gently blow over liquid remaining in siphon. Remove siphon, and with Pasteur pipet, rinse with 1 mL diethyl ether–petroleum ether mixture (1 + 1). Collect rinsings in flask.

Evaporate solvent on boiling-water bath (**d**). Collect solvent in Soxhlet extractor. Alternatively, evaporate solvent in round-bottom flask, using rotatory evaporator (**d**); avoid explosive boiling by not immersing flask in water bath until fat extract has cooled down completely as result of evaporation.

Add 30 mL diethyl ether–petroleum ether mixture (1 + 1) to tube. Turn tube upside down and shake vigorously for 30 s. Let phases separate completely (upper phase must be completely clear and transparent). Transfer upper phase to flask, and rinse siphon as described above. Repeat extraction once more.

After complete evaporation of solvent, dry flask in oven (**e**) for ca 2 h. Cool in desiccator, and weigh to nearest milligram. Repeat drying operation until difference between 2 consecutive weighings is <0.5 % (w/w) of dry residue.

To obtain blank value, perform run as described above, using reagents only.

Calculation

$$\text{Fat, g/100 g} = (a - b)/c \times 100$$

where a = mass (g) of dried extracted fat; b = mass (g) of blank run; c = mass (g) of weighed sample.

Results and Discussion

The results received from all 12 participants are listed in Table 1. The data were statistically evaluated according to the IUPAC 1987 Harmonized Protocol (2, 3). Neither the single nor any of the double Grubb's tests at $P < 0.01$ flagged any outlying laboratories. The precision estimates are presented in Table 2.

All except one of the precision estimates of the method meet the requirements issued by U.S. Department of Agriculture, Food Safety and Inspection Service (4) concerning the preci-

Table 2. Statistical results for the interlaboratory study on determination of fat (g/100 g) in meat and meat products

Statistical parameter	Ham	Beef trimmings	Black pudding	Sausage	Liver paté	Pork loin
No. of laboratories	12	12	12	12	12	12
Mean of matched pairs	2.63	4.94	14.80	23.15	32.45	44.20
Repeatability SD (s_r)	0.12	0.19	0.47	0.36	0.37	0.41
Repeatability relative SD (RSD_r), %	4.5	3.9	3.2	1.6	1.1	0.93
Repeatability value, r ($2.8 \times s_r$)	0.33	0.53	1.3	1.0	1.0	1.2
Reproducibility SD (s_R)	0.16	0.24	0.62	0.73	0.45	0.55
Reproducibility relative SD (RSD_R), %	6.2	4.8	4.2	3.2	1.4	1.2
Reproducibility value, R ($2.8 \times s_R$)	0.46	0.66	1.7	2.0	1.3	1.5

sion of methods of analysis for the control of meat: the repeatability standard deviation (s_r) should be less than 0.63; the reproducibility standard deviation (s_R) should be less than 0.66. The relative standard deviations for reproducibility (RSD_R) agree well with those found in a large number of method performance studies involving a wide range of analytes, matrixes, and measurement techniques. It was found that the RSD_R values generally can be predicted from the so-called Horwitz equation (5), $RSD_R = 2\exp(1 - 0.5 \log C)$ where C is the concentration as a decimal fraction. The ratio between observed RSD_R values and the RSD_R values predicted by this equation, designated HORRAT, can be regarded as an indication of the acceptability of a method with respect to its precision, according to Horwitz et al. (6). The HORRAT values of this method, calculated from the observed reproducibility relative standard deviation for individual test samples, are presented in Table 3. According to Horwitz et al. (6), a series of ratios close to 1.0 or consistently smaller indicates acceptable precision of methods. Correspondingly, HORRAT values near or above 2 probably indicate an unacceptable method with respect to precision. In a survey of hundreds of collaborative analytical studies (6) for the determination of nutrients and related substances, 105 assays (sets of data) were identified as relating to the determination of fat in foods. The fat concentrations of the test materials ranged from 3 to 100 g/100 g. About 90% of the studies showed reproducibility relative standard deviations ranging between 0.5 and 65%, resulting in HORRAT values between 0.2 and 12. The average reproducibility relative standard deviation was 14%. For the method investigated in this work, 11 of 12 HORRAT values were below 2, and over half of the values were below 1.5; therefore, the precision of the method is acceptable.

The same acceptability criterion, "An RSD_R value twice the predicted value indicates an acceptable method," has been adopted by IUPAC and is contained in the IUPAC 1989 harmonized protocol for the adoption of standardized analytical

Table 3. Observed relative reproducibility standard deviations (RSD_R values) for individual test samples and calculated HORRAT values[a]

Material	Fat content, g/100 g	RSD_R, %	HORRAT value
Ham	2.55	7.2	2.1
Ham	2.71	5.2	1.5
Beef trimmings	4.7	5.2	1.6
Beef trimmings	5.1	4.4	1.4
Black pudding	14.4	5.2	1.9
Black pudding	15.2	3.0	1.1
Sausage	20.0	3.6	1.4
Sausage	26.3	2.8	1.2
Liver paté	32.0	1.5	0.65
Liver paté	32.9	1.2	0.52
Pork loin	44.0	1.2	0.54
Pork loin	44.4	1.3	0.55

[a] Ratio between observed RSD_R values and $RSDR_R$ values predicted by the Horwitz equation. HORRAT values below 2 indicate acceptable precision of the methods.

methods (7). This protocol suggests that acceptable values for the relative standard deviation for reproducibility would be between 1 and 4% for a concentration ratio of 1 (=100%) and between 2 and 8 % for a concentration ratio of 0.01 (=1%). The concentration ratios of the test materials in this study were between 0.02 and 0.44. The acceptable range for RSD_R values for these concentrations would then be 1.1–6.9%. The RSD_R values actually observed in this study were between 1.2 and 6.2%. The lowest value was estimated for a fat level of approximately 44.4 g/100 g, and the highest, for a level of approximately 2.6 g/100 g. The ratio between repeatability and reproducibility varied between 0.5 and 0.8. It has been proposed that a ratio greater than 0.3 should generally be interpreted as an indication of a satisfactory method (8).

Collaborators' Comments and Remarks

Initially, 3 laboratories reported values for the reagent blank that were higher than the limit of 0.5 mg (w/w) of the dry residue prescribed in the method. The highest value recorded was 5.5 mg; others were 0.8, 1.6, and 3.3 mg. Detailed inquiries about the received blank values were sent to all the participating laboratories. Five of the 12 laboratories had obtained blank values less than the prescribed 0.5 mg, whereas 5 laboratories had obtained values higher than 0.5 mg. Two laboratories did not present blank values. On the basis of these results, it is considered possible to achieve blank values of <0.5 mg, and no amendments to the method are suggested.

Ten of the 12 participating laboratories found the revised method easier to work with than the original NMKL method from 1974. One laboratory did not find the method easier, and one laboratory preferred the older method. Because the majority of the laboratories clearly preferred the new method, it is suggested that this method be introduced to replace the earlier method.

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MEAT AND MEAT PRODUCTS

Gravimetric Determination of Moisture and Ash in Meat and Meat Products: NMKL[1] Interlaboratory Study

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Fourteen laboratories participated in a collaborative study of gravimetric methods for determination of moisture and ash in meat and meat products. The determination of moisture by drying without sand at 102–105°C for 16–18 h and to constant weight was compared with the official NMKL method using drying with sand to constant weight. The incineration was performed at 550°C. The method was tested on 16 blind duplicate samples of fresh meat and meat products ranging in moisture content from 44.1 to 74.8 g/100 g, and in ash content from 1.0 to 5.4 g/100 g. Drying without sand for 16–18 h and to constant weight gave no significant difference. Therefore, the labor-intensive drying to constant weight is not necessary to obtain reliable results. In comparison with the official NMKL method based on drying with sand, the mean values, repeatability, and reproducibility of the drying method without sand for 16–18 h agree very well. Therefore, there is no reason to retain the more complicated and labor-intensive sand drying method. The conditions of the proposed method differ only slightly from the conditions used in the official AOAC method, 950.46 B(a). The collaborative results for the determination of both moisture and ash gave estimates for precision according to the HORRAT values.

Determinations of moisture and ash are among the most frequent analyses in the food laboratory. Therefore, it is of great importance to simplify these analyses and to reduce the actual working hours without decrease of accuracy. Several official methods for determination of moisture in meat and meat products require mixing the sample with sand before drying and then drying to constant weight (1, 2).

In the present work, a method based on drying the sample at 102–105°C for 16–18 h (overnight) was evaluated. The analytical conditions of this method agree very well with the conditions used in AOAC method **950.46 B(a)** (*Official Methods of Analysis*, 1990, 15th Ed.): same drying time and sample amount, but somewhat higher drying temperature (102–105°C instead of 100–102°C) and larger dishes (60–80 mm diameter instead of ≥50 mm) without lids. The mixing step with sand and the repeated weighings were eliminated. The method was compared with the official NMKL method based on drying with sand (1).

The incineration was performed at 550°C. The temperature was increased gradually in the course of 5–6 h (temperature increase, 100°C/h). A muffle furnace with time/temperature regulator can be used. The proposed method can hardly be compared with the 2 corresponding AOAC methods, **31.012** and **31.013** (*Official Methods of Analysis*, 1984, 14th Ed.), which use different analytical techniques depending on product type.

The methods were subjected to an NMKL[1] collaborative study in 1991. This paper describes the results of this study.

Collaborative Study

Eight materials presented as 16 blind duplicate samples of fresh meat and meat products were distributed to 14 collaborating laboratories from Denmark, Finland, Norway, and Sweden. Each laboratory received a training sample and the method descriptions 3 weeks before the start. The study included the following products: (blind duplicate samples 1 and 13) smoked ham; (2; 19) sirloin; (3; 14) falu sausage (bologna-type sausage); (4; 16) blood sausage; (5; 11) liver paste; (6; 15) salami; (7; 10) ham; (8; 12) pork trimmings. The materials were homogenized in a food processor and frozen in airtight plastic

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[1] Nordic Committee on Food Analysis (Secretariat General, c/o Technical Research Centre of Finland, Food Research Laboratory, Biologinkuja 1, SF-02150 Espoo, Finland).

These methods were accepted as official NMKL methods at the 45th NMKL Annual Meeting, August 27–30, 1991, at Bergen, Norway.

Table 1. Interlaboratory results (g/100 g) for determination of moisture in blind duplicate samples of meat and meat products (drying with sand at 102–105°C to constant weight)

Coll.	Blind dup.	1 Smoked ham	2 Sirloin	3 Falu sausage	4 Blood sausage	5 Liver paste	6 Salami	7 Ham	8 Pork trimmings
1	1	71.80	74.97	62.55	48.76	58.12	44.48[a]	69.42	58.61
	2	71.41	74.80	62.14	49.25	58.38	44.54[a]	69.25	58.92
2	1	71.70	75.13	62.07	49.04	57.48	43.93	69.25	58.42
	2	71.60	75.23	61.83	49.62	57.54	43.75	69.20	58.57
3	1	71.75	74.60	61.72	49.74	57.78	43.90	68.81	58.64
	2	71.59	74.70	62.11	48.53	56.82	43.79	68.75	58.36
4	1	71.30	74.75	61.60	49.12	57.35	43.71	68.75	59.02
	2	71.51	74.84	62.21	48.72	57.12	43.68	68.91	58.35
5	1	71.31	74.95	62.64	49.47	57.53	43.87	68.94	58.26
	2	71.58	74.87	62.00	49.21	57.32	43.81	68.89	58.03
6	1	71.39	75.12	61.56	48.27	57.74	43.80	69.70	58.16
	2	70.96	74.92	61.55	49.01	57.84	43.54	69.36	58.24
7	1	71.32	74.18	61.67	48.82	57.81	44.12	69.18[b]	58.85
	2	71.14	74.50	61.90	48.80	57.73	43.53	68.01[b]	57.76
8	1	71.30	75.12	61.46	48.56	57.39	43.68	69.06	58.82
	2	71.61	74.96	61.85	49.40	58.33	43.77	69.17	58.28
9	1	—[c]	74.70	61.17	—[c]	—[c]	43.78	68.58	58.62
	2	—[c]	74.75	61.04	—[c]	—[c]	43.81	68.65	58.73
10	1	71.31	74.46	62.04	47.68	57.97	44.02	69.25	58.21
	2	71.51	74.54	61.78	48.54	58.01	43.83	69.56	58.24
11	1	71.06	74.41	61.60	48.92	58.38	43.92	68.85	58.27
	2	71.03	74.08	60.99	48.97	57.79	44.11	68.46	58.69
12	1	70.93	74.28	60.93	48.20	57.15	43.93	68.88	58.85
	2	70.90	74.76	61.03	48.29	57.08	43.71	68.75	58.23
13	1	71.32	74.94	61.81	47.76	57.44	43.32[b]	69.16	58.04
	2	71.39	74.93	62.39	48.49	57.78	44.82[b]	69.49	58.12
14	1	71.09	73.90	61.33	48.60	57.35	43.66	68.26	58.39
	2	70.87	74.05	60.71	48.11	57.44	43.45	68.24	58.62

[a] Rejected by Grubb test.
[b] Rejected by Cochran test.
[c] Constant weights were not obtained.

bags. Each participant received 2 identical, randomly coded samples of each material. The samples were distributed in an insulated box containing ice boxes. The collaborators were instructed to analyze each of the samples once.

METHOD

Both methods, i.e., determination of moisture without sand and determination of ash, were accepted as official NMKL methods at the 45th NMKL Annual Meeting, August 27–30, 1991, at Bergen, Norway (Nordic Committee on Food Analysis, No. 23, 3rd Ed., 1991).

Scope and Field of Application

This method describes the gravimetric determination of moisture and ash in meat and meat products. The method is applicable to all types of meat and meat products, but only to wet materials.

Definition

Moisture is the loss in mass obtained after drying meat and meat products as described in this method. Ash is defined as the inorganic residue obtained after removal of moisture and organic matter by heat treatment.

Table 2. Interlaboratory results (g/100 g) for determination of moisture in blind duplicate samples of meat and meat products (drying without sand at 102–105°C)

		1 Smoked ham		2 Sirloin		3 Falu sausage		4 Blood sausage		5 Liver paste		6 Salami		7 Ham		8 Pork trimmings	
Coll	Blind dup.	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight	16–18 h	Constant weight
1	1	71.77	71.77	75.59	75.59	62.96	62.96	48.72	48.72	58.92	58.92	44.59	44.59	69.70	69.70	58.48	58.51
	2	71.49	71.49	74.97	74.97	62.25	62.28	48.85	49.47	58.99	59.02	44.44	44.44	69.56	69.56	58.79	58.89
2	1	71.59	71.68	75.14	75.20	62.43	62.48	49.47	49.52	58.25	58.33	44.32	44.39	69.43	69.46	58.65[a]	58.71
	2	71.59	71.66	75.20	75.23	62.09	62.12	49.83	49.88	58.04	58.11	44.41	44.45	69.09	69.14	58.80	58.87
3	1	71.88	71.90	74.66	74.68	61.97	61.97	50.00	50.03	58.33	58.38	44.16	44.20	69.30	69.30	58.39	58.48
	2	71.71	71.71	75.26	75.29	61.75	61.75	49.02	49.04	57.51	57.54	44.26	44.29	69.13	69.13	58.47	58.49
4	1	71.27	71.29	74.83	74.83	62.09	62.10	49.27	49.27	57.57	57.61	43.95	43.96	69.07	69.07	58.39	58.40
	2	71.26	71.26	74.68	74.69	61.79	61.79	49.09	49.11	56.99	57.02	43.87	43.88	68.91	68.94	58.20	58.23
5	1	71.67	71.67	74.53	74.53	62.19	62.19	49.65	49.71	58.06	58.06	44.11	44.11	68.93	68.93	58.57	58.57
	2	71.35	71.35	74.80	74.80	61.95	61.95	49.28	49.28	57.92	57.95	44.29	44.29	68.64	68.64	58.44	58.44
6	1	70.86	70.89	74.69	74.69	60.96	60.96	48.61	48.61	58.22	58.22	43.78	43.78	69.10	69.10	58.17	58.19
	2	70.78	70.78	74.77	74.77	61.34	61.34	49.32	49.32	58.09	58.09	43.73	43.73	69.38	69.38	58.56	58.58
7	1	71.49	71.50	74.52	74.52	62.23	62.25	48.85	48.85	58.31	58.32	44.20	44.22	69.15	69.15	57.61	58.19
	2	71.17	71.17	74.92	74.97	61.71	61.72	48.55	48.63	58.28	58.40	43.75	43.78	68.22	68.29	57.44	58.02
8	1	71.39	71.39	75.06	75.06	61.87	61.87	48.89	48.89	58.04	58.04	44.08	44.08	69.05	69.05	58.17	58.17
	2	71.45	71.45	74.87	74.87	62.07	62.07	49.93	49.93	58.50	58.50	43.99	43.99	69.11	69.11	57.77	57.77
9	1	71.45	71.45	74.71	74.71	61.61	61.61	48.98	48.98	57.49	57.49	44.08	44.08	68.29	68.29	58.65	58.65
	2	71.30	71.30	74.69	74.69	61.44	61.44	49.61	49.61	58.22	58.22	43.86	43.86	68.59	68.59	58.76	58.76
10	1	71.48[a]	71.50[a]	74.46	74.46	61.17	61.17	48.86	48.90	58.58	58.58	44.03	44.07	68.71	68.72	58.35	58.53
	2	72.80[a]	72.87[a]	73.36	74.97	61.19	61.20	48.99	49.14	58.16	58.17	43.93	43.99	68.55	68.57	58.04	58.26
11	1	70.98	70.98	74.66	74.66	61.82	61.90[a]	49.02	49.07	58.54	58.61	44.27	44.38	68.87	68.87	58.43	58.49
	2	70.79	70.80	74.60	74.60	59.94	60.06[a]	48.95	48.98	58.30	58.33	44.22	44.32	68.60	68.60	58.12	58.22
12	1	71.54	71.54	74.78	74.78	64.47	64.47[a]	48.79	48.79	57.86	57.86	44.10	44.10	69.34	69.34	58.51	58.51
	2	71.34	71.34	75.12	75.12	62.41	62.41[a]	48.58	48.58	57.63	57.63	43.89	43.89	68.87	68.87	58.37	58.37
13	1	71.50	71.50	74.95	74.96	62.01	62.01	48.71	48.72	57.84	57.85	43.90	43.90	69.17	69.18	58.43	58.43
	2	71.71	71.73	75.08	75.09	62.26	62.26	48.29	48.29	58.14	58.14	44.05	44.05	69.10	69.11	58.44	58.44
14	1	71.31	71.31	75.47	75.49	60.67	60.68	48.98	49.03	58.37	58.40	44.18	44.21	68.30	68.30	57.56	57.73
	2	71.12	71.17	74.83	74.84	61.55	61.55	49.14	49.16	57.86	57.89	43.74	43.89	69.02	69.04	57.76	57.94

[a] Rejected by Cochran test.

Table 3. Statistical summary—moisture analysis[a]

Statistic	1 Smoked ham	2 Sirloin	3 Falu sausage	4 Blood sausage	5 Liver paste	6 Salami	7 Ham	8 Pork trimmings
	Drying with sand at 102–105°C to constant weight							
$\bar{X}$, g/100 g	71.33	74.69	61.70	48.76	57 64	43.80	68.98	58.44
s_r	0.17	0.15	0.30	0.44	0 31	0.16	0.15	0.32
RSD_r, %	0.23	0.20	0.49	0.90	0 53	0.37	0.21	0.54
s_R	0.28	0.36	0.50	0.53	0 41	0.17	0.39	0.32
RSD_R, %	0.39	0.48	0.81	1.1	0 71	0.38	0.56	0.54
r	0.46	0.41	0.85	1.2	0.86	0.46	0.41	0.89
R	0.77	1.0	1.4	1.5	1.2	0.47	1.1	0.89
	Drying without sand at 102–105°C for 16–18 h							
$\bar{X}$, g/100 g	71.38	74.83	61.86	49.08	58.11	44.08	68.97	58.30
s_r	0.14	0.32	0.60	0.36	0.30	0.15	0.28	0.17
RSD_r, %	0.19	0.42	0.96	0.73	0.51	0.34	0.40	0.29
s_R	0.30	0.40	0.80	0.43	0.44	0.23	0.38	0.38
RSD_R, %	0.42	0.54	1.3	0.88	0.75	0.52	0.56	0.65
r	0.39	0.88	1.7	1.0	0.83	0.41	0.78	0.47
R	0.83	1.1	2.2	1.2	1.2	0.64	1.1	1.1
HORRAT	0.20	0.26	0.60	0.40	0.35	0.23	0.33	0.30
	Drying without sand at 102–105°C to constant weight							
$\bar{X}$, g/100 g	71.39	74.90	61.88	49.13	58.13	44.10	68.98	58.39
s_r	0.14	0.26	0.59	0.39	0.30	0.13	0.27	0.17
RSD_r, %	0.20	0.35	0.95	0.79	0.52	0.30	0.39	0.29
s_R	0.30	0.29	0.79	0.44	0.44	0.23	0.38	0.30
RSD_R, %	0.42	0.38	1.3	0.89	0.75	0.53	0.56	0.51
r	0.39	0.73	1.7	1.1	0.84	0.38	0.75	0.47
R	0.84	0.80	2.2	1.2	1.2	0.65	1.1	0.83

[a] Outlier results not included in statistics.

Principle

Determination of moisture involves drying of the sample 16–18 h at 102–105°C. The incineration is performed at 550°C.

Apparatus

(**a**) *Mechanical meat chopper.*—With plate openings of 2 or 3 mm.

(**b**) *Food processor.*—1500–3000 rpm.

(**c**) *Metal, porcelain, or glass dishes.*—Such as aluminum, nickel, stainless steel, Duran glass; 60–80 mm diameter; ca 25 mm high.

(**d**) *Drying oven.*—102–105°C.

(**e**) *Porcelain or quartz crucibles.*—30 mm diameter.

(**f**) *Muffle furnace.*—550 ± 5°C. If furnace has no time/temperature regulator, an electric hot plate is also needed.

(**g**) *Desiccator.*—Containing silica gel, blue-indicating, or other efficient desiccant.

Sampling and Sample Preparation

Sampling.—Use a representative sample of at least 200 g. Put sample into plastic bag, airtight container, or similar airtight wrapping. Store sample at maximum of 5°C until further treatment or at maximum of −18°C for periods longer than 3 days. If sample is not frozen, analyses must be performed within 3 days.

Sample preparation.—Remove sample quantitatively from bag. Include gravy, jelly, fat, or anything else that has separated from product in package. Thaw frozen material in refrigerator. Chop sample immediately after removing from refrigerator. Cut sample into small pieces. Pass pieces twice through chopper (**a**). For very fatty samples, use 3 mm plate openings to prevent sample from greasing in chopper. Mix thoroughly after each grinding. Alternatively, sample can be homogenized in food processor (**b**). Samples with a very loose consistency can be mashed and mixed well with a fork. Homogeneity of minced sample can be confirmed by adding a spoonful of charcoal to chopper or food processor before starting homogenization of a control sample and then checking color of the material.

Procedure

Determination of moisture content.—Spread out ca 6 g sample evenly over bottom of a dry and weighed dish (**c**). Weigh to nearest 0.0001 g. Perform 2 determinations from each sample. Dry sample in drying oven (**d**) 16–18 h at 102–

Table 4. Differences between the drying methods (mean values)

Sample No.	Moisture ($\bar{X}_s$), g/100 g, drying with sand at 102–105°C to constant weight	Differences (% units) from $\bar{X}_s$	
		Drying without sand at 102–105°C	
		16–18 h	Constant weight
1	71.33	+0.05	+0.06
2	74.69	+0.14	+0.21
3	61.70	+0.11	+0.12
4	48.76	+0.32	+0.37
5	57.64	+0.47	+0.49
6	43.80	+0.28	+0.30
7	68.98	±0	±0
8	58.44	−0.14	−0.05

105°C. Let sample cool to room temperature in desiccator (**g**), and weigh to nearest 0.0001 g.

Determination of ash content.—To nearest 0.0001 g, weigh duplicate 1.5–2.0 g samples into incinerated and weighed crucible (**e**). If muffle furnace with time/temperature regulator is not available, preash sample on electric hot plate so that temperature increases over 5–6 h to 550°C (increase of temperature, 100°C/h). Ash in furnace at 550°C until ash has gray-white appearance. If muffle furnace with time/temperature regulator is available, perform incineration procedure described previously in furnace. Let crucible cool to room temperature in desiccator (**g**), and weigh to nearest 0.0001 g.

Calculation

Calculate moisture and ash contents of sample by using the following formula:

$$\text{g moisture/100 g} = [(a - b) \times 100]/a,$$
$$\text{g ash/100 g} = (c \times 100)/a$$

where a = mass of sample (g), b = mass of dried sample (g), and c = mass of ash (g).

Reliability of Method

The method was collaboratively studied in 14 laboratories. The study included 16 samples of meat and meat products presented as blind duplicates. Materials ranged in moisture content from 44.1 to 74.8 g/100 g and in ash content from 1.0 to 5.4 g/100 g.

Repeatability (r) and reproducibility (R) of the moisture and ash methods were not markedly dependent on the concentration of analytes in the sample, as can be seen from the following equations, which were calculated for all samples:

For moisture:

$$r = 0.593 + 0.0017 \times \text{moisture content},$$
$$R = 0.797 + 0.00471 \times \text{moisture content}$$

For ash:

$$r = 0.0990 + 0.00933 \times \text{ash content},$$
$$R = 0.138 + 0.0046 \times \text{ash content}$$

Average repeatability for all samples was 0.7 g/100 g for moisture results and 0.12 g/100 g for ash results. Repeated analyses of all samples by the same analyst gave a corresponding repeatability of 0.4 and 0.08 g/100 g, respectively.

Results and Discussion

Moisture

The collaborative results are given in Tables 1 and 2. Laboratory 9 did not achieve constant weight after 8 rounds of drying with sand (Table 1).

The data were analyzed according to the AOAC guidelines (3). Cochran, Grubbs, and double Grubbs outliers were removed at the 1% level of significance.

Mean values, standard deviations, repeatability and reproducibility values, and relative standard deviations of repeatability and reproducibility were calculated for each sample (Table 3). A comparison of the mean values obtained by drying with and without sand is given in Table 4.

Drying without sand was completed after 16–18 h. Repeated drying increased the moisture result by >0.1% unit. No difference in results could be observed at the 95% confidence level. Therefore, drying to constant weight is not necessary to obtain reliable results. There was a significant difference at the 95% confidence level for samples 4, 5, and 6 between drying with and without sand.

The biases in Table 4 are significant and indicate that the comparison method has a negative bias relative to the proposed method. This suggests that the use of sand is contraindicated, retarding rather than aiding the moisture loss. There seems to be no reason to retain the more complicated and labor-intensive sand drying method.

Drying without sand at 16–18 h showed a repeatability value ranging from 0.39 to 1.7 g/100 g (average, 0.81 g/100 g). Repeated analysis in our laboratory gave repeatability values from 0.26 to 0.56 g/100 g (average, 0.40 g/100 g).

Sample 3 in Table 3 shows fairly excessive repeatability and reproducibility standard deviations of 0.60 and 0.80, respec-

Table 5. Collaborative results (g/100 g) for determination of ash in blind duplicate samples of meat and meat products[a]

Coll.	Blind dup.	1 Smoked ham	2 Sirloin	3 Falu sausage	4 Blood sausage	5 Liver paste	6 Salami	7 Ham	8 Pork trimmings
1	1	3.86	1.11	2.07	1.98	2.36	5.51	1.15[b]	3.08
	2	3.85	1.16	2.04	2.02	2.42	5.46	1.62[b]	3.10
2	1	3.80	1.10	2.07	1.93	2.30	4.97[c]	0.89	3.00
	2	3.68	1.03	1.90	1.78	2.28	5.13[c]	0.96	2.87
3	1	3.75	1.07	1.97	1.93	2.38	5.29	1.02	3.05
	2	3.86	1.09	2.03	1.95	2.42	5.52	0.98	3.09
4	1	3.78	1.11	2.01	1.93	2.30	5.39	1.03	3.11
	2	3.70	1.17	1.98	1.87	2.41	5.41	1.02	2.97
5	1	3.77	1.19	2.00	1.89	2.25	5.35	0.97	3.03
	2	3.80	1.20	2.03	1.88	2.24	5.39	0.98	2.98
6	1	4.05[b]	1.10	1.98	2.00	2.29	5.36	0.99	3.06
	2	3.70[b]	1.04	1.96	1.89	2.33	5.47	0.98	3.01
7	1	3.83	1.07	2.02	1.88	2.32	5.44	1.00	3.05
	2	3.77	1.05	2.01	1.91	2.31	5.40	0.97	3.07
8	1	3.77	1.11	1.98	1.99	2.31	5.42	0.99	3.04
	2	3.86	1.10	1.99	1.83	2.31	5.36	1.02	3.10
9	1	3.80	1.14	1.92	2.04	2.26	5.43	1.01	3.01
	2	3.65	1.10	1.94	1.87	2.36	5.39	1.04	3.00
10	1	3.68	1.09	1.89	1.89	2.27	5.41	1.08	2.97
	2	3.75	1.18	1.99	1.88	2.29	5.38	1.07	2.98
11	1	4.26[c]	1.53[c]	2.36[c]	2.39[c]	2.75[c]	5.66[d]	1.38[c]	3.46[c]
	2	4.24[c]	1.49[c]	2.27[c]	2.28[c]	2.60[c]	5.50[d]	1.49[c]	3.43[c]
12	1	3.81	1.04	1.98	1.92	2.26	5.30	1.02	3.02
	2	3.73	1.07	1.93	1.85	2.28	5.40	1.03	3.07
13	1	3.85	1.13	1.99	1.90	2.35	5.46	1.16[c]	3.05
	2	3.88	1.14	2.01	1.98	2.34	5.39	1.27[c]	3.05
14	1	3.85	1.06	2.02	1.94	2.26	5.45	1.00	3.04
	2	3.85	1.07	1.87	1.88	2.31	5.48	1.00	3.03
$\bar{X}$, g/100 g		3.789	1.105	1.984	1.916	2.316	5.11	1.002	3.032
s_r		0.058	0.032	0.052	0.065	0.036	0.043	0.021	0.044
RSD_r, %		1.5	2.9	2.6	3.4	1.5	0.79	2.1	1.5
s_R		0.066	0.048	0.052	0.065	0.052	0.049	0.040	0.053
RSD_R, %		1.7	4.4	2.6	3.4	2.3	0.91	4.0	1.7
r		0.162	0.089	0.147	0.183	0.100	0.120	0.059	0.123
R		0.185	0.135	0.147	0.183	0.146	0.137	0.113	0.148
HORRAT		0.53	1.1	0.72	0.86	0.64	0.29	2.0	0.51

[a] Outlier results not included in statistics.
[b] Rejected by Cochran test.
[c] Rejected by Grubb test.
[d] Rejected because of systematic error.

tively, for the proposed method, mostly depending on the results of Laboratory 12. The duplicate error amounts to 2.06% units, and the mean value of 63.44 differs considerably from the average mean value of 61.88%. This laboratory observed inhomogeneity in sample 3.

The acceptability of the RSD_R values was evaluated according to the method of Horwitz et al. (4). HORRAT ratios greater than 2.0 suggest unacceptable precision. All HORRAT ratios were well below 1.0, which indicates a quite acceptable precision compared with other collaborative studies.

AOAC method **950.46 B(a)**, using almost the same analytical conditions as the proposed method (100–102° for 16–18 h), has been studied collaboratively together with 3 other methods (5): vacuum drying at 98–100°C for 6 h and at 69–71°C for 16–17 h and drying in a conventional air oven at 125 ± 5°C for 2.5–3.5 h. The 100°C vacuum drying method caused loss of fat in high-fat products. The 70°C vacuum method gave reliable results but caused problems due to the small capacity of most vacuum ovens. The 125°C air oven method gave satisfactory results if the drying time was adjusted to the different product types. The 100–102°C air oven method gave reliable results for all products. However, the results, which depended on very limited statistical analysis, can hardly be compared with the collaborative results in the present study. Besides, the different methods have not been compared with drying of the same sample to constant weight.

Ash

Data from the collaborators and the statistical evaluation are given in Table 5. The statistical methods used are described above. The results from Laboratory 11 indicate a systematic error. The results are all biased high relative to the other laboratories, but the duplicate error agrees well with the other laboratories. Unfortunately, we could not find an explanation. Probably the error arose from deviations in time or temperature conditions.

The repeatability values ranged from 0.059 to 0.183 g/100 g (average, 0.13 g/100 g). Repeated analysis in our laboratory gave repeatability values of 0.041–0.098 (average, 0.083 g/100 g). The HORRAT values were lower than 1.1 in all samples except sample 7, which showed a ratio of 2.0. This indicates that the method is acceptable with respect to precision.

Collaborators' Comments

Five of the 14 collaborators reported difficulties in drying the samples with sand. After the first drying step, the samples were hard in consistency and were therefore difficult to mix again with the rod. Laboratory 3 used a muffle furnace without time/temperature regulator.

Summary

The determination of moisture in meat and meat products by drying without sand at 102–105°C for 16–18 h gave reliable collaborative results. Both drying to constant weight and drying with sand can be eliminated. The analytical conditions of the proposed method differ only slightly from the conditions used in AOAC method **950.46 B(a)**.

The determination of ash in meat and meat products, based on the gradual increase of the temperature up to 550°C in the course of 5–6 h, gave reliable collaborative results with the exception of one laboratory, which showed a systematic error.

Both methods gave estimates for precision according to the HORRAT values and were accepted as official NMKL methods at the 45th Annual Meeting, August 27–30, 1991, at Bergen, Norway.

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METALS AND OTHER ELEMENTS

Determination of Total Mercury in Seafood and Other Protein-Rich Products

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A previously developed wet-digestion method for the determination of total mercury in plants by cold vapor atomic absorption spectroscopy (CVAAS) was extended to the analysis of seafood and other products rich in proteins. Oxidation of matrixes is accomplished by $K_2Cr_2O_7$ in the presence of diluted H_2SO_4; a simple air condenser is used to reflux vapors released from the boiling mixture. The original procedure (A) and 2 modifications (B and C), which differ with respect to the mode of acidification and/or digestion time and the types of condensers used, were compared for precision and accuracy by means of National Institute of Standards and Technology Research Material 50 Albacore Tuna and proved to be reliable (Hg present, 0.95 ± 0.1 μg/g; Hg found, 0.97 ± 0.029 μg/g [A], 0.98 ± 0.018 μg/g [B], and 0.94 ± 0.025 μg/g [C]). The modified procedures were tested further in Hg recovery experiments on a variety of biological matrixes with different spiking substances and again showed good analytical characteristics (overall average recoveries = 98 ± 5.1% for seafood and 100 ± 3.6% for protein-rich baby foods).

An estimated 10 000 tons of mercury are released every year into our environment as a consequence of human activity (1). The danger posed to human health by this form of contamination comes mainly from food and stems from the ability of this element to enter the natural alimentary chains, to accumulate in progressively larger quantities at each trophic stage, and to reach highly toxic concentrations in the tissues of organisms that play a role in the human diet (2).

Concern over mercury contamination of the environment has promoted an intensive search for methods aimed at the determination of this metal. Today, mercury is most commonly quantitated by means of cold vapor atomic absorption spectroscopy (CVAAS) following the wet digestion of the sample (3–5).

Among those digestion procedures aimed at limiting the length of the analysis by using high temperatures, most are based on the use of a mixture of nitric and sulfuric acids at its boiling point (6–14). Choosing nitric acid as the oxidant imposes practical and analytical difficulties. Besides limiting the working space to the fume hood, the copious emissions of nitrogen oxides call for both a careful initial control of the heating process and the use of special water condensers to avoid losses of mercury (3).

With some food substances, the oxidizing properties of nitric acid are insufficient, and oxidation must be implemented with the aid of catalysts (3, 8–13) and/or with the addition of a stronger oxidant to finish off mineralization (6–8, 11–13). The residual nitrogen oxides in the digest disturb the spectrophotometric measurement and give a positive bias (15). To eliminate these oxides, hydroxylamine, a selective reducing substance different from that used to reduce the mercuric ions, is usually used. Therefore, the entire process is characterized by the frequent intervention of the analyst, the small number of simultaneous analyses allowed by unwieldy condensers, the need for a fume hood, and the increase in labor and in the danger of contamination dictated by the several reagents involved. A new procedure of high-temperature digestion that takes advantage of the excellent oxidizing properties of potassium dichromate in an acidic environment has been recently proposed for mercury analysis in vegetable materials (16). Free from the drawbacks caused by nitric acid, its validity is now being investigated in the analysis of fish, shellfish, egg, and meat-based baby food. Moreover, some practical modifications of the initial procedure are being suggested, because the oxidation of these materials brings about a less intense foaming than that of products with a high content of carbohydrates.

Experimental

Sample Preparation

Fresh, canned (no oil added), frozen, and freeze-dried products were all purchased off the shelf. Except for freeze-dried products, which were analyzed in their original state, all other samples were first devoided of inedible parts and homogenized in a blender after the addition of an equal weight of water (unnecessary in the case of eggs). Homogenates were frozen at –20°C and freeze-dried for a minimum of 48 h. The tissues were pulverized in the blender to improve mixing further. About 50–150 g of unsieved sample was prepared and kept under mild vacuum to maintain constant humidity (≤0.5%).

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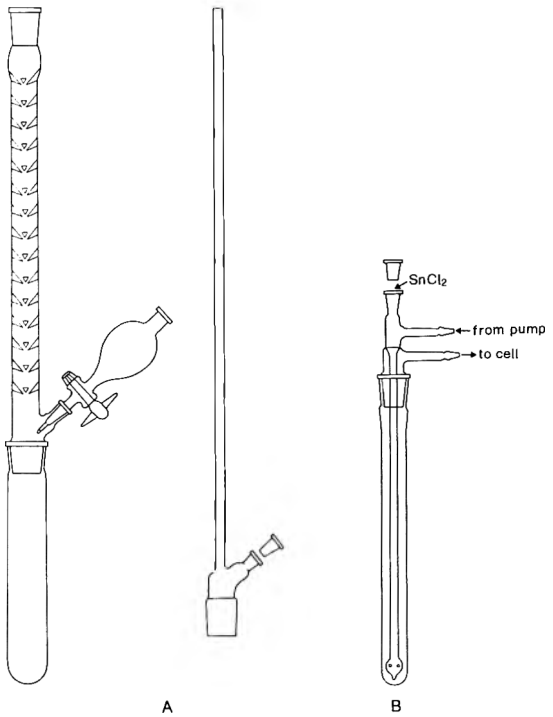

Figure 1. (A) Digestion tube with alternative refluxing heads: Vigreux column (mounted) and glass tube; (B) reduction vessel with bubbler.

Table 1. Instrumental conditions and analytical curve for mercury determination by CVAAS

Parameter	Value
Wavelength, nm	253.7
Slit width, nm	0.7
Absorbance expansion factor	1
Background corrector	Not needed
Range of linearity (m), μg	0.001–0.2
Calibration curve[a]	
$A = a^b + bm$	$A = (1.6 \pm 0.11)m$
Correlation coefficient (r)	0.9998
Mean standard residual deviation ($s_{y/x}$), %	2.3
Detection limit (m), μg[c]	0.001
Detection limit (m/m), μg/g[d]	0.008

[a] The confidence interval for the slope corresponds to the 95% probability level.
[b] The H_0 hypothesis on the intercept (H_0: a = 0) was accepted at the $p = 0.05$ level of significance.
[c] Calculated as the concentration giving a signal equal to 3 times the standard deviation of the absorbance readings of the reagent blank (n = 20).
[d] This value refers to a sample of 0.5 g dry wt and takes into account the volume (25 mL) of digest analyzed.

This procedure guaranteed preservation of the samples and maintenance of their homogeneity until the moment of analysis and recovery tests.

Apparatus

(**a**) *Reaction glassware.*—See Figure 1A. 250 × 32 mm id round-bottomed 100 mL calibrated Pyrex tube, 29/32 joint. 400 × 22 mm id Vigreux column, 29/32 joint or, alternatively, 500 × 6 mm id glass tube, 29/32 joint; both condensers bear a 10/19 sideneck in their lowest part for introduction of acid. Dropping funnel (50 mL) with Teflon stopcock and 10/19 joint to be connected to condenser.

(**b**) *Hot plate magnetic stirrer.*—Provided with continuously variable speed adjustment and temperature regulation.

(**c**) *Hot block digestor.*—Techne DG-1 programmable block digestion system with capacity for 20 digestion tubes.

(**d**) *Atomic absorption spectrophotometer.*—Model 603 equipped with Hg electrodeless discharge lamp (EDL), a D_2 background corrector, and a 140 mm path length, quartz widowed absorption cell (Perkin-Elmer). Instrument settings are reported in Table 1.

(**e**) *Reduction/aeration recirculating apparatus.*—An Hg Analysis System 303-0832 (Perkin-Elmer) was modified to reduce the dead volume of the original loop to a viable minimum.

The dessicant (magnesium perchlorate) was tightly packed inside a 12 mL drying tube. The reduction vessel (Figure 1B) consisted of a 280 × 20 mm id test tube with a 24/29 joint to fit the bubbler. The bubbler's air inlet sidearm was provided with a 10/19 aperture for the introduction of reductant, which is kept closed with a stopper during measurements.

Reagents and Standard Solutions

Distilled deionized water was used throughout. Gilson air-displacement pipets were used to dispense liquids up to 5 mL, and 1-mark Class A Pyrex pipets were used for higher volumes.

(**a**) *Potassium dichromate.*—AR grade (No. 4865 Merck, maximum Hg content 1×10^{-6} % m/m). Distribute salt evenly in a Pyrex crystallizing dish, heat in muffle furnace at 350°C for 4 days, and then increase temperature to 410°C to melt. Keep temperature for 24 h. Cool and grind crystals in ball mill or equivalent to obtain a powder.

(**b**) *Sulfuric acid.*—AR grade, d = 1.84 (No. 732 Merck, maximum Hg content 5×10^{-7} % m/m).

(**c**) *Dilute sulfuric acid (1.5 + 1).*

(**d**) *Stannous chloride solution.*—AR grade (No. 485005 Carlo Erba). Dissolve 25 g $SnCl_2 \cdot 2H2O$ in 80 g 10% v/v $H2SO_4$. Bubble N_2 (20 min) through solution to preserve it from oxidation and to eliminate eventual traces of Hg.

(**e**) *Mercury standard solutions.*—(*1*) *Stock solution.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ (No. 4419 Merck) in 100 mL H_2O. (*2*) *Working solution.*—1 μg/mL. Dilute 0.1 mL stock solution and 1 mL 0.05% $K_2Cr_2O_7$ solution to 100 mL with 10% v/v H_2SO_4.

(**f**) *Methylmercury chloride standard solutions.*—(*1*) *Stock solution.*—250 μg/mL. Dissolve 62.6 mg CH_3HgCl (No. 806100 Merck-Schuchardt) in 100 mL ethanol, and dilute to 200 mL with H_2O. (*2*) *Working solution.*—1 μg/mL. Dilute 0.2 mL stock solution to 50 mL with ethanol (1 + 1).

(**g**) *Phenylmercury acetate standard solutions.*—(*1*) *Stock solution.*—250 μg/mL. Dissolve 83.9 mg $C_6H_5HgOOCCH_3$ (No. 807588, Merck-Schuchardt) in 100 mL ethanol, and dilute

to 200 mL with H_2O. (*2*) *Working solution.*—1 μg/mL. Dilute 0.2 mL stock solution to 50 mL with ethanol (1 + 1).

(**h**) *Reference materials.*—National Institute of Standards and Technology Research Material 50 (NIST RM50) Albacore Tuna ("most probable" Hg content = 0.95 ± 0.1 μg/g); NIST Standard Reference Material 1549 Non-Fat Milk Powder (Hg content = 0.0003 ± 0.0002 μg/g).

Digestion Method

Procedure A.—Deposit an amount of sample corresponding to ca 0.50 g dry mass at the bottom of digestion tube containing a magnetic stirring bar. Add 4.40 g $K_2Cr_2O_7$ and 20 mL H_2O. Assemble apparatus as in Figure 1A using either type of indicated condensers. Dip digestion tube in tilted position into an ice water bath placed on a magnetic stirrer, and start vigorous stirring. Run 20 mL H_2SO_4 from dropping funnel along tube's walls dropwise so as to end operation in ca 5 min. Remove funnel, and insert stopper in place. Transfer apparatus to hot block preheated at 175°C, and mineralize for 60 min. Cool tube, rinse condenser and stirring bar with H_2O, and run washings into digest. Swirl tube to mix, and dilute to mark after cooling. (**Note**: If visual examination of digest indicates that all $K_2Cr_2O_7$ was consumed [a residual quantity as little as 0.1 g imparts a distinct yellow shade to the green digest], repeat digestion with an increased amount of oxidant.)

Procedure B.—Operate as in Procedure A in all respects, but omit use of ice water bath, and slow down addition of acid to ca 15 min to prevent foam from rising.

Procedure C.—With just sample and oxidant (no water) in the digestion tube, add 15 mL H_2SO_4 (1.5 + 1), and quickly connect the tube with the condenser with its sideneck stoppered. Keep stirring the mass until foam subsides (5 min), then introduce through sideneck a second 15 mL portion of the same acid and replace stopper. Heat tube 15 min in hot block at 100°C, increase temperature to 185°C, and end digestion in 90 min. Follow Procedure A for subsequent manipulations.

Determination of Mercury

(**a**) *Sample analysis.*—Pipet 25.0 mL digested sample into the reduction vessel (if a smaller volume has to be used to remain within the linearity range of the calibration graph, dilute to 25.0 mL with reagent blank). Start the recirculating pump, connect bubbler with stopper in place, and zero spectrophotometer on resulting signal. Remove stopper, add 3 mL stannous chloride solution, and replace stopper. Read absorbance when maximum constant value is attained. Disconnect loop's tubing at inlet port of pump, and vent mercury vapors to exhaust hood.

(**b**) *Reagent blank.*—Prepare blank by digesting a solid food item having a mercury content lower than the detection limit of analysis; the same treatment and a comparable diminution of the oxidation potential is, thus, assured to blank and analysis (see *Results and Discussion*). Use Procedure A if matrix is high in carbohydrates, or use any of the above-described procedures if the matrix is proteinaceous in nature. Keep the stated quantitative ratios between product and reagents, and process enough material to prepare a volume of final solution sufficient to work out calibration graph(s) and to adjust volume of sample aliquot taken to analysis. Perform digestion in an Erlenmeyer flask of suitable capacity heated on a hot plate magnetic stirrer set to a surface temperature of 180°C.

A suitable matrix of SRM 1549 Non-Fat Milk Powder was initially used but was very expensive. Therefore, a stock of egg, one of the products under examination that had a nondetectable Hg content, was produced and subsequently used.

(**c**) *Calibration graph.*—Add 0, 0.025, 0.050, 0.100, and 0.200 μg Hg to 25.0 mL reagent blank, and measure absorbances as for samples. Construct a new calibration curve after every fourth determination. The instrumental conditions and a typical standard curve with regression statistics are given in Table 1.

Results and Discussion

Amount of Oxidant

This method requires that the quantity of $K_2Cr_2O_7$, placed in one step in the digestion tube before acidification, be in excess with respect to the stoichiometric quantity required for the full ashing of the sample. This measure has been combined with the precaution of selecting operating conditions that do not allow concentrated H_2SO_4 to come into direct contact with the sample and that, furthermore, avoid localized overheating when H_2SO_4 mixes with water (Procedures A and B) and the heat of the energetic initial oxidation develops. Strong oxidizing conditions are ensured throughout the digestion process, and even the most imperceptible and transitory carbonization effect of the sample is avoided. As a result, the possibility of reduction of mercury to metal with the consequent irreversible escape from the liquid phase (8) is minimal, although it necessarily increases with the decline of the dichromate concentration. This phenomenon has to be reproduced in much the same way in the blank. However, the excess of dichromate must be moderate to limit the amount of stannous chloride needed for its reduction, which will add up to that assigned to the reduction of the mercuric ions. Consequently, all the matrixes considered were previously tested, and their consumption of oxidant was found to range from 6.23 to 8.39 g/g of dry product. Hence, for the sake of simplicity, the conditions of mercury analysis were made identical for all the matrixes, and 8.8 g of $K_2Cr_2O_7$/g of dry product was used, which corresponds to a 40% and a 5% oxidant excess for products having the minimum and maximum reducing power, respectively. This excess of $K_2Cr_2O_7$ is also useful to protect mercury from a premature reduction when the digest comes into contact with the reduction vessel and the bubbler, which carry persistent traces of stannous chloride from the preceding analysis even after repeated acid washings (17). This between-runs treatment is skipped in our case, and a brief washing of glassware with H_2O is all that is necessary.

Digestion Time

The digestion process has to achieve a 2-fold task: the complete mineralization of the matrix to avoid not only mercury

Table 2. Effect of time of digestion of NIST RM50 Albacore Tuna by Procedure A on amount of matrix oxidized and mercury recovery

Digestion time, min[a]	$K_2Cr_2O_7$ consumed, %[b]	Hg recovered, %[c]
10	84.8	44.7
20	90.3	73.8
30	93.5	85.5
40	96.1	92.0
50	97.0	97.9
60	100.0	102.2
70	101.0	99.5
85	100.5	103.8

[a] The 10 min digest was filtered through a 0.45 μm pore size, MF type Millipore filter. No solid residue was detected by weighing.
[b] Data refer to the same sample weight and are relative to the value found at 60 min taken as 100.
[c] As percent of certified Hg content.

trapping within residual solid particles (18) but also the interferences of soluble organic compounds forming stable bonds with mercury ions (e.g., the Hg–S bonds between cysteine and mercury) (19) and the breakdown of Hg–C bonds of those organomercurials (e.g., mono- or dimethyl, ethyl, phenyl mercury) present in the sample. In fact, stannous chloride has difficulty reducing organomercurial mercury (20). Therefore, the "complete dissolution" of the sample to give a "clear digest," as expressed by several authors, is by no means a guarantee of good results. To explore these aspects and to establish the shortest heating time of the digestion tubes that will produce the maximum effect in terms of mercury recovery, we analyzed the research material NIST RM50 Albacore Tuna according to the procedures proposed and quantified at the same time the amount of organic substance gradually oxidized. Table 2 reports the results relative to Procedure A. This shows that after only 10 min the organic moiety was completely solubilized and more than 80% oxidized, and more than 55% of the mercury was still present in a nondeterminable form. Subsequently, the transformation of the mercury gained momentum and was completed, together with the oxidation of the substrate, in 50–60 min. This pattern was verified for Procedure B as well; therefore, a total boiling time of 60 min was chosen in both cases. Procedure C, which required 90 min at maximum temperature to bring digestion to completion, was found to be less time-efficient. These time spans were applicable to all the products examined.

Analytical Features of the Method

The precision and accuracy of the method were determined in 2 ways: analyzing NIST RM50 and performing both recovery assays and replicate analyses of a wide variety of food samples.

NIST RM50, for which an Hg content of 0.95 ± 0.1 μg/g is indicated, was analyzed by Procedures A, B, and C to establish the day-to-day reproducibility. Two initial series of determinations were simultaneously run by applying Procedure A, using the Vigreux column as condenser in one case and the glass tube in the other. This allowed us to compare their efficiencies in refluxing the vapors of volatile Hg compounds at the actual room temperature of 27–31°C. The results are summarized in Table 3. Because the data indicated that the behaviors of the 2 condensers are identical, the glass tube was subsequently preferred because the condensate can be easily washed down from the tube's inner walls. The given 95% confidence limits of NIST RM50 are 0.85–1.05 μg/g: Table 3 shows that all the mean values found fall within the confidence interval of this material and are affected by a slight bias of +2.1, +3.2, and −1.1% for Procedures A, B, and C, respectively. None of the values obtained from the individual determinations falls outside the declared confidence interval. For all 3 procedures, the relative standard deviations (RSD, %), ranging between 1.8 and 3.0, can be considered satisfactory. These reproducibilities compare favorably with the within-laboratory repeatability data reported by Munns and Holland (21) at the conclusion of a collaborative study on Hg analysis in fish performed with a digestion procedure now accepted by AOAC (3). Next our efforts were directed at assessing the individual contribution of the digestion and the measurement phases to the total variabil-

Table 3. Determination of mercury (μg/g) in NIST RM50 Albacore Tuna[a] ("most probable" Hg content = 0.95 ± 0.1 μg/g) on different days by digestion Procedures A, B, and C and comparison of refluxing abilities of 2 condensers (Vigreux column and glass tube)[b] with digestion conducted by Procedure A

	Procedure			
	A[c]			
Day	Vigreux column	Glass tube	B[d]	C[d]
1	0.93	0.94	0.96	0.97
2	0.92	0.95	1.00	0.91
3	0.98	0.88	1.02	0.96
4	0.98	0.94	0.98	0.96
5	0.94	0.94	0.96	0.96
6	0.98	0.99	0.99	0.93
7	0.99	0.98	0.97	0.92
8	0.98	0.97	0.99	0.93
9	0.96	0.99	0.99	0.90
10	1.01	1.01	0.99	0.97
Mean	0.97	0.97	0.98	0.94
Range	0.92–1.01	0.88–1.01	0.96–1.02	0.90–0.97
SD	0.018	0.029	0.018	0.025
RSD, %	1.8	3.0	1.8	2.6
e, %[e]	+2.1	+2.1	+3.2	−1.1

[a] NIST supplies this material in lyophilized form and reports its Hg content as μg Hg/g freeze-dried weight.
[b] See Figure 1A.
[c] The 2 condensers were compared by running the 2 daily analyses simultaneously.
[d] With glass tube as condenser.
[e] Error (e), % was calculated as 100 $(Hg_{found} - Hg_{present})/Hg_{present}$.

Table 4. Determination of endogenous mercury in various seafoods and recovery of mercury from samples spiked with NIST RM50 Albacore Tuna (0.237 μg Hg)[a], methylmercury chloride (0.3465 μg Hg), and phenylmercury acetate (0.3520 μg Hg): samples (0.5 g and 0.25 g[a]) were digested by Procedure B with glass tube as condenser

Sample[b]		Endogenous Hg, μg/g dry wt[c]		Rec., %[d]		
		Mean ± SD	RSD, %	NIST RM50	CH_3HgCl	C_6H_5HgOAc
Fish						
Salmon	(canned)	0.070 ± 0.0042	6.0	96	97	102
Trout	(fresh)	0.076 ± 0.0084	11.0	97	95	101
Mackerel	(canned)	0.083 ± 0.0040	4.9	88	87	101
Hake	(frozen)	0.256 ± 0.0033	1.3	98	99	104
Plaice	(frozen)	0.280 ± 0.0041	1.5	100	101	104
Tuna	(canned)	2.71 ± 0.080	2.8	106	95	94
Swordfish	(fresh)	3.59 ± 0.064	1.8	103	88	102
Porbeagle[e]	(fresh)	14.9 ± 0.35	2.3	—	—	—
Shellfish						
Clam	(fresh)	0.052 ± 0.0037	7.1	100	100	103
Mussel	(fresh)	0.053 ± 0.0058	10.9	97	100	105
Crab	(canned)	0.162 ± 0.0072	4.4	95	98	104
Shrimp	(fresh)	0.189 ± 0.0077	4.1	87	98	102
Mean ± SD				97± 5.7	96 ± 4.5	102 ± 2.8

[a] As 0.25 g standard added to 0.25 g sample.
[b] The state of the products when purchased is given in parentheses.
[c] Average values were estimated from 5 independent determinations.
[d] Recovery percentages were determined from 2 independent experiments and calculated as Rec., % = 100 $(Hg_{found} - Hg_{present})/Hg_{added}$.
[e] The high content of Hg naturally present in this species made spiking meaningless.

ity of the analytical process. Using Procedure B, we mineralized in a single run enough NIST RM50 for the preparation of diluted digest sufficient for 10 determinations, each performed in a different day. The results were as follows: mean Hg content, 0.96 μg/g; range, 0.91–1.00 μg/g; RSD, 3.6 %. The comparison with Table 3 clearly shows that the contribution of digestion to data dispersion is irrelevant, and virtually all the variability is ascribable to, in our experience, the dynamics of the aeration step and the Hg adsorption phenomena inside the loop.

The commercial food samples were analyzed unspiked and with 3 different spikes, namely NIST RM50, standard solutions of methylmercury chloride and phenylmercury acetate for fish and shellfish tissues (SF), and standard solutions of mercuric chloride, methylmercury chloride, and phenylmercury acetate for the other protein-rich foods (PRF).

Table 5. Recovery of mercury (%) from egg and various meat and vegetable baby foods spiked with mercuric chloride (0.4515 μg Hg), methylmercury chloride (0.1733 and 0.3465 μg Hg), and phenylmercury acetate (0.1760 and 0.3520 μg Hg): samples (0.5 g) were digested by Procedure C with glass tube as condenser

Sample[a,b]		$HgCl_2$	CH_3HgCl		C_6H_5HgOAc	
			Spiking level		Spiking level	
			Low	High	Low	High
Whole egg	(fresh)	103[c]	103	99	96	103
Chicken + vegetables	(freeze-dried)	104	95	97	94	104
Lamb + vegetables	(freeze-dried)	104	97	97	93	102
Veal + vegetables	(freeze-dried)	102	104	97	106	99
Beef + vegetables	(freeze-dried)	104	101	99	102	100
Mean ± SD		103 ± 0.89	100 ± 0.89	98 ± 1.1	98 ± 5.6	102 ± 2.1

[a] Endogenous Hg in samples was lower than the detection limit.
[b] The state of the products when purchased is given in parentheses.
[c] Recovery percentages were determined from 2 independent experiences and calculated as in Table 4.

Procedure B was adopted for the first group of samples, and Procedure C was used for the second. The results of this study are shown in Tables 4 and 5. RSDs for Hg in unspiked SF specimens (Table 4), here a measure of repeatability, have generally the same order of magnitude (about 4% or less) as those previously found for the standard material. In 5 cases, RSDs were higher (5–11%). One of these samples (mussel) was reground, passed through a 32 mesh screen, and analyzed in quadruplicate. Because variability did not substantially improve, RSDs higher than 4% were shown not to stem from a nonhomogeneous distribution of Hg in those samples but rather from their low analyte content (≤0.08 μg/g). In our analytical scheme, this meant measuring spectrophotometrically a minimum absolute quantity of Hg of only 0.006 μg.

However, hoping to improve precision by submitting the entire amount (100 mL) of diluted digest to reduction/aeration would be illusory. The comparison of the actual calibration curve with the one established in the previous work (16), where the whole volume of 100 mL of diluted digest was processed, confirms that sensitivity (absorbance units/mass of Hg) drastically decreases by increasing the volume of the solution liberating Hg vapors (22). We concluded that approximately 0.05 μg/g Hg in the sample is the lowest limit allowed by this digestion method and instrumental setup that is compatible with an acceptable precision.

The fortification studies were conducted by keeping the same spike level for all SF samples, and 2 different amounts of Hg were added to PRF products in both methylmercury chloride and phenylmercury acetate spiking experiments. In SF specimens, Hg concentrations after spiking varied from 0.74 to 4.29 μg/g. Recoveries were in the 87–106% range, and an over-all mean recovery of 98 ± 5.1% was obtained. Recoveries lower than 90% (4 cases) do not seem to be positively correlated with the amount of Hg analyzed, and no particular matrix appears to be responsible for consistently high or low values. A better consistency of data was observed in spiking experiments with PRF samples (Table 5) that did not contain measurable Hg of their own and whose Hg concentrations after spiking were 0.9030 μg/g (as $HgCl_2$), 0.3466 and 0.6930 μg/g (as CH_3HgCl), and 0.3520 and 0.7040 μg/g (as C_6H_5HgAc). The overall mean recovery was 100 ± 3.6%.

Conclusions

Many countries still determine mercury toxicity in food by evaluating total mercury. Consequently, those analytical procedures not aimed at the speciation of Hg maintain their validity. The digestion method presented in this paper for the determination of Hg by CVAAS was tested on a variety of food samples, mostly fish species and other marine organisms. Three versions of the method, which differ in certain practical features, were compared, and the analyst can choose which method to use. All allow a high output, require extremely simple glassware, and can be run anywhere on a laboratory's benchtop with very little surveillance from the operator. In all cases, very good precision and adequate accuracy were experienced for Hg levels below and above the most commonly accepted tolerance levels in food (0.5–1.0 mg Hg/kg fresh wt ≅ 0.7–1.4 mg Hg/kg dry wt).

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METALS AND OTHER ELEMENTS

Determination of Arsenic in Vegetable Samples by Hydride Generation Atomic Absorption Spectrometry

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A procedure is described for the determination of arsenic in vegetable samples by hydride generation atomic absorption spectrometry. The samples are mineralized in a microwave acid digestion bomb with nitric acid in the presence of small amounts of vanadium pentoxide. The determination of arsenic is made by the standard addition method. A certified reference sample is analyzed, and the result obtained agreed well with the certified value. The detection limit (dry weight) was about 0.020 μg/g. Reproducibility relative standard deviations ranged from 6.45% at 0.152 μg As/g to 8.31% at 0.059 μg As/g . The concentrations of arsenic in vegetable samples ranged from 0.029 to 0.444 μg/g (fresh weight).

Large amounts of arsenic enter the environment each year because of the use of arsenic compounds in agriculture and industry as pesticides and wood preservatives. The prolonged intake of even low concentrations of arsenic can cause serious toxic effects to appear (1–3). Therefore, interest in analyzing arsenic levels in foodstuffs has grown in recent years (4–11).

As analytical samples, plants are a complex matrix, and most methods of analysis proposed require previous mineralization (12). Currently, the most widely accepted technique involves accelerated digestion by placing the sample in a sealed recipient subjected to acid and high pressure and bombarded with microwaves (microwave acid digestion bomb) (13–18).

The most common procedures for the determination of arsenic in plants are atomic emission, fluorescence, and absorption spectrometry. Wheras electrothermal atomic absorption spectrometry may suffer from interferences by the accompanying elements present in plants, hydride generation atomic absorption spectrometry should provide more freedom from those interferences, because the volatile metal hydride is separated from the interfering matrix. Therefore, the hydride generation technique has become the most widely used approach for arsenic analyses in vegetable samples (11, 19–22), and we chose to use this technique in the determination of arsenic.

