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MARCH/APRIL 1991
VOL. 74, NO. 2

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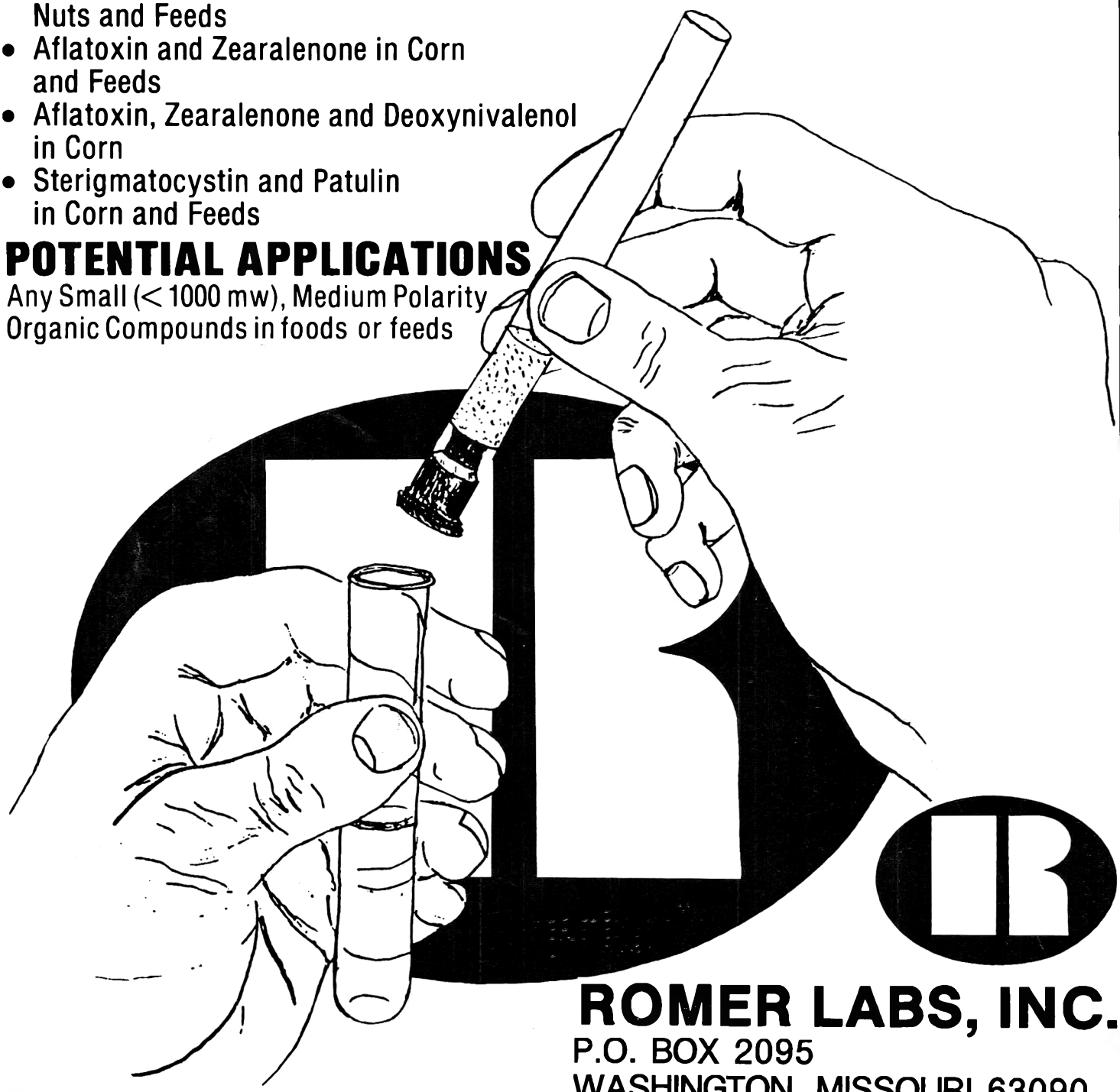

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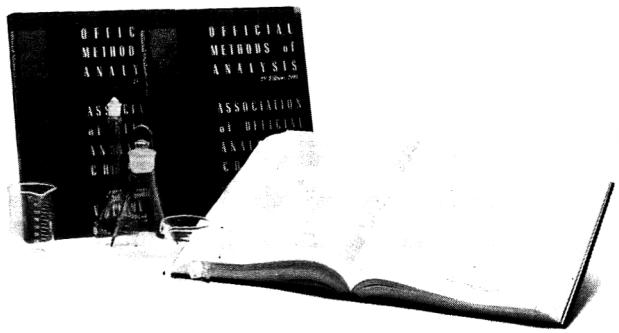

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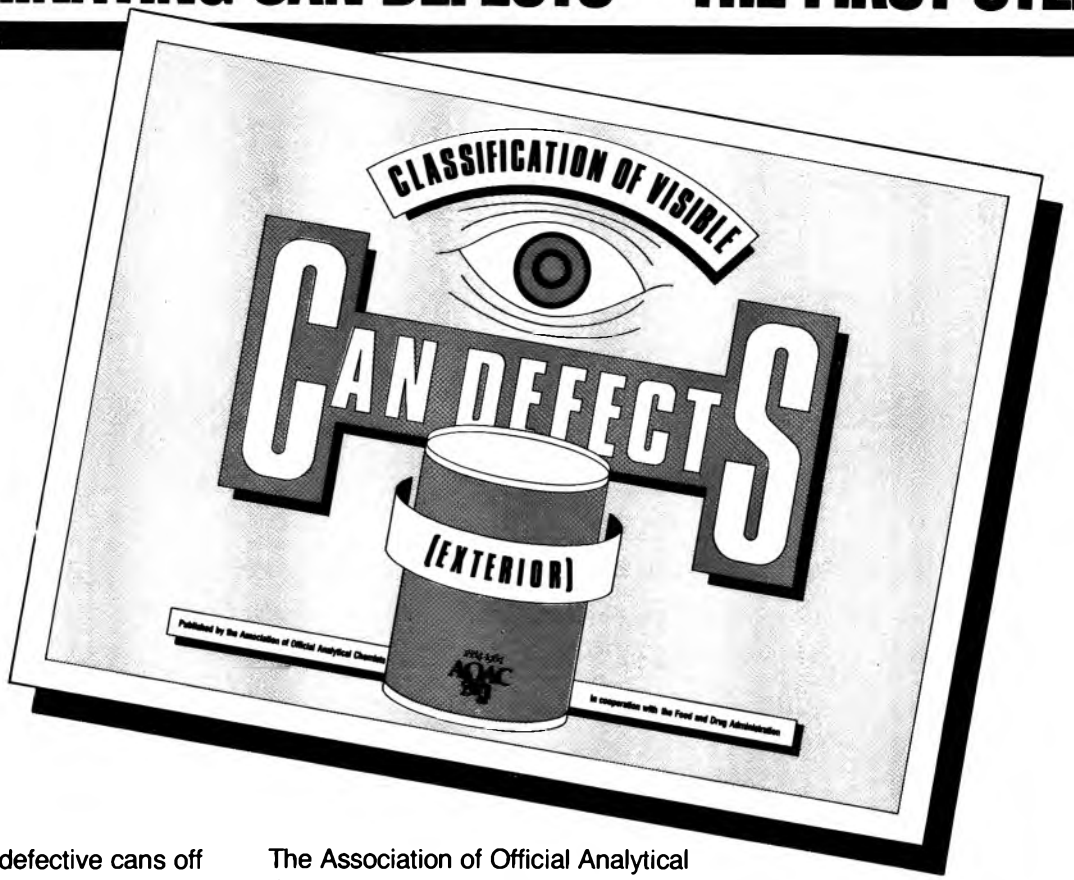

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J A O A C

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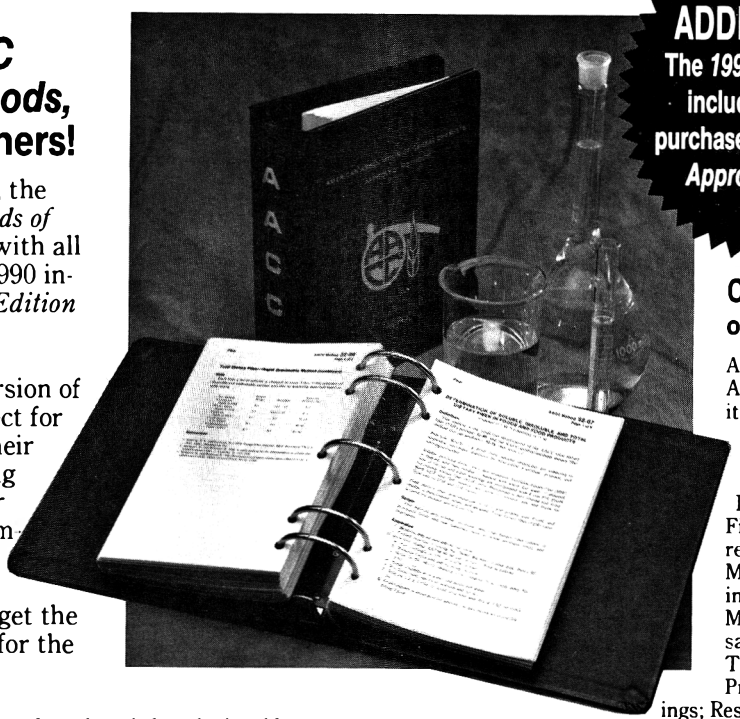

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April 28-May 3, 1991: AOAC Short Courses, Delta Chelsea Inn, 33 Gerrard St West, Toronto, Ontario M5G 1Z4, Canada. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

April 30, 1991: New York-New Jersey AOAC Regional Section Meeting. Busch Campus Center, Piscataway, NJ. Contact: Jeff Singer, Dupont Pharmaceuticals, 1000 Stewart Ave, Garden City, NY 11530, telephone 516/832-2171.

May 13-15, 1991: Northeast AOAC Regional Section Meeting. Sheraton Islander, Newport, RI. Contact: Penelope Lynn, New York State Agricultural Experiment Station, Geneva, NY 11454-0462, telephone 315/787-2280.

June 3-5, 1991: Midwest AOAC Regional Section Meeting, Sioux Falls, SD. Contact: Nancy Thiex, Olson Biochemistry Laboratories, South Dakota State University, PO Box 2170, Brookings, SD 57007, telephone 605/688-6171.

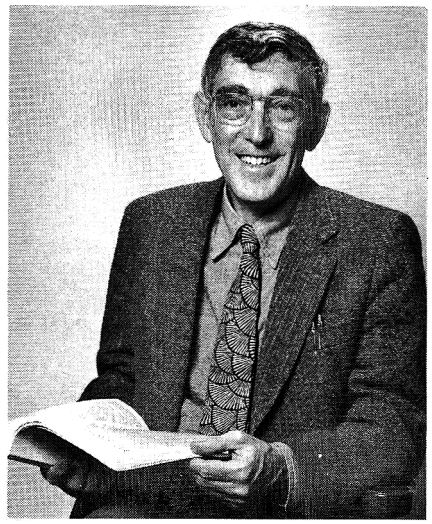
June 27-28, 1991: Pacific Northwest AOAC Regional Section Meeting, Evergreen State College, Olympia, WA. Contact: Charles A. Laubach, Puget Sound Naval Shipyard, Code 134.3, Bremerton, WA 98314, telephone 206/442-0370.

August 12-15, 1991: 105th AOAC Annual International Meeting and Exposition. The Pointe at South Mountain, Phoenix, AZ. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

November 12-13, 1991: AOAC/Europe Regional Section Meeting, State University of Limburg, Maastricht, The Netherlands. Contact: Ellen Jan De Vries, Duphar B.V., PO Box 900, 1380 DA Weesp, The Netherlands, telephone 31-2940-79296.

Bruce Ames to be Keynote Speaker at AOAC Annual Meeting

Bruce Ames, Director of the National Institute of Environmental Health Sciences Center at the University of California, Berkeley, will be the keynote speaker at the opening session of the 105th AOAC Annual International Meeting in Phoenix, AZ, on August 12, 1991.



Internationally known for developing the Ames Test, Ames' work in *Salmonella* established him as a leader in the field of regulation of gene function. His test is a simple and inexpensive test system for the detection of mutagens and potential carcinogens. One of Ames' current research interests is in the area of cancer risk assessment, and annual meeting attendees should look forward to his presentation on the use of analytical data in the determination of risk assessment.

"In view of his illustrious career, no introduction may be necessary," says AOAC Meetings, Symposia, and Educational Programs Committee Chairman Samuel W. Page. The Ames Test itself may be responsible for the generation of more analytical chemical data than any other in the past several decades. "And to a considerable degree," says Page, "AOAC members are responsible for generating the numbers often used in risk assessment. I look forward to his insights into the use – and misuse – of such analytical data."

Ames' research also involves the correlation between diet and cancer, natural carcinogens, and oxidative DNA damage and defenses against oxidation as they relate to the causes of aging. The idea that DNA damage is related to carcinogens has received major support from the research work done by Ames and his colleagues.

Department Chairman of the Division of Biochemistry and Molecular Biology at the University of California, Berkeley, from 1983 to 1989, Ames also served on the National Cancer Advisory Board, is a member of the National Academy of Sciences, and has been elected to the Royal Swedish Academy of Sciences and the Japan Cancer Association. His numerous awards include the General Motors Cancer Research Foundation Prize, the most prestigious award given for cancer research, and the Tyler Prize, the highest award given for achievement in the environmental area.

AOAC President H. Michael Wehr, who invited Ames to speak, adds, "I am extremely pleased that Dr. Ames will be our keynote speaker. Ames' research has been instrumental in developing risk assessment analytical procedures that are frequently used by AOAC members in the development of toxicological data. Dr. Ames' visit is most timely, not only because of the international interest in this field, but also because it is linked to AOAC's planned symposia on Natural Toxins, Biotechnology in the Environment, and Risk Management in the Laboratory."

Workshop on Antibiotics and Drugs in Feeds

To review and update AOAC methods for antibiotics and drugs in animal feeds, AOAC is presenting a workshop on August 11, 1991, on Antibiotics and Drugs in Feeds, designed specifically for bench analysts. Providing a forum for exchange between antibiotic and drug analysts, this workshop is cosponsored by the Association of American Feed Control Officials, the U.S. Food and Drug Administration, and AOAC.

The workshop will be held at The Point at South Mountain, in Phoenix, AZ, immediately preceding the 105th AOAC Annual International Meeting. The workshop offers an opportunity to discuss methods being developed and problems with current methods.

AOAC's General and Associate Referees and other experts will lead discussions on the analytes bacitracin, carbadox, lasalocid, neomycin, and tylosin, and on quality assurance and sample preparation. The following is a full listing of topics and authors:

"Review of Approaches to HPLC Analysis of Carbadox," Alicia Henks, Pfizer, Inc.

"Application of the Chemical and Physical Properties of Lasalocid for Assay in Feeds," Alexander MacDonald, Hoffmann la Roche Inc.

"Evaluation of HPLC Methods for Lasalocid," Harold Campbell, Agriculture Canada

"The Antibiotics and Drug Laboratory GLP Audit," Fred Bond, FDA

"Microbiological Plate Assay of Bacitracin in Feeds," Anil Desai, A.L. Laboratories, Inc.

"Microbiological Assay Systems for Tylosin in Feeds," Mark Coleman, Lilly Research Laboratory

"Observations on the Microbiological Assay of Neomycin in Feeds," Hussein Ragheb, Office of the State Chemist, Purdue University; Gerald Stahl, Upjohn Company; and Stanley Katz, Rutgers University

The workshop will also include a general discussion session on particle size as it relates to assay replication and analyte recovery and stability, moderated by Mark Coleman, Mary Lee Hasselberger, Nebraska State Department of Agriculture Laboratory, and Robert Smallidge, Office of the State Chemist, Purdue University.

"Many of the antibiotic and drug methods in *Official Methods of Analysis* have been on the books for a number of years, and many analysts have introduced modifications to these methods," says Hasselberger, chairman of the workshop. Workshop participants want to learn what modifications of methods are being used, help evaluate the validity of these modifications, and start to incorporate the modifications into the official methods, if warranted.

Participants in the workshop will each receive a copy of the proceedings, which will include a section for each analyte. The material submitted by the workshop presenters for the proceedings is being organized by Audrey Gardner of the New York State Agricultural Experiment Station in Geneva, NY, who will also serve as a program moderator. Other moderators will include Austin R. Long, Animal Drug Research Center, FDA, and Denise Riley Moore, Woodson-Tenent Laboratories.

The registration fee will be a $60 ($70 for nonmembers) add-on to the regular AOAC registration, and will include Sunday lunch and breaks, and a copy of the proceedings. Pre-registration is necessary to guarantee the lunch and pre-meeting correspondence.

For more information, contact Mary Lee Hasselberger, Nebraska State Department of Agriculture Laboratory, 3703 S. 14th St, Lincoln, NE 68502, (402) 471-2176. Analysts are encouraged to mail in questions or topics for the general discussion session before the meeting so that potential responders can be better prepared to reply.

Harvey W. Wiley Awards Fund Contributors

The following members of AOAC have contributed to the Harvey W. Wiley Awards Fund: Juan Carlos Medina Bravo, Odette L. Shotwell, and W. Wesley Weeks.

The Harvey W. Wiley Award Fund was established in 1956 to honor Harvey W. Wiley, "Father of the Pure Food Laws" and founder of AOAC. This fund supports the Harvey W. Wiley Award for the Development of Analytical Methods and the Harvey W. Wiley Scholarship Award. Contributions to sustain the Harvey W. Wiley Awards Fund will be appreciated and should be sent to AOAC.

New Sustaining Member

AOAC welcomes the following new sustaining member: Spencer Laboratorios S.A. DE C.V.

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April 30, 1991 -- NEW YORK-NEW JERSEY REGIONAL SECTION -- Piscataway, NJ
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Contact: Jeff Singer, (516) 832-2171

May 13-15, 1991 -- NORTHEAST REGIONAL SECTION -- Newport, RI
Mass Spectroscopy, Food Analysis, and Pitfalls of Lab Computerization
Contact: Penelope Lynn, (315) 787-2280

June 3-5, 1991 -- MIDWEST REGIONAL SECTION -- Sioux Falls, SD
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Instructions to Authors

Scope of Articles and Review Process

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

General Information

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

1. Write in clear, grammatical English.
2. To Managing Editor, AOAC, 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, acknowledgments, 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.
4. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.
5. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused. Once a manuscript has been accepted for publication, authors will receive instructions for submitting the final version of their accepted manuscript to AOAC on diskette. AOAC accepts MS-DOS-based files from most word processing packages or ASCII text files on MS-DOS-formatted diskettes. (DO NOT SEND DISKETTE WITH ORIGINAL MANUSCRIPT SUBMISSION.)

Format and Style

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

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

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

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

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

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   Journal Article Reference

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

   Book Chapter Reference

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

   Book Reference

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

   Official Methods Reference

   (4) *Official Methods of Analysis* (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.
5. **Footnotes** (separate sheet, **double spaced**): Avoid use of footnotes to text. Include "Received . . . Accepted . . ." line; location/date of presentation, if appropriate; present address(es) of author(s); identification of corresponding author, if not senior author; proprietary disclaimers; institution journal series numbers.
6. **Tables** (one per page, **double spaced**): Refer to recent issue of *Journal* for proper layout and style, especially use of horizontal lines. Do not draw in vertical lines. Include descriptive title sufficient that table stands alone without reference to text. Provide heading for *every* vertical column. Abbreviate freely; if necessary, explain in footnotes. Indicate footnotes by lower case superscript letters in alphabetical order. Do not use one-column tables; rather, incorporate data in text.
7. **Figures:** The *Journal* does not publish straight line calibration curves; state such information in text. Do not duplicate data in tables and figures. Submit original drawings or black/white glossy photographs with original manuscript; photocopies are acceptable only for review. Prepare drawings with black India ink or with drafting tape on white tracing or graph paper printed with nonreproducible green ink. Use a Leroy lettering set, press-on lettering, or similar device; use type at least 2 mm high to allow reduction to page or column size. Identify ordinate and abscissa and give value in *Journal* style (e.g., "Wavelength, nm," "Time, min"). Label curves with letters or numbers; avoid all other lettering/numbering on face of figure (see **Figure captions**). Identify each figure on back with number and authors' names.

Miscellaneous

Abbreviation for liter is L; abbreviation for micron is μm. Do not italicize common Latin expressions such as et al. and in vitro; for nomenclature of spectrophotometry, gas chromatography, and liquid chromatography, follow practice of American Society for Testing and Materials (in particular, do not use "high performance," "high pressure," or the abbreviation "HP" with "liquid chromatography").

1/89

Books in Brief

Environmental Sampling and Analysis: A Practical Guide. By Lawrence H. Keith. Published by Lewis Publishers, Inc., 2000 Corporative Blvd, NW, Boca Raton, FL 33431, 1991. 120 pp. Price: U.S. $39.95/Outside U.S. $48.00. ISBN 0-87371-381-8.

This concise book covers all the critical aspects of environmental sampling and analysis. Extensively peer-reviewed by scientists from the U.S. Environmental Protection Agency and other government agencies, industry, and academia, it is packed with practical advice and tips from renowned experts. Planning, sampling, analysis, QA/QC, and reporting are discussed for air, water, solid liquid, and biological samples, with emphasis on the interdependence between sampling and analytical activities. Special requirements for sampling devices, containers, and preservatives are provided with convenient checklists for sampling plans and protocols. New and revised recommendations involving method detection levels, reliable detection levels, and levels of quantitation are discussed in conjunction with laboratory reports and user presentations of data near analytical detection limits.

Multiple Chemical Interactions. By Edward J. Calabrese. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1990. 1,200 pp. Price: U.S. $95.00/Outside U.S. $112.00. ISBN 0-87371-146-7.

A true classic work on a vitally important and new topic, this book has over 1200 pages of complete information on chemical interactions as it pertains to environmental and medical concerns. It illustrates principles of interactions so you will have the conceptual basis for predicting responses to complex environmental mixtures. Apply it to specific situations - theoretical and practical. To date, environmental standards regulate the effects of a single pollutant. *Multiple Chemical Interactions* provides exhaustive coverage of how the multitude of chemicals in our world interact with human health. This book gives us critically new and complete data on an emerging field that will be of interest to all environmental scientists, environmental and public health professionals, toxicologists, industrial hygienists, regulators, and libraries.

Sample Introduction in Atomic Spectroscopy. Edited by J. Sneddon. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1990. 360 pp. Price: U.S. $133.25/Dfl. 260.00. ISBN 0-444-88229-4.

A comprehensive and detailed description of the most widely used sample introduction techniques in atomic spectroscopy is presented in this volume. Comprising 12 separate chapters, the book describes the theory in detail, and gives an account of techniques and selected applications of sample introduction systems. The first chapter is an overview on sample introduction. The remaining 11 chapters are each devoted to a specific sample introduction and deal with the basic principles, describe the system, advantages, disadvantages, and selected applications. Systems described are: pneumatic nebulization, electrothermal vaporization, laser ablation, impaction/electrostatic precipitation, slurry atomization, ultrasonic and thermospray nebulization, hydride generation, chromatographic, spark and arc, low-pressure discharges, flow injection analysis, and direct solid introduction.

Comprehensive Analytical Chemistry. Edited by G. Svehla. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1990. 392 pp. Price: U.S. $177.00/Dfl. 345.00. ISBN 0-444-87376-7.

This monograph deals with theory and practical applications of trace metals preconcentration. It gives general characteristics of the process and describes in detail the methods of preconcentration: solve extraction, sorption, co-precipitation, volatilization, and others. Special attention is given to preconcentration in combination with subsequent determination methods. The use of preconcentration in analysis of environmental and biological samples, mineral raw materials, high purity substances, and various industrial materials is also considered.

Flavour Science and Technology. Edited by Y. Bessière and A.F. Thomas. Published by John Wiley and Sons,

mation, random walk processes, criteria of band broadening and resolution, steady-state zones, the statistics of overlapping peaks, 2-dimensional separations, and so on. Chapter 7 is a transition chapter comparing and classifying methods, and dissecting the multifaceted role of flow in separations. The final 5 chapters use the general principles of the first part to better understand families of techniques and specific methods. Coverage highlights electrophoretic and sedimentation technqiues, field-flow fractionation, and especially chromatography.

Thanks to Reviewers

Each year at this time, we take the opportunity to convey our gratitude to the hundreds of individuals who voluntarily set aside valuable time to assist the *Journal of the AOAC* by reviewing submitted manuscripts. The reputation of a scientific journal rests on the quality of the science reported therein. Those of you listed below have helped immeasurably to assure the reputation of the *Journal of the AOAC* by insisting on high standards of experimental design, method execution, and data interpretation. Please accept our thanks for your contribution to fulfilling AOAC's mission.

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SPECIAL REPORT

Immunoassay of Pesticides

BENNETT M. KAUFMAN[1] and MARION CLOWER, JR
Food and Drug Administration, Division of Contaminants Chemistry, Washington, DC 20204

Determination of the presence and levels of pesticide residues in food is fundamental in monitoring and regulatory programs. Residues are separated from the food matrix by solvent extraction, followed by cleanup steps. The residues are most often identified and quantitated by instrumental analysis, usually liquid or gas chromatography. Extraction and cleanup are often laborious and time-consuming; determination requires expensive, sophisticated instrumentation. There is a need for rapid, easily performed tests, such as immunoassays, that could be used for screening under field conditions or for quantitation of residues in foods in the laboratory. Although a large number of immunoassays have been developed for pesticide chemicals, very few have been specifically applied to foods, and only a very small number are currently available commercially. The agencies charged with monitoring and regulatory responsibilities—Environmental Protection Agency, Food and Drug Administration, and U.S. Department of Agriculture—as well as professional societies such as AOAC and the International Union of Pure and Applied Chemistry, are investigating and developing guidelines for test kit evaluation and standards to be met before a kit can be accepted as a practical and useful method of analysis for use in their programs.

The Food and Drug Administration (FDA) is responsible for monitoring foods and feeds for the presence of pesticide residues [except for meat and dairy products, which are monitored by the U.S. Department of Agriculture (USDA)]. The current approved FDA methods for determination of pesticide residues in foods are codified as Pesticide Analytical Manual, Vol. I, *Methods Which Detect Multiple Residues*, and Vol. II, *Methods for Individual Residues*. Methods generally involve extraction, cleanup, and determination by such techniques as gas chromatography (GC), mass spectrometry (MS), liquid chromatography (LC), thin-layer chromatography (TLC), or other methods. These analyses require well-equipped laboratories and trained personnel, and are often laborious and time-consuming.

To increase the number of samples analyzed, and to broaden the scope of analyses, rapid and simple tests are needed. Attention has focused on the development, testing, validation, and adoption of immunoassay (IA) systems, including both radioimmunoassay and enzyme-immunoassay techniques. Application of IA technology to regulatory analysis of pesticide residues in food is on the horizon. An IA may be used as an initial "screening" assay to determine whether a residue is present before the food is subjected to a more complex analysis. IAs may also be used in a quantitative fashion, as an adjunct to a quantitative instrumental analysis.

The present paper includes a general account of the efforts to develop IAs for identification and quantitation of pesticide residues and the development of official guidelines for the adoption of assay kits for pesticide detection in food and water, and also describes current commercially available pesticide assay kits.

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Immunoassay Technology

The basic theory and methodologies of IA have been the subject of previous reviews (1, 2), and the general requirements, advantages, and disadvantages of using immunoassay and immunochemical systems for analysis of foods have been described (3–7). An IA must have several important characteristics: It should be specific, sensitive, preferably quantitative, rapid, and relatively easy to perform. Obviously, specificity (the ability to detect only the target analyte) may depend on whether a specific residue (or even isomer) is to be determined, or whether cross-reactivity for an entire class of compounds is required. Likewise, the needed determination limit is related to the residue level that needs to be measured; it may be unnecessary for an assay to have the ability to measure, for instance, as little as one-thousandth of the tolerance level. There may very well be a place in a monitoring program for a rapid assay as a simple screening step, the results of which determine whether further quantitative instrumental analysis needs to be conducted. For this type of screening assay, the determination limit could be "adjusted" so that a positive result would be produced only if the level of pesticide present exceeds some predetermined level below the tolerance. Of obvious concern in such assays would be the frequencies of both false positives (leading to unnecessary repeat analysis by a more time-intensive method of an analytical sample containing no residue) and false negatives (failure to identify a violative sample).

IAs have the benefit of relatively rapid analysis times, due partly to their reduced requirement for extensive cleanup, which also renders the analysis easier. One important aspect of IA is the size of the analytical portion required. The sensitivity of IA is such that an analytical portion of less than 10 g of a solid material needs to be analyzed. Whether this small an analytical portion is (or can be made) statistically representative of the 20 lb composite normally prepared for regulatory analyses is not yet known. Homogeneity studies indicate that a 5–10 g analytical portion is as statistically representative of the laboratory sample as the larger 100 g portion used for most other methods (S. Young and W. Trotter, FDA, unpublished observations, 1985).

Immunoassays rely on antibodies produced by an animal's immune system in response to exposure to a foreign substance or molecule. Antibodies are proteins with the ability to recognize and bind tightly to the foreign substance (the "antigen") that elicited them. Antibodies are normally produced only in response to relatively large antigens, whereas small molecules (haptens) are unable by themselves to elicit an immune response. Pesticide molecules are generally quite small; therefore, the animal is immunized with a conjugate composed of the pesticide chemically coupled to a large carrier protein. The resultant immune response produces antibod-

ies directed against the large carrier, but also antibodies against the small pesticide hapten. Sera derived from animals immunized in this manner contain a large mixture of antibodies, some of which will recognize the pesticide and bind to it to differing degrees. However, such a polyclonal antiserum will vary in its characteristics not only from animal to animal, but also between different bleedings from the same animal; furthermore, the amount of sera produced by even a group of animals is limited.

One way to obtain an essentially unlimited supply of a single type of antibody is by production of hybridomas, a technique in which a normally short-lived antibody-producing cell is fused with a long-lived cancer cell to produce a new hybrid cell with the characteristics of both parents (8). The resultant monoclonal antibody produced by a population derived from such a cell represents a homogeneous collection of antibodies of a single type that can be produced in large quantities for a long time.

Many IAs either were developed for clinical use, to determine pesticide levels in serum, urine, or tissues of people exposed to pesticides (e.g., see section on *Paraquat*), or were initially developed to measure pesticide levels in water or soil extracts (e.g., see section on *Triazines*). Application of IA to measuring pesticide levels in foods involves modification of existing techniques, for extraction of the residue from the food matrix or the development of new techniques. Most IAs function only in aqueous solution, so that many current extraction procedures that use organic solvents would have to be modified to increase the percentage of water present. Likewise, IAs might be modified to tolerate some level of organic solvents. These problems and others will have to be addressed before IAs become adopted for routine pesticide analysis.

Types of Enzyme Immunoassays

The first practical IAs were based on radioactively labeled reagents and are referred to as radioimmunoassays, or RIAs (9, 10). Concerns about the use and disposal of radioactive materials, and the need to prepare fresh, labeled reagents on a frequent basis, have led to the increasing use of enzyme immunoassays (EIA), involving either a homogeneous liquid phase (enzyme-multiplied immunoassay technique, EMIT) or a heterogeneous solid phase (enzyme-linked immunosorbent assay, ELISA) (11). In an enzyme immunoassay, the detecting reagent, such as an antibody, is chemically coupled to an enzyme (usually horseradish peroxidase or alkaline phosphatase). When an appropriate substrate is added to the reaction mixture, the enzyme's action on the substrate produces a colored product that can be measured spectrophotometrically.

Enzyme immunoassays used for pesticide detection are overwhelmingly of the competitive type. In one variation (Figure 1), the antibody capable of binding the analyte is mixed with the test solution and a known amount of radioactive- or enzyme-labeled tracer analyte. The presence of analyte in the solution reduces the amount of labeled material bound; the reduction may be quantitated by reference to a standard curve produced by different known concentrations of unlabeled analyte. These assays may be performed with the antibody immobilized on a solid phase, such as tubes, plates, or beads (a "capture" assay), or the antigen-antibody complex may be absorbed out of solution by charcoal, or precipitated by polyethylene glycol, Protein A, or other means.

In an alternative approach (the "sandwich" assay, Figure 2), the analyte, coupled to a carrier protein to facilitate binding, is attached to the solid phase. The analytical portion is then mixed with antibody (which may be labeled) specific for the analyte, and combined with the bound analyte. The amount of antibody reacting to the fixed analyte is then determined, either directly if the primary antibody was labeled, or by addition of a labeled second antibody directed against the primary antibody.

In a manner analogous to that of the first type of assay, the amount of the antibody bound to the fixed analyte is reduced in a manner proportional to the amount of soluble analyte present in the analytical portion. A dose-response (standard) curve relating amount of signal (color, for EIA, or disintegrations/min, for RIA) to amount of analyte present can then be generated. In most cases, the dose-response curve is nonlinear. One standard method for linearizing the standard curve is the logit transformation, in which the transformed re-

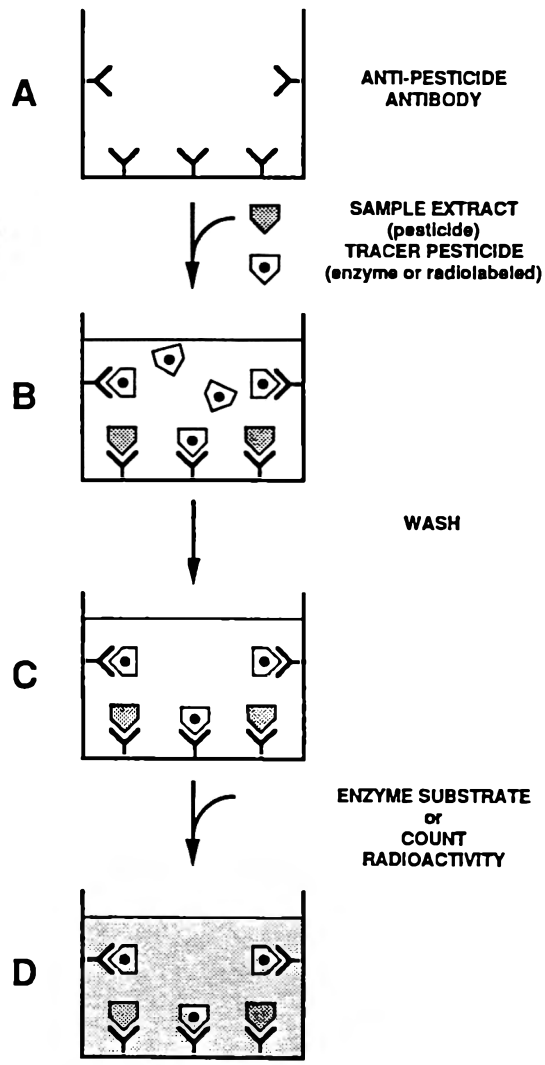

Figure 1. Capture-type competition immunoassay (radio or enzyme assay). (A) Antibody against the pesticide of interest is coated on wells of a microtiter plate. (B) Extracts are placed in well along with known amount of enzyme- or radiolabeled tracer pesticide. (C) After incubation, the plate is washed. (D) Wells are either counted or enzyme substrate is added and the resultant color measured. Pesticide present in the extract reduces the amount of the tracer bound.

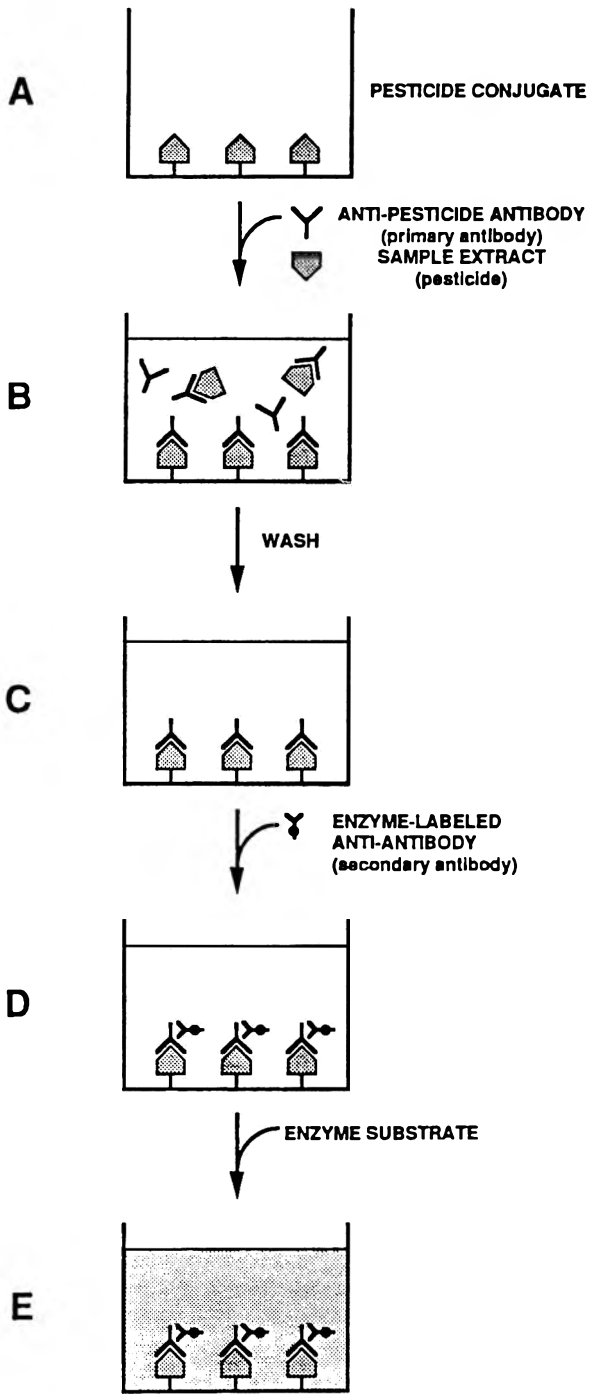

Figure 2. Sandwich-type competition enzyme immunoassay. (A) Pesticide (usually conjugated to a carrier protein) is bound to the wells of a microtiter plate. (B) A limiting amount of antibody (primary) to the pesticide, and analytical sample extract, which may contain pesticide, are added. Pesticide in the extract competes with bound pesticide for the antibody. (C) The plate is then washed. (D) Bound antibody is detected by addition of an enzyme-labeled antibody (secondary) capable of binding the primary antibody. (E) An enzyme substrate, which produces a color upon reacting with the enzyme on the secondary antibody, is then added. Pesticide present in the extract reduces the amount of subsequent color produced.

sponse (absorbance, in the case of EIA) is plotted as a function of [log(concentration)] (2, 12):

$$\text{logit } p = \ln[p/(1-p)]$$

$$p = \frac{(A_x - NSB)}{(B_o - NSB)}$$

where A_x is the response (signal) produced by the analyte, NSB is a nonspecific, or reagent blank, and B_0 is response in the absence of added test analyte (i.e, the maximum response).

Use of Immunoassays for Pesticide Determination

The major classes of pesticides are listed in Table 1. Throughout this review, the term "pesticide" will be used to describe insecticides, fungicides, rodenticides, herbicides, and other agents that are active against pests causing damage to food crops and to dairy and meat products.

Paraquat

Paraquat and diquat are bipyridinium herbicides widely used as contact weed killers. Mortality from paraquat poisoning is high (13); therefore, the ability to monitor the presence of paraquat in clinical specimens, as well as to detect paraquat residues on food crops, is quite important.

One of the first immunoassays for determination of a pesticide was an RIA developed for determination of paraquat in blood specimens of people who had ingested commercial formulations of paraquat solutions (14, 15). This assay had a determination limit of 0.6 μg/L in plasma or urine and a correlation with GC determinations of paraquat of 0.998. Comparison to a dithionite colorimetric assay (16) likewise yielded an excellent correlation. The radioassay was applied to measurement of serum paraquat in poisoned patients in order to develop a correlation between paraquat levels and prognosis (17). Indeed, the reason for developing this assay appears to have been to help differentiate the patients who required minimal treatment (levels below 1 mg/L) from those for whom treatment would not be beneficial (above 1 mg/L) and those for whom aggressive treatment could be efficacious (18).

These findings were extended in a study using plasma and urine paraquat levels as prognostic indicators, in which the RIA described above was compared with another colorimetric dithionite test (19).

Table 1. Major types of chemical pesticides

Type of compound	Examples
Organophosphorus	parathion, malathion, paraoxon, fenamiphos
Organohalogen	DDT; 2,4,-D; alachlor
Cyclodiene	endrin, dieldrin, chlordane
Hexachlorocyclohexane	lindane, BHC
Carbamate	carbaryl, benomyl, carbofuran
Rotenoid	rotenone
Pyrethroid	allethrin
Triazine	atrazine, prometryne, ametryne, simazine
Substituted urea	diflubenzuron, chlorsulfuron
Bipyridyliums	paraquat, diquat
Arsenicals	ammonium and sodium salts of methanearsonate (MAMA, MSMA, DSMA)
Fluorine rodenticides	fluoroacetate, fluoroacetamide
Nicotinoid	nicotine
Warfarin anticoagulants	warfarin

Fatori and Hunter (20) developed 2 RIAs for serum paraquat. One used a ^{3}H tracer, gave results in 20 min, and could determine 10–2500 ng/mL. A second assay, using ^{125}I, required 2 h, but could determine as little as 0.1 ng/mL. Both assays used rabbit antiserum coupled to an insoluble carrier, and were based on the competition between labeled paraquat solutions and serum specimens—a competitive RIA.

An enzyme-based assay for serum paraquat has been described (21) that could detect 0.3–10 ng/mL; this assay has been refined by the use of a murine monoclonal antibody (mAb) (22) offering a 0.8–50 ng/mL sensitivity range. This monoclonal-based assay has been used to measure paraquat in soils (23); extraction required a 5 h reflux with 6N sulfuric acid followed by neutralization. Van Emon and coworkers (24, 25) developed an antiserum-based EIA in which test materials could be extracted by sonication in 6M HCl before analysis. Paraquat residues could even be extracted from some matrices (e.g., cloth) by using the antiserum itself, although this method was quite wasteful of antibody.

This assay has also been used to determine paraquat in milk, beef, and potatoes (26), with average determination limits in these matrices of 2.5 ppb and recoveries of 65–80%, and has shown excellent correlation between the amount of paraquat found by ELISA and that determined by standard GC methodology.

Additionally, there are reports that murine mAbs directed against paraquat can be produced by both in vivo immunizations of mice and in vitro immunization of murine spleen cells (27, 28). These reagents were actually developed to be used in a model system to study antigen-antibody interactions and to measure affinity (association) coefficients; they were not used to develop any immunoassay methodology for paraquat. However, useful protocols for the generation and purification of murine monoclonal anti-paraquat antibodies from ascites fluids were described (29).

Nagao and coworkers (30) used rabbit antiserum to develop an RIA for paraquat that was applicable to serum, urine, and acid extracts of human tissues from cases of paraquat poisoning. This assay could determine 1 ng paraquat in 1 mL liquid or 1 g tissue. Subsequently, these workers reported (31) murine monoclonal anti-paraquat antibodies that were used to develop a competition ELISA with a linear determination range of 1–10 ng paraquat/mL. This assay was not applied to the analysis of paraquat-containing foods or soils.

A fluorometric immunoassay for serum paraquat has also been reported (32). This assay was of the competition type, in which fluorescein-labeled paraquat was mixed with the serum, and sheep anti-paraquat antibodies were added. The antibody-bound paraquat was precipitated from solution by addition of donkey anti-sheep antibodies, followed by centrifugation; the precipitate was redissolved and its fluorescence determined. The amount of fluorescent paraquat bound was inversely proportional to the amount of unlabeled paraquat in the serum, with a linear determination range of 20–2000 μg paraquat/L. The coefficients of variation (CVs) of the assay range from 6 to 9%, depending on the paraquat concentration. Quantitative recovery of paraquat added to serum was reported. This assay, like several of those described above (14, 15, 17, 18, 21, 22), was developed for clinical use in cases of human paraquat poisoning, and was not applied to the analysis of crop or food materials.

Organophosphorus Compounds

The organophosphorus (OP) pesticides are anticholinesterase (anti-AChE) agents operating at cholinergic neuromuscular junctions; these compounds can cause both acute and chronic toxicity in humans (33). Although OP compounds are among the most widely used types of pesticides, little work has been done on developing IAs for these materials.

An abstract describing a rabbit anti-malathion antiserum has appeared (34); reactivity of the serum was demonstrated only by the formation of a precipitating complex of antigen and antibody. Sternberger et al. (35) raised antibody in rabbits by injection of parathion oxygen analog-hemocyanin conjugates. Their brief report mentions the use of RIA to determine antibody levels and the ability of immunized rabbits to survive challenge with up to 3 times the lethal dose of the pesticide. Because these authors were not attempting to develop IAs for pesticides, no assay data were presented.

Haas and Guardia (36) obtained rabbit antiserum by immunizing rabbits with conjugates of either DDA [2,2-bis(*p*-chlorophenyl)acetic acid] or the monoester of malathion. Again, IA development was not the goal of the experiments; in fact, because the sera did not react in a precipitating fashion with the haptens (presumably because the univalent haptens could not form a precipitating complex), an indirect passive agglutination technique had to be used to measure specific antibody.

Ercegovich et al. (37) developed an RIA for parathion with a determination limit of 0.1–0.2 ppm parathion in blood plasma or vegetable extracts. The assay was of the type in which the test portion is mixed with antibody, radiolabeled analyte is added, and the antibody-bound material is separated from the free material by addition of dextran-coated charcoal; the antibody-bound material is left in the supernate. Although as little as 80 ng/mL could be measured in standard solutions, the determination limit of the assay with actual sera and plant extracts was 200 ng/g, an effect the authors attributed to the ability of the test materials to produce nonspecific inhibition of antibody; i.e., a "matrix effect."

An EIA for the oxygen analogue of parathion was developed (38) that could determine the compound at levels of 280 pg/mL in serum or 28 pg/mL in buffer solution (note the 10-fold decrease in determination level because of the "matrix effect" of serum).

The U.S. Army has done a great deal of research on the immunology of OP nerve agents (sarin and soman). Both rabbit (39) and chicken (40) polyclonal and murine monoclonal (41–43) antibodies have been raised against these materials, and used to develop IAs capable of determining as little as 200 ppb soman in standard solutions (41) or 40 ng/mL in serum or milk (43). The cross-reactivities of the antibodies with OP pesticides are unknown but represent an interesting area of investigation.

Organohalogen Pesticides

Despite the long and widespread used of chlorinated organic pesticides, little work has been done on the development of IAs for these compounds. As previously mentioned, Centeno and Johnson (34) described production of a rabbit antiserum reactive with DDT, which they used in an assay system (44). Results were expressed only as a titer (i.e., the reciprocal of the dilution of antiserum giving a measurable agglutination reaction). Although the serum reacted well with the dichlorodiphenyl acetic acid (DDA) derivative used as the original immunogen, little or no reactivity was obtained with parent DDT. Sera from immunized rabbits and normal or "DDT-sensitive" (allergic) humans were tested;

although high titers were found in the immunized animals, the titers of the allergic individuals were not significant. This assay was not applied to determination of residues in foods, however.

Likewise, the anti-DDA serum developed by Haas and Guardia (36) was specific only for DDA and not for DDT, as was found in the similar studies of Centeno and coworkers, noted above. This system was not developed any further.

Langone and Van Vanukis (45) developed a precipitating competitive RIA for dieldrin and aldrin (determination limits of 50 pg/mL and 2 ng/mL, respectively), which required 13 times as much heptachlor and 26 times as much chlordane to achieve 50% inhibition as dieldrin. The assay required a high protein concentration in the buffers (up to 10% horse serum) to yield reproducible results. The effect was attributed to higher solubility of dieldrin in serum than in water. This assay was not applied to crop materials, however.

Banerjee (46) reported an ELISA for DDA analysis in urine. The assay had a determination limit of 5–10 ng/mL of urine and recoveries of 96% from fortified urine, and results correlated almost completely with values found by GC or colorimetric analysis. This assay also has not been applied to foods or crops.

An RIA method for determining 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in water was described by Rinder and Fleeker (47). This assay was a competition RIA and required the precipitation of the antigen-antibody complex by Protein A. The respective limits of determination and recoveries for 2,4-D and 2,4,5-T were 1 ng/10 mL and 10 ng/10 mL water and 90 and 94%, respectively. However, the method could not distinguish between the 2 compounds if both were present.

The method was superseded by an EIA specific for 2,4-D that involved 2 different antisera prepared with different immunogens (48). One system had a limit of determination of 1 ng/mL water, the other of 5 ng/mL; recoveries for both exceeded 94%. Detailed protocols for production of immunogens, antisera, enzyme labeling of reagents, and assay protocols were given, and although the assay gave excellent results with water, it was not used for food or crop materials.

A similar RIA for 2,4-D was developed by Knopp et al. (49). In the assay, the test material, ^{3}H-labeled tracer, and antiserum were incubated for 24 h; then the immune complex was precipitated with polyethylene glycol and human gamma globulin and the radioactivity of the precipitate was counted. The assay could determine 250 pg 2,4-D/mL and was fairly specific for this analyte, showing below 10% cross-reactivity with 2,4,5-T and even less with other 2,4-D derivatives. The intra- and interassay CVs were 5.3 and 13.1%, respectively. The assay was also applicable to serum, urine, or water; recoveries from serum were 96%. The authors stated that "... the applicability for plant tissues should be investigated," although they did not do so themselves.

A competition ELISA and an RIA for 2,4-D with a range of determination of 100–10 000 ng/mL river water and 50–10 000 ng/mL urine have been reported (50). An RIA using antipicloram antiserum was capable of determining picloram at 50–5000 mg/mL in water and urine. The RIA method used tritiated glycine coupled to the herbicides, yielding a stable, long-lived reagent; this method was quicker and easier than the ELISA technique. However, because a scintillation counter was required to measure the radioactivity, the RIA method was limited to laboratories so equipped.

An ELISA for the chloroacetanilide herbicide alachlor in water has been reported recently (51). The assay had a determination range of 0.2–8.0 ppb in 1 mL water and a 0.84 correlation with values determined by GC/MS (although the interassay CVs for the ELISA, at 10–40%, were reported to be much higher than those of the GC/MS analyses). However, determination of alachlor is complicated by the rapid degradation of the parent compound in the environment (52), so that an assay able to determine the degradation products would be more appropriate.

A capture-type enzyme immunoassay for the insecticide endosulfan capable of determining the compound at a level of 3 ppb in aqueous solutions (soil extracts and water) has been described (53); the assay had a useful measurement range of 3–400 ng/mL, and was capable of determining both the parent compound and its degradation products. Although cross-reactivity with most other organochlorine pesticides was low, endrin reacted twice as much as endosulfan.

Carbamates

One of the first IAs for the carbamate pesticide benomyl measured the change in fluorescence polarization upon the binding of anti-benomyl antibody to fluorescently labeled antigen (54). The rate of change of polarization of the fluorescence emitted by the labeled antigen was a function of the amount of unlabeled competing antigen present in the material being analyzed. Although this method could determine benomyl at the subnanogram/mL level, it could be used only in a neutral aqueous environment and required sophisticated equipment with which to measure the fluorescence polarization.

An RIA for benomyl was developed in which rabbit antisera were used against 2-succinamidobenzimidazole, which reacted with the benomyl decomposition product methyl 2-benzimidazole carbamate (MBC) (55). Homogenized cucumber or melon was mixed with ethyl acetate and sodium carbonate, the mixture was refluxed and filtered, the filtrate was dried, and aliquots were redissolved in buffer for assay. A competition radioassay with ^{14}C-MBC was used; unbound material was removed with charcoal. Recoveries ranged from 80 to 90%, and the correlation with LC methodology was 0.99 at the 1 ppm level. The assay was later converted to an EIA, using a microtiter plate format (56). The revised assay was capable of determining 0.35 ppm benomyl and 0.03 ppm thiabendazole in ethyl acetate/sodium carbonate extracts of pears, potatoes, lemons, grapefruit, and apples. The antiserum and fruit or vegetable extract were premixed 15–30 min before addition to the plate coated with MBC or thiabendazole. No "matrix effect" was observed in this system.

Triazines

Several EIAs have been developed for determination of the triazine herbicide atrazine. Huber (57) described the development of an enzyme immunoassay applicable to the determination of atrazine in water. The assay system was a "capture" type in which the antibody (either crude ammonium sulfate-precipitated or affinity-purified) was bound either to microtiter plate wells or to polystyrene spheres, enzyme-labeled tracer analyte and analyte-containing water were combined with the antibody, and the amount of bound enzyme-labeled tracer was determined (the greater the concentration of atrazine in the water, the less tracer bound). The affinity-purified antibody bound to microspheres allowed determination over a range of 2.1–10 500 ng/L water, or 0.11–550 ppt; the microtiter assay could determine 0.011–33 ppb. No attempts were made to apply this assay to other materials.

Table 2. Foods analyzed by atrazine immunoassay[a]

Milk	Corn oil
Fruit juices	Corn meal
Soft drinks	Pineapple (fresh, canned)
Molasses	Corn (fresh, canned, frozen)
Corn syrup	Potato and corn chips
Sugar	Macadamia nuts

[a] Data from Ref. 59.

Another EIA for atrazine determination in water and soil (58) and in foodstuffs (59) has also been described. This is a tube type of capture assay, in which the analyte in the test portion competes with enzyme-labeled tracer analyte for binding to antibody bound to the walls of a plastic tube. Although the number of test portions that can be analyzed by this type of assay is limited because of the difficulties of handling more than just a few tubes, a portable photometer can be used in the quantitation step, and thus the test is field-adaptable. The assay was able to determine 0.5–10 ng atrazine/mL (or other triazines, because the antiserum used cross-reacted) in water or soil extracts. Recoveries ranged from 70 to 90+%, and compared favorably with those determined by LC.

This assay has been applied to determination of residues in a variety of foods (Table 2), ranging from fruit juices and milk to nuts, pineapple, potato, and fresh, canned, and frozen corn (acetonitrile–water extracts of solid foods were used). The interassay CV for these assays was quite good, ranging from 2.4 to 13.7% in solid foods and from 3.8 to 10.9% in liquid materials. This assay represents a good general screening technique for total triazines, because the antiserum cross-reacts with prometryn, ametryn, propazine, dipropetryn, and others. The assay is available commercially from Immuno-Systems, Inc., Scarborough, ME, as the RES-I-MUNE Atrazine kit for the detection of triazines in water (see *Commercial Immunoassay Kits for Pesticides*, below). Adaptation of this kit for determination of triazines in foods is currently being investigated (C. Thorpe, Food and Drug Administration, personal communication, 1989; see discussion below). The RES-I-MUNE triazine assay kit has been used to monitor exposure of workers using towed atrazine spray applicators (60), by measuring pesticide levels in washings from their protective clothing and respirator mask filters.

Schlaeppi et al. (61) have also developed 1 monoclonal-based ELISA for atrazine and a second, using a different mAb, for its metabolic product, hydroxyatrazine. Cross-reactivities for propazine and hydroxypropazine, respectively, were noted. The assays could determine 0.05 ppb of the triazines in 0.85 mL water or 50 ppb in methanol–water extracts of soils (2 g/20 mL solvent). Good recoveries from fortified analytical samples were reported, and the correlation with an LC method for atrazines in water was 0.91. No food or crop materials were analyzed, however.

Dunbar et al. (62) have described a competition ELISA for atrazine/propazine capable of determining these herbicides at the 0.1 ng/mL level. Cross-reactivity with other triazine and nontriazine compounds was low or nil. This assay was not applied to determination of atrazine/propazine in water or crop materials.

Pyrethroids

An antiserum against the pyrethroid insecticide *S*-bioallethrin was produced by Wing et al. (63) and used to develop an RIA that was specific for the active *S*-isomer (64). Although the assay was reportedly able to determine 1.2 nmol of pure material, no food or plant extracts were analyzed.

Miscellaneous Pesticides

An EIA for the herbicide diclofop-methyl has been described by Schwalbe et al. (65). Enzyme-labeled antigen was mixed with rabbit antiserum; the bound material was precipitated with goat antirabbit secondary antibody and 7% PEG. The serum showed a higher activity against diclofop than against the methyl ester, probably because of the use of the acid to produce the hapten-carrier conjugate immunogen. Extracts from a variety of fortified biological materials (Table 3) were analyzed. It should be noted that the materials were fortified immediately before immunoassay; so, although recoveries of >99% for diclofop and 100% for diclofop-methyl were reported, no data were presented for naturally incurred analytes.

Newsome (66) reported an ELISA which was used for determination of residues of the fungicide metalaxyl in fortified cucumber, avocado, potato, squash, and tomato. Pesticide residues were extracted from homogenized crop materials with methanol; aliquots were incubated with rabbit antiserum for 30 min and then placed in wells of metalaxyl-ovalbumin-coated plates for 30 min. The plates were then washed, and enzyme-labeled antirabbit antiserum was added for 30 min, after which the plates were developed by addition of substrate. The CVs between replicates for this assay were 5%, and the minimum amount that could be determined was 63 pg/mL, or 0.025 ppm in the crop; overall recoveries averaged 87%. No matrix effect was observed in this system.

Newsome also described a similar ELISA for the fungicide triadimefon (Bayleton) in foods, applicable to methanol extracts of apple, pear, and pineapple and ethyl acetate extracts of grape (the grape ethyl acetate extract was dried and redissolved in methanol for assay) (67). Plates were coated with a succinyl triadimefon-ovalbumin conjugate. The fruit extracts were preincubated for 30 min with rabbit antiserum (prepared against a human serum albumin-succinyl triadimefon conjugate) and then added to the plate for 1 h. The assay was then developed as described above. Recoveries ranged from 77% (pineapple) to 112% (apple) when the fruits were fortified at the 0.5 ppm level and above (CV 5.1%), and compared favorably with recoveries by a GC method.

Kelley et al. (68) described an ELISA for determination of the herbicide chlorsulfuron in soils. Aqueous alkaline extracts required no cleanup before analysis, and cross-reaction by degradation products was minimal. The sensitivity of the assay was 0.1–0.3 ng/mL (equivalent to 0.4–1.2 ppb). Serum from only 1 rabbit was used, so the supply of the reagent is quite limited. As previously discussed, this limitation represents one of the factors to be considered in the adoption of any immunoassay for widespread use.

An RIA (69), and subsequently an ELISA (70), have been reported for determination of the benzoylphenylurea insect growth regulators diflubenzuron and BAY SIR 8514. The

Table 3. Samples analyzed by diclofop-methyl immunoassay[a]

Soil	Sugar beets
Milk	Soybeans
Urine	Wheat shoots
Serum	Wheat grains

[a] Data from Ref. 65.

Table 4. Commercial pesticide detection kits

Manufacturer	Assay	Pesticide(s) detected	Type of assay[a]	Sensitivity[b]	Matrix[c]
ImmunoSystems, Inc. 4 Washington Ave. Scarborough, ME 04074	Res-I-Mune Atrazine	atrazine, simazine, propazine	ET	1 ppb	water, acetonitrile extracts of food
	Res-I-Mune Aldicarb	aldicarb	ET	N/S[d]	N/S
	Res-I-Mune Chlordane	chlordane, heptachlor, dieldrin, aldrin, endrin, endosulfan	ET	25 ppb	water, food extracts
	Res-I-Mune Carbofuran	carbofuran	ET	1 ppb	water, food extracts
	Res-I-Mune 2,4-D	2,4-dichlorophenoxyacetic acid	ET	1 ppb	water, food extracts
	Res-I-Quant Alachlor	alachlor, metachlor, metalaxyl	EM	0.5–20 ppb	water
	Res-I-Quant Benomyl	benomyl (as carbendazim)	EM	0.4–10 ppb	water, juices
	Res-I-Quant Triazine	atrazine, prometryn, simazine	EM	0.03–3 ppb	water
Environmental Diagnostics P.O. Box 908 2990 Anthony Rd Burlington, NC 27215	EZ-Screen	paraquat	EC	100 ppb	urine, serum, saline extracts of food, crops
ACTIO, Inc. 1127 57th Ave. Oakland, CA 94621	Pesticide Detection Kit	chlorinated hydrocarbons: parathion, phosmet, azophene, carbaryl	CC	2–5 ppm	water, chloroform extracts of crops
Enzytec, Inc. 425 Volker Blvd. Kansas City, MO 64110	ENZYTEC Pesticide Detector Ticket	organophosphorus and carbamate pesticides	CC	1–10 ppm	water

[a] Assay type: ET = competition ELISA in tubes; EM = competition ELISA in microwells; EC = EIA on card; CC = colorimetric cholinesterase inhibition on card.
[b] Claimed by manufacturer.
[c] Suggested by manufacturer.
[d] N/S = not stated by manufacturer.

RIA used a charcoal precipitation step to separate the antibody-bound from the unbound ligand. No determination limits were reported; the assay was used to determine whether serum from immunized animals reacted with the immunizing antigen, and was not quantitative.

The ELISA was developed by using antisera from 3 different rabbits. One serum reacted much better with diflubenzuron than with BAY SIR 8514; another reacted equally well with both. The third, reactive with diflubenzuron, was directly labeled with alkaline phosphatase to produce a single-step direct assay. Except for differences in antigen specificity, all 3 sera produced equivalent results. The best antiserum could determine 1 ppb in water directly, with 100% recovery from fortified materials, and was able to determine 40 ppb in whole milk diluted 1:4 in buffer. Again, it should be noted that the "matrix effect" reduced determination in milk by some 40-fold.

The exquisite specificity possible with immunoassays was demonstrated in the report of an RIA developed for plasma warfarin by using antisera specific for either the *R*-isomer or *S*-isomer (71). This specificity paralleled that reported for the antiserum to *S*-bioallethrin, described above. The warfarin antisera were generated by immunization of animals with pure isomer conjugated to bovine serum albumin; cross-reactivity was 0.3% for *S*-antiserum with *R*-isomer and 3.3% for *R*-antiserum with *S*-isomer. The linear range of the assay was 25–1600 pg standard in 0.7 mL buffer (equivalent to 25 ng/mL plasma). CVs were 3% intraassay and 9% interassay. This assay was not applied to food or soil analysis.

Commercial Immunoassay Kits for Pesticides

Availability

As of April 1990, only a small number of commercial IA kits are available for determination of pesticide residues. The kits, their manufacturers, and some of the characteristics of the kits are listed in Table 4. Most of the kits were originally developed for determination of residues in water; techniques to adapt them to food and crop extracts are being developed. Also included in Table 4 are descriptions of colorimetric (non-IA) field tests for determining organophosphorus and carbamate pesticide residues.

The commercial IA kits with sensitivities of 1–100 ppb (as claimed by their manufacturers) would appear to be useful as first-step screening tests. As supplied, the kits are at best only semiquantitative. Likewise, because most kits were developed for detection of residues in water, extraction and perhaps cleanup steps would be necessary before crop materials could be assayed.

The RES-I-MUNE kit assays are performed in plastic tubes. The tube format limits the number of analytical sam-

ples that can be determined simultaneously. Although the card format of the EZ-Screen kit might be more manageable, it is difficult to quantitate results. A better test format would use the 96-well microtiter plate often used for EIA (which would allow a standard curve to be run with each set of test samples, thus facilitating quantitation) and would provide room for a larger number of test portions to be analyzed, along with replicates. The recently introduced RES-I-QUANT immunoassay kits for alachlor and benomyl offered by ImmunoSystems (see Table 4) use a microwell format (eight 12-well strips). It is claimed that they are capable of quantitating pesticides at levels of 0.5–20 ppb. However, the alachlor kit determines only the parent compound, and, as noted above (52), this particular compound degrades rapidly, so determination of the parent alone is of only limited use. Battery-powered portable spectrophotometers are available (such as the MR250, from DYNATECH, Chantilly, VA 22021), so that hard copy printouts of quantitative results can be generated in the field.

Official Evaluation/Acceptance of IA Kits

Immunoassay methods are not well characterized, and although validation methods are not yet firmly established, the Environmental Protection Agency (EPA) (72), AOAC (73, 74), and the Food Safety and Inspection Service (FSIS) of the USDA (75) have published proposed guidelines for the evaluation of IA test kit applications, setting up collaborative studies, data review, and adoption of IA methods.

An immunoassay submitted to EPA for evaluation must be optimized and documented by the developer and should be accompanied by detailed protocols as well as descriptions of reagent preparations; assurances of adequate pools of reagents, especially antibodies employed in the test; quality control procedures; and, if possible, comparisons to other analytical methods. The test will be evaluated by EPA on the basis of performance with known materials, including different matrices, the occurrence of systematic errors, variability, and comparison with existing tested analytical methods, using a 3–5 laboratory collaborative study. EPA will then formulate recommendations regarding the usefulness and acceptability of the test.

In addition to its draft guidelines on collaborative study procedures (73), AOAC also established a task force on test kits and proprietary methods to draft recommendations on the use of reagents, descriptions in *Official Methods of Analysis*, and validation required before a change or improvement in a protocol can be made (74). Recommendations included the following: (a) specifications for components must be included as part of the method, and future batches of the test must meet the specifications; (b) kit manufacturers must notify AOAC when any changes to components or protocols are made, and the General Referee will determine if such changes require a new validation protocol; (c) for pass/fail (qualitative) methods, acceptable rates of false positives and false negatives need not be specified, but will be considered on a case-by-case basis (it should be noted that there are no criteria for evaluation of qualitative methods proposed by any organization responsible for validating test kits for official use); and (d) because proper validation is AOAC's goal, no limits will be set on the number of methods validated for a given analyte.

The FSIS has proposed a review and approval process with 4 major steps: (a) a decision by the deputy administrator for Technical Services to review a submitted test; (b) approval by the coordinator (the director of Technology Transfer and Assessment) of the test's laboratory performance; (c) acceptance by the coordinator of a collaborative study; and (d) final consideration by the Test Review Steering Committee and approval by the deputy administrator. Applications are first sent to the Test Review Steering Committee for an evaluation of the need for, and interest in, the submitted test. If there is need/interest, the information then goes to a technical reviewer, who reviews the scientific basis of the test. The review is forwarded to the deputy administrator, who approves or rejects further action on the test application. An application would give background information (purpose, matrices to be used, etc.), the equipment and reagents needed, the laboratory characteristics of the test (quantitation, standard curves, recoveries), especially specificity, and a discussion and interpretation of results. Approval of a test requires prior information about the costs of the test, the assurances of at least a 1-year supply of special reagents such as antibodies, complete protocols, and a quality control/quality assurance plan. A collaborative study using representative laboratories is required to validate test performance before final approval; the applicant plans, executes, and bears the costs of the entire test development process. Approvals expire after 1 year, so that tests found unsatisfactory during field testing may be eliminated; approvals will be reissued if test data are satisfactory.

Summary

A large number of IAs potentially capable of measuring a wide range of pesticide compounds have been reported in the literature. Some, such as IAs for atrazines, paraquat, parathion, benomyl, diclofop, and metalaxyl, have been specifically applied to water, soil, and food matrices. Others, although not originally developed to be used for determination of residues in foods, such as IAs for malathion, 2,4-D and 2,4,5-T, dieldrin and aldrin, and pyrethroids, could possibly be developed into assays useful in food analysis.

Agencies involved in testing for pesticide residues, such as the EPA and USDA, as well as AOAC, have proposed guidelines to be used in the evaluation of assays submitted to them.

The growing need for faster, easier methods that allow screening of larger numbers of analytical samples, and the need to determine which test samples require more extensive analysis, means that IAs will become an important part of the analytical requirements necessary to maintain FDA's regulatory responsibilities.

References

(1) Voller, A., Bidwell, D. E., & Bartlett, A. (1979) *The Enzyme Linked Immunosorbent Assay*, Dynatech Laboratories, Alexandria, VA
(2) Kurstak, E. (1986) *Enzyme Immunodiagnosis*, Academic Press, New York, NY
(3) Newsome, W. H. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 919–923
(4) Ellis, R. L. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 521–524
(5) Hammock, B. D., & Mumma, R. O. (1980) in *Recent Advances in Pesticide Analytical Methodology*, ACS Symposium Series **136**, J. Harvey & G. Zweig (Eds), American Chemical Society, Washington, DC, pp. 321–352
(6) Schwalbe-Fehl, M. (1986) *Int. J. Environ. Anal. Chem.* **26**, 295–304
(7) Harrison, R. O., & Hammock, B. D. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 981–987

(8) Kohler, G., & Milstein, C. (1975) *Nature* **256**, 495-497
(9) Ratcliffe, J. G. (1974) *Br. Med. Bull.* **30**, 32-37
(10) Walker, W. C. (1977) *Clin. Chem.* **23**, 384-402
(11) Monroe, D. (1984) *Anal. Chem.* **56**, 920A-931A
(12) Oellerich, M., Haeckel, R., & Haindl, H. (1982) *J. Clin. Chem. Clin. Biochem.* **20**, 765-772
(13) Proceedings of the 2nd European Symposium on Paraquat Poisoning, January 27, 1986, London, in *Human Toxicol.* **6**, 1987
(14) Levitt, T. (1977) *Lancet* **ii**, 358
(15) Levitt, T. (1979) *Proc. Analyt. Div. Chem. Soc.* **16**, 72-76
(16) Stewart, M. J., Levitt, T., & Jarvie, D. R. (1979) *Clin. Chim. Acta* **94**, 253-257
(17) Proudfoot, A. T., Stewart, M. S., Levitt, T., & Widdop, B. (1979) *Lancet* **ii**, 330-332
(18) Braithwaite, R. A. (1987) *Human Toxicol.* **6**, 83-86
(19) Scherrman, J. M., Houze, P., Bismuth, C., & Bourdon, R. (1987) *Human Toxicol.* **6**, 91-93
(20) Fatori, D., & Hunter, W. M. (1980) *Clin. Chim. Acta* **100**, 81-90
(21) Niewola, Z., Walsh, S. T., & Davies, G. E. (1983) *Int. J. Immunopharm.* **5**, 211-218
(22) Niewola, Z., Hayward, C., Symington, B. A., & Robson, R. T. (1985) *Clin. Chim. Acta* **148**, 149-156
(23) Niewola, Z., Benner, J. P., & Swaine, H. (1986) *Analyst* **111**, 399-403
(24) Van Emon, J. M., Seiber, J. N., & Hammock, B. D. (1985) in *Bioregulators for Pest Control*, ACS Symposium Series **276**, P. Hedin (Ed.), American Chemical Society, Washington, DC, pp. 307-316
(25) Van Emon, J., Hammock, B., & Seiber, J. N. (1986) *Anal. Chem.* **58**, 1866-1873
(26) Van Emon, J., Seiber, J., & Hammock, B. (1987) *Bull. Environ. Contam. Toxicol.* **39**, 490-497
(27) Hogg, P. J., Johnston, S. C., Bowles, M. R., Pond, S. M., & Winzor, D. J. (1987) *Molec. Immunol.* **24**, 797-801
(28) Johnston, S. C., Bowles, M., Winzor, D. J., & Pond, S. M., *Fundam. Appl. Toxicol.* **11**, 261-267
(29) Bowles, M., Johnston, S. C., Schoof, D. D., Pentel, P. R., & Pond, S. M. (1988) *Int. J. Immunopharm.* **10**, 537-545
(30) Nagao, M., Takatori, T., Terazawa, K., Wu, B., Wakasugi, C., Masui, M., & Skeda, H. (1989) *J. Forensic Sci.* **34**, 547-552
(31) Nagao, M., Takatori, T., Wu, B., Terazawa, K., Gotouda, H., & Akabane, H. (1989) *J. Immunol.* **10**, 1-17
(32) Coxon, R. E., Rae, C., Gallcher, G., & Landon, J. (1988) *Clin. Chim. Acta* **175**, 297-306
(33) Koelle, G. B. (1981) *Fundam. Appl. Toxicol.* **1**, 129-134
(34) Centeno, E. R., & Johnson, W. J. (1967) *Fed. Proc.* **26**, 704
(35) Sternberger, L. A., Cuculis, J. J., Meyer, H. C., Lenz, D. E., & Kavanagh, W. G. (1974) *Fed. Proc.* **33**, 728
(36) Haas, G. J., & Guardia, E. J. (1968) *Proc. Soc. Exp. Biol. Med.* **129**, 546-551
(37) Ercegovich, C. D., Vallejo, R. P., Gettig, R. R., Woods, L., Bogus, E. R., & Mumma, R. O. (1981) *J. Agric. Food Chem.* **29**, 559-563
(38) Hunter, K. W., & Lenz, D. E. (1982) *Life Sci.* **30**, 355-361
(39) Sternberger, L. A., Sim, V. A., Kavanagh, W. G., Cuculis, J. J., Meyer, H. G., Lenz, D. E., & Hinton, D. M. (1972) *Army Science Conference Proceedings*, Vol. 3, Dept. of the Army, Washington, DC
(40) Schmidt, P., Kuhlmann, R., & Losch, U. (1988) *Z. Naturforsch.* **43c**, 167-172
(41) Hunter, K. W., Lenz, D. E., Brimfield, A. A., & Naylor, J. A. (1982) *FEBS Lett.* **149**, 147-151
(42) Buenafe, A. C., & Rittenberg, M. B. (1987) *Mol. Immunol.* **24**, 401-407
(43) Erhard, M. H., Schmidt, P., Kuhlmann, R., & Losch, U. (1989) *Arch. Toxicol.* **63**, 462-468
(44) Centeno, E. R., Johnson, W. J., & Sehon, A. H. (1970) *Int. Arch. Allergy* **37**, 1-13
(45) Langone, J. J., & Van Vanukis, H. (1975) *Res. Commun. Chem. Pathol. Pharmacol.* **10**, 163-171
(46) Banerjee, B. D. (1987) *Bull. Environ. Contam. Toxicol.* **38**, 798-804
(47) Rinder, D. F., & Fleeker, J. R. (1981) *Bull. Environ. Contam. Toxicol.* **26**, 375-380
(48) Fleeker, J. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 874-878
(49) Knopp, D., Nuhn, P., & Dobberkau, H.-J. (1985) *Arch. Toxicol.* **58**, 27-32
(50) Hall, J. C., Deschamps. R. J. A., & Kreig, K. K. (1989) *J. Agric. Food Chem.* **37**, 981-984
(51) Feng, P. C. C., Wratten, S. S., Horton, S. R., Sharp, C. R., & Logusch, E. W. (1990) *J. Agric. Food Chem.* **38**, 159-163
(52) Sharp, D. B. (1988) in *Herbicides, Chemistry, Degradation, and Mode of Action*, Vol. 3, P.C. Kearney & D.D. Kaufman (Eds), Marcel Dekker, New York, NY, pp. 301-333
(53) Dreher, R. M., & Podratzki, B. (1988) *J. Agric. Food Chem.* **36**, 1072-1075
(54) Lukens, H. R., Williams, C. B., Levison, S. A., Dandliker, W. B., Murayama, D., & Baron, R. L. (1977) *Environ. Sci. Technol.* **11**, 292-297
(55) Newsome, W. H., & Shields, J. B. (1981) *J. Agric. Food Chem.* **29**, 220-222
(56) Newsome, W. H., & Collins, P. G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 1025-1027
(57) Huber, S. J. (1985) *Chemosphere* **14**, 1795-1803
(58) Bushway, R. J., Perkins, B., Savage, S. A., Lekousi, S. J., & Ferguson, B. S. (1988) *Bull. Environ. Contam. Toxicol.* **40**, 647-654
(59) Bushway, R. J., Perkins, B., Savage, S. A., Lekousi, S. J., & Ferguson, B. S. (1989) *Bull. Environ. Contam. Toxicol.* **42**, 899-904
(60) Reed, J. P., Hall, F. R., & Krueger, H. R. (1990) *Bull. Environ. Contam. Toxicol.* **44**, 8-12
(61) Schlaeppi, J.-M., Fory, W., & Ramsteiner, K. (1989) *J. Agric. Food Chem.* **37**, 1532-1538
(62) Dunbar, B., Riggle, B., & Niswender, G. (1990) *J. Agric. Food Chem.* **38**, 433-437
(63) Wing, K. D., Hammock, B. D., & Wustner, D. A. (1978) *J. Agric. Food Chem.* **26**, 1328-1333
(64) Wing, K. D., & Hammock, B. D. (1979) *Experientia* **35**, 1619-1620
(65) Schwalbe, M., Dorn, E., & Beyermann, K. (1984) *J. Agric. Food Chem.* **32**, 734-741
(66) Newsome, W. H. (1985) *J. Agric. Food Chem.* **33**, 528-530
(67) Newsome, W. H. (1986) *Bull. Environ. Contam. Toxicol.* **36**, 9-14
(68) Kelley, M. M., Zahnow, E. W., Petersen, W. C., & Toy, S. T. (1985) *J. Agric. Food Chem.* **33**, 962-965
(69) Wie, S. I., Sylwester, A. P., Wing, K. D., & Hammock, B. D. (1982) *J. Agric. Food Chem.* **30**, 943-948
(70) Wie, S., & Hammock, B. D. (1982) *J. Agric. Food Chem.* **30**, 949-957
(71) Cook, C. E., Ballentine, N. H., Seltzmann, T. B., & Tallent, C. R. (1979) *J. Pharmacol. Exp. Therap.* **210**, 391-398
(72) Van Emon, J. (1989) Abstr. 46, 198th National Meeting, American Chemical Society, Miami Beach, FL, September 1989
(73) Anonymous (1989) *J. Assoc. Off. Anal. Chem.* **72**, 694-704
(74) Mastrorocco, D., & Brodsky, M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 331-332
(75) Anonymous (1989) *Fed. Regist.* **54**, 33920-33923

ALCOHOLIC BEVERAGES

Gas Chromatographic Determination and Pattern Recognition Analysis of Methanol and Fusel Oil Concentrations in Whiskeys

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A more efficient determination of methanol and fusel oil in 4 types of whiskeys was accomplished using gas chromatography and temperature programming. Carbopack C with 0.2% Carbowax 1500 (Supelco, Inc., Bellefonte, PA) produced baseline separation of 13 aliphatic alcohols containing 5 carbons or less. A comparison of methanol and fusel oil concentrations between various types of whiskeys indicates that bourbon and sour mash whiskeys contain considerably more fusel oil (275 ± 12 and 265 ± 5 mg/100 mL, respectively) than blended and scotch whiskeys (47 ± 5 and 114 ± 7 mg/100 mL, respectively). All concentrations were corrected to 100 proof. A statistical pattern recognition analysis of the data showed that fusel oil components (1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) and methanol could be used to distinguish between bourbon-sour mash, blended, and scotch whiskeys. Bourbon and sour mash whiskeys were indistinguishable by fusel oil–methanol analysis. A significant increase in methanol content occurred in bourbon and sour mash whiskeys after bottles were opened and resealed for up to 58 months; fusel oil content remained essentially unchanged.

Analysts often need a simple method for chemically distinguishing between various types of aged whiskeys. The most common types of aged whiskey are bourbon, sour mash, blended, and scotch whiskeys. Gas chromatographic (GC) analysis is the method of choice; however, the complete volatile component profile is quite complex and difficult to interpret. The major volatile compounds, other than ethanol, are found in the fusel oil fraction. This fraction contains 1-propanol (*n*-propyl alcohol), 2-methyl-1-propanol (isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), and 3-methyl-1-butanol (isoamyl alcohol) in various concentrations. Small amounts of methanol (generally less than 25 mg/100 mL) and phenylethyl alcohol (less than 130 mg/100 mL) are also found; however, these alcohols are not generally included as part of the fusel oil fraction.

Individual components of fusel oil are not commonly used to distinguish between various types of whiskeys. However, this study was initiated to determine the feasibility of using GC analysis of fusel oil components combined with a statistical pattern recognition computer program as tools for classifying an unknown whiskey.

The direct GC method is undoubtedly best for determination of methanol and fusel oil; it is the simplest and fastest as well as the most sensitive and accurate. The efficiency and resolution of gas chromatography has improved with the development of better supports and liquid phases. Liquid phases such as Carbowax have been most useful for determining fusel oil (1). A combination of 2 liquid phases on 1 support to resolve fusel oil has been reported (2, 3). These liquid phases have certain disadvantages such as thermal instability, especially when temperature programming is used. It is difficult to apply these mixed phases homogeneously to the solid support. Graphitized carbon black (Carbopack B) coated with PEG 20M and trimesic acid (4) or acid-washed Carbopack B coated with PEG 20M have been used in the separation of fusel oil in whiskey (5). AOAC methods use 23% Carbowax 1500 on Chromosorb W or 2% glycerol and 2% 1,2,6-hexanetriol on Gas-Chrom R (6). More recently, a 5% phenylmethylsilicone fused silica capillary column (7) has been used and liquid chromatographic (LC) methods have been developed (8, 9). Most separations suffer from lack of complete resolution of 2-methyl-1-butanol and 3-methyl-1-butanol.

Pattern recognition has been applied frequently to GC analysis of alcoholic beverages. For example, Rapp et al. (10) used this technique to differentiate between varieties of wines and grapes. They did not use a computer program but relied on manual comparison of a few components of the GC profile. Kwan and Kowalski (11) examined some 137 components with computerized pattern recognition to classify wine as to geographic origin and correlate various sensory qualities with the GC profile. Noble et al. (12) conducted similar work on the varietal classification of wine. Schreier and Reiner (13) used multiple discriminant analysis to distinguish between French and German grape brandies and French cognacs. Saxberg et al. (14) applied pattern recognition techniques to distinguish a well-known brand of scotch whiskey from less expensive whiskeys to detect counterfeit whiskey. Simpkins (15) used congener profiles in the detection of illicit spirits. Other studies (16–18) have included multiple discriminant analysis in the classification of Venetian white wines.

The present study describes a single direct injection procedure for separating and quantitating methanol and fusel oil in whiskey using temperature programming with 0.2% Carbowax 1500 on Carbopack C (Supelco, Inc., Bellefonte, PA).

The data were analyzed using a statistically based pattern recognition program (EINSIGHT) to determine whether the fusel oil fraction and methanol concentrations of 4 whiskeys could be used to distinguish one type of whiskey from another.

Experimental

Chromatographic system.—A Varian Model 3700 gas chromatograph equipped with dual flame ionization detectors was used for all studies. All chromatograms and calculations were obtained and recorded using a Perkin-Elmer Model 3600 computer with CHROM II software. The column was a 6 ft × 2 mm id glass column packed with 0.2% Carbowax 1500 on Carbopack C (80–100 mesh) (Supelco,

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Table 1. Gas chromatographic retention times of alcohols relative to 1-butanol

IUPAC name	Common name	RRT[a]
methanol	methyl alcohol	0.03
ethanol	ethyl alcohol	0.08
2-propanol	isopropyl alcohol	0.18
1-propanol	*n*-propyl alcohol	0.28
2-methyl-2-propanol	*tert*-butyl alcohol	0.34
2-butanol	*sec*-butyl alcohol	0.60
2-methyl-1-propanol	isobutyl alcohol	0.72
1-butanol	*n*-butyl alcohol	1.00
3-pentanol		1.43
2-pentanol	*sec*-amyl alcohol	1.58
2-methyl-1-butanol	active amyl alcohol	1.69
3-methyl-1-butanol	isoamyl alcohol	1.81
1-pentanol	*n*-amyl alcohol	2.08

[a] RRT = 1.0 for 1-butanol.

Inc.). Helium at 30 mL/min and 50 psi inlet pressure was the carrier gas. The column oven temperature was held at 55°C for 4 min., then programmed to increase at 2°C/min to a final temperature of 95°C. The detector block and injection port temperatures were 150°C.

Reagents and standards.—The calibration standard solution for quantitation consisted of a mixture of methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol at concentrations of 2.40, 2.40, 9.60, 10.95, and 18.36 mg/100 mL, respectively, in ethanol–water (5 + 95, v/v). 1-Butanol was added as an internal standard to the calibration standard solution and all whiskey samples at a final concentration of 8.10 mg/100 mL. All alcohol standards were GC grade chemicals (Chem Service, West Chester, PA). The 3-methyl-1-butanol standard contained 8.7% 2-methyl-1-butanol as a contaminant. The standard 2-methyl-1-butanol contained 5.5% 3-methyl-1-butanol. A correction factor was used for the concentration of each of these alcohol standards. An aqueous solution of 7–9 mg/100 mL of each of the alcohols listed in Table 1 was used for the standard solution for relative retention time measurements.

Samples.—122 bottles of bourbon, sour mash, scotch and blended whiskeys were purchased from retail liquor stores. The whiskeys were placed into 1 of the 4 categories (bourbon, sour mash, scotch or blended) according to their original labels. The scotch group includes whiskeys identified by the manufacturer as being blended scotch as well as those scotch whiskeys not identified as such. Seventy-nine whiskey samples had been opened then resealed for 12 months, 26 months, and 58 months.

Analytical method.—The gas chromatographic system was calibrated by injecting 2 μL of the calibration standard. Internal standard was added to each sample and 2 μL of each was injected on column without further sample preparation. Retention times, peak areas, and concentrations were recorded by the CHROM II software.

Results and Discussion

Fusel Oil and Methanol Analysis

Baseline resolution (Figure 1) was obtained for each of the 13 alcohols in the retention time standard listed in Table 1. They were eluted within 25 min. 1-Butanol was chosen as the internal standard because of its intermediate retention time and its absence in appreciable concentration in the 4 kinds of whiskey analyzed. The relative retention time (RRT) was calculated based on a RRT of 1.00 for 1-butanol.

Figure 2 shows a typical GC separation for a well-known sour mash whiskey. The unnumbered peak at RRT 0.59 is ethyl acetate; it appears in all samples but was not included in the quantitation because we were interested primarily in the alcohols.

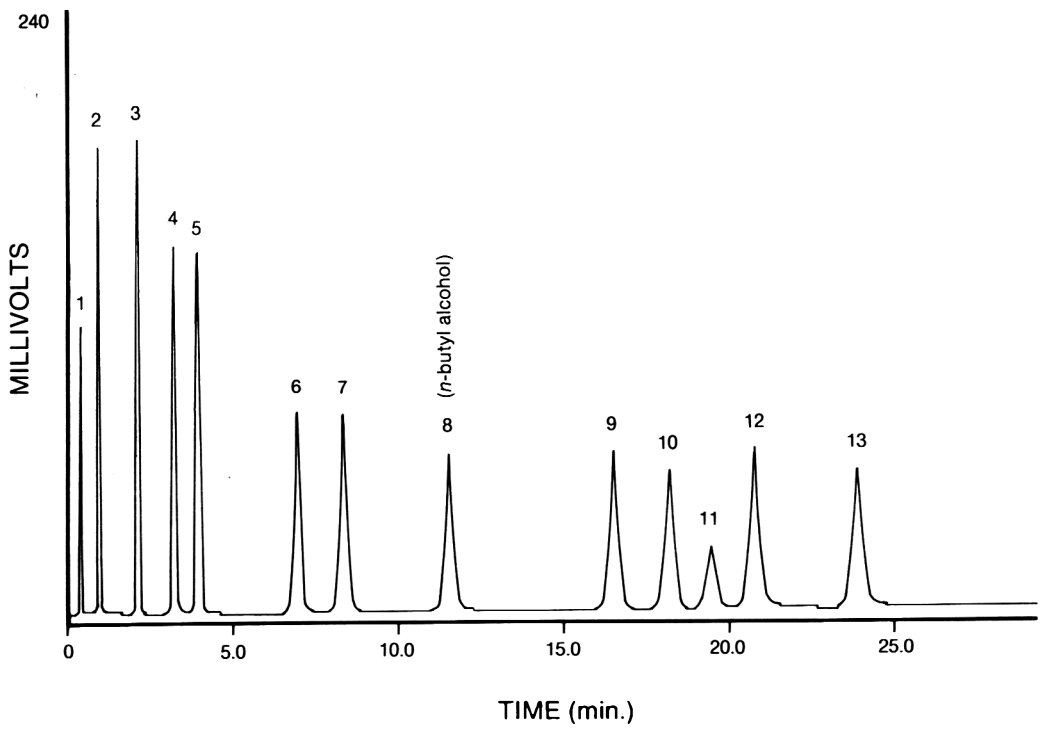

Figure 1. Chromatogram showing baseline resolution of the 13 alcohols listed in Table 1. Chromatographic conditions: 6 ft × 2 mm id glass column packed with 0.2% Carbowax 1500 on Carbopack C (80–100 mesh); column temperature held at 55°C for 4 min., then programmed to 95°C at 2°C/min.

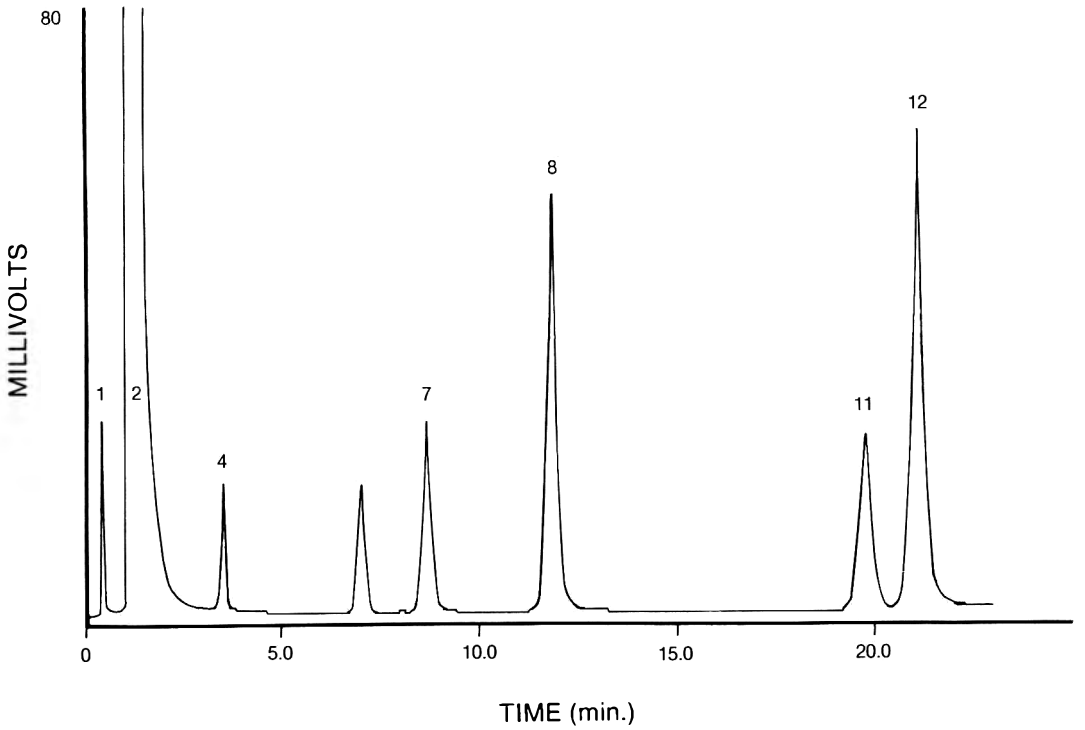

Figure 2. Chromatogram of a typical whiskey sample under the same conditions as in Figure 1 with a shorter run time.

The detector demonstrated a linear response to these concentration ranges: 0.08–8.00 mg/100 mL methanol and 1-propanol; 0.32–32.0 mg/100 mL 2-methyl-1-propanol; 0.36–36.5 mg/100 mL 2-methyl-1-butanol; 0.61–61.2 mg/100 mL 3-methyl-1-butanol. These ranges do not necessarily reflect the upper limit of linearity but, rather, cover the ranges of each component normally found in various types of whiskey. The correlation coefficient in each case was 1.00, demonstrating the linearity over this concentration range. To determine variation in instrument response and in injection techniques, 7 to 20 injections of 1-butanol standard were made each day over a 10-day period. The coefficient of variation each day was, in all determinations except one, less than 2.6%, which would indicate acceptable instrument response and injection technique.