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Experimental

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer Model 2380 with hollow cathode lamp (Perkin-Elmer Corp., Norwalk, CT 06856); Bausch & Lomb D-5000 chart recorder (Bausch & Lomb, Rochester, NY 14692); air–acetylene burner with cell mounting bracket.

(b) *Hydride generator.*—Perkin-Elmer Model MHS-10 (Perkin-Elmer Corp.).

(c) *Quartz absorption cell.*—Heated by air–acetylene flame (extra lean) (Perkin-Elmer Corp.).

(d) *Microwave oven.*—Moulinex Model FM-460 with 15–100% full power (600 W) capability in 25% increments.

(e) *Microwave acid digestion bomb.*—Parr Model 4782 (Parr Instrument Co., Moline, IL 61265).

Reagents

(a) *Nitric acid.*—Reagent grade, 65% (E. Merck, Darmstadt, Germany).

(b) *Hydrochloric acid.*—Reagent grade, 32% (E. Merck).

(c) *Vanadium pentoxide.*—Analytical reagent grade (E. Merck).

(d) *Sodium borohydride solution.*—3.0 g $NaBH_4$ (E. Merck)/100 mL 1% NaOH (E. Merck).

(e) *Arsenious oxide standard solutions.*—(*1*) *Stock solution.*—1 mg/mL. Dissolve 1.320 g As_2O_3 (E. Merck) in 25 mL 20% KOH solution, neutralize with 20% (w/v) HCl, and dilute to 1 L with 1.5% (w/v) HCl solution. (2) *Intermediate solution.*—10 μg/mL. Dilute 10 mL stock solution to 1 L with 1.5% (w/v) HCl solution. (*3*) *Working solution.*—1 μg/mL. Dilute 100 mL intermediate solution to 1 L with 1.5% (w/v) HCl solution.

(f) *Water.*—All solutions were prepared from deionized water with a specific resistivity of 18 MΩ-cm, obtained by filtering distilled water through a Millipore Milli-Q Model RO15 purifier immediately before use.

Digestion

Transfer ca 0.200 g lyophilized and homogenized sample to the microwave acid digestion bomb, and add 2.5 mL 65% HNO_3 and 50 μg V_2O_5 as a catalyst. Mineralization was com-

Table 1. Recoveries of arsenic from vegetable samples

Sample	Scientific name	Concn, μg/g			Rec., %
		Present	Added	Found	
Sugar cane-16	*Saccharum officinarum*	0.152	0.000	0.147	96.71
		0.152	0.032	0.177	96.20
		0.152	0.064	0.211	97.69
		0.152	0.129	0.274	97.51
Green bean-2	*Phaseolus vulgaris*	0.059	0.000	0.056	94.92
		0.059	0.015	0.072	97.30
		0.059	0.030	0.088	98.88
		0.059	0.045	0.108	103.85

plete in 90 s with the oven at its highest setting. The digests were cooled, and the resulting solutions were diluted to a total volume of 25 mL with deionized water.

Determination

Set up spectrophotometer according to manufacturer's recommendations, using peak hold mode and triplicate 5 s readings. Set wavelength at 193.7 nm and slit at 0.7 nm. Prepare reductant of 3% $NaBH_4$ and 1% NaOH. Analyze the samples by the standard addition method by adding amounts ranging from 0.0 to 4.0 μg/L to 4 sample aliquots of the original sample solution and diluting to volume with 1.5% (w/v) HCl depending on the original concentration of arsenic in the sample.

Results

Accuracy and Precision

The sensitivity found (equivalent to 0.0044 unit of absorbance) was 0.094 ng/mL.

For instrumental conditions used in sample analyses, our calculated analytical detection limit (23) was 0.020 μg/g (dry weight). Concentrations of arsenic in all samples exceeded the analytical detection limit.

Table 2. Accuracy of measured concentrations of arsenic in NBS standard reference material (μg/g dry weight)

Material	*n*	Concentration	
		Measured[a]	Reported[b]
SRM 1572 citrus leaves	10	3.094 ± 0.160	3.100 ± 0.300

[a] Mean ± SD.
[b] 95% confidence interval about mean.

The accuracy of the method was tested on the basis of recovery experiments, which were made by adding the quantities of arsenic(III) before the treatment of the solid samples. The results are listed in Table 1. Tests with SRM 1572 gave a value of 3.094 ± 0.160 μg/g (dry weight) (Table 2). Seven determinations of 2 different samples were treated statistically as described by Stiel (24), and the results of the precision tests are summarized in Table 3.

Analysis of Samples

The described procedure was applied to the analysis of arsenic in 51 vegetable samples from the area of Motril, province of Granada, Spain. The concentrations of arsenic ranged from 0.029 to 0.444 μg/g (fresh weight), and the results for all samples tested are shown in Table 4.

Discussion

Mineralization with the microwave acid digestion bomb technique was complete within 90 s, which represents a significant savings in time when compared with other methods that require hours or even days. The use of a small volume of acid and the simplicity of the entire procedure reduce the risk of contamination, an important factor in the determination of trace elements.

The application of this method resulted in satisfactory recoveries for the samples investigated. As shown by the results reported in Table 2, the microwave digestion technique does not affect the precision or accuracy of the arsenic determinations. The reported relative standard deviations are acceptably small (Table 3), and the analytical concentration agrees well with the certified value of the SRM 1572 (Table 2). These results confirm that this procedure is suitable for the determination of total arsenic in vegetable samples.

The limits of detection and sensitivity were comparable with those reported by other authors and were adequate for the sample concentrations of the samples analyzed in this study.

In conclusion, the decomposition method is less complicated and time-consuming than conventional dissolution tech-

Table 3. Precision of method for determination of arsenic in vegetable samples

Sample	Scientific name	*n*	Concn, μg/g[a]	RSD, %
Sugar cane-16	*Saccharum officinarum*	7	0.152 ± 0.0098	6.45
Green bean-2	*Phaseolus vulgaris*	7	0.059 ± 0.0049	8.31

[a] Fresh weight.

Table 4. Concentrations of arsenic in vegetable samples (μg/g fresh weight)

Sample	Scientific names	*n*	Range of concn, μg/g
Sugar cane	*Saccharum officinarum*	24	0.057–0.326
Maize	*Zea mais*	11	0.194–0.444
Potato	*Solanum tuberosum*	6	0.078–0.167
Green bean	*Phaseolus vulgaris*	4	0.059–0.073
Banana	*Musa paradisiaca*	2	0.048–0.114
Lettuce	*Lactuca scariola*	2	0.036–0.067
Tomato	*Lycopersicum esculentum*	2	0.029–0.035

niques. This method can be applied to any routine laboratory analysis used for quality control of vegetables.

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MICROBIOLOGICAL METHODS

Polyclonal Enzyme Immunoassay Method for Detection of Motile and Non-Motile *Salmonella* in Foods: Collaborative Study

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A new enzyme immunoassay (EIA) method for detection of motile and non-motile *Salmonella* was examined in a multilaboratory collaborative study. This method uses a proprietary formulation of polyclonal antibodies to *Salmonella* and is controlled to maintain specificity. Sensitivity is enhanced with an additional antibody reaction designed to minimize false-negative reactions attributable to steric interference that can occur during conjugate binding in immunoassay procedures. Thirty-two laboratories participated in this evaluation, which included 6 food types: nonfat dry milk, dry egg, black pepper, soy flour, chocolate, and ground poultry. Of the 1020 samples analyzed, there was a 97.2% agreement rate between the EIA method and the AOAC/*Bacteriological Analytical Manual* (BAM) culture method, 967.26. False-negative rates for the 2 methods were comparable for all foods and all *Salmonella* levels except ground poultry, where the EIA method detected significantly more confirmed positive samples than did the AOAC/BAM method. Nineteen samples were positive by EIA but negative by the culture method, and 10 samples were negative by EIA but positive by the culture method.

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A new polyclonal enzyme immunoassay (EIA) for *Salmonella* is configured in a microwell plate format. It is designed for the rapid detection of motile and non-motile *Salmonella* in all finished food products, raw food materials, and environmental samples.

Specificity and sensitivity are key parameters in determining a microbiological assay's performance. A number of evaluators have examined the specificity of various rapid methods (1–4), and a recent study compared 3 commercially available methods for both specificity and sensitivity performance (5). The present method addresses specificity by using a complex proprietary blend of *Salmonella*-specific antibodies that are reactive with motile and non-motile strains. Sensitivity is enhanced through the addition of another antibody that immunochemically links the bound *Salmonella* antigens with the enzyme conjugate. This is desirable because the conjugates used in EIA assays are very large polymers of several hundred thousand dalton molecular weight. The size and shape of such molecules limit the binding efficiency to specific epitopes on the cell surface. This physical limitation is called steric interference. The additional antibody in the present method functions as a linking reagent to improve the binding efficiency, because its small size and configuration facilitate efficient immunochemical coupling.

The new method was evaluated in a precollaborative study using 20 food groups, each inoculated with a low level and a high level of *Salmonella*. In total, 1000 samples were analyzed by the EIA method and the AOAC/*Bacteriological Analytical Manual* (BAM) culture method, **967.26**. For all food types, 17 samples were negative with EIA but positive by the culture method. Twenty-seven samples were negative by the culture method but confirmed as positive by EIA. This resulted in an overall false-negative rate of 2.3% for the EIA and 3.7% for the

Table 1. Foods analyzed in the collaborative study and inoculating microorganisms

Food type	Inoculated *Salmonella*	*O* Group	Somatic antigens	Flagellar antigens
Nonfat dry milk	*S. cubana*	G2	1, 13, 23	Z29: —
Dry egg	*S. enteritidis*	D1	1, 9, 12	g, m: (1, 7)
Pepper	*S. luciana*	F	11	a: e, n, Z_{15}
Soy flour	*S. montevideo*	C1	6, 7, 14	g, m, s: —
Chocolate	*S. anatum*	E1	3, 10	e, h: 1, 6
Raw poultry	*Salmonella* spp.[a]	B, C1	—	—

[a] Naturally contaminated.

culture method. There were no false-positive isolates detected in the precollaborative study.

A multilaboratory collaborative study was performed to validate the EIA method. The study was designed to meet AOAC requirements for collaborative studies (6).

Collaborative Study

The multilaboratory collaborative was conducted using nonfat dry milk. dry egg, black pepper, soy flour, chocolate, and ground poultry. This selection represents a variety of food types that require different enrichment conditions and are recognized as potential sources of *Salmonella*.

Preparation of Inoculum

All strains were grown in brain heart infusion (BHI) broth for 18–24 h at 35°C, centrifuged, and suspended in 10% nonfat dry milk for freeze-drying. Lyophilized cultures were stored at −20°C until used.

Inoculated nonfat dry milk, dry egg, black pepper, and soy flour samples were prepared by adding the lyophilized culture listed in Table 1 to approximately 5 g food. This concentrated sample was stomached for 10 min and completely transferred to 200 g food. The 200 g quantity was vigorously shaken 3–4 min in a nonbreakable bottle. The entire quantity was then added to about 1000 g food and vigorously remixed. Most probable number (MPN) levels were then estimated. A final dilution was made into the appropriate quantity of food to achieve the target inoculation level. Low moisture foods received lyophilized cultures of *Salmonella* at 2 levels: 1–5 cells/25 g (low level) and 10–50 cells/25 g (high level).

Ground poultry samples were naturally contaminated. Several lots of ground poultry were procured and tested individually to estimate contamination levels. On the basis of these estimations, lots were combined, thoroughly mixed to approximate target contamination levels, subdivided, and frozen. Samples remained frozen throughout storage, during distribution to collaborators, and upon receipt until the day the test was initiated.

Chocolate was melted and then inoculated at 55°C with a broth culture.

Sample Distribution

Collaborators received a set of 15 samples for each food containing approximately 25 g test material (Table 2). Samples were identified by food type and sample number. For ground poultry products, collaborators received sufficient additional product to perform direct selective enrichment according to the AOAC method. Five samples of each processed food type were uninoculated controls, 5 samples were inoculated with low levels of *Salmonella*, and 5 samples were similarly inoculated with high levels. These products were distributed via an overnight delivery service. Test products were sent to participating collaborators during the week preceding the Monday that analyses were to be initiated. Ground poultry products were frozen before shipment, transported with dry ice, and maintained in a frozen state upon receipt by collaborators before initiating testing. Collaborators were instructed to analyze each sample by both the *Salmonella* EIA and the AOAC/BAM cultural method, thereby allowing for a paired comparative analysis of results.

Collaborative study instructions, EIA test kits, and test samples were supplied by BioControl Systems, Inc. All other materials were the responsibility of the collaborators.

The *Salmonella* strains used to inoculate products are listed in Table 1. Levels were determined by MPN analysis. MPNs were initiated on the same day the food type was analyzed. Triplicate samples of 100, 10, 1, and 0.1 g were preenriched with 900, 90, 9, and 10 mL of the medium appropriate for the food on the basis of the culture method (7). Enrichment broths were analyzed for *Salmonella* according to AOAC culture method, and levels were determined from MPN tables.

Sample Analysis

The culture method for the detection of *Salmonella* was performed as previously described (7, 8). The EIA procedure is described below.

Data Analysis

Test sample optical density data were analyzed by comparison with replicate positive control readings. Positive controls were scrutinized for reproducibility and then multiplied by a factor to determine the cut-off reading upon which a presumptive positive determination was made. In the analysis of optical density data, 2 factors, 0.20 and 0.25, were used to determine the optimal factor to minimize the incidence of both false-negative and false-positive reactions. Tabulated data (Tables 3–9) reflect the use of the 0.20 factor. Sample optical densities were then analyzed using the 0.25 factor, and changes, if any, were noted in *Results*.

Table 2. Food matrix analyzed by each collaborating laboratory[a]

Laboratory	Nonfat dry milk	Dry egg	Black pepper	Soy flour	Chocolate	Ground poultry
1	y	y	y	y	y	y
2	y	y	y	—	—	y
3	y	y	y	y	y	y
4	y	y	y	y	y	y
5	y	y	y	y	—	y
6	y	y	y	y	—	y
7	y	y	y	y	y	y
8	y	y	y	y	—	y
9	y	y	y	y	y	y
10	y	—	—	y	—	y
11	y	—	y	—	—	—
12	y	y	—	—	y	—
13	y	y	y	—	—	—
14	—	—	—	—	—	y
15	—	—	—	—	—	y
16	—	—	—	y	—	y
17	—	—	—	y	y	—
18	—	—	—	—	y	y
19	—	—	—	—	—	y
20	—	y	—	—	—	—
21	—	y	y	—	—	—
22	—	y	—	—	—	—
23	—	y	—	—	y	—
24	—	y	—	—	—	—
25	—	y	—	—	—	—
26	—	—	—	y	—	—
27	—	—	—	—	y	—
28	—	—	—	—	y	—
29	—	—	—	—	y	—
30	—	—	—	—	y	—
31	—	—	—	—	y	—
32	—	—	—	—	y	—

[a] Key: y = collaborator analyzed this food type; — = collaborator did not analyze this food type.

Pair-wise statistical analyses of the proportions positive for the methods were performed for each food group and each inoculation level by using previously published methods (9–11). A chi square value (χ^2) of greater than 3.84 indicated a statistically significant difference of the proportions positive at the 5% probability level.

992.11 Motile and Non-Motile *Salmonella* in Foods—Polyclonal Enzyme Immunoassay Method

First Action 1992

(Applicable to the detection of motile and non-motile *Salmonella* in all foods)

Method Performance:

See Table 992.11 for method performance data.

A. Principle

In polyclonal enzyme immunoassay (EIA) method, proprietary antibodies with high specificity to somatic and flagellar *Salmonella* antigens are bound to microwell plates. Appropriately enriched samples and positive controls are added to plates; any *Salmonella* antigens present will bind to prepared microwells, forming antibody–antigen complex. Nonreactive sample material is washed away. Another *Salmonella*-specific antibody is added, which enhances sensitivity of this method by immunochemically linking bound *Salmonella* antigens to enzyme conjugate. An alkaline phosphatase antibody conjugate is added, and, after incubation, unbound conjugate is washed away. The substrate, *p*-nitrophenylphosphate, is added; absorbance of resulting colored complex is read at 405–410 nm.

B. Reagents

(**a**) *Wash solution concentrate.*—2% polyoxyethylene 20 sorbitan monolaurate (Tween 20) in H_2O.

(**b**) *Substrate tablets.*—5 mg *p*-nitrophenylphosphate per tablet.

(**c**) *Substrate diluent.*—1M diethanolamine in H_2O.

(**d**) *Positive control.*—Stabilized, inactivated *Salmonella* antigen.

(**e**) *Antibody solution.*—Specific polyclonal flagellar and somatic antibodies to *Salmonella* serotypes (ASSUR-LINK® antibody solution is suitable).

(**f**) *Conjugate solution.*—Specific antibodies to antibody, **B(e)**, conjugated to alkaline phosphatase.

(**g**) *Stop solution.*—20% ethylenediaminetetraacetic acid (EDTA) in H_2O.

(**h**) *Antibody-coated microtiter wells.*—12-well microtiter well strips, wells coated with polyclonal *Salmonella* antibody, 96-well holder and cover.

(**i**) *M-broth.*—Broth containing 5.0 g yeast extract, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g K_2HPO_4, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L H_2O, and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ±0.2.

(**j**) *Diagnostic reagents.*—For culture confirmation of presumptive positive EIA tests; see **967.25B**.

Items (**a**)–(**h**) are available as Assurance® *Salmonella* Enzyme Immunoassay (EIA) test kit for detection of motile and non-motile *Salmonella* from BioControl Systems, Inc., 19805 North Creek Parkway, Bothell, WA 98011.

C. Apparatus

(**a**) *Incubator.*—Capable of maintaining 35–37°.

(**b**) *Water bath.*—Capable of maintaining 42 ±0.5° and 100 ±2° (or flowing steam autoclave set at 100°).

(**c**) *Microplate washer or plastic squeeze bottle.*—For washing 12-well microtiter strips.

(**d**) *Microplate reader.*—Photometer with 405–410 nm filter, capable of reading microtiter plates. May include optional printer.

D. General Instructions

Reagents must be stored at 2–8° when not in use. Let reagents warm to room temperature before use. Include duplicate positives and one blank test well with each run of test samples. Use separate pipet for each sample and reagent to avoid cross-contamination. Kit reagents and components must be used as an integrated unit and may not be mixed with components from other manufacturing batches or sources. Use dedicated trough or glassware for each reagent to avoid cross-contamination. Do not use reagents beyond stated expiration date. Do not reuse microwells.

E. Preparation of Sample

(**a**) *Preenrichment.*—Prepare samples according to procedures described in **967.26A** or *Bacteriological Analytical Manual*, 6th ed., AOAC, Arlington, VA, Chapter 7, sec. C, with following exception: *Raw or highly contaminated meat.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (20 000 rpm), and decant blended homogenate into sterile, wide-mouth, screw cap 500 mL jar. Cap jars securely and let stand 60 min at room temperature. Mix well by shaking and determine pH with test paper. Adjust pH to 6.8 ±0.2, if necessary, using sterile 1N NaOH or HCl; cap jar securely and mix well before determining final pH. Loosen jar caps 1/4 turn.

For all samples, incubate 24 ±2 h at 35–37°.

(**b**) *Selective enrichment.*—Following preenrichment incubation, transfer 1.0 mL preenrichment broth to one 10 mL tube tetrathionate broth (TT) and 1.0 mL preenrichment broth to one 10 mL tube selenite cystine broth (SC) as in **967.26B(a)**. Label tubes. Incubate according to sample type: *Processed foods.*—Incubate selective enrichment broths 6–8 h at 35–37°. *Raw foods.*—Incubate tetrathionate enrichment broths 18–24 h in 42 ±0.5° water bath and selenite cystine enrichment broths 18–24 h at 35–37°.

(**c**) *Postenrichment.*—Following selective enrichment incubation, transfer and combine 0.5 mL of each paired TT and SC broths into single tube containing 10 mL M-broth, **B(i)**. Label tubes. Incubate according to sample type: *Processed foods.*—Incubate M-broth enrichments 14–18 h at 35–37°. *Raw foods.*—Incubate M-broth enrichments 6–8 h in 42 ±0.5° water bath.

(**d**) *Preparation of sample for EIA analysis.*—Following M-broth incubation, mix, by vortex mixer, tube contents and transfer 1.0 mL to tube. Retain M-broth sample tubes for confirmation of presumptive positive results.

Inactivate microorganisms by submersing tube containing 1 mL M-broth in boiling water bath for 20 min. Cool tubes to 25–37° before testing. Boiled samples can be stored at 2–8° up to 3 days prior to testing.

F. Enzyme Immunoassay Procedure

(**a**) Prepare reagent dilutions and let reagents and components reach room temperature before starting assay. (*1*) *Wash*

Table 992.11. Method performance for 992.11, testing of motile and non-motile *Salmonella* in foods

Results	%
Agreement[a]	97.2
False negative (EIA)[b]	2.1
False negative (AOAC/BAM)[c]	3.9
False negative (EIA)[d]	1.8
False negative (AOAC/BAM)[e]	3.5

[a] Proportions of samples that agree between AOAC/BAM culture method and EIA method expressed as percentage.

[b] Proportions of samples confirmed as positive by AOAC/BAM culture method but negative by EIA method expressed as percentage of total number of correctly classified negative samples plus number of false negatives by this method.

[c] Proportions of samples confirmed as positive by EIA method but negative by AOAC/BAM culture method expressed as percentage of total number of correctly classified negative samples plus number of false negatives by this method.

[d] Proportions of samples confirmed positive by AOAC/BAM culture method but negative by EIA method expressed as percentage of total number of positive samples.

[e] Proportions of samples confirmed as positive by EIA method but negative by AOAC/BAM culture method expressed as percentage of total number of positive samples.

solution preparation.—Add 1.0 mL wash solution concentrate, **B(a)**, to 100 mL H_2O (sufficient to wash 48 wells). Label container. Stable for 30 days at 2–8°. (*2*) *Substrate solution preparation.*—Add 1 substrate tablet **B(b)** to 5.0 mL substrate diluent, **B(c)**, (sufficient for 48 wells). Label container. Prepare only amount needed for immediate use. Discard unused solution.

(**b**) Prepare reader by installing 405 or 410 nm filter in photometer.

(**c**) Fit required number of microwell strips into holder, allowing for 2 positive controls and 1 blank. Reseal unused microwells in foil pouch. Carefully record positive controls, blank, and sample positions in holder.

(**d**) Vortex mix samples and positive control before pipetting. Pipet 100 µL of each sample into sample wells. Pipet 100 µL positive control, **B(d)**, into each positive control well. Leave blank well empty.

(**e**) Cover microplate and incubate 30 min at 35–37°. Do not stack anything on microwell holder during incubation.

(**f**) Wash each well 3× as follows: (*1*) *Washing procedure.*—Completely remove contents of wells with microwell washer. Immediately fill wells with 250 µL wash solution, **F(a)***(1)*. Repeat 2×. Avoid overfilling wells. Avoid underfilling wells (to prevent ineffective washing). Effective washing is critical to obtaining accurate data. (*2*) *Alternative washing procedure.*—Remove contents of well by inverting and vigorously tapping plate. Wash wells 3× using wash bottle (precleaned) containing wash solution and completely filling each well.

(**g**) Immediately after aspiration of 3rd wash, invert antibody solution bottle, **B(e)**, several times to gently mix. Add 100 µL antibody solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°.

(**h**) Wash each well 3×, as in (**f**).

(**i**) Immediately after aspiration of 3rd wash, invert conjugate solution bottle, **B(f)**, several times to gently mix. Add 100 µL conjugate solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°.

(**j**) Wash each well 3×, as in (**f**).

(**k**) Immediately after aspiration of 3rd wash, add 100 µL substrate solution, **F(a)***(2)*, to each well, including control and blank wells. Cover and incubate 30 min at 35–37°.

G. Reading Results

(**a**) Immediately after incubation, **F(k)**, read absorbance at 405 or 410 nm. Microwell plate reader must be calibrated against blank well before reading samples and control. Standardize reader by adjusting optical density (OD) of blank well to 0. Next, read 2 positive control wells, and then sample wells. (**Note**: Certain samples may read <0; this is not uncommon and indicates a negative result.)

(**b**) If reading will be delayed, add 50 µL stop solution, **B(g)**, to each well. Read within 1 h.

H. Interpretation of Test Results

(**a**) *Control value.*—Positive control reading should be 0.8–2.5 OD units. Readings not within this range may indicate problems with washing procedure.

(**b**) *Cutoff value.*—Calculate average value of 2 positive control readings (in OD units) and multiply by 0.25 to determine cutoff value.

(**c**) *Negative results.*—Samples with OD readings less than cutoff value are negative.

I. Confirmation of Positive EIA Samples

Samples with readings greater than or equal to cutoff value are presumptively positive. Positive samples must be confirmed using culture methods by streaking from M-broth tubes as described in **967.26B**. Typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: *J. AOAC Int.* (1992) **75**, November/December issue

Results

Thirty-two laboratories participated in the study by agreeing to analyze at least one food type. Five laboratories analyzed all 6 food types. For each food, 15 samples were analyzed by each participating laboratory. At the end of the study, there were valid data from 1020 samples, which were statistically analyzed.

The result agreement between the EIA method and the AOAC/BAM method was 97.2%. For the 1020 samples, 517 were *Salmonella* positive by both methods and 474 were negative by both methods, 19 were positive by EIA and negative by AOAC/BAM, and 10 were negative by EIA and positive by AOAC/BAM.

Salmonella was recovered from 546 valid samples; the EIA method detected 536 confirmed positives, and the culture method detected 527 positives. The false-negative rates for the 2 methods were comparable for all foods and all *Salmonella* levels, except ground poultry where the EIA method detected significantly more confirmed positive samples than the AOAC/BAM procedure.

Data from each laboratory were scrutinized for compliance with protocol requirements, performance of assay according to directions for use, and precision of the positive control data. Results of laboratories were eliminated from an individual food type for the following reasons: uninoculated controls were positive for *Salmonella* where biochemical and serological confirmation data indicated that the *Salmonella* isolated from the uninoculated control was the same microorganism used to seed the food type, laboratory initiated testing on the wrong day, laboratory failed to follow protocol or directions, sample confirmation data were not submitted by the laboratory, positive control data were invalid, equipment malfunctioned, and/or laboratory resigned from the study. The data set from any laboratory meeting any of these criteria was omitted from the analysis for that particular food type and appropriately annotated in the data table.

The approved collaborative study protocol required that each food type must have at least 10 laboratories submitting valid data. The number of valid laboratories for each of the 6 food types ranged from 10 to 14 after exclusion of invalid results. In all cases, more than 10 laboratories began the analysis. For chocolate, 11

Table 3. Individual sample results by collaborator for nonfat dry milk

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
9	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
10[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
13	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
	EIA[c]														
1	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
2	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+
3	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
6[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
8	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
9	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
12	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
13	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

[a] Decoded sample identification: 1–5, uninoculated; 6–10, high seed, 2.4 cells/g; 11–15, low seed, 0.24 cell/g.
[b] Data excluded for analysis. Laboratory did not confirm results and report final data.
[c] First entry is presumptive result, second entry is confirmed result, i.e., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

laboratories started. After elimination of invalid data, only 7 laboratories remained for this food; therefore, more collaborators were recruited, and the analysis of this food type was repeated. Upon rerun, 14 valid data sets were reported.

Tables 3–8 present individual collaborator results by food type. These tables are annotated to document all laboratories for which individual data sets were excluded from the analysis. Results are analyzed by individual food type in the following sections.

Nonfat Dry Milk

Thirteen laboratories analyzed nonfat dry milk. Data for laboratories 6 and 10 were omitted from the analysis. Laboratory 6 did not submit data for the confirmation of *Salmonella* by the culture method. Laboratory 10 did not complete the confirmation process for either method. All other laboratories reported all 55 uninoculated samples as *Salmonella* negative and correctly confirmed presumptively positive reactions (Table 3).

All nonfat dry milk samples inoculated at the low level were positive by both the EIA and AOAC/BAM methods. The *Salmonella* level in these samples was 0.24 cell/g. For the high level samples containing 2.4 cells/g, 54 were positive by both methods, and 1 sample was positive according to the AOAC/BAM method and negative according to the EIA method. No false-positive reactions were reported for the EIA method, and χ^2 analysis for all nonfat dry milk samples was 0. Analysis of the EIA data using the 0.25 cutoff calculation factor did not change the results for any sample.

Dry Egg

Seventeen laboratories analyzed dry egg. Eleven laboratories completed the analysis and submitted valid data. Laboratory 6 reported 1 uninoculated control sample as positive for *Salmonella*. Laboratory 25 resigned from the study without completing the analysis. Laboratories 1 and 8 submitted valid data but started on the incorrect date. Laboratory 5 reported invalid positive control values, and laboratory 24 had a

Table 4. Individual sample results by collaborator for dry egg

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1[b]	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
2	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
3	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
4	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
5[c]	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
6[d]	+	+	+	+	+	–	+	–	–	–	+	+	+	+	+
7	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
8[b]	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
9	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
12	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
13	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
20	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
21	+	–	–	+	+	–	–	–	–	–	+	+	+	+	+
22	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
23	+	–	+	+	+	–	–	–	–	–	+	+	+	+	+
24[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
25[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	EIA[g]														
1	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
2	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
3	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
4	+/+	+/+	+/+	+/+	+/+	+/–	+/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
5[c]	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
6[d]	+/+	+/+	+/+	+/+	+/+	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
7	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
8[b]	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
9	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+
12	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
13	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
20	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
21	+/+	–/–	+/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
22	–/–	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
23	+/+	–/–	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
24[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
25[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Decoded sample identification: 1–5, low seed, 0.15 cell/g; 6–10, uninoculated; 11–15, high seed, 1.1 cells/g.
[b] Data excluded from analysis. Laboratory initiated testing on incorrect day.
[c] Data excluded from analysis. Invalid positive control readings.
[d] Data excluded from analysis. Uninoculated controls reported as positive.
[e] Data excluded from analysis. Microplate reader malfunctioned.
[f] No data submitted. Laboratory resigned from study.
[g] First entry is presumptive result, second entry is confirmed result, e.g., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

microplate reader that would not calibrate. These 6 laboratories were omitted from the analysis. The 11 other laboratories reported all 55 uninoculated samples as negative (Table 4).

Samples inoculated at the low level contained 0.15 cell/g. At this inoculation level, 51 samples were positive by both methods, 1 sample was negative by the EIA method but positive by AOAC/BAM method, and 3 samples were confirmed as negative by both methods. The high level samples contained 1.1 cells/g. Fifty-four samples were positive by both methods, and 1 sample was negative by the EIA method and positive by the culture method; χ^2 analysis for all dry egg samples was 0.5.

False-positive assays were observed on 1 low level sample and 2 control samples. No false positives were observed on the high level samples. When the EIA data was analyzed using the

Table 5. Individual sample results by collaborator for black pepper

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
2	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
3	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
4	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
5[b]	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
6	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
7[c]	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–
8	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
9	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
11	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
13	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
21	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+
	EIA[d]														
1	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
2	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
3	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
4	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
5[b]	+/+	–/–	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	–/–	+/+
6	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
7[c]	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	–/–	–/–
8	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
9	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+
11	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
13	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
21	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+

[a] Decoded sample identification: 1–5, high seed, 0.46 cell/g; 6–10, uninoculated; 11–15, low seed, 0.093 cell/g.
[b] Data excluded for analysis. Invalid positive control readings.
[c] Data excluded from analysis. Uninoculated controls reported as positive.
[d] First entry is presumptive result, second entry is confirmed result, i.e., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

0.25 cut-off calculation factor, no confirmed *Salmonella*-positive samples became negative; however, the 2 false-positive control samples were eliminated.

Black Pepper

Twelve laboratories analyzed black pepper. Data from 2 laboratories were not acceptable, resulting in 10 valid sets of data. Laboratory 7 was eliminated because 2 uninoculated control samples were reported as positive; laboratory 5 reported invalid positive control values. The 50 uninoculated samples from the 10 remaining laboratories were all negative (Table 5).

Forty-nine black pepper samples inoculated at the low level of 0.093 cells/g were positive by both the EIA and the AOAC/BAM methods. One sample at this level was negative by both methods. For the high inoculation level samples, containing 0.46 cell/g, 44 samples were positive by both methods, 5 were negative by both methods, and 1 was EIA negative and AOAC/BAM positive. The χ^2 analysis for all black pepper samples was 0.

No false-positive reactions were reported for the EIA method. Analysis of the EIA data using the 0.25 cutoff calculation factor produced one additional false-negative reaction, elevating the χ^2 value to 0.5. There were no false-positive reactions using this factor.

Soy Flour

Twelve laboratories analyzed the soy flour samples. Laboratory 10 reported invalid positive control readings. Laboratory 26 reported an uninoculated sample as positive for *Salmonella*. Both laboratories were eliminated. Ten laboratories reported all 50 uninoculated samples as negative (Table 6).

Confirmed sample agreement for soy flour was 99.3%; only one discrepant sample was detected by EIA but not by AOAC/BAM. Twenty-seven low and 28 high samples were positive by both the EIA and AOAC/BAM methods. Twenty-two low and 22 high samples were negative by both methods. One low sample was positive in the EIA method and negative in the AOAC/BAM method.

Table 6. Individual sample results by collaborator for soy flour

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	+	+	–	+	+	+	+	+	–	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	–	–	+	+	+	+	–	+	+	+
5	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
6	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10[b]	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17	–	–	–	–	–	+	–	+	+	+	–	+	+	+	–
26[c]	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	EIA[d]														
1	–/–	+/–	+/–	–/–	–/–	+/+	+/+	+/–	+/+	+/+	+/+	+/+	+/+	–/–	+/+
3	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	–/–	–/–	–/–	+/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+
5	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–
6	+/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
7	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
8	+/–	–/–	+/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
9	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–
10[b]	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+
16	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
17	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	–/–
26[c]	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–

[a] Decoded sample identification: 1–5, uninoculated; 6–10, low seed, 0.004 cell/g; 11–15, high seed, 0.15 cell/g.
[b] Data excluded for analysis. Invalid positive control readings.
[c] Data excluded from analysis. Uninoculated controls reported as positive.
[d] First entry is presumptive result, second entry is confirmed result, i.e., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

The low level test samples contained 0.004 cell/g, and high level samples contained 0.15 cell/g, which were both significantly below the target inoculation levels of 0.04–0.2 and 0.4–2.0 cells/g. These very low contamination levels may have caused differences in laboratory recovery efficiency, because samples were subdivided in a totally random manner across all collaborators. More important, agreement was excellent within each laboratory. The χ^2 analysis of all soy flour samples was 0.

One low level sample, 1 high level sample, and 6 uninoculated samples were positive in the EIA assay but were not confirmed by culture isolation. The possibility that they were true positives cannot be eliminated. More rigorous analysis may have revealed the presence of *Salmonella*, especially in the inoculated samples. Analysis of the EIA data using the 0.25 cutoff calculation factor eliminated 1 uninoculated false-positive sample. No confirmed *Salmonella*-positive samples became negative.

Chocolate

Fifteen laboratories analyzed chocolate. Fourteen laboratories completed the analysis and submitted valid data. Laboratory 31 reported 2 uninoculated control samples as positive for *Salmonella*. The remaining laboratories reported all 70 uninoculated samples as negative for *Salmonella* (Table 7).

Low level *Salmonella* contained 0.24 cell/g. All 70 samples were positive for *Salmonella* by both the EIA and the AOAC/BAM methods. The high inoculation level was 1.10 cells/g. All 70 samples were positive by both the EIA and the AOAC/BAM methods. No false-negative reactions were reported by either method; χ^2 analysis of all chocolate samples was not applicable because results were identical for both methods.

False-positive optical density readings were observed on 13 uninoculated samples. Analysis of the EIA data using the 0.25

Table 7. Individual sample results by collaborator for chocolate

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
9	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
17	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
18	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
23	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
27	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
28	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
29	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
30	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
31[b]	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
32	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
	EIA[c]														
1	+/–	–/–	–/–	–/–	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
7	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
9	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
12	–/–	+/–	+/–	+/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
17	–/–	+/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
18	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
23	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
27	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
28	+/–	+/–	–/–	–/–	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
29	+/–	+/–	–/–	+/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
30	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
31[b]	–/–	+/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
32	–/–	–/–	–/–	–/–	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

[a] Decoded sample identification: 1–5, uninoculated; 6–10, high seed, 1.1 cells/g; 11–15: low seed, 0.24 cell/g.
[b] Data excluded for analysis. Uninoculated controls positive.
[c] First entry is presumptive result, second entry is confirmed result, i.e., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

cutoff calculation factor eliminated 5 uninoculated false-positive reactions. No confirmed *Salmonella*-positive samples became negative.

Ground Poultry

Fifteen laboratories analyzed the ground poultry samples. Twelve laboratories submitted valid data (Table 8). Laboratory 6 did not submit data for the confirmation of *Salmonella* by the culture method. Laboratory 10 did not perform the complete confirmation procedure on either method. Laboratory 19 had an invalid EIA assay, resulting in no appreciable optical density readings.

Ground poultry samples were naturally contaminated with low levels of *Salmonella*. The high, medium, and low levels were 0.24, <0.003, and <0.003 cell/g, respectively. For the high, medium, and low levels, 15, 0, and 0 samples were positive by both methods, respectively. Twenty-six, 59, and 56 samples were negative by both methods. Eighteen, 0, and 0 samples were EIA positive but AOAC/BAM negative; and 1, 1, and 4 samples were EIA negative but AOAC/BAM positive, respectively.

The χ^2 analysis of all ground poultry samples was 5.04, indicating a statistically significant difference; the EIA method detected more confirmed *Salmonella* than did the culture method. This difference may be attributed to both the very low

Table 8. Individual sample results by collaborator for ground poultry

	AOAC/BAM														
	Sample[a]														
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	–	–	+	+	+	–	–	–	–	–	+	–	–	–	–
15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	–	+	+	+	–	–	–	–	+	–	+	+	–	–	+
18	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	EIA[d]														
1	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
2	–/–	+/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
3	+/+	+/+	+/+	+/–	–/–	–/–	+/–	+/–	–/–	+/–	+/–	–/–	–/–	–/–	–/–
4	–/–	–/–	–/–	+/–	+/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–
5	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
6[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	+/–	–/–
8	–/–	–/–	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
9	–/–	+/+	+/+	+/+	+/+	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
10[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	–/–	–/–	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–
15	+/+	+/+	+/+	+/+	+/+	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
16	–/–	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
18	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
19[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Decoded sample identification: 1–5, naturally contaminated high level, 0.24 cell/g; 6–10, naturally contaminated medium level, < 0.003 cell/g; 11–15, naturally contaminated low level, < 0.003 cell/g.

[b] Data excluded for analysis. Laboratory did not confirm results and report final data.

[c] Data excluded from analysis. Proper test procedure not followed, no reactions occurred.

[d] First entry is presumptive result, second entry is confirmed result, i.e., +/– is presumptive positive but not confirmed, +/+ is presumptive positive and confirmed.

levels of natural contamination found in this product and a high level of competitor microflora.

False-positive assays were observed for the high (4), medium (8), and low (4) levels. These reactions are attributed to the very low levels of naturally contaminated *Salmonella* found in the samples and a high level of competitive microflora. When EIA data were analyzed using the 0.25 cut-off calculation factor, no confirmed *Salmonella*-positive samples became negative; however, false-positive results for high, medium, and low were reduced to 3, 7, and 2, respectively.

Discussion

The analysis of all valid data for each food type/inoculation level resulted in 536 samples that were confirmed positive by the EIA assay. In comparison, there were 527 positives by the AOAC culture method. The overall false-negative rate for the EIA method was 1.8%, and the false-negative rate for the culture method was 3.5%.

Analysis of data by each food type/inoculation level and by all types/levels combined indicated equivalence of the 2 meth-

Table 9. Summary and analysis of *Salmonella* EIA collaborative study results

Product	CFU/g	Samples negative				EIA		AOAC/BAM		Samples positive				EIA		AOAC/BAM		Agreement, %[e]
		Total	EIA	AOAC/ BAM	χ^2	FN rate, %[a]	95% CI, %[b]	FN rate, %[a]	95% CI, %[b]	Total	EIA	AOAC/ BAM	χ^2	FH rate, %[c]	95% CI, %[d]	FN rate, %[c]	95% CI, %[d]	
Nonfat dry milk	2.40	56	56	55	0	1.8	0–5.0	0	0	55	54	55	0	1.8	0–5.3	0	0	98.2
	0.24	55	55	55	—[f]	0	0	0	0	55	55	55	—[f]	0	0	0	0	100.0
Dry egg	1.10	56	56	55	0	1.8	0–5.0	0	0	55	54	55	0	1.8	0–5.3	0	0	98.2
	0.15	59	59	58	0	1.7	0–4.7	0	0	52	51	52	0	1.9	0–5.6	0	0	98.2
Black pepper	0.46	56	56	55	0	1.8	0–5.0	0	0	45	44	45	0	2.2	0–6.5	0	0	98.0
	0.093	51	51	51	—[f]	0	0	0	0	49	49	49	—[f]	0	0	0	0	100.0
Soy flour	0.15	72	72	72	—[f]	0	0	0	0	28	28	28	—[f]	0	0	0	0	100.0
	0.004	73	72	73	0	0	0	1.4	0–3.9	28	28	27	0	0	0	3.6	0–10.5	98.0
Chocolate	1.10	70	70	70	—[f]	0	0	0	0	70	70	70	—[f]	0	0	0	0	100.0
	0.24	70	70	70	—[f]	0	0	0	0	70	70	70	—[f]	0	0	0	0	100.0
Ground poultry	0.24	45	27	44	13.5	2.2	0–6.1	40.0	18.7–61.3	34	33	16	10	3.0	0–8.7	47.0	30.2–63.8	68.0
	<0.003	60	60	59	0	1.7	0–4.7	0	0	1	0	1	0	100.0	NA[g]	0	0	98.3
	<0.003	60	60	56	1.0	6.7	0–15.9	0	0	4	0	4	2.3	100.0	NA[g]	0	0	93.0
Total		503	484	493	2.21	2.1		3.9		546	536	527	2.21	1.8		3.5		97.2

[a] False-negative (FN) rate was calculated according to McClure (10) as number of false-negative results divided by total number of negative samples.
[b] 95% confidence limit (CI) calculated according to McClure (10).
[c] False-negative (FN) rate was calculated according to Zweig and Robertson (11) as number of false-negative results divided by number of positive samples.
[d] 95% confidence limit (CI) calculated by binomial method.
[e] Rate reflects number of samples read identically between EIA and AOAC/BAM.
[f] Statistical analysis not applicable. Methods give identical results.
[g] Confidence interval not applicable, note small base.

ods. Table 9 presents a summary of all results. The overall χ^2 analysis for all foods/levels combined was 2.2. No statistically significant difference between methods occurred, except for ground poultry where the EIA method produced significantly more confirmed positive samples than the AOAC/BAM culture method.

In general, contamination levels were below the target levels of 0.04–0.2 and 0.4–2.0 cells/g for low and high levels, respectively. For ground poultry and soy flour, in particular, levels were approximately one logarithm low, resulting in fewer numbers of confirmed positive tests in both the EIA method and the AOAC/BAM culture method than produced in other foods.

EIA optical density data were analyzed using 2 positive control multiplier factors (0.20 and 0.25) to calculate the presumptive positive cutoff value. Using the 0.20 factor, there was 97.2% agreement between the 2 methods, with an overall χ^2 of 2.2. With the 0.25 factor, the agreement was 97.1%, with an overall χ^2 of 1.63. The final method description includes the 0.25 factor.

Conclusion

Analysis of all valid data submitted by 32 laboratories indicates that the studied *Salmonella* EIA and the culture method are equivalent, with the exception of ground poultry. For this food, the EIA method detected significantly more confirmed positive samples. We recommend that the EIA method be adopted by AOAC as first action.

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MICROBIOLOGICAL METHODS

Using Experimental Design To Optimize a Microbial Diffusion Assay

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As a prerequisite to validation of the cylinder plate diffusion assay for the antibiotic colistin sulfate in plasma, the assay needed to be optimized. *Bordetella bronchiseptica* (ATCC 4617) was the test organism for optimization. Ionic strength of buffer, quantity of test organism, duration of prediffusion, and volume of agar layer were studied. The effects of each factor were examined according to an experimental design. A 4 h prediffusion at 4°C increased sensitivity. Repeatability was improved by using 100 mL agar per bioassay dish, dilution of the plasma with buffer (3 + 1), and prediffusion. The slope of the standard response line was steeper with prediffusion. The reduced quantity of test organism contributed to better sensitivity and good repeatability. No significant interaction between these factors was found. Experimental designs were used successfully to rapidly set up a microbial method requiring a limited number of runs.

Colistin is an antibiotic currently used in calves. Because this antibiotic consists of a mixture of polypeptides (1), only microbial methods can be validly used to determine colistin concentrations in plasma, and the method selected must be validated. The selection of a suitable microbiological method requires the examination of many factors (2), such as incubation temperature, inoculum size, medium, agar thickness, ionic strength of buffer, and prediffusion.

Method optimization is a prerequisite to validation. The parameters examined for optimization of a colistin assay were quantitation limit, slope of the standard curve, and repeatability. Experimental procedures based on experimental designs (3, 4) may be used to improve analytical methods, thus limiting the number of runs required.

This study used a 2-level experimental design with 4 factors to optimize a method for quantitative determination of colistin sulfate in calf plasma. The required limit of quantitation was <0.5 IU/mL (5).

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Experimental

Materials

(a) *Cylinder.*—Stainless steel cylinders, 6 ± 0.1 mm id, 10 ± 0.1 mm high (**957.23 C**) (6).

(b) *Dishes.*—Bioassay dishes (243 × 243 × 18 mm) (Nunc, Denmark).

(c) *Water bath.*—Salvis (SALVIS AG, Switzerland).

(d) *Incubator.*—Memmert (Biolock Scientific, France).

(e) *Zone reader.*—AMS 40-10 (precision, 0.1 mm) (Système analytique, France).

Reagents and Culture Media

(a) *Water.*—Millipore filter and reagent-grade water system were used to obtain ultrapure and sterile water (Millipore S.A., France).

(b) *Phosphate buffer, pH 6.0, 20%.*—Dissolve and dilute 160.0 g monopotassum phosphate and 40.0 g bipotassium phosphate to 1 L with water (**957.23 B**) (6).

(c) *Antibiotic standard.*—Colistin sulfate, 20 260 IU/mg (Roger Bellon, France).

(d) *Assay medium.*—Medium 10 (Difco, France) was found satisfactory.

(e) *Microorganisms.*—A strain of *Bordetella bronchiseptica* ATCC 4617 was stored in Trypticase soy broth (Difco, France) with 10% DMSO (dimethylsulfoxide) at −80°C. The inoculum had an optical density of 1.

Preparation of Standard Curve

Dissolve 39.0 mg collistin sulfate standard, and dilute to 40 mL with water to obtain 2000 IU/mL. Prepare range of colistin sulfate solutions: 80, 40, 20, and 10 IU/mL. Mix 1 mL of each solution with 9 mL plasma to prepare 8, 4, 2, and 1 IU/mL solutions, and dilute with phosphate buffer (Table 1).

Preparation of Plate

Melt assay agar and cool to 40°C for inoculation. Mix thoroughly with 0.05 or 0.1% of inoculum, according to experimental conditions (Tables 1 and 2). Spread 70 or 100 mL agar into bioassay dishes. Place cylinders on each plate in a Latin Square arrangement. Charge each cylinder with 250 µL (7, 8) colistin sulfate preparation by using a Pipetman.

Table 1. Experimental design: levels of each factor

Factor	Level, "–"	Level, "+"
A = Buffer dilution	1/2	3/4
B = Inoculum, %	0.05	0.1
C = Prediffusion, h	0.5	4 at 4°C
D = Volume of agar, mL	70	100

Assay Design

Eight runs were performed; 5 plates constituted 1 run. Each plate contained 2 sets of colistin standards at concentrations of 1, 2, 4, and 8 IU/mL and 24 sets of colistin at 2 IU/mL. For these plates, prediffusion time of 30 min at room temperature or 4 h at 4°C was observed. Plates were incubated at 37°C for 16–18 h.

Measurement of Potencies

Diameters of zones of inhibition were measured with AMS 40-10 zone reader connected to computer so that the following parameters could be calculated according to international standards (9): mean and standard deviation of zone diameter, slope and its standard deviation, repeatability, and reproducibility of method (9, 10). Average of 10 zone diameters was calculated for each test level of standard. Straight line regression with least squares from relationship between log dose and response was produced. Test of linearity was performed on obtained regression line. Then average of data for 5 inhibition zone diameters at 2 IU/mL allowed extrapolation to 24 doses from regression line with log transformation according to AOAC procedures. Rejection was based on Dixon test for outliers (11). Selected data were used to calculate repeatability.

Validation of Method

Method was validated by performing 2 runs of 5 plates at 8-day intervals. Each plate contained 2 standard ranges of colistin at 5 test levels, namely, 0.5, 1, 2, 4, and 8 IU/mL, and 22 sets of median test level (2 IU/mL).

Experimental Design

Base design was 2-level factorial design for 3 factors (ionic strength, inoculum, and prediffusion, respectively, noted by letters A, B, and C). Experimental runs were coded as (–) for low level and (+) for high level, with the values defining limits of experimental domain (Table 1).

A temperature of 4°C was selected to inhibit microorganism growth during 4 h prediffusion period. In preliminary study, influence of ionic strength of pH 6 phosphate buffer with 1, 10, and 20% potassium was studied. Phosphate buffer, pH 6, with 20% potassium improved microbiological results, as described in FAO/OMS report (12). Five plates per run were, therefore, used to minimize errors (5).

A fourth factor may be studied by adding negligible interaction to the experimental design. Quantity of agar (noted by letter "D") was studied at levels defined by interaction between 3 factors of base design (noted by letters "ABC"). "D" and "ABC" were alias structure. Design selected was 2-level fractional factorial design for 4 factors. Table 2 shows design matrix.

Experimental Design Calculation

(**a**) *Effects and interactions calculation.*—Each response (zone diameter, slope, or repeatability) was assigned the mark of corresponding level of factor or interaction of the run. These values were then added together. The result was divided by the number of runs (8 in this case). A quantitative value of the effect of a factor in relation to average of runs (column noted "I" in Table 2) was then obtained (Table 3).

(**b**) *Significance test.*—A statistical graphics system (Statgraphics, STSC, MD 20852) was used with normal probability plot to determine which effects or interactions were significant. Effects are represented by gausso-arithmetic graphic. Nonsignificant effects usually tend to fall along a straight line passing through zero. Distribution is normal, and quantitative values represent estimations of experimental error. Nonaligned dots express nonrandom distribution. Because effects or interactions corresponding to these dots are significant, they influence the method.

Table 2. Average zone diameter, slope of standard response curve, and repeatability for each assay

	Levels										
Run	I	A[a]	B[b]	C[c]	D[d] = ABC	AB + CD	AC + BD	BC + AD	Diameter, mm	Slope	Repeatability, %
1	+	–	–	–	–	+	+	+	11.94	5.89	37.65
2	+	+	–	–	+	–	–	+	11.28	4.85	12.33
3	+	–	+	–	+	–	+	–	10.28	4.69	29.81
4	+	+	+	–	–	+	–	–	11.26	5.19	27.76
5	+	–	–	+	+	+	–	–	13.15	7.60	29.89
6	+	+	–	+	–	–	+	–	14.99	6.50	18.06
7	+	–	+	+	–	–	–	+	13.03	7.38	21.87
8	+	+	+	+	+	+	+	+	12.86	6.09	24.35

[a] Buffer dilution.
[b] Inoculum.
[c] Prediffusion.
[d] Volume of agar.

Table 3. Mean of runs: effects of factors and interactions

	Factor[a]				Interaction			Mean
Response	A	B	C	D = ABC	AB + CD	AC + BD	BC + AD	I
Zone diameter	0.25	−0.49[b]	1.16[b]	−0.46[b]	−0.05	0.17	−0.07	12.35
Slope	−0.37[b]	−0.18	0.87[b]	−0.22	0.17	−0.23	0.03	6.84
Repeatability	−4.59[b]	0.73	−1.67[b]	−1.12[b]	4.70	2.25	−1.17	25.22

[a] A, buffer dilution; B, inoculum; C, prediffusion; D, volume of agar.
[b] Significant effect.

Results and Discussion

The standard deviation of zone diameter did not differ significantly between each assay. The average of the standard deviaton was 0.25 mm. The standard deviation of the slope was also constant (0.27). Only significant effects and interactions of values ranking above the standard deviation of responses were retained.

Table 2 presents the data responses for each run. Table 3 shows the effects and interactions data. These were used to determine the best conditions of microbiological diffusion assay for colistin in plasma. We looked for the following ideal conditions: (*1*) a zone diameter of inhibition as large as possible, which expressed good method sensitivity; (*2*) a high slope of the response curve, which helps to minimize the standard deviation of interpolated concentrations from zone diameters and consequently improves repeatability; and (*3*) good method repeatability, which is the consequence of the low variability of the obtained values.

Influence on zone diameter.—The inhibition zone diameter increased with prediffusion (+1.159 mm), with the mean zone diameter being 12.35 mm. The zone diameter decreased slightly with the quantity of agar (−0.456 mm). The diffusion phenomenon was the result of a horizontal diffusion vector and a vertical diffusion vector. A thicker agar layer increased the vertical diffusion to the detriment of the horizontal diffusion. Consequently, the diameters of the inhibition zones decreased, which is in agreement with studies by Brady and Katz (7). A larger quantity of inoculum also decreased zone diameter (−0.491 mm). The buffer dilution had no significant effects on the zone diameters. There was no interaction between the studied factors.

Influence on slope.—Prediffusion was the most influential factor. The standard curve slope was increased by +0.87 when the duration of prediffusion was increased. In contrast, the phosphate buffer at the high level (+) slightly reduced the slope.

Table 4. Validation of microbial diffusion assay

	Run 1	Run 2	Runs 1 and 2
Zone diameter, mm[a]	13.44	13.62	—
Slope of standard curve	5.98	6.23	—
Repeatability, %	23.80	23.65	24.37
Intralab. reproducibility, %	—	—	24.37

[a] For 2 IU/mL of colistin sulfate.

The other effects and the interactions were not significant to this criterion.

Influence on repeatability.—Three factors were significant. The most important of these was a decrease of −4.59% induced by diluting the plasma with phosphate buffer (3 + 1) for a mean of runs of 25.215%. The repeatability improved with prediffusion (−1.67%) and with a thick agar layer (−1.12%).

The ionic strength of pH 6 phosphate buffer acted favorably on zone diameter and on the repeatability, indicating that the high level (+) should be selected. The increase of the inoculum acted unfavorably on the 3 selected criteria, indicating that the low level (−) should be selected. Prediffusion at the high level (+) improved sensitivity (by increasing zone diameter), the slope of the standard curve, and repeatability. A thick agar layer slightly decreased zone diameter but improved repeatability. Because repeatability is the preponderant criterion for validation of our method, the high level (+) should be selected. We were able to corroborate the fact that the "ABC" interaction was negligible before the "D" factor (thickness of the agar layer) by suppressing this alias structure (the data are not shown here). The effects obtained, which are the sum of the effects of factor "D" and interaction "ABC," reflected the influence of the thickness of the agar layer.

The assay should be carried out as follows: The plasma should be diluted with pH 6 phosphate buffer (3 + 1) with 20% potassium, and 100 mL agar containing 0.05% inoculum should be distributed in each dish. A 4 h prediffusion at 4°C is necessary before incubation at 37°C for 16–18 h.

After optimization was completed, the method was then validated. The relationship between zone diameter and log dose was linear for the standard curve, and the residual standard deviation was not significant. The threshold of quantitation that could be reached was thus 0.5 IU/mL. The average zone diameter at this test level of the standard was 9.8 mm. Table 4 shows the data of the mean zone diameter obtained for the 2 IU/mL test level, the slope of the standard curve, the repeatability of each run, and the intralaboratory reproducibility of the method (9–11). The values obtained for the diameter of inhibition zones and the slope of the standard straight line met our requirements. The repeatability was not significantly different from one run to another. The repeatability and the reproducibility of the method were satisfactory for this kind of assay.

The experimental design we selected for this optimization permitted 4 factors to be tested at 2 levels in 8 runs, instead of 16 runs for a full design. Experimental designs are useful in several sorts of experiments and improve the precision and the

quality of the conclusions while reducing the number of tests required.

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MYCOTOXINS

Determination of Type A and B Trichothecenes in Cereals by Gas Chromatography with Electron Capture Detection

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A quantitative method has been developed for determination of nonmacrocyclic trichothecenes in cereals. The mycotoxins are extracted with acetonitrile–ethyl acetate–water, and the extracts are quickly defatted with hexane and purified on a Sep-Pak Florisil cartridge. The trichothecenes are then silylated with Tri-Sil/TBT and quantitated by capillary gas chromatography with electron capture detection. High recoveries of 13 tested trichothecenes were achieved in experiments on wheat at the 250 μg/kg level. The method was also tested on barley, maize, oats, and rye with good results.

The trichothecene mycotoxins are a group of closely related, secondary metabolites produced by various strains of *Fusarium*, *Trichoderma*, *Myrothecium*, and some other fungi. Some of the most common toxicological effects caused by these toxins are necrosis, diarrhea, and vomiting, but even more severe injuries have been reported. The toxicological potency varies strongly among individual toxins (1–3). Because the trichothecene-producing strains are often capable of producing more than one toxin, there is the possibility that synergistic effects can occur, and these have to be duly considered in toxicological evaluation (1, 2, 4, 5). Several reports of natural contamination by trichothecenes in cereals and feedstuffs indicate that the widespread occurrence of the toxins can be a potent food safety problem (6–10).

Published gas chromatographic methods for determination of trichothecenes in cereals are usually limited to only a few toxins or to their hydrolyzed parent alcohols (11–17). It is desirable to increase the number of toxins that can be determined by the same method, and studies have been undertaken to develop such a method. The method presented in this paper has been used to determine the recovery from wheat of 13 trichothecenes of different polarities. In view of the variation in polarity of these trichothecenes, the method probably can be used for other nonmacrocyclic trichothecenes besides those tested in these recovery experiments.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard 5890 equipped with ^{63}Ni electron capture detector, HP 7673 autosampler, and 25 m OV-1701 capillary column, 0.32 mm id, 0.21 μm film thickness (Chrompack); or 25 m capillary OV-1 column, 0.32 mm id, 0.25 μm film thickness (Chrompack).

(b) *Table centrifuge.*—IEC clinical centrifuge or equivalent for 15 mL test tubes with screw caps.

Reagents

(a) *Solvents.*—Acetonitrile, chloroform, ethyl acetate, hexane, methanol, all ACS grade, used as received but checked for impurities on the GC-ECD; distilled water.

(b) *Trichothecene standards.*—Deoxynivalenol (DON) (Myco Lab Co., Chesterfield, MO); diacetoxyscirpenol (DAS), fusarenon-x (FUS-X), nivalenol (NIV), and T-2 toxin (Romer Labs Inc., Washington, MO); 3-acetyldeoxynivalenol (3-Ac-DON), HT-2 toxin, trichothecin, trichothecolone, and T-2 tetraol (Sigma Chemical Co., St. Louis, MO); neosolaniol (NEO) (Wako Chemical Inc., Dallas, TX).

(c) *Internal standard.*—*p,p*-DDE (U.S. Environmental Protection Agency, Research Triangle Park, NC).

(d) *Phosphate buffer, pH 7.0.*—Mix 50 mL 0.1M potassium dihydrogen phosphate and 29 mL 0.1M sodium hydroxide and adjust pH to 7.0, using pH meter.

(e) *Tri-Sil/TBT.*—Pierce Chemical Co., Rockford, IL.

(f) *Sep-Pak Florisil cartridge.*—1 g (Waters Associates, Inc., Milford, MA).

Extraction and Cleanup

Add 250 mL ethyl acetate–acetonitrile (4 + 1) and 10 mL water to 50 g ground sample in 500 mL flask with Teflon-lined screw cap. Shake vigorously for 30 min on flask shaker. Filter extract through fluted paper filter, and pipet 10 mL filtrate into 15 mL screw-capped test tube. Evaporate to dryness on 45°C water bath under stream of nitrogen. Add 4 mL hexane, 3 mL acetonitrile, and 2 mL water to tube, and shake on Vortex mixer for 30 s. Centrifuge briefly to separate phases, discard upper hexane layer, and repeat last step with another 4 mL hexane. Transfer acetonitrile layer to 50 mL round-bottom flask. Wash tube with 1 mL acetonitrile and transfer to same round-bottom

Table 1. Recoveries of 5 levels of trichothecenes (μg/kg) added to wheat flour

	Rec., % (mean ± SD, n = 5)				
Toxin added	25	100	250	1000	2500
DON	111 ± 4.7	87 ± 3.4	89 ± 1.8	—	92[a]
FUS-X	95 ± 3.0	87 ± 3.7	87 ± 1.5	—	95[a]
NIV	110 ± 8.8	73 ± 4.0	72 ± 2.4	—	77[a]
3-Ac-DON	106 ± 3.4	91 ± 2.9	93 ± 2.3	—	98[a]
DAS	—	—	89 ± 4.9	91 ± 4.5	90 ± 7.3
Trichothecin	—	—	115 ± 11.7	93 ± 4.7	87 ± 12.0
NEO	—	—	97 ± 7.7	92 ± 5.3	87 ± 5.1
HT-2 toxin	—	—	94 ± 5.4	90 ± 2.2	86 ± 7.0
T-2 toxin	—	—	84 ± 7.5	90 ± 1.7	93 ± 5.9

[a] Single analysis.

flask. Evaporate to dryness, using vacuum evaporator and water bath held between 50 and 60°C.

Using gas-tight syringe, activate Sep-Pak Florisil cartridge (1 g) with 5 mL methanol, followed by 5 mL chloroform–methanol (90 + 10). Dissolve sample residue in 2.5 mL chloroform–methanol (90 + 10), and press slowly through cartridge. Rinse tube with another 2.5 mL chloroform–methanol (90 + 10), and press slowly through cartridge. Elute Florisil cartridge with another 15 mL of the same mixture. Collect whole eluate (20 mL) in 100 mL round-bottom flask, and evaporate to dryness (vacuum evaporator and 45°C water bath). Dissolve evaporated sample with three 1 mL portions of chloroform and transfer to 5 mL reaction vial. Evaporate to dryness on 45°C water bath under a stream of nitrogen. Sample is now ready for derivatization.