Table 2 shows results of methanol and fusel oil analysis, including statistical evaluation, using the methods and equipment described for the 122 samples. As the table indicates, all 4 kinds of whiskey contain similar concentrations of methanol, but quite different levels of 2-methyl-1-butanol and 3-methyl-1-butanol and total fusel oil. Figures 3 and 4 show the distribution of methanol and 2-methyl-1-butanol + 3-methyl-1-butanol in the samples, respectively. (See figure captions for legend.) Sour mash and bourbon whiskeys usually contain more than 200 mg of fusel oil/100 mL and 140 mg of 2-methyl-1-butanol + 3-methyl-1-butanol/100 mL; however, blended and scotch whiskeys contain less than 170 mg of fusel oil/100 mL and 120 mg of 2-methyl-1-butanol + 3-methyl-1-butanol/100 mL. Therefore, sour mash and bourbon whiskeys can be distinguished from blended and scotch whiskey according to the concentration of 2-methyl-1-butanol + 3-methyl-1-butanol and total fusel oil. However, scotch whiskey contains more 1-propanol and 2-methyl-1-propanol than blended whiskey. A plot of 2-methyl-1-butanol against 3-methyl-1-butanol concentration in scotch and blended whiskeys (Figure 5) indicates a linear relationship between these 2 components. The correlation coefficient was 0.97; the slope 3.0.

The most definitive plot for distinguishing between the 4 whiskeys is shown in Figure 6. When 2-methyl-1-butanol concentration is plotted vs 1-propanol, 3 distinct clusters of data points appear for scotch, blended, and sour mash–bourbon.

Variation in Methanol and Fusel Oil Concentration After Atmospheric Exposure

Concentrations of methanol and fusel oil in sour mash and bourbon whiskeys were measured for 4 groups of samples:

Table 2. Gas chromatographic determination of methanol and fusel oil concentrations (mg/100 mL, 100 proof) in whiskey (mean ± standard error)

Component	Sour mash	Bourbon	Blended	Scotch
methanol	16.1 ± 0.7	19.1 ± 0.6	11.5 ± 0.6	13.6 ± 0.9
1-propanol	12.8 ± 0.6	12.5 ± 0.5	4.8 ± 0.6	28.7 ± 1.6
2-methyl-1-propanol	52.6 ± 4.5	44.0 ± 1.6	9.9 ± 1.1	38.3 ± 1.9
2-methyl-1-butanol	50.6 ± 2.2	54.9 ± 1.7	8.0 ± 1.1	10.5 ± 1.4
3-methyl-1-butanol	159 ± 6.8	152 ± 2.2	24.6 ± 3.0	36.5 ± 4.5
Total fusel oil	275 ± 12	265 ± 5	47.2 ± 5.3	114 ± 7
Number of samples	37	41	26	18

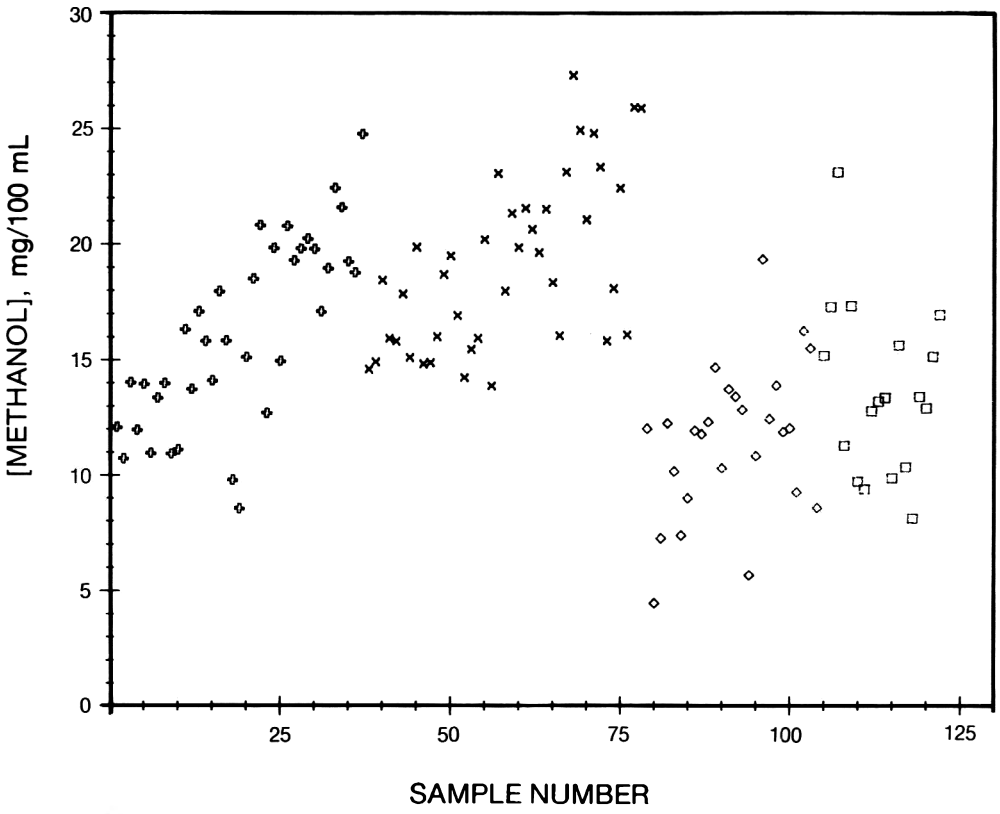

Figure 3. Distribution of methanol in whiskey samples. Sour mash (+), bourbon (x), blended (♦), and scotch (■). Note that all whiskeys contain similar concentrations of methanol.

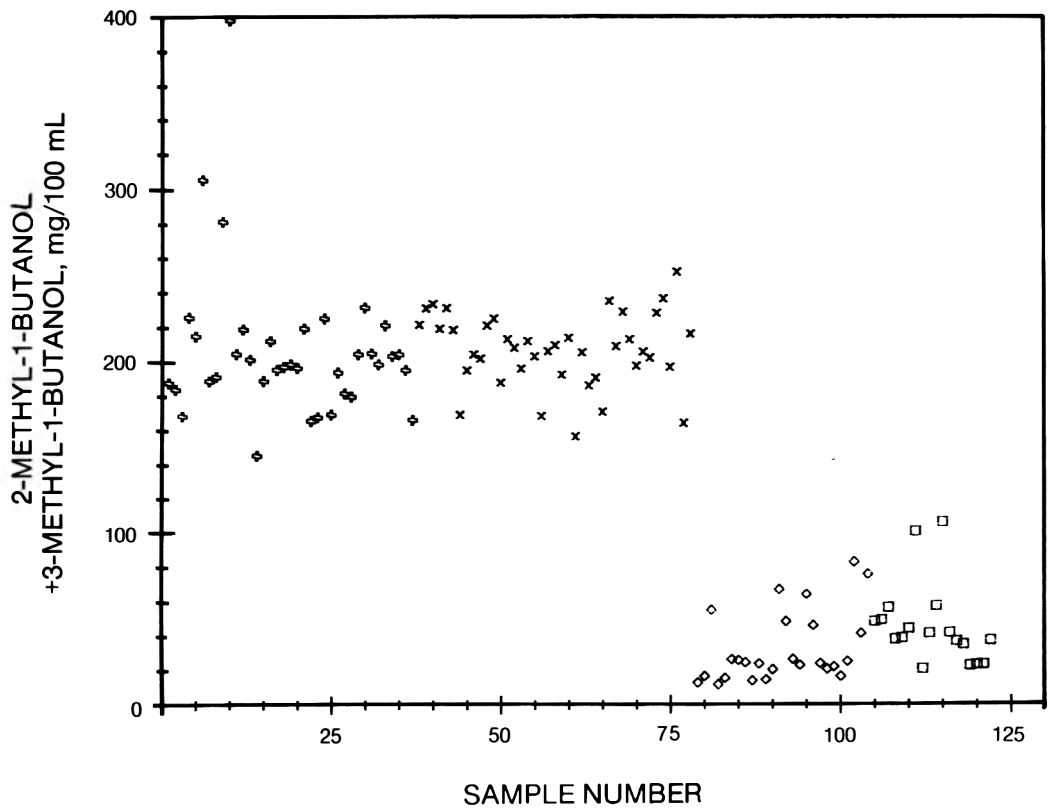

Figure 4. Distribution of 2-methyl-1-butanol + 3-methyl-1-butanol in whiskey samples. Sour mash (+), bourbon (x), blended (♦), and scotch (■). Note that scotch and blended whiskeys have much lower concentrations of these 2 alcohols than sour mash and bourbon whiskeys.

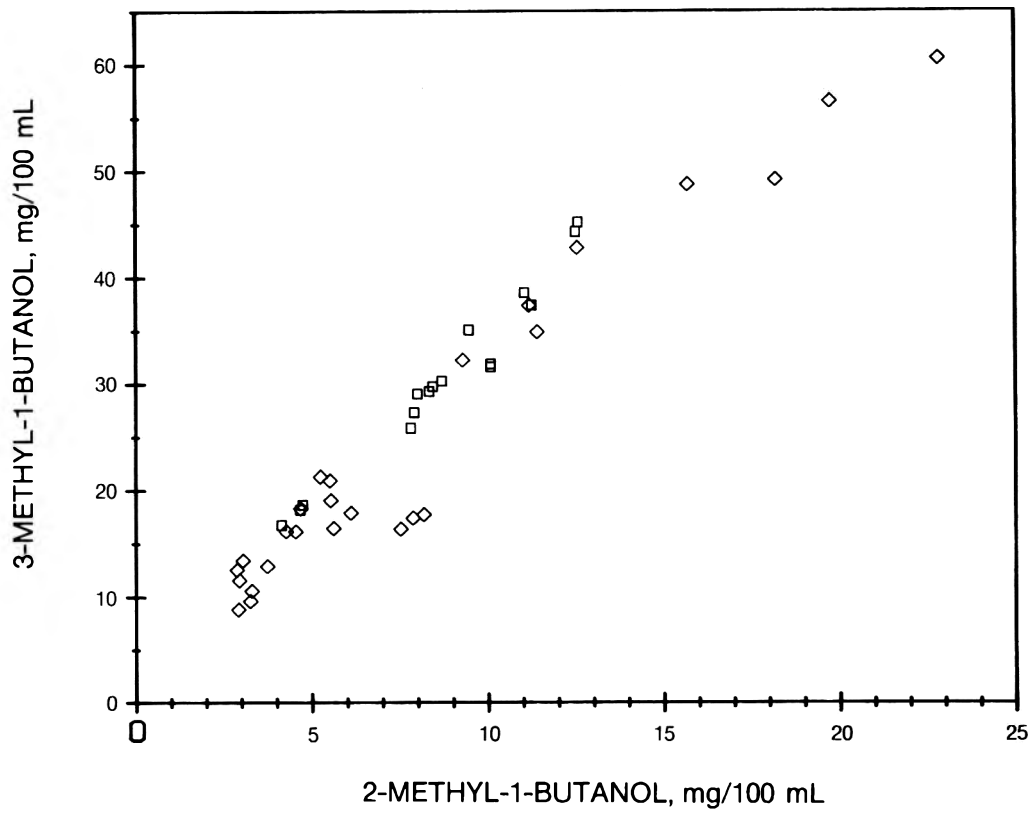

Figure 5. Plot of 2-methyl-1-butanol vs 3-methyl-1-butanol concentration. Scotch (■) and blended (♦). Note the linear relationship (correlation coefficient of 0.97) for these 2 alcohols in scotch and blended whiskeys.

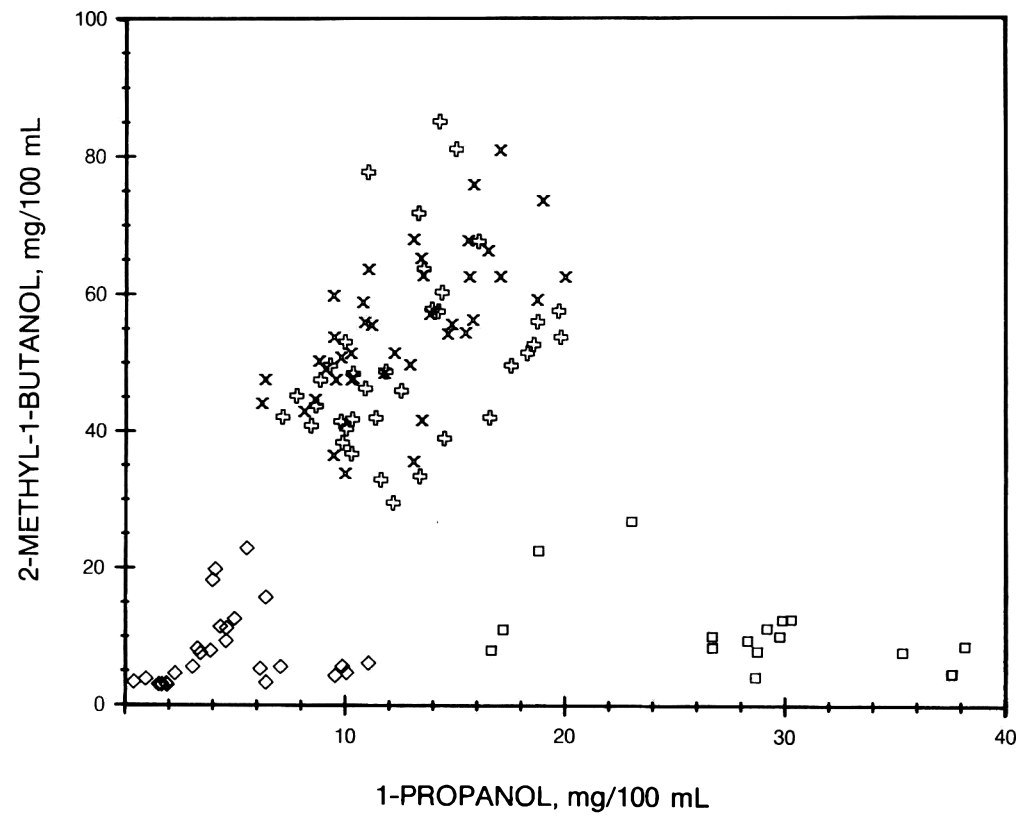

Figure 6. Plot of 2-methyl-1-butanol vs 1-propanol. Scotch (■), blended (♦), sour mash (+), and bourbon (x). Note apparent clustering into 3 groups: sour mash–bourbon, blended, and scotch.

Table 3. Change in concentrations (mg/100 mL, 100 proof) of methanol and fusel oil in whiskey after cap seal was broken

Component	Interval	Sour mash (av.)	Bourbon (av.)
methanol	1st day	13.80	16.66
	12 months	15.20	17.57
	26 months	18.70	19.86
	58 months	21.34	23.74
1-propanol	1st day	12.09	12.68
	12 months	12.49	11.85
	26 months	13.06	10.90
	58 months	15.42	14.54
2-methyl-1-propanol	1st day	44.84	43.73
	12 months	46.73	47.28
	26 months	44.74	44.28
	58 months	55.22	44.83
2-methyl-1-butanol	1st day	48.56	55.27
	12 months	49.04	55.42
	26 months	46.03	51.22
	58 months	47.32	56.94
3-methyl-1-butanol	1st day	146.39	157.37
	12 months	146.39	154.21
	26 months	150.08	143.08
	58 months	150.94	150.31

samples that had been opened (with varying amounts of the contents withdrawn) for 12 months, 26 months, 58 months, and 1 group opened just before analysis. Analysis for all samples was during the same 2- to 3-week time period. Results are shown in Table 3.

Table 4. Eigenvector loadings from principal component analysis of methanol plus fusel oil

Variable	PC[a] 1	PC 2	PC 3	PC 4	PC 5
methanol	0.418	0.020	0.883	−0.214	−0.016
1-propanol	0.103	0.900	0.032	0.419	0.056
2-methyl-1-propanol	0.483	0.296	−0.409	−0.710	−0.093
2-methyl-1-butanol	0.538	−0.223	−0.153	0.448	−0.661
3-methyl-1-butanol	0.541	−0.229	−0.171	0.273	0.742
Eigenvalues	3.014	1.153	0.597	0.193	0.043
Total variance = 5.000					

[a] PC = principal component.

Although these averages represent 4 different groups of samples (i.e., 1st day average, 12 month average, 26 month average, and 58 month average), it appears that there is a significant increase in methanol content after opening then resealing samples and allowing them to stand for the period of time listed. A linear relationship between concentration of methanol and time interval after opening was observed. The correlation coefficient for sour mash is 0.97 and for bourbon is 1.00. No reasonable explanation for the apparent increase in methanol concentration is evident at this time.

With the exception of the 58 month sour mash average for 2-methyl-1-propanol, a significant variation was not found in the fusel oil components for either sour mash or bourbon whiskeys. These data would indicate that fusel oil was

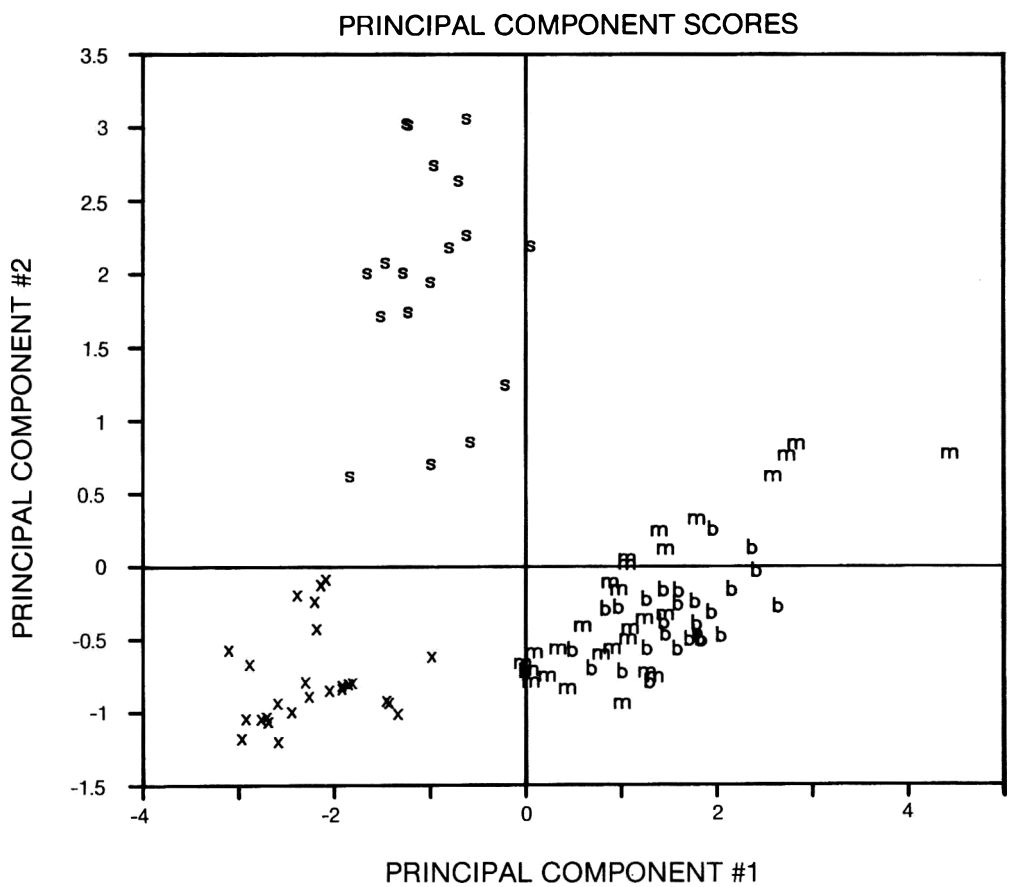

Figure 7. Principal component plot for 100 whiskey samples in which the 5-dimensional data are projected onto 2 axes while retaining 83% of the variance. Note the 3 major groupings of the whiskeys. Scotch (s), blended (x), sour mash (m), and bourbon (b).

formed only during fermentation and ethanol production and did not continue after ethanol formation ceased (19).

Statistical Analysis Using a Pattern Recognition Program

Data for whiskey samples in this study was subjected to a computerized pattern recognition program called EINSIGHT (ver. 2.0 and 2.5) (Infometrix, Seattle, WA). This program uses an expanded memory IBM-PC microcomputer and the support of Symphony (Lotus Development Corp.) or VP-Planner (Paperback Software) software. Two types of data analysis were performed: principal component analysis, and centroidal hierarchical cluster analysis. The aforementioned whiskey data yield 5 variables for each sample. Each sample can then be represented by a point in 5-dimensional space (the number of dimensions equals the number of variables). Principal component analysis is a technique for reducing the dimensionality of the data. This technique reduces the data to 2 or more principal components that are linear combinations of the variables. A projection of the data onto axes defined by these principal components produces a plot that yields the largest amount of information possible in any 2-dimensional plot of the data.

Data for 100 of the 122 samples (version 2.0 of the software was limited to 100 samples at one time) were autoscaled (each variable is adjusted to a standard deviation of 1.0 and a mean of 0.0) and subjected to principal component analysis. Figure 7 is a plot of principal component number 2 vs principal component number 1 using the following labels: scotch (s), blended (x), sour mash (m), and bourbon (b).

A grouping of the data is immediately apparent with blended and scotch whiskeys forming groups that are separate from the sour mash and bourbon whiskeys. Sour mash and bourbon whiskeys were so similar in the constituents chosen for analysis that they could not be distinguished from one another. Table 4 is a listing of eigenvector loadings; it reveals coefficients of the variables in the linear combination of these variables that generates each principal component. As shown in the table, principal component number 1 is composed primarily of a relatively equal combination of 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol with a somewhat smaller contribution by methanol and very little contribution by 1-propanol. Principal component number 2 is composed primarily of 1-propanol with much smaller contributions made by the other alcohols. Total variance for this data is 5.000 (because there are 5 variables). Thus, a plot of principal component 2 vs principal component 1 (1.15 + 3.01) produces a plot (Figure 7) that preserves 83% of the variance contained in the original data set. The loadings also show that, for principal component number 2, 2-methyl-1-butanol and 3-methyl-1-butanol vary inversely with 2-methyl-1-propanol and 1-propanol (they are of opposite signs), thus yielding better separation of the clusters.

Hierarchical cluster analysis evaluates similarity of data in terms of the euclidian distance between data points in 5-dimensional space. This analysis helps to identify outlying points in the original patterns. Cluster analysis of the data is illustrated in Figure 8. The program assigns different letters to different clusters. This shows that the lowermost 2 scotch whiskeys (s in Figure 7; b in Figure 8) are outliers that would be classified in the blended (x, Figure 7; b, Figure 8) catego-

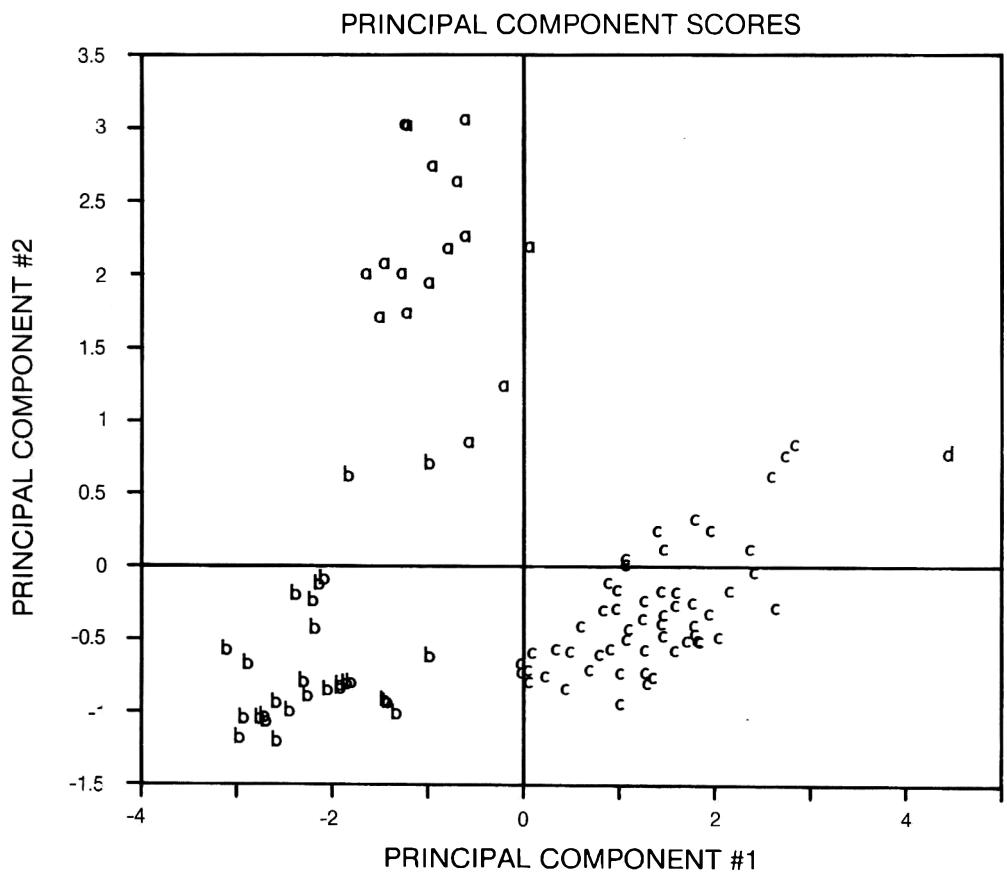

Figure 8. Centroidal hierarchical cluster analysis plot for 100 whiskey samples. Each letter represents a different cluster assignment as a result of cluster analysis. Scotch (a); blended (b); and sour mash–bourbon (c, d). Note that the lowermost 2 scotch whiskeys (s, Figure 7; b, this figure) are assigned to the blended category and 1 sour mash–bourbon is placed in a separate cluster (d).

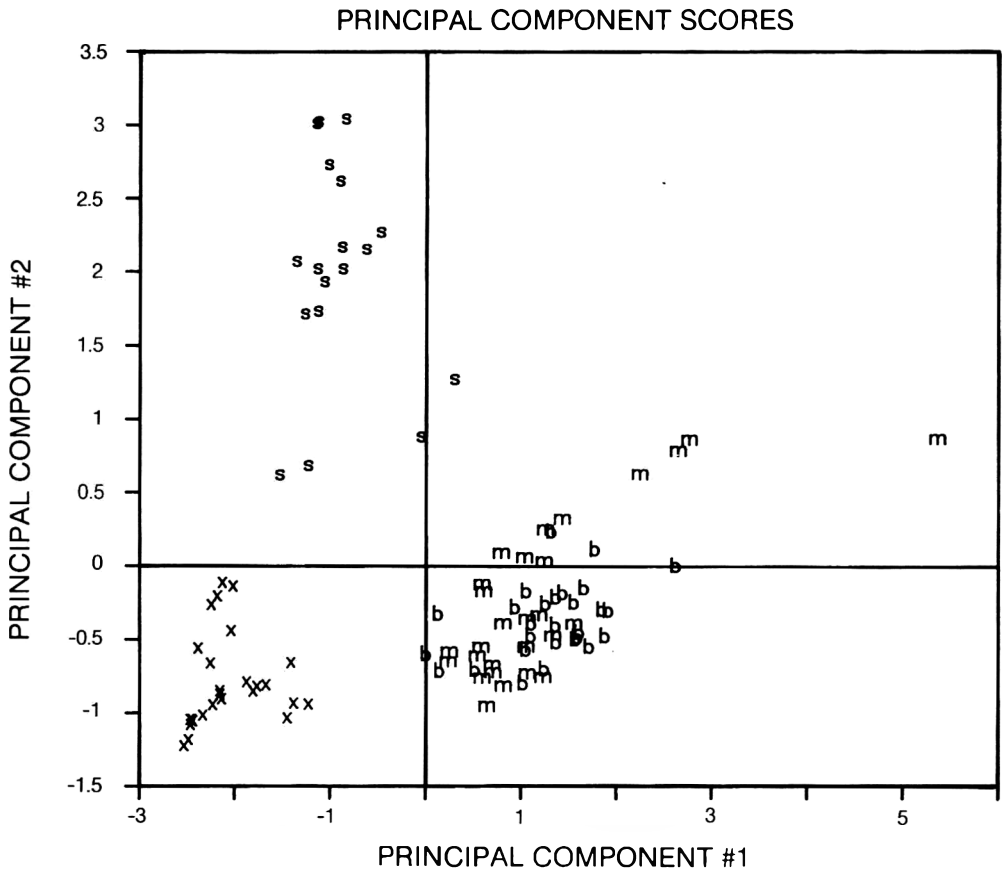

Figure 9. Principal component plot of 100 whiskey samples with methanol eliminated from the data set. Sour mash (m), bourbon (b), scotch (s), and blended (x). The apparent groupings are essentially the same as in Figure 7.

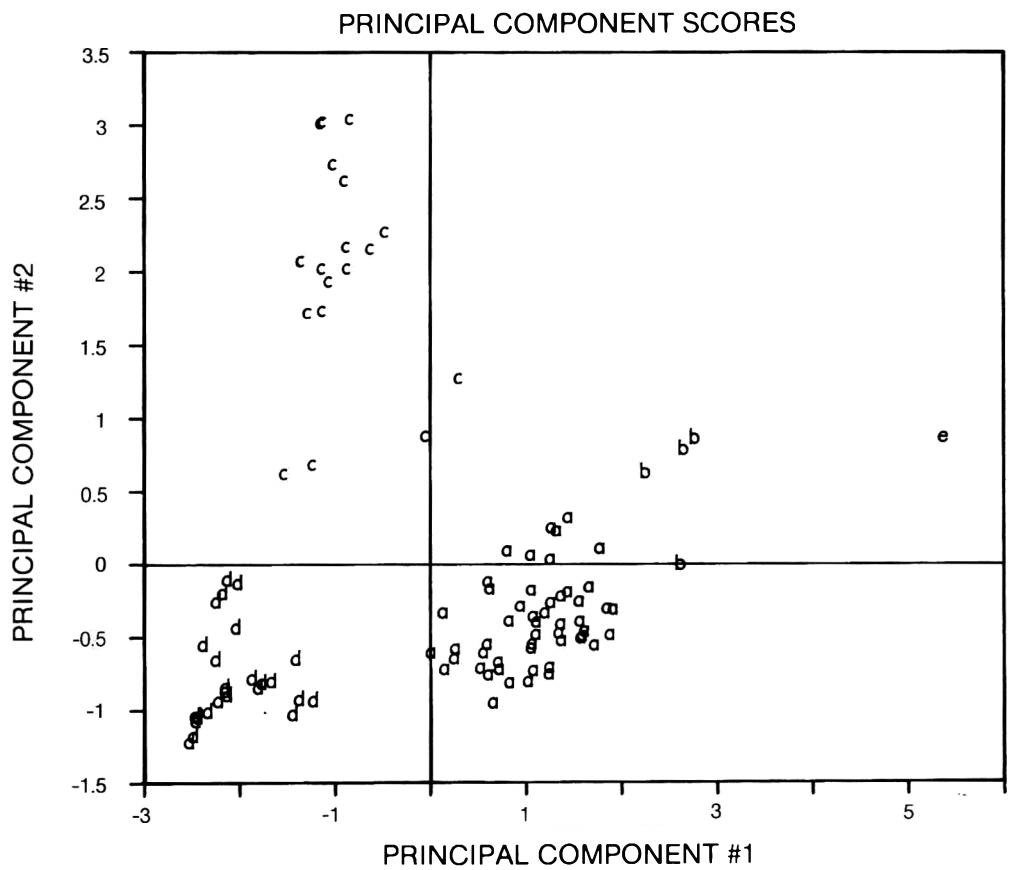

Figure 10. Centroidal hierarchical cluster analysis plot of 100 whiskey samples with methanol eliminated from the data set. Each letter represents a separate cluster as assigned by cluster analysis. Scotch (c); blended (d); and sour mash–bourbon (a, b, e). Note that the 2 scotches (b, Figure 8; c, this figure) are now classified correctly and more sour mash/bourbon samples are placed in separate groups.

ry. In addition, 1 sour mash whiskey (m, Figure 7; d, Figure 8) was classified in a separate category, indicating that it, too, was an outlier. Thus, 89% of scotch whiskeys and 100% of blended whiskeys were classified accurately, with the sour mash–bourbon group having possibly 2 subgroups. The importance of the outlying points is that any unknown sample that falls into these regions must be classified with care.

Next, it was decided that only the fusel oil fraction of the data be examined. This data set (with methanol values eliminated) was subjected to the same analysis (Figures 9 and 10). Note that the grouping of the data is essentially the same; however, this time 100% of scotch (s, Figure 9; c, Figure 10) and blended whiskeys (x, Figure 9; d, Figure 10) are classified correctly and more of the sour mash–bourbon group are identified as outliers (b and e, Figure 10), possibly belonging to 2 additional subgroups. Elimination of the methanol from the data set improved classification of the samples, because no samples were classified incorrectly. Apparently, methanol contributed to the initial misclassification of the 2 scotches. To test this classification scheme, the remaining 22 samples were added to the data set and pattern recognition analyses repeated (using ver. 2.5 of EINSIGHT, which can analyze data from more than 100 samples). Essentially the same results were obtained as with 100 samples.

Based on these results, we can conclude that analysis of the fusel oil fraction is sufficient to classify whiskeys as scotch, blended, or sour mash/bourbon. It is also evident that, to distinguish between sour mash and bourbon whiskeys, 1 or more other variables must be included in the analysis. With a data set of only 4 variables, one could plot each variable against the other, yielding 6 plots. From these plots, one could determine which 2 variables would produce the best data clusters. This would be very laborious as the number of variables increases (5 variables yield 10 plots). Most importantly, this approach will not succeed if a combination of variables is necessary to exhibit clustering of the data. For example, in the whiskey data set, one can obtain adequate clustering of the data by plotting 1-propanol vs 2-methyl-1-butanol or 3-methyl-1-butanol (see Figure 6). This puts the groups closer together, making the correct classification of the outlying samples more difficult. The software program we selected uses a combination of variables to yield better separation of the clusters, making it possible to examine a large number of samples and variables to determine which variables contribute to the clustering, thus, perhaps, limiting future quantitation to only those variables. It also allows one to collect large quantities of data and find a pattern without having known it beforehand.

Acknowledgments

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REFERENCES

(1) Martin, G. E., Burggraff, J. M., Dyer, R. H., & Buscemi, P. C. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 186–190
(2) Kahn, J. H., Trent, F. M., Shipley, P. A., & Vordenbery, R. A. (1968) *J. Assoc. Off. Anal. Chem.* **51,** 1330–1333
(3) Burgett, C. A. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 1176–1179
(4) Di Corcia, A., Samperi, R., & Severini, C. (1979) *J. Chromatogr.* **170,** 245–248
(5) Di Corcia, A., Samperi, R., & Severini, C. (1980) *J. Chromatogr.* **198,** 347–353
(6) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **9.091–9.096**; 15th Ed. (1990), secs **968.09–972.10**
(7) Masuda, M., Yamamoto, M., & Asakura, Y. (1985) *J. Food Sci.* **50,** 264–265
(8) Takeuchi, T., Murase, K., & Ishii, D. (1988) *J. Chromatogr.* **445,** 139–144
(9) Neale, M. E. (1988) *J. Chromatogr.* **447,** 443–450
(10) Rapp, A., Hastrich, H., Engel, L., & Knipser, W. (1978) in *Flavor of Foods and Beverages: Chemistry and Technology*, G. Charalambous & G. E. Inglett (Eds), Academic Press, New York, pp. 391–417
(11) Kwan, W., & Kowalski, B. (1980) *J. Agric. Food Chem.* **28,** 356–359
(12) Noble, A. C., Flath, R. A., & Forrey, R. R. (1980) *J. Agric. Food Chem.* **28,** 346–353
(13) Schreier, P., & Reiner, L. (1979) *J. Sci. Food Agric.* **30,** 319–327
(14) Saxberg, B. E. H., Duewer, D. L., Booker, J. L., & Kowalski, B. R. (1978) *Anal. Chim. Acta* **103,** 201–212
(15) Simpkins, W. A. (1985) *J. Sci. Food Agric.* **36,** 367–376
(16) Scarponi, G., Moret, I., Capodaglio, G., & Cescon, P. (1982) *J. Agric. Food Chem.* **30,** 1135–1140
(17) Moret, I., Di Leo, F., Giromini, V., & Scarponi, G. (1984) *J. Agric. Food Chem.* **32,** 329–333
(18) Moret, I., Scarponi, G., & Cescon, P. (1984) *J. Sci. Food Agric.* **35,** 1004–1011
(19) Reazin, G., Scales, H., & Andreason, A. (1973) *J. Agric. Food Chem.* **21,** 50–54

CEREAL PRODUCTS

Enzyme Immunoassay for Determination of Gluten in Foods: Collaborative Study

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A collaborative study was performed in 15 laboratories to validate a monoclonal antibody-based enzyme immunoassay (EIA) for determination of gluten in foods. The study included 13 samples: maize starch, "gluten-free" baking mixes, wheat flours, cookies, cooked meats, and a soup. Gluten was present in these samples at either zero or 0.02 to 10% by weight, i.e., over almost 3 orders of magnitude. The mean assay values for the foods varied from 88 to 105% of the actual amounts. The assay was quantitative for cereal products and the soup with repeatability (RSD_r, relative standard deviation) and reproducibility (RSD_R) of 16–22% and 24–33%, respectively. The assay was semiquantitative for the processed meat products (RSD_r 14 and 26% and RSD_R 46 and 56%), probably because gluten was unevenly distributed in the small (1 g) samples that were analyzed. The ELISA method produced no false positive results, and false negatives obtained with tannin-containing foods could be avoided by use of a modified sample extractant. None of the collaborators reported problems in following the protocol. The method has been adopted official first action by AOAC for determination of wheat gluten in foods.

Most developed countries have legally defined maximum levels of cereal proteins and other nonmeat proteins allowed to be incorporated into processed meats. Cereal proteins function in processed meat products as binders to improve texture and aid water and fat retention, and as extenders to lower the cost of the finished product. Their levels are difficult to monitor because foreign cereal proteins must be distinguished from meat protein, a problem that becomes more complicated when the products have been heated during processing, which changes protein solubilities and other properties (1).

Furthermore, a significant number of individuals cannot tolerate certain cereals in their diets. The most common cereal intolerance is celiac disease, in which certain cereals damage the absorptive villi of the lining of the small intestine. The toxic components from wheat, rye, triticale, and barley grain have been shown to reside in the aqueous ethanol-soluble proteins (prolamins) of the water- and salt-insoluble protein moiety, often termed gluten. Susceptible persons must eliminate these cereal proteins from their diets (2, 3); however, these proteins are often found in unexpected sources (4, 5). Wheat flour and starch of poor quality (with appreciable gluten content) are frequently used as thickening agents in soups and desserts. Residual barley proteins from malting may be found in beer and in milk drinks and as a flavoring in breakfast cereals. Gluten is often present in confectionery and as a tablet binder in pharmaceuticals.

These 2 concerns, enforcement of food-composition requirements and strict control of the diet of gluten-intolerant individuals, have led to a need to identify or quantitate gluten protein in foods. Amino acid analysis and liquid chromatography are subject to interferences from other food components and are often slow and yield rather equivocal results, especially with cooked foods (6). Protein electrophoresis on polyacrylamide gels has only limited usefulness after cereal products are baked or processed, and extensive sample preparation may be required to detect trace quantities of gluten proteins. Differentiation of protein patterns may require use of gel densitometers and prior knowledge of the electrophoretic patterns of likely contaminants. Only rough quantitation of gluten is possible and electrophoresis is less suited for analyzing large numbers of samples (7).

Immunodiffusion methods have been applied to identification of gluten in uncooked foods and some bakery products, but the low aqueous solubilities of gluten proteins, slowness of the methods, and consumption of antisera have limited acceptance of these methods (8–12). Several radioimmunoassays and enzyme immunoassays using rabbit or sheep (polyclonal) antisera to gliadin have been developed, and these have yielded accurate results with (uncooked) flour blends or starches, but not with cooked or processed foods (13–19). Furthermore, most of these antisera do not detect gluten-like proteins in rye or barley, cereals known to be toxic to celiac and other gluten-intolerant individuals. Recently, some monoclonal antibodies with appropriate cross-reactivity have been described, but the specificities of these antibodies are such that results would not be quantitative after cooking or processing (18).

After several hundred monoclonal antibodies were screened, five were identified with appropriate specificity, affinity, and stability characteristics. The advantage of the antibodies selected for this assay is that they bind to proteins that are not denatured by heat when foods are processed or cooked (20–24). This enables accurate quantitation of gluten in all types of foods. These antibodies form the basis of a proposed test method as an immunoassay kit (20, 21). The present report describes a collaborative study of the proposed method.

Received for publication July 11, 1990.
This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Foods II. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9–13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal Chem.* 74, January/February issue.

Collaborative Study

Each of the 15 collaborators were supplied with 5 prestudy samples, 13 collaborative study samples, and test reagents (a)-(j) in the form of a test kit. None of the laboratories had prior exposure to the method. The prestudy samples were 3 wheat starches containing known amounts of gluten: A, <0.016% (not detectable); B, 0.041% gluten; C, 0.10% gluten; and 2 other samples, a meat-gluten blend (1.7% gluten) and a bread wheat flour (11% gluten). In general, analyses for the prestudy samples were required to be within 25% of the specified amount before laboratories were allowed to proceed with the collaborative study.

Each laboratory received 4 g of each prestudy and collaborative study sample. All samples, except for the meats and soup (stored at −20°C), were stored at ambient temperature. Test kits were stored at 4°C. Collaborators also received specific instructions and a detailed copy of the method, and were required to submit all raw data and results to the Associate Referee.

Collaborators were required to perform 2 separate, complete duplicate analyses on different days, including separate extractions and analyses of 1 g subsamples. The aqueous alcohol-soluble fraction of gluten, gliadin, was used as the reference standard. Separate gliadin standard curves were also required, using newly reconstituted lyophilized antigen (gliadin) standard.

Preparation of Collaborative Study Samples

Meat-gluten blends.—Various amounts of commercial vital wheat gluten (previously analyzed for protein) were blended with pure beef mince (500 g) for 3 min at 20°C, using a Morton (Morton Machinery Co., Wishaw, Scotland) mixer. Samples (100 g) were then cooked 5 min in a domestic microwave (750 W). Samples were cooled and reblended 2 min in domestic food blender. Gluten contents were calculated, accounting for water loss and the protein content of the gluten used. Four-gram subsamples were provided to collaborators.

Wheat flours.—Flours (four 10-g subsamples) were machine-washed in water, using a Glutomatic (Falling Number AB, Stockholm, Sweden) and freeze-dried. Gluten content was determined by Kjeldahl analysis (25).

Cookies.—Cookies were prepared using wheat flour, water, yeast, shortening, salt, sugar, and sodium biocarbonate. The gluten content was calculated, accounting for the proportion of flour present and water and fat losses during baking. Baked cookies were ground 2 min in a domestic coffee grinder.

Baking mixes labeled "gluten free."—Two commercial baking mixes, intended for use by gluten-intolerant (celiac) individuals, were obtained. These mixes contained wheat starch, milk powder, gluconadeltalactone, carboxymethyl cellulose, sodium bicarbonate, ammonium chloride, salt, and sucrose. Thus, differences in their gluten contents were due to differences in the wheat starch used.

Starch.—Sample 8 was a maize starch and thus absolutely gluten-free.

Soup.—Sample 11 was a cooked pumpkin soup thickened with wheat flour.

The 13 collaborative study samples included 3 blind duplicates: baking mixes 4 and 5, cookies 8 and 9, and flours 11 and 13.

991.19 Gluten in Foods

Colorimetric Monoclonal Antibody Enzyme Immunoassay Method

First Action 1991

Method Performance:

Cereal products, soup product

Precision RSD_r 16.4–25.5%; RSD_R 23.3–34.7%

Accuracy determined values 88–102% of actual (where known)

Cooked meat products

Precision RSD_r 13.6–25.5%; RSD_R 46.4–55.9%

Accuracy determined values 105% of actual (where known)

A. Principle

Monoclonal antibodies used in this enzyme-linked immunosorbent assay (ELISA) bind to proteins from celiac-toxic cereals (wheat, rye, triticale, barley) but not nontoxic cereals (rice, maize) and show little difference in binding to different varieties of wheats, barleys, triticales, and ryes. Advantage of particular antibodies used in test is that they bind to proteins that are not denatured by heat during processing or cooking of foods. In method, sample is extracted with aqueous ethanol and centrifuged. Gluten is quantified in supernate by 2-step sandwich method of ELISA. First, gluten analyte (antigen) is incubated with monoclonal antibody immobilized onto the microwell strip to form gluten antigen-antibody complex, which is then incubated with enzyme-labeled antibody. Gluten in sample forms a complex sandwiched between antibody attached to well and antibody labeled with enzyme. Amount of analyte is determined by adding chromogenic substrate. Washing steps incorporated after each interaction stage remove any nonimmobilized species. Response is compared with that observed with gliadin standard, starch samples, and suitable blanks.

B. Apparatus

Apparatus specified here (as guide) has been tested and used for collaborative studies; equivalent apparatus may be used.

(a) *Blender or homogenizer.*—For sample extraction [e.g., IKA Ultra-Turrax Disperser T18/10 with 18N shaft (Janke and Kunkel, D-7813 Staufen, FRG), Ystral Type X1020 (Ystral, D-7801 Dottingen, FRG), Omnimixer (Sorvall, E.I. du Pont de Nemours & Co.), blender (Waring Products, New Hartford, CT 06057)]. With sample containers (e.g., 45 mL type UC, Mallinkrodt).

(b) *Bench-top centrifuge.*—Capable of 2500 rpm (e.g., Hermle Z320, Hermle GmbH, D-7209 Gosheim, FRG).

(c) *Tubes.*—For dilution of sample extracts. Polypropylene, 15 mL (e.g., Type 25319, Corning Glass Works).

(d) *Enzyme-immunoassay reader or visible-light photometer.*—Preferably, use photometer with 405–420 nm screening filter that reads through microtiter plates. Alternatively, use photometer with 405–420 nm filter, and adaptable for 1.5 mL cuvets (Bio-Rad is suitable).

(e) *Micropipet.*—Capable of accurately delivering 50 and 100 μL.

(f) *Glassware.*—Wash bottle, 500 mL; 3 graduated pipets, 10 mL; 3 graduated cylinders, 500 mL.

C. Criteria for Antibodies

Antibodies must satisfy following criteria: (*1*) Bind to proteins that are stable to heating, cooking, baking, or processing. In wheat, such proteins include the aqueous alcohol-soluble omega-gliadin fraction of gluten.

(*2*) Bind to proteins from celiac-toxic cereals (wheat, rye, triticale, barley) but not nontoxic cereals (rice and maize).

(*3*) Show little or no inherent differences in binding to different varieties of wheats, rye, triticales, and barleys, providing they contain similar gluten contents.

(*4*) Bind with high affinity to gluten proteins, such that <0.02% gluten by weight is detectable in food under analysis.

(*5*) Show negligible loss of activity after storage for 12 months at 4°, either after adsorption to polystyrene solid phases or after conjugation with marker enzymes such as horseradish peroxidase.

(*6*) Show reproducible affinity, specificity, and stability among batches.

Most suitable are high-affinity IgG antibodies produced following immunization of experimental animals with purified omega-gliadins. Although polyclonal antisera may be used, obtaining suitable specificity properties and batch-to-batch reproducibility has proven difficult. Thus, monoclonal antibodies are preferred.

D. Reagents

Items (**a**)-(**j**) are available as a test kit (Medical Innovations Limited, 11 Technology Dr, Labrador, 4215 Qld, Australia; Cortecs Diagnostics, Newtech Square, Deeside, Clwyd, CH5 2NT, UK; Transia SA, 8 rue Saint Jean de Dieu, F-69007 Lyon, France). All kit components are stable at least 12 months at 4°. Alternatively, equivalent antibodies may be used for (**a**) and (**e**) providing they satisfy criteria in C. Prepare other components as described.

(**a**) *Antibody-coated microwell strips.*—Monoclonal antibodies to heat-stable gluten components are coated in 50mM sodium carbonate buffer, pH 9.6, onto set of six 16-microwell strips (Nunc, Roskilde, Denmark). Contains 0.01% sodium azide as preservative. (*Caution:* Flush microwell contents thoroughly down a sink with water immediately before use.)

(**b**) *Wash buffer concentrate.*—(100 mL/bottle, 5× concentration). Contains 27.3 g Na_2HPO_4, 9.0 g $NaH_2PO_4 \cdot 2H_2O$, 45 g NaCl, and 0.5 g thimerosal as preservative per liter.

(**c**) *Antibody and sample diluent buffer* concentrate.—(100 mL/bottle, 5× concentration). Contains wash buffer concentrate plus 5 g/L of gelatin from *Teleostean* fish skin (Sigma, St Louis, MO).

(**d**) *Gliadin antigen standard.*—(1 vial). Prepared by extracting bread wheat flour with 10 mL/g of 40% (v/v) ethanol. Then extract is dialyzed against 10mM acetic acid, and lyophilized.

(**e**) *Peroxidase-conjugated monoclonal antibody to gliadin.*—(1 vial, 0.75 mL, 20× concentration).

(**f**) *Peroxidase substrate.*—(1 vial). Buffered 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) 1.1 g/L in 100mM sodium citrate, pH 4.5, containing 0.003% (v/v) H_2O_2 (24 mL).

(**g**) *Stopping solution.*—(1 vial, 11 mL/vial). Contains 3% oxalic acid. (*Caution:* Avoid contact with skin. If contact occurs, wash area with water.)

(**h**) *Reference wheat starches.*—(3 vials, ca 6 g/vial). Vial A, acceptably low gluten content, 0.29% total protein content; Vial B, mid-range gluten protein content, 0.33% total protein; Vial C, high gluten protein content, 0.46% total protein.

(**i**) *Package insert.*

(**j**) *Data record sheets.*

(**k**) *Sample extractant.*—Ethanol–water, 40% v/v.

E. General Instructions

Include gliadin standards A–F, **F(a)**(*3*), and reference starch controls in duplicate with each group of test samples. Add diluted antibody (sample diluent buffer) to additional pair of wells per group of samples. Use these wells, filled with substrate, as blank assay for reader or photometer. Alternatively, blank for assay reader can be set vs air and mean absorbance value of blank assay wells can be subtracted from standard, reference starch, and sample absorbance values. *Do not reuse* wells of plate. Use separate pipet for each sample and kit reagent to avoid cross-contamination. Take special care not to contaminate conjugate or substrate. Components and procedures of this test kit have been standardized for use in the test procedure. Substitutions must be pretested for equivalence.

F. Preparation of Standards and Samples

Let all kit components come to 18–25° before use.

(**a**) *Standards.*—(*1*) Shake bottle well; then dilute wash buffer concentrate, **D(b)**, 1-in-5 with water (add complete contents of bottle to 400 mL H_2O).

(*2*) Dilute diluent buffer concentrate, **D(c)**, 1-in-5 with water (add contents of bottle to 400 mL H_2O, making 500 mL sample and antibody diluent).

(*3*) Add 2.80 mL 40% (v/v) ethanol to vial of gliadin antigen standard, **D(d)**, and gently mix until dissolved. This yields 500 μg/mL gliadin standard, which is further diluted for preparation of standard curve. To prepare calibration standards, dilute the 500 μg/mL gliadin solution 1-in-100 in diluent (100 μL plus 9.9 mL diluent) to give 5 μg gliadin/mL (standard A). By 2-fold serial dilution (1 mL plus 1 mL), prepare additional standards B–F containing 2.5, 1.25, 0.625, 0.313, and 0.156 μg/mL of gliadin, respectively. Diluted gliadin standards A–F must be freshly prepared each day from the reconstituted gliadin standard.

(**b**) *Samples.*—Carry out extractions at 18–25°. Weigh 1 g portions of samples and, if appropriate, starch samples, into labeled sample containers and add 10 mL extractant. Homogenize 30 s. (*Note:* To avoid cross-contamination, apparatus must be thoroughly rinsed with 40% (v/v) ethanol between each extraction.) Centrifuge sample mixture at 2500 rpm for 10 min at 18–25°. Remove supernate (extract) and keep for testing. Before addition to antibody-coated plate, dilute extracts 50-fold, 500-fold, 2500-fold, or 5000-fold depending on expected gluten content:

1/50 dilution. Starch quality control or foods labeled "gluten free," e.g., special dietary breads, baking mixes, baby foods

1/500 dilution. Foods, 0.25–2.5% (w/w) gluten, e.g., soups, some processed meats

1/2500 dilution. Foods, 2–10% (w/w) gluten, e.g., some flours and baked goods, cookies, crackers

1/5000 dilution. Foods, 5–50% (w/w) gluten, e.g., most flours and baked goods

To prepare a 1/50 dilution, add 100 μL extract to 4.9 mL diluent. To prepare 1/500, 1/2500, and 1/5000 dilutions,

first prepare a 1/100 dilution, and then dilute serially. It is important to mix the 1/100 dilution *immediately* after the sample extract is added to the diluent; then perform the second dilution.

G. Determination

Let all reagents come to 18–25° before use. Perform analyses in duplicate.

(*1*) Select number of test strips required. Cover and store remainder at 2–8°. Just before use, remove tape from antibody-coated plate. Discard preservative solution by inverting plate and shaking out contents. (*Caution:* See **D(a)**.) Blot plate dry on absorbent paper or tissue.

(*2*) For blank pair of wells, add diluent alone (100 μL). Add 100 μL of each standard concentration (in duplicate) to wells of microwell plate. Add 100 μL (in duplicate) of diluted sample extracts and reference starches to wells. Cover plate and incubate 30 min at 18–25°.

(*3*) Invert plate and shake out contents. Wash plate 3 times with wash buffer and blot dry as in step *1*.

(*4*) Prepare enzyme-labeled antibody solution. For each strip of 16 wells, add 100 μL enzyme-labeled antibody stock, **D(e)**, to 1.9 mL diluent, **F(a)**(*2*). Swirl gently but mix well (avoid frothing). Add 100 μL diluted enzyme-labeled antibody to wells of plate, including blank pair. Cover and incubate 30 min at 18–25°.

(*5*) Invert plate and shake to remove contents. Wash plates 4 times with wash buffer as in step *3*. Blot plate on absorbent paper before addition of substrate solution.

H. Reading

Results may be read with microplate reader or spectrophotometer.

To use microplate reader, add 100 μL substrate solution, **D(f)**, to each well. Incubate 10 min at 18–25°. Positive wells should develop green color, which indicates presence of gluten. Stop reaction by adding 50 μL stopping solution, **D(g)**, to each well. Color development times for all microwells should be equal (within 15 s). Read optical denisty of each well at 405–420 nm (414 nm is optimum).

For alternative use of spectrophotometer, add 200 μL substrate solution to each well. Incubate 30 min at 18–25°. Stop reaction by adding 50 μL stopping solution to each well. Mix plate contents gently. Immediately transfer 200 μL of this solution to cuvet; then add 800 μL H_2O. Read optical density at 414 nm.

I. Calculation

Determine gluten content for each set of duplicate sample wells by reference to standard curve prepared for each assay using gliadin antigen. On semilogarithmic graph paper, plot optical density of standards (linear scale) vs gliadin content of standards (logarithmic scale). Plot should be virtually linear over middle 4 dilutions (Fig. **991.19**). It is assumed that 50% of protein in gluten is gliadin.

Calculate gluten content as follows:

$$\text{Gluten, \%} = \text{gliadin, } \mu\text{g/mL} \times (D/500)$$

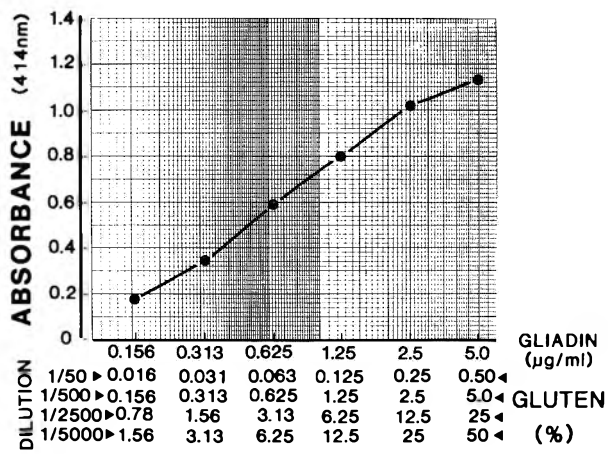
where gluten, % = % by weight of original sample; gliadin, μg/mL = concentration read from standard curve; and D = dilution factor.

Using sample extraction procedure described, gluten content can be read from curve directly. For example, if a 1/50 sample dilution was used, samples that yielded an optical density of 0.74 would contain 0.1% gluten. Optical density readings of 0.74 for sample dilutions of 1/500, 1/2500, and 1/5000 would indicate gluten contents of 1.0, 5, and 10%, respectively.

Optical densities below 0.1 imply that the solution assayed was too dilute or that no gluten was present in the sample; lower dilutions should be chosen for a second ELISA. Optical densities within 0.1 of that of the 5 μg/mL gliadin antigen standard value imply that the solution assayed was too concentrated; higher dilutions should be chosen for a second ELISA.

Ref.: JAOAC **74,** March/April issue (1991).

FIG. 991.19—Typical absorbance curve for gliadin standard



Results

Data from the 15 collaborators are presented in Tables 1–4. The collaborators used either high-speed, shaft-type homogenizers with an internal rotating blade (e.g., Ultraturrax, Polytron, Ystral) (11 of 15) or a homogenizer/blender with a free rotating blade (e.g., Omnimixer, Silverson, or Waring-type) (4 of 15). Although the latter treatment involves lower frequency homogenization, none of the 4 laboratories obtained systematically different results. Fourteen of the 15 collaborators used a microwell reader for absorbance determinations, four with a 405 nm filter, seven with a 410 nm filter, and three with a 414 nm filter. The other collaborator used a conventional spectrophotometer at 414 nm.

Precision

Analysis of 3 supposedly "gluten-free" baking mixes (Table 1) revealed that each laboratory found detectable gluten in each analysis of 2 baking mixes (samples 4 and 5), which were independently found to cause adverse reactions in certain gluten-intolerant individuals (Skerritt, unpublished). The gluten contents determined (means 0.070 and 0.074%) were between those for a 0.32% protein starch (0.041%) and a 0.46% protein starch (0.10%). One other mix (sample 6), acceptable for celiacs, contained just detectable amounts of gluten on 23 of 26 assays (range 0.016–0.033% gluten). In the 3 other analyses, gluten was not detectable (<0.016%), an amount less than that produced by the lowest concentration gliadin standard. Each laboratory obtained mean values below those obtained for starch B (0.32% protein, 0.041% gluten), which is just unacceptable for the celiac diet. No gluten was detectable in the maize starch on 29 of 30 determinations. The precision data for gluten detection in 3 flours and 2 cookies (mean range 3.4–10% gluten) were similar,

Table 1. Collaborative results of ELISA determinations of gluten in gluten-free baking mixes and maize starch[a]

	"Gluten-free" baking mix						Maize starch	
	No. 4		No. 5		No. 6		No.7	
Coll.	1	2	1	2	1	2	1	2
1	0.052	0.068	0.054	0.072	0.019	0.016	<0.016	<0.016
2	0.063	0.046	0.059	0.048	0.016	0.016	<0.016	<0.016
3	0.066	0.081	0.074	0.078	0.024	0.027	<0.016	<0.016
4	0.070	0.073	0.054	0.068	<0.016	0.017	<0.016	<0.016
5	0.090	0.090	0.100	0.100	0.040[b]	0.050[b]	<0.016	<0.016
6	0.069	0.107	0.072	0.101	<0.016	0.021	<0.016	<0.016
7	0.055	0.060	0.076	0.063	<0.016	0.018	<0.016	<0.016
8	0.100	0.11	0.076	0.100	0.021	0.033	<0.016	0.020
9	0.080	0.069	0.120	0.069	0.028	0.022	<0.016	<0.016
10	0.062	0.054	0.070	0.063	<0.016	0.016	<0.016	<0.016
11	0.080	0.060	0.080	0.070	0.020	0.020	<0.016	<0.016
12	0.040	—	0.030	0.080	<0.016[c]	0.170[c]	<0.016	<0.016
13	0.078	0.070	0.080	0.070	0.028	0.020	<0.016	<0.016
14	0.070	0.063	0.079	0.066	0.028	0.018	<0.016	<0.016
15	0.087	0.061	0.072	0.060	0.028	0.021	<0.016	<0.016
Mean	0.0705		0.0735					
s_r	0.0116		0.0162					
s_L	0.0126		0.0075					
s_R	0.0171		0.0179					
RSD_r, %	16.4		22.1					
RSD_R, %	24.3		24.4					
N	29		30		26		30	

[a] Data are % gluten by weight, on an as-is basis.
[b] Mean rejected by Grubbs outlier test; maximum normed residual. $P < 0.01$, substituting x = 0.016 for values <0.016.
[c] Rejected on the basis of inhomogeneity of range.

with repeatabilities (RSD_r) of 19–22% and reproducibilities (RSD_R) of 24–33% (Table 2).

Gluten was not detectable in cooked pure ground beef in 26 of 30 analyses. One laboratory reported 0.25% gluten for one determination and <0.016% for the other; the former result may be due to contamination of the extraction device or a pipeting error. Three other laboratories reported trace levels (0.018, 0.020, 0.028%) for one of 2 determinations (Table 3). For the other samples (2 and 3), which were cooked gluten-meat blends (sample 3 was prepared using 10 times the addition of gluten as used for sample 2), assay repeatability (RSD_r) was 14 and 26% and reproducibility (RSD_R) was 46 and 56%. The considerably poorer reproducibility in the presence of reasonable repeatability and good accuracy (see below) suggests that the lower precision may result from uneven distribution of gluten in the different 4 g subsamples that were provided to the collaborators for duplicate 1 g analyses. Subsequent work in our laboratory has shown that precision is greater with 10–20 g samples. However, customs and quarantine limitations did not allow use of meat samples larger than 1 g in this international collaborative study.

A soup (sample 10, Table 3), which contained a similar level of gluten to that in meat sample 3, was analyzed with superior precision. The liquid form of this matrix, together with use of "prediluted" gluten in the form of wheat flour (rather than gluten) as the additive in this food would contribute to little sampling variation. Although they are not blind samples, data for the wheat reference starches (reanalyzed in the collaborative study) are presented in Table 4. Starch A, which contains approximately 0.01% gluten was detected (<0.016% gluten) to contain gluten in only 3 determinations (0.019%, 0.020%, 0.028%). Starch B had 0.041% gluten, about 2.5 times the limit of detection of the assay. The precision estimates were lower than for starch C, which had 2.5 times more gluten.

Six samples were paired by means of Youden matched pairs, representing different products and levels of gluten. They were samples 4 and 5 (gluten-free baking mixes), samples 8 and 9 (cookies), and samples 11 and 13 (flours). The analyses of these matched pairs generated parameters that agreed well with the parameters of the analysis of the known duplicates (*see* Tables 1 and 2). Precision analysis of Youden matched pairs from each collaborator yielded repeatability (RSD_r) values of 9.8, 8.6, and 12%, respectively, and reproducibility (RSD_R) values of 21, 20, and 27%, respectively.

Accuracy of Gluten Determination

Actual gluten contents were known for most samples, except for the 3 "gluten-free" baking mixes and the soup, because these were obtained commercially. In addition, "standard values" for each sample were obtained by analyzing the set of samples in our laboratory, 8 separate times over 4 months. Means from collaborators' data were compared with the actual data values by the significance test, t-test = $[(\bar{x} - \mu)\sqrt{n}]/s$ (Table 5). In one case (flour sample 11), the collaborators' mean value was lower by 12%; this was not significantly different from the actual value at the 1% level. Sample 13 was much closer to the actual value. In no other case was systematic error noted; indeed accuracy was excellent with each mean determined by the collaborators within 12% of the actual value (where known). Furthermore, with the exception of a gluten-meat blend (sample 2) where the

Table 2. Collaborative results of ELISA determinations of gluten in flours and cookies[a]

	Flour						Cookies			
	No. 11		No. 12		No. 13		No.8		No. 9	
Coll.	1	2	1	2	1	2	1	2	1	2
1	9.0	9.9	5.7	6.0	11.0	13.0	3.2	3.5	2.6	3.4
2	6.3	11.0	3.7	6.6	—	—	2.4	4.6	2.3	4.4
3	6.0	7.0	4.2	3.6	7.4	8.0	1.9	2.8	2.3	2.8
4	8.2	7.3	6.2	4.6	8.3	7.8	3.1	3.1	3.4	2.9
5	16.0[b]	13.6[b]	8.6	7.1	15.6	15.0	4.3	4.0	4.2	3.4
6	7.6	10.3	5.2	7.0	6.9	11.5	4.4	4.6	3.7	4.2
7	10.0	10.5	4.8	5.5	9.1	10.2	3.2	4.1	3.9	2.7
8	11.0	10.0	6.7	7.6	13.0	12.0	3.6	5.0	3.7	6.0
9	11.0	9.7	5.8	5.6	10.0	4.6	3.7	3.3	3.4	3.4
10	5.9	5.5	5.5	4.9	6.2	7.0	3.5	3.6	3.9	4.3
11	15.0	8.8	5.9	6.3	16.0	15.0	3.5	3.5	3.4	3.0
12	6.5	8.1	3.7	5.3	5.1	9.6	1.65	2.65	1.4	2.4
13	9.6	9.8	7.8	8.4	9.2	12.4	5.65	3.8	4.05	3.2
14	10.5	6.8	7.0	5.2	11.5	7.5	3.5	3.4	3.8	3.4
15	10.5	5.3	7.6	3.9	10.5	5.8	3.7	2.5	3.7	2.3
Mean	9.22		5.87		9.97		3.53		3.39	
s_r	2.02		1.13		2.14		0.699		0.759	
s_L	1.74		0.811		2.46		0.512		0.425	
s_R	2.66		1.39		3.26		0.866		0.870	
RSD_r, %	21.9		19.3		21.4		19.9		22.45	
RSD_R, %	28.9		23.8		32.7		24.6		25.7	
N	30		30		28		30		30	

[a] Data are % gluten by weight, on an as-is basis.
[b] Mean rejected by Grubbs outlier test.

Table 3. Collaborative results of ELISA determinations of gluten in cooked ground beef and a vegetable soup[a]

	Cooked beef						Soup	
	No. 1		No. 2		No.3		No. 10	
Coll.	1	2	1	2	1	2	1	2
1	<0.016	<0.016	0.28	0.23	2.4	2.9	2.0	1.6
2	<0.016[b]	0.25[b]	0.19	0.18	1.9	2.1	1.3	2.3
3	<0.016	<0.016	0.063	0.066	0.80	0.94	1.4	1.3
4	<0.016	<0.016	0.042	0.024	0.51	0.25	2.1	1.6
5	<0.016	<0.016	0.20	0.20	1.4	0.86	2.0	2.1
6	<0.016	<0.016	0.098	0.13	1.1	1.3	1.9	1.6
7	<0.016	<0.016	0.21	0.20	3.5	2.5	2.2	1.9
8	<0.016	<0.016	0.26[b]	0.11[b]	2.7	1.5	1.8	2.8
9	0.020	<0.016	0.15	0.13	1.4	1.4	1.8	1.4
10	<0.016	<0.016	0.12	0.14	0.54	0.77	2.8	3.1
11	<0.016	<0.016	0.20	0.17	1.4	0.85	1.8	1.8
12	<0.016	<0.016	0.17	—	1.9	1.9	1.7	1.8
13	<0.016	<0.016	0.086	0.04	1.6	1.0	1.7	1.1
14	<0.016	0.018	0.16	0.14	1.1	0.80	2.3	2.4
15	0.028	<0.016	0.072	0.11	0.74	0.68	1.7	1.9
Mean	<0.016		0.142		1.42		1.91	
s_r			0.0193		0.363		0.331	
s_L			0.0629		0.703		0.325	
s_R			0.0658		0.791		0.464	
RSD_r, %			13.6		25.5		17.3	
RSD_R, %			46.4		55.9		24.3	
N	28		27		30		30	

[a] Data are % gluten by weight, on an as-is basis.
[b] Rejected on the basis of inhomogeneity of range.

Table 4. Collaborative results of ELISA determinations of gluten in wheat starch standards[a]

	A		B		C	
Coll.	1	2	1	2	1	2
1	<0.016	<0.016	0.030	0.023	0.11	0.006
2	<0.016	<0.016	0.040	0.038	0.094	0.092
3	<0.016	<0.016	0.031	0.038	0.090	0.12
4	<0.016	<0.016	0.040	0.039	0.12	0.11
5	<0.016	<0.016	0.050	0.050	0.11	0.13
6	<0.016	<0.016	0.037	0.066	0.098	0.13
7	<0.016	<0.016	0.028	0.033	0.083	0.10
8	<0.016	<0.016	0.031	0.051	0.092	0.11
9	0.020	<0.016	0.040	0.039	0.11	0.10
10	<0.016	<0.016	0.032	0.033	0.096	0.11
11	<0.016	<0.016	0.040	0.070	0.14	0.11
12	<0.016	<0.016	—	0.030	0.070	0.090
13	<0.016	<0.016	0.065	0.039	0.115	0.080
14	0.019	<0.016	0.069	0.039	0.13	0.096
15	0.028	<0.016	0.063	0.020	0.135	0.044
Mean	<0.016		0.0411		0.103	
s_r			0.0142		0.0240	
s_L			0		0	
s_R			0.0142		0.0240	
RSD_r, %			34.7		23.3	
RSD_R, %			34.7		23.3	
N	30		29		30	

[a] Data are % gluten by weight, on an as-is basis.

Table 5. Accuracy of gluten determination[a]

	Coll. data		Standard values (within-laboratory)		Actual values	
Sample	Mean	Sample SD (S)	Mean (N = 8)	SD	%	*t*
1	<0.016	—	<0.016	—	0	
2	0.14	0.061	0.22	0.074	0.135	0.429
3	1.42	0.73	1.6	0.43	1.35	0.371
4	0.071	0.015	0.074	0.012	na[b]	—
5	0.074	0.013	0.074	0.012	na	—
6	0.021[c]	0.005	0.022[d]	0.003	na	—
7	<0.016	—	<0.016	—	—	—
8	3.5	0.69	3.5	0.44	3.5	0
9	3.4	0.66	3.5	0.44	3.5	−0.587
10	1.9	0.39	2.0	0.24	na	—
11	9.2	2.2	11.1	2.5	10.5	2.28[e]
12	5.9	1.1	6.1	1.3	5.8	0.352
13	10.0	2.8	11.1	2.5	10.5	0.692

[a] Data are % gluten by weight, on an as-is basis.
[b] na = not available.
[c] Mean SD of 23/30 determinations, 4/30 <0.016, 3 outliers.
[d] Mean SD of 7/8 determinations, 1/8 <0.016.
[e] $0.01 < P < 0.05$; Student's *t*-test.

sample analyzed in our laboratory appeared slightly high, no other significant differences were found by *t*-test analysis of differences between collaborators' means and standard values established by 8 separate determinations in our laboratory.

Discussion

In this collaborative study, we did not intend to establish the maximum possible performance of the procedure but rather to determine its likely performance in typical food analysis laboratories. Accordingly, experience in enzyme-

linked immunosorbent assay (ELISA) techniques could be rated as extensive by 4 of the 15 laboratories, moderate by 6, and little or none by 5 of the 15 laboratories. In addition, 11 of the 15 laboratories had little prior experience in handling the analyte, wheat gluten. No laboratory had experience in both ELISA and cereal technology.

The method was not developed to quantify gluten with extreme precision or accuracy over a small range of values, but rather to estimate gluten over several orders of magnitude, in this study, from 0.016 to 11%. For several reasons, estimates of gluten content to 1 (or at most, 2) significant figures are appropriate when foods are assayed for this component. First, the analytical method uses a gliadin standard prepared from flour of one particular genotype or variety. Gliadin, the aqueous alcohol-soluble fraction of gluten, is used as the standard in this [and other biochemical gluten testing methods (13–18)] because of the very low aqueous solubility of the other gluten fraction, glutenin (26). Nonetheless, estimation of "gluten" is possible because the proportion of gliadin in gluten from different wheat genotypes closely approximates 50% (27, 28). However, *slight* differences between wheat varieties in the proportion of gliadin to glutenin and the relative proportion of omega-gliadin (the heat-stable fraction) to other gliadins, probably of the order of 20% (20, 21, 27) would contribute to a systematic error of up to 20% for certain samples. Second, legislation relevant to the maximum amount of gluten in meat products that have specified labeling is drafted either in terms of total wheat protein (rather than gluten) or in terms of total cereal (whole meal or flour) content. Flour used as a meat extender typically contains 8–12% protein, of which 70–80% is gluten. Third, with respect to functional (baking) performance, differences of 10% or more are required before quality differences can be detected (29). Variation in methods for manufacturing wheat starch also results in wheat starches with 0.3% protein, the upper limit for use in foods that may be labeled as "gluten-free" (30, 31), which contain between about 0.016% and 0.022% gluten (20, 21, 31).

The precision parameters reported in this study (repeatability RSD_r 14 and 26%, reproducibility RSD_R and 56%) are poorer than would be expected for a chromatographic analysis of a small molecule, such as the active ingredient of a pesticide formulation at a particular concentration. However, the ELISA technique requires several pipeting steps using small (100 μL) volumes, and microwell readers typically produce results of relatively low precision compared with results for spectrophotometers. This is caused in part by microwell location-dependent biases with different types of readers (32). An imprecision of ± 0.05 AU could lead to imprecision of up to $\pm 20\%$ in gluten determined over much of the standard curve (Figure **999.19**). One other immunoassay method for "foreign" vegetable protein (soy) in foods has been collaboratively studied on 3 occasions. Although only meat products were tested, and the analyte was present over a 5-fold rather than a 500-fold concentration range, the 3 studies reported precision parameters of repeatability (RSD_r) 25–68%, 27–60%, and 25–90%, and reproducibility (RSD_R) 48–80%, 46–109%, and 65–97% respectively (33–35). Elimination of outliers and reanalysis of data for a selection of samples for one trial improved repeatability and reproducibility to values similar to those reported in this study (36).

Some imprecision may result from use of only a single extraction step for gluten. The alternative of analyzing pooled extracts from 2 or 3 sequential extractions was not collaboratively studied, because a preliminary survey of analysis indicated that they did not want the method to become longer nor was additional precision required. Because a single extraction yielded a reasonably constant proportion of gluten from a wide range of food types, and because the gliadin standard was calibrated using a single gluten extraction (21), the accuracy of the method would not be affected by use of a single extraction.

No false positive values were seen in the study that were reproducibly obtained by the collaborators. Collaborator 12 obtained a high value for a gluten-free baking mix (sample 3) and Collaborator 2 for cooked beef (sample 1), on only one of the 2 determinations. Both collaborators attributed the high values to accidental cross-contamination of the particular samples. We have not observed false positives with any of several hundred food samples analyzed by ourselves or by other Australian and European laboratories using the method. The only false positives noted were for samples containing under 3% gluten, together with a polyphenol-containing food component, such as cocoa, coffee, or hops. The false negative values are due to binding of gliadin by the polyphenols which prevents its extraction from the food, and can be avoided by extraction of the appropriate foods using 40% (v/v) ethanol–5% (w/v) *Teleostean* fish skin gelatin–2% (w/v) polyvinylpyrrolidone in water. Gliadin standards used for analysis of these samples (only) should be prepared in the modified extractant (Skerritt and Hill, unpublished).

In conclusion, the collaborative study confirmed that the method provides useful estimates of wheat gluten in a variety of food types. Alternative technology to quantitate gluten exists only for wheat flours, in which gluten may be extracted by washing dough prepared from a known mass of flour, and the protein content ($N \times 5.7$) determined by Kjeldahl analysis of the dried gluten (37). The current method, in contrast, is simpler and faster for flours. It provided accurate results with heat-treated foods such as cooked meats and baked cookies. Finally, the method also detects gluten-like proteins (toxic to gluten-intolerant individuals) in rye, barley, and triticale (20, 21); these can be quantified in foods if the cereal source of the gluten is known and standards prepared from the corresponding cereal are used.

Recommendation

On the basis of this collaborative study, it is recommended that the enzyme immunoassay for gluten be adopted official first action.

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References

(1) Skerritt, J. H. (1990) in *Development and Application of Immunoassay for Food Analysis*, J. Rittenburg (Ed.), Elsevier, London, UK, pp. 81–125
(2) Cooke, W. T., & Holmes, G. K. T. (1984) *Coeliac Disease*, Churchill Livingstone, Edinburgh, Scotland
(3) Cole, S. G., & Kagnoff, M. F. (1985) *Ann Rev. Nutr.* **5,** 241–266
(4) Bell, L., Hoffer, M., & Hamilton, J. R. (1981) *J. Can. Diet. Assoc.* **42,** 143–158
(5) Campbell, J. A. (1987) *World Rev. Nutr. Diet.* 51, 189–233
(6) Windemann, H., Meier, P., & Baumgartner, E. (1986) *Z. Lebens. Unters. Forsch.* **186,** 26–32
(7) Wrigley, C. W. (1977) *Food Technol. Aust.* **29,** 17–20
(8) Zareba, J. (1968) *Pol. Tyg. Lek.* **23,** 1962–1963
(9) Lietze, A. (1969) *J. Assoc. Off. Anal. Chem.* **52,** 995–998
(10) Keyser, J. W., & Mahler, R. F. (1973) *Lancet*, i, 678
(11) Guenther, H. O. (1977) *Ann. Nutr. Aliment.* **31,** 179–181
(12) Kaczkowski, J., Kurowska, E., & Moskal, M. (1985) *Acta Aliment. Polon.* **11,** 177–183
(13) Meier, P., Windemann, H., & Baumgartner, E. (1984) *Z. Lebens. Unters. Forsch.* **178,** 361–365
(14) Ciclitira, P. J., Ellis, H. J., Evans, D. J., & Lennox, E. S. (1985) *Clin. Exp. Immunol.* **59,** 703–708
(15) Fritschy, F., Windemann, H., & Baumgartner, E. (1985) *Z. Lebens. Unters. Forsch.* **181,** 379–385
(16) McKillop, D. F., Gosling, J. P., Stevens, F. M., & Fottrell, P. F. (1985) *Biochem. Soc. Trans.* **13,** 486–487
(17) Troncone, R., Vitale, M., Donatiello, A., Farris, E., Rossi, G., & Auricchio, S. (1986) *J. Immunol. Methods* **92,** 21–23
(18) Freedman, A. R., Galfre, G., Gal, E., Ellis, H. J., & Ciclitira, P. J. (1987) *J. Immunol. Methods* **98,** 123–127
(19) Ayob, M. K., Rittenburg, J., Allen, J. C., & Smith, C. J. (1988) *Food Hydrocolloids* **2,** 39–49
(20) Hill, A. S., & Skerritt, J. H. (1990) *Food Agric. Immunol.* **2,** 21–35
(21) Skerritt, J. H., & Hill, A. S. (1990) *J. Agric. Food Chem.* **38,** 1771–1778
(22) Schofield, J. D., Bottomley, R. C., Timms, M. F., & Booth, M. R. (1983) *J. Cereal Sci.* **3,** 103–109
(23) Skerritt, J. H., & Smith, R. A. (1985) *J. Sci. Food Agric.* **36,** 980–986
(24) Menkovska, M., Pomeranz, Y., Lookhart, G. L., & Shogren M.D. (1988) *Cereal Chem.* **65,** 198–201
(25) American Association of Cereal Chemists (1985) Method 46-10 Crude Protein-Improved Kjeldahl Method, *Official Methods*, **8th Ed., American Association of Cereal Chemists, St.** Paul, MN
(26) Lee, J. W., & MacRitchie, F. (1971) *Cereal Chem.* **48,** 620–625
(27) Huebner, F. R. (1970) *J. Agric. Food Chem.* **18,** 256–259
(28) Kent, N. L. (1975) *Technology of Cereals*, 3rd Ed., Pergamon Press, Oxford, UK, p. 39
(29) Finney, K. F. (1943) *Cereal Chem.* **20,** 281–284
(30) Codex Alimentarius Commission (1981) Codex Standard for "Gluten-Free" Foods, STAN 118–198, Food and Agriculture Organization/World Health Organization, Rome, Italy
(31) Ciclitira, P. J., Ellis, H. J., Evans, D. J., Lennox, E. S., & Dowling, R. H. (1983) *Gut* **24,** A487
(32) Harrison, R. O., & Hammock, B. D. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 981–987
(33) Crimes, A. A., Hitchcock, C. H. S., & Wood, R. (1984) *J. Assoc. Publ. Analysts* **22,** 59–78
(34) Olsman, W. J., Dobbelaere, S., & Hitchcock, C. H. S. (1985) *J. Sci. Food Agric.* **36,** 499–507
(35) Hall, C. C., Hitchcock, C. H. S., & Wood, R. (1987) *J. Assoc. Publ. Analysts* **25,** 1–27
(36) McNeal, J. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 443
(37) American Association of Cereal Chemists (1985) Methods 38-10 Gluten-Handwashing, *Official Methods*, 8th Ed., American Association of Cereal Chemists, St. Paul, MN

CHEMICAL CONTAMINANTS MONITORING

Food and Drug Administration Pesticide Residue Monitoring of Foods: 1978–1982

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Pesticide residues in foods are reported for the 5-year period 1978–1982 [fiscal years (FY) 78–82]. Results were compiled from the 2 complementary elements that comprise the Food and Drug Administration's (FDA) program for monitoring pesticide residues in foods. Under regulatory monitoring, which focuses on residues in raw agricultural commodities, a total of 49 877 samples (30 361 domestic and 19 516 import) that included fresh fruits and vegetables, grains, milk and dairy products, seafoods, and a variety of processed foods were analyzed. No residues were found in about 55 and 44% of the domestic and import samples, respectively. About 3% of the domestic and 7% of the import samples were classed as violative. Data from the Total Diet Study, which is conducted to determine dietary intakes of a variety of chemicals, showed that residues of 42 pesticides were found in 1044 composites of table-ready foods. Results of FDA's monitoring for FY78–82 demonstrate that pesticide residue levels in the U.S. food supply were generally well below regulatory limits, and dietary intakes were manyfold lower than the Acceptable Daily Intakes established by international agencies.

Three federal agencies share the responsibility for regulating pesticides. The Environmental Protection Agency (EPA) registers or approves the use of pesticides, and establishes tolerances if use of the pesticide may lead to residues in foods (1). With the exception of meat, poultry, and egg products, for which the U.S. Department of Agriculture is responsible, the U.S. Food and Drug Administration (FDA) is charged with enforcing tolerances for residues in foods shipped in interstate commerce under the Federal Food, Drug, and Cosmetic Act.

FDA monitors pesticide residues in the food supply through 2 different but complementary approaches: (*1*) regulatory or commodity monitoring, which focuses on raw agricultural commodities and measures levels in individual lots of domestically produced and imported foods for determining compliance with EPA tolerances and (*2*) the Total Diet Study, in which dietary intakes of pesticides are determined by analyzing foods as consumed.

The results of these monitoring efforts for earlier years have been published. Findings for 1963–1969 [fiscal years (FY) 64–69] and for 1969–1976 (FY70–76) were compiled by Duggan et al. (2, 3). Information on monitoring for FY77 has also been summarized (4), as has that for FY87 (5), FY88 (6), and FY89 (7). The present paper and a companion paper (8) fill in the intervening years by reporting the results of monitoring for 1978 through 1982 (FY78–82) and FY83–86, respectively. The information is presented as 2 separate articles because of the amount of data involved and because of the changes made in the Total Diet Study in mid-1982.

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Programs and Methods

Regulatory Monitoring

The chief objective of this phase of the program is to enforce EPA tolerances for pesticide residues in foods, and prevent foods that contain illegal residues from entering interstate commerce. Samples from individual lots of domestically grown and imported foods are generally collected as near as possible to the point of production or entry into the United States. FDA can initiate various sanctions such as seizure or injunction when illegal residues are found in domestic samples. When illegal residues are found in imports, shipments are detained at the point of entry. In either case, sampling at this early stage gives FDA the best opportunity to identify shipments that may contain illegal residues. As an important by-product of this monitoring activity, information is acquired on the incidence and levels of pesticide residues, and provides FDA with a national overview of their occurrence. The data obtained through this monitoring effort are made available to EPA for its continuing assessment of pesticide exposure, and are published in the scientific literature, usually in the *Journal of the Association of Official Analytical Chemists*.

In FY79, FDA initiated an evaluation system called the Surveillance Index (SI) to establish monitoring priorities for individual pesticides (9, 10). By systematically evaluating the potential health risk represented by each pesticide chemical if present as a residue in food (or feed), and by assigning it to 1 of 5 levels of potential risk, FDA established a rational basis for directing monitoring. Even before initiation of the SI, FDA monitoring was based on consideration of such factors as pesticide usage volume, toxicological risks, and potential dietary exposure; however, actual evaluations were less formal and detailed.

The SI evaluation is one of the tools used in setting priorities for directed assignments, including selective national or regional surveys of certain commodities or particular pesticides. These surveys are initiated in response to the factors listed above as well as incidents involving misuse or contamination; requests from EPA for data on a pesticide or change in usage that may indicate a greater potential risk; or a lack of information on particular pesticides, commodities, or geographic areas (domestic regions or other countries).

Domestic and import samples are classified as either surveillance or compliance. Surveillance samples are objective samples, collected from shipments for which there is no suspicion of illegal residues. Compliance samples are collected when inspection, previous sampling, or other evidence indicates that a residue problem may exist in a particular shipment. In this report, data from surveillance and compliance samples have been combined because recording of data during FY78–82 did not distinguish between the 2 types. Also, reporting of violative samples in this paper is based solely on preliminary classification of the sample assigned by the FDA

field laboratory that performed the analysis; that is, the sample contained a residue that exceeded the tolerance or a residue for which no tolerance had been established. In addition, with the data system in use at the time, some samples may have been classified as violative based on a nonpesticide-related finding (e.g., microbial contamination). FDA did not necessarily take regulatory action in all instances preliminarily classified as violative; further review by agency compliance officers may have indicated that the residues were not violative or the circumstances did not support regulatory action. Thus, retrievable data for the time covered by this report impose some limitations on specific tabulation of the violative findings.

In FY79, a separate import program on Mexican produce was initiated. Previous FDA sampling had shown a relatively high violation rate for pesticide residues in produce imported from Mexico. Most violations involved pesticide/crop combinations for which there were no EPA tolerances. These findings and the results of marketing forecasts and other reports indicated that a substantial and increasing supply of fresh produce was being imported into the United States from Mexico. FDA concluded that surveillance of imported Mexican produce should be expanded to reduce the amount of violative produce entering the United States. In this report, data on imported foods from Mexico are included with those of imports from other countries.

Total Diet Study

The second major element of the FDA pesticide program is the Total Diet Study, or Market Basket Study, which FDA initiated in May 1961 primarily in response to concern about levels of radionuclides in foods resulting from atmospheric nuclear testing (11). The first Total Diet Study estimated the dietary intakes for young males of strontium-90 and cesium-137 as well as organochlorine and organophosphorus pesticides and selected nutrients. The program has been subsequently expanded to include additional radionuclides, pesticide residues, essential minerals, toxic elements, and industrial chemicals and a greater number of age/sex groups to represent a broader segment of the population.

The Total Diet Study provides a direct measure of pesticide residue intake via the diet. Foods are purchased from supermarkets, prepared ready to eat, and analyzed. During FY78–82, food items were collected throughout the United States to ensure geographic representation; foods were chosen to represent the diets of 3 population groups: the 6-month-old infant, 2-year-old toddler, and 15–20-year-old male.

During the period covered by this report, the Total Diet Study used the "composite sample" approach. Foods representative of a particular diet were purchased, prepared as for consumption in the home, and divided into either 11 or 12 commodity groups (e.g., dairy products, grains and cereal products, etc.) depending on the population group; each commodity group was composited and analyzed. [Changes in the Total Diet Study program over the years have been reviewed by Pennington and Gunderson (11).]

Analytical Methods

Selective monitoring must be used because of the large number of possible pesticide residue/commodity combinations. Thus, although FDA does not analyze all foods for all pesticides, it does cover most chemicals of current concern.

The lower limit of residue measurement in FDA's analysis for a particular pesticide is well below the EPA tolerance. In general, residues present at 0.01 ppm (part per million) and above are measurable; however, for some individual pesticides, because of their analytical characteristics, this limit may vary from about 0.005 to 1 ppm.

FDA laboratories most commonly use multiresidue analytical methods to analyze foods for pesticide residues. Such methodology permits the separation and presumptive identification of a large number of pesticides. The use of multiresidue methods is necessitated by the fact that more than 300 pesticides have tolerances established for one or more food commodities. Single residue methods are used to determine residues that are not amenable to determination by multiresidue methods. The principal methods used are given in FDA's *Pesticide Analytical Manual* (12) or in the AOAC *Official Methods of Analysis* (13). Because the Total Diet Study is conducted to determine levels of chemicals in foods as normally eaten, and because levels of pesticide residues present are usually low, the analytical methods have been modified to achieve quantitation at levels 5–10 times lower than those used for regulatory monitoring (11). Also, the identity of each pesticide residue found is confirmed by an alternative analytical technique.

Detailed information on FDA pesticide residue monitoring programs, sampling protocol, and analytical methods used has been published (2, 3, 10–15).

Results and Discussion

Appendix A shows the results of regulatory monitoring of domestically produced foods by commodity group (grains and grain products, milk/dairy products/eggs, fish/seafoods/other meats, fruits, vegetables, and other commodities) for FY78–82. During that time, 30 361 samples of commercial food shipments were analyzed. In more than half of the samples (55%), no pesticide residues were found by the methods used for analysis.

The vegetables group had the largest number of samples (12 685, 42% of the total), followed by fruits (5268, 17%) and the milk/dairy products/eggs group (5140, 17%).

For each of the individual years, more than half of the domestic samples analyzed had no residues detected (range 51–59%). The number of domestic samples analyzed each year did not vary appreciably, ranging from 5666 in FY78 to 6848 in FY80. The percentage of domestic samples classed as violative for FY78–82 is also shown in Appendix A. The overall violation rate was 3% (commodity group range 1–6%). Grains and grain products and fish/seafoods/other meats had the highest percentage of violative samples; fruits had the lowest.

The data reporting system in effect during the period covered by this report did not provide information on the nature of the violations found. Thus, the violation rates given represent upper limits of the pesticide-related violation rates because substances other than pesticide residues may have rendered some of the samples actionable. The violation rates are not reflective of the status of the general food supply because the data do not distinguish between surveillance and compliance samples or whether violations pertain to pesticide-related findings. The reporting system has been modified since that time to provide more specific data on findings of violative pesticide residues.

Appendix B shows the results of regulatory monitoring of imports by commodity group for FY78–82. Over that period,

Table 1. Foreign countries and number of samples collected and analyzed in FY78-82

Country	No. of samples	Country	No. of samples	Country	No. of samples	Country	No. of samples
Mexico	9653	Brazil	188	Uruguay	70	Iceland	20
Canada	1347	France	183	Kenya	68	Poland	18
Taiwan	686	Denmark	179	Honduras	61	Sudan	18
Dominican Republic	557	Portugal	151	Norway	55	Morocco	17
China, People's Rep. of	472	Australia	150	Colombia	49	Sweden	17
Hong Kong	432	India	147	El Salvador	49	Ireland	16
Chile	428	Turkey	133	Switzerland	49	Indonesia	15
Argentina	384	Costa Rica	129	Austria	44	Malaysia	15
Japan	374	United Kingdom	127	Nicaragua	44	Yugoslavia	15
New Zealand	345	Philippines	121	Jamaica	43	Malawi	14
Spain	268	Belgium	118	Mali	38	Tanzania	14
The Netherlands	246	South Africa	113	Singapore	34	Panama	13
Italy	241	Israel	102	Germany, Dem. Rep. of	30	Unspecified	284
Peru	224	Korea, Rep. of	100	Greece	30		
Thailand	216	Ecuador	71	Finland	24		
Guatemala	190	Germany, Fed. Rep. of	70	Haiti	21		

Ten or fewer samples collected from the following:

Angola	Chad	Gaza Strip	Lebanon	Pakistan	Trinidad & Tobago
Bahamas	Cocos Islands	Germany, Berlin	Liberia	Papua New Guinea	Uganda
Bangladesh	Congo	Ghana	Luxembourg	Paraguay	Venezuela
Belize	Cyprus	Guyana	Macao	Rumania	Wallis & Fortuna Islands
Bolivia	Czechoslovakia	Hungary	Madagascar	Rwanda	Western Samoa
British Virgin Islands	Dominica	Iran	Malta	Sierra Leone	Zaire
Brunei	Egypt	Iraq	Mozambique	South West Africa	
Burma	Ethiopia	Ivory Coast	Nepal	Soviet Union	
Burundi	Faeroe Islands	North Korea	Netherlands Antilles	Sri Lanka	
Cameroon	Fiji Islands	Laos	Nigeria	Surinam	

19 516 samples were collected and analyzed. In 44% of the samples, no residues were found.