Derivatization

Add 50 μL Tri-Sil/TBT to vial containing evaporated sample or standard, and shake 30 s on Vortex mixer. Cap vial and heat 30 min in oven at 80°C. Cool to room temperature, and add 500 μL hexane and 1.0 mL phosphate buffer, pH 7.0. Shake on Vortex mixer 30 s, and let phases separate. Transfer 200 μL of the hexane layer to separate screw-capped tube. Add 100 μL of the internal standard in hexane to tube, and mix on Vortex mixer for few seconds. Derivatized sample is now ready for injection into gas chromatograph.

Gas Chromatography

Inject 1 μL derivatized sample into capillary gas chromatograph in splitless mode under following conditions: Use helium as head carrier gas at 15 psi; nitrogen as makeup gas at 60 mL/min; temperature, injector 200°C and detector 350°C; oven temperature 60°C for 2 min, then increase to 150°C at 40°C/min, then 5°C/min up to 250°C. Keep final temperature for 10 min before cooling for next run.

For analysis on OV-1 capillary column, use conditions as above, but reduce head carrier gas to 10 psi.

Standard Solutions

Dissolve stock solutions of trichothecenes in chloroform or methylene chloride. Store in freezer until use. For simultaneous detection on EC detector, prepare working standard mixture containing 1 μg each of DON, FUS-X, NIV, and 3-Ac-DON per mL, and 10 μL each of DAS, trichothecin, NEO, HT-2

Table 2. Recoveries of 5 levels of trichothecenes (μg/kg) added to rye flour

	Rec., % (mean ± SD, n = 5)				
Toxin added	25	100	250	1000	2500
DON	100 ± 1.3	102 ± 3.0	86 ± 1.1	—	—
FUS-X	ip[a]	93 ± 2.6	84 ± 1.1	—	—
NIV	ip[a]	78 ± 7.2	68 ± 1.8	—	—
3-Ac-DON	92 ± 2.4	98 ± 3.0	89 ± 0.8	—	—
DAS	—	—	105 ± 4.8	95 ± 5.0	102 ± 3.4
Trichothecin	—	—	81 ± 4.9	92 ± 3.0	76 ± 5.7
NEO	—	—	93 ± 3.2	95 ± 2.8	80 ± 1.5
HT-2 toxin	—	—	77 ± 5.8	84 ± 6.4	71 ± 3.9
T-2 toxin	—	—	79 ± 3.4	84 ± 4.8	76 ± 4.8

[a] ip = interfering peaks.

Table 3. Summary of recovery of trichothecenes added to barley, maize, oats, rye, wheat, and wheat bran at 250 ppb level (one analysis per commodity)

Toxin	No. analyses	Mean value, %	Range, %
DON	6	100	92–114
FUS-X	5[a]	100	92–115
NIV	6	78	69–86
3-Ac-DON	6	100	93–118
DAS	6	91	82–103
Trichothecin	6	79	66–94
NEO	6	97	84–124
HT-2 toxin	6	88	78–100
T-2 toxin	6	94	88–102

[a] Interfering peak in wheat bran sample.

toxin, and T-2 toxin per mL; 500 µL evaporated and derivatized standard mixture gives a final solution with toxin concentrations corresponding to 250 and 2500 µg/kg.

Recovery Experiments

Recovery experiments were performed primarily on wheat flour and rye flour spiked with 3 different levels of trichothecenes. Five separate analyses were performed at each level. In addition, the analytical method was tested on other cereal products such as wheat bran and milled whole kernels of barley, maize, oats, wheat, and rye. In these cereals, the recovery experiments involved single analysis at the 250 µg/kg level.

The recovery experiments were performed as follows: To 50 g finely ground sample in a flask, 1.0 mL of a trichothecene mixture in chloroform was spread over the surface. The flask was shaken manually to distribute the added standards as evenly as possible. It was then left open at room temperature for 2 h. Any remaining chloroform was evaporated under a stream of nitrogen. The samples were then analyzed with the method presented above.

Results and Discussion

The method described above was intensively tested by running recovery experiments with wheat flour and rye flour. The 9 trichothecenes used in the recovery experiments gave different responses on the electron capture detector (ECD) after derivatization. The response on ECD is much higher for the silylated type B trichothecenes than for the silylated type A (18, 19). An exception is trichothecin, which belongs to type B but possesses no free hydroxyl group to silylate. To obtain approximately similar peak heights on the ECD, the recovery experiments were performed with different concentration intervals for the toxins. The most ECD-sensitive trichothecenes, DON, 3-Ac-DON, FUS-X, and NIV, were tested in the concentration range 25–250 µg/kg; the less sensitive DAS, NEO, HT-2 toxin, T-2 toxin, and trichothecin were tested in the concentration range 250–2500 µg/kg. All the trichothecenes were determined at 3 different concentrations, with a range that varied by a factor of 10. In addition to these analyses, single recovery experiments were carried out on wheat flour at the 2500 µg/kg level for the most ECD-sensitive trichothecenes. The results of the recovery experiments for wheat flour and rye flour are presented in Tables 1 and 2, respectively, where the mean values and standard deviations are shown. As can be seen, high recoveries were found for all trichothecenes tested. The variations in recovery are generally small in spite of the different concentration ranges tested. There is good agreement in the recoveries for the different trichothecenes in both the wheat flour and rye flour samples. The results for NIV generally show somewhat lower recoveries, and the recovery values for trichothecin vary more than those for the other toxins in the tested matrices.

Table 4. Recoveries of some other trichothecenes added to wheat flour (single analyses at 250 µg/kg)

Toxin	Rec., %
T-2 tetraol	79
Trichothecolone	86
Scirpentriol	77
Verrucarol	82

The analytical method was also tested on other cereal products; the results of these tests are shown in Table 3. The recovery experiments were performed at a trichothecene level of 250 µg/kg in barley, maize, oats, rye, wheat, and wheat bran. Even though only single tests were made on each product, the results show that they compare well with the results in Tables 1 and 2. This implies that the proposed methodology is probably suitable for a much greater variety of products than those tested here. In addition to the 9 trichothecenes mentioned above, an additional 4 compounds, namely, scirpentriol, trichothecolone, T-2 tetraol, and verrucarol, were tested by the method. The results of these recovery experiments are reported in Table 4. They were run as single tests on wheat flour with a spiking concentration corresponding to 250 µg toxin/kg. The results show good agreement with those indicated for the other trichothecenes.

The extraction and cleanup procedures for the cereal products that have been described, as well as the small amount of filtrate used, all contribute to ensure that the final extract, after derivatization, results in a gas chromatogram that is free from interferences. The use of a Sep-Pak Florisil cartridge (12, 16, 18) eliminates most of the remaining impurities after the defatting step. Figure 1A shows a typical chromatogram from an extract of blank wheat flour that has been run on an OV-1701 column, and Figure 1B shows the same wheat flour sample spiked with the toxins DON, 3-Ac-DON, FUS-X, NIV, DAS, trichothecin, NEO, T-2 toxin, and HT-2 toxin. Silylation of trichothecenes produces derivatives (20, 21) that are stable at room temperature for several days. The sensitivity on the ECD varies for the derivatized toxins that were studied, and this implies that the limits of detection for these components also vary.

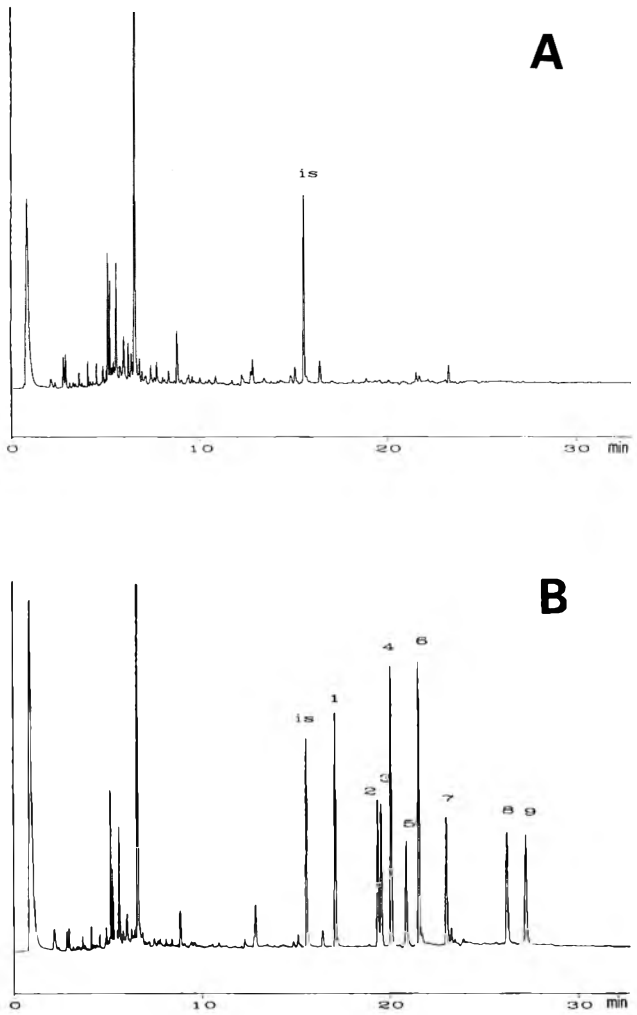

Figure 1. Gas chromatograms on OV-1701 of (A) blank wheat flour sample and (B) the same wheat flour sample spiked with toxins. Concentrations of the added trichothecenes are 250 μg/kg for DON (1), FUS-X (2), NIV (3), and 3-Ac-DON (4) and 2500 μg/kg for DAS (5), trichothecin (6), NEO (7), HT-2 toxin (8), and T-2 toxin (9). Internal standard (IS) added to both chromatograms: 100 μL 0.5 μg *p,p*-DDE/mL.

The lowest concentrations that were used in the recovery experiments, reported in Tables 1 and 2, are approximately double the lowest levels that can be detected in a sample. The GC conditions for the 2 different capillary columns used are described in the method. As can be seen from Figure 1B, the conditions for the OV-1701 column have been optimized to obtain complete separation between the 9 silylated trichothecenes. The separation of peaks obtained on the OV-1 column is somewhat poorer, but this column can be used as an alternative for identification if a GC/MS is not available. Table 5 shows the retention times and order of elution on both columns for the toxins in question.

A simplified and somewhat faster version of the method, in which the cleanup on the Sep-Pak Florisil cartridge is omitted, can be used for screening purposes. Some tests at the 250 μg/kg level on wheat flour and rye flour resulted in relatively interference-free gas chromatograms but with recovery values that varied more than did those for the original method. In the screening procedure, the quantities of sample, solvents, etc., were reduced 10-fold. Thus, a 5 g sample that has been extracted with a 25 mL mixture of the extractants and 1 mL water resulted in a volume of filtrate that was adequate for performing an analysis.

Table 5. Retention times of all tested trichothecenes and elution order of the 9 trichothecenes presented in Figure 1

	OV-1701		OV-1	
Toxin	Peak No.	Min	Peak No.	Min
DON	1	17.10	1	22.75
FUS-X	2	19.35	5	24.66
NIV	3	19.54	6	25.56
3-Ac-DON	4	20.07	3	24.33
DAS	5	20.89	4	24.40
Trichothecin	6	21.56	2	24.22
NEO	7	23.01	7	27.25
HT-2	8	26.22	9	33.54
T-2	9	27.23	8	33.06
Verrucarol	—	16.46	—	21.47
Trichothecolone	—	16.92	—	20.24
Scirpentriol	—	17.42	—	23.16
T-2 tetraol	—	19.37	—	25.76

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Interlaboratory Study on the Analysis of Chlorobiphenyl Congeners

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An intercomparison exercise on the determination of individual chlorobiphenyls (CBs) in marine media was organized by the International Council for Exploration of the Sea, the Intergovernmental Oceanographic Commission, and the Oslo and Paris Commissions. Sixty-two laboratories from 16 countries participated in the first part of this exercise, which consisted of determining 10 CBs in a standard solution. Considerable emphasis was placed on optimization of the gas chromatographic analysis, which has led to better agreement than former intercalibration exercises on CB analysis. Standard errors of 1.10–1.12 for the reproducibility were obtained for all CBs, except CB 52 for a group of 47 laboratories. It was demonstrated that the linear range of the electron capture detector was restricted to a factor of 5 in the concentration range 15–750 pg. About half of the participants used calibration solutions with CB concentrations outside the linear range. More than half of the participants had difficulties in separating the peaks of CB 28 and CB 31. Results based on peak heights showed better reproducibility than results based on peak areas.

For more than 20 years the contamination of different parts of the environment by polychlorinated biphenyls (PCBs) has been a major source of concern of institutes and organizations dealing with marine pollution problems. At the same time there have been problems with the comparability of PCB determinations; these problems could not be solved until recently. The International Council for Exploration of the Sea (ICES) attempted to improve comparability through a series of interlaboratory exercises, at first based on total PCB determination (1–3) and later, when chromatographic techniques improved, with individual chlorobiphenyl (CB) congeners (2, 3). The comparability of measurements of CBs or Aroclor was found to be poor. Interlaboratory coefficients of variation (CVs) varied from 25–50% (1) to 9–98% (3). Suggested causes of error were poor preparation of standard solutions, interfering materials in analytical solutions due to insufficient cleanup, and calculation errors (3).

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A group of laboratories experienced in CB determination and involved in a collaborative interlaboratory project organized by the Community Bureau of Reference (BCR) of the European Community showed that a stepwise approach resulted in better agreement (4, 5). After careful optimization of the different stages of the CB determination, the CVs for 6 CBs (IUPAC No. 28, 52, 101, 138, 153, and 180) were reduced to 11–24% at concentrations of 0.04–0.3 mg/kg in cleaned eel-fat extracts (4) and to 3.1–12.7% in standard solutions (5). According to the model of this BCR study, a stepwise approach was also chosen for a large interlaboratory study involving laboratories concerned with marine environmental analysis.

The objectives of this exercise were defined in the following way: to determine the variation in the results for CB determination among the participating laboratories, to identify the sources that cause this variation, and to reduce this variation by means of a learning process through a step-by-step approach.

The exercise was to be conducted in 4 steps: (*1*) analysis of standard solutions, (*2*) analysis of cleaned extracts of marine sediment and seal blubber, (*3*) analysis of uncleaned extracts of marine sediment and seal blubber, and (*4*) analysis of raw marine sediment material and seal blubber.

In the different stages of the exercise, advice would be given to the participants on the optimization of their instruments, extraction and cleanup techniques, etc. The first step of the exercise is evaluated in this paper.

Experimental

The following 10 CBs were selected for this study: CB 28 (2,4,4′-trichlorobiphenyl), CB 31 (2,5,4′-trichlorobiphenyl), CB 52 (2,5,2′,5′-tetrachlorobiphenyl), CB 101

(2,4,5,2′,5′pentachlorobiphenyl), CB 105 (2,3,4,3′,4′-pentachlorobiphenyl), CB 118 (2,4,5,3′,4′-pentachlorobiphenyl), CB 138 (2,3,4,2′,4′,5′-hexachlorobiphenyl), CB 153 (2,4,5,2′,4′,5′-hexachlorobiphenyl), CB 180 (2,3,4,5,2′,4′,5′-heptachlorobiphenyl), and CB 189 (2,3,4,5,3′,4′,5′-heptachlorobiphenyl). The choice was based on importance for legal tolerance levels in different countries (CBs 28, 52, 101, 118, 138, 153, and 180), chromatographic separation difficulties (CBs 28/31 and 153/105), and possible toxicological relevance (CBs 105, 118, and 189).

One ampoule (A) with known concentrations of these CBs, each around 750 ng/mL in isooctane, was supplied to the participants together with 1 ampoule (B) with the same CBs in an unknown concentration (ca 0.1 of the known standard solution). Four CBs with identities unknown to the participants were added to this second ampoule. An internal standard solution was supplied, which contained octachloronaphthalene at 2.3 μg/mL in isooctane and a blank solution (D), 5 mL isooctane. The internal standard had to be added to the calibration solutions, the unknown solution, and the blank solution in equal volumes and concentrations, so that injection errors and errors due to concentration or dilution could be corrected.

The stock CB solutions were >98% pure in electron capture detection (ECD) chromatograms, but sometimes contaminants were apparent in flame ionization detection chromatograms. Participants were advised to use these CB solutions, which were prepared from commercially obtained, noncertified CB crystals, only for this test and not for any other quantitative purpose. All ampoules were weighed before sending. Each participant had to check the weights upon receipt to control possible losses due to leakage during transport. The basic question of the exercise was to determine the concentrations of 10 CBs in the B solution by using the A solution as a standard.

As an optional exercise, participants were asked to identify and quantitate the added "unknown" CBs and to analyze the solution on 2 columns of different polarity. One column should be an SE-54 column or a column with a comparable polarity to SE-54 (5% phenyl–95% dimethylpolysiloxane). This is the only stationary phase on which the chromatographic properties of all 209 CBs are known (6). The following stationary phases were accepted as equivalent to SE-54: DB-5, HP Ultra 2, RSL-200, BP-5, 5% phenyl, CP-Sil 8, RTX-5, SE-52, SPB-5, and HP-5.

The choice of the second column was left to the participants. Injection on a second column was required for the analysis of peaks that coeluted on the first column. A number of different stationary phases, which were sometimes more polar or sometimes less polar than the SE-54 column, were used: RSL-200, NB-1701, CP-Wax 58, CP-Sil 19, OV-1701, OV-3, SPD-608, DB-17, OV-120, DB-1701, OV-1, RTX-20, HP-Ultra 1, DB-1301, SPB-5, HP-Ultra 2, CP-Sil 20, OV-101, SE-30, RSL-300, and DB-210. No special criteria were used for selection of these columns, nor were any requirements set for film thickness or separation efficiency.

A number of suggestions about gas chromatographic (GC) conditions were given in the guidelines. They concerned internal diameters (preferably ≤0.25 mm) and lengths (minimum 25 m, preferably 50 m) of the capillary columns, carrier gas (preferably hydrogen, otherwise helium), injection volume (fixed volume ≤1 μL), optimization of the injection system for splitless as well as on-column injection, the use of balances or syringes for the preparation of dilutions, and the use of isooctane. Participants were requested to optimize the temperature program of the oven by injecting the A solutions, using different programs, and to complete a form listing the chosen optimum GC conditions. Participants were also requested to perform a linearity test with 8 different dilutions of the A solution for the CBs 52, 101, and 180; to add the internal standard, octachloronaphthalene (OCN), to the different dilutions; to construct response curves; and to identify the linear range of the detector. By plotting the detector response divided by the injected mass vs the injected mass, an S-shaped curve is normally found for ECD. The horizontal part of this curve shows the linear range of the detector (Figure 1), which is defined by the intersection points of the S-shaped curve with 2 horizontal lines at ±10% of the peak height/mass value of the center of the S-shaped curve (5).

Participants were further requested to choose 2 dilutions from the linearity series, embracing the concentrations of the CBs in the B solution, previously estimated by a test injection, and to add the internal standard OCN to the unknown. During each of 6 days (3 days per column), the blank D, 2 of the A standards, and the unknown B were to be injected. The CB concentrations in the B solution were calculated by using the 2 A standards as calibration to give 1 value per day for each CB in B.

Participants were requested to quantitate the concentrations of the 10 CBs in the B solution based on both peak heights and peak areas. Peak heights were used to evaluate this study (Tables 1 and 2) and were compared with peak areas (see *Chromatographic performance*). The chromatograms were to be sent to the coordinators for inspection.

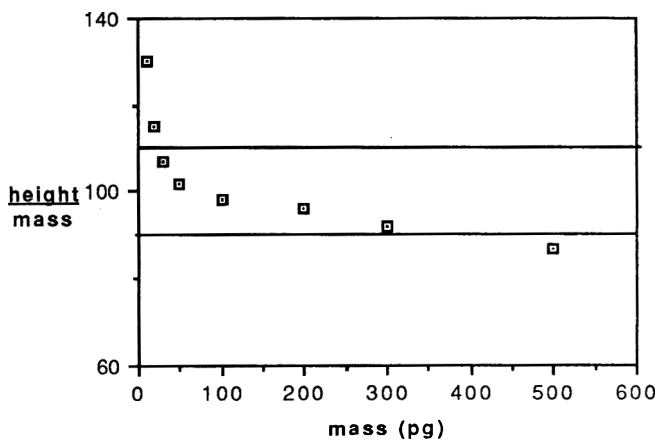

Figure 1. Example of typical response curve of the electron capture detector for CB 153, which shows the relationship between the ratio of the detector response/mass to the mass of the injected CB.

Table 1. Summary of results[a]

CB	Data set	No. labs	No. cols	*n*	s^2 (%) labs	s^2 (%) cols	s^2 (%) within	r	R	s_r	s_R	Mean, measured, pg/μL	Spike, pg/μL
28	2	43	64	189	77	9	14	1.22	1.71	1.07	1.21	71.9	75
31	2	41	60	175	70	9	21	1.24	1.59	1.08	1.18	75.1	75
52	2	48	80	235	85	8	6	1.28	2.74	1.09	1.43	592	791
101	2	55	97	286	79	5	16	1.27	1.80	1.09	1.23	81.2	75
105	2	54	95	280	79	6	14	1.26	1.82	1.08	1.24	69.8	75
138	2	55	97	286	74	7	18	1.26	1.73	1.09	1.22	70.3	75
153	2	53	94	276	81	8	11	1.26	2.02	1.09	1.29	76.9	75
180	2	55	97	286	75	10	16	1.24	1.70	1.08	1.21	70.1	75
189	2	55	97	285	76	13	11	1.23	1.85	1.08	1.24	70.5	75
28	1	37	55	164	42	19	39	1.22	1.38	1.07	1.12	73.5	75
31	1	36	53	156	42	15	43	1.23	1.38	1.08	1.12	75.6	75
52	1	39	69	204	81	11	8	1.29	2.38	1.09	1.36	592	791
101	1	45	82	243	35	15	50	1.26	1.38	1.08	1.12	84.6	75
105	1	44	80	237	26	29	45	1.23	1.36	1.08	1.12	72.8	75
138	1	45	82	243	18	28	54	1.25	1.35	1.08	1.11	72.8	75
153	1	43	79	233	13	45	42	1.23	1.38	1.08	1.12	81.6	75
180	1	45	82	243	16	42	42	1.21	1.33	1.07	1.11	71.5	75
189	1	45	82	243	7	58	34	1.20	1.36	1.07	1.12	72.0	75
28	0	27	39	117	35	31	34	1.20	1.37	1.07	1.12	73.7	75
31	0	27	39	115	36	26	38	1.22	1.39	1.07	1.12	76.2	75
52	0	27	49	147	78	7	16	1.25	1.74	1.08	1.22	597	791
101	0	27	49	147	32	21	47	1.22	1.34	1.07	1.11	83.6	75
105	0	27	49	147	19	38	43	1.19	1.30	1.06	1.10	72.3	75
138	0	27	49	147	18	39	43	1.20	1.33	1.07	1.11	72.4	75
153	0	27	49	146	25	41	35	1.20	1.37	1.07	1.12	81.8	75
180	0	27	49	147	18	47	35	1.17	1.31	1.06	1.10	71.8	75
189	0	27	49	147	16	59	25	1.17	1.37	1.06	1.12	72.5	75

[a] In data set 2, all data are used; in data set 1, the 10 outlying laboratories are removed (see Figure 1); in data set 0, only those laboratories are included that measured all 9 CBs and were not outlying; No. labs, No. cols, and *n* are, respectively, the number of laboratories, columns, and observations involved; s^2 is the relative contribution of the variance components: s^2 labs, among laboratories; s^2 cols, among columns of 1 laboratory; s^2 within, within 1 column; r, R, s_r, and s_R are the back-transformed repeatability and reproducibility values and standard errors.

Table 2. Quantitation of the additional CBs 49, 77, 110, and 149 (pg/μL) by 13 laboratories[a]

Lab.	CB 49 mean ± SD	*n*	CB 77 mean ± SD	*n*	CB 110 mean ± SD	*n*	CB 149 mean ± SD	*n*
1	1574 ± 404	12	—	—	140 ± 20	12	931± 230	12
2	—	—	—	—	—	—	1396 ± 133	12
3	1666 ± 204	12	—	—	130 ± 91	12	1363 ± 141	12
4	1460 ± 49	12	—	—	—	—	1518 ± 61	6
5	1367 ± 107	12	50 ± 3	6	102 ± 8	6	1425 ± 97	12
6	1231 ± 31	12	52 ± 2	6	109 ± 3	6	1458 ± 38	9
7	739 ± 208	12	368 ± 84	12	—	—	2100 ± 179	6
8	1739 ± 88	12	—	—	114 ± 7	12	1659 ± 92	12
9	1236 ± 84	12	—	—	—	—	1356 ± 97	12
10	1200 ± 98	12	234 ± 31	12	—	—	—	—
11	1651 ± 54	12	—	—	—	—	1415 ± 61	12
12	1536 ± 20	6	60 ± 4	7	—	—	—	—
13	1766 ± 112	2	98 ± 0	2	108 ± 0	1	1468 ± 0	1
Mean	1493 ± 209	11	54 ± 5.3	3	108 ± 4.9	4	1450 ± 93	9
Spike	1500	—	60	—	100	—	1500	—

[a] SD, standard deviation; *n*, number of observations.

Results and Discussion

Results were received from 62 laboratories, which made this exercise one of the largest ever organized on CB analysis. The majority of the participants submitted full data sets, but some participants delivered data based on the use of only one GC column. Two laboratories used GC/mass spectrometric (MS) detection; all others used ECD. Splitless injection was the most used injection technique (73 sets of results), followed by on-column injection (20 sets), direct injection (5 sets), and split injection (3 sets). No inventory was made of the number of manual injections and the number of autoinjections used. Twenty-nine laboratories identified 1 or more of the 4 "unknown" CBs, and only 13 laboratories quantitated at least 1 of these CBs.

Statistical evaluation.—The statistical evaluation was mainly based on the international standard ISO 5725 for interlaboratory tests (7). Because the error in this exercise appeared to show a relative character (which means that the standard deviation is proportional to the mean) in which an additive error was assumed, a model with a multiplicative error structure was assumed, in contravention of the ISO standard. This means that the logarithms of the CB concentrations were used in the statistical analysis. However, the back-transformed repeatability value r and the back-transformed reproducibility value R are given. The back-transformed repeatability value can be interpreted as the value below which the ratio of 2 single test results (i.e., the maximum of these 2 results divided by the minimum) obtained by the same method on identical test material under the same conditions (same operator, same apparatus, same laboratory, and within a short interval of time) may be expected to lie within a probability of 95%. R is the value below which the ratio of 2 single test results obtained by the same method on identical test material under different conditions (different operators, different apparatus, different laboratory, and not necessarily within a short interval of time) will fall within a probability of 95%. Because some readers might prefer the repeatability standard error (s_r) and reproducibility standard error (s_R) to be given, these values are also shown in Table 1. It should be recalled that the relations between r and s_r and R and s_R are, respectively, $2.8 \log s_r = \log r$ and $2.8 \log s_R = \log R$. Thus, both standard errors can be interpreted in the same way as explained above, except that the accompanying probability now equals 68%. For small standard errors, the values $s_r - 1$ and $s_R - 1$ may roughly be compared with the values of the CVs, which are usually calculated for intercomparison exercises in which an additive error structure is assumed.

Because laboratories used 2 different columns, a 2-level nested analysis of variance (8) was applied to calculate the following different variance components: among laboratories, among columns within laboratories, and within columns. The repeatability is based on the within-column variance component; the reproducibility is based on the sum of the 3 variance components. Table 1 also gives the relative proportion of these 3 components to their sum. Finally, a principal component analysis was performed on the laboratory means of 6 CBs. This figure includes all laboratories, all columns used, and all replicates. The first axis accounted for a major part of the total variance: 85%. This axis was highly correlated with 5 of the 6 CBs: 101, 105, 138, 180, and 189. The second axis explained another 13% and was mainly correlated with CB 153. Consequently, this analysis, graphically displayed in Figure 2, shows that the biases of the laboratories for the different CBs are very similar. In other words, laboratories measure consistently too high or too low. Outlying laboratories, defined as those laboratories that show large absolute values for the first (<–0.14, >0.17) or second principal component (<–0.05, >0.05), are easily detected.

In the BCR study (5), CVs for the reproducibility (CV[R]) of 3.1–12.7% were found for the analysis of CBs in standard solutions. However, this BCR exercise was performed by a group of 14 selected laboratories, all experienced in CB analy-

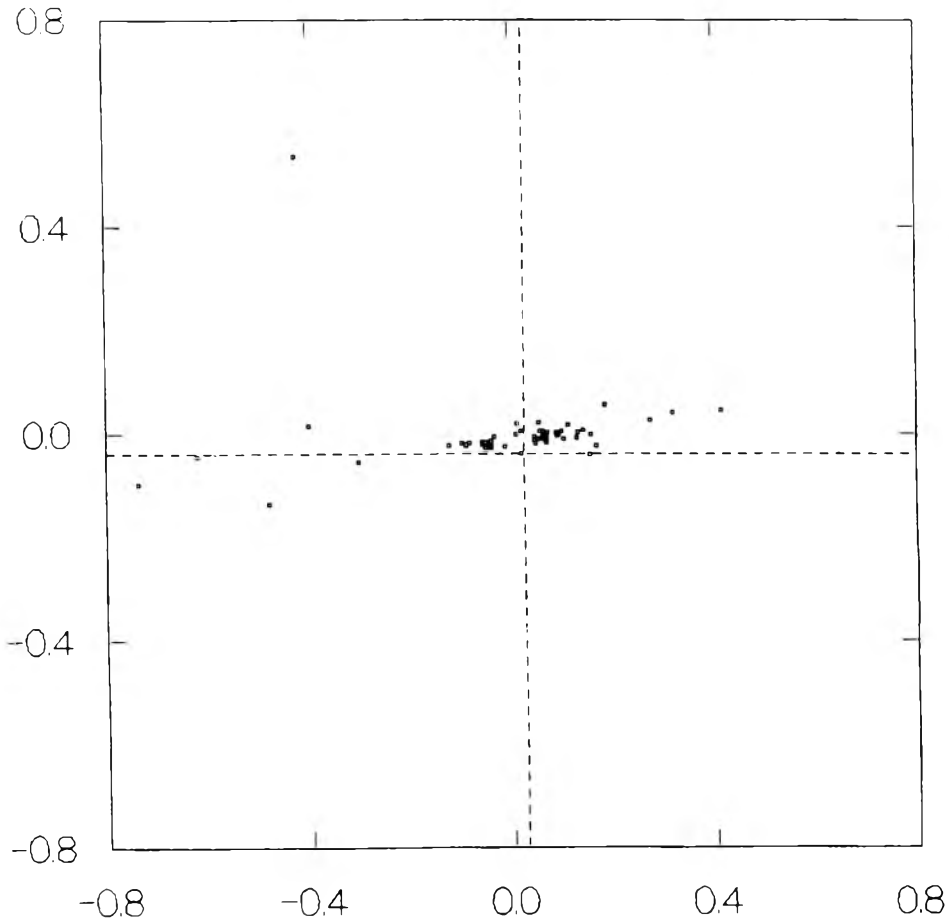

Figure 2. Principal component analysis: percentage of total variance explained by both axes. The vertical axis is particularly correlated with CB 153; the horizontal axis is correlated with CBs 101, 105, 138, 153, 180, and 189.

sis. Also, in the BCR test, the internal standard was already added to the ampoules containing the solutions, which may have reduced the CV(R) in comparison with the present exercise. Other intercomparison exercises usually have been carried out with fat extracts or fish oils, which makes the comparison more difficult. CVs in an ICES intercomparison exercise of CBs in commercial herring oil ranged from 9 to 98% (mean 39%) (3). A small intercomparison exercise in which 11 Dutch laboratories determined CBs in eel fat resulted in CV(R)s of 14–23% (9). A recent international intercalibration exercise of CBs in tuna homogenate (6 CBs), organized by the International Atomic Energy Agency, showed CVs from 15 to 88% (mean 41%) (10). Apparently, the attention paid in this exercise to the optimization of GC conditions resulted in a reduction of CVs.

Linearity.—One of the features of this intercomparison exercise was the identification of the linear range of the ECD system. The construction of the linearity response curve (Figure 1) appeared to be a surprise to a number of participants. Linear ranges of ECD systems have been provided by chromatography manufacturers for 4 or 5 decades. This may be partly true for the higher concentration range, but it is certainly not true for the range below 50 pg. Because the ECD system is only intended to measure concentrations of halogenated substances in this low picogram range, the user must be aware of a continuous linearity problem.

No one brand of ECD system performed better than others. The cell volume may differ among the different ECD types. This can cause differences in the linear range. Further differences may be caused by differences in optimization, e.g., temperature choice and flow rate of makeup gas. Even changes in the linear range may occur with time (11).

In this exercise, all participants identified the linear range of the detector to be not more than 5 times the concentration range of 15–750 pg; however, an exception of 20 times was identified. Only half of the participants brought standards and sample into the linear range of ECD by concentration or dilution. Apparently, the participants were not accustomed to the practice of dilution or concentration of the samples before injection.

In normal practice, this restricted linearity will be a problem for many laboratories, especially those working with very low concentrations. Samples will have to be concentrated in many cases, or if it is not possible to achieve an injected mass >50 pg by concentration, multiple standard calibration will be necessary.

CBs 28 and 31.—Only 28 laboratories were able to quantitate CBs 28 and 31. Their results, however, were comparable with the results for most other CBs, with s_Rs of 1.21 for CB 28

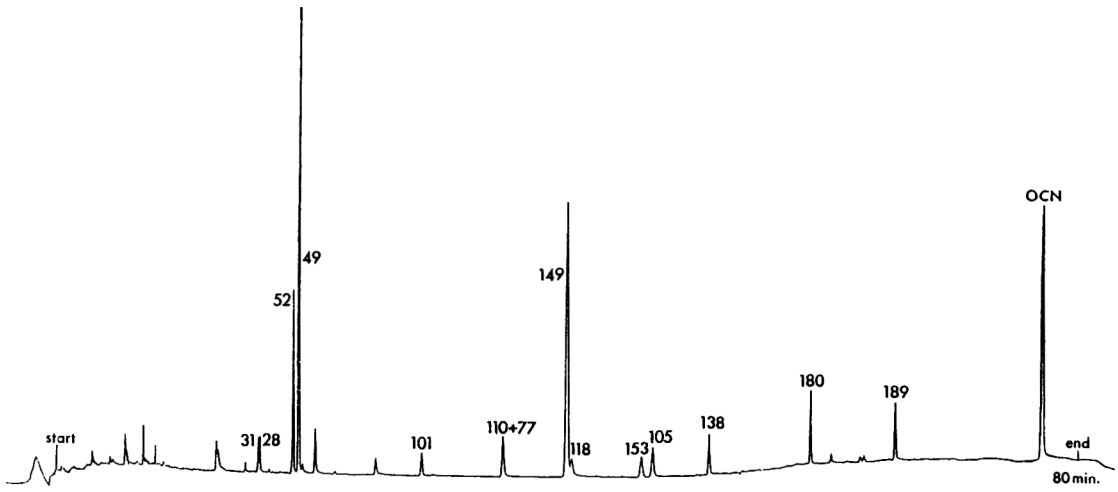

Figure 3. Chromatogram of the unknown solution (B) on a WCOT CP Sil 8 CB column (50 m × 0.15 mm × 0.22 μm).

and 1.18 for CB 31 (Table 1). The separation of these CBs is the most difficult of all CB separations in this exercise. Polar columns are unsuitable for the separation, except when the length is >50 m and the diameter is <0.15 mm (12). On SE-54 columns with proper dimensions and under proper conditions, 2 peaks can just be made visible. A baseline separation is never obtained. A chromatogram of the unknown (B) solution in Figure 3 shows the close elution of CBs 28 and 31 on a narrow-bore SE-54 column. Apolar phases like SE-30 are the best choice, but these columns cause separation problems for the other CBs. Multidimensional GC may solve this dilemma (13, 14). Considering the minor importance of these CBs, especially when marine mammals are concerned, improving this separation may not be worth the effort.

CB 52.—Results obtained for CB 52 showed a large s_R (1.43) for all data (Table 1) and a mean deviating 25% from the spiked concentration. There are 2 reasons for this bad performance: (*1*) the concentration in the B solution was 10 times as high as the concentration of the other CBs, and (*2*) the close-eluting CB 49 was added as an unknown, also in a very high concentration (Figure 3; Table 2). Analytical results are significantly lower than the nominal concentration. This clearly shows that results are biased, because they are not in the linear range of the detector.

Although this situation with very high concentrations of only 1 or 2 CBs does not occur normally, it is striking that almost all participants had problems with this determination. The best choice would have been an extra dilution of the B solution to bring the concentration of CB 52 into the linear range of the electron capture detector. Although in Figure 3 the peaks of CB 52 and CB 49 are almost baseline-resolved, chromatograms of many participants showed partial coelution of these peaks. At a lower concentration, the resolution will improve. From the results of other intercomparison exercises (3, 4), results for CB 52 are expected to be comparable with those of other CBs.

CB 101.—The determination of CB 101 has been rather successful, with an s_R of 1.23 for all data (Table 1). The mean values for all results (81.2), as well as for the selected groups (84.6 and 83.6), are on the high side. No coelution problems were met, although this does not mean that no identification problems would occur in determining actual samples. Near-eluting or coeluting congeners like CB 84 (15), which were not present in this exercise, may hinder such a quantitation.

CB 118.—By adding CB 149 in a 20-fold concentration, an extra obstacle was added to the determination of CB 118. The s_R for all data of CB 118 (1.24) does not deviate greatly from most other s_Rs, but it is based on a much smaller group of laboratories. Therefore, results for CB 118 are not included in Table 1. This means that this small group has a good mutual agreement, with a very acceptable mean of 75.1. Most of the other laboratories quantitated the peaks of CBs 118 and 149 together as CB 118, which resulted in very high concentrations. For a sample extract, it is expected that CB 118 can be quantitated by most of the participants, possibly with a slightly higher s_R. SE-54-like columns and more polar columns are suitable for the separation of the CBs 118 and 149.

CB 105 and 153.—The s_Rs for CB 105 and CB 153 are 1.24 and 1.29, respectively, for all data with corresponding means of 69.8 and 76.9 (Table 1). Some participants were expected to have difficulties with the separation of these 2 CBs, but only 2 laboratories were not able to separate them. However, this does not mean that the same agreement will be achieved for a normal practice sample. The presence of CB 132, in particular, will hinder the quantitation (16, 17). For toxicological reasons, CB 105 is considered one of the more important CBs (18). To perform an acceptable analysis, the diameter of the capillary columns may not exceed 0.2 mm, with column lengths of 50 m (11); otherwise, a more polar stationary phase is required.

CBs 138, 180, 189.—The results for CBs 138, 180, and 189 are encouraging, with means of 70.3, 70.1, and 70.5, respectively, and s_Rs of 1.22, 1.21, and 1.24, respectively, for all data (Table 1). The s_R of CB 189, also for the selected group of 28 laboratories, is slightly higher than the other s_Rs, perhaps because CB 189 is the latest eluting CB in this series. Although the splitter closing time has been optimized by all participants, some discrimination might have increased the s_R. In the se-

lected data (data set 0, Table 1), especially for CBs 180 and 189, the contribution of s^2 columns to the reproducibility is greater than the contribution of s^2 within. This column effect cannot be explained by lack of resolution, because these 2 peaks elute as baseline-separated peaks on most columns. Differences in discrimination between columns may explain this phenomenon.

On SE-54 columns and various columns of different polarity, CB 138 will coelute with CB 163, which may be present in environmental samples (19, 20). Only with a very polar stationary phase (SP-2330–biscyanopropylphenyl polysiloxane) (20) or multidimensional GC (21) will it be possible to separate these 2 compounds.

Unknown CBs.—CBs 49, 77, 110, and 149 were added to the B solution, and participants were requested to identify and quantitate them. Twenty-four laboratories were able to identify CB 49, 23 laboratories identified CB 149, 15 laboratories identified CB 110, and 15 laboratories identified CB 77. Only 8 laboratories were able to identify both CB 77 and CB 110. These 2 CBs coelute on an SE-54 column. Table 2 shows the results of the quantitative analysis. With a criterion of ±25% deviation from the mean, 11 participants were able to quantitate CB 49. Nine laboratories quantitated CB 149, 4 laboratories quantitated CB 110, and only 3 laboratories quantitated CB 77. For identification and quantitation, each laboratory used its own standard. The agreement of the mean results of the laboratories without the outliers shows that the working standards of these laboratories are comparable.

Chromatographic performance.—As mentioned earlier, many laboratories have made progress in capillary GC analysis. In general, GC performance was acceptable, although tailing peaks were noticed in several chromatograms. The classification of laboratories as outliers in most cases resulted from a nonoptimized GC system. Next to linearity problems, the use of nitrogen as a carrier gas, tailing peaks, negative spiking, baseline drift, and working with old instruments unsuitable for capillary GC were the major reasons for unreliable results. Two laboratories submitted data obtained by MS detection. Both were classified as outliers. Apparently, GC/MS cannot be considered as a reliable technique for the quantitation of CBs.

All 32 different types of stationary phases used in this test were suitable, in principle, for the determination of most CBs. However, for the analysis of actual samples with more difficult separations, the number of suitable columns will probably be smaller. The SE-54-like columns (5% phenyl–95% dimethylpolysiloxane) were acceptable for determination of the CBs in this exercise. Under optimum conditions with the proper length, diameter, and film thickness, the CBs 28/31 and 118/149 can be separated on these columns. With more polar columns, the separation of CBs 28 and 31 will soon become impossible, whereas the separation of CBs 118 and 149 improves. A reverse effect is observed with less polar columns. Recent developments in stationary phases, e.g., those based on a different principle like liquid-crystalline phases (14, 16, 22, 23), may be able to provide broader possibilities for the analysis of CBs. Multidimensional GC (13–15, 21) may also provide solutions for separation problems in CB determination.

Positive identifications of CBs in the blank solution were reported by many participants. Concentrations up to 10% of the CB concentrations in the B solution were reported by several laboratories, and in exceptional cases concentrations of 40 pg/μL were found in the blank. This shows that background contamination and cross-contamination, even if only in the stage of injecting standard solutions, are severe problems for many laboratories. It is essential to check the purity of solvents and the cleanliness of syringes and glassware. Another source of contamination may be the septa of autosampler vials. Contaminants from the septa may be extracted through the vapor inside these vials, particularly if samples are reinjected from the same vial with the same pierced septum.

Figure 1 shows that, except for the group of outliers, no dominating effects were caused by either injection technique or column diameter used. Quantitation based on peak heights was better than that based on peak areas. The calculated s_Rs for CBs 101 and 180 were 1.27 and 1.30, respectively, for peak areas, and 1.23 and 1.21, respectively, for peak heights. This difference contradicts the theoretical expectation but may be explained by the use of integrators, which may not define the peak area as accurately as necessary.

Conclusions

In comparison with former interlaboratory exercises, an encouraging result has been achieved. The stepwise approach has shown that, if encouraged to examine a procedure such as linearity testing of the detector, laboratories can perform better. Between-laboratory standard deviations of 1.10–1.13 were obtained for all CBs except CB 52. A group of 11 laboratories was identified as an outlier group, for which reconsideration of the optimization process is advised. However, some important problem areas were identified. Difficulties were met in identifying the linear range of the electron capture detector and in bringing the concentrations of the samples and standards into this linear range. CBs 28 and 31 could be separated by only half of the participants. Most participants should be able to separate CBs 52 and 49 and CBs 118 and 149 in normal practice samples. Relatively high concentrations of CBs 52, 49, and 149 influenced the determination in this study in a negative way. On the other hand, determination of CBs 105 and 153 may be expected to cause more problems in environmental samples than in this study. The presence of CB 132 and the low concentration of CB 105 will require capillary columns with a high resolution.

A significant difference in results between peak height and peak area calculation was observed; reproducibility was worse for results based on peak areas. This is explained by inaccurate integration. When using peak areas to calculate CB concentrations, laboratories should improve the software for the integration.

Many participants reported positive identifications of CBs in the blank solution, in some cases up to 60% of the CB concentrations in the unknown standard solution. This shows that background contamination is a problem in many laboratories,

even in this case where extraction and cleanup are not involved.

One of the objectives of this exercise was to reduce the variation in the determination of CBs in marine media to enable the laboratories to conduct collaborative studies on trends in CB concentrations in the marine environment. An s_R of 1.11, for example, which is found for CB 138 (Table 1), means that in joint studies on CB levels the differences between 2 values will be within a factor of 1.35 and have a probability of 95%. Cleanup and extraction will increase this ratio. Consequently, in identifying trends of CB concentrations in the environment, changes in CB concentrations will be identified only when they are approximately 50% or greater. Developments in chromatographic techniques, like multidimensional GC and new stationary phases, and improvements as a result of this exercise, such as linearity of the ECD system, lower blank values, and better integration of the chromatograms, may lead to a further reduction of the CVs of the CB determination.

As a second step in this exercise, participants will be requested to analyze cleaned extracts of seal blubber and marine sediment. According to this stepwise approach, laboratories classified as outliers in this first step will be requested to perform an extra test with standard CB solutions before entering the second stage to demonstrate the improvement in their performance.

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The following laboratories participated in this study:

Rijksstation voor Zeevisserij, Oostende, Belgium; Instituut voor Hygiëne en Epidemiologie, Brussels, Belgium; Laboratory for Ecotoxicology, Free University, Brussels, Belgium; Laboratory ECCA, Zwijnaarde, Belgium; Bedford Institute of Oceanography, Darthmouth NS, Canada; Freshwater Institute, Winnipeg, Manitoba, Canada; West Vancouver Laboratory, Vancouver, BC, Canada; Institute of Marine Environmental Protection, Dalian, China; National Environmental Research Institute, Charlottenlund, Denmark; National Agency of Environmental Protection, Soborg, Denmark; National Board of Waters and the Environment, Helsinki, Finland; Finnish Institute of Marine Research, Helsinki, Finland; Service des Eaux de L'Institut Pasteur de Lille, Lille, France; Laboratoire Municipal et Region de Rouen, Rouen, France; Laboratoire DERO/MR, Nantes, France; Laboratoire Municipal de Bordeaux, Bordeaux, France; Laboratoire Municipal d'Hygiene, Le Havre, France; Laboratoire Municipal de Brest, Brest, France; IFREMER Centre de Brest, Plouzané, France; Laboratoire Municipal de Toulon, Toulon, France; Institut Bouisson Bertrand, Montpellier, France; Bundesforschungsanstalt für Fisherei, Hamburg, Germany; Alfred-Wegener-Institut für Polar und Meeresforschung, Bremerhaven, Germany; Deutsches Hydrographisches Institut, Hamburg, Germany; Veterinäruntersuchungsamt Cuxhaven, Cuxhaven, Germany; Institut für Küsten- und Binnenfischerei, Cuxhaven, Germany; Institute Schumacher, Heide, Germany; Landesamt für Wasserhaushalt und Küsten, Schleswig-Holstein, Kiel, Germany; Fisheries Research Centre, Dublin, Ireland; Netherlands Institute for Fisheries Research, IJmuiden, The Netherlands; CIVO-TNO, Institute for Fishery Products, IJmuiden, The Netherlands; Rijkswaterstaat, Tidal Water Division, The Hague, The Netherlands; Rijksinstituut voor Natuurbeheer, Arnhem, The Netherlands; Delta Institute, Yerseke, The Netherlands; Netherlands Institute for Sea Research, Den Burg, Texel, The Netherlands; Senter for Industriforskning, Oslo, Norway; Norwegian Institute for Water Research, Oslo, Norway; Institute of Marine Research, Bergen, Norway; National Veterinary Institute, Oslo, Norway; Insituto de Ciencias del Mary y Limnología, Mexico City, Mexico; Instituto Hydrográfico, Lisbon, Portugal; Instituto National de Investicacao das Pescas, Lisbon, Portugal; Dereccao-Geral Qualidade do Ambiente, Lisbon, Portugal; Instituto Español de Oceanografía, Centro Costero de Vigo, Vigo, Spain; Centro de Estudios Avanzados, Barcelona, Spain; Laboratorio de Contaminación Toxicología, Madrid, Spain; National Environmental Protection Board, Solna, Sweden; Swedish National Food Administration, Uppsala, Sweden; MAFF Fisheries Laboratory, Burnham on Crouch, United Kingdom; SOAFD Marine Laboratory, Aberdeen, United Kingdom; Clyde River Purification Board, Glasgow, United Kingdom; Forth River Purification Board, Edinburgh, United Kingdom; Strathclyde Regional Council Chemists Department, Glasgow, United Kingdom; Pesticide Research Laboratory, Pennsylvania State University, University Park, PA, United States; Battelle Ocean Sciences, Duxburry, MA, United States; GERG Texas A & M University, College Station, TX, United States; Science Applications Int. Corporation, San Diego, CA, United States; UCSC-CDFG, Trace Organics Facility, Santa Cruz, CA, United States; NIST, Center for Analytical Chemistry, Gaithersburg, MD, United States; University of Massachusetts-Boston, Boston, MA, United States; National Marine Fisheries Service, Gloucester, MA, United States; Institute of Applied Geophysics, Moscow, Russia.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Optimization, Automation, and Validation of the Solid-Phase Extraction Cleanup and On-Line Liquid Chromatographic Determination of *N*-Methylcarbamate Pesticides in Fruits and Vegetables

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A fully automated liquid chromatographic (LC) method was developed for the determination of 21 *N*-methylcarbamates and 12 of their metabolites. Our previously developed solid-phase extraction (SPE) cleanup method can now be performed using an automated SPE cleanup apparatus. The cleaned-up extract is injected on-line into the LC carbamate analysis system, which is based on a reversed-phase LC separation, postcolumn hydrolysis of the carbamates on a solid-phase catalyst, reaction of the methylamine formed with *o*-phthalaldehyde reagent, and fluorescence detection of the derivatives. The optimized method was evaluated. Recovery and repeatability data were collected for 13 representative carbamates and 12 metabolites on 12 different food product types. The reliability of the method in routine analysis of fruit and vegetable samples is demonstrated. Registration of the analytical results of control samples in Shewhart charts during a year of routine analysis has demonstrated the excellent performance of this complete method. The limits of determination are in the 5–50 μg/kg (ppb) range. Sample throughput is about 20 per 24 h. Data are presented on real residues of *N*-methylcarbamates found in fruits and vegetables during 4 years of routinely applying this multiresidue method.

In recent years, both legislators and consumers have shown increased interest in the safety of food products. Events such as the appearance of pesticide residues in fruits and vegetables have impelled governments to set up monitoring programs (1). These programs determine the contamination levels in food products and trace possible occasions in which residue tolerance levels were exceeded because of incorrect agricultural practice. To execute such monitoring programs efficiently, a set of multiresidue methods must be able to detect as many pesticides as possible in all crop types available on the market.

In addition to the traditional multiresidue methods for the gas chromatographic (GC) determination of organochlorine, organophosphorus, and organonitrogen pesticides with element-specific detectors (electron capture, flame photometric, and nitrogen–phosporus, respectively), liquid chromatography (LC) is now becoming a useful tool for the determination of the relatively polar *N*-methylcarbamate pesticides. These pesticides are routinely monitored by LC only in the United States (2, 3). Surprisingly, introduction of this method in European control laboratories has lagged, possibly because there is less interest in this group of pesticides and, undoubtedly, because of the relatively high investment required for instrumentation, qualified personnel, and time. The method involves laborious extraction and cleanup procedures.

Five years ago, our group (4) succeeded in replacing the traditional cleanup of the Krause method by a quick and practical cleanup using the solid-phase extraction (SPE) technique on aminopropyl-bonded silica cartridges. In a subsequent study (5), the principle of solid-phase catalysis was applied to the postcolumn hydrolysis of *N*-methylcarbamate pesticides. This modification improved and simplified the postcolumn system considerably. This method was elaborated for the entire class of *N*-methylcarbamates and their most important metabolites, and the method was subsequently implemented in the routine pesticide screening program of the pesticide analysis laboratory of our regional food inspection service in the years 1987–1990. Although the method appeared to be very satisfactory in daily routine work, the cleanup step still remained the rate-determining step in sample throughput. This was even more true when compared with capillary GC multiresidue methods in which a cleanup can be omitted without deleterious results.

The introduction of apparatus for automated solid-phase extraction (SPE) by various analytical instrument vendors gave us an important opportunity to reduce analysis time and make the procedure more economical by increasing sample throughput. This paper describes the conversion of the manual method into an automated SPE cleanup method by means of the com-

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mercial automated SPE cleanup (ASPEC) apparatus (Gilson). By the on-line coupling of the SPE and LC analysis steps, the complete SPE cleanup step can be performed during the LC analysis run time of the previous sample in the series.

The automated method was validated and subsequently applied and evaluated in routine practice for nearly a year. Data are given on recoveries, repeatability, analysis of control samples (Shewhart charts), and residues on real practice samples.

Experimental

Apparatus

(a) *Liquid chromatograph.*—LC high pressure gradient system (Gilson), consisting of 2 Model 305 pumps, Model 811B dynamic mixer, and Model 805 manometric module (Gilson Medical Electronics, Middleton, WI 53562).

(b) *ASPEC apparatus.*—Gilson ASPEC provided with thermo-unit and valve-switching unit (both from Meyvis, Bergen op Zoom, The Netherlands), Model 401 diluter, extraction rack for 100 mg cartridges, sample vial rack, and elution rack, which can be heated.

(c) *Injection valve.*—Model 7010, electrically activated 6-port injection valve with 100 µL sample loop (Rheodyne, Cotati, CA 94928), included in ASPEC apparatus.

(d) *Postcolumn reactor oven.*—Model 7910 column heater (Jones Chromatography, Littleton, CO 80162).

(e) *Reagent delivery pump.*—L-6000 isocratic LC pump (Merck, Darmstadt, Germany), provided with low-pressure pulse dampener (Free University, Amsterdam, The Netherlands).

(f) *Fluorescence detector.*—F-1000 (Merck) dual monochromator fluorescence detector equipped with 12 µL flow cell, with 10 bar back pressure regulator (Beckman, Fullerton, CA 92634) installed at outlet capillary.

(g) *Analytical column.*—LiChroCART 250 × 4 mm id cartridge column (Merck), packed with Superspher RP-8 (4 µm).

(h) *Guard column.*—LiChroCART 4 × 4 mm id cartridge column (Merck), packed with LiChrosorb RP-18 (5 µm).

(i) *Postcolumn catalytic reactor.*—50 × 4 mm id stainless steel column, packed with Aminex A-27 (15 µm) strong anion exchange resin (Bio-Rad, Richmond, CA 94804).

(j) *o-Phthalaldehyde (OPA) reaction capillary.*—25 cm × 0.25 mm id poly(tetrafluoroethylene) connection capillary between mixing tee and fluorescence detector.

(k) *Data acquisition.*—Macintosh IIcx computer with Dynamax HPLC Method Manager software (Rainin, Woburn, MA 01801) for LC gradient control and data acquisition, Rainin control/interface module, and Hewlett-Packard DeskWriter printer.

(l) *Homogenizer.*—Polytron (Kinematica, Luzern, Switzerland) Model PT 10–35.

(m) *Centrifuge.*—Varifuge GL Model 4100 (Heraeus-Christ, Osterode, Germany).

(n) *Food chopper.*—Model UM 12 (Stephan, Hameln, Germany).

(o) *Water purification system.*—Elgastat UHQ (Elga, High Wycombe, UK).

Reagents

(a) *Acetonitrile.*—LC-grade (Lab-Scan, Dublin, Ireland).

(b) *Solvents.*—Dichloromethane, acetone, methanol, light petroleum (bp 40–60°C), all of residue analysis quality (Merck, Darmstadt, Germany).

(c) *Water.*—Tap water purified with Elgastat UHQ purification system (Elga).

(d) *Disodium tetraborate solution.*—0.01M in purified water. Dissolve 3.8 g anhydrous disodium tetraborate (Merck) in 1 L water in volumetric flask.

(e) *OPA reagent.*—Dissolve 250 mg *o*-phthalaldehyde for fluorescence analysis (Merck) in 2 mL acetonitrile, transfer to 1 L volumetric flask, add 0.1 mL 2-mercaptoethanol (Merck), and dilute to volume with 0.01M disodium tetraborate solution. Mix well, filter through 0.45 µm membrane filter, and degas by vacuum before use.

(f) *SPE cartridges.*—100 mg (1 mL) Bond-Elut aminopropyl-bonded silica extraction columns (Varian/Analytichem, Harbor City, CA 90710).

(g) *N-methylcarbamate standard stock solutions.*—Dissolve 10 mg carbamate standard (U.S. Environmental Protection Agency, Research Triangle Park, NC, or Promochem, Wesel, Germany) in 10 mL dichloromethane to give 1 mg/mL stock solution, which is stable for at least 1 year in freezer at −18°C. Prepare standard mixture in dichloromethane by transferring 100 µg (in 100 µL) of each standard in 100 mL volumetric flask, and dilute to volume with dichloromethane. This gives 1 µg/mL standard mixture.

Prepare diluted standard mixture by transferring 5.0 mL 1 µg/mL solution into 100 mL volumetric flask, and dilute to volume with dichloromethane to give 0.05 µg/mL concentration. Standard mixtures in dichloromethane are stable for at least 1 year if refrigerated.

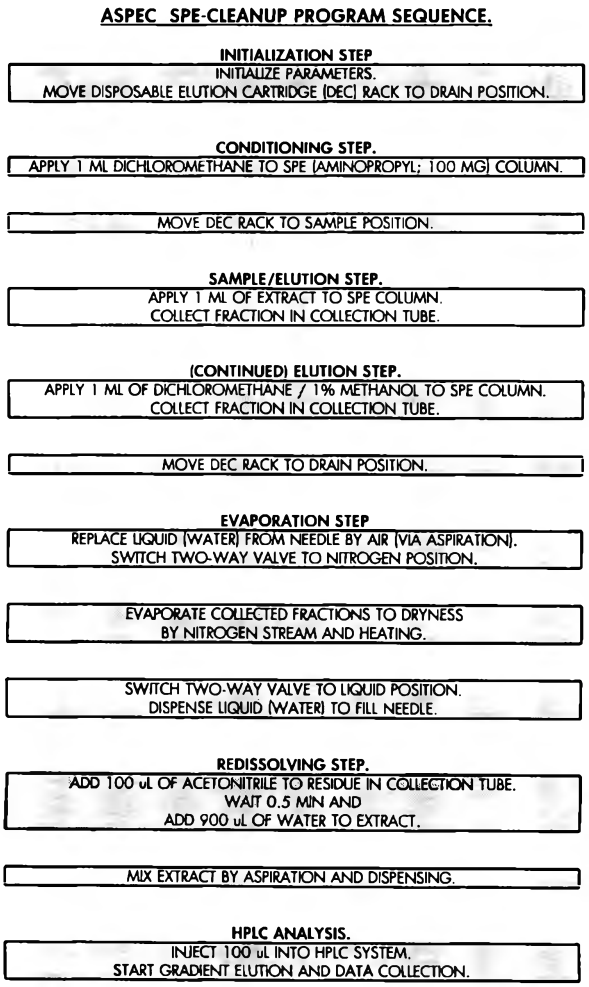
(h) *Standard working solution.*—Prepare working solution just before LC analysis. Transfer 1 mL 0.05 µg/mL standard solution in sample vial, evaporate to dryness, and redissolve in 1 mL starting LC mobile phase.

Extraction

Fruits and vegetables.—Homogenize 15 g well-mixed, chopped crop sample with 30 mL acetone in centrifuge tube (14 × 5 cm id, 250 mL volume) for 30 s with Polytron. Add 30 mL dichloromethane and 30 mL light petroleum, and extract by homogenizing another 60 s. Centrifuge tube 2 min at 4000 rpm. Decant upper (organic extract) layer, which can also be used for GC multiresidue methods, into Erlenmeyer flask.

Transfer 2 mL of this extract to ASPEC sample vial. Place series of sample vials (in ASPEC rack) in water bath at 60°C, and gently evaporate extraction solvent to near dryness. Allow remaining solvent to evaporate in air. Redissolve residue in 1 mL dichloromethane.

Grains.—Weigh 25 g grain sample in Erlenmeyer flask, and add 100 mL acetone–dichloromethane. Let stand overnight



Scheme 1. ASPEC program sequence.

(16 h) for extraction. Transfer 1 mL extract to ASPEC sample vial, and proceed as described above for fruits and vegetables.

Automated Cleanup

Place sample rack with redissolved extracts in ASPEC apparatus, which executes complete SPE cleanup automatically, followed by on-line injection of 100 µL cleaned-up extract into LC system. Flowchart of various steps of ASPEC (cleanup, evaporation, and redissolution) is shown in Scheme 1.

ASPEC apparatus has to be reprogrammed for this particular application. Software program is available from ASPEC representative (Meyvis, Bergen op Zoom, The Netherlands).

LC Analysis

Use following conditions for LC analysis: Injection volume, 100 µL; mobile phase flow-rate, 0.8 mL/min; gradient run, linear 5% solvent mixture B (acetonitrile–water, 80 + 20, v/v)–95% solvent mixture A (acetonitrile–water, 10 + 90, v/v) at time 0, to 80% mixture B–20% mixture A at 60 min, and equilibrate at initial conditions for 10 min; analytical column temperature, 30°C; catalytic reactor temperature, ca 120°C (**Note:** Hydrolysis reactor temperature should be high enough to obtain about the same detector response for oxamyl and methomyl; when applying low flow-rate [minimum 0.1 mL/min] through reactor column, e.g., overnight, let cool down completely before decreasing flow rate); derivatization: OPA-reagent flow-rate, 0.2 mL/min; detection: excitation wavelength, 340 nm; emission wavelength, 455 nm.

Data acquisition and processing was performed via computer with Dynamax HPLC Method Manager software. *N*-Methylcarbamates and metabolites were quantitated by external standard method. In second part of research work, internal standard method was applied. At redissolution step, just before ASPEC procedure, residue was dissolved in 1 mL landrin solution in dichloromethane (0.05 µg/mL).