As in domestic sampling, the vegetables group comprised the largest number of samples, 10 845 (56% of the total), followed by fruits (3268, 17%) and the fish/seafoods/other meats group (1967, 10%).

For each of the individual years, almost half of the import samples analyzed had no residues detected (range 42–49%). The total number of import samples analyzed each year was somewhat greater in the later 3 years than in the first 2 years of the time span. This reflects the gradually increasing emphasis on sampling of imported foods, especially in the vegetables and fruits groups.

Appendix B also gives the percentage of import samples classed as violative for FY78–82. The overall average was 7% (commodity group range 3–15%). The commodity group with the highest violation rate was grains and grain products; the lowest was fish/seafoods/other meats.

Imported foods that were sampled in FY78–82 originated in 116 different countries (Table 1). Mexico had the largest number of samples analyzed, reflecting the importance of produce that is shipped to the United States from that country.

In FY70–76 (3), about 33 000 domestic and 18 000 import samples were analyzed. The total number of samples analyzed during FY78–82 was 49 877 (30 361 domestic and 19 516 import, Table 2), again demonstrating the growing attention to imports.

The results of the FY70–76 monitoring (3) showed that no residues were found in 44% of the 51 345 samples, compared with 50% for FY78-82. However, these figures are not directly comparable because the data for FY70-76 represent only *surveillance* samples of domestic foods. (At that time, import samples were not identified separately as surveillance or compliance.) Also, the results reported for FY70-76 include data for animal feeds; these data are not included in the present report.

In FY77, 12 911 samples (7870 domestic and 5041 import) were analyzed (4). No residues were found in 41% of the samples. Although the 12 911 samples represent both surveillance and compliance sampling for domestic and imported foods, commodities such as animal feeds, hay, and silage are included. Also, the FY77 data include analyses for metals as well as pesticide residues. Therefore, these results also cannot be compared directly with the FY78–82 data.

Table 3 lists the 128 different pesticides that were found in domestic and import commodities during FY78–82. It is not possible to develop a table that would accurately reflect all the pesticides that were detectable during this time period because of FDA's evolving development of analytical methods, compounds covered, recoveries, and coding of methods

Table 2. Domestic and import samples collected and analyzed in FY78–82

	Year					
Samples	1978	1979	1980	1981	1982	Total
Domestic	5666	5790	6848	6127	5930	30361
Import	3193	3554	4454	4324	3991	19516
Total	8859	9344	11302	10451	9921	49877

Table 3. Pesticides found as residues in FY78–82 regulatory monitoring

Acephate	Chlordecone	Dicloran	Iprodione	Paraquat	Quintozene
Alachlor	Chlorfenvinphos	Dicofol	isobenzan	Parathion	Ronnel
Aldicarb	Chlornitrofen	Dicrotophos	Lindane	Parathion-methyl	Silvex
Aldrin	Chlorobenzilate	Dieldrin	Linuron	Pentachlorophenol	Sulfallate
Allethrin	Chloroform	Dimethoate	Malathion	Permethrin	Sulfotep
Anilazine	Chlorothalonil	Diphenylamine	Maleic hydrazide	Perthane	2,4,5-T
Atrazine	Chlorpropham	Disulfoton	Mercury (as fungicide)	Phenthoate	TDE
Azinphos-ethyl	Chlorpyrifos	EBDCs[a]	Merphos	Phenylphenol, *o*-	Tecnazene
Azinphos-methyl	Chlorthiophos	Endosulfan	Methamidophos	Phorate	TEPP
Benomyl	Cyhexatin	Endrin	Methidathion	Phosalone	Terbufos
BHC	Cypermethrin	EPN	Methiocarb	Phosmet	Tetradifon
Biphenyl	2,4-D	Ethion	Methomyl	Phosphamidon	Thiabendazole
Bromide, inorganic	Daminozide	Ethoprop	Methoxychlor	Pirimicarb	Toxaphene
Bromopropylate	DCPA	Ethylene dibromide	Methyl trithion	Pirimiphos-methyl	Triazophos
Captafol	DDT	Ethylene dichloride	Mevinphos	Profenofos	Trichloroethylene
Captan	DEF	Fenitrothion	Mirex	Pronamide	Trichloronat
Carbaryl	Demeton	Fenthion	Monocrotophos	Propargite	Trifluralin
Carbofuran	Diazinon	Fenvalerate	Naled	Propoxur	Vinclozolin
Carbon tetrachloride	Dibromochloropropane	Folpet	Nitrofen	Prothiofos	
Carbophenothion	Dichlofluanid	Fonofos	Omethoate	Pyrazophos	
Carboxin	Dichlone	Heptachlor	Ovex	Pyrethrins	
Chlordane	Dichlorvos	Hexachlorobenzene	Oxamyl	Pyrethroids, synthetic	

[a] Includes amobam, mancozeb, maneb, metiram, nabam, and zineb.

used in monitoring. The evolution of analytical monitoring over the years has, in some cases, resulted in improvements in detection limits. In the FY70–76 regulatory monitoring (3), 124 different residues were found in the surveillance samples of foods and animal feeds.

A good deal of FDA effort involves costly, time-intensive analyses to broaden the scope of coverage for important pesticides not covered by routine monitoring. Table 4 lists the selective surveys conducted during FY78–82, mostly using single residue methods. Some of these were directed toward a single pesticide in a particular commodity (e.g., coumaphos in milk); others involved a number of related chemicals in several different foods (e.g., triazine herbicides in various commodities such as corn, peaches, and small fruits). Violative residues were rarely found.

The description of and detailed data on the Total Diet Study findings for FY78–82 have been published (16–22) and will not be repeated here. One aspect of historical interest, however, is a comparison of the most frequently found pesticide residues in Total Diet Study analyses. During FY70–76 (3), the residues most frequently found in the adult Total Diet composites were BHC, DDE, DDT, diazinon, dieldrin, heptachlor epoxide, lindane, and malathion. In FY78–82, most of these same compounds were still among the most frequently found of the residues from 42 different pesticides found in the adult Total Diet (Table 5). Of these, the organochlorine pesticides BHC, DDT, and dieldrin were no longer registered for use on foods in the United States by 1978. However, because of their slow degradation in the environment, residues continued to occur, although at low and decreasing levels. For example, in FY70–76 (3), intake levels of BHC, DDT (total), and dieldrin were 0.03, 0.1, and

Table 4. Selective surveys conducted in FY78–82

Pesticide	Commodity
Aldicarb	potatoes
Carbendazim/benomyl/thiophanate-methyl	bananas, citrus, apples, grapes
Carbofuran	potatoes
Coumaphos	milk
Daminozide	apples
Dibromochloropropane	various
Disulfoton, phorate, terbufos (including metabolites)	various
EBDCs[a]/ethylenethiourea	various vegetables
Ethylene dibromide	various
Fumigants, various	grains
Maleic hydrazide	potatoes
Mirex	eggs, milk, root crops
N-Methyl carbamates[b]	various fruits & vegetables
Organochlorine/organophosphorus	coffee beans
Paraquat	various
Pentachlorophenol	eggs, milk, crustacea, fish, mollusks
Pronamide	lettuce
Triazine herbicides	various

[a] Includes amobam, mancozeb, maneb, metiram, nabam, and zineb.
[b] Covered the entire chemical class.

Table 5. Frequency of occurrence of pesticides in adult Total Diet Study in FY78–82

Pesticide[a]	Total no. of findings[b]	Percent occurrence
Dieldrin	246	24
BHC, alpha & beta	236	23
DDT, total	231	22
Malathion	187	18
Hexachlorobenzene	184	18
Heptachlor, total	128	12
Diazinon	113	11
Lindane	112	11
Quintozene, total	89	9
Chlorpropham	79	8
Endosulfan, total	77	7
Pentachlorophenol	76	7
Dicloran	75	7
Chlordane, total	70	7
Parathion	50	5
DCPA	44	4
Chlorpyrifos	33	3
Ethion	32	3
Dicofol	26	2
Tecnazene, total	25	2
Toxaphene	20	2
Fonofos	19	2
Linuron	19	2
Methoxychlor	16	1
Dimethoate	13	1
Carbaryl	11	1
Phosalone	10	1

[a] "Total" designates the finding of any isomers, metabolites, or alteration products of that pesticide.
[b] Based on 1044 composites.

0.04 μg/kg body wt/day, while in FY81–82, the levels were 0.01, 0.03, and 0.02 μg/kg body wt/day, respectively, for the teenage male. For all residues present, the amounts found were many times lower than the Acceptable Daily Intakes (ADIs) set by the World Health Organization (WHO) (19).

Summary

FDA continued its wide-scale monitoring of the food supply for pesticide residues. During FY78–82, residues of 128 different pesticides were found in the 49 877 commodity samples. No residues were found in 55% of the domestic samples and 44% of the import samples. In the Total Diet Study, residues from 42 different parent pesticides were found; their calculated dietary intakes were well below WHO ADIs.

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References

(1) *Code of Federal Regulations* (1988) Title 40, U.S. Government Printing Office, Washington, DC, Parts 152, 180, 185, and 186
(2) Duggan, R. E., Lipscomb, G. Q., Cox, E. L., Heatwole, R. E., & Kling, R. C. (1971) *Pestic. Monit. J.* **5**, 73–212
(3) Duggan, R. E., Corneliussen, P. E., Duggan, M. B., McMahon, B. M., & Martin, R. J. (1983) *Pesticide Residue Levels in Foods in the United States from July 1, 1969 to June 30, 1976*, Food and Drug Administration and Association of Official Analytical Chemists, 240 pp
(4) *Compliance Program Report of Findings. FY77 Pesticides and Metals Program* (1981) Food and Drug Administration, Washington, DC, 19 pp
(5) *Food and Drug Administration Pesticide Program—Residues in Foods–1987* (1988) *J. Assoc. Off. Anal. Chem.* **71**, 156A–174A
(6) *Food and Drug Administration Pesticide Program—Residues in Foods–1988* (1989) *J. Assoc. Off. Anal. Chem.* **72**, 133A–152A
(7) *Food and Drug Administration Pesticide Program—Residues in Foods–1989* (1990) *J. Assoc. Off. Anal. Chem.* **73**, 127A–146A
(8) Yess, N. J., Houston, M. G., & Gunderson, E. L. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 273–280
(9) Reed, D. V. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 122–124
(10) Reed, D. V., Lombardo, P., Wessel, J. R., Burke, J. A., & McMahon, B. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 591–595
(11) Pennington, J. A. T., & Gunderson, E. L. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 772–782
(12) *Pesticide Analytical Manual* (1968 and revisions) Vols. I and II, Food and Drug Administration, Washington, DC (available from National Technical Information Service, Springfield, VA 22161)
(13) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA
(14) McMahon, B., & Burke, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 640–650
(15) *Inspection Operations Manual* (February 29, 1988) Pesticide Sampling, Food and Drug Administration, Rockville, MD, pp. 1–3
(16) Podrebarac, D. S. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 176–185
(17) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 862–875
(18) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1184–1197
(19) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 146–161
(20) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 842–861
(21) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1163–1183
(22) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 123–145

Appendix A. Analysis of domestic samples by commodity group in FY78–82

Commodity group	Year											
	1978–82			1978		1979		1980		1981		1982	
	Total no. of samples	Percent samples with no residues found	Percent viol.[a]	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found
A. Grains and Grain Products													
Corn, popcorn	196	62	6	17	12	4	100	64	73	40	63	71	62
Rice	236	74	2	36	83	38	79	67	61	40	65	55	85
Soybeans	282	47	5	–	–	39	38	64	41	78	42	101	57
Wheat	728	45	3	157	61	127	35	163	30	132	51	149	46
Other whole grains	207	55	5	33	6	22	91	84	58	50	76	18	78
Bakery & cereal products/snack foods	71	58	21	15	13	15	60	21	86	18	72	2	0
Grain products	147	30	27	35	14	31	10	45	36	9	67	27	48
Total	1867	51	6	293	46	276	46	508	48	367	57	423	58

Appendix A. (continued)

Commodity group	1978–82 Total no. of samples	1978–82 Percent samples with no residues found	1978–82 Percent viol.[a]	1978 Total no. of samples	1978 Percent samples with no residues found	1979 Total no. of samples	1979 Percent samples with no residues found	1980 Total no. of samples	1980 Percent samples with no residues found	1981 Total no. of samples	1981 Percent samples with no residues found	1982 Total no. of samples	1982 Percent samples with no residues found
B. Milk/Dairy Products/Eggs													
Butter/butter products	106	14	0	31	16	11	55	16	0	23	15	25	8
Cheese/cheese products	567	48	<1	98	57	52	8	140	53	133	41	144	60
Eggs	1977	76	3	319	71	321	70	556	74	447	81	334	83
Ice cream/ice cream products	24	71	0	1	100	2	50	3	100	9	33	9	100
Milk & cream	2450	55	1	577	50	548	47	447	57	447	67	431	58
Filled & imitation milk	16	75	6	2	50	–	–	4	75	9	89	1	0
Total	5140	62	2	1028	57	934	53	1166	64	1068	68	944	66
C. Fish/Seafoods/Other Meats													
Fish & shellfish	3689	24	6	913	19	732	19	900	29	690	23	454	28
Other meats	26	42	12	13	54	4	25	5	20	1	100	3	33
Total	3715	24	6	926	20	736	19	905	29	691	23	457	28
D. Fruits													
Blackberries	40	68	0	–	–	13	92	14	57	6	33	7	71
Blueberries	82	77	0	8	88	14	71	37	84	9	78	14	57
Boysenberries	13	77	0	–	–	–	–	1	0	5	80	7	86
Cranberries	104	30	1	12	83	18	6	24	25	14	21	36	31
Grapes	259	62	<1	63	65	71	46	64	75	40	73	21	48
Raspberries	123	33	2	1	0	12	17	36	25	37	41	37	38
Strawberries	748	28	1	115	35	131	26	184	24	166	33	152	27
Other berries	26	58	4	2	50	1	0	5	60	9	44	9	78
Grapefruit	193	26	0	32	44	43	42	20	5	83	11	15	53
Lemons	122	37	0	28	29	34	44	36	33	13	38	11	45
Oranges	281	21	0	45	47	48	19	40	33	120	4	28	39
Other citrus fruits	78	42	0	12	100	33	21	8	13	15	33	10	80
Apples	1155	52	<1	195	62	193	41	220	50	268	56	279	51
Pears	303	64	0	66	62	46	76	34	59	79	57	78	67
Other core fruits/mixed fruits	12	50	0	1	100	1	0	3	67	7	43	–	–
Apricots	25	8	0	–	–	–	–	15	13	2	0	8	0
Cherries	288	43	1	52	60	59	58	54	33	53	49	70	23
Nectarines	50	30	2	–	–	–	–	25	20	16	6	9	100
Peaches	668	35	1	205	31	155	30	116	35	97	43	95	41
Plums & prunes	50	68	0	–	–	1	100	1	100	35	63	13	77
Other pit fruits	12	83	0	–	–	2	50	2	100	5	80	3	100
Papaya	28	96	0	–	–	–	–	10	90	10	100	8	100
Pineapples	54	98	0	8	88	10	100	18	100	5	100	13	100
Other tropical fruits	9	78	0	–	–	–	–	–	–	3	33	6	100
Cantaloupe	219	72	<1	21	71	49	57	91	78	20	80	38	74
Honeydew	20	80	0	6	100	1	100	4	100	2	100	7	43
Watermelon	43	93	5	4	100	12	100	5	100	13	92	9	78
Other vine fruits	18	89	0	4	100	–	–	1	100	6	83	7	86
Other fruits	3	100	0	–	–	–	–	–	–	2	100	1	100
Fruit jams & toppings	67	66	0	8	100	4	50	19	68	16	44	20	70
Fruit juices	45	96	0	2	50	1	100	7	100	5	100	30	97
Fruits, dried or paste	130	80	2	12	100	24	75	19	79	35	89	40	70
Total	5268	47	1	902	52	976	42	1113	47	1196	44	1081	51
E. Vegetables													
Blackeyed peas	30	83	0	3	67	2	50	22	90	3	67	–	–
Corn	872	97	<1	185	96	187	94	188	99	129	99	183	99
Garden/green/sweet peas	262	83	5	60	73	55	84	70	80	44	95	33	88
String beans	537	75	<1	102	77	118	76	115	69	83	71	119	82
Other beans, peas, & corn	248	64	1	79	52	15	67	51	53	55	82	48	73
Cucumbers	539	62	1	125	63	114	64	115	49	96	73	89	62
Eggplant	102	86	1	3	67	14	93	18	94	28	75	39	90
Okra	158	95	1	36	97	46	96	25	96	3	100	48	92
Peppers	496	72	1	86	79	95	72	100	65	95	85	120	63
Pumpkins	51	80	0	5	60	1	100	15	67	5	80	25	92
Squash	443	58	2	18	89	64	63	113	48	90	61	158	57
Tomatoes	1004	73	4	206	78	162	77	254	63	190	77	192	71
Artichokes	97	38	5	3	100	28	29	39	44	11	36	16	31
Asparagus	110	93	0	24	79	19	95	25	96	19	100	23	96
Broccoli	263	70	0	69	83	37	68	50	74	44	61	63	62
Broccoli raab	8	25	0	–	–	2	0	–	–	3	0	3	67
Brussels sprouts	24	92	0	13	100	3	100	1	100	3	67	4	75
Cabbage	903	76	1	150	81	152	74	216	75	210	73	175	78
Cauliflower	208	93	0	50	90	35	91	52	92	38	95	33	100
Celery	359	22	3	52	19	48	21	112	14	82	30	65	26
Chinese cabbage	39	62	13	1	100	13	77	7	86	6	50	12	33
Collards	178	61	8	3	100	37	54	61	69	37	68	40	50
Dandelion greens	11	55	9	3	100	–	–	2	50	4	50	2	0
Endive/chicory	114	53	4	7	86	26	62	38	58	25	36	18	39
Kale	34	47	3	3	67	7	29	5	40	7	71	12	42
Lettuce	1348	40	4	140	49	124	39	335	41	382	38	367	39
Mustard greens	124	54	8	13	69	30	57	19	58	24	46	38	50
Parsley	119	31	22	22	32	24	46	24	38	31	13	18	33
Spinach	265	41	19	51	33	20	25	58	43	67	51	69	41
Swiss chard	12	67	0	–	–	–	–	5	40	5	80	2	100
Turnip greens	134	60	7	5	40	42	57	18	61	31	58	38	68
Other leaf/stem vegetables	102	72	9	15	93	17	94	22	64	27	70	21	52
Mixed vegetables	1	100	0	–	–	–	–	–	–	–	–	1	100
Mushroom/truffle products	129	19	1	12	0	16	0	47	26	24	8	30	33
Carrots	537	42	2	122	41	82	23	126	48	101	45	106	49
Leeks	8	63	13	–	–	–	–	5	80	2	0	1	100
Onions	358	71	<1	77	78	94	66	83	75	62	69	42	67
Parsnips	14	50	0	3	30	–	–	2	50	2	0	7	71
Potatoes	1159	56	1	287	49	187	57	253	43	204	67	228	67
Radishes	45	53	2	1	0	9	33	5	60	7	71	23	56
Red beets	81	73	2	3	67	17	88	19	79	20	65	22	64
Rutabagas	39	38	0	24	33	–	–	7	71	3	33	5	20

Appendix A. (continued)

Commodity group	Year											
	1978-82			1978		1979		1980		1981		1982	
	Total no. of samples	Percent samples with no residues found	Percent viol.[a]	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found
Sugar beets	64	97	0	7	100	1	0	16	100	13	92	27	100
Sweet potatoes	369	66	1	61	66	102	65	87	68	57	58	62	71
Turnips	77	70	1	8	38	24	75	9	78	9	78	27	70
Other root/tuber vegetables	30	67	7	7	57	4	100	13	62	1	0	5	80
Vegetable juices	49	67	0	12	100	1	100	7	86	21	62	8	13
Vegetables, dried or paste	96	46	2	12	25	3	100	25	20	15	53	41	61
Vegetables with sauce	390	66	4	76	50	66	61	86	78	85	62	77	78
Other vegetable-related products	45	82	4	25	92	–	–	3	67	8	75	9	44
Total	12685	63	3	2269	66	2143	65	2968	60	2511	63	2794	64
F. Other													
Spices & flavorings	79	71	9	–	–	25	56	4	100	35	77	15	73
Peanuts	319	45	2	84	55	80	35	52	46	46	93	57	40
Pecans	78	99	0	–	–	24	96	–	–	20	100	34	100
Sunflower seeds	115	65	7	4	25	17	100	27	78	50	52	17	59
Other nuts, seeds, & related products	146	75	3	33	70	5	40	20	95	44	75	44	75
Refined vegetable oil	25	84	0	1	0	5	100	1	100	15	87	3	67
Vegetable oil seed stock	35	49	9	1	0	3	33	2	100	20	55	9	33
Other vegetable oil products	72	57	24	23	48	7	43	6	100	25	40	11	100
Alcoholic beverages	20	85	0	4	100	–	–	16	81	–	–	–	–
Bottled water, other waters, & soft drinks	54	94	2	12	100	7	86	11	100	12	100	12	83
Chocolate & cocoa products	31	10	19	2	0	6	50	21	0	–	–	2	0
Food sweeteners	53	96	4	15	87	9	100	5	100	15	100	9	100
Other food products	87	63	2	13	46	37	76	7	86	12	25	18	67
Unspecified	572	50	4	56	48	500	49	16	50	–	–	–	–
Total	1686	60	5	248	58	725	53	188	64	294	66	231	68
A-F Total	30361	55	3	5666	53	5790	51	6848	54	6127	56	5930	59

[a]Classified as violative by laboratory performing analysis; does not necessarily represent final agency conclusion on compliance status of sample.

Appendix B. Analysis of import samples by commodity group in FY78-82

Commodity group	Year											
	1978-82			1978		1979		1980		1981		1982	
	Total no. of samples	Percent samples with no residues found	Percent viol.[a]	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found
A. Grains and Grain Products													
Rice	22	41	14	4	0	3	67	3	67	5	40	7	43
Wheat	3	67	0	1	100	–	–	–	–	1	0	1	100
Other whole grains	35	63	3	14	64	4	0	5	80	4	50	8	75
Bakery & cereal products/snack foods	24	42	29	5	40	3	67	2	50	11	18	3	100
Grain products	119	45	17	11	55	54	26	21	52	17	65	16	75
Pasta products	198	43	15	15	13	18	28	109	45	29	52	27	52
Total	401	45	15	50	40	82	28	140	48	67	48	62	63
B. Milk/Dairy Products/Eggs													
Butter/butter products	29	31	0	5	100	9	56	5	20	7	43	3	0
Cheese/cheese products	1046	23	4	335	14	255	27	178	20	169	33	109	36
Eggs & egg products	453	24	30	59	5	87	3	149	27	76	26	82	51
Milk & cream	52	81	6	2	100	4	75	8	63	5	100	33	82
Filled & imitation milk	3	67	0	1	0	–	–	2	100	–	–	–	–
Total	1583	26	12	402	13	355	22	342	25	257	32	227	48
C. Fish/Seafoods/Other Meats													
Fish & shellfish	1949	57	3	546	50	390	55	477	58	344	60	192	68
Other meats	18	39	0	7	71	1	0	2	0	4	25	4	25
Total	1967	56	3	553	50	391	54	479	58	348	60	196	67
D. Fruits													
Blackberries	2	0	0	–	–	–	–	1	0	1	0	–	–
Blueberries	25	92	0	12	100	6	83	2	100	2	100	3	67
Boysenberries	4	0	0	1	0	1	0	–	–	–	–	2	0
Cranberries	8	25	0	3	0	–	–	1	100	1	0	3	33
Grapes	196	64	5	8	38	38	68	36	47	82	74	32	56
Raspberries	20	30	0	2	50	4	0	6	0	7	57	1	100
Strawberries	777	29	13	212	32	87	39	285	27	103	26	90	20
Other berries	58	14	22	–	–	25	20	30	3	1	100	2	100
Grapefruit	39	85	0	1	100	6	100	18	89	8	75	6	67
Lemons	18	50	6	–	–	3	100	1	0	1	0	13	46
Limes	70	84	0	2	100	14	71	14	79	13	92	27	89
Oranges	103	62	0	6	83	23	74	31	77	25	24	18	67
Tangerines	56	59	0	–	–	8	88	8	100	25	24	15	80
Other citrus fruits	20	65	5	2	50	3	0	9	89	4	50	2	100
Apples	205	59	2	24	67	31	68	60	55	44	55	46	57
Pears	39	51	8	11	45	3	33	6	83	8	50	11	45
Other core fruits/mixed fruits	12	67	8	1	0	6	83	2	100	–	–	3	33
Apricots	6	50	0	2	50	1	100	–	–	1	0	2	50
Cherries	8	63	0	–	–	–	–	3	67	2	100	3	33
Nectarines	47	28	4	2	0	9	56	9	11	13	23	14	29
Olives	38	45	3	7	57	11	73	1	0	11	9	8	50
Peaches	43	42	2	6	67	9	56	7	0	13	38	8	50
Plums & prunes	29	69	0	2	100	7	57	5	40	6	83	9	78
Other pit fruits	27	89	4	1	0	5	100	12	92	2	100	7	86

Appendix B. (continued)

Commodity group	Year: 1978-82 Total no. of samples	1978-82 Percent samples with no residues found	1978-82 Percent viol.[a]	1978 Total no. of samples	1978 Percent samples with no residues found	1979 Total no. of samples	1979 Percent samples with no residues found	1980 Total no. of samples	1980 Percent samples with no residues found	1981 Total no. of samples	1981 Percent samples with no residues found	1982 Total no. of samples	1982 Percent samples with no residues found
Bananas	100	90	0	7	100	22	68	16	88	29	97	26	100
Mangoes	81	91	0	–	–	11	100	16	88	29	86	25	96
Papaya	22	86	5	1	0	6	100	8	75	5	100	2	100
Pineapples	123	96	1	14	100	20	90	20	95	27	93	42	100
Plantains	24	100	0	–	–	11	100	5	100	4	100	4	100
Other tropical fruits	195	37	9	15	40	30	47	42	43	72	25	36	44
Cantaloupe	209	43	1	14	43	24	33	57	44	58	50	56	39
Honeydew	104	53	0	10	90	32	38	20	60	25	60	17	41
Watermelon	94	71	3	24	54	23	78	18	89	5	60	24	71
Bitter melons	17	71	6	1	100	4	75	5	80	1	100	6	50
Other vine fruits	29	76	0	2	0	4	100	5	80	6	83	12	75
Other fruits	14	43	0	–	–	5	80	1	0	1	0	7	29
Fruit jams & toppings	99	76	7	19	74	10	70	33	61	23	96	14	86
Fruit juices	88	93	1	14	79	22	91	16	100	9	100	27	96
Fruits, dried or paste	219	52	6	12	58	56	50	66	42	21	71	64	56
Total	3268	54	7	438	49	580	60	875	48	688	55	687	60
E. Vegetables													
Blackeyed peas	50	28	8	2	50	5	40	24	4	12	42	7	71
Corn	51	88	2	2	100	11	73	13	92	13	92	12	92
Garden/green/sweet peas	320	61	15	29	79	115	70	68	50	44	61	64	45
Mung beans	166	32	34	13	15	37	35	19	42	76	28	21	43
String beans	304	38	6	63	60	64	36	77	23	55	34	45	38
Other beans, peas, & corn	546	56	9	68	59	136	49	107	63	107	61	128	52
Cucumbers	930	25	5	139	31	166	22	196	29	178	22	251	22
Eggplant	300	32	8	47	51	47	32	46	35	98	20	62	34
Okra	283	74	6	23	61	33	64	58	71	100	84	69	71
Peppers	1758	24	9	392	23	223	18	336	23	373	30	434	24
Pumpkins	20	80	0	1	100	2	50	6	100	8	63	3	100
Squash	872	48	5	139	74	174	61	140	32	184	39	235	40
Tomatoes	2359	28	4	210	36	279	37	677	25	614	19	579	35
Other fruits used as vegetables	41	68	2	7	43	2	100	8	88	10	60	14	86
Artichokes	47	98	2	2	100	11	100	11	100	9	89	14	100
Asparagus	83	96	0	9	78	8	88	17	100	21	100	28	100
Bamboo sprouts	29	100	3	3	100	6	100	4	100	10	100	6	100
Broccoli	102	53	1	2	0	16	63	34	41	27	74	23	43
Broccoli raab	2	0	0	–	–	–	–	1	0	–	–	1	0
Brussels sprouts	81	59	6	5	60	21	48	17	76	13	62	25	56
Cabbage	141	76	8	25	72	29	79	22	95	17	76	48	67
Cauliflower	100	78	0	13	92	26	62	15	73	13	92	33	82
Celery	39	13	13	17	6	10	0	2	0	4	75	6	17
Chinese cabbage	2	100	0	–	–	–	–	–	–	–	–	2	100
Endive/chicory	48	73	0	4	75	15	67	16	75	7	71	6	83
Lettuce	109	31	2	10	40	16	44	26	46	11	18	46	20
Parsley	16	31	25	10	20	1	100	1	100	1	0	3	33
Spinach	25	32	24	2	50	5	0	8	25	7	29	3	100
Other leaf/stem vegetables	122	64	5	12	67	17	53	28	79	25	68	40	55
Mixed vegetables	11	73	9	1	100	3	33	2	100	2	50	3	100
Mushroom/truffle products	179	44	2	36	25	24	29	37	16	38	47	44	89
Carrots	201	33	8	49	51	47	28	52	17	35	29	18	50
Leeks	6	33	17	–	–	4	50	–	–	1	0	1	0
Onions	154	73	1	26	85	38	79	29	62	21	67	40	73
Potatoes	92	62	1	10	40	3	33	26	69	36	53	17	88
Radishes	66	36	5	5	20	4	75	23	17	17	35	17	59
Red beets	16	50	0	4	75	3	67	5	60	2	0	2	0
Shallots	13	85	0	–	–	4	75	5	100	3	100	1	0
Sweet potatoes	21	81	0	1	100	7	71	7	86	5	80	1	100
Turnips	21	57	0	2	0	1	100	9	67	4	25	5	40
Other root/tuber vegetables	295	91	2	45	98	46	83	91	90	62	90	51	92
Vegetable juices	19	79	5	2	100	3	100	3	33	5	80	6	83
Vegetables, dried or paste	681	45	7	152	48	154	36	127	43	128	50	120	52
Vegetables with sauce	96	59	3	48	56	14	71	15	33	11	73	8	88
Other vegetable-related products	28	57	4	4	50	2	0	6	67	10	40	6	67
Total	10845	41	7	1634	45	1832	44	2414	38	2417	39	2548	43
F. Other													
Whole coriander	136	54	14	1	0	3	67	1	0	43	42	88	61
Other whole spices	199	60	10	7	71	50	48	44	61	52	63	46	67
Ground spices	29	17	0	4	0	10	20	1	0	7	14	7	29
Other spices & flavorings	29	41	17	2	100	7	29	5	60	7	29	8	38
Peanuts	321	16	18	–	–	1	0	–	–	316	16	4	25
Other nuts & related products	131	52	14	1	100	42	36	34	50	37	49	17	100
Sesame seeds	126	30	17	5	0	104	24	12	75	4	100	1	0
Other seeds & related products	29	59	14	1	100	2	0	10	60	7	43	9	78
Refined vegetable oil	16	75	13	5	80	1	100	4	75	3	67	3	67
Other vegetable oil products	28	64	4	–	–	1	100	6	67	13	54	8	75
Alcoholic beverages	26	100	0	1	100	–	–	4	100	2	100	19	100
Beverage bases	12	92	8	1	100	2	100	3	67	3	100	3	100
Coffee & tea	170	58	9	45	53	28	32	18	67	35	74	44	57
Bottled spring & mineral water	2	100	0	–	–	–	–	1	100	–	–	1	100
Other waters & soft drinks	9	78	22	1	0	2	50	2	100	3	100	1	100
Chocolate & cocoa products	37	38	5	16	38	11	36	6	50	2	50	2	100
Food sweeteners	11	64	9	3	67	2	50	3	33	3	100	–	–
Other food products	63	57	3	15	87	16	31	12	42	10	90	10	40
Unspecified	78	44	9	8	75	32	75	38	55	–	–	–	–
Total	1452	45	13	116	57	314	32	204	59	547	34	271	66
A-F Total	19516	44	7	3193	43	3554	44	4454	43	4324	42	3991	49

[a] Classified as violative by laboratory performing analysis; does not necessarily represent final agency conclusion on compliance status of sample.

Food and Drug Administration Pesticide Residue Monitoring of Foods: 1983–1986

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Pesticide residues in foods are reported for the 4-year period 1982–1986 [fiscal years (FY) 83–86]. Results were summarized from the 2 complementary approaches that make up the Food and Drug Administration's (FDA) pesticide residue monitoring program. Under regulatory monitoring, which focuses on residues in raw agricultural commodities, a total of 49 055 samples (27 700 domestic and 21 355 import) that included fresh fruits and vegetables, grains and grain products, milk and dairy products, seafoods, and a variety of processed foods were analyzed. No residues were found in 60 and 48% of the domestic and import samples, respectively, compared with 55 and 44% in FY78–82. About 3% of the domestic and 5% of the import samples were violative. In FY78–82, about 3 and 7% were violative, respectively. The other FDA monitoring approach, the Total Diet Study, was revised in April 1982 to expand coverage of age/sex groups, use updated diets, and provide for analysis of individual foods. Results from monitoring under this modified approach and from regulatory monitoring continued to demonstrate that pesticide residues in the U.S. food supply were well below regulatory limits, and dietary intakes were many times lower than the Acceptable Daily Intakes established by international agencies.

In a companion article (1), the 2 complementary approaches of the Food and Drug Administration (FDA) to monitoring the U.S. food supply for pesticide residues were described. The results of regulatory monitoring for fiscal years (FY) 1978–1982 were summarized and compared with those from other years. The second approach to monitoring, the Total Diet Study, was described as it was carried out during those years and some historical perspective was given to the findings. (The detailed data have been published, 2–8.)

In the present paper, data on regulatory monitoring for the 4-year period FY83–86 are presented, and the results are compared with those for FY78–82 (1), a 5-year span. In addition, the extensive changes made in mid-1982 in the Total Diet Study are described and information similar to that given for FY78–82 is included.

Results and Discussion

Regulatory Monitoring

In examining the data compiled under regulatory monitoring, 2 points that were explained in the companion paper (1) should be kept in mind. One is that selective monitoring was and continues to be used by FDA in order to cover the large number of possible pesticide/commodity combinations. The other is that the lower limit of measurement of a specific pesticide, as analyzed by FDA, has always been well below the Environmental Protection Agency (EPA) tolerance. This limit may range from about 0.005 to 1 ppm (part per million).

Appendix A shows the results of regulatory monitoring of domestically produced foods by commodity group (grains and grain products, milk/dairy products/eggs, fish/seafoods/other meats, fruits, vegetables, and other commodities) for FY83–86, a 4-year period. A total of 27 700 samples (surveillance plus compliance) were analyzed. In 60% of the samples, no residues were found. During FY78–82, a 5-year period, 30 361 samples were analyzed; no residues were found in 55% (1).

As in the FY78–82 survey, the vegetables group had the largest number of samples (13 092, 47% of the total), followed by fruits (6005, 22%) and the milk/dairy products/eggs group (4220, 15%).

For each of the individual years, the percentage of domestic samples with no residues detected ranged from 56 to 65 (the range in FY78–82 was 51–59%). The number of samples in each of the 4 years did not vary appreciably, ranging from 6664 in FY83 to 7306 in FY85 (average 6925)—somewhat higher than the average of 6072 for FY78–82 (1).

As explained in greater detail in the companion paper (1), the reporting of violative samples is based on the preliminary classification of the sample assigned by the laboratory that performed the analysis; that is, the sample contained a residue that exceeded the tolerance or a residue for which no tolerance had been set. Also, as noted in the companion paper (1), some samples may have been classified as violative based on substances other than pesticide residues. The percentage of domestic samples classed as violative for FY83–86 is shown in Appendix A. The overall violation rate was about 3% (the same as in FY78–82). Fish/seafoods/other meats had the highest percentage of violative samples, while milk/dairy products/eggs and fruits had the lowest. The violation rate for fish/seafoods/other meats was attributed to a number of violations involving chlorinated hydrocarbon pesticides (especially dieldrin) in bottom-feeding species (e.g., carp, catfish) and in fresh raw and fresh smoked chub and whitefish collected from areas of the Great Lakes known to be contaminated with these pesticides.

Appendix B shows the results of regulatory monitoring of imports by commodity groups for FY83–86. During that 4-year period, 21 355 samples (surveillance plus compliance) were collected and analyzed (19 516 in the 5-year period FY78–82). In 48% of the samples, no residues were detected, compared with 44% in FY78–82.

As in domestic sampling, the vegetables group had the largest number of samples (12 957, 61% of the total), followed by fruits (6193, 29%). In FY78–82 monitoring, these 2 commodity groups comprised 56 and 17% of the total import samples.

In each of the individual years, no residues were found in 45 to 49% of the import samples. The numbers of import samples analyzed per year continued to increase (the average was 3903 for FY78–82 and 5339 for FY83–86). In FY87, the number reached 7989 (9); in FY88, 10 475 (10); and in FY89, 11 100 (11). These figures reflect the continuing, increasing emphasis on monitoring of imported foods, especially raw vegetables and fruits.

About 5% of the import samples were classed as violative (Appendix B) [commodity group range 3–11%; in FY78–82 the average was 7%, commodity group range 3–15% (1)]. The highest violation rate was for the "other" commodities group; the lowest was for the grains and grain products and fish/seafoods/other meats groups.

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Table 1. Foreign countries and number of samples collected and analyzed in FY83–86

Country	No. of Samples	Country	No. of Samples	Country	No. of Samples	Country	No. of Samples
Mexico	13514	Germany, Fed. Rep. of	144	Honduras	62	Iceland	22
Chile	1088	Thailand	142	South Africa	61	Indonesia	22
Canada	852	France	140	United Kingdom	60	Greece	21
New Zealand	679	Belgium	127	Jamaica	51	Norway	18
Dominican Republic	528	Brazil	123	El Salvador	42	Poland	17
The Netherlands	480	Japan	117	Philippines	32	Uruguay	17
Taiwan	409	India	104	Peru	31	Yugoslavia	17
China, People's Rep. of	339	Argentina	101	Kenya	30	Germany, Dem. Rep. of	16
Spain	258	Israel	91	Portugal	28	Panama	14
Italy	209	Turkey	83	Ecuador	24	Finland	13
Hong Kong	208	Austria	82	Korea, Rep. of	24	Hungary	13
Australia	179	Costa Rica	81	Malawi	24	Mozambique	11
Guatemala	167	Denmark	65	Colombia	23	Unspecified	60
Haiti	163						

Ten or fewer samples collected from the following:

Afghanistan	Cameroon	Guadeloupe	Martinique	Pakistan	Switzerland
Albania	Central African Republic	Guyana	Morocco	Romania	Tanzania
Bahamas	Cocos Islands	Iran	Nepal	Rwanda	Tunisia
Bangladesh	Cuba	Ireland	Netherlands Antilles	Senegal	Uganda
Belize	Cyprus	Ivory Coast	Nicaragua	Singapore	Venezuela
Bolivia	Czechoslovakia	Liberia	Nigeria	Sri Lanka	Western Samoa
Bulgaria	Egypt	Madagascar	North Korea	Sweden	Zaire
Cambodia	Fiji Islands	Malaysia			

Over the 4-year period, samples of imported foods from 97 countries were analyzed (Table 1); 116 countries were represented during the 5-year period FY78–82. Mexico continued to be the major exporter of produce to the United States, and was sampled accordingly.

In FY78–82, the total number of samples (domestic and import) analyzed per year ranged from 8859 in FY78 to 11 302 in FY80 (average 9975) (1). During FY83–86, the number ranged from 11 766 in FY83 to 12 911 in FY85 (Table 2) (average 12 264), a 23% increase over the FY78–82 average.

Table 3 lists the 135 different pesticides that were found in domestic and import commodities during the 4-year period FY83–86. As noted in the companion paper (1), it is not possible to develop a table that would accurately reflect all the pesticides detectable during this time period. In the 5-year FY78–82 survey (1), 128 pesticides were found.

Table 4 lists the selective surveys conducted in FY83–86, mostly using single residue methods. As is usual, some of these involved a single residue in 1 commodity (e.g., cyhexatin in strawberries), while others targeted a number of related chemicals in several commodities (e.g., various fumigants in grain products). As in the selective surveys conducted in FY78–82 (1), violative residues were rarely found.

During FY83–86, several episodes occurred that warranted intensive monitoring efforts. The approximately 11 500 samples that were analyzed in response to these special situations are not included in the tabulations of domestic and import samples provided elsewhere in this report. These episodes are briefly described below.

In FY84, EPA revoked the exemption from the requirement of a tolerance for the fumigant ethylene dibromide (EDB). From FY83 through FY86, FDA collected and analyzed approximately 9000 samples for EDB to gather information on its levels in foods and then to enforce the new tolerances established for that chemical.

In FY85 and FY86, FDA participated, in conjunction with EPA and the National Marine Fisheries Service, in a Congressionally mandated survey of polychlorinated biphenyls (PCBs) in Atlantic coastal bluefish. FDA analyzed approximately 800 samples collected in the 2-year survey not only for PCBs, which are industrial chemicals, but also for pesticides.

Findings of illegal residues of aldicarb in FY85 prompted widespread monitoring for that chemical in domestic produce. Approximately 700 samples were collected and analyzed.

In FY86, findings of illegal organochlorine pesticides, such as heptachlor epoxide residues, in animal feed led FDA to conduct intensive monitoring of milk and cheese. Approximately 1000 samples were analyzed.

Total Diet Study

Since it was initiated in 1961, there have been many changes, refinements, and expansions in the Total Diet Study regarding diet basis, population groups covered, collection sites, foods collected, analytes, and analytical methodology. The Total Diet Study underwent its most extensive changes

Table 2. Domestic and import samples collected and analyzed in FY83–86

	Year				
Samples	1983	1984	1985	1986	Total
Domestic	6664	7061	7306	6669	27700
Import	5102	4928	5605	5720	21355
Total	11766	11989	12911	12389	49055

Table 3. Pesticides found as residues in FY83–86 regulatory monitoring

Acephate	Chloropropylate	EBDCs[a]	Methomyl	Pirimiphos-ethyl
Aldicarb	Chlorothalonil	Endosulfan	Methoxychlor	Pirimiphos-methyl
Aldoxycarb	Chlorpropham	Endrin	Methyl bromide	Procymidone
Aldrin	Chlorpyrifos	EPN	Metolachlor	Profenofos
Allethrin	Chlorthiophos	Ethion	Mevinphos	Pronamide
Anilazine	Coumaphos	Ethoprop	Mirex	Propargite
Atrazine	Cyhexatin	Ethylene dibromide	Monocrotophos	Propoxur
Azinphos-ethyl	Cypermethrin	Ethylene dichloride	Nicotine	Prothiofos
Azinphos-methyl	Daminozide	Fenbutatin oxide	Norea	Pyrethroids, synthetic
Benomyl	DCPA	Fenitrothion	Omethoate	Quintozene
BHC	DDT	Fensulfothion	Oxamyl	Rotenone
Bromide, inorganic	DEF	Fenthion	Paraquat	Simazine
Bromophos-ethyl	Demeton	Fenvalerate	Parathion	Sulfotep
Bromopropylate	Diazinon	Folpet	Parathion-methyl	Sulfur dioxide
Captafol	Dichlofluanid	Fonofos	Pentachlorophenol	TDE
Captan	1,3-Dichloropropene	Gardona	Permethrin	Tecnazene
Carbaryl	Dichlorvos	Heptachlor	Perthane	Terbufos
Carbofuran	Dicloran	Hexachlorobenzene	Petroleum oils	Tetradifon
Carbon disulfide	Dicofol	Imazalil	Phenthoate	Thiabendazole
Carbon tetrachloride	Dicrotophos	Iprodione	Phenylphenol, *o*-	Toxaphene
Carbophenothion	Dieldrin	Lindane	Phorate	Triadimefon
Chlordane	Dimethoate	Linuron	Phosalone	Triadimenol
Chlordecone	Dinocap	Malathion	Phosmet	Triallate
Chlorfenvinphos	Dioxathion	Metalaxyl	Phosphamidon	Triazophos
Chlorobenzilate	Diphenylamine	Methamidophos	Phosphine	Trichloronat
Chloroform	Disulfoton	Methidathion	Picloram	Trifluralin
Chloropicrin	Diuron	Methiocarb	Pirimicarb	Vinclozolin

[a] Includes amobam, mancozeb, maneb, metiram, nabam, and zineb.

in mid-1982. At that time, data from the U.S. Department of Agriculture's (USDA) 1977–1978 Nationwide Food Consumption Survey (12) and the National Center for Health Statistics' Second National Health and Nutrition Examination Survey of 1976–1980 (13) became available and were used to select a group of commonly consumed foods and to develop nationally representative diets for 8 age/sex groups based on these foods (14). The caloric content of these diets represents typical intakes according to age and sex. From 1975 to mid-1982, the bases for the Study had been 1965 USDA/Household Food Consumption Survey data (15), 3 age/sex groups, and a food commodity group composite system for analysis of samples chosen in 4 regions of the country (14). The extensive mid-1982 revisions resulted in the use of an updated diet basis; expansion of the age/sex groups from 3 to 8 to include 60–65-year-old females and males, 25–30-year-old females and males, 14–16-year-old females and males, 2-year-old toddlers, and 6–11-month-old infants; and analysis of 234 individual representative foods.

Under the revised plan, foods are collected 4 times per year, once from each of 4 geographical areas of the United States. Each collection consists of identical foods purchased from grocery stores in 3 cities within a geographical area. The 3 subsamples of each food (from the 3 cities) are prepared table-ready, then combined to form a laboratory sample, which is analyzed for pesticide residues as well as industrial chemicals, radionuclides, essential minerals, and toxic elements. The results of these analyses are summarized and published in the *Journal of the Association of Official Analytical Chemists*.

Table 5 lists the most frequently found of the residues from 62 different pesticides detected in the Total Diet Study analyses for FY82–86. These data reflect the time period from mid-1982 through mid-1986 because modifications in the Total Diet Study were put into effect in April 1982. Several residues, malathion, dieldrin, diazinon, and DDT, remain among the most frequently found over a number of years, including FY78 (1) through FY86. Although DDT and dieldrin were no longer registered for use on foods in the United States during the time covered by these reports, residues continued to occur because of their slow degradation in the environment. Residue levels and dietary intakes, however, have shown a decrease with time. For example, dieldrin, the only pesticide whose estimated dietary intake ever approached its Acceptable Daily Intake (ADI) (5), in 1986

Table 4. Selective surveys conducted in FY83–86

Pesticide	Commodity
Aldicarb	canned tomatoes, potatoes
Captan, folpet	grapes, strawberries
Cyhexatin	strawberries
Daminozide/UDMH	apples and apple products
Disulfoton, phorate, terbufos (including metabolites)	various
Ethylene dibromide	various
EBDCs[a]/ethylenethiourea	various vegetables
Fluazifop-butyl	potatoes
Fumigants, various	grain products
Methiocarb	wine
Methyl bromide	citrus products, grain, and grain products
Paraquat	various
Pentachlorophenol	food-grade gelatine
Permethrin (including metabolites)	tomato products
Vinclozolin (including metabolites)	green beans, kiwi fruit, strawberries

[a] Includes amobam, mancozeb, maneb, metiram, nabam, and zineb.

Table 5. Frequency of occurrence of pesticides in adult Total Diet Study in FY82-86[a]

Pesticide[b]	Total no. of findings[c]	Percent occurrence
DDT, total	910	24
Malathion	824	22
Dieldrin	521	14
Pentachlorophenol	471	13
BHC, alpha & beta	441	12
Diazinon	378	10
Hexachlorobenzene	352	9
Chlorpyrifos	295	8
Heptachlor, total	270	7
Chlordane, total	218	6
Dicloran	209	6
Lindane	189	5
Endosulfan, total	178	5
Methamidophos	144	4
Chlorpropham	137	4
Carbaryl	135	4[d]
Dicofol, total	109	3
Dimethoate	99	3
Parathion	98	3
Ethion	89	2
Toxaphene	74	2
DCPA	72	2
Quintozene, total	65	2
Phosalone	63	2
Tecnazene	61	2
TDE, total	60	2

[a] Reflects the 16 market basket collections made between April 1982 and April 1986. See text for explanation of mid-year starting date.
[b] "Total" designates the finding of any isomers, metabolites, or alteration products of that pesticide.
[c] Based on 3744 samples.
[d] Reflects overall incidence; however, only 1152 foods were analyzed for *N*-methyl carbamates.

occurred at a level one-twentieth that found 20 years earlier (14).

Summary

This article, together with the companion paper (1), completes the reporting of pesticide residues in the U.S. food supply from FY78 through FY86 [the data for FY87 (9), FY88 (10), and FY89 (11) have also been published]. During FY83-86, residues of 135 different pesticides were found in 49 055 samples of various commodities. No residues were found in 60% of the domestic and 48% of the import samples compared with 55 and 44% in FY78-82. In the Total Diet Study, residues from 62 different parent pesticides were found. The results from the Total Diet Study, which was considerably revised in mid-1982, continued to demonstrate that the levels of pesticide residues found in the diet were well below the ADIs set by the World Health Organization (5).

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References

(1) Yess, N. J., Houston, M. G., & Gunderson, E. L. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 265-273
(2) Podrebarac, D. S. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 176-185
(3) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 862-875
(4) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1184-1197
(5) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 146-161
(6) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 842-861
(7) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1163-1183
(8) Gartrell, M. J., Craun, J. C., Podrebarac, D. S., & Gunderson, E. L. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 123-145
(9) *Food and Drug Administration Pesticide Program—Residues in Foods-1987* (1988) *J. Assoc. Off. Anal. Chem.* **71**, 156A-174A
(10) *Food and Drug Administration Pesticide Program—Residues in Foods-1988* (1989) *J. Assoc. Off. Anal. Chem.* **72**, 133A-152A
(11) *Food and Drug Administration Pesticide Program—Residues in Foods-1989* (1990) *J. Assoc. Off. Anal. Chem.* **73**, 127A-146A
(12) Nationwide Food Consumption Survey Data Tapes. Spring, Summer, Fall, and Winter Quarters 1977-78 (1980, 1981) U.S. Department of Agriculture, PB80-190218, PB80-197429, PB80-200223, PB81-118853, National Technical Information Service, Springfield, VA
(13) Second National Health and Nutrition Examination Survey, 1976-80. Data Tape 5704 (24-Hour Recall, Specific Food Item) (1982) National Center for Health Statistics, PB82-142639, National Technical Information Service, Springfield, VA
(14) Pennington, J. A. T., & Gunderson, E. L. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 772-782
(15) *Food Consumption of Households in the United States, Spring 1965* (1967) Household Food Consumption Survey 1965-66, Report No. 1, Consumer and Food Economics Research Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, DC

Appendix A. Analysis of domestic samples by commodity group in FY83–86

Commodity group	1983–86 Total no. of samples	1983–86 Percent samples with no residues found	1983–86 Percent viol.[a]	1983 Total no. of samples	1983 Percent samples with no residues found	1984 Total no. of samples	1984 Percent samples with no residues found	1985 Total no. of samples	1985 Percent samples with no residues found	1986 Total no. of samples	1986 Percent samples with no residues found
A. Grains and Grain Products											
Corn, popcorn	150	42	5	11	36	29	45	73	37	37	51
Rice	268	69	1	65	78	118	58	68	81	17	65
Soybeans	145	72	1	62	69	36	69	33	82	14	71
Wheat	420	28	5	56	34	99	39	162	22	103	25
Other whole grains	55	56	11	17	47	17	47	10	90	11	55
Bakery & cereal products/snack foods	48	56	2	21	86	23	26	2	100	2	50
Grain products	153	43	4	33	36	70	27	29	86	21	48
Total	1239	48	4	265	58	392	46	377	48	205	40
B. Milk/Dairy Products/Eggs											
Butter/butter products	76	37	0	27	11	22	45	17	35	10	90
Cheese/cheese products	441	67	<1	123	49	103	65	134	75	81	83
Eggs	1651	82	3	434	79	401	77	470	84	346	87
Ice cream/ice cream products	55	89	0	28	79	20	100	7	100	–	–
Milk & cream	1974	69	<1	529	64	448	62	519	75	478	73
Filled & imitation milk	23	96	0	2	100	3	100	16	94	2	100
Total	4220	73	1	1143	68	997	69	1163	78	917	79
C. Fish/Seafoods/Other Meats											
Fish & shellfish	1759	33	7	511	40	353	24	494	34	401	32
Other meats	70	49	11	38	37	12	58	17	65	3	67
Total	1829	34	8	549	39	365	25	511	35	404	32
D. Fruits											
Blackberries	12	58	0	1	100	–	–	8	50	3	67
Blueberries	71	82	0	14	93	11	91	22	82	24	71
Boysenberries	17	71	0	8	88	7	43	1	100	1	100
Cranberries	116	17	<1	18	33	35	17	15	27	48	8
Grapes	253	68	0	64	64	72	76	58	71	59	59
Raspberries	126	49	0	24	42	56	41	9	56	37	65
Strawberries	874	29	3	185	28	200	34	229	35	260	19
Other berries	75	73	0	36	78	21	71	16	63	2	100
Grapefruit	116	33	0	22	18	42	52	30	27	22	18
Lemons	80	56	0	12	17	25	92	25	68	18	17
Oranges	334	65	0	51	47	96	69	119	71	68	60
Other citrus fruits	71	46	0	9	22	29	52	23	48	10	50
Apples	1259	44	1	221	45	245	56	342	47	451	36
Pears	268	51	0	80	51	53	45	62	69	73	41
Other core fruits/mixed fruits	11	27	0	–	–	–	–	1	100	10	20
Apricots	115	63	0	18	72	50	72	23	65	24	33
Cherries	288	41	1	81	41	85	36	36	64	86	35
Nectarines	141	67	0	16	38	66	79	32	94	27	26
Peaches	443	44	1	105	23	160	63	71	51	107	31
Plums & prunes	190	85	0	22	91	74	84	52	90	42	76
Other pit fruits	46	91	0	9	78	18	100	12	92	7	86
Papaya	34	88	0	2	100	25	84	5	100	2	100
Pineapples	50	92	0	9	100	22	95	10	90	9	78
Other tropical fruits	48	83	10	1	100	24	71	17	94	6	100
Cantaloupe	277	84	<1	41	80	43	93	89	96	104	72
Honeydew	61	75	0	14	64	8	67	14	79	25	80
Watermelon	143	92	2	14	100	9	100	71	85	49	98
Other vine fruits	55	75	0	10	20	9	78	6	100	30	87
Other fruits	26	92	0	–	–	–	–	22	95	4	75
Fruit jams & toppings	166	71	0	15	33	72	74	65	86	14	36
Fruit juices	119	87	0	7	86	36	100	40	98	36	61
Fruits, dried or paste	120	89	<1	39	79	52	94	27	100	2	0
Total	6005	54	1	1148	48	1645	62	1552	64	1660	43
E. Vegetables											
Blackeyed peas	28	96	0	20	95	1	100	6	100	1	100
Corn	308	97	<1	108	97	87	98	58	93	55	98
Garden/green/sweet peas	512	73	3	47	74	261	73	100	71	104	77
String beans	276	66	4	71	75	90	52	47	70	68	74
Other beans, peas, & corn	272	86	1	82	78	65	85	79	90	46	93
Cucumbers	298	66	2	108	67	57	67	73	59	60	73
Eggplant	78	85	4	23	78	21	95	20	80	14	86
Okra	50	94	2	21	100	10	70	10	100	9	100
Peppers	371	66	3	85	68	96	71	102	64	88	60
Pumpkins	21	67	0	7	86	4	75	4	50	6	50
Squash	420	75	1	132	71	82	76	111	74	95	80
Tomatoes	671	73	<1	167	65	193	88	162	70	149	68
Artichokes	87	67	1	29	45	29	62	14	86	15	100
Asparagus	149	97	0	36	97	42	100	19	95	52	96
Broccoli	524	84	0	68	91	134	83	159	91	163	76
Broccoli raab	25	72	0	10	80	8	75	3	67	4	50
Brussels sprouts	71	94	0	1	100	27	96	30	93	13	92
Cabbage	561	79	1	150	80	135	86	141	83	135	65

Appendix A. (continued)

Commodity group	Year 1983–86 Total no. of samples	1983–86 Percent samples with no residues found	1983–86 Percent viol.[a]	1983 Total no. of samples	1983 Percent samples with no residues found	1984 Total no. of samples	1984 Percent samples with no residues found	1985 Total no. of samples	1985 Percent samples with no residues found	1986 Total no. of samples	1986 Percent samples with no residues found
Cauliflower	364	96	<1	60	97	109	96	95	98	100	95
Celery	331	24	<1	82	22	80	31	86	27	83	16
Chinese cabbage	98	81	3	26	77	23	70	22	95	27	81
Collards	200	36	35	38	34	32	63	42	33	88	27
Dandelion greens	31	39	48	5	40	6	33	9	22	11	55
Endive/chicory	135	51	10	25	32	53	53	36	58	21	57
Kale	98	38	21	12	33	10	60	22	32	54	37
Lettuce	2579	45	4	772	33	655	54	639	50	513	46
Mustard greens	148	52	9	30	50	42	55	32	59	44	45
Parsley	127	54	13	20	70	15	53	40	50	52	50
Spinach	324	52	11	82	60	68	51	95	47	79	51
Swiss chard	44	43	30	4	100	6	33	19	32	15	47
Turnip greens	108	53	8	21	62	25	72	28	43	34	41
Other leaf/stem vegetables	215	69	15	43	79	44	70	56	61	72	69
Mixed vegetables	4	75	0	–	–	–	–	1	100	3	67
Mushroom/truffle products	109	63	0	43	58	27	56	30	67	9	100
Carrots	554	53	4	137	55	132	45	163	60	122	52
Leeks	41	83	2	2	100	11	82	15	80	13	85
Onions	281	70	1	77	62	55	75	64	73	85	72
Parsnips	73	36	29	13	38	12	33	26	27	22	45
Potatoes	1130	60	1	324	61	228	54	366	67	212	53
Radishes	157	62	6	28	50	48	73	44	64	37	54
Red beets	109	53	19	36	50	27	63	28	50	18	50
Rutabagas	56	38	4	6	67	14	14	19	37	17	47
Sugar beets	71	90	0	19	95	10	100	27	96	15	67
Sweet potatoes	227	74	0	46	57	52	75	44	80	85	79
Turnips	93	41	2	19	47	19	32	22	59	33	30
Other root/tuber vegetables	49	80	2	4	50	12	92	18	94	15	60
Vegetable juices	35	54	9	11	27	14	79	7	43	3	67
Vegetables, dried or paste	194	73	2	62	81	60	60	32	81	40	73
Vegetables with sauce	315	73	4	41	63	120	78	118	74	36	61
Other vegetable-related products	70	91	1	13	85	19	89	27	100	11	82
Total	13092	63	4	3266	59	3370	67	3410	66	3046	61
F. Other											
Spices & flavorings	191	68	22	21	90	35	100	55	84	80	38
Peanuts	233	37	<1	47	21	77	30	56	52	53	45
Pecans	45	89	9	16	69	9	100	8	100	12	100
Sunflower seeds	59	90	0	6	100	23	83	13	100	17	88
Other nuts, seeds, & related products	236	90	<1	79	94	76	82	53	92	28	96
Refined vegetable oil	23	87	0	5	100	13	85	3	67	2	100
Vegetable oil seed stock	54	59	2	25	52	6	83	11	91	12	33
Other vegetable oil products	18	83	6	10	90	5	80	5	80	1	100
Alcoholic beverages	180	36	0	10	20	4	0	15	47	151	36
Bottled water, other waters, & soft drinks	64	98	3	14	93	8	100	13	100	23	100
Chocolate & cocoa products	3	100	0	1	100	–	–	1	100	–	–
Food sweeteners	77	74	6	12	67	15	93	46	70	4	75
Other food products	129	64	1	44	66	21	52	14	100	54	59
Unspecified	3	100	0	3	100	–	–	–	–	–	–
Total	1315	65	4	293	69	292	69	293	78	437	52
A–F Total	27700	60	3	6664	57	7061	63	7306	65	6669	56

[a] Classified as violative by laboratory performing analysis; does not necessarily represent final agency conclusion on compliance status of sample.

Appendix B. Analysis of import samples by commodity group in FY83–86

Commodity group	1983–86			Year 1983		1984		1985		1986	
	Total no. of samples	Percent samples with no residues found	Percent viol.[a]	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found	Total no. of samples	Percent samples with no residues found
A. Grains and Grain Products											
Rice	26	73	8	2	0	6	67	1	100	17	82
Wheat	10	60	0	–	–	–	–	5	60	5	60
Other whole grains	47	79	9	9	78	10	70	13	54	12	92
Bakery & cereal products/snack foods	23	91	0	1	100	8	100	8	75	5	100
Grain products	101	74	2	26	69	32	88	27	70	20	70
Pasta products	61	54	0	11	73	25	44	20	45	5	100
Total	268	71	3	49	69	81	74	74	61	64	81
B. Milk/Dairy Products/Eggs											
Butter/butter products	7	43	0	2	50	4	50	1	0	–	–
Cheese/cheese products	262	76	2	74	68	62	68	63	83	63	86
Eggs & egg products	266	47	12	63	38	100	44	65	62	38	47
Milk & cream	21	100	0	12	100	4	100	2	100	3	100
Total	556	63	7	151	58	170	54	131	72	104	72
C. Fish/Seafoods/Other Meats											
Fish & shellfish	379	52	1	121	65	79	51	68	40	111	47
Other meats	37	24	24	9	22	12	17	15	27	1	100
Total	416	50	3	130	62	91	46	83	37	112	47
D. Fruits											
Blackberries	62	24	35	18	33	16	13	25	24	3	33
Blueberries	78	62	3	11	64	8	38	37	54	22	82
Boysenberries	16	13	13	3	33	3	33	6	0	4	0
Cranberries	15	13	0	1	100	4	0	7	14	3	0
Grapes	1500	54	5	330	50	181	68	131	60	858	52
Raspberries	166	46	7	10	50	28	50	89	48	39	36
Strawberries	281	12	5	28	14	93	9	73	10	87	17
Other berries	93	22	2	19	26	12	17	22	32	40	15
Grapefruit	24	58	0	2	100	7	43	4	100	11	45
Lemons	15	33	20	–	–	8	63	–	–	7	0
Limes	82	84	0	23	96	17	76	16	81	26	81
Oranges	158	50	1	18	67	51	59	23	57	66	36
Tangerines	112	47	0	15	100	35	31	15	87	47	30
Other citrus fruits	21	86	5	3	100	–	–	3	33	15	93
Apples	168	49	2	45	47	37	59	44	52	42	40
Pears	129	53	5	10	70	23	39	53	74	43	33
Other core fruits/mixed fruits	11	55	0	1	0	4	25	4	75	2	100
Apricots	28	71	0	4	100	7	100	6	83	11	36
Cherries	25	40	0	2	100	6	0	9	33	8	63
Nectarines	79	25	1	23	30	21	29	15	20	20	20
Olives	30	63	0	7	71	7	43	4	25	12	83
Peaches	92	50	1	16	56	23	52	22	32	31	58
Plums & prunes	73	49	0	17	59	13	77	19	32	24	42
Other pit fruits	13	85	0	3	100	3	33	3	100	4	100
Bananas	181	96	0	65	95	68	99	21	100	27	85
Mangoes	335	33	28	55	95	32	78	219	10	29	38
Papaya	16	94	0	6	100	2	100	2	50	6	100
Pineapples	231	44	18	31	68	59	41	36	36	105	42
Plantains	12	83	0	4	100	6	83	–	–	2	50
Other tropical fruits	122	61	7	23	39	31	52	26	85	42	67
Cantaloupe	735	26	2	132	22	181	19	227	21	195	42
Honeydew	263	26	3	40	23	61	21	61	18	101	35
Watermelon	321	75	2	107	70	34	82	37	100	143	70
Bitter melons	55	80	7	20	75	18	89	10	70	7	86
Other vine fruits	64	48	5	5	40	23	22	18	67	18	67
Other fruits	27	44	0	4	25	3	100	11	64	9	11
Fruit jams & toppings	86	81	5	9	89	33	70	28	89	16	88
Fruit juices	335	87	0	10	100	48	98	263	84	14	93
Fruits, dried or paste	139	78	2	49	71	25	76	26	73	39	90
Total	6193	50	5	1169	56	1231	50	1615	47	2178	50
E. Vegetables											
Blackeyed peas	34	56	3	15	47	1	0	18	67	–	–
Corn	38	87	5	14	100	14	79	2	100	8	75
Garden/green/sweet peas	487	63	6	105	68	130	65	119	62	133	59
Mung beans	71	63	4	8	63	18	61	26	65	19	63
String beans	453	51	5	92	65	134	44	125	49	102	48
Other beans, peas, & corn	392	65	11	174	61	84	63	93	71	41	76
Cucumbers	1300	21	7	399	24	318	12	298	21	285	29
Eggplant	415	38	4	116	40	93	44	111	32	95	38
Okra	300	69	10	61	74	64	56	109	67	66	80
Peppers	2557	24	8	617	20	610	24	719	24	611	28
Pumpkins	18	94	0	4	100	8	100	2	100	4	75
Squash	1316	57	3	330	54	338	49	360	65	288	60
Tomatoes	2768	29	<1	862	38	656	28	663	24	587	24
Other fruits used as vegetables	60	85	5	13	69	15	80	16	94	16	94
Artichokes	13	92	0	6	83	1	100	2	100	4	100

Appendix B. (continued)

Commodity group	Year: 1983–86 Total no. of samples	1983–86 Percent samples with no residues found	1983–86 Percent viol.[a]	1983 Total no. of samples	1983 Percent samples with no residues found	1984 Total no. of samples	1984 Percent samples with no residues found	1985 Total no. of samples	1985 Percent samples with no residues found	1986 Total no. of samples	1986 Percent samples with no residues found
Asparagus	117	92	0	35	89	13	85	41	93	28	100
Bamboo sprouts	15	93	0	5	80	6	100	2	100	2	100
Broccoli	108	73	1	23	61	40	68	29	76	16	100
Broccoli raab	18	67	0	2	50	–	–	9	67	7	71
Brussels sprouts	150	77	6	26	77	42	83	59	81	23	57
Cabbage	278	48	15	32	53	192	38	36	83	18	83
Cauliflower	85	87	0	24	83	25	92	24	88	12	83
Celery	25	16	0	1	0	–	–	9	22	15	13
Chinese cabbage	44	73	0	11	91	10	30	10	80	13	85
Endive/chicory	96	61	0	12	17	8	63	27	48	49	80
Lettuce	193	51	3	36	50	28	68	93	42	36	64
Parsley	29	62	3	11	55	1	100	6	67	11	64
Spinach	48	50	17	6	50	3	100	20	55	19	37
Other leaf/stem vegetables	158	53	11	35	60	25	52	57	39	41	66
Mixed vegetables	6	67	0	2	50	1	0	2	100	1	100
Mushroom/truffle products	117	77	5	69	83	21	71	9	67	18	67
Carrots	105	50	1	18	44	16	50	32	44	39	56
Leeks	11	91	0	–	–	1	100	3	100	7	86
Onions	181	83	<1	23	83	57	82	51	90	50	76
Potatoes	60	77	0	8	50	6	100	19	68	27	85
Radishes	134	69	8	29	41	24	75	45	78	36	75
Red beets	29	69	10	2	50	6	50	15	73	6	83
Shallots	15	100	0	4	100	2	100	3	100	6	100
Sweet potatoes	21	100	0	6	100	2	100	7	100	6	100
Turnips	28	82	0	3	100	5	80	13	85	7	71
Other root/tuber vegetables	160	92	1	39	87	25	88	49	96	47	94
Vegetable juices	8	88	0	1	0	3	100	3	100	1	100
Vegetables, dried or paste	347	76	3	86	65	108	74	80	86	73	81
Vegetables with sauce	127	65	4	9	56	36	72	51	53	31	81
Other vegetable-related products	22	73	5	8	75	7	71	3	67	4	75
Total	12957	44	5	3382	44	3197	41	3470	45	2908	46
F. Other											
Whole coriander	164	72	10	34	41	34	88	45	84	51	71
Other whole spices	197	62	13	37	62	17	59	26	88	117	56
Ground spices	26	46	23	9	56	3	33	6	33	8	38
Other spices & flavorings	65	34	48	6	83	9	22	5	40	45	31
Peanuts	32	28	47	11	73	17	0	2	0	2	50
Other nuts & related products	122	94	2	63	100	21	81	21	86	17	100
Sesame seeds	20	55	5	5	60	9	67	2	0	4	50
Other seeds & related products	29	69	0	6	67	6	100	8	63	9	56
Refined vegetable oil	9	78	0	3	100	2	100	1	100	3	33
Other vegetable oil products	9	56	0	4	50	1	0	2	50	2	100
Alcoholic beverages	23	91	0	1	100	1	100	–	–	21	90
Beverage bases	39	67	0	–	–	5	80	26	69	8	50
Coffee & tea	80	71	4	27	44	17	94	14	79	22	82
Bottled spring & mineral water	9	56	11	–	–	–	–	–	–	9	56
Other waters & soft drinks	16	75	0	–	–	2	100	3	100	11	64
Chocolate & cocoa products	9	44	0	4	50	1	100	3	0	1	100
Food sweeteners	90	91	1	2	100	9	100	68	93	11	73
Other food products	26	46	4	9	33	4	50	–	–	13	54
Total	965	68	11	221	68	158	69	232	80	354	61
A–F Total	21355	48	5	5102	49	4928	45	5605	48	5720	49

[a]Classified as violative by laboratory performing analysis; does not necessarily represent final agency conclusion on compliance status of sample.

DAIRY PRODUCTS

Direct and Indirect Determination of True Protein Content of Milk by Kjeldahl Analysis: Collaborative Study

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Currently, the reference procedure for determination of the "protein" content of milk is based on measurement of the total nitrogen content of milk by the Kjeldahl method (AOAC method, 920.105). About 6% of the total nitrogen content of milk is nonprotein nitrogen. Therefore, total nitrogen multiplied by the conversion factor 6.38 overestimates the true protein content of milk on average by about 6%. In the present study, new direct and indirect methods were developed for measurement of the true protein content of whole milk by Kjeldahl nitrogen determination. Both new methods are sample preparation procedures used to fractionate the nitrogen-containing compounds in milk prior to measurement of the nitrogen content of these fractions by Kjeldahl analysis. The collaborative study consisted of 9 pairs of blind duplicate milk samples that were analyzed for total nitrogen, nonprotein nitrogen, and protein nitrogen by each of 10 laboratories. Both methods for true protein measurement (direct and indirect) gave acceptable statistical performance characteristics and good agreement between methods. The new direct method requires about half the laboratory analysis work of the indirect method (i.e., total minus nonprotein nitrogen). The methods have been adopted official first action by AOAC as (*1*) a new method for nonprotein nitrogen determination in milk, (*2*) a new method (direct) for determination of protein nitrogen content of milk, and (*3*) an alternative method (indirect) for determination of protein nitrogen content of milk.

As the value of milk fat decreases because of lower consumer demand for high fat dairy products and the value of milk protein increases, the importance of milk protein measurement will increase. Historically, total nitrogen (determined by Kjeldahl analysis) has been used as a reference method for measurement of the "protein" content of milk. However, when the "protein" content of milk is based on Kjeldahl total nitrogen multiplied by 6.38, it assumes that all nitrogen present in milk is associated with protein. It has been documented that this assumption is not correct and that approximately 6% of the nitrogen content of milk (1) is present in the form of nonprotein nitrogen (NPN). The NPN content of milk varies from farm-to-farm and has been reported to range from about 2 to 10% of the total nitrogen content (2–4). The major NPN components of milk are urea, creatine, creatinine, amino acids, and other minor nitrogen-containing compounds (1, 5). It has been estimated that urea contributes about half of the NPN content of milk (1, 5).

NPN and true protein components in milk are not of equal value nutritionally for the consumer or functionally for the dairy product manufacturer. In the future, the dairy industry may need to place more emphasis on the protein content of milk as an index of the relative values of milks produced by different farms. The true protein nitrogen content of milk is a more correct basis for establishing the protein value of different milks than is the total nitrogen content of milk because the proportion of total nitrogen that is due to NPN varies from farm to farm (3, 4).

The Kjeldahl method for measurement of the total nitrogen content of milk was recently optimized and collaboratively studied (6). This nitrogen measurement method (6), which received AOAC interim official first action approval and has been adopted by AOAC, was utilized in the present study for measurement of the nitrogen content of fractions prepared from milk.

Development of sample preparation procedures for standardized NPN and standardized true protein nitrogen determinations in the present study were based on the fundamental work done by Rowland (7) on fractionation of nitrogen-containing compounds of milk. Trichloroacetic acid (TCA) was used to selectively precipitate the proteins in milk. Two approaches, an indirect and a direct method, were investigated for determination of true protein content of milk. In the indirect method, total nitrogen and NPN contents of a milk sample are determined and the true protein nitrogen content is obtained by difference. In the direct method, the milk protein is precipitated, collected, and analyzed directly for nitrogen content.

The objectives of the present research were to (*1*) develop, optimize, and standardize the sample preparation procedures for determination of the NPN and true protein nitrogen contents of milk and (*2*) collaboratively study the within- and between-laboratory performance of the indirect and direct methods of measurement of the true protein content of milk.

Method Development

Traditionally, when analysts measured the NPN or true protein content of milk, a final concentration of 12% TCA in solution with milk was used to precipitate protein. The fil-

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The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Foods I. The methods were approved interim official first action by the Chairman of the Official Methods Board and were adopted official first action at the 104th AOAC Annual International Meeting, September 10–13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* 74, January/February issue.
[1] J. Richard Fleming is Chairman, Test Procedures Committee of the Federal Milk Marketing Orders.

trate contained the NPN compounds. The total nitrogen content of the original milk and the nitrogen content of the TCA filtrate (i.e., nonprotein nitrogen) obtained from the same milk sample were measured. The true protein content of milk was calculated by difference between total nitrogen and NPN. There has never been an AOAC method for determination of NPN in milk. Therefore, the first phase of our work was to develop a standardized procedure for measurement of NPN in milk.

The general approach for the NPN determination was as described by Rowland (7), using a 15% TCA solution mixed with milk to obtain a final concentration of TCA in the mixture of approximately 12%. Other concentrations of TCA and different volume ratios of milk to TCA solution have been used over the years and were evaluated in preliminary work by the Associate Referee prior to the collaborative study. Higher concentrations of TCA mixed with smaller volumes of milk gave consistently lower values for NPN than did the classical method developed by Rowland (7). Therefore, the 15% solution described by Rowland was selected as the standard TCA concentration.

All of the NPN contained in 10 mL of milk was collected in the TCA filtrate, which resulted from mixing 10 mL of milk with 40 mL of 15% TCA solution. However, only a 20 mL aliquot of that filtrate was used for NPN measurement. An equation for calculation of the NPN content of milk was developed based on the assumption that milk contains about 3.5% fat and 3.0% true protein. The denominator of the equation (shown in the method) for calculation of the NPN content of milk represents the weight of milk in the 20 mL aliquot of the TCA filtrate. The 0.065 constant factor in the denominator was used as an estimate of the weight of the fat/protein precipitate that was removed from the filtrate. If the 0.065 constant factor were used for a milk sample that had 1.0% higher or lower total fat plus protein (i.e., 7.5 or 5.5% instead of 6.5%), then the final result would be lower or higher by 0.0004%, respectively, on a protein basis (nitrogen × 6.38). This error is extremely small and is of no practical significance for most purposes. In the present study, the sum of true protein and fat for the 9 individual sample materials ranged from 6.04 to 7.17%, with an average of 6.51%.

Measurement of both total nitrogen and NPN to determine the true protein content of milk doubles the amount of analysis work compared with only measuring the total nitrogen. The additional work and expense to determine true protein has been one of the reasons the dairy industry has preferred the use of total nitrogen to estimate the protein content of milk. Therefore, a method was developed for direct determination of the true protein nitrogen content of milk by the Kjeldahl method. The principle is exactly the same as for the measurement of NPN. The most important detail of the new method is that coagulation of milk protein with TCA must be done in the Kjeldahl flask that will be used for analysis of that sample. This allows for complete recovery of the precipitated milk protein. The direct approach is half the work and cost for true protein determination compared to the more traditional indirect approach.

Collaborative Study

Each of 10 laboratories received 2 sets of 18 raw milk samples (9 pairs of blind duplicates). Each pair of blind duplicates represented milk from 1 farm. One set of samples was used for determination of total nitrogen and NPN (indirect method); the other set of samples was used for the direct protein nitrogen determination method. The sample numbering on the 2 sets of samples received by each laboratory was different as was the analysis order. Thus, the analyst did not know which samples corresponded between or within sets. Sample coding was designed such that all samples had different identification numbers in all laboratories. Sample testing order was randomized between laboratories.

A 3-digit sample coding system was used. A computer program was prepared to translate the codes and match blind duplicate test results, as well as to calculate AOAC statistical parameters and conduct outlier tests (8, 9). Data collection forms, preprinted with sample code numbers listed in the order of sample analysis, were sent to each laboratory to minimize data recording errors. Each laboratory submitted a copy of final test values, plus a copy of all raw data (i.e., weights and titration volumes). All calculations were checked by the staff in the Associate Referee's laboratory.

A form was provided for the analyst to comment about each individual sample, and a questionnaire was provided for each test method. Information, such as sample arrival time and actual testing conditions during the analysis, was requested to help assure that analysts followed all details of the procedures.

Raw milks (5 L) were collected from 9 different farms on Monday (day 1) and transported on ice to the central laboratory. On day 2, milk samples were cold-split in the central laboratory: milk from each farm was mixed in 1 large plastic container, agitated continuously with a motor-driven stirrer, and milk was drawn from a spout directly into coded 6-oz Whirl-Pak sample bags. Forty-six or more 6-oz Whirl-Pak bags of milk were prepared (80 mL/bag). Samples were refrigerated immediately after splitting. Sample splitting uniformity was verified with a Dairylab 2 infrared milk analyzer (**972.16**) (10) by assaying fat and protein content of milk in the first, middle, and last sample bag.

On Tuesday afternoon, when milk from all 9 farms had been split, the samples were put into appropriate groupings, packed in ice, placed in insulated shipping containers, and sent by overnight air delivery to participating laboratories. On Wednesday (day 3), samples arrived at the laboratories and testing was initiated as soon as possible. Arrival temperature of the milks was always ≤4°C. All testing was completed by Monday (day 8). Test results and completed questionnaires arrived at the Associate Referee's laboratory by Wednesday (day 10). All data were summarized and returned to individual testing laboratories by day 16. Collaborators verified that the data used by the Associate Referee for the statistical analysis for their laboratory were entered into the computer correctly. Milk quality was verified as described previously (11).

991.21 Nonprotein Nitrogen in Whole Milk
Kjeldahl Method
First Action 1991

Method Performance:

$s_r = 0.006$; $s_R = 0.012$; $RSD_r = 2.817\%$; $RSD_R = 5.707\%$

A. Principle

Protein is precipitated from milk by addition of trichloroacetic acid (TCA) solution. Final concentration of TCA in the mixture is ca 12%. Precipitated milk protein is removed by filtration. Filtrate contains nonprotein nitrogen components of milk. Nitrogen content of filtrate is determined as in **991.20.**

B. Apparatus

See **991.20B** or **991.20I**.

C. Reagents

See **991.20C** or **991.20J** and in addition:

(a) *Trichloroacetic acid solution.*—15% w/v, analytical grade CCl_3COOH. (*Caution:* See safety note on trichloroacetic acid.) TCA is soft, white, deliquescent crystal, which should be stored in container protected from light and moisture.

(b) *Hydrochloric acid standard solution.*—0.010N HCl. Prepare as in **936.15**. Alternatively, use premade solution of certified specification range 0.0101–0.0099N and use 0.010 for calculation.

D. Preparation of Sample

Warm milk to 38 ± 1°. Mix milk as in **925.21**. Immediately pipet milk (10 ± 0.1 mL) into preweighed 125 mL Erlenmeyer flask and weigh. Record all weights to nearest 0.0001 g. Add 40 ± 0.5 mL 15% TCA solution to flask. Weigh flask and contents. Swirl to mix. Let precipitate settle (ca 5 min). Filter (Whatman No. 1 paper, 15 cm, N-free; or equivalent) and collect entire filtrate. Filtrate should be clear and free of particulate matter; if it is not, repeat sample preparation. Swirl filtrate to mix. Pipet 20 ± 0.2 mL filtrate into a 50 mL beaker and weigh. Pour filtrate from beaker into Kjeldahl digestion flask that contains boiling chips, K_2SO_4, and $CuSO_4 \cdot 5H_2O$ catalyst solution as in **991.20D** or **991.20K**. Immediately reweigh empty beaker. Add H_2SO_4 as in **991.20D** or **991.20K**. Flask may be stoppered and held for digestion at later time. Digest and distill a blank solution (16 ± 0.5 mL 15% TCA and no sample) each day samples are analyzed. Keep record of blank values. If blank values change, identify cause.

E. Determination

Proceed as in **991.20E** or **991.20L**, substituting 0.010N HCl solution for 0.100N HCl solution as titrant in **991.20E(c)** or **991.20L(b)**.

F. Calculation

Calculate results as follows:

$$\text{Nitrogen, \%} = [1.4007 \times (V_s - V_b) \times N]/\{(W_f \times W_m)/[W_t - (W_m \times 0.065)]\}$$

where V_s and V_b = mL titrant used for sample and blank, respectively; N = normality of HCl solution; W_f = weight, g, of 20 mL filtrate; W_m = weight, g, of milk; and W_t = weight, g, of milk plus 40 mL 15% TCA solution. *Note:* Factor 0.065 in denominator assumes that milk contains about 3.5% fat and 3.0% true protein (i.e., 0.035 + 0.030). Factor may need to be adjusted if liquid dairy products of different composition are analyzed (i.e., concentrated or fractionated skim or whole milk products, etc.).

$$\text{"Protein equivalent," \%} = \text{\% nitrogen} \times 6.38$$

which is nonprotein nitrogen expressed as protein equivalent.

G. Repeatability and Reproducibility

For method performance parameters obtained in collaborative study of this method, r value = 0.016 and R value = 0.033.

Ref.: JAOAC **74,** March/April issue (1991).

991.22 Protein Nitrogen Content of Milk
Kjeldahl Method
First Action 1991
Direct Method

Method Performance:

$s_r = 0.008$; $s_R = 0.021$; $RSD_r = 0.285\%$; $RSD_R = 0.702\%$

A. Principle

Protein is precipitated from milk by trichloroacetic acid (TCA) solution. Precipitation *must be* done in Kjeldahl flask or tube. Final concentration of TCA in mixture is ca 12%. The 12% TCA solution, which contains nonprotein nitrogen components of sample, is separated from protein precipitate by filtration. Nitrogen content of protein precipitate is determined as in **991.20.**

B. Apparatus

See **991.20B** or **991.20I**.

C. Reagents

See **991.20C** or **991.20J** and in addition:

(a) *Trichloroacetic acid solution.*—15% w/v, analytical grade CCl_3COOH. (*Caution:* See safety note on trichloroacetic acid.) TCA is soft, white, deliquescent crystal, which should be stored in container protected from light and moisture.

D. Preparation of Sample

Warm milk to 38 ± 1°. Mix milk as in **925.21**. Immediately place weighed sample (5 ± 0.1 mL) in Kjeldahl digestion flask. Record all weights to nearest 0.0001 g. Add 5 ± 0.1 mL H_2O, rinsing any milk on neck of flask into bulb. Add 40 ± 0.5 mL 15% TCA solution to flask. Swirl mixture. Let precipitate settle (ca 5 min). Pour mixture from Kjeldahl flask through filter paper (Whatman No. 1, 15 cm, N-free; or equivalent) and collect filtrate. (Some protein precipitate will remain in Kjeldahl flask and some will be collected on paper. It is not necessary to remove precipitate from flask.)

Immediately after pouring mixture (do not let precipitate dry on neck of Kjeldahl flask), use pump dispenser to add 10 ± 0.5 mL 15% TCA to Kjeldahl flask and rinse any precipitate on neck of flask down into bulb. Swirl to mix. Pour mixture from Kjeldahl flask through same filter paper, and add filtrate to that previously collected. Immediately rinse neck of Kjeldahl flask with another 10 ± 0.5 mL rinse of 15% TCA solution. Swirl to mix and pour mixture from flask through same filter paper used earlier. Collect entire filtrate. Filtrate should be clear and free of particulate matter. At this point, filtrate is no longer needed and may be discarded in an appropriate manner.

Wearing TCA-resistant gloves, pick up filter paper; take care not to lose any precipitate. Pinch paper at top and twist sides and bottom to form oblong shape. If any precipitate remains on either inner or outer lip of Kjeldahl flask, wipe with filter paper so precipitate adheres to paper. Drop filter paper into Kjeldahl flask. Add boiling chips, K_2SO_4, $CuSO_4 \cdot 5H_2O$ catalyst solution, and H_2SO_4 as in **991.20D** or **991.20K**. Flask may be stoppered and held for digestion at later time. Digest and distill a blank (filter paper) each day that samples are analyzed. Keep record of blank values. If blank values change, identify cause.

E. Determination

Proceed as in **991.20E** or **991.20L**.

F. Calculation

Calculate protein nitrogen in milk as in **991.20G**.

G. Repeatability and Reproducibility

For method performance parameters obtained in collaborative study of this method, r value = 0.024 and R value = 0.059.

Ref.: JAOAC **74**, March/April issue (1991).

991.23 Protein Nitrogen Content of Milk
Kjeldahl Method
First Action 1991
Indirect Method

Method Performance:
s_r = 0.014; s_R = 0.031; RSD_r = 0.483%; RSD_R = 1.051%

A. Principle

Total nitrogen and nonprotein nitrogen contents of milk sample are determined separately. Difference between results of these 2 determinations is protein nitrogen content of milk.

B. Determination

(a) *Total nitrogen.*—Determine as in **991.20**.
(b) *Nonprotein nitrogen.*—Determine as in **991.21**.

C. Calculation

Subtract nonprotein nitrogen content from total nitrogen content of milk sample and multiply result by 6.38.

D. Repeatability and Reproducibility

For method performance parameters obtained in collaborative study of this method, r value = 0.040 and R value = 0.088.

Ref.: JAOAC **74**, March/April issue (1991).

Results and Discussion

The data for determination of total nitrogen, NPN, protein nitrogen determined indirectly, and protein nitrogen determined directly are presented in Tables 1, 2, 3, and 4, respectively. Invalid data and statistical outliers are identified in these tables. The total amount of invalid and outlier data for this study are within the acceptable limits for collaborative studies (9).

The AOAC statistical parameters for repeatability and reproducibility were summarized by sample material. In general, the statistical parameters for total nitrogen determination (Table 5) were similar to those reported in an earlier study (6). The between-laboratory agreement for total nitrogen in this study was not quite as good as that observed in the previous study (6); s_R = 0.026 and 0.017, RSD_R = 0.822 and 0.504%, respectively.

Statistical performance characteristics for determination of the NPN content of milk have not been reported previously. The concentration of NPN in milk is low, about 6% of the total nitrogen (1). Therefore, it was expected (and observed) that values for RSD_r and RSD_R would be much larger for NPN than for total nitrogen. Statistical parameters for milk NPN determination are presented in Table 6. The average value for NPN as a percent of total nitrogen for the milk samples used in this study was 6.4%.

Statistical performance characteristics of indirect and direct methods of determination of true protein content of milk are shown in Tables 7 and 8, respectively. The repeatability and reproducibility of the direct method for true protein (Table 8) were better than these values for the indirect method (Table 7). This was expected because the indirect method requires 2 separate measurements (total and nonprotein nitrogen), which will increase within-laboratory and between-laboratory variation of results. The indirect method would be useful when separate direct determination of the NPN content of milk is required in addition to the protein content.

The mean test values for true protein in milk by the indirect and direct methods were similar (2.976 and 2.995%, respectively). The difference in mean protein concentration

Table 1. Total nitrogen in raw milk (blind duplicates) expressed on % protein basis (nitrogen × 6.38)

	Laboratory									
Material	A	B	C	D	E	F	G	H	I	J
1	3.206	3.176	3.216	3.151	3.199	3.160	3.181	3.226	3.148	3.182
	3.204	3.178	3.224	3.183	3.213	3.170	3.198	3.222	3.148	3.175
2	3.197	3.203	3.203	3.166	3.213	3.159	3.200	3.224	3.146	3.099
	3.184	3.182	3.210	3.173	3.182	3.158	3.204	3.225	3.131	3.110
3	3.132	3.121	3.145	3.105	3.086	3.127	3.135	3.121	3.076	3.110
	3.142	3.114	3.135	3.107	3.132	3.099	3.128	3.153	3.102	3.032
4	3.180	3.153	3.098	3.154	2.952[a]	3.139	3.172	3.197	3.133	3.171
	3.172	3.123	3.174	3.143	3.168[a]	3.133	3.162	3.185	3.121	3.121
5	3.127	3.119	3.154[a]	3.120	3.145	3.097	3.144	3.119	3.097	3.120
	3.137	3.132	3.038[a]	3.130	3.111	3.106	3.162	3.156	3.092	3.125
6	3.492	3.483	3.471	3.445	3.505	3.439	3.478	3.493	3.437	3.452
	3.487	3.481	3.492	3.463	3.478	3.454	3.474	3.513	3.432	3.426
7	2.950	2.933	2.944	2.902	2.934	2.913	2.946	2.971	2.920	2.926
	2.941	2.926	2.921	2.894	2.935	2.906	2.949	2.949	2.908	2.910
8	3.181	3.180	3.204	3.167	3.186	3.156	3.188	3.200	3.140	3.161
	3.177	3.151	3.182	3.146	3.186	3.145	3.195	3.196	3.126	3.126
9	3.316	3.294	3.329	3.290	3.315	3.264	3.322	3.336	3.271	3.281
	3.308	3.303	3.342	3.267	3.320	3.274	3.317	3.328	3.262	3.281

[a] Invalid data (incorrect amount of NaOH delivered).

Table 2. Nonprotein nitrogen in raw milk (blind duplicates) expressed on % protein basis (nitrogen × 6.38)

	Laboratory									
Material	A	B	C	D	E	F	G	H	I	J
1	0.206	0.206	0.183	0.199	0.163[a]	0.189	0.194	0.201	0.215	0.212
	0.218	0.209	0.198	0.200	0.193[a]	0.201	0.192	0.208	0.209	0.206
2	0.230	0.228	0.213	0.216	0.183[a]	0.213	0.221	0.245	0.236	0.269[b]
	0.226	0.234	0.216	0.236	0.233[a]	0.234	0.226	0.220	0.232	0.256[b]
3	0.241	0.238	0.217	0.228	0.151[a]	0.224	0.228	0.227	0.256	0.259
	0.242	0.242	0.213	0.228	0.224[a]	0.225	0.227	0.234	0.243	0.266
4	0.203	0.192	0.192[c]	0.187	0.186	0.186	0.190	0.194	0.208	0.199
	0.200	0.195	0.173[c]	0.190	0.180	0.187	0.192	0.196	0.205	0.204
5	0.211	0.207	0.196	0.206	0.197	0.197	0.201	0.203	0.223	0.215
	0.213	0.213	0.184	0.198	0.210	0.195	0.200	0.207	0.230	0.228
6	0.197	0.187	0.164	0.179	0.179[a]	0.175	0.187	0.178	0.202	0.199
	0.193	0.185	0.170	0.192	0.178[a]	0.182	0.187	0.193	0.198	0.191
7	0.195	0.194	0.177	0.183	0.183[a]	0.178	0.186	0.187	0.210	0.219
	0.197	0.199	0.181	0.187	0.179[a]	0.181	0.191	0.197	0.212	0.236
8	0.206	0.197	0.180	0.194	0.183[a]	0.185	0.189	0.196	0.213	0.210
	0.202	0.205	0.186	0.187	0.184[a]	0.212	0.186	0.197	0.205	0.224
9	0.197	0.198	0.177	0.184	0.182[a]	0.183	0.182	0.202	0.205	0.206
	0.198	0.193	0.174	0.192	0.168[a]	0.186	0.187	0.193	0.212	0.218

[a] Invalid data (analysis performed on twice warmed samples).
[b] Statistical outlier (Grubbs test).
[c] Statistical outlier (Cochran test).

between the 2 methods is even smaller when the data are recalculated to include only laboratories (n = 5) where complete data were available for both the indirect and direct methods (2.983 and 2.987%, respectively). The difference in mean test value between methods is much smaller than the difference in mean test value between laboratories within either method. Therefore, the small difference in mean test value between methods is of little practical significance.