Method Validation

Twelve food product types, chopped and well-homogenized samples, in centrifuge tube, were spiked at 0.13 mg/kg with mixture of 13 *N*-methylcarbamates and 12 metabolites by adding 200 µL 10 µg/mL standard mixture in dichloromethane to 15 g sample. Sample was mixed and allowed to stand 1 h before extraction.

Results and Discussion

Automated SPE Cleanup

In one of the previous studies (4), a rapid and convenient cleanup method was developed, based on an SPE of a dichloromethane extract on aminopropyl-bonded silica cartridges. A strong feature of this cleanup is the normal-phase mode of the SPE, which adds extra specificity and selectivity to the reversed-phase model LC separation. However, the incompatibility of the SPE eluant, dichloromethane–1% methanol, with the LC mobile phase, acetonitrile–water, necessitates an evaporation and a redissolution step. A few years ago, this appeared to be the bottleneck for complete automation with the commercially available ASPEC instrument (Gilson) for automated SPE. The standard ASPEC apparatus could be adapted rather easily to include the solvent-exchange step. Technically, this has been realized by the addition of an electrically driven 2-way switching valve, which can select between the solvent for SPE or nitrogen for evaporation. In this way, a nitrogen stream is directed to the sample extract via the same needle used for solvent delivery and movement of the cartridge racks. To accelerate the evaporation step, a heating module was used to heat the aluminum block containing the vials with the eluted SPE fractions to about 40°C. From those vials, an aliquot of the cleaned, redissolved extract is injected on-line into the LC system by means of this same needle.

The original software program for the various SPE steps and evaporation/redissolution step was based exclusively on a straightforward reversed-phase cleanup step; therefore, essential changes in the basic software were required so that SPE could be performed with, for example, a deviating order of cartridge rack movements. The programming work is rather time-consuming and requires programming language skills of an experienced analyst, which makes the procedure setup very difficult. After the programming changes, some trial-and-error

experiments (e.g., to determine the optimal needle positions for the evaporation step) were performed to obtain the optimized ASPEC procedure.

The major advantage of the ASPEC is that the cleanup is no longer the rate-determining step in the whole procedure, because during the LC run time of a sample, the next sample is being cleaned up. Thus LC analysis and data processing determine the sample throughput. The recoveries of the automated SPE were determined for a standard mixture of 25 *N*-methylcarbamates and metabolites in dichloromethane. Recoveries ranged from 75 to 100%, and the coefficients of variation (CVs) were invariably between 1 and 3% ($n = 5$).

LC Separation

To increase the resolution of the LC separation of a total of 33 analytes (21 parents and 12 metabolites), gradient elution was evaluated. The optimal linear binary gradient (see *Experimental*) resulted in the retention behavior shown in Table 1. Five critical pairs of compounds remained unresolved when a Supersphere C_8-bonded silica stationary phase was used. Some of these pairs could, however, be well separated on a C_{18}-bonded silica phase, although some other peaks then coincide.

Figure 1a is an example of a liquid chromatogram of a mixture of 23 well-separated carbamates and metabolites. The average detection limit, based on a signal-to-noise ratio of 3:1, is 100 pg (except for thiofanox, 500 pg) when gradient elution LC is used.

The total run time is 60 min, plus an extra 10 min for equilibration at the initial mobile phase of the gradient. This means that a sample throughput of 20 per 24 h is possible. If an optimal resolution, especially of the early eluting carbamates, is not required, throughput may be increased to 30 or 40 samples per 24 h by running an isocratic LC separation with acetonitrile–water (35 + 65).

Catalytic Solid-Phase Hydrolysis

In our previous paper (5), Aminex A-27 (a strong anion exchanger) and magnesium oxide were used for the postcolumn solid-phase hydrolysis reaction instead of the classical NaOH basic hydrolysis. During the last 2 years, much more experience has been gained in using these materials in daily routine work.

Magnesium oxide is a very cheap and rigid material, besides having favorable kinetics/retention characteristics for the postcolumn reaction. The lack of commercially available, uniform, spherical MgO particles with a narrow particle size range, however, forms the major drawback for a definitive application of this solid-phase material in routine analysis. Experiments with different packing procedures for the coarse MgO material have revealed that special skills are still required to pack reliable reactor columns that will not clog during prolonged use.

On the other hand, we have noticed that the quality and/or properties of the newer polymer-based anion exchangers have been improved over time. Columns packed with Aminex can now be used continuously at 120°C for up to 6 months, provided necessary cleanup of samples is executed. Surprisingly, the use of gradient elution LC is also no longer a problem. We concluded that, at present, the Aminex A-27 is a more reliable material than MgO in the long run, which justifies the higher price of the former material.

Table 1. Relative retention times (RRT) of 21 *N*-methylcarbamates and 12 of their metabolites (in italics) in the reversed-phase LC system Supersphere RP-8 (4 μm), with acetonitrile–water gradient[a]

No.	Carbamate/metabolite	RRT
1	*Butocarboxim sulfoxide*	0.21
2	*Aldicarb sulfoxide*	0.23
3	*Butoxycarboxim*	0.30
4	*Aldicarb sulfone*	0.32
5	Oxamyl	0.33
6	Methomyl	0.39
7	*Ethiofencarb sulfoxide*	0.44
8	*Thiofanox sulfoxide*	0.46
9	*3-Hydroxycarbofuran*	0.52
	Ethiofencarb sulfone	0.52
10	*Methiocarb sulfoxide*	0.55
11	Dioxacarb	0.60
	Thiofanox sulfone	0.60
	Tranid	0.63
12	*Methiocarb sulfone*	0.75
	Butocarboxim	0.75
13	Aldicarb	0.80
14	*3-Ketocarbofuran*	0.82
	Cloethocarb	0.97
15	Propoxur	0.97
16	Carbofuran	1.00
	Bendiocarb	1.00
17	Carbaryl	1.09
	Thiofanox	1.10
18	Ethiofencarb	1.11
19	Isoprocarb	1.16
	Landrin	1.17
20	Carbanolate	1.22
21	Methiocarb	1.34
22	Promecarb	1.39
23	Bufencarb	1.57
	Mexacarbate	>1.60
	Aminocarb	>1.60

[a] Absolute retention time of carbofuran = 35.0 min.

Method Validation

Recoveries and Repeatability

The accuracy and precision of the automated method were determined via analysis of 12 types of fruits and vegetables, each type representing a typical class of crops. The homogenized samples were fortified with a mixture of 13 *N*-methylcarbamates and 12 metabolites at the 0.13 mg/kg level. Although the recoveries and repeatability data depend on the matrix/pesticide combination, they are quite acceptable. The

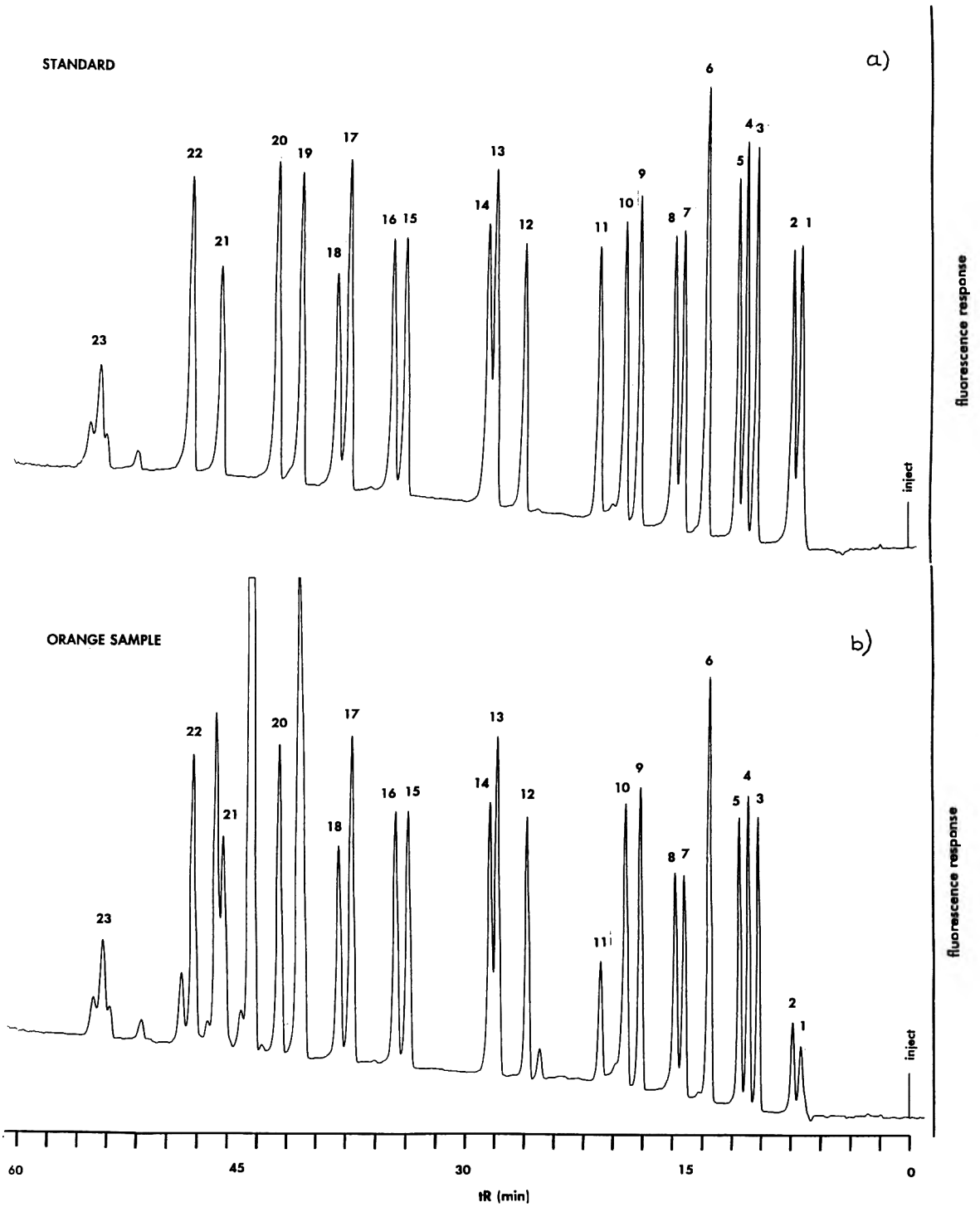


Figure 1. Liquid chromatograms of (a) a standard mixture of 23 *N*-methylcarbamates, and (b) an orange sample fortified with 23 *N*-methylcarbamates at 0.13 mg/kg level after extraction and cleanup on aminopropyl-bonded silica SPE column (by ASPEC). Numbers above the peaks correspond with those given in Table 1 (see *Experimental* for further details).

data are summarized in Table 2. Five replicate analyses were performed each time. A typical example of a liquid chromatogram from a fortified orange sample extract after SPE cleanup is shown in Figure 1b.

For 13 parent *N*-methylcarbamates, recoveries were in the range 65–100%, with a CV range of 0.9–6.7%. Ten of the metabolites had a recovery range of 54–96%, with a CV range of 0.7–7.2%. The 2 most polar carbamates, butocarboxim sulfox-

Table 2. Average recoveries (%) and repeatability coefficients of variation (CV, $n = 5$) of *N*-methylcarbamates and metabolites from fortified samples at the 0.13 ppm level

	Apple		Beans		Carrot		Cauliflower		Endive		Onion		Orange		Paprika		Peach		Potato		Strawberry		Rice	
Carbamate	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV	Rec.	CV
Butocarboxim sulfoxide	20.0	5.00	21.0	5.83	18.8	5.83	17.0	8.32	17.6	8.62	19.0	3.72	22.6	8.04	20.4	7.43	18.4	6.20	20.2	4.14	16.6	5.39	91.4	2.07
Aldicarb sulfoxide	33.4	4.54	28.6	3.99	27.6	6.58	23.6	7.70	25.8	6.37	29.2	2.87	27.6	4.86	27.4	4.90	27.2	4.79	27.8	4.69	25.2	6.52	97.8	1.92
Butoxycarboxim	73.0	2.17	70.0	2.26	67.6	2.24	67.8	2.84	67.4	2.70	71.8	3.33	71.4	2.54	64.8	2.01	63.2	2.06	69.0	2.29	65.4	2.78	98.6	1.34
Aldicarb sulfone	76.8	2.14	71.8	2.29	69.0	2.05	72.6	2.50	68.2	2.17	70.2	2.34	69.8	1.20	67.4	3.08	67.2	1.94	69.8	2.56	68.2	0.66	98.6	1.95
Oxamyl	74.8	2.57	72.2	2.48	70.0	2.86	71.6	2.54	70.0	1.75	71.0	3.59	68.8	1.90	70.8	1.55	66.8	1.64	70.8	2.53	66.4	1.72	101.2	2.05
Methomyl	79.2	2.43	87.8	1.49	78.8	2.09	78.2	1.67	82.8	2.32	83.6	1.81	76.6	1.98	81.4	2.23	77.6	1.15	80.6	2.25	78.0	3.01	91.0	1.87
Ethiofencarb sulfoxide	69.4	2.81	73.6	2.65	62.6	3.68	63.2	3.43	68.2	2.41	62.2	3.67	65.6	2.31	65.8	1.27	68.4	1.67	68.0	2.08	62.6	3.11	97.8	1.48
Thiofanox sulfoxide	60.8	4.26	66.4	1.35	60.0	3.12	53.6	3.87	58.2	3.07	57.0	2.77	65.8	2.72	65.6	2.77	62.2	2.38	62.6	2.90	56.0	2.53	93.2	2.28
3-Hydroxycarbofuran	72.6	3.17	79.6	1.12	76.2	2.85	76.4	2.38	79.4	1.91	74.0	2.53	71.2	3.35	77.0	8.90	77.2	3.93	73.2	4.03	68.4	3.37	93.0	3.74
Ethiofencarb sulfone	78.2	2.29	85.4	2.82	76.8	6.00	83.2	2.15	81.8	2.51	83.2	2.61	79.2	2.43	78.6	1.93	76.0	3.84	78.2	2.10	76.0	1.86	95.0	3.39
Methiocarb sulfoxide	77.0	3.05	78.6	2.48	75.6	2.21	77.6	2.34	77.0	3.18	71.0	2.63	72.6	2.30	76.4	2.71	71.0	2.23	73.2	2.24	71.8	2.29	95.2	2.86
Dioxacarb	90.8	2.85	91.0	2.06	87.2	1.70	96.6	1.88	92.0	2.31	83.2	1.78	90.0	2.22	89.0	1.12	84.0	2.23	90.8	2.74	85.8	1.92	100.8	1.79
Thiofanox sulfone	89.6	2.57	94.8	1.89	83.6	2.75	96.2	2.25	93.2	1.40	86.4	1.55	87.6	2.07	86.8	1.50	86.2	1.27	86.6	2.53	83.6	1.07	101.6	1.82
Methiocarb sulfone	79.8	2.41	80.6	1.66	73.0	2.74	83.0	2.41	79.4	2.46	84.0	2.66	75.0	7.18	76.2	2.85	75.4	2.91	77.0	2.43	76.4	0.72	102.4	2.61
Aldicarb	86.6	2.66	93.8	2.31	82.4	2.20	92.6	2.49	90.2	2.65	82.8	1.32	89.6	1.27	85.6	1.33	82.8	1.57	82.0	2.99	82.8	1.01	92.2	1.48
3-Ketocarbofuran	87.0	3.54	94.0	2.81	85.2	2.54	92.6	1.81	89.0	2.38	88.6	1.71	85.6	2.42	88.0	2.13	84.4	2.31	89.6	1.69	83.8	2.13	100.4	1.34
Propoxur	90.2	1.45	94.6	1.92	87.8	2.33	94.8	1.16	91.6	2.95	90.0	1.92	89.4	1.50	89.2	0.94	88.0	2.13	88.8	1.47	89.2	0.94	95.2	1.30
Carbofuran	93.2	1.59	99.4	1.53	90.0	1.36	93.6	1.79	93.8	2.31	91.8	2.36	90.2	1.21	91.2	1.43	90.2	2.40	92.8	1.93	91.2	1.20	94.4	1.82
Carbaryl	94.8	1.38	100.0	2.24	92.0	2.03	94.0	1.50	93.8	1.91	94.4	2.32	90.6	1.85	94.2	1.74	95.8	1.36	93.8	2.31	92.2	1.61	91.0	1.87
Ethiofencarb	73.4	5.15	65.8	6.75	83.4	4.21	91.4	0.98	76.6	5.88	77.2	2.13	87.2	3.18	88.0	2.13	73.2	4.14	63.4	3.96	71.8	3.02	30.8	4.87
Isoprocarb	84.0	3.04	91.8	1.42	83.4	2.34	87.6	2.07	84.0	3.04	81.4	2.39	—[a]	—	88.4	2.21	79.8	2.57	79.4	2.90	85.4	1.34	89.4	2.51
Carbanolate	89.6	1.69	93.8	3.57	88.8	2.01	91.4	2.13	90.2	2.65	90.0	1.57	88.0	2.78	90.6	2.54	89.6	2.03	92.0	2.17	88.6	1.51	84.8	3.11
Methiocarb	92.4	2.82	96.8	2.36	89.2	2.56	93.2	1.76	92.0	2.17	93.6	1.79	—	—	95.6	1.75	91.2	0.92	91.6	1.98	89.2	2.01	79.4	2.88
Promecarb	92.8	3.09	93.6	2.34	86.8	1.71	—	—	87.8	3.45	90.6	1.67	87.0	3.15	87.2	0.96	88.4	1.29	92.8	2.79	85.4	2.13	79.4	4.28
Bufencarb	83.0	2.69	92.4	2.11	82.4	1.09	83.0	1.90	87.2	2.05	82.2	2.18	86.2	3.00	83.2	3.34	83.6	2.62	83.6	2.62	82.8	2.62	74.4	4.51

[a] —, Recovery could not be calculated because of sample interference.

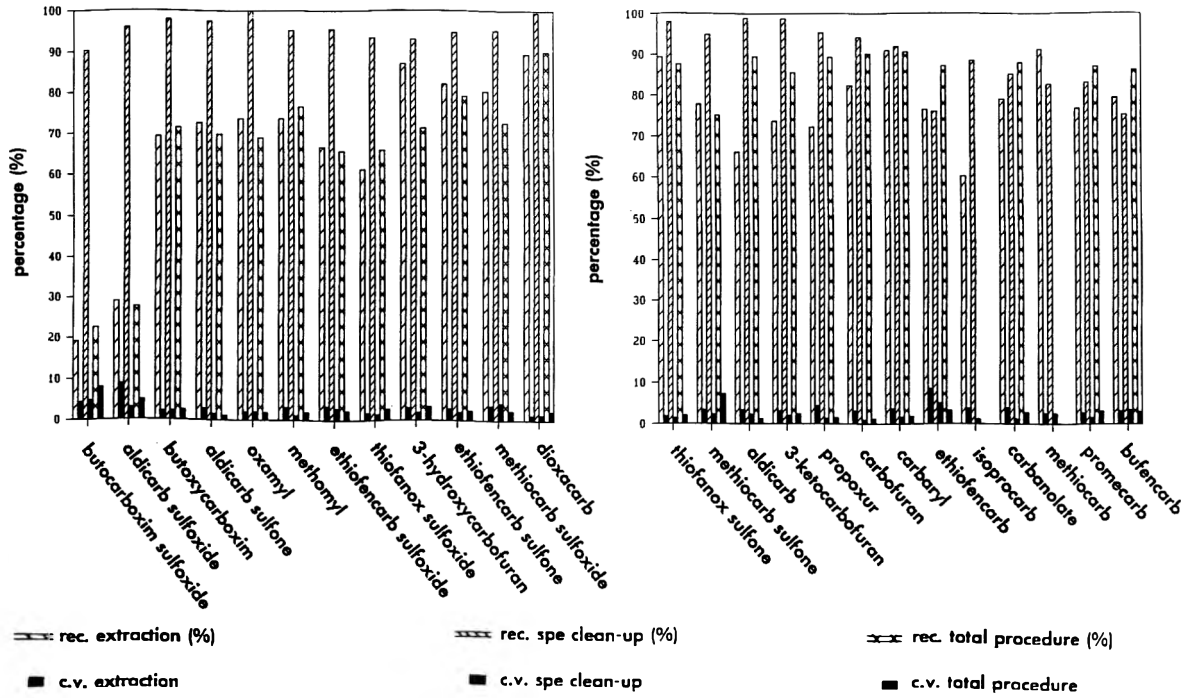


Figure 2. Average recoveries (%) and repeatability coefficients of variation (CV, $n = 5$) of the extraction step, the SPE cleanup step (by ASPEC), and the total procedure for fortified (0.13 mg/kg) orange samples.

ide and aldicarb sulfoxide, were recovered only at the 17–33% level, although CVs were still reasonably good, between 2.9 and 8.6%. By performing extractions with spiked water solutions, we proved that the lower recoveries for these latter compounds were due to loss in the liquid–liquid partitioning step because of their relatively high polarity. Higher recoveries may be obtained by repeated extractions. Because no partitioning is necessary for rice, recoveries were in the 74–102% range.

A closer look at Table 2 shows that, apart from the absolute recovery, 90% of the CVs are in the 1–4% range. Thus, the method has excellent repeatability, despite homogenization, extraction, evaporation/redissolution, SPE, a second evaporation/redissolution, LC analysis, and data analysis steps.

To determine the contributions of the major steps in the method to the overall recoveries and CVs, the extraction and the SPE were considered separately for spiked orange samples. The results are shown graphically in Figure 2. This display reveals clearly that recovery losses are mainly caused by incomplete extraction or partitioning due to the relatively polar nature of these typical carbamates. Extraction/partitioning is also the major contribution to the overall values of the repeatability CVs. The CV ranges were 1.3–8.5% and 0.6–4.7% for the extraction step and SPE step, respectively.

Stability of Carbamates

An easily overlooked critical point in the method, and one that deserves special attention, concerns the stability of the various *N*-methylcarbamates in the LC mobile phase and/or water. We observed that a large number of the *N*-methylcarbamates are degraded in aqueous media. These include ethiofencarb, ethiofencarb sulfoxide, ethiofencarb sulfone, methiocarb sulfoxide, methiocarb sulfone, and oxamyl. Therefore, after evaporation of solvent from the SPE eluant fraction, the time between its redissolution into LC solvent and injection in the LC should be as short as possible. Fortunately, in the fully automated, on-line method, this short interval is guaranteed, but in laboratories applying this method in the nonautomated mode, that is, via off-line SPE in batch and LC analysis of a series of sample extracts in water–acetonitrile that are lined up in an autosampler, analysts must be aware of the degradation of the labile carbamates in the later sample vials. In that case, the extracts should be stabilized, e.g., via acidification (pH = 3) of the aqueous solutions. For more details, the reader is referred to our recent paper on the determination of *N*-methylcarbamates in aqueous samples (6).

Linearity

The linear dynamic range of the detector response for the *N*-methylcarbamates was checked and appeared to be from 0.1 to 100 ng injected on-column. This is also the practical working range. The average correlation coefficient was 0.995.

Control Sample

As a quality assurance measure, a control sample was continuously analyzed in each series of routine samples. Green pepper was chosen as a relevant matrix, and residues of 4 *N*-methylcarbamates (methomyl, propoxur, carbaryl, and methiocarb) spiked to green pepper are stable for at least a year if the sample is kept in a freezer at −18°C. The analytical data of the control sample, spiked at the 0.1 mg/kg level, are displayed on

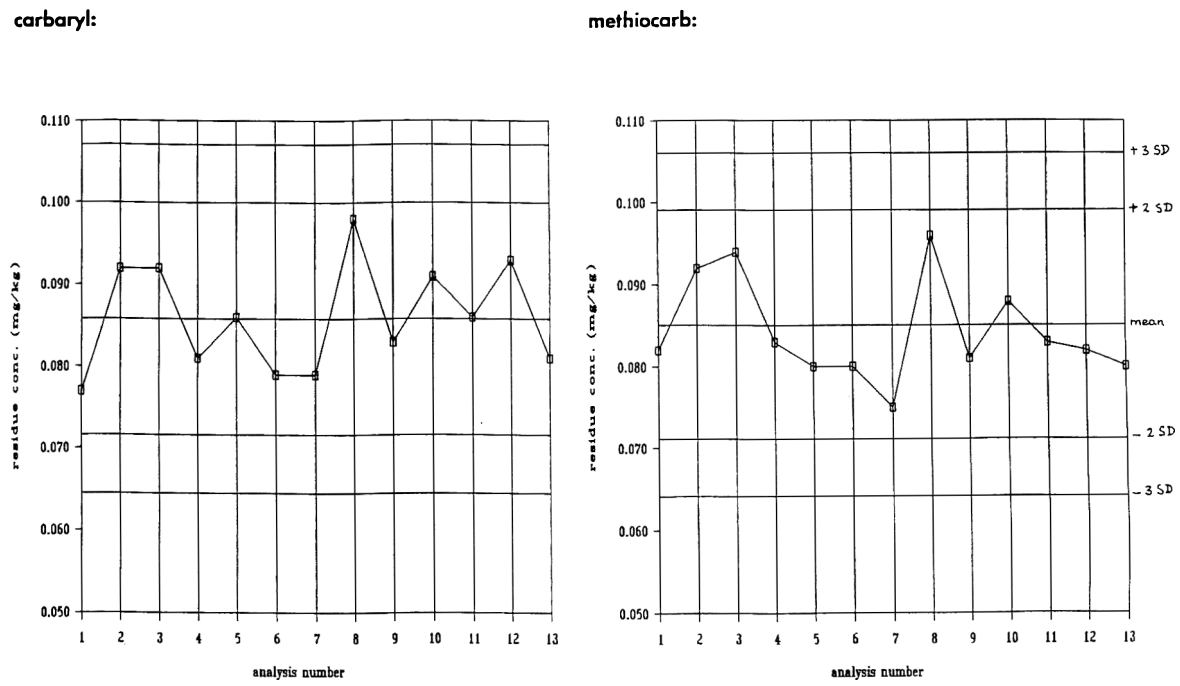


Figure 3. Example of Shewhart control charts for the *N*-methylcarbamates carbaryl and methiocarb. The analytical results were collected over a yearlong period.

a Shewhart control chart. The results for carbaryl and methiocarb are shown in Figure 3, where the time interval between succeeding analysis numbers is a month. The horizontal lines in the chart represent the warning (2 × SD) and action (3 × SD) limits. The results in Figure 3 confirm the overall reliability of the automated method in routine analysis. When the automated method was introduced into routine analysis, the internal standard method was chosen as the quantitation method, a measure that further improves the repeatability of the method.

Routine Crop Analysis

In our laboratory, the LC method for the determination of *N*-methylcarbamate residues in fruits and vegetables has now been in continuous use for more than 4 years. During this period, the method has evolved from the conventional isocratic LC separation with NaOH-based postcolumn hydrolysis to the automated SPE cleanup coupled on-line with gradient-run LC and postcolumn hydrolysis on a solid phase. During more than a year of routine application, the method in this latter form has demonstrated a very high sensitivity, selectivity, and precision, predominantly because of the efficient SPE cleanup and specific reaction detection system. Even the most difficult sample types, such as citrus fruits, may now be analyzed conveniently, because matrix interferences are reduced to a minimum and/or are well separated from the carbamates through the optimized LC gradient run.

However, some extra effort is required to confirm the positive residues detected, especially at low concentration levels. LC/mass spectrometry (MS) is not generally sensitive enough nor available in a routine analysis environment. Therefore, alternative methods should be used. Although more research is needed, various confirmation methods can be mentioned here. Capillary GC/nitrogen–phosphorus detection or, preferably, GC/MS is the best confirmation method for the thermostable carbamates. These methods may also be used after the carbamate has been converted to a thermostable derivative. If LC is the only choice because of the incompatibility of the analyte with GC, analysis on a second column packed with a stationary phase with a different selectivity and/or the application of a different detector, such as a UV or electrochemical detector, is a good alternative. In routine analysis, sulfoxide and/or sulfone metabolites have been detected in combination with the parent carbamate, which already provides confirmation. Conversion of an oxime carbamate or its sulfoxide into the corresponding sulfone product via reaction with potassium permanganate is a useful method to confirm the identity. Finally, it should be kept in mind that running the standard LC analysis with the postcolumn reactor at room temperature and/or the OPA reagent flow rate turned off gives extra information on the identity of a peak observed in the original chromatogram.

Residue data obtained in The Netherlands over a 4-year period (July 1987–July 1991) are summarized in Table 3. Positive residues were detected in nearly 10% of all samples analyzed. Methomyl, oxamyl, carbaryl, propoxur, carbofuran, methiocarb, and butocarboxim occurred most frequently; ethiofencarb and aldicarb were detected only occasionally. The latter 4 carbamates were sometimes accompanied by or appeared as their sulfoxide and/or sulfone metabolites. The number of

Table 3. *N*-Methylcarbamate residues in fruits and vegetables in the years 1987–1991

Product	Aldicarb[a]	Butocarboxim[a]	Carbaryl	Carbofuran	Ethiofencarb[a]	Methiocarb[a]	Methomyl	Oxamyl	Propoxur	Total analyzed	Positive residues	No. exceeding residue tolerances
Berries	—	1	1	1	—	—	—	—	6	153	9	1
Potatoes	1	—	2	—	—	1	—	—	—	267	4	1
Strawberries	—	1	3	1	—	2	3	3	7	231	20	1
Apricots	—	—	1	—	—	—	—	—	—	13	1	—
Pineapples	—	—	2	—	—	—	—	—	1	15	3	—
Endive	—	—	—	—	—	1	1	—	1	183	3	1
Apples	—	—	36	—	—	1	4	4	14	285	59	—
Artichokes	—	—	—	—	—	—	1	—	—	3	1	—
Eggplant	—	1	—	—	—	1	3	2	—	18	7	2
Cauliflower	—	—	—	—	—	2	—	—	1	61	3	—
Beans	—	—	—	—	—	1	6	1	1	149	9	2
Cabbages	—	—	—	—	1	—	—	—	—	23	1	—
Lemons	—	1	—	—	—	—	—	—	—	53	1	—
Grapes	—	—	12	—	—	—	10	—	2	208	24	7
Raspberries	—	—	—	—	—	—	—	—	2	10	2	—
Grapefruits	—	—	1	—	—	—	—	—	—	78	1	—
Iceberg lettuce	—	—	—	—	2	1	3	1	3	42	10	—
Kiwis	—	—	3	—	—	—	1	—	—	57	4	—
Celery	—	2	—	—	—	—	—	1	1	21	4	—
Cucumber	—	—	—	—	1	2	12	7	3	64	25	10
Mandarins	—	2	1	—	—	—	—	1	—	51	4	2
Lettuce	—	—	1	—	—	—	2	4	5	115	12	1
Melons	—	1	1	1	—	1	6	5	—	85	15	1
Nectarines	—	—	1	—	—	—	1	1	—	36	3	2
Peppers	2	—	1	—	—	13	22	5	2	92	45	32
Pears	—	—	1	—	—	—	—	—	1	66	2	—
Peaches	—	—	1	1	—	—	1	2	—	69	5	—
Zucchini	—	—	—	—	—	1	4	1	—	16	6	2
Plums	—	—	1	2	1	—	1	—	4	156	9	—
Radish	—	—	—	3	—	—	—	—	1	24	4	—
Rice	—	—	1	—	—	—	—	—	—	75	1	—
Oranges	—	1	10	3	—	—	—	2	—	229	16	1
Spinach	—	—	—	—	—	—	—	—	1	141	1	—
Wheat	—	—	—	—	—	—	—	—	1	139	1	—
Tomatoes	—	1	—	—	—	—	1	4	—	117	6	—
Total	3	11	80	12	5	27	82	44	57	3345	321	66

[a] Including sulfoxide and sulfone metabolites.

samples exceeding the Dutch maximum residue limits for *N*-methylcarbamates reached about 2% of all samples analyzed or 20% of all samples that were found to contain residues of *N*-methylcarbamates. These data, representing the first comprehensive survey undertaken in Europe, demonstrate that it is worthwhile to include the multiresidue method for *N*-methylcarbamates in routine pesticide residue analysis schemes.

Conclusion

A very selective and sensitive multiresidue method for fruit and vegetable samples covering 21 *N*-methylcarbamates and 12 of their metabolites was developed based on an ASPEC apparatus coupled on-line with an LC separation with fluorescence detection after catalytic solid-phase hydrolysis and OPA derivatization. The minimum detectable amount is about 100 pg for each pesticide, which means that residue levels in real crop samples down to 5 µg/kg can be detected.

The sample throughput was increased considerably (about 1 h) by the automated method. The application and quality assurance in a routine laboratory were demonstrated. During 4 years of monitoring *N*-methylcarbamates in fruits and vegetables, a significant number of positive residues have been detected in a variety of crop samples. In view of the number of *N*-methylcarbamate residue concentrations exceeding the tolerance levels (about 2%), inclusion of this multiresidue method in routine pesticide monitoring is justified.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of *N*-Methylcarbamate Insecticides in Vegetables, Fruits, and Feeds Using Solid-Phase Extraction Cleanup in the Normal Phase

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A liquid chromatographic (LC) method is described for the simultaneous determination of 8 carbamate pesticides in vegetable, fruit, and feed crops. Carbamates are extracted and then partitioned from vegetable, fruit, and feed crop samples using procedures previously developed. An aliquot is taken from the sample partitioning, evaporated by a nitrogen stream, and reconstituted in 5 mL 5% methylene chloride–hexane. Sample cleanup is accomplished by aspirating through an aminopropyl Bond Elut (NH_2) solid-phase extraction column. Following aspiration, the sample is eluted with 10 mL 2% methanol–methylene chloride. The eluate is evaporated, again under nitrogen, and reconstituted in methanol for fluorometric LC analysis. The method provides an excellent cleanup for all matrixes studied. Initial recoveries of 98, 90, and 91% were obtained for fortifications of 0.05, 0.5, and 5.0 ppm, respectively. Linear range recovery studies demonstrated results that agreed with the initial recovery study. An intralaboratory study of the method was conducted in quadruplicate, using 10 assorted vegetable, fruit, and feed crops. With no exceptions, the overall average pesticide recovery for this study was 92%, with a standard deviation of 7.1 (n = 253). Carbamate recoveries varied from 80 to 105%, with coefficients of variation (CVs) that compared favorably to the intralaboratory Horwitz CV. Detection limits of less than 0.01 ppm are obtainable with the described method.

Many agricultural regulatory agencies are faced with the problem of analyzing a large number of samples for a wide variety of pesticides. Typically, the classes of compounds most often analyzed by these agencies include the organochlorine, organophosphorus, organonitrogen, and a class of nonpersistent systemic insecticides, known as the *N*-methylcarbamates. A multiresidue method based on an acetone extraction was described by Luke et al. (1, 2) and later collaboratively studied by Sawyer (3). This method, known as the "Luke method," became a widely accepted standard multiresidue method for analyzing chlorinated, phosphated, and nitrogen pesticide residues in agricultural products (4, 5).

Methods for the analysis of *N*-methylcarbamate insecticides by gas–liquid chromatography (GLC) proved to be problematic because of their heat-labile character. A multiresidue method, based on Luke's procedures, for determining the parent phenyl *N*-methylcarbamate insecticides (i.e., carbofuran, carbaryl) on crops was introduced (6). The method required derivatization of the parent *N*-methylcarbamate to a 2,4-dinitrophenyl ether that, subsequently, was separated by GLC and detected by electron capture. Unfortunately, the carbamoyl oximes (i.e., oxamyl, methomyl) could not be determined by this method and required special procedural considerations that included liquid chromatographic (LC) analysis (7).

Moye et al. (8) were the first to adapt an LC, fluorogenic labeling technique to the detection of *N*-methylcarbamate insecticides. Their technique involved a reversed-phase separation followed by a postcolumn base hydrolysis that liberated methylamine, which further reacted with *o*-phthalaldehyde (OPA)–mercaptoethanol to form a highly fluorescent isoindole. The chromatography (9, 10), the recovery, and the sample preparation were further defined by Krause (11) and Krause and August (12). This work was collaboratively studied (13, 14) and is widely used by regulatory agencies (15). Muth and Erro later improved and simplified cleanup by substituting an octadecyl (C_{18}) bonded silica Sep-Pak SPE column for Krause's charcoal-silanized Celite column (16). At present, our laboratory uses a modification of the method described by Muth and Erro (16).

Other investigators (17, 18) attempted to expand the present multiresidue methods to include *N*-methylcarbamates. These investigators were interested in combining Luke's multiresidue extraction and partition procedures (1, 2) with the solid-phase extraction (SPE) cleanup and postcolumn determinative procedures described by Krause (9, 10). The methods developed from their work are valuable, because they have eliminated the need for a separate extraction procedure and expanded the util-

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ity of Luke's multiresidue method to include the carbamate pesticides. Unfortunately, these methods require steps that include specific apparatus such as a charcoal–Celite cleanup column and a vacuum evaporator, and additional Florisil cleanup steps that proved to be very labor-intensive and do not offer satisfactory cleanup of some vegetable or fruit crops (17).

An in-depth look at carbamate cleanup methods was described by de Kok et al. (19). They investigated the patterns of carbamate elution and matrix interferences of some agricultural crops using a variety of SPE bonded silica columns in the reversed- and normal-phase modes. A significant finding was that matrix compounds that typically coextracted with the C_{18} cleanup method were greatly reduced when the normal-phase mode was used. de Kok et al. (19) showed that the best cleanup was with an aminopropyl-bonded silica cartridge. Similar work in animal organ tissues (20) further supported the effectiveness of normal-phase mode cleanup in the analysis of carbamate pesticides with Luke's procedures.

The proposed method successfully uses Luke's extraction and partition procedures (1, 2) in combination with a normal-phase aminopropyl-bonded silica SPE column cleanup and LC postcolumn fluorogenic determination. It allows for the simultaneous analysis of 8 carbamate insecticides in vegetable, fruit, and feed crops with a small aliquot of the partitioned sample. With the remaining volume of partitioned sample extract, the organochlorine, organophosphate, and organonitrogen compounds can be determined.

METHOD

Reagents

(a) *Solvents.*—Methanol, methylene chloride, and hexane; all Optima grade or equivalent pesticide residue grade (Fisher Scientific Co., Orlando, FL 32809).

(b) *LC acetonitrile.*—UV grade (Fisher Scientific Co.). Before use, filter LC acetonitrile through 0.45 μm membrane and degas by helium sparge continuously throughout the day.

(c) *LC water.*—Prepare using NANOpure II water purification system (Barnstead, Newton, MA 02159). Before use, degas water by helium sparge as described for LC acetonitrile.

(d) *Eluting solution 2%.*—Prepare using Optima grade methanol and methylene chloride by pipetting 20 mL methanol into 1 L volumetric flask with methylene chloride. (**Note**: Composition of this solution is critical to the method.)

(e) *Reconstituting solution 5%.*—Prepare using Optima grade methylene chloride and hexane by diluting 50 mL methylene chloride to 1 L with hexane. (**Note**: Composition of this solution is critical to the method.)

(f) *NaOH solution 0.05N.*—Dissolve 80 g NaOH pellets in 200 mL LC water and mix. Pipet 5 mL 10N NaOH into 1 L volumetric flask. Dilute to 1 L with LC water.

(g) *Borate buffer solution 0.2M.*—Weigh 24.8 g ACS grade boric acid granular (H_3BO_3; Catalog No. A73-3, Fisher Scientific Co., or equivalent), and dissolve in ca 1800 mL LC water with aid of magnetic stirrer. Adjust pH to 10.4 with 10N NaOH aqueous solution, and dilute to volume with LC water.

(h) *Stock OPA solution.*—Add 5 g OPA [C_6H_4–1,2$(CHO)_2$; Catalog No. O4241-5, Fisher Scientific Co., or equivalent] into 100 mL volumetric flask, and dilute to volume with methanol.

(i) *Working OPA solution.*—Add 1975 mL 0.2M borate buffer solution into 2 L volumetric flask, and add 10 mL stock OPA solution. Dilute to volume. Store unused portion in refrigerator. Before use, filter appropriate volume of working OPA solution through aqueous 0.45 μm membrane, and direct addition of 2-mercaptoethanol ($HSCH_2CH_2OH$; Catalog No. O3446I-100, Fisher Scientific Co.) to achieve final concentration of 80μL/100 mL working OPA solution.

(j) *Feed extraction solution.*—Add 350 mL acetone into 1 L volumetric flask, and dilute to volume with LC water.

(k) *Na_2SO_4.*—Anhydrous granular; ACS certified (Fisher Scientific Co.)

(l) *Carbamate standards.*—Aldicarb sulfoxide, oxamyl, methomyl, 3-hydroxycarbofuran, propoxur, carbofuran, carbaryl, and methiocarb; U.S. Environmental Protection Agency (EPA)/U.S. Food and Drug Administration (FDA) Reference Standards (EPA, Research Triangle Park, NC 27711).

Apparatus and Equipment

(a) *Blender.*—Stainless steel, explosion-proof laboratory blender with 3 L capacity for blending of samples (Catalog No. 14-509-7C, Fisher Scientific Co.).

(b) *Centrifuge tubes.*—Conical, graduated, stopper size 13, Pyrex brand glass, 13 mL capacity, 141 mm × 17 mm od (Fisher Scientific Co.), or equivalent.

(c) *Solvent filter unit.*—Glass filter apparatus, 47 mm fritted glass filter base (Catalog No. 85116, Waters Chromatography Div., Millipore Corp., Milford, MA 01757).

(d) *SPE cartridge.*—Aminopropyl Bond Elut cartridge, 500 mg sorbent mass and 3 mL volume (Analytichem International, Inc., Harbor City, CA 90710).

(e) *Evaporator.*—Any device capable of focusing stream of nitrogen gas in water bath at 40–45°C. These units can be purchased or homemade.

(f) *Sample filters.*—Acro LC 13 disposable 13 mm diameter filter assembly with 0.2 μm poly(vinylidene fluoride) (PVDF) membrane (Gelman Sciences, Inc., Laboratory/Diagnostics, Ann Arbor, MI 48106), or equivalent.

(g) *pH meter.*—Fisher Acumet, Model 420 digital pH/ion meter (Fisher Scientific Co.), or equivalent.

(h) *Graduated cylinders.*—250 mL graduated to deliver, stopper size 19 (Catalog No. 08-566-10E, Fisher Scientific Co.).

(i) *Membrane filters.*— 0.45 μm, 47 mm diameter, PVDF (Catalog No. N04SP04700, Micron Separations, Inc. [ordered from Fisher Scientific Co.]), used for organic solvents; and Supor-450, 0.45 μm, 47 mm diameter (Product No. 60173, Gelman Sciences, Inc.), used for aqueous solutions.

(j) *Separatory tumbler rack.*—Any device capable of handling twelve 1000 mL separatory funnels, certified safe with flammable solvents (Gulf Technical Industries, Tallahassee, FL 32301). (**Note**: No differences were observed when manual shake out of sample was performed. In addition, use of separatory tumbler rack will save significant time in preparation.)

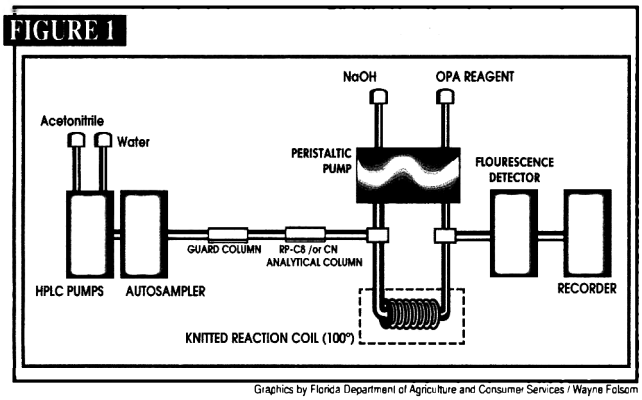


Figure 1. LC postcolumn fluorometric system.

(**k**) *SPE vacuum chamber.*—Any vacuum chamber that is designed to withstand negative pressure of 25–30 psi and has capacity to extract 12 SPE cartridges simultaneously (Catalog No. 9400 DK, Baxter Health Care Corp. Scientific Products Division, McGaw Park, IL 60085-6787).

(**l**) *Buchner filter funnel.*—150 mL glass filter funnel with course fritted filter bottom (Part No. 10-358M, Fisher Scientific Co.).

(**m**) *Graduated cylinder, 50 mL.*—Calibrated to deliver (Part No. 08-562A, Fisher Scientific Co.).

LC Apparatus (See Figure 1)

(**a**) *Mobile-phase delivery system.*—Vista Model 5500 LC programmable gradient pump with column oven, (Varian Instrument Group, Sunnyvale, CA 94089).

(**b**) *Injector.*—Model 8085 automatic sampler with 10 µL injection loop (Varian Instrument Group).

(**c**) *Guard column.*—Guard-PAK guard column holder (Part No. 88141, Waters Chromatography Div.), fitted with Guard-PAK C_{18} or cyanopropyl precolumn inserts (Part No. 88070 and 26750, respectively, Waters Chromatography Div.).

(**d**) *Analytical column.*—25 cm × 4.6 mm id, containing 5 µm Zorbax C_8 spherical particles; or 25 cm × 4.6 mm id, containing 5 µm Zorbax CN spherical particles (MAC-MOD Analytical, Chadds Ford, PA 19317).

(**e**) *Carbamate hydrolysis chamber.*—Unit consists of hot plate/stirrer (Model PC-351, Corning) with temperature controller (Fisher Scientific Co.) and water bath made from 2 L stainless steel Bain Marie food container (American Restaurant Supplies, Tallahassee, FL 32310). Drill two 1/4 in. holes into container lid, and insert each end of reaction coil (**i**) through hole and fit with 0.02 in. id PEEK compression screw (Part No. P-200X, Upchurch Scientific, Inc., Oak Harbor, WA 98277-9986) so that coil hangs 3–4 in. from bottom of food container when lid is in place. Systemically connect each end of reaction coil into LC system using 0.021 in. id PEEK "Tee's" (Part No. P-712, Upchurch Scientific Inc.).

(**f**) *Fluorescence detector.*—Kratos/Schoeffel FS-970 fluorescence detector with 10 µL cell; excitation wavelength set at 340 nm, emission wavelength filtered at 455 nm with Schoeffel KV 418 filter (Applied Biosystems, Inc., Foster City, CA 94404).

(**g**) *Data system.*—Vista CDS 402 integrator, printer/plotter (Varian Instrument Group).

(**h**) *Reaction reagent delivery system.*—Gilson Minipuls 2 rabbit, peristaltic pump (Rainin Instrument Co. Inc., Woburn, MA 01801) fitted with Fisherbrand Accu-rated pump tubes; flow rate, 0.10 cc/min (Catalog No. 14-190-73, Fisher Scientific Co.). Set delivery speed to 0.5 cc/min. Place inlet of NaOH solution pump tube into reagent reservoir, and connect outlet of NaOH solution pump tube to first in-line PEEK tee of carbamate reaction chamber (**e**) with ca 10 in. connecting tubing (**j**). Similarly, place inlet of working OPA solution into reagent reservoir, and connect outlet to second in-line PEEK tee with 10 in. connecting tubing. (**Caution**: Prime reaction reagent lines with water before beginning delivery of mobile phase to prevent NaOH solution backflushing into end of analytical column.)

(**i**) *Reaction coil.*—10 ft knitted 1/16 × 0.021 in. id, high-pressure Teflon tubing (24). (**Note**: Reaction coils may be purchased, pre-knitted, through Supelco, Inc., Bellefonte, PA 16823-0048; Part No. 5-9206M, or hand knitted with aid of Boy Scout "Knitting Knobby," available through any local official Boy Scout supplies dealer.)

(**j**) *Connecting tubing.*—Use 1/16 × 0.021 in. id, high-pressure Teflon tubing to connect NaOH and working OPA solution reaction reagent pump lines to PEEK tee's. Use 1/16 × 0.01 in. id, high-pressure Teflon tubing to connect analytical column outlet to first in-line PEEK tee and to connect second in-line PEEK tee to detector. (**Note**: Length of tubing connecting column to carbamate hydrolysis chamber [**e**] should be no greater than 10 in. Optimal length of tubing to connect second in-line tee to detector is 27 in.) Use 1/16 × 0.01 in. id PEEK tubing to connect injector, guard column, and analytical column inlet.

(**k**) *Tubing connectors.*—0.02 in. id "nipple connectors" for linking peristaltic tubing and high-pressure Teflon tubing in reaction reagent delivery system (**h**) (Alpkem Corp., Clackamas, OR 97015).

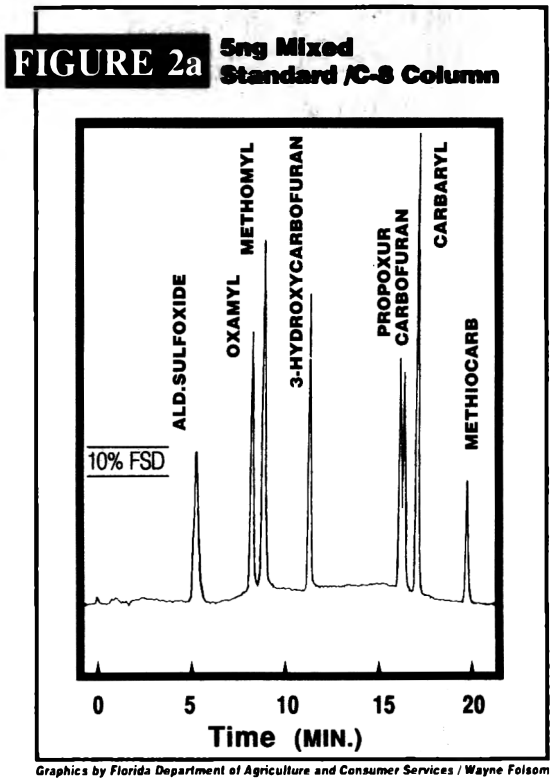
LC Standard Solutions

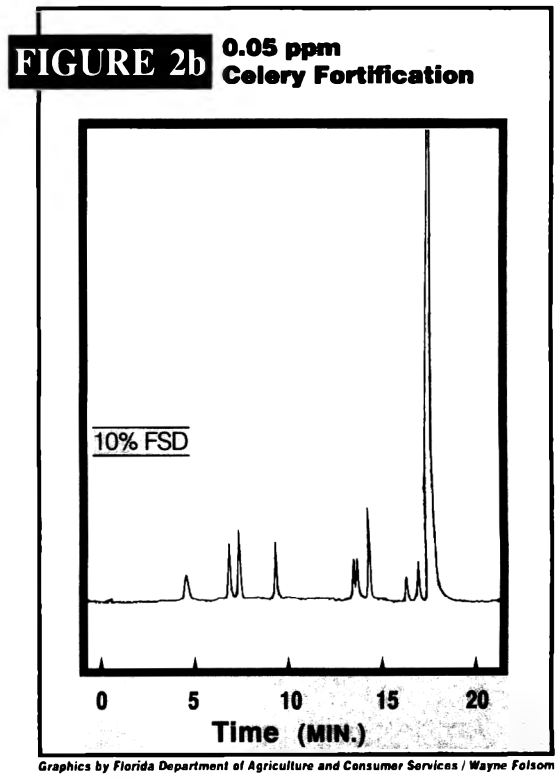
(**a**) *Stock solutions (1000 ng/µL).*—Weigh 100 mg reference standard into 100 mL volumetric flask. Dissolve standard and dilute to volume with methanol. Stock reference standards are stored at 0°C. Stock standards are made biannually.

(**b**) *Working solutions (10 ng/µL, 1 ng/µL).*—Dilute 0.5 mL stock standard to 50 mL with LC grade methanol to make 10 ng/µL spiking standard. Dilute 5.0 mL 10 ng/µL solution to 50 mL with LC grade methanol to make 1 ng/µL standard. Store at 4°C, and replace as new stock solutions are made.

(**c**) *Working mix standard (0.5 ng/µL).*—Pipet 0.5 mL each of stock standard solutions into 1000 mL volumetric flask, and dilute to volume. Split solution into 6 equal volumes, and store unused portions at 4°C. Replace expired solutions monthly.

(**d**) *Mix spiking solution (25 ng/µL).*—Pipet 5 mL each of stock standard solutions into 200 mL volumetric flask, and di-





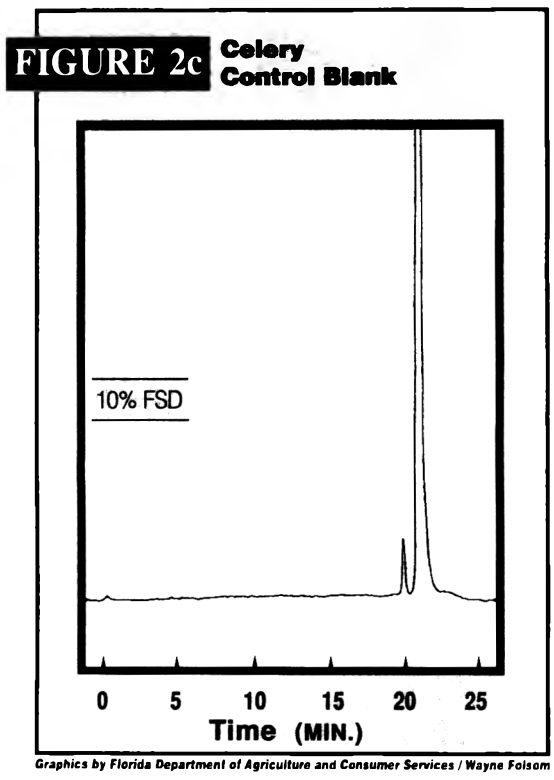


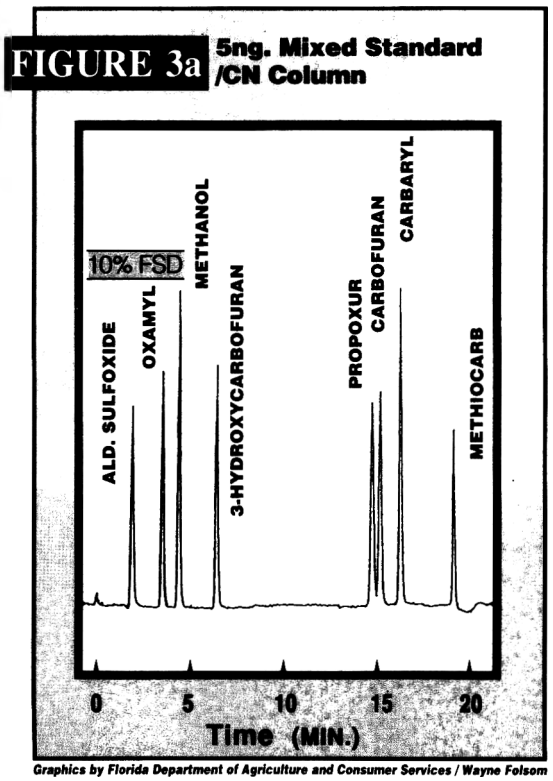
Figure 2. (a) Carbamate mixed standard profile for C_8 column; (b) 0.05 ppm level celery fortification recovery profile; and (c) celery control blank. Postcolumn OPA fluorescence, excitation at 340 nm and emission at 455 nm.

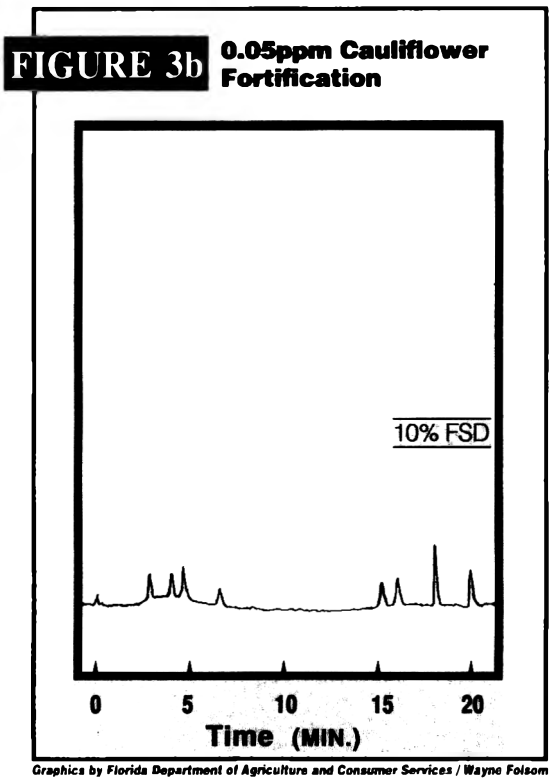
lute to volume with methanol. Remake biannually, or as necessary if exhausted before scheduled date. Store at 4°C.

LC Operating Conditions

Adjust binary pump mobile-phase flow to 1.50 ± 0.02 mL/min at 10% acetonitrile in water. Adjust flow of both NaOH and working OPA solutions to 0.1 ± 0.02 mL/min. Maintain carbamate hydrolysis chamber at 100°C and column oven at 30°C. Set fluorescence detector as follows: excitation wavelength, 340 nm; emission wavelength, 455 nm; sensitivity, high; slit width, 10; range, 1; and time constant, 2. Adjust integrator sensitivity so that 5 ng carbofuran gives $50 \pm 0.5\%$ full-scale response on Vista 402 printer/plotter set at attenuation of 8. Baseline noise should be <2% (usually, baseline noise is approximately ≥1 mm width).

Let system fitted with C_8 analytical column equilibrate at 10% acetonitrile in water for 8 min, and inject sample; begin 18 min linear gradient to 70% acetonitrile in water, and finish with 1 min linear gradient to 10% acetonitrile in water. When using CN analytical column, equilibrate system for 6 min at 15% acetonitrile in water, inject sample, and begin gradient. Maintain 15% acetonitrile in water for 8 min, increase to 60% acetonitrile in water linearly in 10 min, and then finish with 1 min linear gradient to 15% acetonitrile in water. (**Note**: We recommend conditioning LC system by running 2 successive gradients before injection and calibration of working standard.) Inject 10 µL 0.5 ng/µL concentration of working mix standard, and immediately initiate gradient as described above. On completion of standard run, check chromatography by noting resolution between propoxur and carbofuran. If resolution is less





than 0.8 (on Zorbax C_8), change guard column. If resolution has not improved, column should be changed. Mobile-phase solvents are degassed by continuous helium sparge.

Carbamate Retention Times

Retention times (min) of 8 carbamates, using above conditions for C_8 column, are as follows: aldicarb sulfoxide, 6.1; oxamyl, 8.0; methomyl, 8.7; 3-hydroxycarbofuran, 10.8; propoxur, 15.8; carbofuran, 16.0; carbaryl, 16.8; and methiocarb, 19.2 (see Figure 2a). Retention times for cyanopropyl column are as follows: aldicarb sulfoxide, 3.5; oxamyl, 4.6; methomyl, 5.3; 3-hydroxycarbofuran, 7.0; propoxur, 14.1; carbofuran, 15.7; carbaryl, 17.5; and methiocarb, 18.6 min (see Figure 3a). Retention of compounds may vary from column to column; however, separation patterns are reproducible under method conditions stated.

Sample Extraction and Partitioning: Luke Procedures

Weigh 100 ± 0.50 g freshly chopped vegetable or fruit into stainless steel, 4-blade blender, and add 200 mL acetone. Weigh 50 ± 0.50 g previously ground feed into stainless steel, 4-blade blender, and add 200 mL feed extraction solution (35% acetone–water). Blend 3 min. Filter through large glass filter funnel containing plug of glass wool tight enough to retain crop particulate, and collect extract in pint jar. (A fluted conical filter may be substituted for glass wool; however, filtering extract is much slower using this device.) Measure 50 mL aliquot of filtered extract in 50 mL graduated cylinder, and add to 1000 mL separatory funnel. Add 3.5 g NaCl to separatory funnel, and tumble in separatory rack until salt dissolves. Add 50 mL each

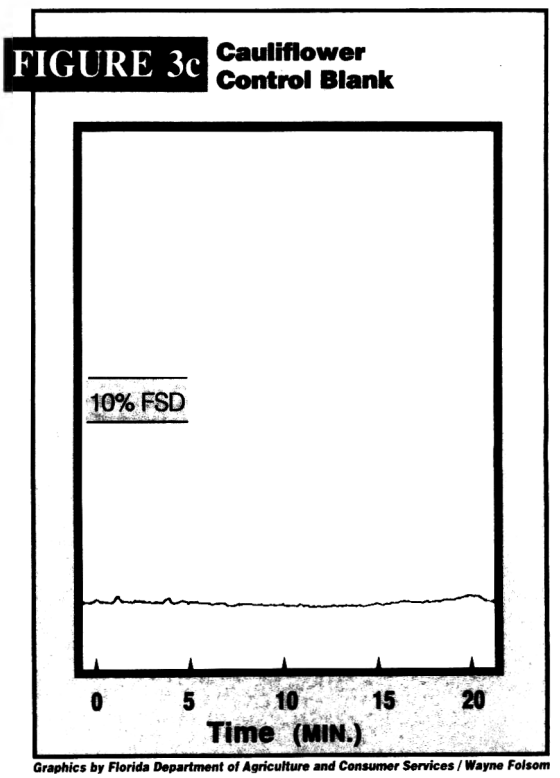


Figure 3. (a) Carbamate mixed standard profile for cyano-propyl column; (b) 0.05 ppm level cauliflower fortification recovery profile; and (c) cauliflower control blank. Postcolumn OPA fluorescence, excitation at 340 nm and emission at 455 nm.

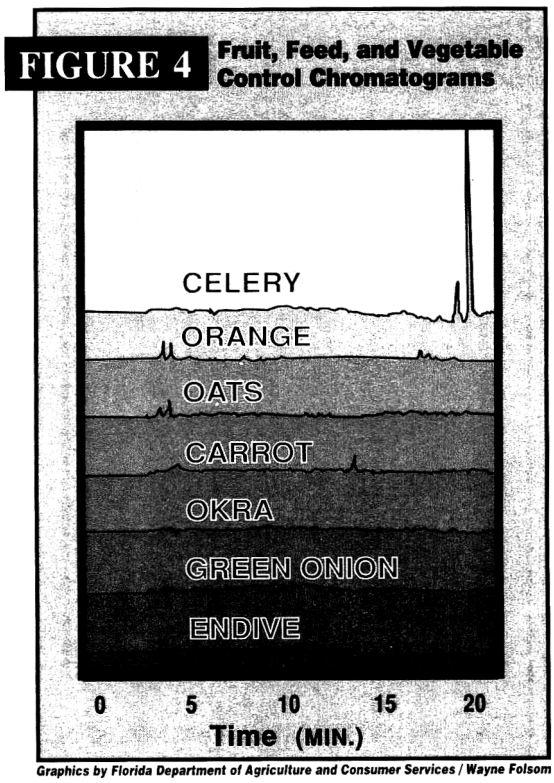


Figure 4. Chromatographic profiles of 7 crop controls. Each control chromatogram represents the equivalent of 15 mg sample injected.