Summary and Conclusions

Results from the indirect and direct methods for measurement of true protein in milk are in good agreement. The repeatability and reproducibility of both methods are acceptable. Within- and between-laboratory performance for the true protein determination was slightly better using the direct method. Repeatability and reproducibility of the direct method for true protein nitrogen determination compared very favorably with performance of method **991.20** for determination of total nitrogen. Therefore, there would not be a significant change in within- and between-laboratory agreement of test results if the basis for estimation of the protein content of milk were changed from total nitrogen to protein

Table 3. True protein nitrogen in raw milk (blind duplicates) determined by *Indirect* method (TN minus NPN) and expressed on % protein basis (nitrogen × 6.38)

	Laboratory									
Material	A	B	C	D	E	F	G	H	I	J
1	3.000	2.970	3.033	2.952	3.036[a]	2.972	2.988	3.025	2.933	2.970
	2.986	2.969	3.026	2.983	3.020[a]	2.969	3.006	3.014	2.940	2.969
2	2.967	2.975	2.990	2.950	3.031[a]	2.945	2.978	2.979	2.909	2.830[b]
	2.958	2.948	2.995	2.937	2.949[a]	2.924	2.978	3.005	2.900	2.853[b]
3	2.891	2.882	2.928	2.877	2.935[a]	2.902	2.907	2.894	2.819	2.852
	2.900	2.873	2.922	2.879	2.908[a]	2.875	2.900	2.920	2.858	2.766
4	2.977	2.961	2.906[b]	2.967	2.766[c]	2.953	2.982	3.003	2.925	2.972
	2.972	2.927	3.002[b]	2.953	2.988[c]	2.946	2.971	2.989	2.916	2.917
5	2.916	2.911	2.958[c]	2.914	2.947	2.900	2.944	2.917	2.874	2.904
	2.923	2.919	2.854[c]	2.932	2.901	2.912	2.962	2.949	2.862	2.897
6	3.295	3.296	3.308	3.266	3.326[a]	3.264	3.291	3.316	3.235	3.252
	3.294	3.296	3.321	3.271	3.300[a]	3.272	3.287	3.320	3.234	3.235
7	2.756	2.739	2.767	2.719	2.751[a]	2.734	2.761	2.785	2.710	2.707
	2.743	2.727	2.740	2.706	2.756[a]	2.724	2.757	2.752	2.695	2.674
8	2.975	2.983	3.024	2.973	3.003[a]	2.971	2.999	3.005	2.927	2.950
	2.975	2.946	2.996	2.959	3.002[a]	2.932	3.010	2.999	2.920	2.902
9	3.120	3.097	3.152	3.106	3.133[a]	3.081	3.139	3.135	3.066	3.075
	3.111	3.110	3.168	3.076	3.152[a]	3.088	3.130	3.136	3.050	3.063

[a] Invalid nonprotein nitrogen data.
[b] Nonprotein nitrogen statistical outlier.
[c] Invalid total nitrogen data.

Table 4. True protein nitrogen in raw milk (blind duplicates) determined by *direct* method and expressed on % protein basis (nitrogen × 6.38)

	Laboratory									
Material	A	B	C	D	E	F	G	H	I[a]	J
1	3.001	3.000	3.030	2.980	2.994	2.960	2.982	3.022	2.949	3.021
	3.011	2.993	3.027	2.989	3.005	2.969	2.996	3.032	2.983	3.028
2	2.973	2.970	2.980	2.980	2.987	2.934	2.981	2.998	2.936	2.995
	2.975	2.962	2.981	2.953	2.995	2.951	2.999	3.004	2.911	2.989
3	2.914	2.895	2.929	2.880	2.932	2.866	2.913	2.917	2.873	2.910
	2.897	2.912	2.920	2.890	2.917	2.847	2.911	2.928	3.052	2.908
4	2.975	2.979	3.003	2.961	3.001	2.950	2.986	3.002	2.919	2.982
	2.971	2.971	2.990	2.958	3.007	2.942	2.991	2.989	2.933	2.980
5	—[b]	2.930	2.952	2.893	2.946	2.900	2.934	2.928	2.874	2.946
	2.926[b]	2.921	2.943	2.917	2.955	2.899	2.955	2.944	2.856	2.937
6	3.303[c]	3.299	3.316	3.283	3.328	3.271	3.305	3.324	3.258	3.303
	3.137[c]	3.308	3.316	3.285	3.327	3.251	3.313	3.331	3.259	3.301
7	2.749	2.751	2.757	2.719	2.786	2.725	2.759	2.721	2.708	2.775
	2.753	2.755	2.749	2.739	2.775	2.722	2.764	2.756	2.736	2.765
8	2.990	2.975	2.986	2.962	3.023	2.955	2.981	2.995	2.937	3.000
	2.994	2.974	3.012	2.976	3.000	2.931	2.979	2.992	2.943	2.986
9	3.125	3.116	3.127	3.116	3.140	3.082	3.131	3.145	3.096	3.145
	3.123	3.109	3.138	3.099	3.145	3.081	3.122	3.142	3.065	3.123

[a] Invalid data (distillate diluted and transferred for titration).
[b] Invalid data (laboratory accident).
[c] Statistical outlier (Cochran test).

Table 5. AOAC statistical parameters by sample material for total nitrogen expressed on % protein basis

	Material									
Statistic	1	2	3	4	5	6	7	8	9	Mean
No. of labs	10	10	10	9	9	10	10	10	10	—
No. of accepted values	20	20	20	18	18	20	20	20	20	—
Mean, %	3.188	3.179	3.115	3.152	3.124	3.470	2.929	3.170	3.301	3.181
s_r	0.009	0.010	0.023	0.024	0.014	0.012	0.009	0.013	0.007	0.014
RSD_r, %	0.292	0.316	0.753	0.745	0.432	0.340	0.315	0.412	0.224	0.425
s_R	0.026	0.037	0.028	0.027	0.020	0.026	0.020	0.025	0.026	0.026
RSD_R, %	0.815	1.155	0.900	0.865	0.644	0.737	0.693	0.787	0.799	0.822
r value	0.026	0.028	0.066	0.066	0.038	0.033	0.026	0.037	0.021	0.038
R value	0.074	0.104	0.079	0.077	0.057	0.072	0.057	0.071	0.075	0.074

Table 6. AOAC statistical parameters by sample material for nonprotein nitrogen expressed on % protein basis

	Material											
		2			4							
Statistic	1	All data	Outlier removed	3	All data	Outlier removed	5	6	7	8	9	Mean[a]
No. of labs	9	9	8	9	10	9	10	9	9	9	9	—
No. of accepted values	18	18	16	18	20	18	20	18	18	18	18	—
Mean, %	0.203	0.231	0.227	0.236	0.193	0.194	0.207	0.187	0.195	0.199	0.194	0.204
s_r	0.006	0.010	0.010	0.004	0.005	0.002	0.006	0.006	0.005	0.008	0.005	0.006
RSD_r, %	3.065	4.262	4.378	1.731	2.514	1.158	2.758	3.066	2.747	4.060	2.391	2.817
s_R	0.009	0.015	0.009	0.015	0.009	0.008	0.012	0.011	0.016	0.012	0.012	0.012
RSD_R, %	4.653	6.527	4.121	6.224	4.621	4.095	5.736	5.721	8.297	6.121	6.399	5.707
r value	0.018	0.028	0.028	0.012	0.014	0.006	0.016	0.016	0.015	0.023	0.013	0.016
R value	0.027	0.043	0.026	0.041	0.025	0.023	0.034	0.030	0.046	0.034	0.035	0.033

[a] Mean values only include data for sample materials 2 and 4 with outliers removed.

Table 7. AOAC statistical parameters by sample material for true protein nitrogen determined by *indirect* method (TN minus NPN) and expressed on % protein basis

	Material									
Statistic	1	2	3	4	5	6	7	8	9	Mean
No. of labs	9	8	9	8	9	9	9	9	9	—
No. of accepted values	18	16	18	16	18	18	18	18	18	—
Mean, %	2.984	2.959	2.880	2.958	2.916	3.281	2.733	2.969	3.106	2.976
s_r	0.010	0.012	0.024	0.017	0.015	0.006	0.014	0.019	0.010	0.014
RSD_r, %	0.327	0.399	0.840	0.586	0.528	0.177	0.522	0.636	0.333	0.483
s_R	0.030	0.031	0.040	0.027	0.026	0.030	0.029	0.035	0.035	0.031
RSD_R, %	0.999	1.060	1.382	0.901	0.887	0.899	1.048	1.172	1.110	1.051
r value	0.028	0.033	0.069	0.049	0.044	0.017	0.040	0.053	0.029	0.040
R value	0.084	0.089	0.113	0.075	0.073	0.084	0.081	0.099	0.098	0.088

Table 8. AOAC statistical parameters by sample material for true protein nitrogen determined by *direct* method and expressed on % protein basis

	Material										
						6					
Statistic	1	2	3	4	5	All data	Outlier removed	7	8	9	Mean[a]
No. of labs	9	9	9	9	8	9	8	9	9	9	—
No. of accepted values	18	18	18	18	16	18	16	18	18	18	—
Mean, %	3.002	2.978	2.905	2.980	2.931	3.294	3.304	2.751	2.984	3.123	2.995
s_r	0.007	0.009	0.009	0.006	0.010	0.039	0.006	0.011	0.011	0.008	0.008
RSD_r, %	0.222	0.310	0.314	0.184	0.349	1.196	0.186	0.381	0.372	0.242	0.285
s_R	0.022	0.019	0.023	0.019	0.021	0.045	0.023	0.020	0.021	0.020	0.021
RSD_R, %	0.738	0.647	0.796	0.642	0.712	1.357	0.693	0.725	0.719	0.650	0.702
r value	0.019	0.026	0.026	0.016	0.029	0.111	0.017	0.030	0.031	0.021	0.024
R value	0.063	0.055	0.065	0.054	0.059	0.127	0.065	0.056	0.061	0.057	0.059

[a] Mean values only include data for sample material 6 with outlier removed.

nitrogen. Protein values based on true protein nitrogen content of milk will be lower than those based on total nitrogen.

Milk "protein" measured using total nitrogen multiplied by 6.38 may be the same for 2 farms (e.g., 3.2%). However, if the portion of total nitrogen present as NPN in milk from one farm is 3% and from the other farm 8%, then the true protein content of these 2 milks will differ (i.e., 3.104 vs 2.944%, respectively). On a total nitrogen basis for payment, the same value would be placed on these 2 milks. However, on a true protein nitrogen basis the milks would have different values. The difference in true protein nitrogen content of milk from these 2 farms reflects more correctly the relative differences in their protein values (i.e., for dairy product manufacture and nutritional value) than the "protein" based on measurement of total nitrogen.

Recommendations

The Associate Referee recommends the following: (*1*) the new method for determination of the nonprotein nitrogen content of milk be adopted official first action, (*2*) the new direct method for measurement of true protein nitrogen content of milk be adopted official first action, and (*3*) the new indirect method for measurement of the true protein nitrogen content of milk be adopted official first action method as an alternative method.

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Eastern Ohio/Western Pennsylvania
Greater Kansas City
New England
New York/New Jersey
Texas
Upper Midwest

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References

(1) Walstra, P., & Jenness, R. (1984) *Dairy Chemistry & Physics*, John Wiley & Sons, New York, NY, pp 137–138
(2) Szijarto, L., Biggs, D. A., & Irvine, D. M. (1973) *J. Dairy Sci.* **56,** 45–51
(3) Bruhn, J. C., & Franke, A. A. (1979) *J. Dairy Sci.* **62,** 1326–1328
(4) Franke, A. A., Bruhn, J. C., & Lawrence, C. H. (1988) *J. Dairy Sci.* **71,** 2373–2383
(5) Webb, B. H., Johnson, A. H., & Alford, J. A. (1974) *Fundamentals of Dairy Chemistry*, 2nd Ed., Avi Publishing Co., Westport, CT, p. 41
(6) Barbano, D. M., Clark, J. L., Dunham, C. E., & Fleming, J. R. (1990) *J. Assoc. Off. Anal. Chem.* **73,** 849–859
(7) Rowland, S. J. (1938) *J. Dairy Res.* **9,** 42–46
(8) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(9) Horwitz, W. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 160–173
(10) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA
(11) Barbano, D. M., Clark, J. L., & Dunham, C. E. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 898–914

DRUG FORMULATIONS

Liquid Chromatographic Determination of Six Sympathomimetic Drugs in Dosage Forms

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A simple and rapid stability-indicating liquid chromatographic method is described for quantitative determination of 6 sympathomimetic drugs in various liquid and solid formulations. Analyses were carried out on a C_{18} reverse phase column using 0.01M 1-octanesulfonic acid, sodium salt in 0.2% acetic acid–methanol (70 + 30) as the mobile phase with photometric detection at 220 nm. Coefficients of variation for 5 consecutive injections of a mixed standards solution ranged from 0.62% for metaraminol to 1.40% for epinephrine. Standard recoveries ranged from 98.8% for metaraminol to 100.8% for epinephrine. The method was linear between 0.2 and 10 μg of drug injected and was used successfully to analyze 17 commercial products in a variety of dosage forms.

The sympathomimetic or adrenergic drugs affect those cell chemicals that mediate sympathetic nerve transmissions (1). They have clinical application primarily as vasoconstrictors, bronchodilators, and nasal decongestants.

Liquid chromatographic (LC) methods have been described for determination of sympathomimetic compounds in pharmaceutical preparations. These methods determine a single compound (2), resolve enantiomers (3–6), or separate various formulations in combination with other drug substances (7–10). Official *United States Pharmacopeia* methods (11) for each drug involve LC analysis with ultraviolet detection.

The present paper describes a single, stability-indicating LC method that can be applied to the sympathomimetics and can distinguish them on the basis of differing retention times. In addition, the method eliminates the time-consuming process of changing chromatographic conditions between samples when a variety of these products are to be tested. The present method is useful for both identification and assay of commercial products.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Tracor model 950 pump, equipped with variable wavelength detector (Tracor Inc., Austin, TX 78725), Spectra Physics model 4100 computing integrator (Spectra Physics, Santa Clara, CA 95051), Rheodyne model 7125 injector valve with 20 μL sample loop (Rheodyne Inc., Cotati, CA 94928), and Whatman Partisil-5, ODS-3, 25 cm × 4.6 mm column, 5 μm particle size (Whatman, Inc., Clifton NJ 07014). Operating conditions.—Column temperature, ambient; mobile phase flow rate, 1.0 mL/min; detection wavelength, 220 nm; range, 0.32 AUFS; recorder range, 1 mV; chart speed, 1 cm/min.

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Reagents

(a) *Chemicals and solvents.*—LC or analytical reagent grade. 1-Octanesulfonic acid, sodium salt (Eastman Kodak Co., Rochester, NY 14650).

(b) *Mobile phase.*—0.01M 1-Octanesulfonic acid, sodium salt in 0.2% glacial acetic acid–methanol (70 + 30) (8), filtered through a 0.45 μm filter and degassed under vacuum.

(c) *Standards.*—USP reference standards, norepinephrine (NE) bitartrate, epinephrine (EPI) bitartrate, phenylephrine (PHE) hydrochloride, and levonordefrin (LEV) (US Pharmacopeial Convention, Inc. Rockville, MD 20853). Isoproterenol (ISO) hydrochloride and metaraminol (MET) bitartrate were in-house working reference standards. All were dried according to USP specifications.

(d) *Standard solutions.*—Prepare solutions in 0.01N HCl in which final concentration of free base is 100 μg/mL for EPI (182 μg/mL EPI bitartrate), NE (199 μg/mL NE bitartrate), and LEV; and 200 μg/mL for ISO (234 μg/mL ISO hydrochloride), PHE (244 μg/mL PHE hydrochloride), and MET (380 μg/mL MET bitartrate).

Samples

Injections of norepinephrine bitartrate (1 mg norepinephrine/mL), epinephrine hydrochloride (1 mg epinephrine/mL), isoproterenol hydrochloride (1 mg isoproterenol HCl/5 mL), metaraminol bitartrate (10 mg metaraminol/mL), phenylephrine hydrochloride (1% phenylephrine HCl) as well as tablets of isoproterenol hydrochloride (10 mg isoproterenol HCl/mL) and phenylephrine hydrochloride ophthalmic solution (10% phenylephrine HCl) were obtained from commercial sources.

Sample Preparation

Tablets.—Weigh and finely powder not less than 20 tablets. Transfer an accurately weighed quantity of powder to a suitable volumetric flask, dissolve in 0.01N HCl to a concentration equivalent to that of the respective standard solution and filter if necessary.

Injections, ophthalmic solutions, and inhalation solutions.—Transfer an accurately measured volume of liquid from a composite of the pooled contents of not fewer than 3 containers to a suitable volumetric flask and dilute in 0.01N HCl as for tablets.

Samples for Recovery Study

Samples and standards were prepared as described above. Equal volumes of corresponding solutions were mixed and aliquots of mixtures were injected into chromatograph.

Determination

Equilibrate LC column with mobile phase for ca 1 h. Proceed with analysis by injecting equal volumes of the corresponding standard and sample preparations.

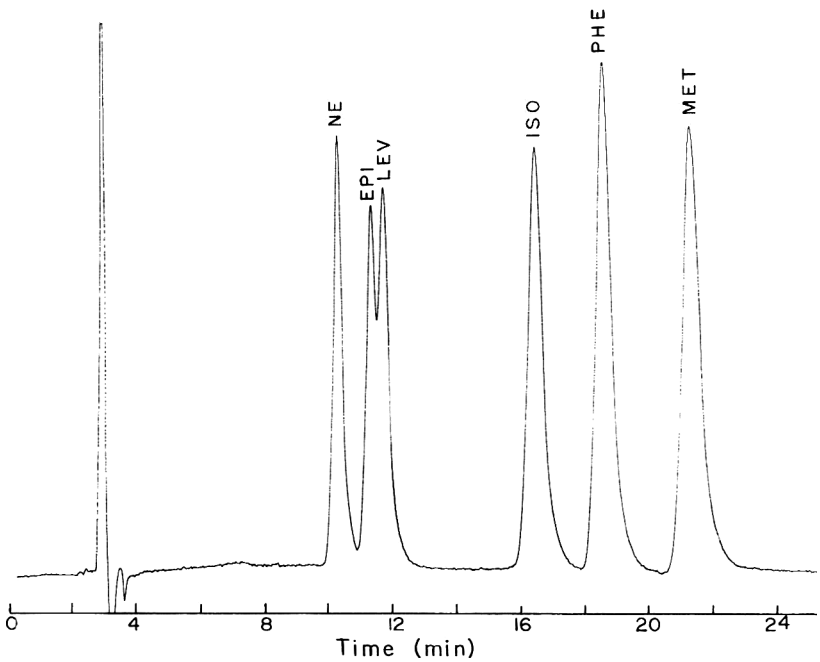

Figure 1. LC separation of a mixture of sympathomimetic drugs (0.32 AUFS). Tartaric acid elutes at the solvent front. NE = norepinephrine, EPI = epinephrine, LEV = levonordefrin, ISO = isoproterenol, PHE = phenylephrine, and MET = metaraminol. See text for chromatographic conditions.

Calculation

Calculate quantity of drug using a comparison of peak responses of samples to those of corresponding standards as follows:

Tablets.—

$$\text{mg/tab.} = (Ru/Rs)(C)(T/S)(D)(F)$$

where Ru and Rs = peak height responses for the sample preparation and standard preparation, respectively; C = concentration of standard, mg/mL; T = average tablet weight; S = weight of sample composite taken for analysis, mg; D = sample dilution; and F = ratio of formula weight of free base to its salt. F = (169.18/319.26) for NE; (183.21/333.29) for EPI; (167.21/317.29) for MET; and 1 for LEV, PHE, and ISO.

Injections, ophthalmic solutions or inhalation solutions.—

$$\text{mg/mL} = (Ru/Rs)(C)(D/V)(F)$$

where V = volume of sample taken; other symbols have the same meanings as above.

Results and Discussion

A typical chromatogram is presented in Figure 1. Tartaric acid from the 3 bitartrate salts elutes at the solvent front. The drugs are baseline separated except for epinephrine and levonordefrin. Because this pair is not a commercially produced combination, the lack of complete resolution is not a practical problem. They are, however, sufficiently well resolved that the presence of at least 10% of one in the other is detectable.

Chromatographic conditions were selected on the basis of applicability to all compounds. A detection wavelength of 265 nm is suitable for each; detection in this region is commonly used for these drugs. However, 220 nm was selected because sensitivity is approximately 4-fold that in the longer wavelength region. Thus, lower limits of detection may be attained, enabling detection of cross contamination.

Results of precision and sensitivity testing are shown in Table 1. Reproducibility of the chromatographic system was determined on the basis of peak area measurements for 5 replicate injections of mixed standards solutions. Sensitivity was determined by establishing the minimum concentration of drug needed to produce a peak twice the amplitude of the baseline noise.

Calibration curves for the sympathomimetics were linear over a range of 0.2 to 10.0 μg of drug injected. The correlation coefficient for each was 0.9996 or greater.

Work was done to determine whether the procedure was stability indicating. Forced degradation of the individual pure drug substances was accomplished by dilution with 1N NaOH for 1 h followed by neutralization with HCl (9). Base degradation was used because these drug substances undergo oxidation at an elevated pH. Discolored solutions were injected with the result that extra peaks appeared only in the region of the solvent front. A decrease in peak responses for the drugs was observed.

Four known impurities were also tested on the system. Their names and retention times relative to the corresponding parent peak are: (*1*) adrenalone, impurity of EPI (11), relative retention time (RRT) = 1.23; (*2*) adrenochrome, oxidation product of EPI (12), RRT = 0.29; (*3*) benzaldehyde, hydrolysis product of PHE (13), RRT = 0.50; and (*4*) *m*-hydroxybenzaldehyde, degradation product of PHE (13), RRT = 0.30.

The USP establishes a limit for norepinephrine, a bio-

Table 1. Precision and sensitivity of 6 sympathomimetics

Compound	RSD, % ±	Sensitivity, ng
EPI	1.40	33
NE	0.65	27
LEV	0.73	36
ISO	0.81	53
PHE	0.93	40
MET	0.62	50

Table 2. Analysis of commercial products and spike recoveries[a]

Compound	Manuf.	Dosage form	Spike recovery, %	Declared, %	
				LC assay[b]	USP limit
EPI	A	Inj	100.8	105.1	90.0–115.0
	A	Inj		111.3	
	B	Inj	99.1	107.8	
	B	Inj		107.3	
NE	C	Inj	100.2	112.0	90.0–115.0
	C	Inj		110.8	
ISO	B	Inj	99.5	104.5	90.0–115.0
	B	Inj		104.3	
	C	Inhal soln	99.2	105.9	90.0–115.0
	C	Inhal soln		110.1	
	C	Tablets	99.6	99.2	93.0–107.0
	C	Tablets		96.9	
PHE	C	Inj	99.4	100.4	90.0–115.0
	C	Inj		98.8	
	C	Ophth soln		102.6	90.0–115.0
MET	D	Inj[c]	98.8	102.7	90.0–110.0
	D	Inj		101.3	

[a] Levonordefrin is not commercially available as a single component drug.
[b] Average of duplicate assays.
[c] Methyl paraben in the injections eluted just before the peak of interest.

chemical precursor of epinephrine, in epinephrine bulk drug of not more than 4.0%. Although no such requirement exists for the epinephrine dosage forms, work was done to determine its detectability. The limit of detection of norepinephrine in epinephrine solutions was 5 mg/mL, corresponding to 0.5% of the epinephrine concentration. Norepinephrine was not detected in any epinephrine injectable solutions.

Commercially available products were assayed for drug content by the proposed LC method. Recoveries on those products were determined for mixed portions of samples and corresponding standards. Recoveries ranged from 98.8% for MET to 100.8% for EPI (Table 2).

In conclusion, this method offers a significant advantage in that it provides a single, accurate, and precise means of assay for sympathomimetic drugs in dosage forms. Because it can be used interchangeably on a variety of these drugs, it eliminates the time normally required to change chromatographic systems.

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References

(1) McLean, R. (1960) in *Medicinal Chemistry*, A. Burger (Ed.), Interscience Publishers, Inc., New York, pp. 595–620
(2) Causon, R. C., Desjardins, R., Brown, M. J., & Davies, D. S. (1984) *J. Chromatogr.* **306,** 257–268
(3) Allgire, J. F., Juenge, E. C., Damo, C. P., Sullivan, G. M., & Kirchhoefer, R. D. (1985) *J. Chromatogr.* **325,** 249–254
(4) Scott, B. S., & Dunn, D. L. (1985) *J. Chromatogr.* **319,** 419–426
(5) Kirchhoefer, R. D., Sullivan, G. M., & Allgire, J. F. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 163–165
(6) Gal, J. (1984) *J. Chromatogr.* **307,** 220–223
(7) Wouters, I., Roets, E., & Hoogmartens, J. (1984) *J. Pharm. Biomed. Anal.* **2,** 481–490
(8) Schieffer, G. W., & Hughes, D. E. (1983) *J. Pharm. Sci.* **72,** 55–59
(9) Ghanekar, A. G., Das Gupta, V. (1978) *J. Pharm. Sci.* **67,** 1247–1250
(10) Alvi, S. U., & Castro, F. (1978) *J. Liquid Chromatogr.* **9,** 2269–2279
(11) *U.S. Pharmacopeia* (1990) 22nd Rev., U.S. Pharmacopeial Convention, Inc., Rockville, MD
(12) *Merck Index* (1983) 10th Ed., Merck & Co., Rahway, NJ
(13) Schieffer, G. W., Smith, W. O., Lubey, G. S., & Newby, D. G. (1984) *J. Pharm. Sci.* **73,** 1856–1858

DRUG RESIDUES IN ANIMAL TISSUES

Matrix Solid Phase Dispersion (MSPD) Isolation and Liquid Chromatographic Determination of Furazolidone in Pork Muscle Tissue

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A method for the isolation and liquid chromatographic (LC) determination of furazolidone in pork muscle tissue is presented. Blank or furazolidone-fortified pork muscle tissue samples (0.5 g) were blended with octadecylsilyl (C_{18}, 18% load, endcapped, 2 g) derivatized silica. A column made from C_{18}/pork matrix was first washed with hexane (8 mL), followed by elution of furazolidone with ethyl acetate. The ethyl acetate extract was then passed through an activated alumina column. The eluate contained furazolidone that was free from interfering compounds when analyzed by LC with UV detection (photodiode array, 365 nm). Detector response with increasing concentrations of furazolidone isolated from fortified samples was linear ($r = 0.998 \pm 0.002$) with an average percentage recovery of 89.5 ± 8.1% for the concentration range (7.8–250 ng/g) examined and resulted in a minimum detectable limit of 390 pg on column, and a detector response of more than 5 times baseline noise. The inter-assay variability was 9.9 ± 5.4% with an intra-assay variability of 1.5%.

Furazolidone, a nitrofuran, has been used in animal production as an antimicrobial agent. As a feed additive, it can increase animal vigor and aid in growth promotion. It is an effective therapeutic drug used to treat bacterial scours, enteritis, and bloody dysentery in swine (1). It can also be used topically to treat bacterial infections resulting from wounds, abrasions, or lacerations as well as generalized and chronic infections of the skin.

Use of furazolidone in food producing animals has been restricted because evidence indicates it is a mutagenic (2) and carcinogenic (3) agent. Furazolidone can be present as a residue in animal-derived pork products from its intentional or inadvertent use. An additional concern relates to the international import/export trade of pork products from countries where furazolidone is not restricted or may be improperly monitored. Thus, the U. S. Department of Agriculture/Food Safety Inspection Service (USDA/FSIS) has included furazolidone as a residue of concern in the *Compound Evaluation and Analytical Capability National Residue Program Plan* (4).

Methods for extracting and analyzing residues should be rapid, specific, and sensitive enough to allow for isolation and detection of target residues below the action level established by regulatory agencies. The U.S. Food and Drug Administration (FDA) has established a zero tolerance for residues of furazolidone in edible tissues of swine. However, for regulatory purposes, FDA has defined a level of 100 ppb (100 ng/g) as the limit of quantification in approving uses of this drug in food producing animals (4). The 100 ppb level is used by FDA for enforcement purposes and the USDA/FSIS uses the identical level for its role in residue monitoring programs (4).

We can improve residue monitoring programs by introducing residue isolation methods that have short sample preparation times and minimize the use of expendable materials, especially solvents. Ideally, these sample preparation protocols should involve few steps and result in extracts that contain the target residue(s) with high recoveries and a minimum of background interference. In this regard, classical isolation techniques for furazolidone have included sample homogenization in large volumes of extracting solvents, back washing, additional solvent partitioning steps, and evaporation of large volumes of extracting solvents (5–7). Thus, the need for simplified furazolidone residue isolation techniques exists.

We recently developed a method for the multiresidue/multiclass extraction of drugs from biological matrixes (8–12) [known as matrix solid phase dispersion (MSPD)] that overcomes many complications associated with classical isolation techniques. We report here the first use of MSPD methodology for isolation of furazolidone from pork muscle tissue.

Experimental

Apparatus and Reagents

(a) *Solvents.*—(LC grade) highest purity available from commercial sources; use without further purification.

(b) *Water.*—For LC analyses; triple-distilled and passed through Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system.

(c) *Furazolidone.*—Sigma Chemical Co., St. Louis, MO.

(d) *Column material.*—Bulk C_{18} (40 μm, 18% load, endcapped; Analytichem International, Harbor City, CA). Clean by making a column (50 mL syringe barrel) of bulk C_{18} material (22 g) and sequentially washing with 2 column volumes each of hexane, dichloromethane (DCM), and methanol. Vacuum-aspirate washed C_{18} until dry. Prepare activated alumina (80–200 mesh Type F-20; Alltech, Inc., Deerfield, IL) columns by placing 0.5 g activated alumina into a 10 mL plastic syringe barrel plugged with a filter paper disc (No. 1; Whatman, Clifton, NJ). Cover column head with filter paper disc (No. 1), compress, and wash with 4 mL ethyl acetate just before use.

(e) *Stock furazolidone solutions.*—1 mg/mL (1 + 1, v/v). Prepare by dissolving standard compound with LC grade methanol–dichloromethane (1 + 1, v/v). Dilute to desired μg/mL levels (0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 μg/mL) with methanol. Place a 10 μL portion of each of these stock solutions into LC vials and add a solution (0.1 mL methanol–0.3 mL 0.015M H_3PO_4, v/v) to each vial to make a final volume of 400 μL.

(f) *Sample extraction columns.*—Ten mL syringe barrels

[1]Address inquiries to this author.

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(Becton-Dickinson, Rutherford, NJ); thoroughly wash and dry before use.

(g) *Protective hood.*—Use during extraction and drying steps.

Sample Preparation, Fortification, and Extraction Procedure

Fresh pork muscle tissue samples were obtained from a local market. Random 0.5 g tissue samples were excised from whole pork tissue and samples were randomized. No attempt was made to select lean tissue only. Sampling was carried out by randomly slicing sections of tissue at various locations within the whole sample and using 0.5 g subsections of the randomized tissue for fortification studies. Two grams C_{18} were placed in a glass mortar. Standard furazolidone (10 μL of 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 μg/mL stock solutions) was randomly injected into tissue (0.5 g of the previously excised tissue samples) and fortified samples were allowed to stand for 4 min before blending into the C_{18}. Blank tissue control samples were prepared similarly except that 10 μL methanol containing no furazolidone was added to the sample. Blank control tissues contained no incurred furazolidone residue.

Samples were gently blended into the C_{18} with a glass pestle until the mixture was homogenous in appearance. A gentle circular motion with very little pressure produced a homogenous mixture. The resultant C_{18}/tissue matrix was placed into a 10 mL plastic syringe barrel plugged with a filter paper disc (Whatman No. 1). The column head was covered with a filter paper disc and the column contents were then compressed to a final volume of 4.5 mL with a syringe plunger that had the rubber end and pointed plastic portion removed. A pipette tip (100 μL plastic Flex Tips; Brinkmann Instruments, Inc., Westbury, NY) was placed on the column outlet to increase residence time of the eluting solvents on the column. The resulting column was first washed with 8 mL of LC grade hexane. If the initial solvent flow through the column was hindered, positive pressure was applied to the column head (pipette bulb) to initiate gravity flow, after which flow was gravity controlled. When flow ceased, excess hexane was removed from the column with positive pressure as described above.

Furazolidone was then eluted with 8 mL ethyl acetate collected in a 10 mL conical glass test tube. The ethyl acetate eluate was passed through a prewashed (ethyl acetate) activated alumina column. The ethyl acetate eluate was collected in a 10 mL conical glass test tube and then dried under a steady stream of dry nitrogen gas. A solution (0.1 mL methanol and 0.3 mL 0.015M H_3PO_4) was added to the test tube contents. The sample was then sonicated (5–10 min) to disperse the residue, which resulted in a suspension. The contents of the tube were then transferred to a microcentrifuge tube and centrifuged (IEC Centra M, International Equipment Company, Needham Hts., MA) at 17 000 × *g* for 5 min. The resultant clear supernatant was filtered through a 0.45 μm filter (Prep Disc, Bio Rad Inc., Richmond, CA) and a portion (20 μL) was analyzed by liquid chromatography.

Liquid Chromatographic Analysis

Analyses of extracted sample and standard furazolidone were conducted with a Hewlett Packard HP 1090 liquid chromatograph (HP 79994A Chemstation) equipped with a photodiode array detector set at 365 nm with a bandwidth of 20 nm and spectrum range of 200–450 nm. The solvent system was 0.015M H_3PO_4–acetonitrile (60 + 40, v/v) at an isocratic flow rate of 1 mL/min. A reverse-phase octadecylsilyl (ODS) derivatized silica column (10 μm, 30 cm × 4 mm id, Micro Pak, Varian, Sunnyvale, CA) maintained at 45°C was used for all determinations.

Recovery Experiments and Statistical Treatment

Standard curves were obtained by plotting integrated peak areas of standards and standards extracted from fortified samples (n = 5 replicates) at each respective concentration (n = 6 levels: 7.8, 15.6, 31.3, 62.5, 125, and 250 ng/mL furazolidone). A comparison of extracted furazolidone-fortified sample areas to areas of pure standards run under identical conditions gave absolute percent recoveries [mean of 30 samples plus or minus the standard deviation (SD)]. Interassay variability was determined as follows: The mean of the areas for 5 replicates of each concentration (7.8, 15.6, 31.25, 62.5, 125, and 250 ng/mL or ng/g pork tissue) was calculated. The standard deviation corresponding to each mean was divided by its respective mean and multiplied by 100, which resulted in the coefficient of variation (CV) for each concentration. The mean of these CVs was calculated along with its SD and was defined as the interassay variability plus or minus the SD. Intra-assay variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean area of 5 replicates of an identical sample (125 ng furazolidone/g pork tissue).

Discussion

Isolation of chemical residues from a complex biological matrix such as tissue can be time-consuming and require large quantities of materials. Isolation techniques that serve to minimize labor and materials, especially solvents, are advantageous because the cost of each analysis can be reduced and sample throughput can be increased.

Sample preparation steps used to isolate a given residue from an animal matrix can consume the majority of the time and materials during total analysis. Further, analytical capability is highly dependent on the cleanliness of the sample extract, which is a direct consequence of the procedure used to isolate the residue. Classical residue isolation techniques (5–7) have traditionally involved extractions of the matrix with large volumes of solvents, solvent backwashing, pH adjustments, additional backwashing, and evaporation of large volumes of extracting solvents to facilitate the isolation of residues free from interferences.

The technique we describe to isolate furazolidone from pork muscle tissue produced extracts that were free from interferences, as can be seen in the LC chromatograms of an extract of a blank control pork muscle sample [Figure 1(A)] and of a furazolidone-fortified pork muscle extract [Figure 1(B)]. Some potential interferences are eliminated by using a UV photodiode array detector set at 365 nm, making many compounds present in the extract transparent to the detector. However, a photodiode array detector allows one to obtain a characteristic UV spectrum of furazolidone (Figure 2), which can serve to further confirm the presence of this compound in the extract.

Table 1 shows recoveries of furazolidone at each concentration. The standard curve correlation coefficients (0.998 ± 0.002) and intra- (1.5%, 125 ppb) and interassay (9.9 ± 5.4%) variabilities were acceptable. Recoveries were consistent at each concentration examined; and standard curves were linear, indicating that this extraction procedure is suitable for furazolidone isolations from tissue. The minimum

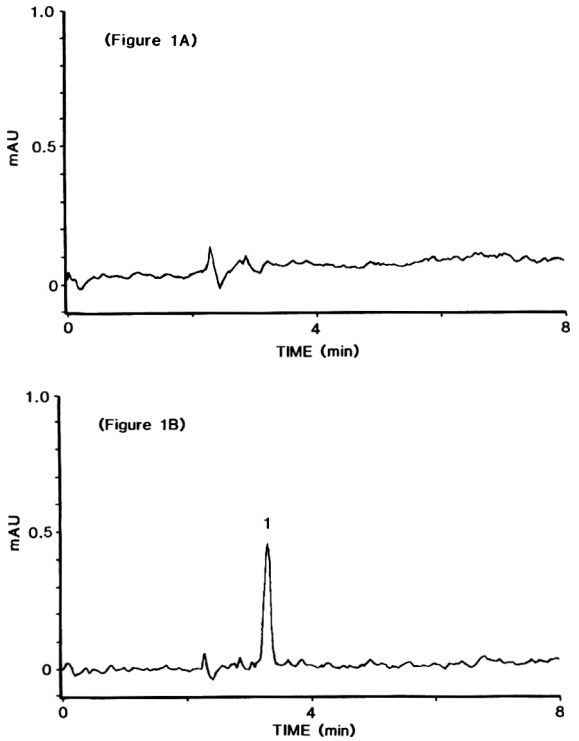

Figure 1. Representative LC chromatograms (UV, photodiode array set at 365 nm) of (A) ethyl acetate extract of blank pork muscle tissue control and (B) furazolidone-fortified (31.3 ng/gm, 782.5 pg on column) (18) pork muscle tissue. Peak number 1 is furazolidone.

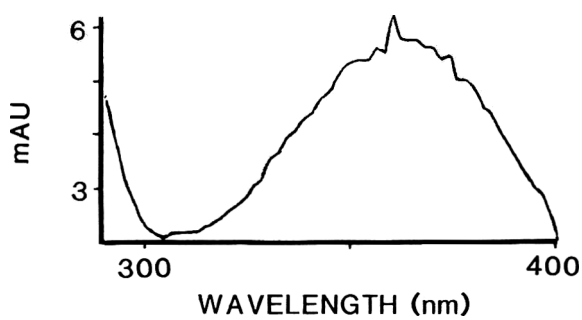

Figure 2. UV spectrum scan (290–400 nm) of furazolidone obtained from LC analysis (UV photodiode array set at 365 nm, 250 ng/g tissue) of ethyl acetate extract of furazolidone-fortified pork muscle tissue.

detectable limit was 195 pg (7.8 ng/g tissue, 20 μL injection volume from a total final volume of 0.4 mL) on column, and, therefore, provides adequate sensitivity. Because the sample extract is free from interferences when monitored at 365 nm, the sensitivity of the assay may be increased by injecting more sample or dissolving the extract residue in a smaller final volume.

The theoretical aspects of the MSPD technique have been the subject of previous papers (8–12); however, this is the first reported use of MSPD for isolation of furazolidone from pork tissue. In the MSPD technique, the sample is dispersed by mechanical and hydrophobic forces over a large surface area (1000 m^2/2 g of C_{18}, theoretical), exposing the entire sample to the extraction process. Even though the washing and extracting solvent volumes are small (8 mL), the process is an exhaustive extraction, whereby a thin layer of sample is extracted with a large volume of solvent. Using a sequential elution protocol, one may remove interferences in one solvent and then elute the residue of interest in a different solvent.

Table 1. Determination of furazolidone in pork muscle tissue by MSPD

Furazolidone added, ng/g	Amt found, ng/g ± SD[a]	Recovery, %	Coeff. of variation
7.8	7.56 ± 0.52	96.9	6.9
15.7	16.09 ± 1.54	102.5	9.6
31.3	26.32 ± 4.37	84.1	16.6
62.5	53.38 ± 4.64	85.4	8.7
125.0	103.38 ± 8.06	82.7	7.8
250.0	213.75 ± 6.86	85.5	3.2
Av. for method		89.5 ± 8.1	

[a] $n = 6$

Use of an activated alumina column to clean the MSPD ethyl acetate extract after extraction is an additional step that removes potential interferences found in the ethyl acetate extract. The alumina cleanup step made the extract drying and residue solubilizing steps easier and provided for a cleaner LC analysis.

The MSPD method for isolation of furazolidone from pork muscle tissue is rapid, minimizes the use of expendable material (especially solvents), and provides a sample extract free of interferences when analyzed by UV detection at 365 nm. The method may be applicable to furazolidone isolations from other matrixes as well.

Acknowledgments

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REFERENCES

(1) *Code of Federal Regulations* (1988) Title 21, Part 558:262, Washington, DC
(2) Klemencic, J.M., & Wang, C.Y. (1978) "Mutagenicity of Nitrofurans," in *Nitrofurans: Chemistry, Metabolism, Mutagenesis and Carcinogenesis*, G.T. Bryan (Ed.), Raven Press, New York
(3) Cohen, S.M. (1978) "Toxicity and Carcinogenicity of Nitrofurans," in *Nitrofurans: Chemistry, Metabolism, Mutagenesis and Carcinogenesis*, G.T. Bryan (Ed.), Raven Press, New York
(4) *Compound Evaluation and Analytical Capability National Residue Program Plan*, (1988) J. Brown (Ed.), U.S. Dept of Agriculture, Food Safety & Inspection Service, Washington, DC
(5) Ernst, G.F., & Van der Kaaden, A. (1980) *J. Chromatogr.* **198**, 526–528
(6) Nakabeppu, H., & Tatsumi, K. (1984) *Chem. Pharm. Bull.* **32**(10), 4193–4196
(7) Vroomen, L.H.M., Van Ommen, B., & Van Bladeren, P.J. (1987) *Toxic. In Vitro.* **1**(2), 97–104
(8) Barker, S.A., Long, A.R., & Short, C.R. (1989) *J. Chromatogr.* **475**, 353–361
(9) Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., & Barker, S.A. (1989) *J. Assoc. Off. Anal. Chem.* **72**(5), 739–741
(10) Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., & Barker, S.A. (1989) *J. Assoc. Off. Anal. Chem.* **72**(5), 813–815
(11) Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., & Barker, S.A. (1989) *J. Liq. Chromatogr.* **475**, 404–411
(12) Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., & Barker, S.A. (1990) *J. Agr. Food Chem.* **38**(2), 423–426

ENVIRONMENTAL QUALITY

Capillary Column Gas Chromatography with Nitrogen-Phosphorus Detection for Determination of Nitrogen- and Phosphorus-Containing Pesticides in Finished Drinking Waters: Collaborative Study

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A joint U.S. Environmental Protection Agency/AOAC interlaboratory method validation study was conducted on EPA Method 507, Determination of Nitrogen- and Phosphorus-Containing Pesticides in Finished Drinking Water by Gas Chromatography with a Nitrogen-Phosphorus Detector, to determine the mean recovery and precision for analyses of 45 nitrogen- or phosphorus-containing pesticides in reagent water and finished drinking waters. The study design was based on Youden's nonreplicate plan for collaborative tests of analytical methods. The waters were spiked with 45 nitrogen- or phosphorus-containing pesticides at 6 concentration levels, prepared as 3 Youden pairs. Ten volunteer laboratories extracted the spiked test waters with methylene chloride, performed a solvent exchange with methyl *tert*-butyl ether, and analyzed an aliquot of each extract by gas chromatography using a nitrogen-phosphorus detector. Results were analyzed using an EPA computer program, which measured recovery and precision for each of the 45 pesticides and compared the performance of the method between water types. Method 507 was judged acceptable for all analytes tested except merphos, which thermally decomposed in the injection port of the gas chromatograph. Five compounds (carboxin, disulfoton, metolachlor, pronamide, and simazine) exhibited statistically significant matrix effects for the finished drinking water. The method has been adopted official first action by AOAC.

The widespread contamination of vulnerable groundwater supplies by pesticides and herbicides became a major concern to the U.S. Environmental Protection Agency (EPA) in the early 1980s. In the fall of 1983, EPA was charged by the Congress of the United States to monitor drinking water supplies in the United States to assess the degree of contamination by these compounds. As a result, EPA initiated the National Pesticide Survey (NPS) to provide a comprehensive assessment of ground water contamination in the 50 states. NPS Method 1 (1), which was developed specifically to analyze the nitrogen- or phosphorus-containing pesticide compounds, uses capillary gas chromatography with a nitrogen-phosphorus detector. EPA Method 507 (2), a slightly modified version of NPS Method 1, was later proposed for measuring the regulated herbicides, alachlor and atrazine (3), as well as 45 other unregulated pesticides and herbicides in vulnerable water supplies.

EPA's Environmental Monitoring Systems Laboratory at Cincinnati, OH (EMSL-Cincinnati), develops or selects analytical methods and provides quality assurance (QA) support for agency programs that involve water and wastewater regulations. In EMSL-Cincinnati, the responsibility for providing QA support is assigned to the Quality Assurance Research Division (QARD). QARD's program provides the QA support to establish the reliability and legal defensibility of water and wastewater data collected by the agency, the state regulating authorities, the private sector, and commercial laboratories that perform compliance analyses. One of QARD's activities is to conduct interlaboratory method validation studies to evaluate analytical methods selected for the agency's operating programs. This paper describes the results of an interlaboratory method validation study performed on EPA Method 507.