Table 1. Vegetables, fruits and feeds analyzed by method

Vegetables		
Asparagus	Broccoli	Beans, green
Cabbage, chinese	Cabbage, green	Carrot
Cauliflower	Celery	Collards, green
Corn, yellow	Cucumber	Eggplant
Endive	Escarole	Lettuce, bibb
Lettuce, iceberg	Lettuce, leaf	Lettuce, romaine
Mushroom	Okra	Onion, green
Pea, green	Pea, snow	Pepper, bell
Potato	Rhubarb	Spinach
Squash, yellow	Tomato	Zucchini
Fruits		
Applesauce	Apricot	Banana
Grape, green	Grapefruit[a]	Lemon[a]
Lime	Peaches	Pear
Prune	Orange[a]	Strawberry
Feeds		
Corn/cottonseed	Oat, wild type	Sunflower seed

[a] Contain significant interferences when analyzed with rind.

of CH_2CL_2 and petroleum ether, rotate rack twice, and vent separatory funnel. Tumble mixture for 3 min, and let stand 3 min or until phases separate. Drain (lower) aqueous layer into 50 mL graduated cylinder used to measure sample extract. Collect (upper) organic phase in 400 mL beaker after passing through Buchner funnel containing bed of anhydrous granular Na_2SO_4 (ca 2.5 in. deep). Return aqueous layer to separatory funnel, and partition twice with 50 mL CH_2Cl_2, each time collecting (lower) organic phase through Na_2SO_4 Buchner funnel. Wash Na_2SO_4 with 25 mL CH_2Cl_2, stretch latex glove over Buchner funnel, and press slightly to force remaining CH_2Cl_2 out of Na_2SO_4. Do not let glove touch salt bed.

Sample Cleanup: Carbamate Analysis

Transfer combined sample extracts to 250 mL graduated cylinder. Ensure complete transfer by rinsing 400 mL beaker 2–3 times with a few milliliters of acetone. Bring final volume in graduated cylinder to 240 mL with acetone, and mix well. With 20 mL pipet, transfer aliquot of combined extracts into 25 mL disposable culture tube. Secure 20 mL sample in warm water bath (45°C), and evaporate to dryness under stream of nitrogen. Remove nitrogen stream immediately after sample is completely evaporated, and add 5 mL reconstituting solution (5% CH_2Cl_2–hexane). (**Note**: Recovery of propoxur, carbofuran, carbaryl, and methiocarb are greatly affected by extended evaporation beyond dryness.) Sonicate sample for 15 s, and then vortex-mix sample for 15 s. (**Note**: Remaining 220 mL combined sample extract may be split equally and analyzed for chlorinated hydrocarbon and organophosphate pesticides.)

Fit aminopropyl (NH_2) SPE column into manifold of SPE vacuum chamber, and attach 25 mL SPE column reservoir to top of NH_2 column. Activate NH_2 column first with 10 column volumes (ca 10 mL) eluting solution (2% methanol–CH_2Cl_2), followed by 10 column volumes of reconstituting solution. Do not let column bed dry during column activation. (**Note**: For more efficient use of time, we recommend performing this step during 20 mL aliquot sample evaporation.) Transfer reconstituted 5 mL sample (above) to SPE column. Using a Pasteur pipet, rinse the sample test tube with an additional 5 mL reconstituting solution, and transfer the volume to the SPE column reservoir to yield a final reconstituted sample volume of 10 mL. Apply light vacuum to aspirate sample through NH_2 column at ca 1.5 mL/min. Elute sample with 10 mL eluting solution, and collect eluate in 13 mL conical centrifuge tube. Evaporate eluate to dryness by using gentle stream of nitrogen, as described earlier. Reconstitute sample with 1 mL methanol. Sonicate sample for 10 s, and then vortex-mix sample for 10 s. Filter sample through 0.2 μm PVDF filter and analyze on LC system.

Analysis

Using 8085 autosampler, inject 10 μL methanol sample extracts onto LC column using LC apparatus and parameters described earlier. Use peak retention times to tentatively identify residue peaks from sample injections. Measure peak height (or peak area), and determine amount of residue by comparing height obtained from sample residues to height obtained from known amount of reference standard(s). For analytical validity,

Table 2. Recoveries (%) of 8 *N*-methylcarbamates from crop samples fortified at 0.05, 0.5, and 5.0 ppm

Compound	No. of recoveries	Mean rec. %	SD	CV, %
		0.05 ppm		
Aldicarb sulfoxide	12	97	6.7	6.8
Oxamyl	12	101	8.6	8.5
Methomyl	11	97	9.1	9.4
3-Hydroxycarbofuran	12	103	9.4	9.2
Propoxur	12	92	10.0	10.9
Carbofuran	12	93	7.1	7.6
Carbaryl	12	94	6.4	6.7
Methiocarb	12	105	17.0	16.2
		0.5 ppm		
Aldicarb sulfoxide	12	87	7.2	8.3
Oxamyl	12	93	6.3	6.8
Methomyl	12	92	8.0	8.7
3-Hydroxycarbofuran	12	96	11.5	11.9
Propoxur	12	85	7.9	9.3
Carbofuran	12	90	7.0	7.8
Carbaryl	12	91	9.1	10.0
Methiocarb	11	85	6.4	7.5
		5.0 ppm		
Aldicarb sulfoxide	12	89	8.6	9.7
Oxamyl	12	93	6.9	7.4
Methomyl	12	93	6.9	7.4
3-Hydroxycarbofuran	12	95	7.6	8.0
Propoxur	12	85	8.5	10.0
Carbofuran	12	91	9.0	9.9
Carbaryl	12	93	8.3	8.9
Methiocarb	12	88	8.3	9.4

both residue amounts and size of peaks should match within ±25% of reference standards. Include fortification of known blank crop matrix and reagent blank for simultaneous analysis of set of 10 samples. Calculate recoveries of oxamyl, methomyl, carbofuran, and carbaryl for each set of samples analyzed. Recoveries must be within ±2 standard deviations (SD) from statistical mean recovery.

Limit of Detection and Quantitation

Criteria established by Parker (26) for detection of the method limit of detection (LOD) and the limit of quantitation (LOQ) were used in this study. Briefly, LOD and LOQ are defined as 3 and 10 times background signal contributed by matrix blank at analyte retention time, respectively (26). Calculate method LODs and LOQs by comparing response (amount/height or amount/area) given by individual carbamate standard to 3 or 10 times, respectively, noise response contributed by crop control at retention time of interest. In general, detection limits are calculated on the basis of carbofuran response at 50% full-scale deflection. Individual LOD and LOQ are determined when residues have been confirmed in samples routinely screened by this method.

Results and Discussion

The determination of carbamate insecticides was accomplished using the LC postcolumn fluorometric labeling technique described by Moye et al. (8) and later evaluated and refined by Krause (9, 10). This technique provides the selectivity, resolution, and sensitivity to detect oxime and phenyl N-methylcarbamates at the nanogram level. Two types of analytical columns were used, interchangeably, with satisfactory results: a C_8 Zorbax 250 × 4.6 mm column and a cyanopropyl (CN) Zorbax 250 × 4.6 mm column. The use of 2 analytical columns provided additional information for residue peak identification by altering the capacity factors of the carbamates. The retention times observed for all carbamates except carbofuran and methiocarb differed by approximately 1.0 min. The 8 carbamates used in the study elute within 20 min on either analytical column (Figures 2a and 3a).

The method presented is simple and quick and allows a technician to prepare 15–20 samples within 1 working day. Determination of carbamate residues can then be accomplished comfortably by a chemist, within the department's 48 h reporting limit. It requires a 20 mL aliquot derived from Luke's ex-

Table 3. Method recovery (%) of carbamates at 0.05 ppm fortification

	N-methylcarbamate[a]							
Crop	ALSFX	OXA	MTH	OHCBO	PXUR	CBO	CYL	MTCB
Broccoli	94	96	102	105	97	100	97	90
Collard	102	101	99	104	91	106	95	101
Endive	91	103	101	100	93	95	97	103
Lemon	103	102	106	99	99	107	80	90
Lime	96	110	109	106	98	106	112	111
Orange	90	96	109	106	93	95	104	82
Potato	99	96	94	108	88	94	103	91
Rhubarb	91	98	94	104	89	93	97	92
Spinach	90	113	106	111	99	107	104	102
Tomato	104	114	102	100	87	95	93	103
Average	96	103	102	104	93	100	98	97
SD	5.6	7.0	5.5	3.8	4.6	6.1	8.5	8.8
Grand average	99							
Grand SD	3.8							

[a] Abreviations are as follows: ALSFX, aldicarb sulfoxide; OXA, oxamyl; MTH, methomyl; OHCBO, 3-hydroxycarbofuran; PXUR, propoxur; CBO, carbofuran; CYL, carbaryl; and MTCB, methiocarb.

traction and partition procedures (1, 2, 4–6). The method effectively eliminates the need for a separate extraction and cleanup method for carbamate analysis and allows for the determination of organochlorine and organophosphorus pesticides through the normal Luke procedures. As a result, our laboratory has realized a lower operating cost and saved a significant number of work hours previously required for carbamate analysis.

A list of vegetables, fruits, and feeds used during the development of this method is given in Table 1. These samples were obtained from growers throughout the state of Florida with no known pesticide use history. To ensure that crops were free of incurred residues before their use in this method's development, all samples used in this study were screened for carbamate residues using a modified version of the method described by Muth and Erro (16). The Muth and Erro carbamate extraction and cleanup method uses a C_{18} SPE column for the cleanup of aqueous vegetable extract. The method was modified to improve the cleanup of crop interferences. Coextractable crop interferences were minimized using a 0.025M phosphate buffer as the extraction solution. Crop coextractants were further min-

Table 4. Method recovery (%) of carbamates at 5.0 ppm fortification

	N-methylcarbamate[a]							
Crop	ALSFX	OXA	MTH	OHCBO	PXUR	CBO	CYL	MTCB
Broccoli	97	87	92	101	88	97	97	102
Collard	104	101	99	101	92	103	106	97
Endive	86	91	91	93	85	90	88	84
Lemon	82	86	86	90	78	84	101	80
Lime	85	94	95	97	90	95	94	88
Orange	90	94	92	88	77	81	95	83
Potato	87	91	87	91	80	87	85	84
Rhubarb	87	96	101	101	91	95	97	90
Spinach	84	88	89	91	83	88	84	81
Tomato	77	97	94	88	73	78	84	84
Average	88	93	93	94	84	90	93	87
SD	7.7	4.8	4.8	5.4	6.6	7.8	7.6	7.2
Grand average	90							
Grand SD	3.6							

[a] Abbreviations are as follows: ALSFX, aldicarb sulfoxide; OXA, oxamyl; MTH, methomyl; OHCBO, 3-hydroxycarbofuran; PXUR, propoxur; CBO, carbofuran; CYL, carbaryl; and MTCB, methiocarb.

Table 5. Performance of method from repeatability data of carbamates from 8 crops at 0.5 the ppm level[a]

	N-methylcarbamate							
Crop	ALSFX	OXA	MTH	OHCBO	PXUR	CBO	CYL	MTCB
Cauliflower	91	98	91	102	86	94	91	90
	7.7	10.4	7.2	15.1	11.0	12.2	10.6	9.6
	7.8	10.6	7.9	14.8	12.8	12.9	11.7	10.7
Cucumber	92	96	92	98	85	93	90	89
	5.8	4.4	3.0	4.3	7.4	6.7	8.7	5.2
	6.4	4.6	3.3	4.4	8.7	7.2	9.6	5.8
Eggplant	90[b]	93	93	101	86	93	91	89
	6.1	2.8	2.2	5.4	5.5	5.0	1.9	1.1
	6.8	3.0	2.4	5.4	6.4	5.4	2.1	1.3
Lettuce	97	101	99	100	97	99	96	92
	4.8	5.0	6.0	5.4	7.4	6.0	5.3	8.3
	4.9	4.9	6.0	5.4	7.6	6.1	5.5	8.9
Squash	88[c]	98	93	100	85	94	96	90
	0.4	5.7	6.9	6.9	6.0	5.9	4.7	11.8
	0.5	5.8	7.4	6.9	7.1	6.3	4.9	13.1
Peach	87	96	92	95	84	91	95	92
	5.3	3.9	7.4	7.8	6.5	4.1	6.3	4.8
	6.0	4.0	8.1	8.2	7.8	4.5	6.6	5.2
Pear	82	93	88	96	80	88	90	82
	10.2	7.5	6.0	9.0	5.3	7.4	2.9	5.3
	12.3	8.1	6.8	9.3	6.6	8.4	3.2	6.5
Oats/feed	93	97	90	99	85	92	91	89
	9.1	12.6	9.1	14.3	9.4	11.0	11.4	11.6
	9.8	12.9	10.1	14.4	11.0	12.0	12.6	13.1
Average	90	97	92	99	86	93	93	89
SD	4.5	2.7	3.2	2.4	4.8	3.1	2.7	3.1
Grand average	92							
Grand SD	4.2							

[a] Abreviations are as follows: ALSFX, aldicarb sulfoxide; OXA, oxamyl; MTH, methomyl; OHCBO, 3-hydroxycarbofuran; PXUR, propoxur; CBO, carbofuran; CYL, carbaryl; and MTCB, methiocarb. Data represent 4 determinations.

[b] $n = 3$ for aldicarb sulfoxide in eggplant.

[c] $n = 2$ for aldicarb sulfoxide in squash.

imized by eluting a 10 mL sample aliquot (equivalent to 1.7 g of the original sample) from the C_{18} SPE column with 10 mL anhydrous ethyl ether. The ether eluant was evaporated to dryness under a stream of anhydrous nitrogen gas and reconstituted in 1.0 mL absolute methanol. The modified Muth and Erro (16) extraction and cleanup procedure has been used successfully in our laboratory since 1986. All crops were screened before their use as controls for the method presented here.

The method's cleanup performance is best illustrated through the 7 crop control chromatograms shown in Figure 4. In our experience, the laboratory's in-house method (16) did not provide an adequate cleanup for these fruits and vegetables. However, using the method described here, matrix interferences are virtually nonexistent within the retention window of the 8 carbamates tested, between 2.5 and 20 min. The celery and carrot controls show minor matrix interferences, and these matrix peaks did not interfere with the carbamate elution patterns of either the C_8 or CN analytical columns used in this method. A celery profile showing the result of a 0.05 ppm fortification is presented in Figure 2b. In this crop profile, all 8 carbamates elute before the observed celery crop interferences. The cleanup obtained for the orange control is a dramatic improvement over that reported by Krause (11). It eliminates almost all of the naturally occurring fluorescent peaks found in the orange matrix. Similar cleanups were achieved for the other citrus fruits listed in Table 1. The okra, green onion, and endive profiles are free of any crop interferences. In addition, cauliflower profiles showing a 0.05 ppm fortification and corre-

Table 6. Analysis of incurred residues: method comparison

Matrix	Amount reported, ppm		Pesticide found
	Muth method[a]	New method	
Pears	ND[b]	0.06	Carbaryl
Eggplant	0.15	0.11	Methomyl
Tomato	0.04	0.05	Propoxur
Broccoli	ND[b]	0.05	Methomyl
Bell pepper	0.10	0.06	Methomyl
Bell pepper	0.06	0.06	Methomyl
Squash	0.02	0.10	Methomyl

[a] The Muth method was modified as follows: a 0.025M phosphate buffer replaced water as an extraction solution; a 10 mL aliquot representing approximately 1.7 g of sample was analyzed rather than the entire sample extract; elution with 10 mL anhydrous ethyl ether replaced elution with 2 mL methanol.

[b] Residues in sample were reported as not detected.

sponding control blank can be seen in Figures 3b and 3c, respectively. Using this method, the cleanup for cauliflower is improved over that reported by de Kok et al. (19). The greatest improvement experienced over de Kok's method seems to be the cleanup provided for the early eluting carbamates. The crop control profiles obtained using this method were reproducible and virtually free from coextracted interferences for all crops listed in Table 1. In each crop profile, approximately 15 mg of the crop control was injected onto the LC column.

Recovery studies were initially accomplished without regard to the type of crop tested. Vegetables such as broccoli, celery, collards, green onion, and most members of the cabbage family, as well as citrus fruits, were of particular interest because they typically contained coextractable interferences with at least one of the 8 carbamate insecticides when routinely screened in our laboratory by the Muth and Erro method (16). Fortifications at the 0.05, 0.5, and 5.0 ppm levels were studied using a random selection of the vegetables and fruits listed in Table 1. Table 2 summarizes the overall method performance at each fortification level for aldicarb sulfoxide, oxamyl, methomyl, 3-hydroxycarbofuran, propoxur, carbofuran, carbaryl, and methiocarb. Recoveries obtained for the 3 fortification levels and each of the carbamate/crop combinations invariably ranged between 85 and 105%. All compounds fortified at the 0.05 ppm level demonstrated recoveries exceeding the 90% level. The average recovery for carbamates tested at this level was 98%, with a SD of 4.8 (n = 96). Recoveries of the 8 carbamates at the 0.5 and 5.0 ppm fortification levels were at or above 85%. The average recoveries for the 0.5 and 5.0 ppm fortifications were 90 and 91%, respectively. SDs of 3.9 (n = 95) and 3.3 (n = 96) were obtained for the 0.5 and 5.0 ppm fortification levels, respectively. The coefficients of variation (CVs) were excellent for all 3 of the tested fortification levels. The highest CV was 4.9% and corresponded to the 0.05 ppm level. CVs for the 0.5 and 5.0 ppm levels were 4.3% and 3.6%, respectively. The recoveries of the individual carbamates mirror one another closely for the 0.5 and 5.0 ppm levels. The average recoveries, SDs, and CVs at these 2 levels were virtually the same. However, when comparing the recoveries of the 0.05 fortification level to the 0.5 and 5.0 ppm levels, slightly higher recoveries were obtained for the individual carbamate insecticides. Overall, CVs obtained in this experiment were excellent and complied favorably with the intralaboratory CV derived from the Horwitz curve (25). The overall average recovery for carbamates at the 3 fortification levels is 93%, with a SD of 8.4 (n = 287).

The majority of the residue determinations observed in our laboratory fell between the range of 0.05 and 5.0 ppm. This method is able to detect residues below the 0.05 ppm level; however, determinations at this level are often not reportable, because they fall below the conservative criteria our laboratory uses to determine the detection and quantitation limits (26). At the other extreme, our experience shows residue determinations at or above the 5.0 ppm level are also not common. However, any determination of a carbamate residue peak of this magnitude should be confirmed through an additional preparation because SPE column breakthrough may have occurred. (The aminopropyl SPE column load limit for the carbamates has not been determined in this study.) Therefore, fortification levels of 0.05 and 5.0 ppm were chosen to demonstrate the linear range of the Bond Elute aminopropyl SPE column used in this method. Ten crops (broccoli, collard, endive, lemon, lime, orange, potato, rhubarb, spinach, and tomato) were fortified with 8 carbamates to see what effect residue concentration would have on carbamate recoverability for specific crops. Single recovery determinations were made along with a crop control. The recovery data are given in Tables 3 and 4. Recoveries for all 8 carbamates at both the 0.05 and 5.0 ppm levels averaged 99 and 90%, with respective SDs of 6.2 and 6.5 (n = 80 for both levels). These results agree with the recoveries observed in the first experiment at similar fortification levels.

A method repeatability study was performed by a team consisting of a technician and chemist from our laboratory. The repeatability was determined by analyzing 8 crops (cauliflower, cucumber, eggplant, lettuce, squash, peach, pear, and oats/feed), in 4-fold with 8 carbamates at the 0.5 ppm level. The performance data are presented in Table 5. With no exceptions, the overall average pesticide recovery and SD for this study were 92% and 7.1 (n = 253), respectively. The range of recoveries was between 80 and 102%. CVs for all carbamate/crop combinations fell within the Horwitz intralaboratory CV for an analyte at this level (25, 26).

An additional study comparing this new method and the one previously used in our laboratory (16) indicated favorable results. When incurred residues were detected in crops screened by the Muth and Erro method (16), the samples were analyzed simultaneously by both of the methods. Table 6 compares results that were obtained from the simultaneous analyses of samples previously determined to contain various incurred carbamate residues. In general, the detections observed between each method agree very closely with one another. Only 2 samples, pears and broccoli, were reported as none detected (ND) using the Muth and Erro method (16). Although these samples contained confirmed residues, the determined values did not meet the laboratory's criteria for reporting determinations

above LOQ (26). However, reportable values were obtained using the new method. The improved cleanup and the improved sensitivity have made it possible to meet the criteria necessary to report residues in these crop samples. In fact, this method cleanup has allowed for an increase in sensitivity that was not realized initially. A 1.5 g sample equivalent (20 mL aliquot) yields a detection limit consistently less than 10 ppb, based on carbofuran at 50% full-scale deflection. With the method previously used in our laboratory (16), a similar gram sample equivalent gave detection limits ≥10 ppb. Moreover, we have experienced success in using an excitation wavelength of 232 nm and an emission wavelength of 434 nm for the determination of carbamates in a variety of crops. Use of 232 nm for excitation was not possible with the prior method used in our laboratory (16), because it resulted in an excessively noisy baseline and the detection of more crop interferences. At present, not enough data have been obtained to substantiate a further increase in sensitivity by using the alternative wavelengths. Use of these higher energy wavelengths for excitation and emission have not yet been adopted for routine analysis in our laboratory.

Conclusion

The method successfully combines the extraction and partition procedures described by Luke et al. (1, 2) with SPE principles to further cleanup crop sample matrixes for subsequent LC analysis by postcolumn fluorometric detection. The carbamate analysis is simple and improves the cleanup for a variety of vegetables, fruits, and feeds. It is capable of detecting carbamates in a range between 5 ppm and 5 ppb with an amount of sample extract equivalent to about 1.5 g of the original sample. The method offers excellent quantitative recovery of 8 carbamate insecticides in a wide variety of agricultural products and still maintains its utility in analyzing for organochlorine, organophosphorus, and organonitrogen pesticides by gas–liquid chromatography.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Trace-Level Quantitation of Sulfonylurea Herbicides in Natural Water

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A novel gas chromatographic/electron capture detection (GC/ECD) technique is described for quantitation of trace sulfonylurea herbicide residues in natural water. In this method, the parent sulfonylurea herbicides are isolated from their aryl sulfonamide metabolites by Florisil chromatography, and the acidic aqueous hydrolysis products of the parent compounds are then quantitated. The method has been validated for both metsulfuron methyl (MET; methyl 2-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl] aminosulfonyl} benzoate), and chlorsulfuron [CHLOR; 2-chloro-*N*-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-aminocarbonyl benzenesulfonamide]. Acidic hydrolysis of the isolated parent compounds MET and CHLOR produced a high yield of the corresponding aryl sulfonamides, methyl 2-(aminosulfonyl)benzoate and 2-chlorobenzenesulfonamide, as confirmed by mass spectral analysis. In-house validation studies confirmed excellent chromatographic behavior, high recovery efficiency (>80%), and generally good precision (<12% coefficient of variation) for quantitation of MET and CHLOR in fortified natural water samples over a wide range of concentrations (50 ppb, 500 ppt, and 50 ppt). Validated limits of quantitation were 50 ppt, with minimum limits of detection conservatively estimated as 10 ppt.

Metsulfuron methyl (MET; methyl 2-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl] aminosulfonyl} benzoate) and chlorsulfuron [CHLOR; 2-chloro-*N*-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-aminocarbonyl benzenesulfonamide] (Figure 1) are members of the sulfonylurea class of herbicides, manufactured and marketed by E.I. du Pont de Nemours, Inc. (1). MET and CHLOR are registered for use on cereals and non-cropland, and they are applied in the 1.8–126 g a.i./ha range, depending upon the specific use pattern (1). Such low rates of applied active ingredient reflect the potent phytotoxicity of sulfonylureas relative to other classes of synthetic herbicides. Because of this broad spectrum of herbicidal activity (2, 3), MET has been investigated as a potential forestry herbicide, primarily for site preparation treatments. This fact, together with a paucity of information pertaining to the fate of MET in Canadian forest environments, prompted the initiation of a series of experiments designed to address gaps in relevant data about its environmental fate.

Before these studies were begun, a review of the scientific literature revealed that a variety of analytical methods have been developed for sulfonylurea herbicides: liquid chromatography/mass spectrometry (LC/MS) (4–7), LC using photoconductivity detection (LC/PCD) (8–12), enzyme-linked immunosorbent assay (13), and bioassay techniques (14–20). Hershberger (21) described adaptation of the basic LC/PCD technique for determination of MET. Although effective for routine analysis at the part-per-billion level, the LC/PCD technique is characterized by poor resolution and insufficient sensitivity for quantitation of trace residues (<100 parts per trillion [ppt]) in environmental substrates. In addition, the photoconductivity detector used in this procedure is not widely available and must be specifically modified for use in sulfonylurea herbicide analysis. A variety of bioassay techniques capable of detecting sulfonylurea residues in the low part-per-trillion range are available; however, these techniques are semiquantitative, nonconfirmatory, and constrained by a limited response range and the long time required (several weeks in some cases) to conduct the analyses (1). LC/MS (4–7) and immunoassay (13) methods, which have been reported for CHLOR and other sulfonylurea herbicides, are either not sensitive enough or not yet widely available for routine residue analysis in environmental matrixes.

Highly sensitive techniques for routine trace determination of sulfonylurea compounds, particularly MET, in environmental matrixes is essential, given the extremely low use rates for these herbicides and their documented phytotoxic activity at such low environmental concentrations (1). The requirement for trace level determination is complicated by the thermal lability and reactivity of the polar bridge nitrogen groups characteristic of sulfonylurea herbicides, which generally prohibit the use of sensitive gas chromatographic (GC)-based methods for quantitation of the intact compounds (1). Our initial investigations, which focused on MET, have confirmed the inability to chromatograph this compound intact by either standard or cool on-column injection techniques. Attempts to derivatize the intact MET molecule by using diazomethane techniques similar

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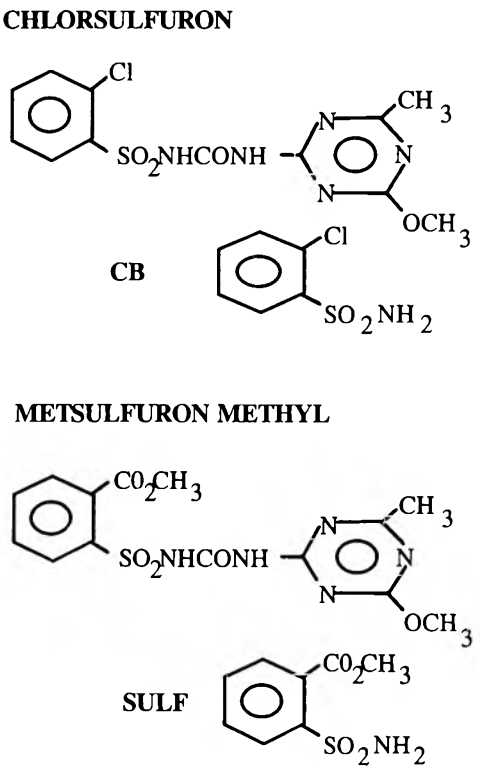

Figure 1. Chemical structure of metsulfuron methyl (MET) (MW 381), its primary degradation product methyl 2-(aminosulfonyl)benzoate (SULF) (MW 215), chlorsulfuron (CHLOR) (MW 358) and its primary degradation product 2-chlorobenzenesulfonamide (CB) (MW 191).

to those applied by Ahmad (22) and Ahmad and Crawford (23) for derivatization of CHLOR also failed. Similarly, a variety of other derivatization reagents and techniques (perfluoroacylation, BF_3 methylation, and silylization) were investigated but were unsuccessful in producing a single analyte amenable to GC/electron capture detection (ECD) in high yield.

Because many of these reactions were successful for the model compound tolbutamide in concurrent studies, we postulated that the benzoate moiety ortho to the sulfonylurea bridge in MET (Figure 1) may have stearic or lability effects that add to the difficulty of derivatizing this compound. Long et al. (24) have similarly noted that full control over reaction conditions during derivatization of CHLOR is necessary to avoid formation of multiple derivatives or derivative degradation products, both of which may impair quantitative analyses. Long et al. (24, 25) have used the characteristic thermal lability of sulfonylurea herbicides to develop a gas chromatographic/nitrogen-phosphorus detection (GC/NPD) technique for quantitation of CHLOR residues in milk. This approach relies upon production of the volatile thermal decomposition product 2-amino-4-methoxy-6-methyl-1,3,5-triazine, and is well suited for the quantitation of part-per-billion levels of CHLOR in milk. In a similar but independent approach, we have exploited the excellent chromatographic and electron capturing characteristics observed for methyl 2-(aminosulfonyl)benzoate (SULF), the acidic hydrolysis product of MET. On the basis of this finding, we report the initial development of a GC/ECD technique for trace-level quantitation of MET in natural waters and the subsequent extrapolation of this technique for use with the sulfonylurea analogue CHLOR, based on its acidic hydrolysis product 2-chlorobenzenesulfonamide (CB).

Experimental

Materials and Methods

(**a**) *Gas chromatograph.*—Modified Hot On-column Injection System, Varian Vista Model 6000 converted for on-column injection and equipped with ^{63}Ni electron capture detector; megabore DB-17 column (15 m × 0.53 μm id; 1.0 μm film) (J&W Scientific, Inc., Rancho Cordova, CA 95670); Varian model 8000 autosampler and Model 604 data system. Operating conditions: injection port, 250°C; column temperature program, 100°C for 1 min to 220°C for 3 min at 20°C/min, followed by second ramp to 250°C for 4 min at 30°C. Nitrogen as carrier gas and makeup gas (7.5 + 22.5 mL/min).

(**b**) *Gas chromatograph/mass spectrometer.*—Hewlett-Packard Model HP5995, in full-scan mode with electron impact ionization; electron energy, 70 eV; ion source temperature, 180°C; analyzer temperature, 180°C; mass range, 40–400; scan rate, 690 amu/s; transfer line temperature, 280°C; perfluorotributylamine used for calibration and tuning. Operating conditions for gas chromatograph interfaced to mass spectrometer: injection port, 210°C in splitless mode; oven temperature, 100°C (1 min) to 220°C at 16°C/min; DB-5 column, 30 m × 0.2 mm id, 0.33 μm film; carrier gas, helium at flow rate of 1 mL/min.

(**c**) *Dry bath.*—Fisher Brand modular heater (Fisher Scientific Co., Pittsburgh, PA 15219).

(**d**) *Sonicator.*—Branson Model B-92-H ultrasonic bath (Branson Ultrasonics Corp., Danbury, CT 06810-1961).

(**e**) *Rotary evaporator.*—Buchi RE20 rotary evaporator (Buchi Co. Flawil, Switzerland).

(**f**) *Analytical standards.*—MET and CHLOR (both 99.9% purity, supplied courtesy of E.I. du Pont de Nemours and Co. (Wilmington, DE 19898). SULF, 99.5% purity (Aldrich Chemical Co., Milwaukee, WI 53201).

(**g**) *Solvents.*—Optima grade methylene chloride, acetonitrile, ethyl acetate, and acetone (Fisher Scientific Co.).

(**h**) *Reagents.*—Acetic acid (CH_3COOH), 5% solution in acetonitrile; aqueous sulfuric acid (H_2SO_4) 5% and 0.1%; Florisil (60–100 mesh), fully activated (Fisher Scientific Co.) and maintained under continuous heat (120°C) until immediately before use.

(**i**) *Natural water.*—Water samples collected from Brook Lake and Greenwater Lake, near Sault Ste. Marie, ON, Canada, and stored at 2–5°C before use. Characteristics of these samples are provided in Table 1.

(**j**) *[^{14}C]MET.*—[^{14}C]MET (DPX T6376; ref. #0173) labeled in the phenyl ring (14.43 μCi, 11.70 mg) was supplied courtesy of E.I. du Pont de Nemours and Co. and used as tracer

Table 1. Greenwater Lake and Brook Lake water characteristics[a]

Characteristic	Greenwater Lake	Brook Lake
pH	6.7	6.0
Alkalinity, ppm Mg equiv.	1.85	0.32
Conductivity, μmhos	32.0	21.8
Ca, ppm	3.15	2.75
Mg, ppm	0.74	0.57
Na, ppm	0.90	0.68
K, ppm	0.58	0.31
Cl, ppm	0.48	0.14
SO_4, ppm	3.75	4.65
NH_3, ppm as N	340.00	9.84
NO_3/NO_2, ppm as N	0.02	0.022
Total organic carbon, ppm	2.78	6.76
Total inorganic carbon, ppm	2.55	0.66
SiO_2, ppm	4.24	1.09

[a] Values are average of 2 samples obtained concurrently.

in monitoring extraction and column chromatography efficiencies.

Extraction and Concentration

After titration of the sample to pH 3.0 with 5% sulfuric acid, CHLOR, MET, and their respective sulfonamides, CB and SULF, were extracted by single liquid–liquid partition from natural water (500 mL) into 100 mL methylene chloride. Emulsions formed during liquid–liquid partition were broken by placing entire separatory funnel in sonication bath for ca 3 min. Extract was concentrated to ca 5 mL by rotary evaporation in Buchi rotary evaporator with water bath at 65°C. Methylene chloride concentrate was transferred to 35 mL centrifuge tube, and round-bottom flask was rinsed with three 5 mL portions of ethyl acetate, which were added to centrifuge tube. Combined methylene chloride–ethyl acetate mixture was concentrated to ca 10 mL by evaporation under stream of pure nitrogen.

Column Chromatography

Concentrated samples were loaded onto Florisil minicolumns (1 g Florisil in glass-wool-plugged disposable Pasteur pipets, 14.5 cm × 7.5 mm id) preconditioned with 7 mL methylene chloride. SULF and CB, which were essentially unretained on columns, were eluted in load volume and additional 5 mL 20% acetone in ethyl acetate. Subsequently, MET and CHLOR were eluted with 5 mL 5% acetic acid in acetonitrile. Fractions containing SULF and CB were evaporated to dryness and recovered in ethyl acetate (≥5 mL) for GC/ECD analysis.

Hydrolytic Conversion

After column chromatographic separation of MET and CHLOR from respective aryl sulfonamides potentially formed via natural environmental degradation, residues of intact, parent herbicides were converted to SULF and CB by acidic, aqueous hydrolysis. Eluates from Florisil column containing MET and CHLOR were collected in 35 mL centrifuge tubes, evaporated to ca 1 mL, and diluted to 5 mL with distilled, deionized water. Sample pH was adjusted to 3.0 by addition of 0.1% sulfuric acid (Brook Lake water extract required ca 0.1 mL). Centrifuge tube was tightly sealed and heated 1 h at 90°C. After hydrolytic conversion, SULF and CB formed in reaction were partitioned into 10 mL ethyl acetate directly in 35 mL tube. Upper organic phase was quantitatively transferred to 15 mL graduated centrifuge tube, evaporated to dryness, reconstituted in exact volume of ethyl acetate (typically 10 mL), and mixed thoroughly before quantitation by GC/ECD.

GC/ECD Quantitation

Under operating conditions noted above, both primary environmental degradation product and acidic hydrolysis product of parent herbicides MET and CHLOR were chromatographed by hot, on-column injection to a 15 m DB-17 megabore column. MET was quantitated by using authentic SULF external standard, 1-point calibration curve, and electronic integration of sample peak area. CHLOR was similarly quantitated against either external standard (authentic SULF or CB prepared by acid hydrolysis). Parent herbicide concentrations in fortified water samples were back-calculated, using conversion factor to correct for respective molecular weight ratios of parent herbicide to analyte (MET:SULF, 381:215 = 1.77; CHLOR:CB, 358:191 = 1.87). When SULF was used as external standard to quantitate CHLOR, a similar molecular weight ratio was applied (CHLOR:SULF, 358:215 = 1.67). All samples were diluted to levels within the predetermined linear range of the detector.

Results and Discussion

Extraction and Concentration

On the basis of ^{14}C-radiotracer experiments and "cold" techniques, extraction efficiencies for both parent herbicides and aryl sulfonamide metabolites were >90% with a single-step extraction. Titration of the sample to pH 3.0, slightly below pK_a 3.3 and 3.6 of the parent compounds, MET and CHLOR, respectively, was essential for high recovery efficiency. Recovery of MET after rotary evaporation was significantly enhanced by repetitive washing with ethyl acetate rather than methylene chloride. Evaporating to a small volume (about 5 mL) rather than to complete dryness also improved recoveries. These results suggest that sulfonylureas may sorb to glass surfaces, which results in poor or variable recovery if preventive measures and appropriate solvents are not used.

Column Chromatography

A column chromatographic method for separation and elution of SULF and MET was identified after extensive investigation of the effects of various parameters, including sorbent type (Florisil, alumina, and silica), bed size, elution solvent mixtures and volumes, preacidification of sample, and column

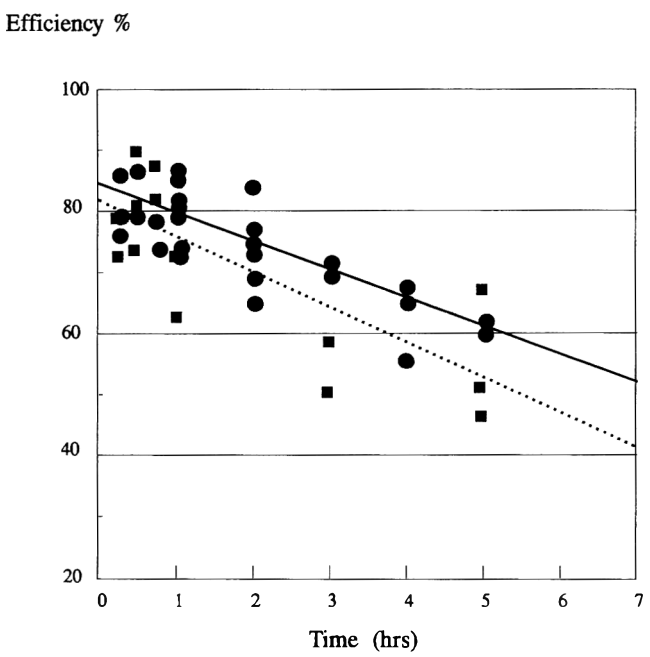

Figure 2. Effect of reaction time and pH on aqueous hydrolysis of metsulfuron methyl at 90°C: –•–, pH = 3; ···■···, pH 4.

conditioning, on the elution patterns of the 2 compounds. Sample pH was critical for effective separation and elution from the Florisil column. For our water type, no further acidification was required, because the pH of the concentrated extract was within the required range of pH 3–4. If the method is to be applied to other water types, the sample pH may have to be verified before the sample is loaded on the column and the elution pattern is verified. Postcolumn sample handling was also observed to be critical. When the eluate (5% acetic acid in acetonitrile) was evaporated to complete dryness, a white precipitate formed that substantially impaired subsequent hydrolysis, presumably because of irreversible sorption of MET within the precipitate. Under these conditions, the parent herbicides MET and CHLOR were completely separated from their respective aryl sulfonamide degradation products, and recoveries of all compounds (MET, CHLOR, SULF, and CB) from the Florisil column were essentially quantitative.

Hydrolytic Conversion

Sabadie (26) described the role of pH, temperature, various salts, and humic acids as variables affecting chemical hydrolysis of MET. Our extensive studies on the hydrolysis reaction support the general conclusions drawn from this comprehensive study: that decreasing pH and increasing temperature significantly enhance the rate of reaction. More importantly, Sabadie (26) also reported 2 hydrolytic pathways for MET in acidic aqueous solution. The first pathway involves hydrolysis of the sulfonylurea bridge, and the second involves hydrolysis of the methoxyl substituent on the heterocyclic portion of the molecule. A total of 4 hydrolytic products (2 heterocyclic compounds, SULF, and a substituted sulfonamide) may be formed in varying proportions depending on the reaction pathway. Although pH did not influence the selectivity of the hydrolytic

Table 2. Mass spectral data for acidic aqueous hydrolysis products of metsulfuron methyl and chlorsulfuron

Metsulfuron methyl		
	Relative abundance, %	
Major ion	SULF std	Hydrolysis product
m/z 215 (M^+)	12.5	12.5
m/z 199 (M^+–NH_2)	100.0	100.0
m/z 184 (199–CH_3)	78.4	71.4
m/z 135 (199–SO_2)	38.6	37.5
m/z 120 (135–CH_3)	36.4	32.9
Chlorsulfuron		
Major ion	CB[a]	Hydrolysis product
m/z 191 (M^+)	43	48
m/z 175 (M^+–NH_2)	23	19
m/z 156 (M^+–Cl)	9	5
m/z 127 (M^+–$SONH_2$)	47	38
m/z 128 (M^+–$SONH_2$+1(H))	69	41
m/z 111 (M^+–SO_2NH_2)	100	100
m/z 92 (127–Cl)	15	14

[a] Relative abundances for the same major ions as reported by Long et al. (25) for 2-chlorobenzenesulfonamide.

pathway, increasing temperature favored cleavage of the sulfonylurea bridge to form SULF in high proportion. Our initial studies indicated that high temperatures (90°C) and low pH (3.0–4.0) resulted in optimal production of the SULF product. Under these conditions, reaction times greater than 2 h resulted in reduced yield (Figure 2). As a result of these studies, standard conditions of pH 3.0, 1 h reaction time, and 90°C were established for the method.

Under these standard conditions, hydrolytic efficiencies for different blank water samples fortified with MET and CHLOR were >85%. Both hydrolytic conversion efficiency and variation were consistently superior in natural waters compared with distilled, deionized water: means (CVs, %) for Greenwater Lake, 84.54 (5.5%); for Brook Lake, 86.43 (3.3%); and for distilled, deionized water, 67.81 (15%); $n = 3$. Because blank natural water samples showed no interfering coextractive peaks at the retention time of the SULF and CB analytes that would explain the higher recovery in natural water, we postulate that some natural constituent of these waters enhances the hydrolytic conversion under these experimental conditions. Although hydrolytic and overall recovery efficiencies were essentially identical for the 2 natural water types studied here, further research to examine the general applicability of the technique to other natural water types is required.

Identity of the hydrolytic products of MET as SULF and CHLOR as CB was confirmed by GC/MS analysis under operating conditions described previously (Table 2). In addition to having identical mass spectra, the hydrolysis product of MET and the authentic SULF standard exhibited identical re-

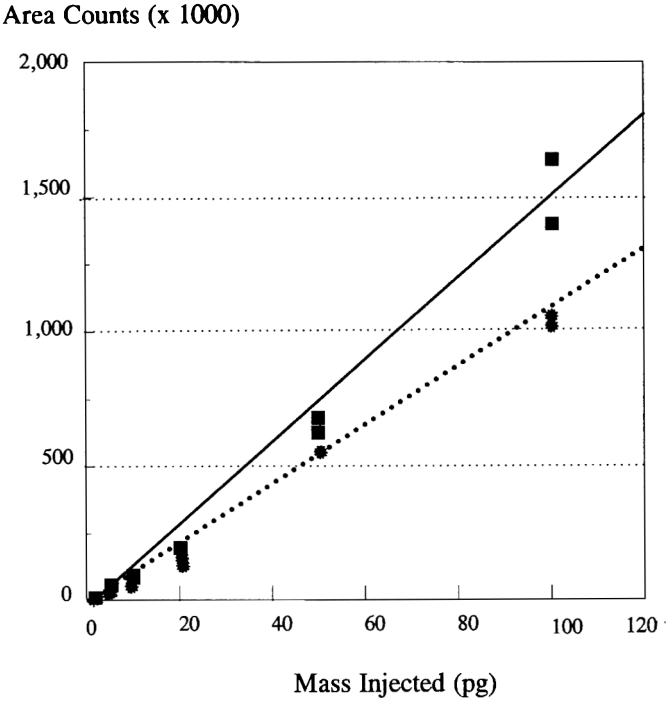

Figure 3. Linearity of electron capture detector response to methyl 2-(aminosulfonyl)benzoate (SULF), —■—, and 2-chlorobenzenesulfonamide (CB), · * ·.

tention times (Rt) on 2 different columns (DB-5, 30 m capillary column, Rt = 10.22 min; DB-17, 15 m megabore column, Rt = 7.3 min). The mass spectrum observed for the single hydrolytic product (CB) of CHLOR was consistent with that recently reported by Long et al. (25), and the product exhibited a retention time of 6.7 min on the DB-17, 15 m megabore analytical column.

Linearity of Detector Response

Linearity of the ECD response to SULF and CB was determined by serial dilution of the authentic SULF standard (100 pg/μL in ethyl acetate) and a CB standard (100 pg/μL in ethyl acetate) prepared in-house by acid hydrolysis of CHLOR under conditions described above. Duplicate 2 μL on-column injections were made for each of 6 standard concentrations (50, 25, 10, 5, 2.5, 1, 0.5, and 0.2 pg/μL), and peak areas were integrated electronically. Regression analysis of the data indicated linearity of the ECD response to SULF ($F = 1030.19$, $P < 0.0001$, and $r^2 = 0.986$) and CB ($F = 2893.43$, $P < 0.0001$, and $r^2 = 0.995$) over the mass range of 1.0–100 pg (Figure 3).

Minimum Limits of Detection and Quantitation

The theoretical minimum limits of detection (MLD = ts; where t = critical value of $t_{0.01,5}$, and s = standard deviation of blank response) were calculated according to the recommendations of Kirchmer (27) and based on the standard deviation of blank response for the complete analytical procedure. On the basis of these principles, the minimum limits of detection for both SULF and CB analytes were conservatively estimated as 10 ppt. In-house method validation studies were conducted on blank natural water fortified with MET, SULF, and CHLOR at various concentrations (Table 3). Results indicated high overall mean recovery efficiencies (>80%) and generally good precision (<12% CV). Chromatographic resolution and peak shape were excellent at parent herbicide concentrations above 100 ppt (Figure 4). On the basis of these in-house validation test results, limits of quantitation (LOQ) for this technique as applied to natural waters were estimated to be 50 ppt for parent herbicides (MET and CHLOR) and 25 ppt for their respective aryl sulfonamide degradation products (SULF and CB). Reinjection of samples at 24 h and 5 days after the original analysis indicated that final samples were stable. An exception to the generally good results was observed for the lowest fortification level of CHLOR. In these highly concentrated samples, a small coextractive peak eluting immediately ahead of CB seriously affected the baseline, negatively influenced resolution, and artificially enhanced CB peak area. In addition, multiple injections of such concentrated sample extracts (for both MET and CHLOR) resulted in reduced sensitivity and peak tailing, which is symptomatic of degradation at the head of the analytical column. However, sensitivity and chromatographic quality were easily restored by simply cutting 5–10 cm from the head of the column, a procedure that could be per-

Table 3. Recovery efficiency and precision for quantitation of metsulfuron methyl (MET), chlorsulfuron (CHLOR), and the aryl sulfonamide degradation product (SULF) from fortified blank natural water[a]

Analyte	Fortification level, ppb	*n*	Mean rec. efficiency, %	SD	CV
MET	50	6	92.0	5.2	5.7
CHLOR	50	6	80.4	6.1	7.6
SULF	25	5	80.4	3.7	4.6
MET	0.5	6	83.9	4.2	5.0
CHLOR	0.5	6	95.4	4.2	4.4
SULF	0.25	6	83.1	6.8	7.6
MET	0.05	6	97.9	10.9	11.1
CHLOR	0.05	5	102.9	40.4	39.0
SULF	0.025	6	110.0	13.0	11.9

[a] Validation results as obtained using fortified samples of Brook Lake water and hot on-column injection to DB-17 column, 15 m × 0.53 μm id; film thickness, 1.0 μm.

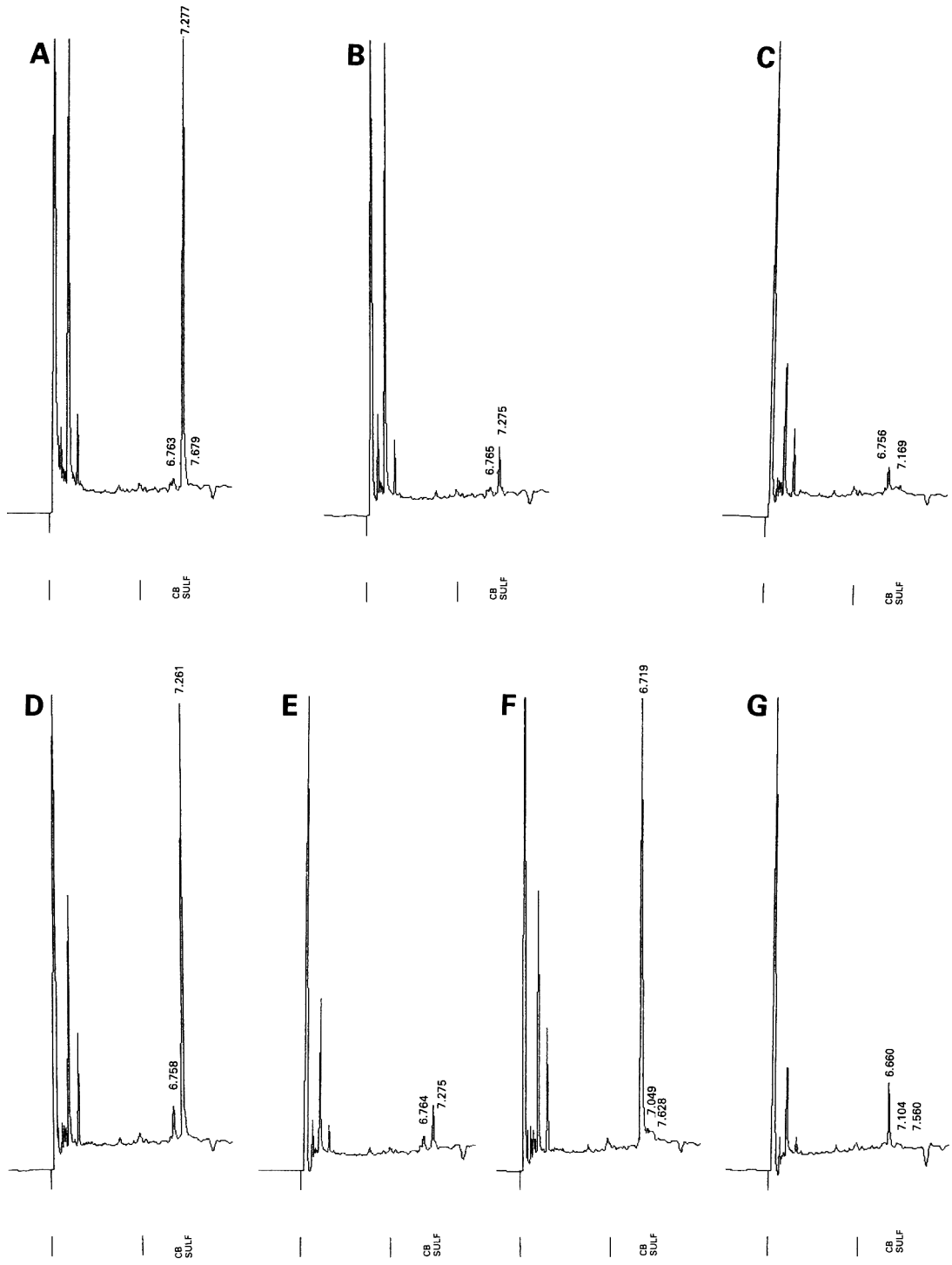

Figure 4. Sample chromatograms obtained under GC/ECD conditions described in text. (A) Methyl 2-(aminosulfonyl)benzoate standard (50 pg injected), (B) methyl 2-(aminosulfonyl)benzoate standard (5 pg injected), (C) natural water procedural blank, (D) metsulfuron methyl concentration equivalent to 500 ppt in natural water (50 pg injected), (E) metsulfuron methyl concentration equivalent to 50 ppt in natural water (5 pg injected), (F) chlorsulfuron concentration equivalent to 500 ppt in natural water (50 pg injected), and (G) chlorsulfuron concentration equivalent to 50 ppt in natural water (5 pg injected).

formed repeatedly without substantial effect on retention or resolution of the column.

The aforementioned analytical problems are considered to be artifacts of the hot on-column injection technique and the megabore column used in this study. Initial tests of septum-programmable injector technology (Varian SPI) to deliver the sample directly onto the chromatographic column resulted in significantly increased sensitivity (about 10-fold greater than that observed on our instrument) and a substantially cleaner baseline. We postulate that the enhanced sensitivity is due to relative inactivity of the "cold" on-column injection technique. Theoretically, use of this technology should provide the capability to detect sulfonylurea herbicide residues at levels as low as 1 ppt in natural water. Further research aimed at testing this hypothesis is continuing.

Conclusions

The method is suitable for isolation and trace-level quantitation of 2 sulfonylurea herbicides, MET and CHLOR, and their respective aryl sulfonamide degradation products in natural water. The relatively simple technique is characterized by high recovery efficiency (>80%), generally good precision (<12% CV), and excellent sensitivity (validated LOQ = 50 ppt; MLD = 10 ppt), as well as excellent chromatographic resolution. The basic method may be used for routine trace-level determination of MET, CHLOR, or both sulfonylurea herbicides. In situations in which the types of sulfonylurea residues in the sample are known, the method is amenable to internal standardization using the other analogue as an internal standard, enhancing sample throughput and reducing error resulting from system variation.

On the basis of initial investigations using temperature-programmable injection technology, further lowering of sensitivity limits to about 1 ppt is feasible. Further experimentation with such new GC technology, as well as extension of the basic method to other sulfonylurea herbicides and other environmental matrixes, is presently ongoing and will be the subject of future publications.

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CAS Registry No. Metsulfuron methyl 74223-64-6

CAS Registry No. Chlorsulfuron 64902-72-3

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Eight Organochlorine Pesticides at Low Nanogram/Liter Concentrations in Groundwater Using Filter Disk Extraction and Gas Chromatography

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Eight organochlorine pesticides may be quantitated routinely at levels below 10 parts per trillion (ng/L) in natural groundwaters. Analytes are extracted rapidly from 1 L groundwater samples by using conditioned Teflon filter disks impregnated with silica containing bonded octadecyl phase. Filter is dried briefly under vacuum, and pesticides are eluted with 10 mL ethyl acetate. The extract is dried with about 1 g anhydrous sodium sulfate and then concentrated to exactly 1 mL. Pesticides are separated in a gas chromatograph equipped with a capillary column, detected by a high-sensitivity electron capture detector, and quantitated by the method of external standards. Certified reporting limit (CRL) values for the 8 pesticides ranged between 2 and 7 ng/L. Preliminary data demonstrated that the organochlorine pesticides could be collected on the filter disks and stored up to 4 weeks in the dark at either refrigerated or ambient temperature without obvious degradation of the sample. Calculated CRL values are comparable (within a factor of 2) to method detection limit values defined by the U.S. Environmental Protection Agency.

Increasingly strict environmental regulations may require legal detection limits as low as 1 part per trillion (i.e., 1 ng/L) in natural groundwaters for a wide variety of carcinogenic pollutants, including the organochlorine pesticide dieldrin, within the next few years. For this reason, existing methods for dieldrin and related species must be improved to satisfy this future need.

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Traditional extraction procedures, such as U.S. Environmental Protection Agency (EPA) Method 3510A, "Separatory Funnel Liquid–Liquid Extraction" (1), are useful for extracting pesticides and other species at sub-microgram-per-milliliter levels, but are not likely to be routinely applicable at levels approaching 1 ng/L. Such procedures involve extraction with large volumes of methylene chloride followed by subsequent Kuderna-Danish concentration to a final volume of 1 mL and solvent exchange to hexane. At analyte concentrations approaching 1 ng/L, these methods are subject to analyte losses via incomplete extraction, sorption onto glass apparatus, and evaporative losses during concentration and solvent exchange. For all of these reasons, the extraction method of choice should use minimal quantities of organic solvent, which reduces the extent of solvent concentration required, and the smallest possible surface area of glassware.

Solid-phase extraction (SPE) of the organochlorine pesticides from groundwaters provides a useful alternative to the traditional liquid–liquid extraction-based methods (2–4). In general, an SPE cartridge containing octadecyl groups chemically bonded to silica is first conditioned with methanol or acetonitrile, followed by water. The groundwater sample is then passed through the cartridge. Organochlorine pesticides retained on the extraction medium are later eluted with a small volume of nonpolar solvent; subsequent concentration may or may not be required. Analytes are later separated and quantitated in a gas chromatograph with an electron capture detector. This method is easily expanded to process several samples simultaneously by using vacuum manifolds, which are currently available commercially from several suppliers. Although such an approach appears reasonable and direct, traditional SPE columns contain small quantities of phthalates and antimicrobials that would produce an unacceptably high blank and subsequently interfere with pesticide quantitations at the desired levels. The problem is described more thoroughly by Junk et al. (5). One obvious solution to these problems is to use SPE columns with glass bodies and Teflon frits, rather than surgical polypropylene bodies and frits; such SPE columns have become available only recently. These columns, and their associated hardware, are probably more appropriate for processing large num-

bers of samples, and they become prohibitively expensive when small numbers of samples are involved.

For all of these reasons, our method uses commercially available filter disks that contain octadecyl groups chemically bonded to silica and impregnated in a Teflon disk. The disk is mounted in a glass filtering funnel and conditioned like the traditional SPE column described above. This approach was originally described by Hagen et al. (6), and it is applicable to groundwaters containing a variety of phthalates and pesticides at concentrations as low as 1 μg/L in groundwater. Briefly, a groundwater sample is passed rapidly through the disk; organochlorine pesticides are later removed with a small volume of organic solvent. After a modest (10-fold) concentration, pesticides are quantitated at the nanogram/liter level with low blank by using a dedicated capillary-column gas chromatograph equipped with a high-sensitivity electron capture detector.

METHOD

Apparatus

(**a**) *Gas chromatograph.*—Model 3500 (Varian, Sugar Land, TX), or equivalent, dedicated capillary/multimethod microprocessor-based gas chromatograph, equipped with electron capture detector. Detector: optimized 100 μL cell geometry, 8 mCi Ni^{63} foil, factory-specified detection limit <50 femtograms of lindane, and linear range $>10^4$. Split/splitless injector, operated in splitless mode. Use instrument's "on-board" printer, stand-alone data station, or stand-alone integrator (e.g., Hewlett-Packard Model 3390A Integrator) for recording chromatograms and integrating peaks.

(**b**) *Automatic injector.*—Model 8100 microprocessor (Varian) controlled low-loss autosampler for gas chromatography (GC).

(**c**) *Liquid chromatography (LC) solvent cleanup assembly.*—Contains 250 mL glass reservoir, 34/45 female tapered funnel base, 1000 mL receiver flask with 34/45 male joint and glass cap, clamp, stainless steel holder, and screen (No. 5-8062M, Supelco, Inc., Supelco Park, Bellefonte, PA, or equivalent).

(**d**) *Glass scintillation vials.*—20 mL.

(**e**) *Vacuum hose.*—To fit suction-receiving flask in LC solvent cleanup assembly.

(**f**) *Tweezers.*—With needle or spaded ends.

(**g**) *Needle valve.*—Placed in line between vacuum source and LC solvent cleanup assembly.

(**h**) *Extraction disks for environmental analysis.*—47 mm diameter, with Empore® bonded octadecyl (C_{18}) phase (No. 1214-5004, Varian Instrument Co., Sample Preparation Products Division, Harbor City, CA).

(**i**) *Kuderna-Danish tube concentrators.*—10 mL capacity, graduated, as required; with rack.

(**j**) *Evaporator.*—Silli-Vap® 6-position micro blowdown equipment (No. 19792, Pierce Chemical Co., Rockford, IL, or equivalent).

(**k**) *Nitrogen gas cylinder.*—Research grade, with regulator and tubing, for Silli-Vap evaporator.

(**l**) *Disposable glass automatic sampler vials.*—2 mL, with screw caps and thick PTFE-lined silicone rubber septa (No. 03-949835-00, Varian Instrument Co., or equivalent).

(**m**) *Water purification system.*—Milli-Q® Four-Bowl, Model No. ZD 20 11584, delivers ASTM Type II water on demand (Millipore Corporation, Bedford, MA, or equivalent).

(**n**) *Amber shell vials.*—Capacity 10 or 15 mL, with solid Teflon-lined screw caps.

(**o**) *Clear shell vials.*—Capacity 40 mL, with solid Teflon-lined screw caps, precleaned according to protocols of EPA. (These include washes with laboratory grade detergent and acid, followed by rinses with deionized water and solvent, and finishing with oven drying. Alternatively, bottles may be purchased precleaned according to these protocols from commercial vendors such as I-CHEM RESEARCH, Hayward, CA.)

(**p**) *MICRO® solution or equivalent.*—For cleaning glassware.

Reagents

(**a**) *Ethyl acetate, methanol, hexane, and acetone.*—Distilled-in-glass, suitable for pesticide analysis (Burdick & Jackson Laboratories, Muskegon, MI; J.T. Baker, Inc., Phillipsburg, NJ; or equivalent).

(**b**) *Sodium sulfate.*—Anhydrous, reagent grade.

(**c**) *Sodium chloride.*—Reagent grade.

(**d**) *ASTM Type II water.*—Obtain from Milli-Q water purification system described above.

(**e**) *Pesticide standards.*—Pesticide Standard Mix A (product No. 32003) and Pesticide Standard Mix B (product No. 32004) (Restek Corp., Bellefonte, PA).

(**f**) *Organochlorine pesticides.*—Aldrin, isodrin, γ-chlordane, α-chlordane, 4,4′-DDE, dieldrin, endrin, 4,4′-DDT. Obtain standard stock solutions from EPA Quality Assurance Materials Bank (Quality Assurance Division, Environmental Monitoring Systems Laboratory, Las Vegas, NV), or purchase similar solutions of known concentration from AccuStandard (New Haven, CT), or equivalent supplier.

(**g**) *Standard solutions.*—Prepare working stock solution containing 1 μg/mL of each of the 8 pesticides in methanol. Store at 4°C in amber screw-cap vial with solid Teflon-lined cap. (**Caution**: Some of the organochlorine pesticides, such as dieldrin, are known or suspected carcinogens. Wear appropriate personal protection such as gloves when preparing standards.) Prepare diluted working standards with concentrations between 0.5 and 25 ng/mL in ethyl acetate; prepare working stock solution of 1 μg/mL. Store diluted working standards at 4°C in amber screw-cap vials with solid Teflon-lined cap.

(**h**) *Check standards.*—Dilute 15 μL Pesticide Standard Mix A to final volume of 25 mL in hexane to form Check Standard A. Check Standard A contains 5 compounds at 4.8 ng/mL each (α-BHC, γ-BHC, heptachlor, Endosulfan I, and surrogate standard tetrachloro-*m*-xylene), 5 compounds at 9.6 ng/mL each (dieldrin, endrin, 4,4′-DDD, 4,4′-DDT, and surrogate standard decachlorobiphenyl), and 1 compound at 48 ng/mL (methoxychlor). Dilute 15 μL Pesticide Standard Mix B to 25 mL in hexane to form Check Standard B. Check Standard B contains 7 compounds at 4.8 ng/mL each (β-BHC, δ-BHC, al-

drin, heptachlor epoxide (isomer B), γ-chlordane, α-chlordane, and surrogate standard tetrachloro-*m*-xylene) and 6 compounds at 9.6 ng/mL each (4,4′-DDE, Endosulfan II, endrin aldehyde, endosulfan sulfate, endrin ketone, and surrogate compound decachlorobiphenyl). Store diluted check standards in amber screw-cap vials with solid Teflon-lined caps at 4°C.

(i) *Air, helium, nitrogen.*—99.999% purity.

Certification Samples for Pesticides in Synthetic Groundwater

Weigh 1.48 g reagent grade anhydrous sodium sulfate into 1 L volumetric flask, and dilute to volume with ASTM Type II water. Weigh 1.65 g reagent grade anhydrous sodium chloride into second 1 L volumetric flask, and dilute to volume with ASTM Type II water. Transfer 100 mL of each of these 2 solutions into 1 L flask, and dilute to volume with ASTM Type II water to prepare simulated 1 L groundwater samples containing ASTM Type II water and sulfate and chloride, each at 100 mg/L, for method certification (7). Spike these samples with enough working stock solution (1 μg/mL) to yield concentrations of 0.5–20 ng/L of each of the 8 pesticides in water. Stir briskly for 10 min before proceeding.

Authentic Groundwater Samples

Store samples of authentic groundwaters in refrigerator at 4°C. Do not add chloride and sulfate to authentic groundwaters before extraction.

Sample Preparation

Conditioning filter disk.—Dismantle LC solvent cleanup assembly. Insert stainless steel screen in its glass holder. Place Empore filter disk onto stainless steel screen; use metal tweezers to handle filter disk. Place 250 mL solvent reservoir on top of filter disk and glass holder. Secure glass holder and reservoir with clamp (provided with assembly); insert assembly on top of 1000 mL receiver. Prewash disk with 10 mL final eluting solvent (ethyl acetate). Apply vacuum to pull half of ethyl acetate through disk. Leave remaining ethyl acetate undisturbed on disk for 1 min; apply vacuum to pull ethyl acetate through disk. Maintain vacuum to pull air through disk for 5 min to dry. (**Caution**: Assembly may leak if holder, disk, and reservoir do not fit properly. If leaking is observed, readjust position of reservoir until leaking stops and repeat this step.) Add 10 mL methanol to assembly and apply vacuum. Pull ca 85% of methanol through disk, releasing vacuum before disk goes completely dry. (**Note:** Do not allow disk to go completely dry until extraction is complete.) Add 10 mL Milli-Q water to assembly and apply vacuum. Pull ca 85% of water through disk, releasing vacuum before disk goes completely dry. (**Note**: Do not allow disk to go completely dry until extraction is complete.)

Organochlorine pesticide collection.—Add ca 250 mL from 1 L groundwater sample to reservoir. Adjust vacuum so that water passes through disk at ca 25–30 mL/min. Continue adding groundwater sample until it passes through filter disk (ca 30–40 min will be needed to extract groundwater sample). (**Note**: Do not allow disk to go completely dry until extraction is complete.) After sample is processed, pull air through disk for ca 5–10 min to remove residual water from disk.

Organochlorine pesticide extraction and concentration.—Remove filter base and attached sample reservoir from 1000 mL receiver. Secure filter base and sample reservoir assembly to ring stand, using clamp. Handle assembly by edge of filter base. (**Caution:** Do not touch or disturb sample reservoir; assembly may leak, causing sample loss.) Place 20 mL liquid scintillation vial into tip of filter base. (Vial should fit snugly into ground glass joint.) Add 10 mL ethyl acetate eluting solvent to filter reservoir and rinse reservoir walls. Pull ca 50% eluting solvent through filter, using vacuum. Let disk and solvent stand undisturbed for ca 1 min. Pull remainder of eluting solvent through disk. Collect ethyl acetate eluate in liquid scintillation vial. Add ca 1 g anhydrous sodium sulfate to ethyl acetate extract to remove excess water. Shake gently; minimize contact with scintillation bottle cap. Let bottle stand undisturbed for at least 30 min. Transfer dried ethyl acetate to 10 mL Kuderna-Danish concentrator tube, using Pasteur transfer pipet. Concentrate extract to final volume of 1 mL, using dry flowing nitrogen passing through Silli-Vap. (Note that 6 tubes may be concentrated simultaneously.) Using Pasteur pipet, transfer final 1 mL concentrate to 2 mL screw-cap vial for GC analysis.

Automatic Sample Injection

Place 2 mL screw-cap vials in autosampler. Accompany each set of sample extracts by a complete set of diluted working standards, one or more diluted working standards analyzed immediately after set of real sample extracts, and one vial each of diluted Pesticide Standards A and B (check samples), analyzed both before and after real sample extracts. Inject samples onto GC column. Operating conditions: injection volume, 2 μL; "hot" needle time, 0.00 min (needle is removed immediately from injector); injection rate, 1.0 μL/s; upper and lower air gaps around sample; operating gas, air, 20 psi.

Gas Chromatography

Analytical column: DB-5, No. 1235033, 30 m × 0.325 mm, film thickness 1.0 μm, (J & W Scientific, Inc., Folsom, CA). Injector temperature, 250°C; detector temperature, 325°C. Carrier gas: ultrahigh purity helium, 4.8 mL/min, linear velocity 58.4 cm/s; tank pressure set at 80 psi. Make-up gas for electron capture detector, ultrahigh purity nitrogen; tank pressure set at 80 psi; flow rate set at 30 ± 1 mL/min. Oven temperature program: initial temperature 80°C, hold for 0.00 min; linear ramp to 180°C at 30°C/min, hold for 0.00 min; linear ramp to 290°C at 5°C/min, hold for 7.00 min; cool to 80°C. Equilibration time to next run is 2 min. Total run time between injections is ca 40 min. Record and integrate component peaks, using either on-board integrator of Varian 3500 gas chromatograph or stand-alone integrator.

Quality Assurance/Quality Control

Prepare at least 1 blank, consisting of 1 L ASTM Type II water containing 100 mg/L each of sulfate and chloride, to accompany each lot of real samples processed. Prepare at least 2

spiked samples, consisting of 1 L ASTM Type II water containing 100 mg/L each of sulfate and chloride and either 5 or 20 μL 1 μg/mL working stock solution (final concentration 5 or 20 ng/L in water), with each lot of real samples. The 2 spiked samples are internal quality control checks used to monitor reproducibility and repeatability. Check standards A and B, described earlier, are external quality control checks used to monitor "in control" status of analytical system.

Calculations

Plot integrated peak for each pesticide against its concentration. Fit linear regression line to standard responses obtained each day. Standard responses obtained after unknown samples have been analyzed may be used to evaluate detector stability over given 24 h period. Calculate organochlorine pesticide concentrations in certification, real, or blank samples, using calibration procedure described above. Calculate certified reporting limit (CRL) for each organochlorine pesticide by using standard certification software (8), or equivalent (9). Calculate method detection limit (MDL) by using its formal definition and Statistical Analysis Software System (9), or equivalent.

Abbreviated Holding Time Study

Prepare 20 aliquots (1 L) ASTM Type II water spiked with 50 μL 8-component working stock solution (final spike level 50 ng/L in each of 8 pesticides in water).

Extract 20 samples as previously described. Place each filter disk in 40 mL screw-cap vial with solid Teflon-lined cap. Store 8 filters in dark at ambient temperature (24.5°C) and 8 filters in monitored refrigerator (4°C). Immediately extract, elute, concentrate, and quantitate organochlorine pesticides from remaining 4 filters to establish recoveries at time = 0. Thereafter, perform same operations weekly for 2 filters taken from each condition for 4 weeks.

Results and Discussion

General Analytical Considerations

Ultratrace concentrations of pesticides considered in this work (nanogram/liter levels in the samples, nanogram/milliliter levels in the final extracts) demand strict cleanliness in the sample collection, preparation, and final chromatographic separation. In most cases, strict cleanliness in the sample collection is ensured by using 1 L amber bottles that have been cleaned according to the protocols established by EPA. These protocols generally specify washes with laboratory grade detergent and acid, followed by rinses with deionized water and solvent, and finally oven drying. Laboratory glassware such as the LC solvent cleanup assembly should be soaked in clean, dilute MICRO solution overnight. Glassware is then scrubbed with a brush, rinsed 3 times with distilled water, and further rinsed 3 times with acetone. Glassware is then heated at 110°C for at least 3 h. Clean capped vials may be stored in a contaminant-free area until needed. Further, the Empore filter disks contribute a low but detectable blank, even though they are usually considered clean enough for general use. The conditioning procedure described, which permits ethyl acetate to remain on the disk for 1 min, removes most of the interfering material from the disk surface. Studies in which ethyl acetate is allowed to remain on the disk longer than 1 min showed no additional reduction in the blank. Finally, even the type of cap and vial used for the final 1 mL extract may contribute to the overall blank. Crimp-caps used with 2 mL vials have a thin, Teflon-coated rubber septum, which could be punctured only once before a substantial increase in the blank was observed. Substituting screw caps, which use a thick silicone rubber and PTFE septum, permitted multiple sampling from the same vial without an increase in blank.

Several additional variations in the procedure were also evaluated. Concentrating the 10 mL ethyl acetate extract to a final volume of 100 μL rather than 1 mL, for example, resulted in improved sensitivity, but at the expense of both elevated blank and excessive variation in the final pesticide recovery values at the nanogram/milliliter level. This problem was particularly severe with aldrin and isodrin, which were the most volatile of the target organochlorine pesticides. Ten-fold concentration (from 10 to 1 mL) of the ethyl acetate extract provided the needed sensitivity with both acceptable blank and a smaller variation in recovery. Final extraction with methyl *t*-butyl ether instead of ethyl acetate improved the extraction recovery of all 8 analytes, particularly aldrin and isodrin. However, studies using LC-grade or distilled-in-glass grade methyl *t*-butyl ether exhibited a substantially elevated blank compared to similar grades of ethyl acetate. Because our method was to be optimized for dieldrin, ethyl acetate was selected as the solvent of choice; it provided a lower blank, and its extraction efficiency for dieldrin was similar to that of methyl *t*-butyl ether.

Figure 1 demonstrates the typical baseline and chromatographic separation for both the extract from a 5 ng/L spiking level in a simulated groundwater sample used for certification (extract concentration of approximately 5 ng/mL) and a 5 ng/mL standard containing all 8 organochlorine pesticides. In general, throughout the entire study all of the analytes were baseline-resolved, including the difficult pair 4,4′-DDE/dieldrin. The baseline still exhibited some small fluctuations and low-level noise but was usually acceptable. The solvent blank frequently exhibited small peaks that eluted in the general region where the 8 organochlorine pesticides eluted; however, the analyte peaks were identified and integrated without interference.

The electron capture detector response toward the 8 organochlorine pesticides was initially established according to the "Precertification" protocol of the standard certification package (8). Briefly, a set of five 8-component standards ranging between 0.5 and 20 ng of each pesticide/mL was analyzed in duplicate overnight. Peak areas were plotted against concentration for each pesticide, and the resulting linear regression lines (both with and without a non-zero intercept) were evaluated for lack of fit. The coefficients of determination (r^2) for the linear regression model with a non-zero intercept ranged between 0.9797 and 0.9992 for the 8 pesticides; only one of these values fell below 0.9935. The r^2 for the linear regression model with a

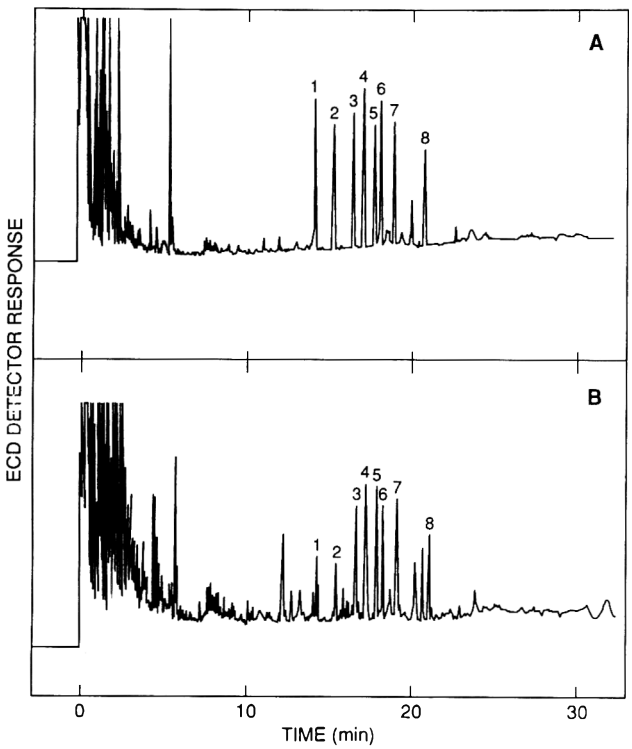

Figure 1. Comparison between (A) an 8-component organochlorine pesticide standard at 5 ng/mL and (B) the extract obtained from an artificial groundwater spiked at 5 ng/L: (1) aldrin; (2) isodrin; (3) γ-chlordane; (4) α-chlordane; (5) 4,4′-DDE; (6) dieldrin; (7) endrin; and (8) 4,4′-DDT.

forced zero intercept ranged between 0.9422 and 0.9953 for the 8 pesticides; only one of these values fell below 0.9861. Linear regression models with non-zero intercepts were used for all future calculations because the r^2, as a whole, were greater than those for linear regression models with forced zero intercepts.

Method Certification and Calculation of CRL

The certification protocol was designed by the U.S. Army Toxic and Hazardous Materials Agency (7) for intensive evaluation of the performance of a method over a 4-day period. Briefly, 1 L samples of ASTM Type II water containing chloride and sulfate (as sodium chloride and sodium sulfate) at 100 mg/L each are spiked with working stock solution to concentrations ranging between 0.5 and 20 ng/L (the "target" concentrations) of each of the 8 pesticides. Extraction, collection, concentration, and quantitation are performed for each spike level on each of the 4 days. Each set of daily certification samples included a blank consisting of unspiked ASTM Type II water fortified with the usual concentrations of sulfate and chloride but containing no pesticide spike. Extracts from each set are accompanied by 2 sets of diluted working standards (concentrations ranging between 0.5 and 25 ng/mL) and 2 pairs of Pesticide Check Standards, as described under *METHOD*.

The daily calibration line for each pesticide is used to calculate the concentration of pesticide found in each extract, and from that value the concentration in the original spiked aqueous sample (the "found" concentration) was calculated. Concentration data obtained from a representative volatile analyte, isodrin, and a representative nonvolatile analyte, 4,4′-DDT, are given in Tables 1 and 2, respectively, for each of the 4 certification days. These data are later entered into the certification software package (8), which in turn calculates a linear regression relationship between "target" and "found" concentrations and tests for both zero intercept and lack-of-fit. The slope of the linear regression line may be taken as the "recovery" of a given analyte through the method. The certification software package also calculates a CRL value, which is the concentration of a substance that can be measured and reported with both a false-positive and false-negative risk of 5% (i.e., $\alpha = \beta = 0.05$). Principles for calculating CRL values are described in detail by Grant et al. (10) and Hubaux and Vos (11). The certification software package (8) also allows the investigator to obtain a graph that shows the linear regression line discussed above, the confidence bands, and the CRL value. The CRL value is located by the following 4-step procedure: (*1*) calculate and plot a regression line, representing the relationship between "found" vs "target" concentrations, with appropriate 2-sided 90% confidence limits for a predicted observation; (*2*) locate intercept of the upper 90% predictive confidence limits with the y-axis ("found" concentrations); (*3*) draw a horizontal line from this intercept until it intersects the lower 90% predictive confidence limits; and (*4*) draw a vertical line from the intercept described in (*3*) to the x-axis ("target" concentrations). This intersection with the x-axis is the CRL. The process is shown graphically in Figures 2 and 3.

An additional consideration of the certification protocol is the instrument response to the daily standards. For the first 7 calibrations, responses must agree within 25% with the mean response for the same concentration, as determined from Precertification and the initial calibration. After 7 calibrations, responses must agree within 2 standard deviations, rather than a percentage (7). Replacing manual sample injections with automatic injections, as described under *METHOD*, not only improves injection-to-injection reproducibility, but also allows complete sets of calibration standards to be analyzed conveniently with every set of samples.

Table 3 presents CRL values for all 8 pesticides, calculated from the entire set of calibration data and daily calibration lines, as well as the line slope or recovery of each pesticide. CRL values range between approximately 2 ng/L (for 4,4′-DDT and α-chlordane) to 7 ng/L (for aldrin). Recovery values generally improved with decreasing analyte volatility and ranged from 48% (aldrin) to 93% (dieldrin). The observed recovery for aldrin, 48%, agreed with that reported by Hagen et al. (6), 51%, for concentrations ranging from 1 to 100 μg/L in groundwater, or some 50–5000 times the highest concentration tested during certification. In addition, day-to-day reproducibility of a given pesticide also improved with decreasing analyte volatility, frequently resulting in lower CRL values. Figures 2 and 3 illustrate the difference between the certification graphs for isodrin, a volatile analyte, and 4,4′-DDT, a nonvolatile analyte. The scatter of the "found" concentrations over the 4-day certification period is clearly less severe for 4,4′-DDT than for isodrin.

Table 1. Comparison of target vs found concentrations on the 4 certification days: isodrin

	Found concn, ng/L			
Target concn, ng/L	Day 1	Day 2	Day 3	Day 4
0.5	-0.18	0.25	0.49	0.060
1	0.15	0.81	0.58	0.34
2	0.96	0.56	1.04	1.96
5	2.92	1.64	2.29	4.03
10	3.70	5.65	7.20	6.61
20	8.49	10.40	10.60	12.50

CRL values reported in Table 3 are in substantial agreement with those found by Wilson and Summer (4), who used conventional SPE technology and reported data for a smaller selection of organochlorine pesticides. These investigators reported CRL values of aldrin, isodrin, dieldrin, and endrin as 20, 6, 6, and 8 ng/L, respectively. The current study produced CRL values of 7, 7, 6, and 4 ng/L, respectively, for the same suite of compounds.

Even though certification samples were prepared and analyzed at concentrations below 2 ng/L, no pesticide could be certified below this concentration, although analytes were frequently detected at these levels. This condition was caused by several factors. First, baseline noise that was manageable at a spike level of 5 ng/L (5 ng/mL in the extract) became significant at spike concentrations <2 ng/L (2 ng/mL in the extract), which caused a high bias in some of the data. Second, nonquantitative recoveries of pesticides sharply reduced the expected response in certain cases, resulting in a negative bias (sometimes even a negative concentration) in some data sets. Third, contaminants that were not observed in neat solvent occasionally became significant and troublesome when extracts were concentrated. Finally, losses of nanograms or even picograms of analyte on the walls of laboratory glassware cannot be overlooked, despite best efforts to minimize such losses. All of these factors taken together yielded nonreproducible detection of pesticides and, hence, lack of certification at spike concentrations below 2 ng/L. Although CRL values at the 1 ng/L level may indeed be possible, such work would involve nonstandard laboratory ware (e.g., expensive custom-prepared

Table 2. Comparison of target vs found concentrations on the 4 certification days: 4,4'-DDT

	Found concn, ng/L			
Target concn, ng/L	Day 1	Day 2	Day 3	Day 4
0.5	-0.35	-0.071	0.35	0.15
1	0.024	0.55	0.13	0.37
2	2.02	1.15	1.08	2.56
5	3.94	4.04	4.63	4.38
10	7.84	8.69	8.75	8.21
20	15.10	15.38	15.70	15.45

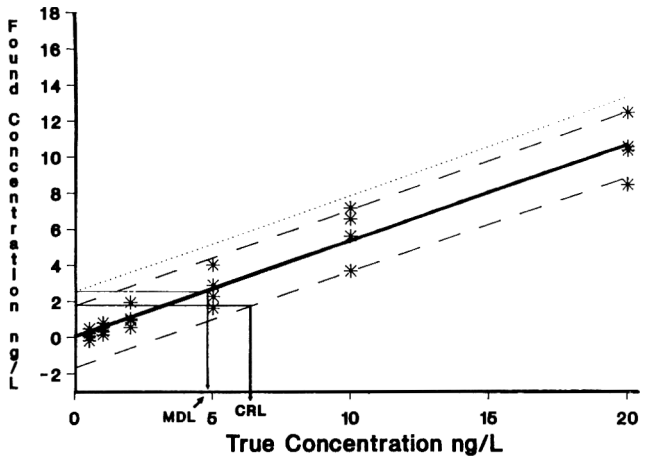

Figure 2. Certification graph (found vs expected concentrations) of isodrin: (····) = 99% confidence band; (——) = 90% confidence band; (———) = calculated regression line; MDL = method detection limit; and CRL = certified reporting limit.

Teflon apparatus or completely silanized glassware, one or both of which may contribute an additional blank of its own) or a dedicated clean room. For all of these reasons, CRL values reported in Table 3 should be taken as limits that can be obtained by most laboratories with reasonable skill and care.

One analyst can prepare about 6 samples in their entirety per 8 h day (including extraction and sample concentration). Eight samples may be extracted (concentration postponed) per 8 h day. The corresponding GC analysis typically requires about 40 min per sample. We strongly recommend that GC determinations be performed overnight with unattended automatic operation.

Method Intercomparison with Authentic Contaminated Groundwaters

The method described above was tested on authentic contaminated groundwaters taken from monitoring wells. An inde-

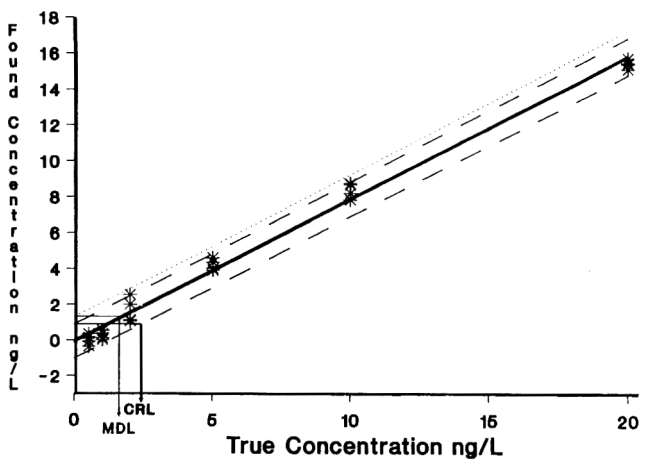

Figure 3. Certification graph (found vs expected concentrations) of 4,4'-DDT: (····) = 99% confidence band; (——) = 90% confidence band; (———) = calculated regression line; MDL = method detection limit; and CRL = certified reporting limit.

Table 3. Certified reporting limits (CRL) and recovery data for 8 organochlorine pesticides

Pesticide	Rec., %	CRL, ng/L
Aldrin	48	6.7
Isodrin	53	6.5
γ-Chlordane	72	2.6
α-Chlordane	76	2.0
4,4′-DDE	66	4.6
Dieldrin	93	6.3
Endrin	86	4.0
4,4′-DDT	79	2.4

pendent testing laboratory had previously characterized similar waters by traditional liquid–liquid extraction procedures (1), and had charted the concentrations of several organochlorine pesticides over time. Table 4 demonstrates that pesticide concentrations found by the proposed method were in generally good agreement with those obtained by more conventional methods. In 7 cases, the conventional method yielded a pesticide concentration less than its detection limit (typically <50 ng/mL); in 6 of these cases, the same pesticides were "not found" by the current method. These results may be taken as equivalent, that is, with no detectable analyte, within the detection limits attributed to each pesticide. Somewhat elevated values were obtained by the current method for dieldrin and 4,4′-DDT, possibly because of the smaller volume of organic solvent used and the reduced extent of concentration compared with the traditional method. Samples were initially screened by using a final extract volume of 1 mL. When several pesticide concentrations exceeded 100 ng/mL, a second 1 L aliquot was extracted; the final extract volume was 10 mL. Discrepancies in concentrations based on either a 1 or 10 mL final volume are probably caused by reduced volatilization losses in the latter determination.

Comparison of MDL with CRL

EPA defines an MDL as "the minimum concentration of a substance that can be measured with 99% confidence that the analyte concentration is greater than zero, and is determined from analysis of a sample in a given matrix containing the analyte" (12). Therefore, this definition specifies only the risk of a false positive as 1%. Because the risk of a corresponding false negative is not specified in the legal definition, pure chance yields a false-negative probability of 50%. By contrast, CRL values, which are calculated according to the procedure of Hubaux and Vos (11), are defined as the concentration of a substance that can be measured and reported with both a false-positive and false-negative risk of 5% (i.e., $\alpha = \beta = 0.05$). In either case, the figure of merit is derived from confidence bands constructed about the regression line by using specified values for the false-positive and false-negative risk.

Both definitions were applied to the entire set of certification data and used to calculate appropriate MDL and CRL values. In general, CRL and MDL values compared very favorably, as shown in Table 5 and Figures 1 and 2. Differences were no greater than 1–2 ng/L (for CRL values ranging between 2 and 7 ng/L) for any of the 8 pesticides. Although CRL and MDL values differ slightly, the method detection limit for a given pesticide may be specified by either procedure without invoking serious errors.

Lingering debate may still exist over which procedure is preferred for calculating a detection limit. There appear to be some important advantages to the CRL approach on the basis of both practical operational and theoretical statistical considerations. The procedure for calculating CRL values specifies a 40-fold range of target concentrations (normally from half to 20 times that of the target value) over a brief but intensive trial period of 4 days. Hence, there is at least a limited opportunity to evaluate day-to-day performance of a new method. By contrast, the procedure for calculating MDL values specifies 7 aliquots taken at a single concentration on 1 day. Clearly, of course, the operator of the MDL method could perform additional determinations at more concentrations on more than 1 day, but the procedure does not make that mandatory. In addition, the statistical definition of the MDL provides for a very rigorous risk of a false positive (1%), but a very weak risk of a false negative (50%). By contrast, the CRL uses a more modest value of 5%, which is used for the specification of both a false

Table 4. Comparison of Oak Ridge National Laboratory and independent analyses of organochlorine pesticides in contaminated groundwaters

	Well A		Well B	
Pesticide	ORNL, ng/L	Traditional, ng/L	ORNL, ng/L	Traditional, ng/L
Aldrin	nf[a]	<58–617	nf	87–265
Isodrin	nf	<51–231	nf	<51–217
γ-Chlordane	253 260[b]	95–459[c]	190 252[b]	95–950[c]
α-Chlordane	30 46[b]	95–459[c]	108 184[b]	95–950[c]
4,4′-DDE	nf	<54–417	nf	<54–540
Dieldrin	971 1010[b]	175–848	2700 2860[b]	2100–2700
Endrin	nf	<50–139	397 849[b]	500–1500
4,4′-DDT	234 562[b]	<49–116	148 156[b]	<49–490

[a] nf = not found.
[b] Final extract volume 10 mL, not the usual 1 mL.
[c] Total chlordanes reported in the traditional procedure.

Table 5. Calculated values of certified reporting limit (CRL) and method detection limit (MDL) for 8 pesticides using the entire certification data set and unweighted or weighted least squares

	CRL, ng/L		MDL, ng/L	
Pesticide	Unweighted	Weighted	Unweighted	Weighted
Aldrin	6.7	6.6	4.9	4.8
Isodrin	6.5	5.9	4.7	4.3
γ-Chlordane	2.6	6.5	1.9	4.7
α-Chlordane	2.0	6.4	1.4	4.7
4,4′-DDE	4.6	5.3	3.4	3.9
Dieldrin	6.3	3.9	4.6	2.9
Endrin	4.0	4.2	2.9	3.1
4,4′-DDT	2.4	6.5	1.8	4.8

positive and a false negative. Hence, the definition of the CRL is actually a little tighter than that for the MDL.

CRL and MDL Calculated by Using Weighted Regression Analyses

Statistical procedures normally used to calculate either CRL or MDL values tacitly assume that the variance is a constant value for "found" concentrations at each "true" concentration (10–12). In this work, the spread of "found" concentrations was clearly not constant but appeared to increase in a roughly linear manner as the "true" concentrations increased. Grant et al. (10) observed that this was a very common situation and discussed several ways to provide defensible CRL values. These included shortening the range of "true" values tested during certification, adding additional target values (in this case, <0.25 ng/L), truncating the data set by eliminating some of the values at higher "true" concentrations, and using a "weighted" least squares (unweighted data normally used). We observed that a shorter range of "true" values would reduce the applicability of the analytical method and that routine reproducible determination of extract concentrations approaching 0.25 ng/mL would not be feasible; neither approach was investigated. Truncation of the data set is routinely used by the certification protocol described in reference 7, but the protocol seems somewhat arbitrary. The "weighted" least squares was the most attractive alternative but has not been widely used (10).

The weighting factor described here is the reciprocal of the estimated variance from the 4 replicate measurements at each "true" concentration (i.e., weighting factor equals $1/s^2$). Such a factor gives additional weight to those concentrations measured with more precision and reduced weight to those measured with less precision. Table 5 compares CRL and MDL values calculated by both the weighted and unweighted least squares procedures. In general, the range of weighted CRL or MDL values overlaps that of the unweighted data. Weighting may or may not reduce the CRL or MDL value reported. CRL or MDL values were reduced slightly for 2 pesticides (isodrin and dieldrin), were increased slightly for 4 pesticides (α-chlordane, γ-chlordane, 4,4′-DDE, and 4,4′-DDT), and were virtually unchanged for 2 pesticides (endrin and aldrin). In this particular application, the benefits of weighting the data appear minimal.

Abbreviated Holding Time Study; Stability of Organochlorine Pesticides on Teflon Filters

The normal protocol for determining organochlorine pesticides in groundwater involves shipping water samples in bottles, typically in coolers maintained at 4°C with reusable blue-ice packs, followed by the extraction, collection, concentration, and quantitation described above. Provided that the analytes are stabilized on the Empore filters, the initial extraction of the groundwaters might possibly be performed in the field and, therefore, only an Empore filter would be shipped to the laboratory for analysis, instead of the entire 1 L sample of water. Potential reductions in shipping costs and improvements in convenience for the field sampling team would be considerable.

The rigorous determination of sample holding times for a given analyte and matrix is an exhaustive and time-consuming process (13). However, an initial or exploratory study was performed in which stabilities of the 8 organochlorine pesticides were evaluated at a single spike concentration. A set of 1 L Milli-Q water samples spiked at 50 ng/L was passed through preconditioned Empore filters, as described previously. Half

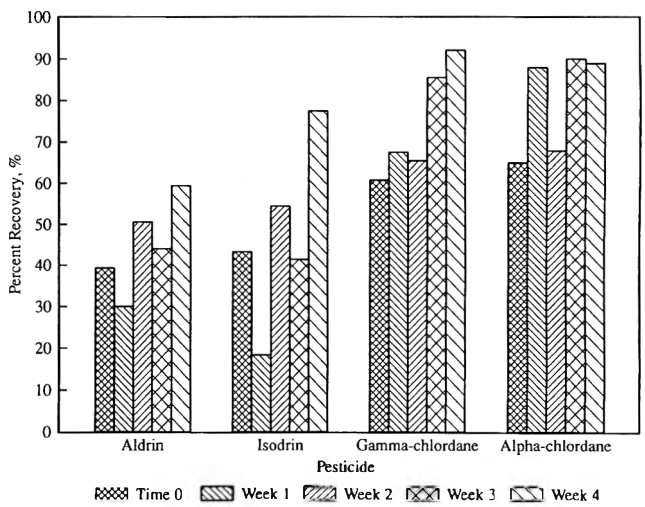

Figure 4. Stability of aldrin, isodrin, γ-chlordane, and α-chlordane on Empore® filter disks over 4 weeks; spike concentration, 50 ng/L in water.

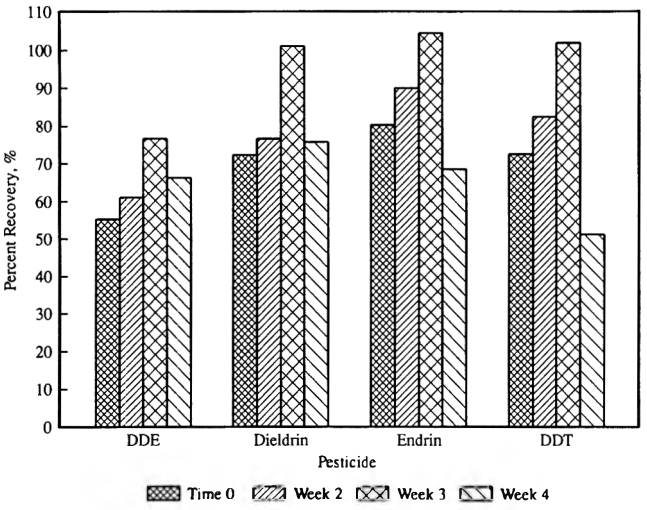

Figure 5. Stability of 4,4′-DDE, dieldrin, endrin, and 4,4′-DDT on Empore® filter disks over 4 weeks; spike concentration, 50 ng/L in water.

the filter disks were stored at 24.5°C, the remainder at 4°C for 4 weeks. Organochlorine pesticides from pairs of filters from each storage condition were extracted, collected, concentrated, and quantitated in the usual manner. Recovery data were collected and compared for each pesticide over the trial period.

Figures 4 and 5 show the stability of the 8 organochlorine pesticides collected on Empore filter disks and stored at room temperature; the behavior of these compounds stored at 4°C is similar. Despite the obvious variation in the recovery data, there was no consistent degradation of the analyte concentrations during the 4-week study. Such information suggests that analytes are stabilized on the Empore filters and that shipping pesticides to the laboratory on a filter, rather than in water, may be a reasonable possibility.

Conclusions

The analytical method described above provided a rapid, quantitative, and convenient procedure for determining 8 organochlorine pesticides at the low parts per trillion level. The extraction, which featured a low-blank reversed-phase Empore filter disk, permitted pesticide recoveries ranging between 48% (aldrin) and 93% (dieldrin). Detection limits, calculated by using either MDL or CRL criteria, ranged between 2 and 7 ng/L for all 8 analytes tested. The method yielded results comparable to conventional methods for authentic contaminated groundwaters. Finally, organochlorine pesticides stored on the disks appeared to be stable for at least 4 weeks when stored in the dark at either room or refrigerator temperatures.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Gas Chromatographic Determination of Acaricide Danjiami in Citrus and Soil

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A method is described for determining total danjiami residues in citrus and soil. Danjiami and its metabolites are hydrolyzed to 2,4-dimethylaniline by refluxing with acid/base, re-extracted with petroleum ether, and cleaned up with acid/base partition. Sample is then derivatized to the heptafluorobutyranilide and quantitated by electron capture gas chromatography. Theoretical detectable limits are 5 ppb for citrus pulp and soil and 1 ppb for citrus peel. Recoveries for control samples fortified with danjiami at 0.25–2.0 ppm averaged 82.4% for citrus and 82.8% for soil.

Danjiami, *N*-(2,4-dimethylphenyl)-*N'*-methyl formamidine hydrochloride, is an acaricide discovered and developed by Zhejiang Institute of Technology, China, in the early 1980s (Figure 1). Danjiami water-soluble concentrate is currently being tested in China as a systemic acaricide to control spider mites and resistance mites of tea, apples, and citrus fruits. It has been registered by the Ministry of Agriculture, People's Republic of China. Because of its wide application in agriculture and animal husbandry, and because little is reported about methods for determination of danjiami residues, the development of an analytical method for danjiami residue in crops and environment is important. The present method was developed to determine residual danjiami and its metabolites in citrus and soil.

Experimental

Apparatus

(a) *Gas chromatograph.*—Model SP-501N equipped with electron capture detection (ECD) system. Column: glass, 150 × 0.4 cm id, packed with 1.5% OV-17 + 2% QF-1 on 80–100 mesh Chromosorb W-HP. Operating conditions: nitrogen carrier, 75 mL/min; injector, 235°C; column, 130°C; detector, 215°C; range, 5; attenuation, 0.5; chart speed, 4 mm/min; and pulse, 100 μs. Under these conditions, retention time for danjiami derivatives was ca 2.4 min.

(b) *Recording integrator.*—Model CDMC-2A (Shanghai Computing Technical Institute, China).

(c) *Water-cooled reflux condenser.*—350 mm long, glass.

(d) *Vacuum apparatus.*—Model ZXZ-1 (Huang Yan Medical Machine Factory, China).

(e) *Blender.*—Mill 2 (Moulinex, Paris, France).

Reagents

(a) *Pesticide standard solutions.*—Danjiami (99.0%) (Zhejiang Institute of Technology) prepared as alcohol solutions, each at 1 μg/mL and 0.1 μg/mL, stored in the refrigerator.

(b) *2,4-Dimethylamiline (DMA) standard solutions.*—DMA (98.5%) (Zhejiang Institute of Technology). *Stock solution.*—5.00 mg DMA/mL *n*-hexane. *Working solution.*—Dilute stock solution with *n*-hexane as needed.

(c) *Heptafluorobutyric anhydride (HFBA).*— >99% GC grade.

(d) *Petroleum ether.*—bp. 60–90°C, AR grade, redistilled in all-glass apparatus.

(e) *Ethanol.*—95%, AR grade.

(f) *Hydrochloric acid.*—1N and 3N; dilute AR grade HCl with deionized water.

(g) *Sulfuric acid.*—0.25N; dilute AR grade H_2SO_4 with deionized water.

(h) *Sodium hydroxide solution.*—10N.

(i) *Anhydrous sodium sulfate.*—AR grade, dried in oven for 4 h at 180°C before use.

(j) *Saturated sodium hydrogen carbonate solution.*—AR grade $NaHCO_3$ prepared with deionized water.

Sample Hydrolysis

Weigh 40 g citrus pulp, 30 g blended citrus peel (100 g chopped peel plus 200 mL distilled water), or 40 g air-dry soil sample into a 250 mL flat-bottom flask. Add 80 mL 2N HCl (for citrus peel, add 100 mL 0.25N H_2SO_4) and 10 mL ethanol (95%); blend the mixture well. Attach flask to a reflux condenser, and then reflux 1 h in a 100°C water bath. Cool the solution to room temperature. Filter the solution through a Büchner funnel fitted with filter paper with the aid of a vacuum.

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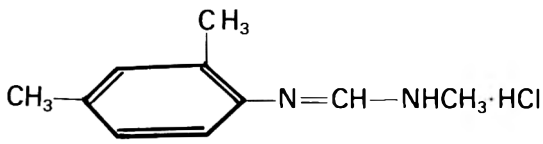

Figure 1. Structure of danjiami.

Rinse filter residues with 10 mL 2N HCl (for citrus peel, rinse with 10 mL 0.25N H_2SO_4). Collect filtrates to another flat-bottom flask, add 40 mL 10N NaOH, and reflux 1 h in a 95°C water bath.

Extraction and Cleanup

Transfer hydrolysis solution to a 250 mL separatory funnel. Extract twice with 2 × 30 mL petroleum ether. Let phases separate, discard lower phase, and collect petroleum ether phase to another 250 mL separatory funnel. Repeat partitioning with 2 × 30 mL 1N HCl, discard organic phase, combine aqueous phase into another 250 mL separatory funnel, and add 10 mL 10N NaOH. Extract twice with 2 × 20 mL petroleum ether, discard aqueous phase, combine petroleum ether extracts to a 50 mL volumetric flask through funnel containing anhydrous sodium sulfate, and adjust to the mark with petroleum ether.

Derivatization

Place a 5 mL aliquot of the above extract in a 20 mL screw-cap tube, add a drop of deionized water and 8 µL HFBA, and treat 1 h in a 50°C water bath. Shake tube gently several times during the procedure. On cooling, add 5 mL saturated $NaHCO_3$ into the tube, and shake mixture 1 min. Let phases separate, and inject 2 µL derivatized sample (organic phase) into the GC system.

Recovery Test

Spike untreated control samples with danjiami standard solution at fortification levels of 0.25, 0.5, 1, and 2 ppm, and analyze each sample by the proposed procedure.

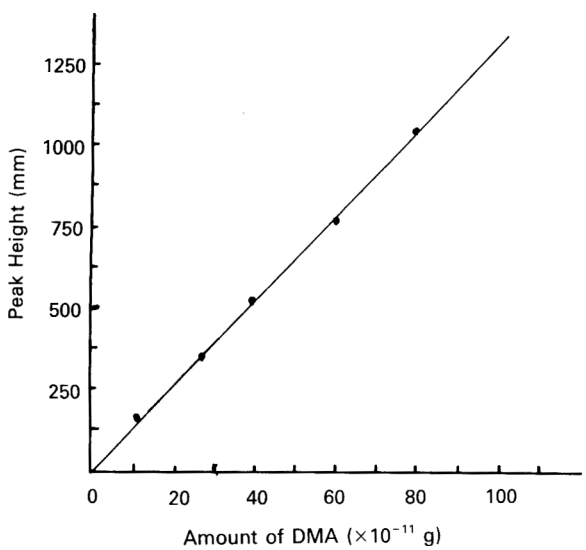

Figure 2. Standard curve for DMA.

Table 1. Recovery from control samples fortified with danjiami

Sample	Level, ppm	Rec., %		
		n	Range	$\bar{X} \pm$ SD
Citrus pulp	0.25	6	78.7–102.9	86.8 ± 10.7
	1.0	6	75.7–79.7	77.2 ± 1.7
	2.0	6	79.2–87.7	83.2 ± 6.3
Av.				82.4 ± 6.2
Citrus peel	0.5	5	74.6–86.2	80.5 ± 4.1
	1.0	5	74.8–85.8	82.4 ± 4.6
	2.0	5	80.7–86.8	84.1 ± 5.0
Av.				82.3 ± 4.6
Soil	0.25	4	86.4–90.8	89.1 ± 2.0
	0.5	4	77.5–82.6	80.0 ± 2.3
	1.0	4	75.6–81.4	79.3 ± 2.6
Av.				82.8 ± 2.3

Calculation

Generate standard curve of the instrument response from the HFBA derivative of DMA by plotting peak height (Y) vs amount on-column (X). The standard curve is plotted in Figure 2. Calculate the residue concentration of danjiami equivalents in the sample by using the following equation:

$$\text{danjiami (ppm)} = (A \times C \times 109)/(B \times D \times 0.609)$$

where A = amount calculated from standard curve, g; B = sample weight, g; C = final volume of extract, mL; D = inject volume, µL; and 0.609 = DMA–danjiami transfer factor.

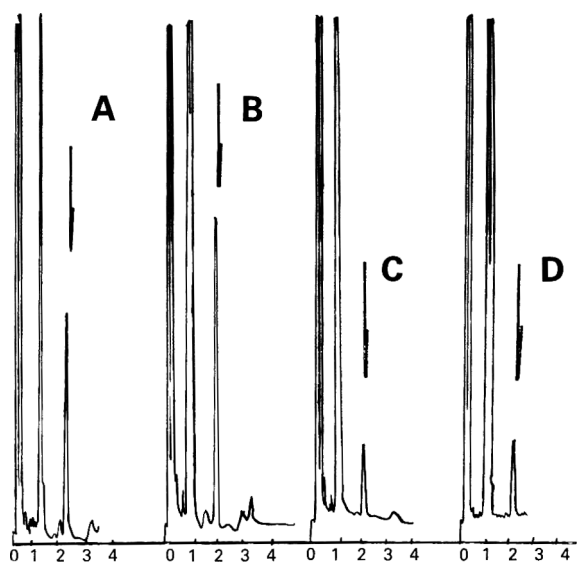

Figure 3. Gas chromatograms of DMA–HFBA and samples fortified with danjiami: A, DMA–HFBA standard (0.24 ng); B, citrus peel sample (0.32 ng); C, citrus pulp sample (0.08 ng); and D, soil sample (0.07 ng).

Table 2. Linear range and theoretical detectable limits of danjiami in citrus and soil

Sample	Linear range, ng	Sample weight, g	Final volume of extract, mL	Inject volume, μL	Theoretical detectable limits, ppb
Citrus pulp	0.01–1	40	50	2	5
Citrus peel	0.01–1	10	50	1	1
Soil	0.01–1	40	50	2	5

Results and Discussion

The mean recoveries, standard deviations, and corresponding coefficients of variation are listed in Table 1. Recovery ranges of danjiami from citrus pulp fortified with 0.25–2 ppm, from citrus peel fortified with 0.5–2 ppm, and from soil fortified with 0.25–1 ppm were 75.7–102.9%, 74.6–86.8%, and 75.6–90.8%, respectively. The average recoveries of danjiami from citrus pulp, citrus peel, and soil at all levels were 82.4 ± 6.2% (n = 18), 82.3 ± 4.6% (n = 15) and 82.8 ± 2.3% (n = 12), respectively. Typical chromatograms of standard sample and citrus and soil samples are shown in Figure 3.

Linear range and theoretical detectable limits in citrus and soil are summarized in Table 2. The ECD peak height response was linear over a 0.01–1 ng range; the average correlation coefficient for the peak height linearity of the standard curve solution was 0. 9908 within 4 days.

In hydrolysis experiments with different acids, danjiami recoveries in citrus peel fortified at 1 ppm averaged 82.4% (n = 5) with 2N H_2SO_4 and 78.0% (n = 5) with 2N HCl; therefore, sulfuric acid is better suited for hydrolysis of citrus peel.

Acknowledgments

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PESTICIDE FORMULATIONS

Liquid Chromatographic Determination of Methazole in Technical and Formulated Products: Collaborative Study

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An isocratic normal-phase liquid chromatographic (LC) method for measuring methazole in technical and formulated products was collaboratively studied by 12 laboratories. Samples of technical or formulated products are dissolved in 1,4-dioxane–hexane containing 2-chloro-5-nitrobenzophenone as internal standard. Following centrifugation and further dilution, the sample is analyzed by LC on a 25 cm × 4.6 mm stainless steel column packed with 5 μm silica, with ultraviolet detection at 254 nm. Premixed 1% 2-propanol in hexane is used as the mobile phase. Peak areas are obtained at the retention times corresponding to the internal standard and methazole. The quantity of active ingredient is determined by comparing the area ratio of active ingredient to internal standard in the sample with the same ratio in a single calibration solution. Collaborators were provided with 1 Youden matched pair for each of the technical, 75% wettable granular, and 4 lb/gal flowable products studied. The reproducibility relative standard deviations (RSD_R) for the product types studied were 1.05% for technical, 1.80% for granular, and 4.76% for flowable. The method has been adopted first action by AOAC International.

Methazole, 2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione, is a selective grass herbicide for use in cotton, onion, and garlic (1). It is particularly effective against pigweed and lambsquarters (2). Methazole is the active ingredient in Probe® (registered trademark of Sandoz Ltd, Basel, Switzerland).

Methods of analysis for methazole include an AOAC infrared (IR) spectrophotometric method (3), a gas chromatographic method (4), and a normal-phase liquid chromatographic (LC) method (5). The present collaborative study evaluated the normal-phase LC method for analysis of technical and formulated methazole products. Of the significant advantages of the LC method over the IR method, increased selectivity and automation of sample analysis are probably the most pronounced.

Submitted for publication November 1, 1991.
The recommendation was approved by the General Referee and the Committee on Pesticide Formulations and Disinfectants and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.*, January issue.

Collaborative Study

Each collaborating laboratory received 1 Youden matched pair for each of the methazole formulations included in the study: a technical product, a 75% wettable granular product, and a 4 lb/gal flowable product. Collaborators were instructed to perform single determinations on each sample by the LC method provided.

992.02 Methazole in Technical and Pesticide Formulations—Liquid Chromatographic Method

First Action 1992

(Applicable to technical products and wettable granular and flowable formulations containing methazole as only active ingredient)

Method Performance:

Technical product, 99.6%

$s_r = 0.81$; $s_R = 1.04$; $RSD_r = 0.81\%$; $RSD_R = 1.05\%$

Wettable granular product, 75%

$s_r = 1.40$; $s_R = 1.40$; $RSD_r = 1.80\%$; $RSD_R = 1.80\%$

Flowable product, 4 lb/gal

$s_r = 1.92$; $s_R = 1.92$; $RSD_r = 4.76\%$; $RSD_R = 4.76\%$

A. Principle

Methazole is determined by normal-phase liquid chromatography (LC), using 2-chloro-5-nitrobenzophenone as internal standard, and premixed isocratic mobile phase of 1% 2-propanol in hexane.

B. Safety

Acute oral LD_{50} is 2501 mg/kg; acute dermal LD_{50} is 12 500 mg/kg (Sandoz Crop Protection).

Technical methazole should be handled carefully; contact may result in chloracne or skin irritation. 1,4-Dioxane is cancer suspect agent; use only in exhaust hood. To minimize formation of peroxides, do not store solutions for prolonged periods of time. Use personal protective equipment, including safety glasses, disposable gloves, and laboratory coats, when handling samples. Perform sample and standard preparation in hood with protective sash. Disposal of samples, standards, and wasted materials must be conducted in compliance with on-site safety policies and procedures.

C. Apparatus

(**a**) *Liquid chromatograph.*—Able to generate >1500 psi, with UV absorbance detector capable of measurement at 254 nm.

(**b**) *Chromatographic column.*—250 × 4.6 mm id, packed with 5 μm silica (Whatman Partisil 5® is suitable). Guard column 3 × 0.125 in., packed with 37–50 μm silica (Corasil®, Waters Associated, is suitable). Operating conditions: column temperature, ambient; mobile phase, 2-propanol–hexane (10 + 1000) premixed; flow rate, 1.0 mL/min; injection volume, 50 μL. Retention times: internal standard, ca 5.4 min; methazole, ca 9.7 min. Pump mobile phase until system is equilibrated (flat baseline). Allow 11 min between injections.

D. Reagents

(**a**) *Solvents.*—2-Propanol and 1,4-dioxane, LC grade; hexane, UV grade.

(**b**) *Internal standard solution.*—Accurately weigh ca 520 mg (to nearest 0.1 mg) 2-chloro-5-nitrobenzophenone (purity ≥98%) into 50 mL volumetric flask. Dissolve and dilute to volume with 1,4-dioxane.

(**c**) *Methazole reference standard solution.*—Accurately weigh ca 75 mg (to nearest 0.1 mg) methazole reference standard (Sandoz Crop Protection Corp., 1300 E Touhy Ave, Des Plaines, IL 60018) into 2 oz bottle. Pipet 5 mL internal standard solution into bottle. Pipet 20 mL hexane into bottle and mix well. Pipet 5 mL of this solution into another 2 oz bottle. Add, by pipet, 25 mL hexane and shake well. Pipet 5 mL of this solution into 4 oz bottle. Add, by pipet, 50 mL hexane. Shake well. Use this solution for response measurements. Ambient temperature should be >17° for effective solvation of methazole.

E. Preparation of Sample

Use mortar and pestle to grind granular formulations (ca 2.0 g). Mix flowable formulations well to ensure sample homogeneity. Accurately weigh portion containing ca 75 mg (to nearest 0.1 mg) methazole into 2 oz bottle. Pipet 5 mL internal standard solution into bottle. Shake well (ca 5 min) until sample is completely dispersed (or dissolved). Pipet 20 mL hexane into bottle. Shake 15 min. Centrifuge 10 min at 1700 rpm to obtain clear supernate. Pipet 5 mL clear supernate (or solution) into another 2 oz bottle. Add, by pipet, 25 mL hexane. Shake well. Pipet 5 mL of this solution into 4 oz bottle. Add, by pipet, 50 mL hexane. Shake well. Use this solution for sample analysis. Prepare sample at ambient temperature (>17°).

F. Determination

Make repetitive 50 μL injections of standard solution into chromatograph. Divide peak area of methazole by that of internal standard to determine response ratio. Response ratios must agree within ± 1%. Average duplicate response ratios obtained with standard solution, **D**(**c**). Inject sample solution in duplicate. Sample response ratios must agree within ± 1%. Average sample response ratios. Inject standard solution in duplicate after 6 or less sample injections. Average response ratios of standard immediately preceding and following sample injection must agree within ± 1%. If not, repeat determination.

G. Calculation

$$\text{Methazole, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = average response ratios for sample and standard solutions, respectively; W and W' = weight (g) of sample and standard, respectively; P = % purity of standard.

Ref.: *J. AOAC Int.* (1992) **75**, November/December issue
CAS-20354-26-1 (methazole)

Results and Discussion

Twelve collaborators returned complete sets of data for each of the 3 products tested in this study (Table 1). Cochran and Grubbs (single and double) tests were used for detection of outliers. Results of collaborator 12 for the technical product were flagged by the Cochran test as outliers. Results of collaborator 7 were flagged by the single Grubbs test as outliers for the same product, after results for collaborator 12 were omitted. Recycling through the Cochran and Grubbs tests, after omitting results for collaborator 7, did not justify omitting results for collaborator 5 for the technical product as outliers. Results of collaborator 7 for the 75% granular product were flagged as outliers by the single Grubbs test. Outliers are marked in Table 1 and were excluded from the calculation of the statistics. The cause of these results was not apparent from chromatograms submitted by collaborators 7 and 12. No outliers were detected for the flowable (4F) product. Overall, RSD_R values for technical and granular products are good. The somewhat larger value for the flowable formulation (4.76%) is consistent with data for this type of formulation, which tends to undergo phase separation rather fast, affecting sample homogeneity and subsequent representative sampling.

Analysis of known synthetic formulations, spike and recovery studies, material balance analysis, and an investigation of impurities commonly found in technical methazole were conducted using this collaborated method. Results indicate this method is free from interferences (Figure 1).

Table 1. Collaborative results for LC determination of methazole (%) in technical product and 4 lb/gal flowable (4F) and 75% wettable granular (WG) formulations

	Technical		4F		75% WG	
Coll.	1	2	1	2	1	2
1	99.36	100.05	43.53	38.70	76.81	76.80
2	100.70	99.12	36.32	42.52	80.41	73.67
3	98.55	98.17	40.84	39.79	78.91	76.96
4	99.57	100.70	42.26	40.80	78.28	78.02
5	101.99	100.71	40.47	39.46	78.33	76.83
6	99.42	99.11	39.88	38.38	77.89	76.54
7	105.53[a]	105.53[a]	40.65	42.16	83.95[a]	82.53[a]
8	98.60	97.98	42.91	39.26	76.60	77.73
9	99.96	99.15	40.47	39.31	78.09	77.13
10	98.60	100.70	39.50	38.90	78.10	77.20
11	99.58	99.96	39.66	39.32	78.85	77.12
12	99.00[b]	112.20[b]	42.00	42.40	77.80	76.70
Mean	99.60		40.40		77.49	
s_r	0.81		1.92		1.40	
s_R	1.04		1.92		1.40	
RSD_r, %	0.81		4.76		1.80	
RSD_R, %	1.05		4.76		1.80	

[a] Outlier by single Grubbs test. Omitted from statistical evaluation.
[b] Outlier by Cochran test. Omitted from statistical evaluation.

Collaborators' Comments

One collaborator reported difficulty dissolving methazole in the internal standard solution. Investigation showed that solvation was attempted in a 10°C laboratory hood. No further problems were experienced when solvation was attempted in a warmer (17°C) laboratory. Therefore, the sample preparation procedure was amended to reflect this. No other problems were reported.

Recommendation

It is recommended that the normal-phase LC method for the determination of methazole in technical and formulated products be adopted first action.

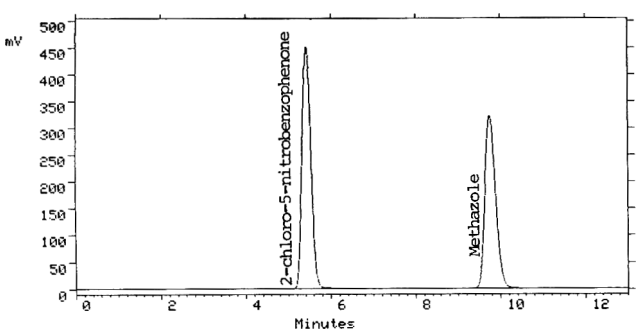

Figure 1. Typical liquid chromatogram of methazole and internal standard.

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SUGARS AND SUGAR PRODUCTS

$\delta^{18}O$ Measurements in Water for Detection of Sugar Beet-Derived Syrups in Frozen Concentrated Orange Juice: Collaborative Study

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Stable isotope ratio mass spectrometry has shown that pure frozen concentrated orange juices (FCOJ) of 63–67° brix possess a mean $\delta^{18}O$ value of +14.28‰, with a standard deviation of 1.80. Beet invert syrups, which are produced using ground water, possess negative values. As a result, $\delta^{18}O$ values decrease on addition of such syrups to FCOJ. Samples with values less than +8.9‰ (3 standard deviations from the mean for pure FCOJs) can confidently be considered as adulterated. A collaborative study was conducted in which a pure FCOJ and 4 samples adulterated to various levels with medium beet invert syrup were sent to each of 6 collaborators. In all but 2 instances, juices containing more than 10% beet syrup would have been classified as adulterated by the collaborators; none would have classified pure juice as adulterated. The plot of mean $\delta^{18}O$ values for all collaborators at each adulteration level has a correlation coefficient >0.999. The method has been adopted first action by AOAC International.

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The recommendation was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.*, January issue.
Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
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Sophisticated approaches have been required to detect some of the concoctions elaborated in recent years for use as fruit juice adulterants. Orange juice has been a popular target for adulteration, especially since 1977, when the first in a series of severe Florida freezes caused the demand for orange juice to exceed its domestic supply. Methods are now available for monitoring the addition to juices of major adulterants, such as sugar syrups derived from corn and sugar cane (1, 2) and orange pulpwash solids (3).

Mixtures of orange juice and the adulterants high fructose corn syrup (HFCS) or cane invert syrups are detected by mass spectrometry using stable carbon isotope ratio analysis (SCIRA). The basis of the test is that most elements have light and heavy stable isotopes, and there are small but measurable source-dependent differences in their ratios. The isotopes ^{13}C and ^{12}C in atmospheric carbon dioxide are fractionated to different extents during photosynthesis by the C_3 (Calvin) and C_4 (Hatch–Slack) pathways. As a result, products from C_3 plants, including orange juice, have lower $\delta^{13}C$ values than do products from C_4 plants, such as HFCS and cane syrups. Adulterated mixtures possess intermediate ratios. A volume (4) on juice adulteration includes 2 chapters on the application of stable isotope ratio variations (5, 6).

Inexpensive syrups prepared by hydrolysis of sugar beet sucrose cannot be detected in orange juice from $\delta^{13}C$ values because sugar beets are C_3 plants. As a result, such syrups have been added by some processors to circumvent their detection by SCIRA. An isotopic approach was suggested from studies of the deuterium content of beet and wine sugars (7). D/H ratios of carbon-bound atoms in beet sucrose were shown to be consistently lower than the ratio in sugar from wines. It was then found that the difference in δD values (8, 9) between beet and orange juice sugars is even greater. $\delta^{18}O$ values were also different, and a discriminatory formula using ratios of both elements was proposed that would describe with 99.99% confidence the province of pure juices (9). Sample preparation and mass spectrometric measurements are not as straightforward as in SCIRA. Also, δD determination requires nitration of a dry

sugar fraction prepared from the juice, so that all exchangeable oxygen-bound D and H atoms are replaced. A suggestion (10) worth evaluating to enhance sensitivity of that approach involves determining δD, $\delta^{13}C$, and $\delta^{18}O$ values in both the pulp (essentially carbohydrate) and soluble sugar fractions of juice. The ratios in pulp serve as markers for the pure juice, and those in the soluble sugars reflect contributions from both juice and adulterant. Differences in ratios between the 2 fractions greater than those found in pure juices would indicate adulteration.

Isotopic analyses of water have been useful for testing orange juice authenticity. δD and $\delta^{18}O$ values in ground (tap) water are a function of environmental variables such as temperature and altitude, but they are invariably negative and less than those in plants growing in this water. This provided the basis for tests to distinguish natural fruit juice from those reconstituted from concentrate by addition of ground water (11). Isotope effects associated mainly with evapotranspiration cause most enrichment in the heavy isotopes to be in plant leaf water, but enrichment also occurs in all fruits and vegetables surveyed (12). These studies suggest that ground water associated with the manufacture of products such as beet syrups and orange pulp wash could be detected in a variety of fruit and vegetable juices and used as a marker for adulteration.

Collaborative Study

The pure frozen juice concentrate (62.6° brix) component of the collaborative samples was prepared after mixing equal proportions of hamlin, valencia, and pineapple orange juices. Medium beet invert syrup (Great Western Sugar Co.) was diluted with water to 62.6° brix and then mixed thoroughly with the orange juice. Mixtures containing 45, 60, 75, and 90% orange juice were prepared. Two ounces of the mixtures and 2 oz pure orange juice were added to fill nalgene bottles, and the bottles were then sealed with tape and frozen. They were sent via express mail under cold packs to the collaborators.

Sophisticated expertise was required of the collaborators, who possess dedicated sample preparation systems and specialized isotope ratio mass spectrometers. Each of the stable isotope ratio analysis (SIRA) laboratories has established what it considers to be optimal experimental protocol, based on extensive experience. These protocols vary in detail among laboratories. The procedures described in the present method have been demonstrated to be very effective for sample preparation (13) and for determining $^{18}O/^{16}O$ values.

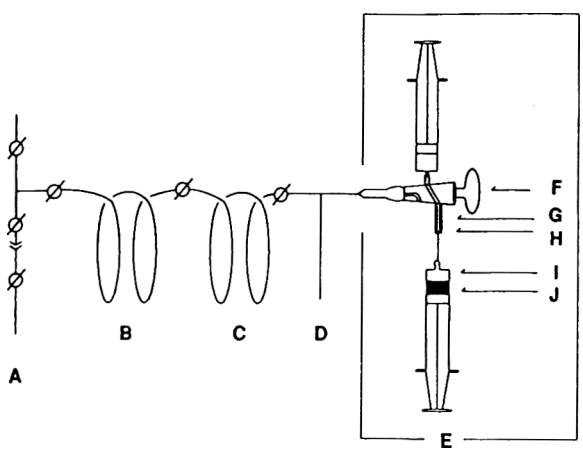

Figure 992.09. Schematic diagram of vacuum line for collection and purification of CO_2: (A) sample bottle for collection of CO_2; (B) multitrap 2; (C) multitrap 1; (D) cold finger; (E) sample inlet, illustrated in detail; (F) 3-way stopcock; (G) capillary glass tubing; (H) silicone rubber septum; (I) equilibrated CO_2 in gaseous phase; (J) water sample.

992.09 Sugar Beet-Derived Syrups in Frozen Concentrated Orange Juice—$\delta^{18}O$ Measurements in Water/Stable Isotope Ratio Mass Spectrometric Method

First Action 1992

(Applicable to classification of frozen concentrated orange juice with $\delta^{18}O$ value < +8.9‰ as diluted with ground water-prepared product.)

Method Performance:

100 and 90% orange juice samples, mean $\delta^{18}O$ value = 12.24‰

$s_r = 0.88$; $s_R = 1.81$; $RSD_r = 7.2\%$; $RSD_R = 14.8\%$

75 and 60% orange juice samples, mean $\delta^{18}O$ value = 6.73‰

$s_r = 0.30$; $s_R = 1.76$; $RSD_r = 4.5\%$; $RSD_R = 26.1\%$

A. Principle

Sample is equilibrated with CO_2 to achieve isotopic equilibrium between oxygen in sample water and oxygen in CO_2. After equilibration, CO_2 is removed and purified, and $^{18}O/^{16}O$ is measured by isotope ratio mass spectrometer. Differences in $\delta^{18}O$ values for pure orange juice concentrate (63–67° brix; mean $\delta^{18}O_{SMOW} = +14.28$‰; SMOW is standard mean ocean water) and ground water used in preparation of sugar beet syrups (usual range of $\delta^{18}O_{SMOW} = -5$ to -10‰) provide a measure of beet syrup in orange juice concentrate.

B. Apparatus

(a) *Equilibration system.*—60 mL plastic syringes, 2.5 cm 22 gauge needles, 1.8 cm diam. steel balls, 40 cm diam. wheel to which 12 syringes can be attached and rotated at 10–12 rpm by electric motor.

(b) *Purification system.*—Vacuum-tight glass manifold including liquid nitrogen traps, sample collection bottle, and high vacuum source (Figure **992.09**).

(c) *Mass spectrometer.*—Instrument designed or modified for isotope ratio measurement and capable of accuracy of 0.01% of abundance at mass 46 (VG Micromass 602, 903, PRISM VG Instruments Inc., 32 Commerce Centre, Cherry Hill Dr, Danvers, MA 01923, or equivalent).

Table 1. $\delta^{18}O$ values (‰) for 63–67° brix orange juices from different growing areas[a]

Source	No. of samples	Mean, ‰	Range, ‰ Low	Range, ‰ High	SD	CV, %
Florida	15	+14.06	+12.3	+16.2	1.12	8.0
California	6	+14.57	+12.0	+17.6	2.67	18.3
Brazil	9	+15.10	+12.8	+18.2	1.98	13.1
Others	4	+12.87	+11.5	+15.0	1.43	11.1
All samples	34	+14.28	+11.5	+18.2	1.80	12.6

[a] From reference 14.

C. Reagents

(a) *Pressurized carbon dioxide.*—100%.

(b) *Reference water standard.*—Standard mean ocean water (SMOW).

(c) *Carbonate standards.*—*(1)* NIST SRM 20 Solenhofen limestone ($\delta^{13}C_{PDB} = -1.06‰$; $\delta^{18}O_{PDB} = -4.14‰$). *(2)* NIST SRM 19 TS limestone ($\delta^{13}C_{PDB} = 1.92‰$; $\delta^{18}O_{PDB} = -2.19‰$), or equivalent. (PDB is Pee Dee belemnite limestone standard.)

(d) *Phosphoric acid.*—100%

D. Preparation of Sample

Place steel ball in barrel of syringe, and pour 30 mL sample into barrel. Insert plunger and expel as much air as possible from syringe. Attach 2.5 cm 22 gauge needle. Use rubber septum attached to pressurized reservoir of pure CO_2 to add 20 cc of CO_2. Remove syringe from septum, and seal needle by piercing a #000 rubber stopper. Attach syringe to wheel, and rotate in vertical plane at 10–12 rpm for ≥2 h. Maintain air (sample) temperature at 25 ±0.5°. Insert needle into septum on preparation line, Figure **992.09**. Transfer CO_2 from syringe into preparation line; move CO_2 into evacuated portion of preparation line up to first stopcock, and cool finger with liquid nitrogen. Pump away any air through multitrap 1, also cooled in liquid nitrogen. Water vapor is retained in multitraps 1 and 2, which are cooled with dry ice–acetone slurry. For each sample set, reference water standard (SMOW) should be analyzed 2×. Analyze a standard before and after samples.

E. Determination

Calibrate reference gas of mass spectrometer using at least 2 carbonate standards. React carbonate standards with 100% H_3PO_4 at 25 ±0.1°. Make any necessary corrections due to instrumental error, such as zero enrichment, peak tailing, or gas mixing. Correct for ^{17}O contribution to mass 45.

F. Calculations

Calculate $\delta^{13}C$ and $\delta^{18}O$ from as follows:

$$\delta^{H}E\ (‰) = [(^{H}E/^{L}E_{Sample})/(^{H}E/^{L}E_{Std}) - 1] \times 1000$$

where ^{H}E and ^{L}E are heavy and light isotopic species of element E, respectively. A frozen orange juice concentrate with $\delta^{18}O$ value of +15‰ would have an $^{18}O/^{16}O$ ratio 15‰ greater than $^{18}O/^{16}O$ ratio in SMOW and would be "heavy" in ^{18}O relative to the standard.

Convert analytical data obtained relative to reference gas to PDB scale using the following equation:

$$\delta_{(x-PDB)} = \delta_{(x-B)} + \delta_{(B-PDB)} + \{ (\delta_{(x-B)}) (\delta_{(B-PDB)}) 10^{-3} \}$$

where $(x - B)$ and $(x - PDB)$ refer to analysis of sample (x) relative to standard *(B)* and to PDB, and $(B - PDB)$ is analysis of standard (B) relative to PDB; all δ values expressed in ‰ (standard B defined as carbon dioxide source used, derived from standard limestone, graphite, or crude oil).

Convert $\delta^{18}O_{PDB}$ values to $\delta^{18}O_{SMOW}$ by adding –0.26‰.

Correct sample values by average deviation from expected value found for water standards by adding or subtracting appropriate ‰ value. A $\delta^{18}O$ value < +8.9‰ for sample of frozen concentrated orange juice indicates the presence of ground water-prepared product (typically sugar beet-derived syrups).

Ref.: *J. AOAC Int.* (1992) **75**, November/December issue

Results and Discussion

Orange juice is normally stored and transported as 63–67° brix concentrate, and is retailed either as a concentrate at about 42° brix or as single strength juice at about 12° brix. Conversion of single strength juices to 63–67° brix concentrates results in the preferential evaporation of "light water" and causes $\delta^{18}O$ values to increase by about 10‰ units. Ground water is almost invariably negative in $\delta^{18}O$, and it is used in the manufacture of beet invert syrups. Adding beet invert syrups to 63–67° brix concentrates causes $\delta^{18}O$ values to decrease. The more beet invert syrup added, the lower the resulting $\delta^{18}O$ value in the adulterated frozen orange juice concentrate.

Earlier we surveyed $\delta^{18}O$ values of water in orange juices (14), and the results for the 63–67° brix orange juice concentrates are listed in Table 1. The mean $\delta^{18}O$ value for 34 samples was +14.28‰, with a standard deviation of 1.80. We recommend a cutoff of +8.9‰ for orange juice concentrates over 62° brix. This is 3 standard deviations from the mean, so it is extremely probable that a pure concentrate will have $\delta^{18}O$ value greater than +8.9‰.

Results of the collaborative study are compiled in Table 2. In all but 2 instances, juices containing more than 10% beet syrup would have been classified as adulterated by the collab-

Table 2. $\delta^{18}O$ values (‰) for collaborative samples of beet invert syrup/orange juice mixtures

	Sample				
Collaborator	5 (pure)	1 (90% OJ)	4 (75% OJ)	2 (60% OJ)	3 (45% OJ)
1	+15.4	+13.1	+10.2	+7.2	+3.7
2	+13.1	+ 8.7	+ 6.1	+3.0	+0.8
3	+11.4	+ 9.6	+ 7.0	+4.3	+1.3
4	+15.7	+13.1	+10.6	+7.0	+3.6
5	+13.5	+10.7	+ 8.2	+5.8	-0.4
6	+11.6	+11.0	+ 7.0	+4.4	-3.0

orators, and none would have been misclassified pure juice as adulterated. From these data, the limit in discriminating between false negatives is between 60 and 75% orange juice. Results from the study were analyzed by using a Youden matched pair analysis to estimate repeatability and reproducibility. Samples 1 and 5 were paired, as were samples 2 and 4. Sample 3, with less than 50% pure frozen orange juice concentrate, did not have a matching sample. For samples 5 and 1, the average and estimates of repeatability and reproducibility are as follows: average, 12.24‰; s_r, 0.88; s_R, 1.81; RSD_r, 7.2%; RSD_R, 14.8%. Values for samples 4 and 2 are as follows: average, 6.73‰; s_r, 0.30; s_R, 1.76; RSD_r, 4.5%; RSD_R, 26.1%.

Both pure orange juice concentrates (Table 1) and beet syrups possess a range of values. Therefore, from $\delta^{18}O$ values in a suspect sample, the level of adulteration cannot be assigned but the degree of certainty as to purity can be. This is also the case for the $\delta^{13}C$ adulteration detection methods.

The plot of the mean $\delta^{18}O$ values from data reported by all collaborators at each adulteration level has a correlation coefficient greater than 0.999 (Figure 1). The cutoff value of +8.9‰ for pure juices is indicated on the x-axis. The range shown at each adulteration level represents twice the standard deviation at that level and indicates that all adulteration levels were significantly different from one another. The intercept at 0% orange juice is −6.2‰, a $\delta^{18}O$ value typical for pure beet invert syrups (14).

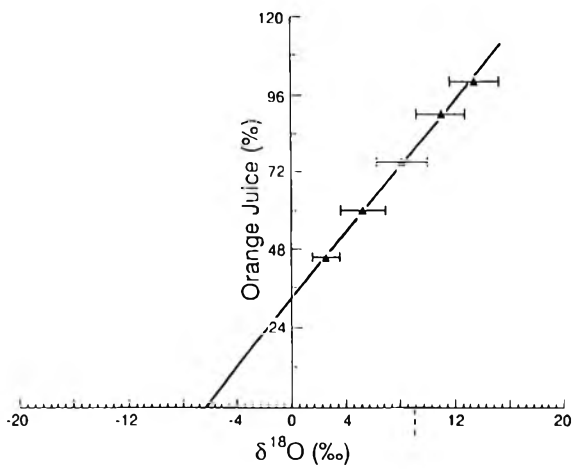

Figure 1. Plot of mean $\delta^{18}O$ value for pure product and each level of adulteration of frozen orange juice concentrate with beet invert syrup for all collaborators. Line is described by Y = 5.0839X + 32.841; r = 0.99907. ⊢⊣ = $t_{0.05} \times (S^2/6)^{1/2}$. Dashes indicate cutoff value at 8.9‰ for pure juices.

Recommendation

We recommend that the stable isotope ratio mass spectrometric method for detection of sugar beet-derived syrups in frozen concentrated orange juice by using $\delta^{18}O$ measurements in water be adopted first action.

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VITAMINS AND OTHER NUTRIENTS

Determination of Tryptophan Content in Infant Formulas and Medical Nutritionals

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A method was developed for determination of total tryptophan content in soy- and milk-based nutritional products. The method uses enzymatic (pronase) digestion of the protein to release tryptophan, which is separated and quantitated by isocratic reversed-phase liquid chromatography with fluorescence detection. Enzymatic digestion is completed for products containing these types of proteins in less than 6 h and is accomplished under chemically mild conditions (pH 8.5, 50°C), which do not significantly degrade tryptophan. Chromatographic separation is complete in about 8 min, including an internal standard. The precision of the method is 1–2% relative standard deviation. Accuracy is demonstrated by agreement with theoretical values for standard proteins (amino acid sequence known) and by quantitative recoveries of overspikes, which use either free tryptophan or a standard protein as the spiking material. The method allows determinations on samples containing a wide range of tryptophan values. Appropriate sample size selection and verification of digestion time requirements should allow the method to be applied to different protein types as well. The method allows 24 h turnaround of tryptophan analyses, and quantitative recoveries represent a significant improvement on existing techniques applied to infant formulas and other nutritional products.

Tryptophan is an amino acid that is essential for proper human nutrition (1). Manufacturers of nutritional products are often required to demonstrate adequate tryptophan content in their products. Several analytical methods for tryptophan determination have been developed. For pure proteins and peptides, classical acid hydrolysis will suffice if antioxidant compounds such as thioglycolic acid (2) are added; but the presence of carbohydrate, as in nutritional products, results in complete or near complete destruction of the analyte (3). Hydrolysis using mercaptoethanesulfonic acid (4) or *p*-toluenesulfonic acid (5) also suffers from this limitation. Chemical derivatization has been suggested for tryptophan analysis (6), but such methods are often tedious, and completeness of reaction may be a problem. Hydrolysis in strong base, such as NaOH (7) or $Ba(OH)_2$ (8), has become the method of choice for tryptophan determinations. However, we found that this approach results in incomplete tryptophan overspike recoveries (typically 85–94%) from product samples, even if protective compounds such as starch, thiodiglycol, or ascorbic acid are added. A method using very mild hydrolysis conditions would allow recovery of tryptophan without the concomitant degradation associated with chemical hydrolysis.

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Several methods using proteases or mixtures of proteases have been reported. Examples include the use of papain (9) and pronase (10). In our experience, use of papain produces a substantial blank from autodigestion, whereas the digestion conditions reported for pronase require very long incubation times and result in incomplete release of tryptophan. In the case of pronase, the long digestion times required may be linked to the use of phosphate buffers. Calcium salts added to stabilize metalloproteases in at least one of the commercially available enzyme preparations would precipitate as calcium phosphate. The probable subsequent losses of protease activity (loss of zinc) suggests an explanation for the incomplete recoveries we have observed and the long digestion times reported (10).

We perceived a need for a generally applicable method for total tryptophan determinations that uses a mild digestion process that results in quantitative, reproducible recoveries from protein-containing nutritional products. The method described in this report uses pronase digestion under conditions in which the stabilizing calcium salts are soluble, and complete release of protein and peptide tryptophan is achieved in less than 6 h from products containing milk concentrates, caseinates, or soy protein isolates. The method also produces accurate data for bovine serum albumin and chicken egg white lysozyme, the other proteins tested thus far. Tryptophan and internal standard (5-methyl-D,L-tryptophan) in the resulting solutions are separated by an isocratic reversed-phase liquid chromatographic (LC) system (fluorescence detection) in about 8 min, with baseline resolution of the analytes. Tryptophan is quantitated by comparison with standards and subsequent subtraction of the blank (there is some autolysis).

Experimental

Apparatus

(a) *Liquid chromatograph.*—Refrigerated autosampler with 20 µL sample loop (AS 100) (BioRad, Richmond, VA

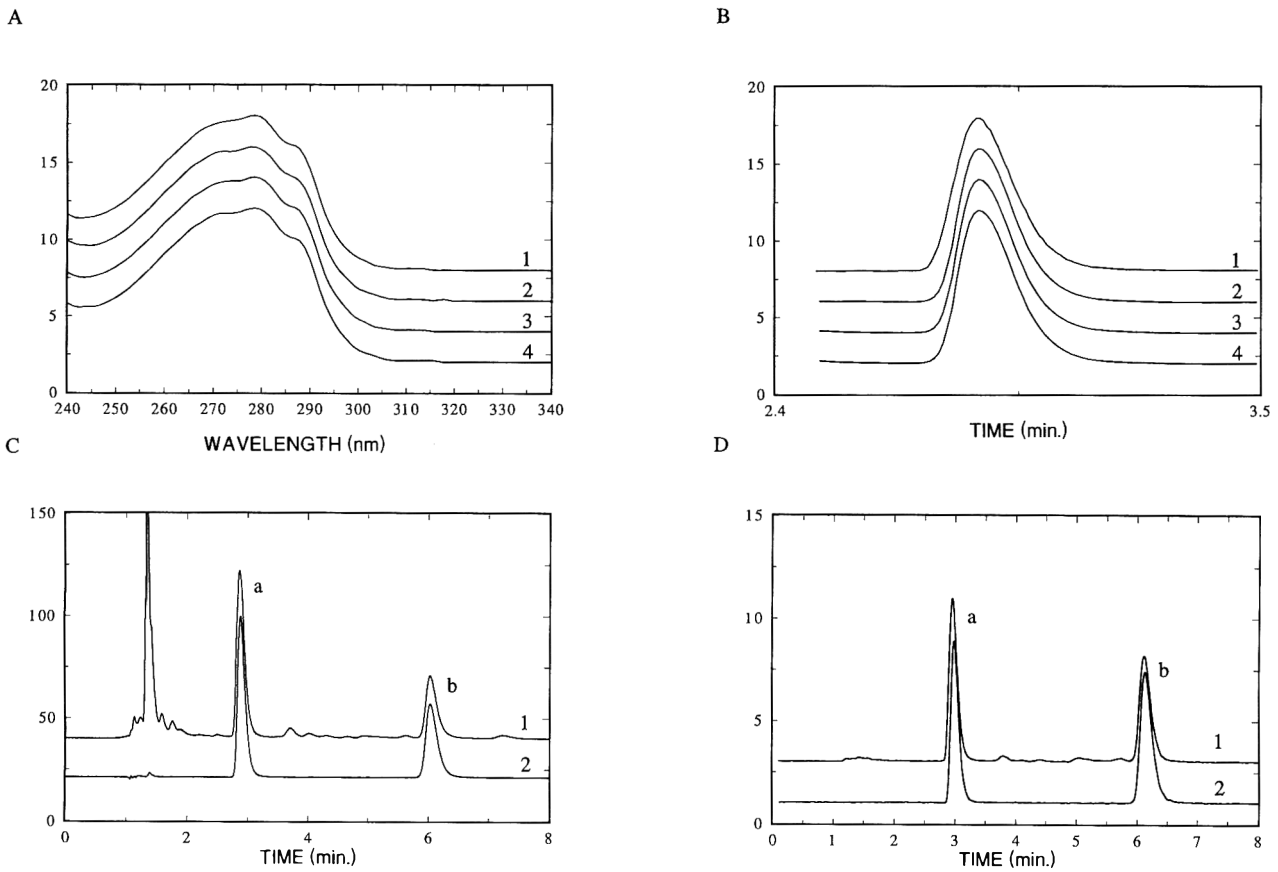

Figure 1. Peak purity. A: Absorbance spectra from the tryptophan peak apex in a standards chromatogram (1) and from the falling edge (2), apex (3), and rising edge (4) of a sample tryptophan peak. B: Peak shapes for tryptophan obtained by monitoring fluorescence (1) or absorbance at 290 nm (2), 280 nm (3), or 270 nm (4). The fluorescence elution profile was shifted by 0.1 min to allow overlay (fluorescence detector was in series with the diode array in this experiment). By monitoring either absorbance (C) at 280 nm or fluorescence (D), elution times were compared for sample (1) and standards (2) for both tryptophan (a) and 5-methyltryptophan (b).

94804), pump (LC-6A), column oven (CTO-6A), and fluorescence detector (RD-535), all from Shimadzu (Columbia, MD 21046). LC data station, equipped with A/D interface, was used for data collection (PE Nelson, Cupertino, CA 95014).

(**b**) *LC column.*—Octyl silica column, APEX II, 10 cm × 4.6 mm, 5 μm packing (Jones Chromatography, Littleton, CO 80162).

(**c**) *Heating block.*—Reacti-Therm Heating Unit, equipped with Reacti-Block T-1 (Pierce, Rockford, IL 61105).

(**d**) *LC sample filters.*—ACRODISC disposable filter assembly, 0.45 μm, 25 mm (Gelman Sciences, Ann Arbor, MI 48106).

Reagents

(**a**) *Solvent.*—LC grade methanol (Baxter Healthcare Corp., Burdick & Jackson Division, Muskegon, MI 49442).

(**b**) *Water.*—From Milli-Q Water System (Millipore Corp., Bedford, MA 01730).

(**c**) *Phosphoric acid, 85%, and sodium phosphate, monobasic.*—LC grade (Mallinckrodt, St. Louis, MO 63134).

(**d**) *L-Tryptophan.*—(United States Pharmacopeia, Rockville MD, 20852).

(**e**) *Other reagents.*—5-Methyl-DL-tryptophan, Pronase (Type XIV Protease, Bacterial), tris(hydroxymethyl)aminomethane (trizma), citric acid, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (hepes), and zinc acetate were obtained from Sigma Chemical Co. (St. Louis, MO 63178).

Solutions

(**a**) *Digestion buffer.*—0.1M Trizma, pH 8.5. Dissolve 6.06 ± 0.02 g trizma base in ca 850 mL water. Adjust pH to 8.50 ± 0.05 with dropwise addition of 1.0N HCl. Dilute to final volume of 1 L with water.

(**b**) *Buffers for pH study.—0.1M Trizma buffers.*—pH 8.0, 8.5, and 9.0; prepared as described above (**a**), except adjust pH to desired target. *Hepes buffers.*—Dissolve 11.91 ± 0.02 g free acid into ca 400 mL water. Adjust pH to 6.8, 7.5, or 8.0 by dropwise addition of 1N NaOH, and dilute to 500 mL with water. *Citrate buffers.*—Dissolve 10.50 ± 0.02 g citric acid monohydrate in ca 400 mL water, adjust pH to 5.0, 6.0, or 6.8

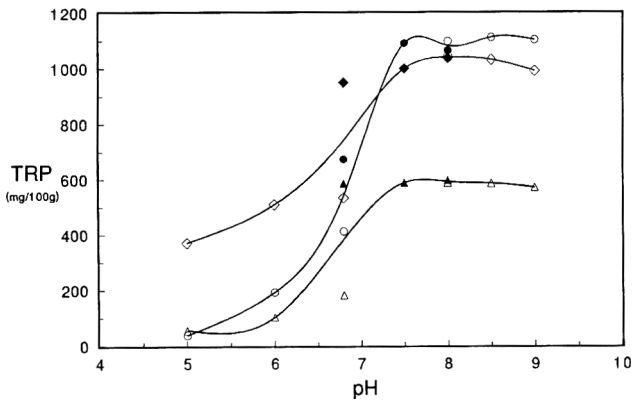

Figure 2. pH dependence of hydrolysis. Samples (50 mg protein) of 3 different proteins were dissolved in buffers of various pH values (see text for details of buffer composition) and digested 24 h with pronase (10 units) at 50°C. Tryptophan was determined as detailed in text. Data shown are averages of 2 replicate determinations. Proteins were soy protein isolate (circles), caseinate (diamonds), and bovine serum albumin (triangles). Open symbols are used for citrate and trizma buffers. Closed symbols are used for hepes buffers.

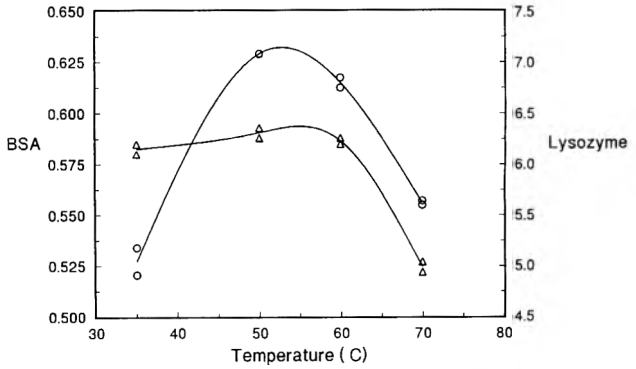

Figure 3. Temperature dependence of hydrolysis. Samples (50 mg protein) of 2 proteins (chicken egg white lysozyme [triangles] and bovine serum albumin [circles]) were dissolved in 0.1M trizma buffer, pH 8.5, and digested 24 h with pronase (10 units) at various temperatures. Tryptophan was determined as detailed in text. Data shown (results are g tryptophan/100 g sample) are individual determinations for each temperature. Expected results for bovine serum albumin (594 mg/100 g sample) and chicken egg white lysozyme (7700 mg/100 g sample) were derived as described in the text (*Results*).

by dropwise addition of 1N HCl, and dilute to 500 mL with water.

(c) *Pronase stock solution.*—Dissolve 0.200 ± 0.002 g pronase (1000 ± 80 units of enzyme activity; 1 unit will hydrolyze casein to produce 1.0 μmol tyrosine equivalents/min at 37°C and pH 7.5) to final volume of 50 mL with digestion buffer. Avoid excessive foaming; do not shake.

(d) *Internal standard stock solution.*—Dissolve 0.400 ± 0.002 g 5-methyl-D,L-tryptophan in water to final volume of 500 mL. Record mass of 5-methyl-D,L-tryptophan for use in calculations.

(e) *Tryptophan stock solution.*—Dissolve 0.100 ± 0.002 g tryptophan in water and dilute to 1 L. Record mass of tryptophan for use in calculations.

(f) *Working standards solutions.*—Prepare 3 standard solutions in 50 mL volumetric flasks. Add 0.5 mL internal standard stock solution and 10 mL methanol to each flask. Then, for tryptophan stock solutions, add 1.0 mL for low standard, 5.0 mL for mid standard, and 10.0 mL for high standard solutions. Dilute with water to 50 mL final volume.

(g) *LC mobile phase.*—Dissolve 13.8 ± 0.1 g sodium phosphate monobasic in exactly 2.0 L water. Add 7.0 mL 85% phosphoric acid with mixing. Add 500 mL methanol, mix thoroughly, and filter through 0.45 μm filter. Place in LC solvent reservoir.

Sample Preparation

Set heating unit to 50°C, and let equilibrate before digestion. Weigh sample containing between 50 and 700 μg tryptophan (typical proteins used in nutritional products contain ca 1% tryptophan, and a sample size of 30–50 mg protein is usually appropriate; a volume of ≤1.5 mL is preferred) directly into duplicate 16 × 100 mm culture tubes. Duplicate blank tubes are also prepared, with no sample added. Add 0.5 mL internal standard stock and 3.0 mL digestion buffer to each tube. Add 0.5 mL pronase stock solution to each tube, and mix thoroughly, taking care to minimize foaming (this can inactivate the enzyme). Cap and place all tubes in 50°C heating block, and incubate 6 h.

Remove samples and let cool. Quantitatively transfer each sample to 50 mL volumetric flask. Add 10 mL methanol, and dilute to 50 mL final volume with water. Mix thoroughly, and filter aliquot through 0.45 μm LC filter into autosampler vial. Samples can be run immediately or stored frozen for up to 1 week.

Chromatography System

(a) *Parameters.*—Set column oven to 50°C and pump flow rate to 1 mL/min. Set autosampler temperature to 8°C, and equilibrate. Set fluorescence detector excitation wavelength to 295 nm and emission wavelength to 345 nm, using low-sensitivity setting with fast response. Condition column with mobile phase for 1 h or until stable baseline is observed. Ignite detector lamp at least 30 min before analyses.

(b) *System suitability check.*—Make injections of standard mixture, such as mid standard solutions (f). Acceptable performance is demonstrated by 2 criteria: (*1*) elution times falling within windows of 2.45–4.10 min for tryptophan and 5.50–8.25 min for 5-methyl-D,L-tryptophan, and (*2*) peak width at half height for 5-methyl-D,L-tryptophan is <15 s. Failure to match elution time windows can usually be corrected by changes in mobile phase composition; in general, a 10% change in organic content (methanol) will result in a 3-fold change in the k′ value for analyte. Failure in peak width crite-

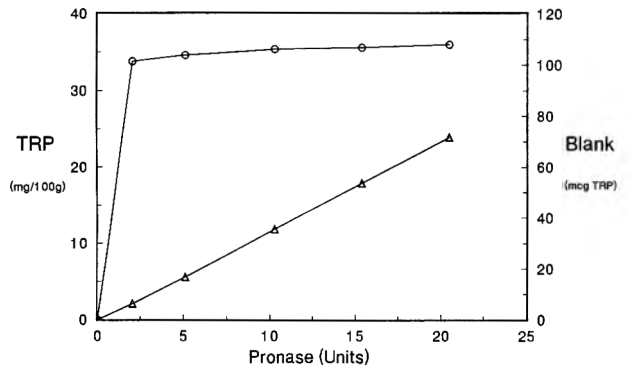

Figure 4. Units of pronase required. Samples of Formula 2 (containing approximately 50 mg protein) were digested 24 h with various amounts of pronase in a pH 8.5, 0.1M trizma buffer system (constant final volume) at 50°C. Tryptophan determinations were completed as detailed in the text. Data shown are averages of 3 determinations for samples (circles) and averages of duplicate blanks (triangles) for each enzyme level.

rion indicates inadequate column performance, which usually requires column replacement.

(c) *Calibration.*—Make duplicate injections of the 3 standards, and determine peak areas. Plot mean peak area ratio vs concentration ratio (tryptophan:5-methyl-D,L-tryptophan in both cases). Slope of this plot (from regression analysis) is response factor, R. Unknown concentrations can be calculated by the following relation:

$$\text{Result (mg tryptophan/100 g sample)} = \frac{TRP/5-MT}{R} \times \frac{IS}{SW} \times 0.05\ \text{L} \times 100$$

where *TRP* = tryptophan peak area; *5-MT* = 5-methyl-D,L-tryptophan peak area; *R* = response factor (slope of standards plot); *IS* = concentration (mg/L) of 5-methyl-D,L-tryptophan in sample preparation; and *SW* = sample weight (g).

Results: Method Development

Separation and Detection System

The internal standard (5-methyl-D,L-tryptophan) used in this work was selected for chemical similarity to tryptophan (hydrophobicity and intrinsic fluorescence). Use of a short column (100 × 4.6 mm) at 50°C produced baseline resolution in approximately 8 min. Peak purity checks were made on both tryptophan and internal standard peaks by using a Hewlett-Packard 1090 diode array detector and the Hewlett-Packard Model 1046A fluorescence detector. Absorbance spectra of rising edge, apex, and falling edge of the tryptophan peak in the sample were compared with one another and with the apex spectrum from a standards injection (Figure 1A). A similar comparison was made for the internal standard peak (data not shown). Tryptophan elution profiles at 270, 280, and 290 nm, as well as fluorescence (excitation, 295 nm; emission, 345 nm), were overlaid, and peak shapes were compared (Figure 1B). Finally, the elution profiles for standards and samples, with both absorbance (Figure 1C) and fluorescence (Figure 1D) at 280 nm, were overlaid to compare peak shape and elution time.

Optimizing Enzyme Digestion

Optimum pH.—Duplicate 1.5 mL samples containing 50 mg protein (bovine serum albumin, soy protein isolate, or caseinate) were hydrolyzed with 10 units of pronase in the presence of 3 mL of one of several buffer systems (buffers described in *Solutions*). Incubation was 24 h at 50°C. Samples were processed and analyzed as described in *Sample Preparation*) and *Chromatography System*, respectively. Analytical results are presented as a function of incubation pH in Figure 2. Addition of 0.5mM zinc acetate or 10mM calcium chloride to the incubation mixture did not affect results (data not shown).

Temperature.—Duplicate 1.5 mL samples (bovine serum albumin or chicken egg white lysozyme in 0.1M trizma buffer, pH 8.5) containing 50 mg protein were hydrolyzed 24 h with 10 units of pronase in 3 mL 0.1M trizma buffer, pH 8.5, at various incubation temperatures (35, 50, 60, and 70°C). Samples were processed and analyzed as described above. Tryptophan results are presented as a function of temperature in Figure 3.

Amount of enzyme.—Duplicate 1.5 mL samples (milk-based formula) containing 50 mg protein were hydrolyzed 24 h with various amounts of enzyme (2, 5, 10, 15, and 20 units) at 50°C in 0.1M trizma buffer, pH 8.5 (total incubation volume was held constant). Samples were processed and analyzed as described above. Tryptophan results (and blank values) are presented as a function of units of added pronase in Figure 4.

Sample size was also varied to contain 10–50 mg protein. Triplicate samples (at each sample size) of a milk-based formula were hydrolyzed 16 h with 10 units of pronase in 0.1M trizma buffer, pH 8.5, at 50°C (total incubation volume was held constant by water addition). In a separate experiment, a stock solution of chicken egg white lysozyme (10 mg/mL) was prepared in 0.1M trizma buffer, pH 8.5. Aliquots containing 5, 10, 20, 30, 40, and 50 mg protein were combined with the same buffer to a constant final volume of 5 mL and hydrolyzed 16 h with 10 units of pronase in 50°C, pH 8.5. Mixtures were processed and analyzed as detailed above. Data for both experiments are presented in Table 1.

Digestion time.—Duplicate samples (chicken egg white lysozyme, infant formulas containing soy protein isolate, casein, or a casein hydrolyzate) containing 50 mg protein in 1.5 mL (sample + 0.1M trizma buffer, pH 8.5) were hydrolyzed with 10 units of pronase at 50°C, pH 8.5, as described above, except that incubation time was varied from 0 to 24 h. After incubation, samples were processed immediately and analyzed as described above. Tryptophan results are presented as a function of digestion time in Figure 5.

Results: Method Validation

Linearity of detector response.—Standard solutions were prepared containing 0–200 μg tryptophan/mL. These solutions were injected in duplicate (20 μL injection), and peak area response was measured. A linear response is observed up to

Table 1. Effects of sample size on tryptophan (TRP) determination

Formula 2 concentrated liquid			Lysozyme[a]		
Sample mass, mg	TRP, mg[b]	Result, mg TRP/100 g sample	Sample mass, mg	TRP, mg[b]	Result, mg TRP/100 g sample
153	0.0561	36.6	5.0	0.385	7.51
150	0.0535	35.6	5.0	0.385	7.58
155	0.0561	36.2	10.0	0.770	7.54
326	0.119	36.6	10.0	0.770	7.48
340	0.125	36.5	20.0	1.54	7.40
315	0.116	36.6	20.0	1.54	7.40
524	0.191	36.5	30.0	2.31	7.15
536	0.194	36.2	30.0	2.31	7.09
499	0.180	36.1	40.0	3.08	6.97
716	0.260	36.3	40.0	3.08	6.94
758	0.270	35.6	50.0	3.85	6.72
762	0.272	35.7	50.0	3.85	6.75

[a] Expected value for chicken egg white lysozyme is 7.70 g tryptophan/100 g sample (see *Results* for derivation of this value).
[b] Tryptophan values were derived from analytical data for the Formula 2 concentrated liquid samples; theoretical values are presented for the samples of chicken egg white lysozyme.

≥4000 ng injected tryptophan. A quantitation limit for this separation and detection system using the low-sensitivity setting of the detector is estimated (10 times noise level) as approximately 0.5 ng injected tryptophan.

Accuracy.—The accuracy of this method was first assessed by analysis of 2 pure proteins, bovine serum albumin and chicken egg white lysozyme. The sample size for bovine serum albumin was 50 mg; the sample size for lysozyme was 5 mg. Four replicate sample preparations (6 h digestion time, 50°C, pH 8.5) on each of 4 days produced the analytical results presented in Table 2.

Overspike recovery studies were performed on 3 different infant formula products. Bovine serum albumin (25 mg) was added as a spike material into product samples (sample sizes were determined to contain approximately 25 mg total protein). Samples were then processed, as described in *Sample Preparation*. Calculations from spiked and unspiked samples showed quantitative recoveries of tryptophan from the spiked protein (Table 3).

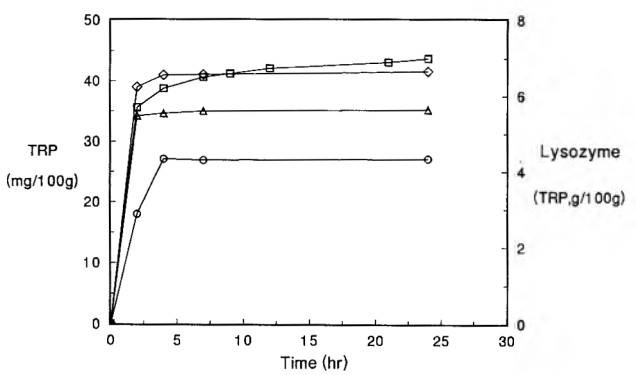

Figure 5. Time course of digestion. Samples of chicken egg white lysozyme (squares), Formula 1 (diamonds), Formula 2 (triangles), or Formula 3 (circles) containing approximately 50 mg protein were digested with 10 units of pronase at pH 8.5 (0.1M trizma) and 50°C for various times. Tryptophan contents of samples were then determined as detailed in the text. Data presented are means of duplicate determinations for each time point.

Finally, analytical results generated from this method were compared with those generated by a base hydrolysis method. Samples of 6 different products were analyzed by the method of Hugli and Moore (7), modified by inclusion of 15 mg ascorbic acid (antioxidant)/mL 8N NaOH used for hydrolysis (final NaOH concentration was 5N). Samples were hydrolyzed under vacuum 22 h at 110°C, cooled on ice, neutralized to pH 4–7 with 6N HCl (ice cold), filtered, and analyzed by using a Beckman 6300 amino acid analyzer. Norleucine was used as an internal standard. The analytical results generated were compared with those obtained by this method (Table 4).

Precision.—Two standard proteins (bovine serum albumin and lysozyme) and 3 nutritional products (milk-based concentrated liquid, soy protein isolate-based concentrated liquid, and hydrolyzed casein-based ready-to-feed liquid) were assayed on each of 4 nonconsecutive days. Four weighings of each sample type were processed each day. Three operators were involved, and only cans from the same case lot (products) or bottle (standard proteins) were used. Three different columns (different lots of packing material) and 2 different lots of enzyme were used. Data are presented in Tables 2 and 5.

Discussion and Conclusions

The separation system presented here allows injection-to-injection times of 8 min with baseline resolution. Considerable variability between lots of columns and mobile phase can be accommodated because of the substantial lack of interfering peaks (Figure 1D). Both peaks of interest are probably chromatographically pure, because several standard peak purity checks failed to reveal spectral or shape differences between standard and sample peaks (Figure 1). The system has more

Table 2. Method performance: analysis of standard proteins

	Analytical result, mg/100 g sample							
	BSA[a]				Lysozyme			
Sample	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
1	576	582	578	582	7288	7889	7480	7527
2	582	580	591	582	7611	7735	7437	7612
3	577	579	583	580	7234	7496	7388	7319
4	574	580	591	583	7758	7504	7596	7604
Mean	577	580	586	582	7473	7656	7474	7516
SD	3.4	1.3	6.4	1.3	253	191	89	136
%RSD	0.6	0.2	1.1	0.2	3.4	2.5	1.2	1.8
Overall mean		581				7530		
Overall SD		4.6				177		
Overall %RSD		0.8				2.4		

[a] Abbreviations used: BSA = bovine serum albumin, lysozyme = chicken egg white lysozyme, SD = standard deviation, %RSD = percent relative standard deviation.

sensitivity (quantitation limit, 0.5 ng injected tryptophan) than is needed for this analysis; therefore, the analysis is limited only by the blank value generated by pronase autolysis. A large linear range (0.5–400 ng injected tryptophan) contributes to ruggedness; even though peak area values must fall within the standard range, this range could be considerably larger than currently described, provided that complete digestion can he demonstrated at the higher tryptophan values (cf. Table 1). Also, it is likely that absorbance at 280 nm could be used for detection instead of fluorescence (Figure 1C), but only fluorescence was validated.

Enzyme digestion procedures should be done under conditions that are optimal for enzyme activity. Factors usually considered include temperature, pH, time of digestion, and enzyme/substrate ratio. Enzyme activation and stabilization requirements must also be evaluated. During method development, the best conditions for protein hydrolysis (releasing tryptophan) were identified by using a variety of proteins, including bovine serum albumin and chicken egg white lysozyme, as well as protein commodities such as soy protein isolate, caseinates, and a partially hydrolyzed caseinate, both in product matrixes and as isolated components. A pH of 8.5 was chosen, using trizma buffer, because maximal tryptophan release was observed from pH 7.5 to 8.5 (Figure 2). The higher pH was used because the hydrolysis process releases acid equivalents, and buffering capacity should be optimized when acid is added (trizma $pK_a = 8.1$). Pronase is known to contain at least 5 zinc metalloproteases and metallopeptidases (11). The pronase source we used contains approximately 25% (w/w) calcium acetate, which is added as a stabilizer. Addition of either zinc or calcium salts did not result in additional tryptophan release, a result indicating that maximum activation had already oc-

Table 3. Method performance: bovine serum albumin spike recovery values from several products; percent recovery of bovine serum albumin tryptophan from product

	Formula 1 CL[a]				Formula 2 CL				Formula 3 RTF			
Sample	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
1	99.5	104.4	96.2	99.4	101.7	101.0	100.4	100.2	101.9	101.0	98.2	100.1
2	101.4	101.2	98.6	99.6	103.0	100.9	100.0	100.4	96.1	100.0	98.8	100.9
3	101.4	100.5	98.1	99.1	100.3	99.1	100.7	98.0	99.9	99.9	108.4	101.2
4	101.2	101.9	97.4	99.1	100.4	100.5	101.2	102.2	97.9	100.4	98.0	98.6
Mean	100.9	102.0	97.6	99.3	101.4	100.4	100.6	100.2	99.0	100.3	100.8	100.2
SD	0.92	1.70	1.04	0.24	1.27	0.88	0.51	1.72	2.50	0.50	5.04	1.16
Overall mean[b]		99.9				100.6				100.1		
Overall SD		1.98				1.15				2.68		

[a] Abbreviations used: CL = concentrated liquid, RTF = ready-to-feed, and SD = standard deviation from the mean.
[b] Overall mean calculated by using all data points ($n = 16$) for each product.

Table 4. Comparison with base hydrolysis in several products

Sample	Protein source	Base hydrolysis[a]	Pronase hydrolysis[a]
Formula 1 CL[b]	Soy protein isolate	40.1 ± 0.6	44.0 ± 0.4
Formula 1 powder	Soy protein isolate	162 ± 4	173 ± 2
Formula 2 CL	Cow's milk	33.6 ± 1.2	37.6 ± 0.3
Formula 2 RTF	Cow's milk	18.1 ± 0.4	19.8 ± 0.5
Formula 3 RTF	Casein hydrolysate	24.6 ± 0.4	27.3 ± 0.4
Formula 4 RTU	Casein	74.0 ± 1.8	81.2 ± 0.9

[a] All analytical results are presented as mg tryptophan/100 g sample. Samples were from the same container of product, analyzed in duplicate on the same day. Data are means ± standard deviation.
[b] Abbreviations used: CL = concentrated liquid, RTF = ready-to-feed, RTU = ready-to-use.

curred. An optimal temperature of 50°C was identified (Figure 3).

Ten units of enzyme was found to be more than adequate for maximum tryptophan release from a sample (milk-based infant formula) containing 50 mg protein (Figure 4). With this amount of enzyme, a linear relationship was demonstrated between sample size and tryptophan release over a 5-fold range of sample size (Table 1), a result indicating the ruggedness of the method to sample size changes. During this experiment, we found that accurate analytical results can be obtained even when the blank signal is about 50% of the total signal. Although a sample signal of about 8–10 times the blank is more typical, this indicates the ability of the method to quantitate tryptophan even in protein sources that are relatively low in this amino acid. Samples containing tryptophan-rich proteins are best analyzed by using a much reduced sample size. An example is lysozyme, as discussed below.

A digestion time of 6 h was selected, even though 2–4 h was adequate (Figure 5) for the proteins commonly used in nutritional products (milk proteins, including caseins, and soy proteins). The excess digestion time contributes further ruggedness to the method as applied in our laboratory, while still allowing complete sample processing and autosampler loading within an 8 h day.

For accuracy evaluations, proteins of known amino acid sequence were selected and analyzed. An expected value of 594 mg/100 g sample for bovine serum albumin was calculated on the basis of sequence data (12) and a 96.4% (w/w) protein sample. An expected value of 7700 mg/100 g sample for chicken egg white lysozyme was calculated from sequence data (13) and a 90% (w/w) protein sample. Protein content of these samples was confirmed by Kjeldahl nitrogen determinations using the standard AOAC method (14). Although analytical values from bovine serum albumin samples were 97.8% of the expected value (Table 2), the result for a 50 mg sample of lysozyme was only 87.5% of the expected value, even with a 16 h hydrolysis time (Table 1). A 6 h hydrolysis time resulted in only 75% recovery for the same sample size (data not shown). However, if a reduced sample size was used (5 mg), essentially quantitative results (97.8% of theoretical) were observed when the standard digestion time of 6 h was used (Table 2). It should be noted that the 5 mg sample size contains

Table 5. Method performance: precision data for several sample types

	Analytical result, mg/100 g sample											
	Formula 1 CL[a]				Formula 2 CL				Formula 3 RTF			
Sample	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
1	40.1	39.7	38.2	40.8	34.8	34.8	37.4	36.5	26.6	26.2	26.8	26.7
2	40.2	39.4	38.8	40.9	35.2	34.6	36.4	36.8	26.3	25.9	27.0	26.8
3	39.9	39.8	38.2	40.8	35.3	34.9	35.7	36.7	26.2	26.3	27.2	27.0
4	40.3	39.7	38.7	41.1	35.1	34.8	36.1	36.5	26.2	26.0	26.9	26.8
Mean	40.1	39.7	38.5	40.9	35.1	34.8	36.4	36.6	26.3	26.1	27.0	26.8
SD	0.17	0.17	0.32	0.14	0.22	0.13	0.73	0.15	0.19	0.18	0.17	0.13
%RSD	0.43	0.44	0.83	0.35	0.62	0.36	1.99	0.41	0.72	0.70	0.63	0.47
Overall mean		39.8				35.7				26.6		
Overall SD		1.0				0.91				0.42		
Overall %RSD		2.51				2.55				1.58		

[a] Abbreviations used: CL = concentrated liquid, RTF = ready-to-feed, SD = standard deviation, and %RSD = percent relative standard deviation.

an amount of tryptophan (0.385 mg) more closely comparable with that in the 50 mg sample of bovine serum albumin (0.297 mg). Sample sizes containing between 50 and 700 μg tryptophan are recommended because of the above data. This indicates a consideration for wide use of this method. Proteins that are highly resistant to proteolysis or have unusually high tryptophan content may give low analytical values unless care is exercised in applying this method. For this reason, appropriate sample sizes and digestion times for other protein sources should be determined on an individual basis.

Precision experiments using highly purified proteins (90–96% [w/w] protein) exhibited relative standard deviations that were consistent with product samples containing only 1.8–3.0% protein by weight (compare Tables 2 and 5). Therefore, a wide range of sample protein concentrations is tolerated by this method, even in relatively complicated matrixes, and no interfering peaks were observed.

Spike recovery studies were performed with either bovine serum albumin or free tryptophan as the spiking material in several different product matrixes. Free tryptophan recoveries (in the presence or absence of a product matrix) ranged from 98 to 103% (data not shown), a result indicating the stability of the analyte under the conditions of hydrolysis and analysis. The quantitative recovery of tryptophan from bovine serum albumin, both in the absence of a product matrix (Table 2) and in 3 different product matrixes (Table 3), indicates that hydrolysis of this protein is complete, and no significant interference with either the hydrolysis or the analytical system is present. The new method gives analytical results that range from 107 to 112% of those generated by using the standard base hydrolysis method for different products (Table 4). These results are in good agreement with our own recovery observations for the base hydrolysis method (85–94% recovery of added tryptophan) and the essentially quantitative recoveries observed for the new method. The results are also consistent with substrate depletion, not enzyme inactivation resulting in the plateaus in our time course studies of product hydrolysis (Figure 5).

In summary, the data presented indicate the validity of applying this method to the determination of tryptophan in infant formulas and nutritional products containing milk concentrates, caseinates, soy protein isolates, or partial hydrolysates generated from these proteins. Further application may be possible, as indicated by the analytical results for bovine serum albumin and lysozyme, but each protein should be evaluated individually for completeness of tryptophan release under these conditions. Although this method may not be applicable to all proteins, its application to these particular protein sources represents a substantial improvement over the currently used methodology, both in speed of sample preparation and in recovery of analyte.

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TECHNICAL COMMUNICATIONS

Rapid Isolation of the Sterol Fraction in Edible Oils Using a Silica Cartridge

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A commercial silica cartridge was used successfully to separate the fraction of the unsaponifiable components of edible oils containing the sterols and some related compounds. Analysis by gas chromatography indicates that there is good agreement between the results from samples obtained by the new rapid method and those isolated using the time-consuming thin-layer chromatographic technique. This paper presents comparison data for 7 vegetable oils (peanut, soy, corn, rapeseed, olive, sesame, and coconut), a 2-component blend (olive and soy), and a commercial milk product erroneously labeled cholesterol-free.

Sterols are structurally related alicyclic alcohols found in the unsaponifiable fraction of fats and oils. The sterol pattern of an oil is characteristic of the product and, together with the fatty acid methyl esters profile, constitutes the basis of the officially sanctioned method for defining the identity of an edible oil (1).

The isolation of the unsaponifiable fraction of an oil, which usually represents 1% or less of the total material, consists of a vigorous saponification followed by ether extraction and extensive water washing to obtain an ether solution free of the alkali salts of the fatty acids. That solution contains the sterols, as well as a number of other compounds such as fatty and triterpene alcohols, tocopherols, carotenoids, and other hydrocarbons.

The present methodology for the isolation of the sterol mixture previous to analysis by gas chromatography (GC) (2, 3) requires 2 consecutive thin-layer chromatographic (TLC) steps requiring 1–1.5 working days. Because the initial saponification, extraction, and washings take another working day, the total time involved in the preparation of the sterols for chromatography is considerably problematic when analyzing samples against a short deadline. The protracted TLC cleanup procedure was successfully circumvented by using an underivatized silica cartridge, thus reducing the time involved in the sterol isolation step to 20–30 min/sample. The method has been applied, with good results, to the analysis of peanut, soybean, corn, rapeseed, sesame, olive oil, coconut, olive–soybean blends, and milk.

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Experimental

Apparatus

(a) *Glass column.*—Approximately 400 × 30 mm.

(b) *Fritted glass filter.*—60 mL, fine porosity.

(c) *TLC plates.*—Silica gel G, precoated, 20 × 20 cm, Uniplate-T (Analtec).

(d) *Silica cartridges.*—Sep-Pak (Waters Associates).

(e) *Syringe.*—10 mL, glass, with Luer tip.

(f) *Gas chromatograph.*—Hewlett Packard 5840A, equipped with a 4 ft × 4 mm id column, packed with Gas Chrom Q Ultra Pak 230/270 coated with 1.5% OV-17 (50% phenyl–50% methylpolysiloxane) (Alltech Associates). Column temperature program: 254°C for 40 min, increasing at 3°C/min to 280°C, and held for 20 min. Injection port temperature: 300°C. Detector: flame ionization, 300°C. Carrier gas: nitrogen at a linear rate of 36–37 mL/min.

Reagents

(All reagents were analytical grade or better.)

(a) *KOH.*—2N in methanol.

(b) *Sodium sulfate.*—Anhydrous.

(c) *Aluminum oxide.*—Anhydrous, neutralized: dry aluminum oxide 3 h in a closed container at 105°C. Add distilled water (6 mL/100 g), and shake vigorously. Let stand 12 h before using (3).

(d) *2′,7′-Dichlorofluorescein.*—0.2% solution in absolute ethanol. Add a few drops of alcoholic KOH to make alkaline.

(e) *Diethyl ether.*—Anhydrous.

(f) *Petroleum ether.*

(g) *Plant sterols standard mixture.*—Supelco, Inc.

(h) *Cholesterol.*—Sigma Chemical Co.

(i) *Stigmasterol.*—Sigma Chemical Co.

(j) *Erythrodiol.*—Atomergic Chemetals Corp.

(k) *Uvaol.*—Atomergic Chemetals Corp.

(l) *Squalene.*—Atomergic Chemetals Corp.

Samples

Saponification and TLC separation.—Saponify 10 g oil by refluxing 1–1.5 h in 2N methanolic KOH. Transfer to a separatory funnel, and extract 3 times with diethyl ether. Wash pooled

Table 1. Composition of sterol fraction (%): comparison of TLC and Sep-Pak isolation[a]

	Cholesterol	Brassicasterol	Campesteroi	Stigmasteroi	β-Sitosterol	Δ^5-Avenasterol	Δ^7-Stigmasterol	Δ^7-Avenasterol	Erythrodiol	Uvaol
	Relative retention time									
Sample	0.61	0.70	0.80	0.87	1.00	1.12	1.18	1.32	2.15	2.29
Peanut										
TLC	—	—	16.4	9.9	64.3	8.6	tr[b]	0.7	—	—
Sep-Pak	—	—	16.5	9.6	64.6	8.6	tr	0.7	—	—
Literature range (4)	—	—	(12.0–16.5)	(9.0–12.1)	(57.7–69.8)	(9.1–12.4)	(tr–0.5)	(tr–0.6)	—	—
Soybean										
TLC	—	—	21.5	20.3	56.6	tr	0.8	0.7	—	—
Sep-Pak	—	—	19.0	19.0	52.7	3.7	4.0	1.6	—	—
Literature range (4)	—	—	(20.1–24.6)	(17.4–20.4)	(54.5–56.9)	(0.4–2.4)	(0–1.8)	(0–1.0)	—	—
Corn										
TLC	—	—	18.9	7.4	69.6	3.7	—	0.4	—	—
Sep-Pak[c]	—	—	18.8	7.6	69.5	4.1	tr	tr	—	—
Sep-Pak[d]	—	—	18.3	8.0	67.8	← 5.6[e]	→	0.3	—	—
Corn										
TLC	—	—	18.5	7.6	68.7	4.5	tr	0.2	—	—
Sep-Pak[f]	—	—	18.0	7.9	67.0	6.8	tr	0.3	—	—
Literature range (4)	—	—	(19.9–20.3)	(5.9–7.0)	(68.7–69.5)	(3.6–4.5)	(0.4–1.0)	(tr–0.4)	—	—
Rapeseed[g]										
TLC	—	7.0	30.8	4.0	53.7	2.7	1.7	tr	—	—
Sep-Pak	—	7.2	30.1	4.2	51.7	3.6	2.5	0.2	—	—
Literature range (4)	—	(7.5–12.2)	(29.3–30.8)	(tr–0.8)	(51.8–54.1)	(1.5–2.6)	(0.1–0.4)	(tr–0.4)	—	—
Olive										
TLC	—	—	2.8	1.6	71.0	←	6.8[e]	→	13.6	3.6
Sep-Pak	—	—	3.0	2.0	62.8	6.1	4.2	3.4	14.9	3.4
Literature range (4)	—	—	(2.4–5.6)	(0.3–3.9)	(82.4–91.4)	(4.1–13.6)	(0–0.3)	(0–0.1)	—	—
Soy–olive (90 + 10)										
TLC	—	—	18.3	20.5	← 54.7[e]	→	4.9	0.7	—	—
Sep-Pak	—	—	18.0	19.6	52.5	3.3	4.5	1.2	—	—
Milk–soy mix										
TLC	25.2	—	12.7	12.5	44.8	1.4	2.7	tr	—	—
Sep-Pak	25.3	—	12.6	12.5	44.1	2.1	2.8	tr	—	—
Sesame										
TLC	—	—	18.1	10.2	55.6	7.7	5.5	3.3	—	—
Sep-Pak	—	—	18.0	10.2	58.0	5.2	6.5	2.0	—	—
Literature values (5)	—	—	19.0	10.0	62.0	7.0	2.0	—	—	—
Coconut										
TLC	—	—	7.4	15.4	53.1	24.0	—	—	—	—
Sep-Pak	—	—	7.5	15.0	43.0	34.2	—	—	—	—
Literature range (4)	—	—	(7.6–8.1)	(13.5–14.8)	(42.7–46.0)	(31.3–33.7)	—	—	—	—

[a] Area relative to total area of relevant peaks.
[b] tr, trace.
[c] Represent duplicate extractions.
[d] Represent duplicate extractions.
[e] Represents unresolved peaks.
[f] Represents a different sample.
[g] Hydrogenated rapeseed from vegetable shortening.

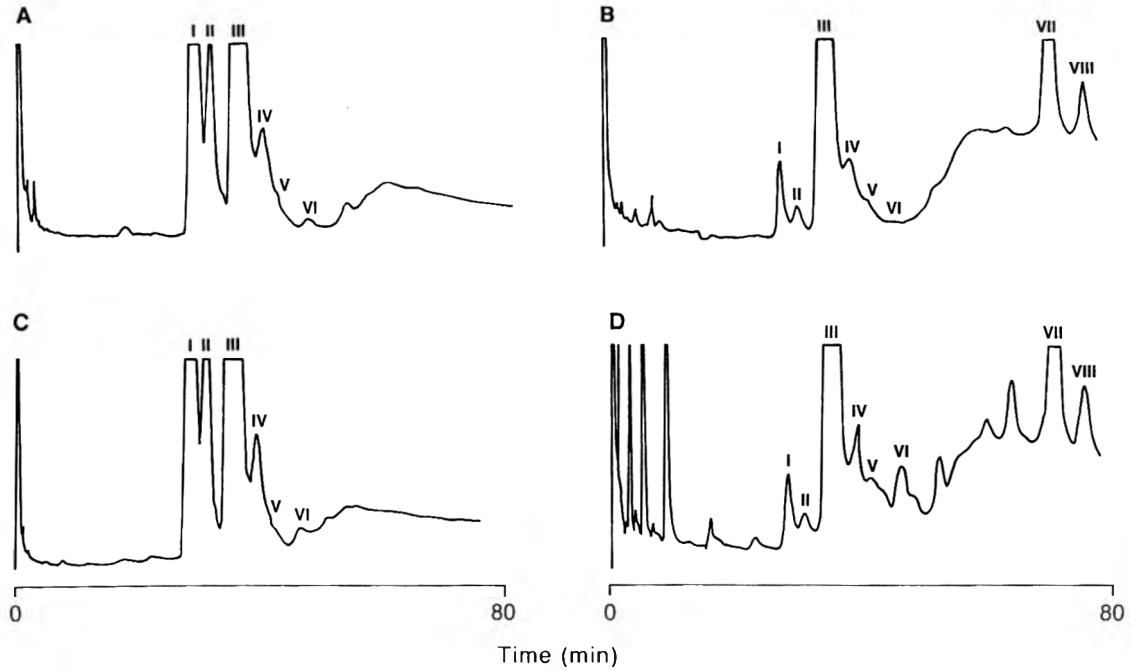

Figure 1. Representative gas chromatograms of sterols of corn and olive oils from a TLC plate (A and B) and from a silica cartridge (C and D). Peak identities are campesterol (I), stigmasterol (II), β-sitosterol (III), Δ^5-avenasterol (IV), Δ^7-stigmasterol (V), Δ^7-avenasterol (VI), erythrodiol (VII), and uvaol (VIII).

ether extracts with water until they are soap free, and dry by passing through a Na_2SO_4 column followed by shaking with neutral Al_2O_3. Filter the dried ether extract, evaporate solvent, and suspend residue in 1 mL chloroform. This solution contains all the unsaponifiable compounds. Apply to a TLC plate, side by side, 0.5 mL chloroform solution and ca 40 μL of ca 1.5 mg/mL β-sitosterol standard as a band and a spot, respectively. Develop in petroleum ether–anhydrous diethyl ether (PE–DEE). Visualize bands by spraying with 2′,7′-dichlorofluorescein and examining the plate under UV light. Scrape the band containing the sterol (the broad band coinciding with the standard), and extract the silica with warm chloroform in a fritted glass filter. Evaporate the extract to dryness, apply to a second TLC plate, and repeat the procedure. Evaporate the second chloroform extract, dissolve the residue in 0.5 mL ethyl acetate, and inject ca 0.5 μL into a gas chromatograph.

Silica cartridge cleanup.—In a small glass test tube, evaporate to dryness 100 μL (50 μL for sesame oil) chloroform solution of the unsaponifiable components, and resuspend residue in 1 mL PE–DEE (95 + 5). Attach the short end of the silica cartridge to the Luer tip of a 10 mL glass syringe, push 3.0 mL PE twice through the cartridge with the plunger, and discard the effluent. Use the air volume in the syringe to dry the cartridge after each solvent application throughout the procedure. Never pull the plunger back while the cartridge is attached. Add the sample to the cartridge through the syringe. Let it penetrate the silica first by gravity and finally by driving it with the plunger. Elute the sample according to the following scheme: 3.0 mL PE–DEE (95 + 5), 2 × 3.0 mL PE–DEE (80 + 20) (for sesame oil, use 3 × 3.0 mL PE–DEE [70 + 30]), and 3.0 mL PE–DEE (30 + 70). Collect the 30 + 70 fraction. Evaporate fraction to dryness under nitrogen, and suspend residue in 100 μL ethyl acetate (200 μL for sesame oil). Inject into the GC system 0.5 μL or a volume giving an area of ca 2–4 × 10^6 for the β-sitosterol peak. Identify the sterols, erythrodiol, and uvaol by chromatographing, under the same conditions, a mixture of plant sterols containing brassicasterol, campesterol, stigmasterol, and β-sitosterol and individual cholesterol, erythrodiol, or uvaol reference material. For Δ^5-avenasterol, Δ^7-stigmasterol, and Δ^7-avenasterol, use the ratio of the peak's retention times to that of β-sitosterol: 1.12, 1.18, and 1.32, respectively.

Results and Discussion

Table 1 shows results obtained by applying the 2 methods to 7 oils, a 2-component blend, and a commercial sample of milk labeled cholesterol-free. This last product consisted of allegedly defatted milk mixed with partially hydrogenated soy bean oil. The analysis demonstrated that, contrary to the label claim, about one-third of the milk fat was still present. Recovery of cholesterol using the cartridge was 87.9%. Milk lipids were extracted using a modification of the Punwar method (6).

In general, the best agreement in the values obtained with the 2 methods appears in the early peaks, from cholesterol to stigmasterol. The recovery of β-sitosterol from the cartridge is consistently lower than from the TLC plate. The greatest variation, both between cartridge runs and between methods, occurs with the Δ sterols. As can be seen in Figure 1, that section of the chromatogram is far from ideal, a factor that will contribute to the poor reproducibility of the peak areas. However, poor reproducibility of those components also characterizes succes-

sive runs of TLC-cleaned samples and in no case has led to misclassification of the oil.

The silica-cartridge-isolated sterol fraction of olive oil shows a number of early emerging components that are eliminated in the TLC plate, where they appear in a discrete band directly above the band containing the sterols and the 2 bands containing dihydroxytriterpenes. Because the peaks have short retention times (below 15 min), they do not interfere with the compounds of interest and can be easily excluded from integration by a time program. A similar situation exists with respect to erythrodiol and uvaol, which in the cartridge fraction are preceded by an unidentified peak mostly eliminated in the TLC plate, where the unidentified component separates in the band beyond the sterols. These triterpenic components come from the skin and seed of the olive and are important in differentiating an extracted from an expressed oil (7). Under the described chromatographic conditions, the extraneous peak is well resolved from erythrodiol.

To test the recovery of erythrodiol and uvaol, known amounts of those compounds were added to a sample of virgin olive oil that was then saponified and processed according to the 2 methods. With the cartridge, the recovery was 83.0% for erythrodiol and 85.3% for uvaol. With the double TLC cleanup, only 18.5% of the added erythrodiol and 18.3% of the uvaol were recovered from the second plate. However, it should be kept in mind that recovery calculations were based on absolute peak area, whereas for the samples, the levels of the individual components are quantitated as percent of total area. In the TLC method, the sterol peaks are also lower, which results in a smaller total area and compensates for the lower absolute recoveries. In general, a considerable loss of sample occurs between the first and the second plate, but this loss does not significantly affect the relative individual values.

Squalene, a C_{30} linear triterpene, has been used to characterize olive oil, because it is olive oil's main hydrocarbon constituent. Squalene is isolated in the PE–DEE (95 + 5) fraction of the cartridge elution and could serve as corroborating evidence of the absence or presence of olive oil in a blend.

This method is now being routinely used for the rapid screening of oil sterols in the Philadelphia District laboratory with satisfactory results.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Bleaching of Methylene Blue as an Index of Lipoxygenase Activity

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An assay of lipoxygenase activity was developed that depends on measurements of the time needed for the bleaching of methylene blue to begin. This time was inversely related to the level of lipoxygenase activity ($y = 114.5132 - 0.0132x$; $r = -0.99$); it was not affected by the concentration of methylene blue, linoleic acid, or hydroperoxide. Lipoxygenase activity can be calculated from the time needed for the bleaching of methylene blue to begin.

Lipoxygenase, an enzyme found in animals and plants (1–6), oxidizes unsaturated fatty acids with a (*Z,Z*)-1,4-pentadiene structure to hydroperoxide (7). Lipoxygenase activity is generally measured by monitoring (at 234 nm) the conjugated diene produced via the enzyme reaction with linoleic acid as the substrate (8–10). However, this method is not appropriate when a large sample must be analyzed. Results can depend on whether the substance being tested is emulsified, dried, or aqueous. In studies of lipid peroxidation, lipoxygenase is often used to prepare hydroperoxide; the added methylene blue bleaches rapidly, and the time that passes until the color begins to change is inversely related to the lipoxygenase activity. To overcome the limitations mentioned above, this phenomenon was quantitated so that it can be used to measure lipoxygenase activity.

Experimental

Reagents and Apparatus

All reagents used were analytical grade.

(**a**) *Linoleic acid.*— >99% pure (Sigma Chemical Co., St. Louis, MO 63178).

(**b**) *Lipoxygenase (from soybeans).*—Type I-B, lyophilized (Sigma).

(**c**) *Methylene blue.*— >98.5% pure (Tokyo Kasei Co., Ltd., Tokyo, Japan).

(**d**) *Silica gel.*—Kisel gel 60, 70–230 mesh, and Kisel gel 60 PF_{254}, 70–230 mesh (Merck, Darmstadt, Germany), used for thin-layer chromatography (TLC).

(**e**) *TLC plate.*—Silica gel 60 plate and silica gel 60 F_{254} plate (Macherey Nagel Co., Duren, Germany).

(**f**) *Mobile phase.*—Mixture of *n*-hexane, diethyl ether, and acetic acid (60 + 40 + 1, v/v/v) (Nacalai Tesque, Inc., Kyoto, Japan), used as TLC solvent.

(**g**) *TLC apparatus.*—0.25 mm gel prepared with TLC apparatus (Yazawa Scientific Apparatus Mfg. Ltd., Tokyo, Japan).

(**h**) *Spectrophotometer.*—Model U-2000 (Hitachi Ltd., Tokyo, Japan).

(**i**) *Recorder.*—Model 200 (Hitachi).

(**j**) *Water bath.*—Type E 15 (Haake, Berlin, Germany), or equivalent.

(**k**) *Oxygen analyzer.*—Model 0260 (Beckman Instruments, Inc., Fullerton, CA).

(**l**) *Vacuum pump.*—Type 4UP-C5 (Hitachi).

(**m**) *Fluorescent inspection lamp.*—Topcon F1-31 (Tokyo Kogaku Kikai K.K., Tokyo, Japan).

Preparation of Reagents

(**a**) *Borate buffer, pH 9.0.*—0.2M. Suspend 12.36 g boric acid in ca 600 mL distilled water. While stirring suspension, adjust pH to 8.0 with 50% NaOH solution. Adjust final pH to 9.0 with 10% NaOH solution. Dilute to 1 L with distilled water.

(**b**) *Substrate solution.*—Put 0.05 mL linoleic acid into 50 mL volumetric flask, and add 1.0 mL 95% undenatured ethanol. Mix gently until solution is homogeneous; then, while stirring slowly, add distilled water to 50 mL. For assays, dilute 5.0 mL of the above solution to 30 mL with 0.2M borate buffer.

(**c**) *Methylene blue solutions.*—Dilute methylene blue solution with 0.2M borate buffer to obtain solutions with concentrations from 2.5 to 15mM.

(**d**) *Enzyme solution.*—Dilute lipoxygenase solution with 0.2M borate buffer to obtain solutions with activities from 10 to 10 000 units.

Preparation of Linoleic Acid Hydroperoxide

Linoleic acid hydroperoxide was prepared by the procedure of Matsuda et al. (11) by enzymatic peroxidation with lipoxygenase. The standard reaction mixture contained 32mM linoleic acid, 0.1% Tween 80 (Nacalai Tesque), 0.2M borate buffer (pH 9.0), and 50 units of the lipoxygenase in a total volume of 20 mL. It was incubated in a conical flask to facilitate flushing with oxygen (Taiyo Yozai Co., Fukuoka, Japan; ≥95% pure). The reaction mixture was stirred mechanically for 40 min at 30°C under a stream of pure oxygen. After incubation, hydroperoxide was extracted with diethyl ether. The formation of linoleic acid hydroperoxide was monitored by mea-

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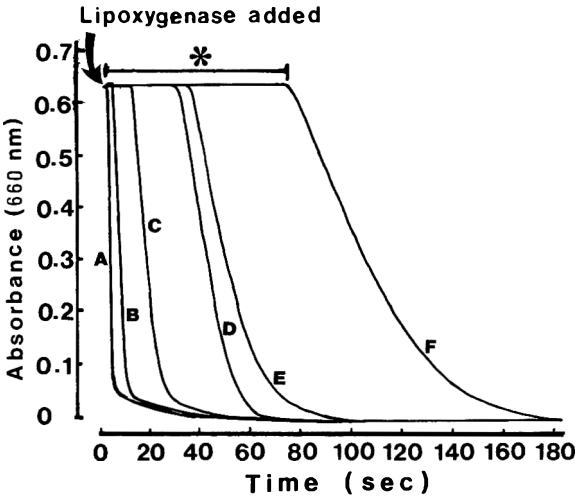

Figure 1. Bleaching of methylene blue during lipid peroxidation by lipoxygenase. The reaction mixture contained 10mM methylene blue and 20mM linoleic acid in 0.2M borate buffer. The reaction was started by adding different amounts of lipoxygenase in a total volume of 3.0 mL. One enzyme unit corresponds to the formation of 1 μmol hydroperoxide/min: A, 8400 units; B, 8200 units; C, 8000 units; D, 6600 units; E, 5800 units; F, 2850 units; *, time until bleaching began.

suring the increase in the absorbance at 234 nm. The hydroperoxide was obtained by TLC with a mixture of *n*-hexane, diethyl ether, and acetic acid (60 + 40 + 1, v/v/v) as the solvent system and was monitored under UV light.

Assay of Lipoxygenase Activity

The reduction of methylene blue through lipid peroxidation by lipoxygenase was assayed with reaction mixtures containing different amounts of linoleic acid, 0.2M borate buffer, and methylene blue. The reaction was started by adding the given amount of the enzyme to a total volume of 3.0 mL. The absorbance at 660 nm was recorded with a spectrophotometer for 2 min at 25°C. The number of seconds from the addition of the enzyme until the absorbance began to decrease was measured. The time at which the absorbance began to decrease was easy

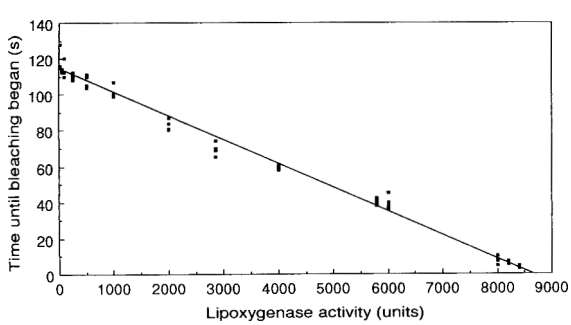

Figure 2. Correlation between lipoxygenase activity and the time required for bleaching of methylene blue to begin.

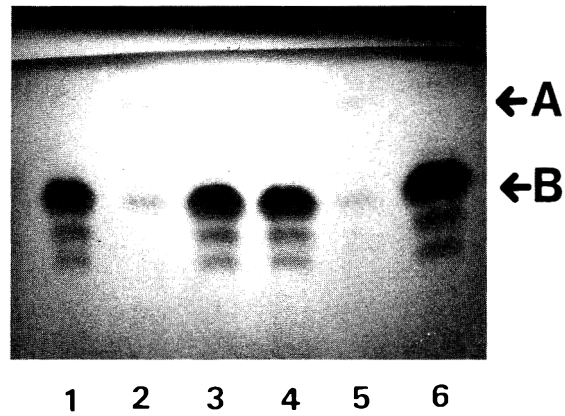

Figure 3. TLC of lipid peroxidation by lipoxygenase under various conditions: A, linoleic acid; B, linoleic acid hydroperoxide. TLC plate was examined under a fluorescent lamp: 1, no treatment; 2, heat; 3, diethyl ether; 4, EDTA; 5, low pressure; 6, acetic acid.

to identify (Figure 1). The purity of the resulting linoleic acid hydroperoxides and other products was checked by TLC with the solvent system described above. The TLC plate was sprayed with 50% sulfuric acid and then heated 20 min at 110°C. The effect of the oxygen concentration on the time until the bleaching of methylene blue began was monitored with a quartz Thunberg absorption cell. The concentration of oxygen before the reaction was measured with an oxygen analyzer.

Results and Discussion

Figure 1 shows the absorbance of the solution at 660 nm after different amounts of lipoxygenase were added. The time needed for the bleaching of methylene blue to start depended on the enzyme activity. The slope of the resulting regression line was negative ($y = 114.5132 - 0.0132x$, $r = -0.99$; Figure 2).

Neither the concentration of methylene blue nor that of linoleic acid affected the time until the start of the bleaching. These findings suggest that lipoxygenase activity can be assayed simply by measuring the time until the start of the bleaching.

The bleaching of methylene blue probably involves a reduction reaction. To determine if lipoxygenase was involved directly in the reduction, or if hydroperoxide products acted as the reducer, the enzyme itself was inactivated by heat, or an inhibitor was added. The time required for the absorbance at 660 nm to reach zero was only slightly affected, except by heat and low pressure; thus, lipoxygenase was not directly involved in methylene blue bleaching. TLC of samples after the reaction showed that hydroperoxide was formed under all conditions except heat and low pressure (Figure 3).

The finding that almost no bleaching occurred under low pressure suggested that oxygen was involved in the bleaching. Therefore, the effect of the concentration of dissolved oxygen was studied. Bleaching was more rapid as the concentration increased (Figure 4).

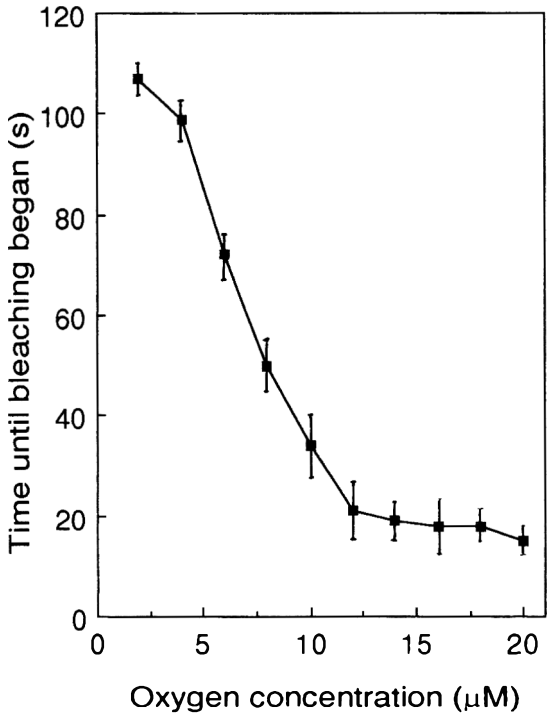

Figure 4. Effect of the concentration of oxygen on the time needed for bleaching of methylene blue to begin. Reaction conditions are described in the legend for Figure 1. The lipoxygenase activity was 6600 units. Each point represents the mean of 4 trials. The bars show standard deviations.

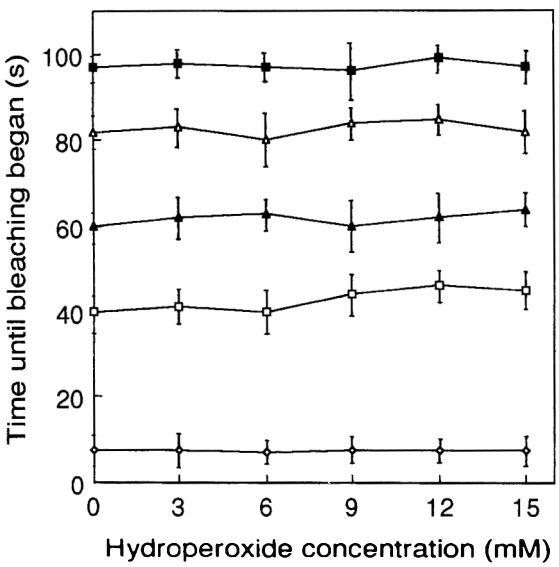

Figure 5. Effect of the concentration of hydroperoxide on the time needed for bleaching of methylene blue to begin at 5 levels of enzyme activity. Reaction conditions are described in the legend for Figure 1. Each point represents the mean of 4 trials. The bars show standard deviations.

If oxygen participated in the bleaching of methylene blue, hydroperoxide production should have been suppressed. However, because hydroperoxides were produced under all conditions except heat and low pressure, it is likely that oxygen was involved only in hydroperoxide production during the reaction. The hydroperoxide that was produced probably affected methylene blue bleaching. Therefore, I examined whether the amount of hydroperoxide was related to the time when bleaching began. There was no such relationship (Figure 5); hence, hydroperoxide itself was probably not involved directly in methylene blue bleaching. Also, hydrogen was not abstracted from hydroperoxide during methylene blue bleaching. Hydroperoxide did not decompose (Figure 3).

The reaction probably occurred as shown below (Equation 1). First, hydrogen was abstracted from the lipid RH (here, linoleic acid). Second, the lipid radical R· was converted to the peroxy radical ROO· by the addition of oxygen. ROO· then abstracted the hydrogen from RH. Methylene blue (MB) was probably bleached when hydroperoxide was produced by this hydrogen abstraction.

$$\begin{aligned} RH &\xrightarrow{\text{Lipoxygenase}} R\cdot \\ R\cdot + O_2 &\rightarrow ROO\cdot \\ ROO\cdot + RH + MB &\rightarrow ROOH + R\cdot + MB\text{–}H \text{ (bleaching)} \end{aligned} \quad (1)$$

This method is different from others, because lipoxygenase activity can be assayed simply by noting the time needed for bleaching of methylene blue to begin. The results are highly reproducible, even with large samples. Furthermore, it is easy to perform, and the equipment is inexpensive. It is not known whether this method can be used to assay lipoxygenase from animals and plants.

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REGULATORY ANALYTICAL METHODS

Introduction

Regulatory agencies approve and promulgate methods of analysis that regulate commodities in their markets. In many instances, these internally approved methods are not widely disseminated outside the agencies. In order to give a method a wider audience, an agency may submit it for publication in the *Journal of AOAC International*. This "Regulatory Analytical Methods" section in the Journal will provide select methods submitted by an agency. Final decisions to publish a method rests solely with the Editor-in-Chief of the Journal.

The methods published under this section have received approval by a national, state/provincial, or international regulatory agency or body and are used to regulate commodities that fall within the scope of the Journal. Each regulatory analytical method published contains its own introduction that explains the origin, nature, and approval that the particular method has undergone.

Having been approved by the regulatory agency submitting the method, the methods published under this section do not undergo the peer review accorded AOAC Journal articles before being published. These methods are ***not*** AOAC Official Methods of Analysis, since they have not been subjected to the full AOAC collaborative study process.

The intent of publishing these regulatory methods in the Journal is to give them a wider distribution and provide them with a publication reference. It is hoped that this section will be used by regulatory bodies of the world to disseminate their methods to scientists everywhere. They are not, however, meant to supplant the AOAC collaborative study process, and AOAC encourages all regulatory bodies and associated industries to support that process.

Animal Drugs

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The U.S. Food and Drug Administration (FDA) is required by the general safety provisions of sections 409, 512, and 706 of the Federal Food, Drug and Cosmetic Act to determine if each food additive, new animal drug, or color additive proposed for use in food producing animals is safe. The pertinent regulations implementing the statutory provisions are found in 21 CFR Parts 70 and 500.

The sponsor of an application for use of an animal drug is required as part of the approval to submit scientific data to demonstrate that the use of the drug is safe for the animals proposed and any edible food to be used for consumption. To demonstrate the safety of the drug, the sponsor must submit acceptable analytical methods. These methods must be capable of determining and confirming the amount and presence of the animal drug or its metabolites in a variety of matrixes.

FDA typically requires analytical methods for finished pharmaceutical and medicated feed dosage forms and for residues of the drug in edible animal tissues. The sponsor must present data or information that demonstrates the method can perform what it purports according to sound analytical principles. In addition, FDA performs a trial of the method according to strict procedures to assure the method is acceptable. The U.S. Department of Agriculture laboratories also participate with FDA in testing tissue residue methods.

The types of methods described above, because they are used for regulatory purposes by FDA in its compliance programs, are available for release to the general public. These methods are releasable under the Freedom of Information Act. 21 CFR 514.11(e)(6) specifically permits the public disclosure of assay methods after approval of the animal drug has been published in the *Federal Register*.

Because of the volume and types of feeds containing approved animal drugs and used for food-producing animals, the FDA Center for Veterinary Medicine (CVM) has made it a policy to make available to the public methods for animal drugs in Type A Medicated Articles (premixes) and the corresponding Type B and C Medicated Feeds and tissue residues. CVM is the unit responsible for evaluating the effectiveness and safety of animal drugs.

Before 1973, the methods were incorporated into a manual called the *Food Additives Analytical Manual*. This manual, which is no longer available, included methods for direct and indirect food additives and animal drugs. In 1985, CVM issued a new manual called *Animal Drug Analytical Manual*. This manual, often referred to as ADAM, contains only methods for animal drugs. The 1985 edition contained only 8 methods. The manual is published and distributed by AOAC International. Information for the manual is furnished by CVM.

Since 1985, there have been no updates to ADAM. CVM, in cooperation with AOAC International, is now introducing a new procedure for making methods more readily available. It is the intention of CVM in conjunction with AOAC International to first publish the methods in the *Journal of AOAC International* and then incorporate the same in a later update to ADAM.

Albendazole

The following methods are approved regulatory methods to determine and confirm residues of albendazole in cattle.

REGULATORY ANALYTICAL METHODS

Method I. Liquid Chromatographic/Fluorescence Quantitative Determination of Albendazole Residues in Cattle Liver

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Albendazole is approved for use as an anthelmintic in cattle.

Regulation Information

21CFR520.45 provides for the approved uses of albendazole: 21CFR520.45a, as a suspension containing 11.36% albendazole (approved June 13, 1989); 21CFR520.45b, as a paste containing 30% albendazole (approved December 15, 1989).

Tolerances: 21CFR556.34

Marker Residue: SK&F 81038, S-(Propylsulfonyl)-1H-benzimidazol-2-amine

A tolerance of 0.2 ppm is established for the 2-aminosulfone metabolite (as the marker residue) in cattle liver (target tissue).

The safe concentrations for total albendazole residues in uncooked edible tissues of cattle are as follows: 0.6 ppm in muscle, 1.2 ppm in liver, 1.8 ppm in kidney, and 2.4 ppm in fat.

Method I. Liquid Chromatographic/Fluorescence Quantitative Determination of Albendazole Residues in Cattle Liver

Scope

Albendazole residues are determined in cattle liver.

Principle

The liver sample is subjected to acid hydrolysis for 1 h at 110°C. After adjusting to pH 8–9.5, the sample is extracted with ethyl acetate. The extract is partitioned between a 1N HCl solution and ethyl acetate. The pH of the aqueous phase is readjusted to 8–9.5. The aqueous phase is washed with toluene and cleaned up on a C_{18} SEP-PAK cartridge, before liquid chromatography (LC), by using a C_{18} column and fluorescence detection. Quantitation of the marker residue, *S*-(propylsulfonyl)-1*H*-benzimidazol-2-amine (SK&F 81038), is based on an internal standard method where 5-(butylsulfonyl)-1*H*-benzimidazol-2-amine (SK&F 101437) is used as the internal standard. If the marker residue is determined in excess of the established tolerance, 80% of the sample extract is used for confirmation by preparing the *t*-butyldimethylsilyl (t-BDMS) derivative followed by gas chromatographic/mass spectrometric-multiple ion detection (GC/MS-MID) (Albendazole Method II). A flow diagram of the procedure is presented in Figure 1.

Limit of Reliable Measurement

The method has been shown to be practical, reproducible, accurate, and sensitive for determining and confirming the marker residue in liver from albendazole-dosed cattle at concentrations of 100–400 ppb.

Apparatus

(All references to commercial apparatus and chemicals in this and the following section are for descriptive purposes only and do not constitute endorsement or recommendation of a product or source by the U.S. Food and Drug Administration (FDA) or any U.S Government agency; equivalent products may be substituted.)

(a) *Glass graduated centrifuge tube.*—50 mL ground glass stoppered (Kimble No. 45176-50).

(b) *Glass graduated centrifuge tube.*—13 mL ground glass stoppered (Kimble No. 45176-13).

(c) *Oven.*—Isotemp Model 176 equipped with thermometer (Fisher Scientific Co.).

(d) *Mechanical shaker.*—Eberbach flatbed (A.H. Thomas No. 8287-E30).

(e) *Centrifuge.*—Equipped with rotors for 15 and 50 mL tubes (Sorvall Model GLC-1).

(f) *N-Evap.*—Organomation Assoc., Inc., Model 112.

(g) *Commercial blender.*—Waring Model 31BL91.

(h) *Pipetter.*—Becton Dickinson Labware Model 6606.

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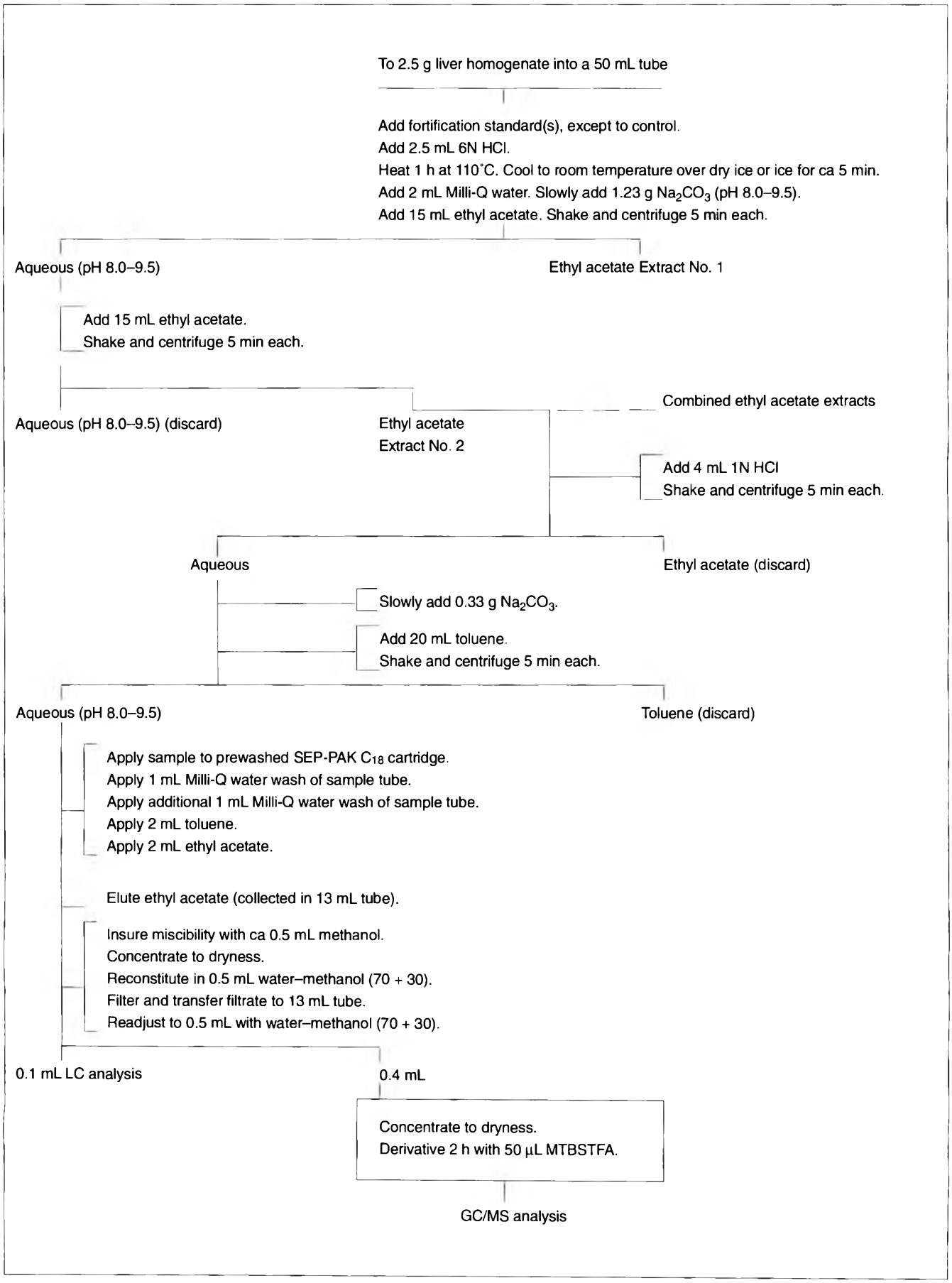

Figure 1. Flow diagram of extraction procedure.

(i) *pH Meter.*—Radiometer Model 22.

(j) *SEP-PAK C_{18} cartridges.*—Waters Assoc. No. 51910.

(k) *Vortex-Genie mixer.*—Model K-550-G (Fisher Scientific Co.).

(l) *Filter assembly.*—MF-1 centrifugal microfilter (Bioanalytical Systems Inc.).

(m) *Filters.*—0.2 μm RC58, regenerated cellulose (Bioanalytical Systems Inc.).

(n) *Balance.*—Top loading (Sartorius Model 1219MP).

(o) *Pipet.*—10 mL glass disposable (A.H. Thomas No. 7536-H85 or Corning No. 7077).

(p) *Pipet.*—Pasteur disposable (A.H. Thomas No. 7760-A10 or 7760-A26).

(q) *Pipet-Aid.*—Drummond Scientific Co.

(r) *Syringe.*—10 mL glass luer tip hypodermic (A.H. Thomas No. 8932-050).

(s) *Electrobalance.*—Cahn Model G-2.

(t) *LC column.*—μ-BONDAPAK C_{18}, 30 cm × 3.9 mm id (Waters Associates No. 27324).

(u) *LC microprocessor/controller.*—Model 420, Altex Scientific Inc.

(v) *Guard column.*—CO:PELL ODS, 5 cm × 2 mm id (Whatman Inc.) or LiChrosorb 10 μm (Brownlee Labs MPLC cartridge).

(w) *LC pump.*—Altex Scientific Inc. Model 100.

(x) *LC injection valve.*—Rheodyne Model 7120 equipped with 20 μL loop, or an automatic sample injector.

(y) *Recording integrator.*—Hewlett Packard Model 5880.

(z) *LC fluorescence spectrophotometer.*—Dual monochromator (Perkin Elmer Model 204A).

(aa) *Syringe.*—Model LC-210, Pressure-Lok, equipped with Rheodyne needle (Precision Sampling Corp.).

(bb) *Xenon lamp power supply.*—Perkin Elmer Model 150.

(cc) *Vacuum filter.*—47 mm glass (Millipore Corp.).

(dd) *Filter.*—Nylon-66 membrane 0.45 μm, 47 mm diameter (Rainin Instrument Co.).

(ee) *Syringe.*—10 μL glass (Hamilton Model 1801).

(ff) *Syringe.*—50 μL glass Pressure-Lok (Precision Sampling Model C-160).

(gg) *Fused silica capillary GC column.*—30 m × 0.25 mm id, DB-5 liquid phase, 0.25 μm film thickness (J & W Scientific, Inc.).

(hh) *GC/MS system.*—Finnigan Corporation Model 4500 mass spectrometer equipped with Model 9610 gas chromatograph.

Reagents and Solutions

(a) *S-(Propylsulfonyl)-1H-benzimidazol-2-amine (marker chemical, SK&F 81038).*—Analytical standard (SmithKline Animal Health Products, West Chester, PA 19380).

(b) *S-(Butylsulfonyl)-1H-benzimidazol-2-amine (internal standard, SK&F 101437).*—Analytical standard (SmithKline Animal Health Products).

(c) *Hydrochloric acid.*—Concentrated (J.T. Baker Chemical Co. No.1-9535).

(d) *Dimethylsulfoxide (DMSO), ethyl acetate, methanol, toluene, acetonitrile.*—Distilled in glass (Burdick & Jackson Laboratories).

(e) *Diethanolamine.*—Fisher Scientific Co. No. D-45.

(f) *Sodium carbonate.*—Anhydrous powder (Mallinckrodt, Inc., No. 7521).

(g) *Water.*—Produced by a Milli-Q/Milli-RO system (Millipore Corp.).

(h) *Potassium phosphate monobasic.*—Fisher Scientific Co. No. P-285.

(i) *Potassium phosphate dibasic.*—Anhydrous (Mallinckrodt Inc. No. 7092).

(j) *Nitrogen.*—Prepurified grade (M.G. Burdett Gas Products Co.).

(k) *Dry ice.*

(l) *N-Methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA).*—Regis Chemical Co.

(m) *Stock standard solutions.*—(*1*) Into a 100 mL volumetric flask, weigh 10.0 mg SK&F 81038 analytical standard. Dissolve and dilute to the mark with DMSO (100 μg/mL). (*2*) Into a 100 mL volumetric flask, weigh 10.0 mg SK&F 101437 analytical standard. Dissolve and dilute to the mark with DMSO (100 μg/mL).

(n) *Working standard solutions.*—(*1*) Pipet 2.0 mL stock solution (*1*) and 8.0 mL DMSO into 13 mL glass stoppered centrifuge tube, and vortex mix to produce 20 μg/mL solution (2.5 g liver fortified with 50 μL this solution is equivalent to 400 ppb concentration of the marker compound). (2) Pipet 5.0 mL working standard (*1*) and 5.0 mL DMSO into 13 mL glass stoppered centrifuge tube, and vortex mix to produce 10 μg/mL solution (2.5 g liver fortified with 50 μL this solution is equivalent to 200 ppb concentration of the marker compound). (*3*) Pipet 5.0 mL working standard (*2*) and 5.0 mL DMSO into 13 mL glass stoppered centrifuge tube, and vortex mix to produce 5 μg/mL solution (2.5 g liver fortified with 50 μL this solution is equivalent to 100 ppb concentration of the marker compound). (*4*) Pipet 1.0 mL stock solution (*2*) and 9.0 mL DMSO into 13 mL glass stoppered centrifuge tube, and vortex mix to produce 10 μg/mL solution (2.5 g liver fortified with 50 μL this solution is equivalent to 200 ppb concentration of the internal standard).

(**Note**: Store all standard solutions in a refrigerator. Because DMSO solidifies under these conditions, standard solutions must be reliquified by placing tubes in a beaker of lukewarm water and vortex mixing solutions before use. Standard solutions are stable in DMSO for a minimum of 6 months.)

Analysis

Isolation procedures.—For liquid–liquid extraction, thoroughly homogenize representative sections of cattle liver in a commercial Waring blender until no visible pieces remain (ca 3–5 min/100 g). Keep homogenates frozen at all times until they are analyzed.

Into the bottom of an unstoppered 50 mL glass centrifuge tube supported in a beaker that is tared on a balance, weigh 2.5 ± 0.05 g homogenized liver using a wide tip disposable pipet. Prepare 4 blank tissue samples, 1 for control and 3 for fortification. Add 100 μL pure DMSO to the control. Add 50 μL DMSO to tissues suspected of containing albendazole residue.

Add 50 μL working standard solution (*4*) to each of the remaining 3 blank tissue samples and tissue samples suspected of containing albendazole residue. Add 50 μL working standard solutions (*1*), (*2*), and (*3*) to separate blank tissue samples from the set of 3 already fortified with working standard solution (*4*). (**Note:** Add fortification solutions to the 50 mL tube just above the homogenate to insure solution when the HCl is added in the next step).

Add 2.5 mL 6N HCl to each sample prepared above, stopper each tube, and place sample tubes (covered with another tube rack to prevent the stoppers from popping) in an oven preset to 110 ± 4°C (at elevations ≤1000 ft above sea level). (**Note**: At elevations ≥ 5000 ft, hydrolysis for 30 min in a 15 psi pressure vessel is required to adjust for atmospheric pressure. Timing for hydrolysis should begin when samples are placed into the pressure vessel with the water already boiling. It is necessary to have the water boiling to reach pressure as quickly as possible [≤4 min].) The hydrolysis time is critical and must be timed accurately.

After 1 h, remove samples from the oven and let tubes cool to room temperature over ice for ca 5 min. Add 2.0 mL Milli-Q water to each sample. While vortex mixing the sample, slowly add enough (ca 1.2 g) sodium carbonate to achieve pH of 8–9.5 when monitored with a pH meter. (**Note**: Add the carbonate over a period of ca 4–5 min to avoid foaming and/or caking. Samples at or near room temperature do not foam as much as colder samples; therefore, the carbonate can be added more quickly. Place the clean pH electrode directly into the sample; after recording pH, dab any drop of solution from the electrode on the inside of the tube and then completely remove the electrode from the tube, rinse it with water, and dry it with a Kimwipe before monitoring the next sample.)

Add 15 mL ethyl acetate to each sample and stopper tubes. Shake samples, supported in a rack in a horizontal position, for 5 min. Remove stoppers and centrifuge samples 5 min at ca 400 *g*. Transfer as much as possible of the ethyl acetate extracts to separate 50 mL glass centrifuge tubes using a disposable pipet: *Do not transfer any of the aqueous phase*. (**Note**: Use of a Pipet-Aid in this step is beneficial.)

Reextract each sample a second time by repeating the last 2 steps and pooling the appropriate extracts in the respective 50 mL glass centrifuge tubes.

Add 4.0 mL 1N HCl to each ethyl acetate pooled extract and stopper tubes. Shake samples, supported in a rack in a horizontal position, for 5 min. Remove stoppers and centrifuge samples ca 5 min.

Remove as much of the ethyl acetate phase as possible by aspiration: *Do not remove any of the aqueous phase*. Evaporate the remaining ethyl acetate (≤1 mL) from the aqueous phase in the N-Evap waterbath at 35 ± 5°C. (**Note**: If necessary, the extraction can be stopped overnight at this point.)

While vortex mixing each sample, add enough (ca 0.33 g) sodium carbonate slowly (ca 1–2 min to avoid foaming and/or caking) to give a pH of 8–9.5, monitored with a pH meter.

Add 20 mL toluene to each sample and stopper tubes. Shake samples, supported in a rack in a horizontal position, for 5 min. Remove stoppers and centrifuge samples ca 5 min.

Remove as much of the toluene phase as possible by aspiration: *Do not remove any of the aqueous phase*. Evaporate the remaining toluene (≤1 mL) from above the aqueous phase in the N-EVAP waterbath at 35 ± 5°C.

Solid-phase extraction.—Attach a SEP-PAK C_{18} cartridge to the luer tip of a 10 mL glass syringe secured to a ring stand with a clamp for each sample (cylinder barrel removed).

Prewash each cartridge with 2 mL methanol followed by 2 mL 0.2M potassium phosphate pH 8.0 buffer. Transport each reagent through the cartridge at 1–2 mL/min by means of gentle nitrogen pressure applied through a rubber stopper fitted into the top of the syringe after addition of the respective reagent. The nitrogen pressure is removed when nitrogen begins to exit the cartridge. Discard eluates. (If necessary, extractions can be stopped overnight at this point.)

Transfer the evaporated aqueous phase from each sample to a separate prewashed syringe cartridge unit. Elute this phase from the cartridge using gentle nitrogen pressure described above, then transfer a 1 mL Milli-Q water wash of each sample tube (vortex-mixed) to the respective syringe-cartridge unit in a similar manner using the respective pasteur pipet. Discard all eluates.

Add an additional 1 mL water wash of each sample tube (vortex-mixed) to the respective syringe-cartridge unit, and elute using gentle nitrogen pressure described above. Discard eluates.

Add 2 mL toluene to each syringe-cartridge unit, and elute by using gentle nitrogen pressure described above. Discard eluates.

Place a 13 mL glass centrifuge tube beneath each syringe-cartridge unit. Add 2 mL ethyl acetate to each unit, and elute using gentle nitrogen pressure described above. This fraction contains the compounds of interest.

Add ca 0.5 mL methanol to each eluate from the last step, and vortex mix to obtain a homogeneous solution. Concentrate these eluates to dryness under dry nitrogen in an N-EVAP waterbath at 35 ± 5°C.

After reaching dryness, add 0.5 mL water–methanol (70 + 30, v/v) solution, and vortex mix to reconstitute the residue.

After ca 30 min, apply each of the reconstituted samples to a separate Bioanalytical System filter unit fitted with a 0.2 μm regenerated cellulose filter. Centrifuge each filter unit containing a sample extract ca 5–10 min.

Determinative Procedures

LC conditions.—Mobile phase: 0.02M KH_2PO_4/0.01M diethanolamine (prepare by placing 200 mL 0.1M KH_2PO_4 and 1.05 g diethanolamine in a 1 L volumetric flask and diluting to the line with water)–methanol–acetonitrile (68 + 20 + 12, v/v/v). Prepare this solution daily, filter through a 0.45 μm Nylon-66 filter, and degas under vacuum before use. Flow rate, 1.8 mL/min. Fluorescence detector: Initially set excitation and emission wavelengths at 300 and 320 nm, respectively, with a slit width of ca 10 nm for both. Inject an appropriate volume of sample (20 μL), and observe the intensity of the response for both SK&F 81038 and SK&F 101437 with a reasonable baseline sensitivity setting. If additional sensitivity is required, pro-

Table 1. Summary of validation trial data

Laboratory	Rec. from fortified tissues, %	CV, %	Dosed tissue values, ppb	CV, %
FDA (HFV-146)	105.3 ±10.5	9.9	300.9 ±26.5	8.8
FDA (HFR-8160)	72.5 ±17.3	23.8	250.4 ±25.4	10.1
USDA (CDLB)	100.4 ±10.2	10.2	211.8 ±6.6	3.1
USDA (EL)	101.7 ±5.2	5.1	182.2 ±16.5	9.1
Interlaboratory rec. value	101.7 ±5.2[a]	5.1	—	—
Interlaboratory dosed tissue value	—	—	226.1 ±48.9	21.6

[a] FDA, HFR-8160 omitted.

ceed as follows: While observing the detector absorption response, adjust the excitation wavelength ca 5 nm to reduce the zero suppression. Reinject the same sample as above and determine if the signal responses for the compounds of interest increase. Continue this procedure of optimizing both the excitation and emission wavelengths to obtain an adequate signal-to-noise ratio (≥25/L) for the analysis.

Temperatures for the column, mobile phase, and detector, ambient (70 ± 2°F).

Under these conditions, retention times of albendazole marker (SK&F 81038) and the internal standard (SK&F 101437) are ca 4.3 and 7.0 min, respectively.

Analysis of samples.—Transfer filtrates from the last step of the isolation procedure to separate 13 mL glass-stoppered centrifuge tubes, and adjust the volume of each filtrate to 0.5 mL with water–methanol (70 + 30, v/v). After vortex mixing, remove 0.1 mL for LC analysis. Use the remaining liquid to conduct confirmation analyses of the residues (Method II). (**Note**: Use of silanized tubes in this step is recommended.)

Calculations.—If an integrator is unavailable, substitute manual peak height measurements and peak height ratio data for peak area and peak area ratio data in the following discussion.

Divide the peak area of the albendazole marker SK&F 81038 by the peak area of the internal standard SK&F 101437 from the LC data for each sample extract to obtain peak area ratios:

peak area ratio = area of albendazole marker (SK&F 81038)/area of internal standard peak (SK&F 101437)

Construct an analytical curve by plotting, on linear axes, the peak area ratio vs the respective ppb value for each fortified control sample. Using the regression equation for the analytical curve, directly calculate the ppb level of albendazole marker in the unknown samples from their peak area ratios using the following equation:

$$x = (y - b)/m$$

where x = ppb concentration in samples, y = peak area ratio of sample extract, b = intercept from regression equation, and m = slope from regression equation.

Recovery procedures.—Analyze control liver fortified with albendazole to determine recovery levels. To determine that a cattle liver can be used as control, proceed as follows: Prepare four 2.5 g liver homogenate samples. Fortify 2 aliquots with 100 µL DMSO and the other 2 aliquots with 50 µL DMSO and 50 µL internal standard solution (200 ppb). Process samples through the extraction method, and analyze using the LC procedure. Nonfortified control samples (DMSO only) should produce either no response or a negligible response at the retention time corresponding to the internal standard, when compared with the response observed for a known control sample fortified at 200 ppb. Providing this background is negligible, calculate the background at the retention time corresponding to the albendazole marker in the control samples fortified with internal standard. If a response is observed, calculate the peak area (height) ratio. If either no response or a peak area (height) ratio less than 5× that calculated for a known control fortified at 200 ppb is determined, the cattle liver is considered a control.

Confirmatory procedures.—Confirm the presence of albendazole by the GC/MS procedure described in Method II.

Reliability of the Method

Validation.—A method trial of the determinative and confirmatory procedures was performed at the 200 ppb level with the following participating laboratories: FDA, New Animal Drug Evaluation, Methods Trial Review Staff, HFV-146; FDA, Denver District Office, HRF-8160; U.S. Department of Agriculture (USDA), Food Safety and Inspection Service, CDLB, Beltsville; and USDA, Food Safety and Inspection Service, Eastern Laboratory. A summary of the validation trial data is found in Table 1.

Albendazole residues were not detected in the control sample by any of the participating laboratories.

Confirmation for the presence of albendazole was obtained by FDA (HFV-146) and USDA (CDLB). Confirmation was not attempted by USDA (EL) due to limitation in their available equipment. FDA, however, recommended that the error limits for *m/z* 189 be changed from ±10% to ±20% due to a compound coeluting with the internal standard.

Overall results of the determinative procedure appeared to be satisfactory. Absolute recoveries of the marker compound vs the internal standard obtained in FDA and USDA laboratories indicated that the marker was recovered consistently at a 10% higher rate than the internal standard. Use of a standard curve generated from fortified tissues helps to correct for this factor. However, for monitoring purposes in the field, this requirement would put an additional burden on the analyst. Because an in-

ternal standard is already used that gives a 1:1 detector response and relative recoveries of the marker vs internal standard can be determined in each laboratory, a correction factor could be used for more accurate results. This would eliminate the need for a standard curve and the samples necessary to generate it. It was stated that the SEP-PAK cleanup is necessary only if GC/MS confirmation is to be performed. Unless an excessive violation rate is found, elimination of this step would undoubtedly increase check sample throughput.

Additional work performed by the Methods Trial Review Staff indicated that the method, with minor modifications, is capable of determining and confirming albendazole residues down to 25 and 50 ppb, respectively. However, before the method could be accepted at a level significantly below 200 ppb (i.e., <100 ppb), it would have to be completely reevaluated.

The validation study verified that the method is capable of determining and confirming albendazole residues at 200 ppb and above in cattle liver. The method is relatively rapid, permitting the analysis of 8 more samples per day.

Discussion

Sample storage.—If frozen cattle liver is used, it must be kept frozen until assay. Liver homogenate stored in small vials is thawed under lukewarm water before each assay. Brief thawing of either the frozen liver or frozen liver homogenate is acceptable if the sample is refrozen as soon as possible.

Isolation procedures.—A ≥75% method recovery is achieved by transferring as much of the extraction solvents as possible. In cases where the solvent is discarded and the aqueous phase containing the compounds of interest is retained, it is imperative to avoid removing any of the aqueous layer. Transferring the 4 mL aqueous phase to the prewashed syringe cartridge unit should be done quantitatively by using a pasteur pipet. The 1 mL washes of the sample tube are transferred to the SEP-PAK C_{18} cartridge by using the same pasteur pipet. When transferring solutions, separate disposable and pasteur pipets are used for each sample to avoid contamination.

Sample stability.—Sample extracts contained in water–methanol (70 + 30, v/v) are stable for at least a month and, if reanalyzed, provide data similar to the previous LC analysis. Sample extracts concentrated to dryness for derivatization and subsequent GC/MS analysis are stable for at least a month when stored in a refrigerator.

Method time requirements.—Assuming analysis of 8 samples (control liver, control liver fortified at 1/2 Rm [Rm = marker residue concentration at withdrawal time], at Rm, and at 2 Rm, and 4 samples), the isolation procedure can be completed in a total time of about 7 h on the first day. The LC determinative (about 1.5 h) and GC/MS confirmatory (about 3–4 h) analyses can be performed the next day, for a total assay time of 2 days.

References

(1) Method was submitted by SmithKline Beecham Animal Health in conjunction with requirements for approval of their new animal drug applications, NADAs 110-048 and 128-070.

(2) The albendazole method trial report, dated November 8, 1984, was submitted by Dr. David R. Newkirk to Dr. Robert C. Livingston, Acting Director, FDA Division of Drug Manufacturing and Residue Chemistry, HFV-140.

REGULATORY ANALYTICAL METHODS

Method II. Gas Chromatographic/Mass Spectrometric Confirmatory Method for Albendazole Residues in Cattle Liver

Co-Edited by: JOHN MARKUS[1]
U.S. Food and Drug Administration, Department of Health and Human Services, Center for Veterinary Medicine, Office of New Animal Drug Evaluation, 7500 Standish Place, Rockville, MD 20855
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Albendazole is approved for use as an anthelmintic in cattle.

Regulation Information

21CFR520.45 provides for the approved uses of albendazole: 21CFR520.45a, as a suspension containing 11.36% albendazole (approved June 13, 1989); 21CFR520.45b, as a paste containing 30% albendazole (approved December 15, 1989) (1, 2).

Tolerances: 21CFR556.34

Marker Residue: SK&F 81038, S-(Propylsulfonyl)-1H-benzimidazol-2-amine

A tolerance of 0.2 ppm is established for the 2-aminosulfone metabolite (as the marker residue) in cattle liver (target tissue).

The safe concentrations for total albendazole residues in uncooked edible tissues of cattle are as follows: 0.6 ppm in muscle, 1.2 ppm in liver, 1.8 ppm in kidney, and 2.4 ppm in fat.

Method II. Gas Chromatographic/Mass Spectrometric Confirmatory Method for Albendazole Residues in Cattle Liver

Scope

Albendazole residues are confirmed in cattle liver.

Principle

Quantitation of the marker residue is based on an internal standard method where SK&F 101437 [5-(butylsulfonyl)-1H-benzimidazol-2-amine] is used as the internal standard. The determinative procedure uses liquid chromatography (LC) for quantifying the marker residue concentration (Method I). If the marker residue is determined in excess of the tolerance, 80% of the sample extract is used for confirmation by preparing the *t*-butyldimethylsilyl (t-BDMS) derivative followed by gas chromatographic/mass spectrometric-multiple ion detection (GC/MS-MID) analysis.

Limit of Reliable Measurement

The method has been shown to be practical, reproducible, accurate, and sensitive for determining and confirming the marker residue in liver from albendazole-dosed cattle at concentrations of 100–400 ppb.

Apparatus

See *Apparatus*, Albendazole Method I.

Reagents and Solutions

See *Reagents and Solutions*, Albendazole Method I.

Standard solutions for GC/MS analysis.—Prepare stock and working standard solutions for GC/MS as described in Method I by using methanol rather than DMSO as the solvent.

Analysis

Isolation procedures.—Follow the isolation and sample analysis procedures of Method I to prepare the solutions of the filtrates in water–methanol (70 + 30, v/v). Use remaining 0.4 mL sample after LC quantitation for confirmation as described below.

Derivatization procedures.—Concentrate remaining 0.4 mL liver extract samples (Method I), along with prepared standard samples, to dryness in an N-EVAP waterbath at 35 ± 5°C. If necessary, reconstitute the residue in 50 µL methanol, and concentrate samples to dryness so that the majority of residue is at or below the 100 µL mark on the tube. Concentrate samples using dry air. Nitrogen may be used in place of air; if so, flush samples with air momentarily if immediately proceeding to the next step.

Add 50 µL *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) reagent to each concentrated sample, stopper tubes, and vortex mix samples. Place stoppered sample tubes (covered with another tube rack to prevent stoppers from popping) in an oven preset to 100 ± 4°C to assure complete

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[1] Current address: 1709 Burning Tree Dr, Vienna, VA 22182.

Table 1. Exact masses and elemental compositions of ions for albendazole marker t-BDMS derivative

Nominal mass	Exact mass	Elemental compostion	Error, ppm
130	130.0507	$C_4H_{12}NSi_2$	1.6
189	189.0721	$C_9H_{11}N_3Si$	4.2
236[a]	—	—	—
246	246.0881	$C_{11}H_{16}N_3Si_2$	2.0
296	296.0886	$C_{12}H_{18}N_3O_2SSi$	−0.2
303	303.1585	$C_{15}H_{25}N_3Si_2$	0.6
340	340.0969	$C_{13}H_{22}N_3O_2SSi_2$	1.4
354	354.1126	$C_{14}H_{24}N_3O_2SSi_2$	7.4
396	396.1596	$C_{17}H_{30}N_3O_2SSi_2$	−7.1
410	410.1752	$C_{18}H_{32}N_3O_2SSi_2$	−4.2
452	452.2221	$C_{21}H_{38}N_3O_2SSi_2$	−10.1
467	467.2455	$C_{22}H_{41}N_3O_2SSi_2$	−11.5

[a] No reasonable compositions.

derivatization. Remove samples from the oven ca every half hour for vortex mixing, then immediately return tubes to the oven. After a total derivatization time of 2 h, remove samples from the oven for GC/MS analysis. (**Notes**: Samples may remain in the oven longer. Two-hour derivatization is a minimum. If a large number of samples are prepared for analysis, it is recommend that each sample remain in the oven until analysis. Sample extracts concentrated to dryness for derivatization and subsequent GC/MS analysis are stable for at least 1 month when stored in a refrigerator.)

Determinative Procedures

Gas chromatograph operating conditions.—Injection port temperature, 260°C; column oven temperature program: initial temperature, 200°C (hold 1 min); final temperature, 300°C (hold for duration of the analysis); temperature program rate, 20°C/min; injection mode, Grob splitless, split vent timing 0.6–1 min, vent flow 100 mL/min; carrier gas, helium, 30–40 psi; linear velocity, 60–70 cm/sec; injection volume, 1.0–3.0 µL.

Approximate retention times under these conditions are ca 8 min for the marker residue (SK&F 81038) and ca 9 min for the internal standard (SK&F 101437).

Mass spectrometer operating conditions.—Temperatures: source, 140°C (Model 4500), 225°C (Model 4000 dial setting 30); separator, 275 ± 5°C; transfer line, 275 ± 5°C; manifold, 120°C (Model 4500), 100 ± 5°C (Model 4000); multiplier gain, 5×10^4–1×10^5; voltage, 2000–2400 eV; electron energy, 70 eV; calibration standard, FC-43 (perfluorotributylamine); emission current, −0.30; preamplifier sensitivity, 10^{-7}–10^{-8}.

Analysis of samples.—After initial instrument calibration to ≥500 amu, perform partial scan analysis (mass range 45–500 amu) with an appropriate amount (ca 3.0 µL) of a standard sample containing 10 µg SK&F 81038 and 10 µg SK&F 101437 as the *t*-butyldimethylsilyl (t-BDMS) derivatives in 50 µL derivatizing reagent. Determine the centroid mass assignments of the ions *m/z* 189, 354, 410, and 467 for SK&F 81038-t-BDMS and *m/z* 189, 368, 424, and 481 for SK&F 101437-t-BDMS by averaging the observed centroid masses over ca 5 scans of the respective chromatographic peaks or by using a procedure applicable to the particular data system used. Calibrate the instrument to obtain a minimum relative intensity of 10% for each ion monitored. Set the MID mode to monitor these 7 ions. Select a beginning and end mass range for each ion and scan time appropriate for the particular instrument used.

Proceed as follows for the MID analysis: Perform 3 analyses of the derivatized standard samples (equivalent to 100, 200, and 400 ppb) with an appropriate injection volume (ca 3.0 µL). From these analyses, determine the relative ion intensity reproducibility for the instrument used.

Analyze an aliquot (ca 3.0 µL) of the derivatization reagent (MTBSTFA) to insure there is no ghosting interference with the monitored ions at the retention times corresponding to the components of interest.

Analyze an appropriate amount (ca 3.0 µL) of the derivatized control and fortified control (100, 200, and 400 ppb) cattle liver extracts, respectively. Determine the relative ion intensity reproducibility from these samples. The relative ion intensity for a particular ion should not vary by more than ±10%.

Repeat the analysis of an aliquot of MTBSTFA.

Providing the relative ion intensity is reproducible to within ±10% and no ghosting interference is apparent, analyze the derivatized cattle liver extracts suspected of containing albendazole residues. Alternate injections of each sample with injections of the derivatization reagent to insure that there is no ghosting interference between analyses of the suspect samples.

Record the relative intensity data for ions *m/z* 189, 354, 410, and 467 for SK&F 81038-t-BDMS and *m/z* 189, 368, 424, and 481 for SK&F 101437-t-BDMS from each analysis. Confirmation of the albendazole marker residue in the suspect cattle liver samples consists of the following: (*1*) identifying a response at the retention time corresponding to the albendazole marker t-

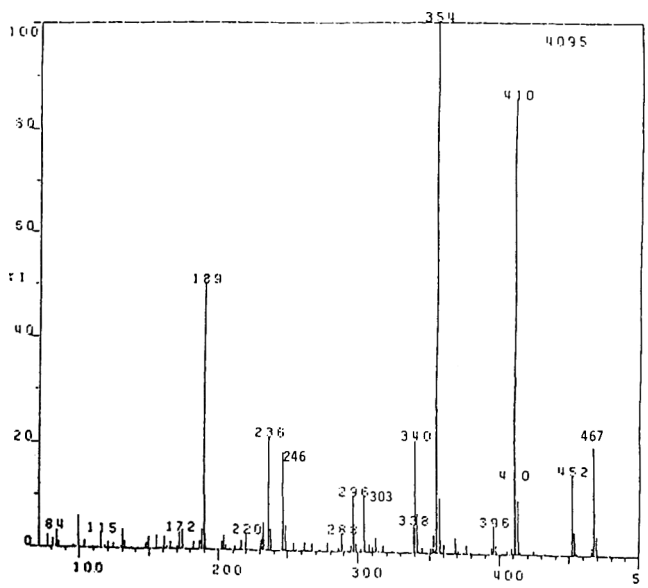

Figure 1. EI mass spectrum of albendazole marker t-BDMS derivative.

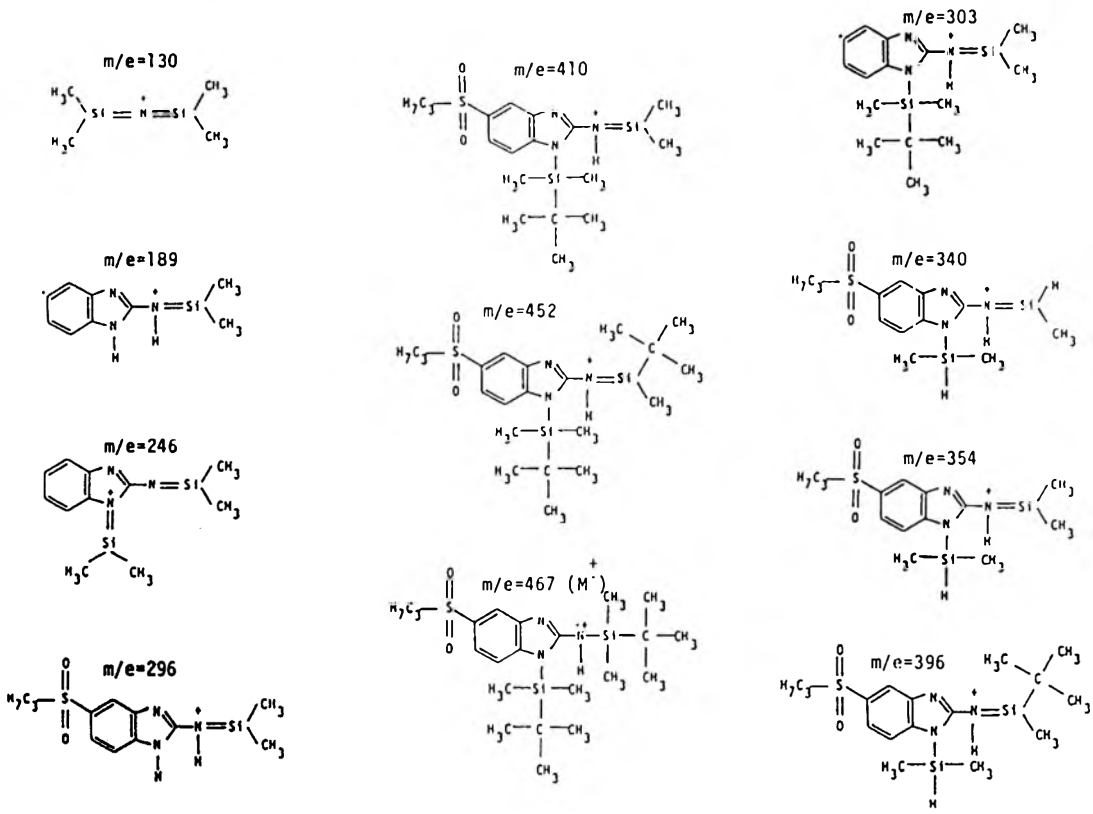

Figure 2. Possible structures for the major ions from the EI mass spectra of albendazole marker t-BDMS derivative.

BDMS derivative, (*2*) identifying the 4 characteristic mass ions (*m/z* = 189, 354, 410, and 467) in this response, and (*3*) the relative ion intensity of each ion is reproducible to within ±10% of that obtained for the ions from the derivatized control cattle liver extracts fortified with the albendazole marker chemical.

Supplemental mass spectral information.—Table 1 lists the most reasonable exact mass assignments and elemental compositions of the ions of interest for the electron impact mass spectrum of the albendazole marker t-BDMS derivative, shown in Figure 1. These data were obtained with a high resolution VG Model 7070 magnetic sector instrument. The *m/z* 354 ion was saturated deliberately to enhance several of the weaker ions. Structures for the elemental compositions in Figure 2 are only hypothetical and should be regarded as such without further data on the sequence of fragmentation. These data are presented merely as supplemental information to support the rationale for choosing the ions to monitor.

Reliability of the Method

See *Validation* in Method I.

References

(1) The method was submitted by SmithKline Beecham Animal Health in conjunction with requirements for approval of their new animal drug applications, NADAs 110-048 and 128-070.

(2) The albendazole method trial report, dated November 8, 1984, was submitted by Dr. David R. Newkirk to Dr. Robert C. Livingston, Acting Director, FDA Division of Drug Manufacturing and Residue Chemistry, HFV-140.

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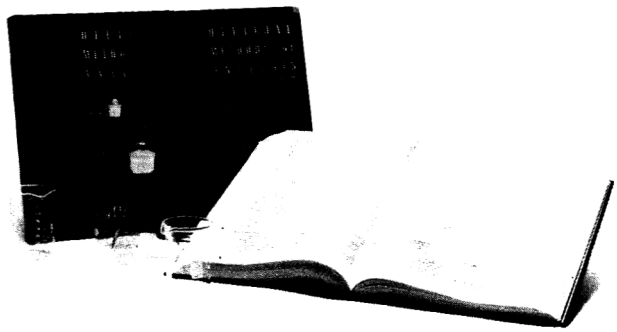

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