AOAC, whose mission is method validation and publication, used its association with state and private laboratories to solicit voluntary participants on methods of common interest to EPA and AOAC. In the spring of 1989, the AOAC Associate Referee provided copies of Method 507 and a description of the study requirements to over 30 laboratories with an invitation to participate in the interlaboratory study. Responses were received from 18 laboratories interested in participating in the study. Study samples were mailed in June 1989; data were returned from 12 laboratories in August 1989.

The study was conducted under the direction of QARD, EMSL-Cincinnati. As primary contractor to QARD, Bionetics Corp. was responsible for the preparation, analysis, and distribution of sample concentrates, user instructions, report forms, review of the returned data, and submission of a final report. The raw data were statistically evaluated by QARD using a computer program, Interlaboratory Method Validation Study (IMVS) (4), designed for these types of studies.

The objective of this study was to characterize multilaboratory performance of Method 507 in terms of recovery, overall and single-analyst precision, and the effect of water type on recovery and precision for 45 of the 47 compounds covered

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The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Environmental Quality. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9-13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* **74**, January/February issue.

in the method. Prometryn was removed from the study because of resolution problems with a compound in each of the 2 mixes used. Demeton-S was unavailable from commercial sources.

Collaborative Study

The study design was based on Youden's nonreplicate design for collaborative evaluation of overall precision, single-analyst precision, and mean recovery for analytical methods (5). Two samples, similar yet different in concentration of the 45 analytes, were analyzed as Youden pairs at each of 3 levels to provide data for estimating single-analyst precision. The 45 analytes were divided into 2 mixes to minimize resolution problems (*see* Table **991.07A**). The collaborating laboratories were directed to extract the samples, analyze each extract, and report 1 value for each analyte present. Analyses of spiked reagent water evaluated the proficiency of the method on a sample free of interferences. Analyses of the spiked finished drinking waters were intended to demonstrate the suitability of the method on a regulated matrix and to compare the results with those for reagent water.

Spiking solutions, calibration standards, and quality control samples were prepared and heat-sealed in ampoules, each containing approximately 1.5 mL solution. Prior to distribution, the ampoule solutions were analyzed against standards freshly prepared from neat materials. At the completion of the study, the ampoules were analyzed again to verify the stability of the solutions over the time of the study.

Each participating laboratory was sent 24 sample ampoules (6 concentrations for each of 2 waters for 2 groups of target compounds), 4 calibration standard concentrates, 2 internal standard concentrates, 2 surrogate standard concentrates, 4 quality control samples with acceptance limits, a copy of Method 507, report forms, and a questionnaire. The collaborating analysts were instructed to analyze the samples in strict accord with the written method and to complete the analyses within 60 days from receipt of the samples.

Treatment of Data

The returned data were grouped by water type, arranged into 6 subsets defined by the 6 different samples, and evaluated analyte by analyte using the EPA IMVS computer program. First, missing data points were replaced by values estimated by interpolation, and "less than" and "nondetect" values were converted to zero. Subsequent outlier tests were those suggested in the ASTM Standard Practice D2777-86 (6). Next, Youden's laboratory ranking procedure was used to reject data from laboratories that had a consistently higher or lower bias in their submitted data for a given analyte compared to that for the other laboratories. If a bias was determined, the 6 analyte values were rejected for that laboratory. This procedure was applied to each analyte data set for each water type at the 5% level of significance. Next, the zeroes and interpolated values were removed before any further analyses. Finally, Thompson's test for individual outliers (7), was applied to the data using a 5% significance level. If an individual datum point was rejected on the basis of this test, it was removed from the subset, and the test was repeated one more time using the remaining data in the subset.

Summary statistics were calculated for the mean recovery and overall method precision for each of the 6 concentration levels. Single-analyst precision was calculated for each of the 3 concentration pairs. The IMVS computer program used these summary statistics to calculate relationships between mean recovery and true concentration and between precision and mean recovery in the form of linear regression equations using the weighted least squares technique (8). Coefficients of determination of the weighted regression equations (COD_w) were also calculated to evaluate the fit of the retained data sets. These weighted equations tend to degrade the calculated COD_w values, which in some cases resulted in very low or negative COD_w values. However, these regression equations can be used to estimate the mean method recovery, overall precision, and single-analyst precision at any value within the concentration range studied. IMVS also determined statistically significant matrix effects between water types for each of the 45 analytes.

991.07 Nitrogen- and Phosphorus-Containing Pesticides in Finished Drinking Water

Gas Chromatographic Method

First Action 1991

(Applicable to determination of 44 nitrogen- or phosphorus-containing pesticides in finished drinking water)

Method Performance:

Collaborative study showed method acceptable for all analytes tested except merphos, which thermally decomposed in GC injection port. Matrix effects were statistically significant for 5 compounds (carboxin, disulfoton, metolachlor, pronamide, and simazine) in finished drinking water. In reagent water, RSD_R for 44 pesticides ranged from 12.8 to 25.4%, and exceeded 25% only for vernolate (25.4%). In finished drinking water, RSD_R for 44 pesticides ranged from 11.5 to 42.6%, and exceeded 25% for tricyclazole (25.1%), terbufos (25.8%), fluridone (28.1%), terbutryn (29.1%), disulfoton (32.5%), and carboxin (42.6%). *See* table of method performance data.

A. Principle

Measured volume of sample (1 L) is extracted with CH_2Cl_2 by shaking in separatory funnel or by mechanical tumbling in bottle. CH_2Cl_2 extract is separated, dried with anhydrous Na_2SO_4, solvent-exchanged with methyl *tert*-butyl ether (MTBE), and concentrated to 5 mL. Pesticides are separated and measured by capillary column gas chromatography using a nitrogen-phosphorus detector. Estimated method detection limits range from 0.075 μg/L for simazine to 5.0 μg/L for mevinphos; estimated method detection limits for 36 pesticides range from 0.13 to 1.0 μg/L.

B. Apparatus

(a) *Grab sample bottles.*—1000 mL, borosilicate glass with TFE-fluorocarbon lined screw caps (Wheaton Media/Lab bottle No. 219820 meets these specifications). Extract liners overnight with methanol before use.

(b) *Separatory funnel.*—2000 mL, borosilicate glass with TFE-fluorocarbon stopcock and ground-glass or TFE-fluorocarbon stopper.

(c) *Tumbler bottle.*—1.7 L, low extractable borosilicate glass with TFE-fluorocarbon lined screw caps (Wheaton Roller Culture Vessel No. 348273 meets these specifications). Cut liners to fit screw cap from TFE-fluorocarbon sheets (Pierce No. 012736 meets these specifications); extract liners overnight with methanol before use.

(d) *Kuderna-Danish (K-D) apparatus.*—(*1*) *Concentrator tube.*—10 or 25 mL, borosilicate glass, graduated, Ŧ 19/22. Check calibration of concentrator tube at volumes used in

Method Performance for **991.07**, Nitrogen- and phosphorus-containing pesticides in finished drinking water[a]

Pesticide	Concn, μg/L[b]	Reagent Water				Finished Drinking Water			
		s_r	s_R	RSD_r, %	RSD_R, %	s_r	s_R	RSD_r, %	RSD_R, %
Alachlor	5.0	0.67	0.78	14.5	16.9	0.68	1.05	14.2	21.9
Ametryn	2.0	0.31	0.28	16.7	15.1	0.19	0.33	9.7	16.3
Atraton	5.0	0.89	0.87	17.8	17.3	0.40	0.62	8.8	13.6
Atrazine	2.0	0.22	0.27	11.8	14.5	0.18	0.31	9.2	15.8
Bromacil	10.0	1.66	1.60	17.4	16.7	0.95	1.84	10.1	19.5
Butachlor	10.0	1.22	1.46	13.0	15.5	1.16	1.59	12.5	17.1
Butylate	5.0	0.74	0.89	19.6	23.4	0.59	0.67	15.4	17.5
Carboxin	10.0	2.04	2.00	21.8	21.4	1.18	3.08	16.3	42.6
Clorpropham	10.0	1.77	1.78	18.2	18.2	0.94	1.87	10.0	19.8
Cycloate	5.0	0.74	0.84	17.3	19.5	0.34	0.63	8.0	14.6
Diazinon	2.0	0.21	0.34	11.8	19.2	0.31	0.44	17.6	24.9
Dichlorvos	5.0	0.74	0.76	15.3	15.6	0.35	0.64	7.8	14.2
Diphenamid	5.0	0.88	0.75	18.7	15.9	0.73	1.20	13.8	22.5
Disulfoton	2.0	0.28	0.33	16.4	19.0	0.22	0.52	14.0	32.5
Disulfoton sulfone	10.0	2.45	2.26	23.6	21.7	1.12	1.94	12.3	21.3
Disulfoton sulfoxide	10.0	1.78	1.86	18.7	19.5	1.02	1.44	10.2	14.4
EPTC	2.0	0.26	0.32	15.3	18.7	0.14	0.23	7.9	13.6
Ethoprop	2.0	0.24	0.37	12.8	20.2	0.18	0.35	9.7	18.8
Fenamiphos	20.0	2.33	3.50	12.9	19.5	2.81	4.32	15.8	24.3
Fenarimol	5.0	1.18	1.09	24.2	22.4	0.57	0.87	12.1	18.4
Fluridone	10.0	2.27	2.47	22.2	24.2	2.08	2.86	20.4	28.1
Hexazinone	5.0	1.11	1.06	22.4	21.3	0.57	0.82	12.2	17.6
Merphos	10.0	0.88	1.80	17.7	36.4	1.01	1.41	18.4	25.6
Methyl paraoxon	10.0	1.11	1.73	11.1	17.2	2.02	2.23	18.8	20.7
Metolachlor	10.0	1.06	1.22	11.4	13.2	0.84	1.24	8.3	12.4
Metribuzin	2.0	0.34	0.38	17.5	19.8	0.19	0.27	9.9	14.5
Mevinphos	10.0	1.52	1.28	15.6	13.2	0.54	1.07	5.8	11.5
MGK-264	10.0	1.48	1.66	16.0	18.0	1.34	1.80	14.4	19.4
Molinate	2.0	0.34	0.34	18.0	18.2	0.14	0.32	8.1	18.3
Napropamide	5.0	0.62	0.69	14.2	15.7	0.49	0.86	11.6	20.2
Norflurazon	5.0	1.27	1.19	26.4	24.8	0.54	0.89	11.8	19.5
Pebulate	2.0	0.32	0.39	17.8	21.8	0.15	0.33	8.8	19.5
Prometon	2.0	0.34	0.33	17.8	17.2	0.22	0.30	11.8	16.4
Promanide	10.0	1.60	1.47	16.4	15.2	0.88	1.38	9.8	15.4
Propazine	2.0	0.32	0.32	17.4	17.2	0.19	0.26	10.4	14.3
Simazine	2.0	0.25	0.28	13.2	14.6	0.19	0.26	9.3	12.9
Simetryn	2.0	0.34	0.34	17.9	18.0	0.18	0.24	9.2	12.8
Stirofos	20.0	3.15	3.87	17.0	20.9	3.04	3.85	15.4	19.5
Tebuthiuron	10.0	1.81	1.79	18.7	18.4	0.81	1.14	8.6	12.0
Terbacil	50.0	5.03	9.35	10.1	18.8	6.41	9.93	13.4	20.7
Terbufos	10.0	0.84	1.50	10.2	18.1	1.28	2.17	15.2	25.8
Terbutryn	2.0	0.22	0.24	11.8	12.8	0.28	0.52	15.6	29.1
Triademefon	2.0	0.37	0.40	18.8	20.3	0.18	0.29	9.8	15.6
Tricyclazole	20.0	3.22	2.95	17.8	16.3	3.65	4.98	18.4	25.1
Vernolate	2.0	0.33	0.41	20.1	25.4	0.14	0.26	8.3	15.7
Average				16.8	18.8			12.0	19.2
Std. dev.				3.8	4.0			3.5	6.0

[a] s_r and s_R = standard deviations for repeatability and reproducibility, respectively. RSD_r and RSD_R = corresponding relative standard deviations.
[b] Concentration value is 10–15 times estimated method detection limit (MDL).

method. Use ground-glass stoppers to prevent evaporation of extracts. (*2*) *Evaporation flask.*—500 mL, borosilicate glass, T 24/40 top, T 19/22 bottom, capable of attachment to concentrator tube with springs. (*3*) *Snyder columns.*—3 ball macro, 218 mm, T 24/40 or 2-ball micro, 170 mm, T 19/22.

(e) *Vials.*—Glass, 5 to 10 mL capacity, with TFE-fluorocarbon lined screw caps.

(f) *Separatory funnel shaker.*—(Optional). Capable of holding 2-L separatory funnels and shaking them with rocking motion to thoroughly mix funnel contents (Eberbach Co., Ann Arbor, MI, or other suppliers).

(g) *Tumbler.*—Capable of holding tumbler bottles end-over-end at 30 rpm (Associated Design and Manufacturing Co., Alexandria, VA, meets these specifications).

(h) *Boiling stones.*—Carborundum, No. 12 granules. Heat 30 min at 400° before use. Cool and store in desiccator.

(i) *Water bath.*—Heated, capable of control ±2°. Use bath in hood.

(j) *Balance.*—Analytical, capable of accurately weighing to nearest 0.0001 g.

(k) *Gas chromatograph.*—Temperature-programmable system for use with capillary columns, including syringes, analytical columns, gases, detector, and strip chart recorder. Data system is recommended for measuring peak areas. Pri-

Table 991.07A Chemical Abstracts Service Registry Numbers, Peak Identification Codes, Retention Times, and Estimated Method Detection Limits for 45 Pesticides

Analyte	CAS No.	Peak No.[a]	Retention Time, min		Estd MDL, μg/L
			Primary[b]	Confirmation[c]	
Alachlor	15972-60-8	B9	35.96	34.1	0.38
Ametryn	834-12-8	A20	36.0	34.52	2.0
Atraton	111-44-4	A13	31.26	29.97	0.6
Atrazine	1912-24-9	B3	31.77	31.23	0.13
Bromacil	314-40-9	A21	37.22	40.0	2.5
Butachlor	23184-66-9	B14	41.45	39.0	0.38
Butylate	2008-41-5	B1	22.47	18.47	0.15
Carboxin	5234-68-5	A27	42.77	42.05	0.6
Chlorpropham	101-21-3	A12	29.09	—[d]	0.5
Cycloate	1134-23-2	A11	28.58	29.67	0.25
Diazinon	333-41-5	B6	33.23	—[d]	0.25
Dichlorvos	62-73-7	A3	16.54	15.35	2.5
Diphenamid	957-51-7	A23	38.87	37.97	0.6
Disulfoton	298-04-4	A17	33.42	30.9	0.3
Disulfoton sulfone	2497-06-5	A24	41.31	42.42	3.8
Disulfoton sulfoxide[e]	2497-07-6	A4	19.08	—[d]	0.38
EPTC	563-12-2	A5	20.07	16.57	0.25
Ethoprop	13194-48-4	B2	28.58	26.42	0.19
Fenamiphos	22224-92-6	B15	41.78	41.0	1.0
Fenarimol	60168-88-9	A30	51.32	50.02	0.38
Fluridone	59756-60-4	A31	56.68	59.07	3.8
Hexazinone	51235-04-2	A29	46.58	47.8	0.76
Merphos[f]	150-50-5	B16	42.35	39.28	0.25
Methyl paraoxon	950-35-6	B7	35.58	34.1	2.5
Metolachlor	51218-45-2	B11	37.74	35.7	0.75
Metribuzin	21087-64-9	A18	35.20	34.73	0.15
Mevinphos	7786-34-7	A6	22.51	21.92	5.0
MGK-264[g]	113-48-4	B12	38.73	36.73	0.5
Molinate	2212-67-1	A10	25.66	22.47	0.15
Napropamide	15299-99-7	A25	41.83	—[d]	0.25
Norflurazon	27314-13-2	A28	45.92	47.58	0.5
Pebulate	1114-71-2	A8	23.41	19.73	0.13
Prometon[e]	1610-18-0	A14	31.58	30.0	0.3
Pronamide[e]	23950-58-5	A16	32.76	32.63	0.76
Propazine	139-40-2	A15	32.01	31.13	0.13
Simazine	122-34-9	B4	31.49	31.32	0.075
Simetryn	1014-70-6	A19	35.72	34.55	0.25
Stirofos	22248-79-9	B13	41.27	39.65	0.76
Tebuthiuron	34014-18-1	A9	25.15	42.77	1.3
Terbacil	5902-51-2	B8	33.79	—[d]	4.5
Terbufos[e]	13071-79-9	B5	32.57	—[d]	0.5
Terbutryn	886-50-0	B10	36.80	34.8	0.25
Triademefon	43121-43-3	A22	38.12	37.0	0.65
Tricyclazole	41814-78-2	A26	42.25	44.33	1.0
Vernolate	1929-77-7	A7	22.94	19.25	0.13

[a] Identification of chromatographic peaks in Figs **991.07A** and **991.07B**. Letters indicate which spiking mixture (A or B) contains the analyte.
[b,c] See method section, **B(k)**, for column description and operating conditions.
[d] Data not available.
[e] Compound shows instability in aqueous solutions.
[f] Merphos is converted to *S,S,S*-tributylphosphorotrithioate (DEF) in the hot GC injection port; DEF is actually detected using the method conditions.
[g] MGK-264 gives 2 peaks; peak identified in this table was used for quantification.

mary column: 30 m × 0.25 mm id DB-5 fused-silica capillary column, 0.25 μm film thickness (J&W Scientific, Inc., meets these specifications). Confirmation column: 30 m × 0.25 mm id DB-1701 fused-silica capillary column, 0.25 μm film thickness (J&W Scientific, Inc., meets these specifications). Operating conditions: injection volume 2 μL splitless with 45 s delay; He carrier gas at 30 cm/s linear velocity; injector 250°; detector 300°; oven programmed from 60 to 300° at 4°/min; nitrogen-phosphorus detector.

C. Reagents

(a) *Standard solutions.*—Use standards of test compounds (Table **991.07A**) with purity >96% to prepare stock solutions at 1 mg/mL in MTBE. Commercially prepared stock standards may be used at any concentration if they are certified by manufacturer or independent source. These stock standards may be available from U.S. Environmental Protection Agency Toxic and Hazardous Materials Repository, Research Triangle Park, NC. Store solutions at room tem-

perature and protect from light. Replace stock solutions after 2 months, or sooner if comparison with laboratory control standards indicates degradation.

(**b**) *Internal standard solution.*—Prepare 2-nitrotoluene (purity >98%) stock solution at 0.25 mg/mL in MTBE. Add 50 μL stock solution to 5 mL sample extract to give final internal standard concentration of 2.5 μg/mL.

(**c**) *Surrogate solution.*—Prepare 1,3-dimethyl-2-nitrobenzene (DMNB) (purity >98%) stock solution at 0.25 μg/mL in MTBE. Add 50 μL stock solution to 1 L sample prior to extraction to produce surrogate concentration of 12.5 μg/L in sample and, assuming quantitative recovery, 2.5 μg/mL in extract.

(**d**) *Instrument performance solution.*—Using standard solutions, (**a**), combine 5 μL vernolate stock solution, 0.5 mL bromacil stock solution, 30 μL prometon stock solution, and 15 μL atrazine stock solution in 100 mL volumetric flask, and dilute to volume with MTBE.

(**e**) *Solvents.*—Acetone, methylene chloride, methyl *tert*-butyl ether (MTBE), distilled-in-glass quality or equivalent.

(**f**) *Phosphate buffer.*—pH 7. Mix 29.6 mL 0.1N HCl and 50 mL 0.1M dipotassium hydrogen phosphate (K_2HPO_4).

(**g**) *Sodium sulfate.*—Granular, anhydrous. ACS grade. Heat in shallow tray for >4 h at 450° to remove interfering organic substances.

(**h**) *Sodium chloride.*—Crystals. ACS grade. Heat in shallow tray for >4 h at 450° to remove interfering organic substances.

(**i**) *Reagent water.*—Water reasonably free of contamination that would prevent determination of any analyte of interest.

(**j**) *Preservative.*—Mercuric chloride solution. 10 mg $HgCl_2$ (ACS grade)/mL reagent water, (**i**).

(**k**) *Sodium thiosulfate.*—$Na_2S_2O_3$. Granular, anhydrous. ACS grade.

D. Preparation of Sample Bottles

Add 1 mL preservative, **C(j)**, to glass sample bottle. If residual chlorine is expected to be present in samples, add 80 mg $Na_2S_2O_3$, **C(k)**, to sample bottle before collection.

E. Sample Collection

Collect 1 L grab samples in glass bottles by conventional sampling practices. Because bottles contain preservative and $Na_2S_2O_3$, do not prerinse bottles with sample before collection. Add sample to bottle containing preservative, seal sample bottle, and shake vigorously 1 min. Refrigerate samples at 4° from time of collection until extracted. Protect from light. Samples are stable for 14 days when stored under these conditions. Extracts, stored at 4° away from light, are stable for 14 days.

F. Sample Preparation

(**a**) *Automated extraction procedure.*—Add preservative, **C(j)**, to any samples not previously preserved. Mark water meniscus on side of sample bottle for later determination of sample volume. Add 50 μL surrogate stock solution, **C(c)**, to sample. If mechanical separatory funnel shaker is used, pour entire sample into separatory funnel. If mechanical tumbler is used, pour entire sample into tumbler bottle. Adjust sample to pH 7 by adding 50 mL phosphate buffer, **C(f)**. Check pH and add H_2SO_4 or NaOH if necessary.

Add 100 g NaCl, **C(h)**, to sample, seal, and shake to dissolve salt. Add 300 mL CH_2Cl_2 to sample bottle, seal, and shake 30 s to rinse inner walls. Transfer solvent to sample contained in separatory funnel or tumbler bottle, seal, and shake 10 s, venting periodically. Repeat shaking and venting until pressure release is not observed during venting. Reseal and place sample container in appropriate mechanical mixing device (separatory funnel shaker or tumbler). Shake or tumble sample for 1 h.

After extraction, pour contents of tumbler bottle into 2 L separatory funnel. Let organic layer separate from water phase for ≥10 min. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation mechanically. Collect CH_2Cl_2 extract in 500 mL erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4. Swirl flask to dry extract; let flask sit 15 min. Determine original sample volume by refilling sample bottle to mark and transferring water to 1000 mL graduated cylinder. Record sample volume to nearest 5 mL.

(**b**) *Manual extraction method.*—Add preservative, **C(j)**, to samples not previously preserved. Mark water meniscus on side of sample bottle for later determination of sample volume. Add 50 μL surrogate stock solution, **C(c)**, to sample. Pour entire sample into 2 L separatory funnel. Adjust sample to pH 7 by adding 50 mL phosphate buffer, **C(f)**. Check pH, and add H_2SO_4 or NaOH, if necessary. Add 100 g NaCl to sample, seal, and shake to dissolve salt. Add 60 mL CH_2Cl_2 to sample bottle, seal, and shake bottle 30 s to rinse inner walls.

Transfer solvent to separatory funnel and extract sample by vigorously shaking funnel for 2 min with periodic venting to release excess pressure. Let organic layer separate from water phase for ≥10 min. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation mechanically. Collect CH_2Cl_2 extract in 500 mL erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4. Add second 60 mL portion of CH_2Cl_2 to sample bottle and repeat extraction procedure a second time, combining extracts in erlenmeyer flask. Perform third extraction in same manner. Swirl flask to dry extract; let flask sit for 15 min. Determine original sample volume by refilling sample bottle to the mark and transferring water to 1000 mL graduated cylinder. Record sample volume to nearest 5 mL.

G. Extract Concentration

Assemble K-D concentrator by attaching 25 mL concentrator tube to 500 mL evaporation flask. Decant CH_2Cl_2 extract into concentrator. Rinse remaining Na_2SO_4 with two 25 mL portions of CH_2Cl_2 and decant rinses into concentrator.

Add 1 or 2 clean boiling stones to evaporation flask and attach macro-Snyder column. Prewet column by adding ca 1 mL CH_2Cl_2 to top. Place K-D apparatus on 65–70° water bath so that concentrator tube is partially immersed in hot water and the entire lower, rounded surface of flask is bathed with hot vapor. Adjust vertical position of apparatus and water temperature as required to complete concentration in 15–20 min. At proper rate of distillation, balls of column will actively chatter, but chambers will not flood. When apparent volume of liquid reaches 2 mL, remove K-D apparatus, let drain and cool ≥10 min.

Remove Snyder column; rinse flask and its lower joint with 1–2 mL MTBE, collecting rinse in concentrator tube. Add 5–10 mL MTBE and fresh boiling stone. Attach micro-Snyder column to concentrator tube and prewet column by adding ca

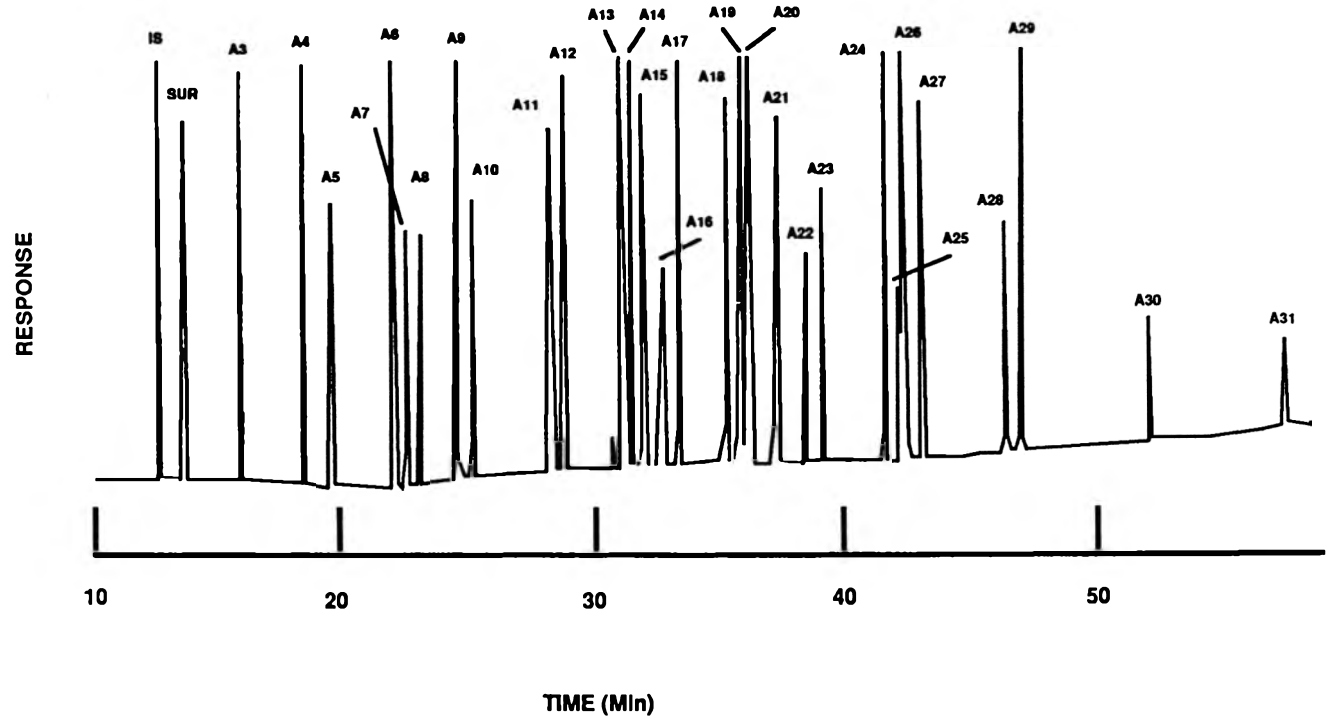

FIG. 991.07A—Reconstructed GC-NPD chromatogram of group A compounds analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column (0.25 μm film). *See* B(k) for operating conditions and Table 991.07A for peak identification. IS = Internal standard; SUR = surrogate standard.

0.5 mL MTBE to top. Place micro K-D apparatus on water bath so that concentrator tube is partially immersed in hot water. Adjust vertical position of apparatus and water temperature as required to complete concentration in 5–10 min. When apparent volume of liquid reaches 2 mL, remove apparatus from bath and let it drain and cool. Add 10 mL MTBE and boiling stone and reconcentrate to 2 mL. When apparent volume of liquid reaches 2 mL, remove apparatus from bath and let it drain and cool. Add second 10 mL MTBE and boiling stone and reconcentrate to 2 mL. When apparent volume of liquid reaches 2 mL, remove apparatus from bath and let it drain and cool. Add third 10 mL MTBE and boiling stone and reconcentrate to 2 mL. Remove micro K-D apparatus from bath and let it drain and cool. Remove micro-Snyder

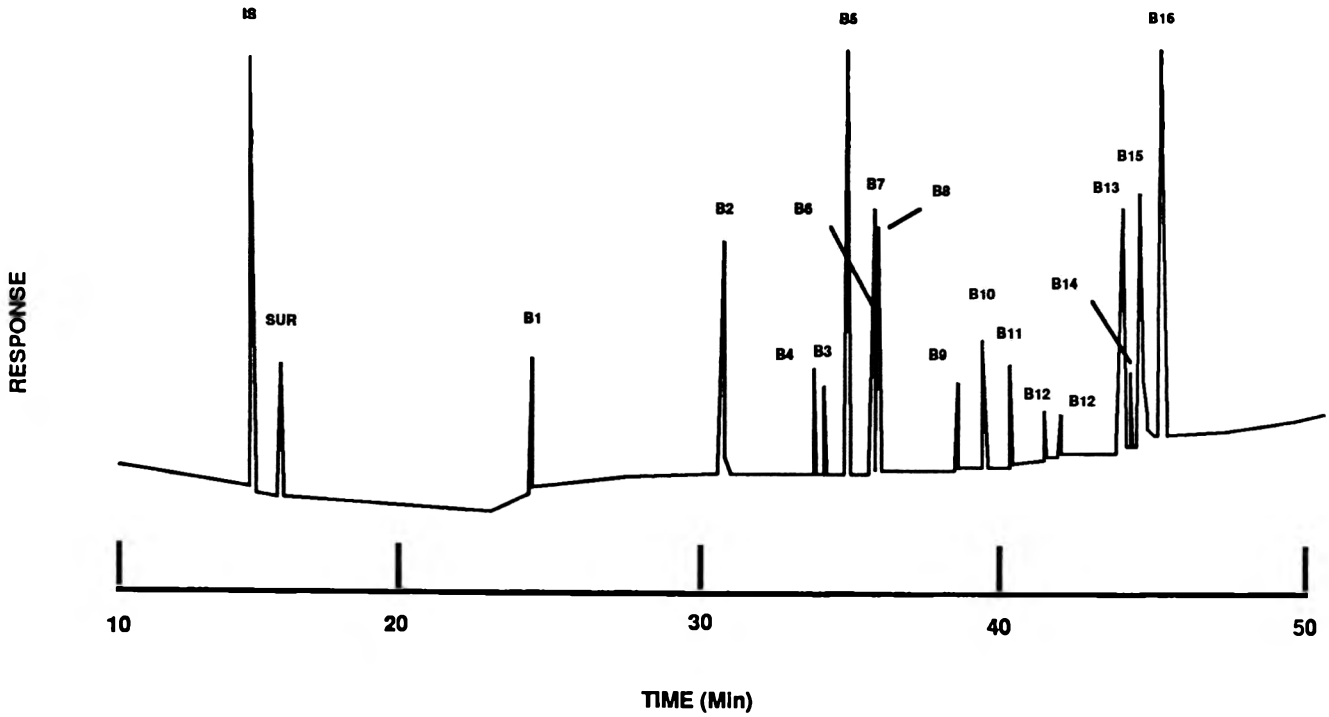

FIG. 991.07B—Reconstructed GC-NPD chromatogram of group B compounds analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column (0.25 μm film). *See* B(k) for operating conditions and Table 991.07A for peak identification. IS = Internal standard; SUR = surrogate standard.

Table 991.07B Laboratory Performance Check Solution

Test	Analyte	Concn, μg/mL	Requirements
Sensitivity	Vernolate	0.05	Detection of analyte; S/N > 3
Chromatographic performance	Bromacil	5.0	0.80 < PGF < 1.20[a]
Column performance	Prometon Atrazine	0.30	Resolution > 0.7[b]

[a] PGF = peak Gaussian factor. Calculate as follows:

$$PGF = (1.83 \times W^{1/2})/W^{1/10}.$$

where $W^{1/2}$ is the peak width at half height and $W^{1/10}$ is the peak width at tenth height.

[b] Resolution between the 2 peaks as defined by the equation:

$$R = t/W$$

where t is the difference in elution times between the 2 peaks and W is the average peak width at the baseline, of the 2 peaks.

column; rinse walls of concentrator tube while adjusting volume to 5.0 mL with MTBE.

Add 50 μL internal standard stock solution, **C(b)**, to sample extract, seal, and shake to distribute internal standard. Transfer extract to appropriate-size TFE-fluorocarbon-sealed screw-cap vial. Store at 4° until analysis. A 14-day maximum storage time is recommended.

H. Calibration of Gas Chromatograph with Nitrogen-Phosphorus Detector

Table **991.07A** summarizes retention times and estimated method detection limits observed using this method. Examples of separations using these conditions are shown in Figs **991.07A** and **991.07B**. Initially, perform 5 level calibration within linear range of detector, using internal standard and relative response factors. If response factor (RF) value over working range is constant (≤10% RSD), average RF can be used for calculations. Verify calibration curve daily using 1 or 2 calibration standards. If response of any analyte varies >20% from average relative response factor for initial calibration, analysis of single-level standard must be repeated with fresh standard. Alternatively, new calibration curve must be prepared.

I. Quality Control

Minimum quality control requirements for this method include (*1*) initial demonstration of method performance, (*2*) analysis of surrogate standard in each sample (acceptable recovery is 70–130%), (*3*) monitoring of internal standard area counts in each sample (area of internal standard should be within 30% of area for calibration standard), (*4*) analysis of method blank with each set of extracted samples as continuing check on sample contamination, (*5*) analyses of spiked reagent water as continuing check on method recovery, and (*6*) analysis of daily instrument QC standard to ensure acceptable instrument performance (Table **991.07B**).

Demonstrate initial method performance by extracting four 1 L samples of spiked reagent water at concentration levels indicated in Table **991.07C** (ca 10 times estimated method detection limit). Calculate average percent recovery and standard deviation of percent recovery. For acceptable performance, relative standard deviation should be <20% and analyte mean recoveries should be within acceptance limits in Table **991.07C**. Demonstrate continuing check on method recovery by analyzing 1 reagent water sample spiked at concentration levels in Table **991.07C** with every 20 samples, or 1 with each set of extracted samples, and compare recovery to performance-based acceptance limits in Table **991.07C**.

Table 991.07C Acceptance Limits (as Percent of Mean Recovery) for Analysis of Laboratory Quality Control Sample

Analyte	Concn Level[a]	Mean Recovery[b]	Overall Std Dev.[b]	Acceptance Limits,[c] %
Alachlor	5.00	4.63	0.78	49.5–150
Ametryn	2.00	1.88	0.28	55.3–145
Atraton	5.00	4.72	0.87	44.9–155
Atrazine	2.00	1.86	0.27	55.9–144
Bromacil	10.0	9.55	1.60	49.7–151
Butachlor	10.0	9.41	1.46	53.2–147
Butylate	5.00	3.81	0.89	29.9–170
Carboxin	10.0	9.37	2.00	35.0–164
Chlorpropham	10.0	9.76	1.78	45.3–155
Cycloate	5.00	4.29	0.84	41.2–159
Diazinon	2.00	1.78	0.34	42.7–157
Dichlorvos	5.00	4.84	0.76	53.1–147
Diphenamid	5.00	4.72	0.75	52.3–148
Disulfoton	2.00	1.73	0.33	42.9–157
Disulfoton sulfone	10.0	10.4	2.26	34.8–165
Disulfoton sulfoxide	10.0	9.51	1.86	41.3–159
EPTC	2.00	1.72	0.32	44.2–156
Ethoprop	2.00	1.84	0.37	39.7–160
Fenamiphos	20.0	18.0	3.50	41.7–158
Fenarimol	5.00	4.86	1.09	32.7–167
Fluridone	10.0	10.2	2.47	27.4–172
Hexazinone	5.00	4.96	1.06	36.1–164
Merphos	[d]			
Methyl paraoxon	10.0	10.0	1.73	48.1–152
Metolachlor	10.0	9.26	1.22	60.5–139
Metribuzin	2.00	1.94	0.38	41.2–159
Mevinphos	10.0	9.70	1.28	60.4–139
MGK-264	10.0	9.23	1.66	46.0–154
Molinate	2.00	1.88	0.34	45.7–154
Napropamide	5.00	4.37	0.69	52.6–147
Norflurazon	5.00	4.80	1.19	25.4–174
Pebulate	2.00	1.78	0.39	34.3–166
Prometon	2.00	1.92	0.33	48.4–152
Pronamide	10.0	9.72	1.47	54.5–145
Propazine	2.00	1.86	0.32	48.4–152
Simazine	2.00	1.90	0.28	55.8–144
Simetryn	2.00	1.93	0.34	47.2–153
Stirofos	20.0	18.6	3.87	37.5–162
Tebuthiuron	10.0	9.72	1.79	44.6–155
Terbacil	50.0	49.8	9.35	43.8–156
Terbufos	10.0	8.30	1.50	45.8–154
Terbutryn	2.00	1.90	0.24	62.1–138
Triademefon	2.00	1.96	0.40	38.8–161
Tricyclazole	20.0	18.1	2.95	51.1–149
Vernolate	2.00	1.62	0.41	24.1–176

[a] Concentration level 10–15 times estimated MDL, μg/L.

[b] Calculated from the regression equations for mean recovery and overall standard deviation obtained in collaborative study of the method for reagent water matrix.

[c] Acceptance limits are defined as the mean recovery ± 3 standard deviations.

[d] Merphos breakdown to DEF was incomplete and resulted in poor recovery and precision.

Ref.: JAOAC **74**, March/April issue (1991).

Results and Discussion

Twelve laboratories returned data for this study. A review of the returned quality control data sets showed 2 laborato-

Table 1. Total number of rejected data, by compound, by water type and in total

Compound	Reagent water[a]	Finished drinking water[a]	Total rejected data[b]
Alachlor	3	0	3
Ametryn	13	10	23
Atraton	1	13	14
Atrazine	7	20	27
Bromacil	14	10	24
Butachlor	12	19	31
Butylate	12	8	20
Carboxin	6	18	24
Chlorpropham	5	7	12
Cycloate	8	8	16
Diazinon	12	6	18
Dichlorvos	13	19	32
Diphenamid	5	0	5
Disulfoton	1	18	19
Disulfoton sulfone	9	6	15
Disulfoton sulfoxide	7	13	20
EPTC	8	12	20
Ethoprop	6	1	7
Fenamiphos	3	13	16
Fenarimol	1	12	13
Fluridone	8	12	20
Hexazinone	7	6	13
Merphos	0	6	6
Methyl paraoxon	18	13	31
Metolachlor	7	14	21
Metribuzin	6	18	24
Mevinphos	19	15	34
MGK-264	6	7	13
Molinate	7	12	19
Napropamide	13	6	19
Norflurazon	5	12	17
Pebulate	13	12	25
Prometon	2	8	10
Pronamide	14	12	26
Propazine	7	12	19
Simazine	6	8	14
Simetryn	1	19	20
Stirofos	21	18	39
Tebuthiuron	3	18	21
Terbacil	21	17	38
Terbufos	12	9	21
Terbutryn	7	1	8
Triademefon	1	12	13
Tricyclazole	14	6	20
Vernolate	7	12	19
Total	371	498	869
(Percentage of total submitted data)	(13.8%)[a]	(18.4%)[a]	(16.1%)[c]

[a] Total submitted data by water type was 6 concentrations × 10 laboratories × 45 analytes = 2700.
[b] Total submitted data by analyte was 6 concentrations × 10 laboratories × 2 water types = 120.
[c] Total submitted data for the study = 5400.

ries with a significant percentage of quality control data outside control limits. These 2 data sets were removed from the study before computer processing.

Rejection of Outliers

For the entire study, the IMVS computer program rejected 16.1% (869) of the 5400 data points submitted. The percentage of rejected data in reagent water was 13.8% and in finished drinking water was 18.4% (Table 1). The statistical procedures identified the largest number of outliers in the stirofos data set, 39, whereas the alachlor set produced only 3. Of the 6 compounds with the highest number of rejected data, the butachlor, methyl paraoxon, stirofos, and terbacil data sets lost a majority of their data as a result of coelution problems reported by several laboratories.

Table 2. Total number of rejected data, by laboratory, by outlier test, and in total

Laboratory code	Laboratory ranking test[a]	Thompson's outlier test[a]	Total rejected data[b]
01	222	15	237
02	102	15	117
03	24	9	33
04	138	29	167
08	114	14	128
09	78	6	84
10	48	4	52
14	6	4	10
16	6	2	8
17	24	9	33
Total	762	107	869

[a] Level of significance 0.05.
[b] Total submitted data by laboratory was 6 concentrations × 45 analytes × 2 water types = 540.

The number of data rejected for each laboratory is presented in Table 2. The laboratory ranking test accounted for 87.7% of all rejected data. Of the 10 laboratories submitting data, laboratory 1 had the highest number of data rejected, 43.9% of its total submitted data, which was very high in comparison with the remaining laboratories. The vast majority of laboratory 1 data was removed by the laboratory ranking procedure, which detected a systematic high bias.

Method Recovery

The summary statistics calculated after removal of outliers are presented in Table 3. The coefficients of determination (COD_w) calculated for the weighted linear regression equations of mean recovery were all above 0.98, which confirms the suitability of these equations for estimating the mean recovery at any concentration level within the range tested. The mean recoveries for the 45 pesticides, as estimated from the slopes of the regression equations, were greater than 85% except for butylate (approximately 74%), merphos (approximately 52%), vernolate (approximately 79%), and carboxin in drinking water. The poor recoveries for butylate and vernolate were consistent with the single-laboratory evaluation data (2). The method failed to measure merphos adequately; decomposition to DEF in the gas chromatograph could not be controlled by the collaborators to produce quantitative data.

Precision

The overall standard deviation (s_R, reproducibility) is the precision associated with measurements generated by a group of laboratories; the single-analyst standard deviation (s_r, repeatability) is the precision associated with performance in an individual laboratory. Weighted linear regression equations presented in Table 3 describe method precision as a function of mean recovery. The COD_w values calculated for these weighted equations show them to be representative of the submitted data sets. Experience has

Table 3. Summary statistics and regression equations for Method 507 collaborative study data sets

		Reagent Water				Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Alachlor	1.50	1.48	0.40	0.46	$X = 0.912C + 0.072$	1.66	0.61	0.28	$X = 0.902C + 0.274$
	2.24	2.05	0.30		$s_R = 0.138X + 0.142$	2.25	0.59		$s_R = 0.155X + 0.308$
	5.98	5.33	1.09	0.70	$s_r = 0.075X + 0.325$	5.45	1.14	0.94	$s_r = 0.139X + 0.013$
	7.48	6.77	1.21			6.94	0.92		
	12.00	11.66	1.39	1.41		11.54	2.50	1.66	
	15.00	13.75	2.04			13.99	2.98		
Ametryn	0.60	0.60	0.13	0.09	$X = 0.911C + 0.063$	0.60	0.12	0.12	$X = 0.863C + 0.071$
	0.90	0.90	0.14		$s_R = 0.130X + 0.040$	0.82	0.14		$s_R = 0.181X + 0.000$
	2.40	2.18	0.13	0.47	$s_r = 0.194X - 0.051$	2.17	0.49	0.20	$s_r = 0.073X + 0.063$
	3.00	2.96	0.63			2.59	0.23		
	4.80	4.30	0.39	0.86		4.34	1.30	0.48	
	6.00	5.50	1.13			5.23	0.58		
Atraton	1.00	1.05	0.21	0.14	$X = 0.922C + 0.107$	1.01	0.17	0.12	$X = 0.887C + 0.114$
	1.50	1.46	0.26		$s_R = 0.185X - 0.006$	1.43	0.23		$s_R = 0.127X + 0.038$
	4.00	3.57	0.26	0.83	$s_r = 0.216X - 0.130$	3.59	0.56	0.33	$s_r = 0.086X + 0.011$
	5.00	4.92	1.19			4.47	0.19		
	8.00	7.35	0.94	1.64		7.35	1.51	0.77	
	10.00	9.66	2.69			9.15	1.13		
Atrazine	0.30	0.31	0.02	0.09	$X = 0.911C + 0.036$	0.42	0.17	0.04	$X = 0.932C + 0.117$
	0.45	0.45	0.14		$s_R = 0.143X + 0.008$	0.49	0.08		$s_R = 0.112X + 0.091$[e]
	1.20	1.08	0.14	0.16	$s_r = 0.089X + 0.057$	1.21	0.39	0.22	$s_r = 0.12$[f]
	1.50	1.40	0.18			1.52	0.25		
	2.40	2.31	0.30	0.30		2.35	0.21	0.10	
	3.00	2.77	0.39			3.08	0.37		
Bromacil	3.01	3.16	0.74	0.82	$X = 0.885C + 0.702$	3.18	1.04	0.41	$X = 0.888C + 0.512$
	4.51	5.18	1.09		$s_R = 0.131X + 0.348$	4.55	1.03		$s_R = 0.136X + 0.558$[e]
	12.00	10.93	1.21	2.94	$s_r = 0.149X + 0.241$	11.14	2.77	1.30	$s_r = 0.096X + 0.047$
	15.00	14.66	3.76			13.70	1.64		
	24.10	21.42	2.79	2.60		21.37	4.46	2.32	
	30.10	26.50	2.82			28.10	3.37		
Butachlor	2.00	1.77	0.44	0.34	$X = 0.950C - 0.088$	2.07	0.43	0.33	$X = 0.907C + 0.232$
	3.00	2.88	0.67		$s_R = 0.132X + 0.223$	2.88	0.42		$s_R = 0.166X + 0.044$
	8.00	7.39	1.33	0.88	$s_r = 0.126X + 0.034$	7.66	1.31	1.17	$s_r = 0.121X + 0.034$
	10.00	9.38	1.04			9.37	2.07		
	16.00	15.51	2.33	2.52		13.85	1.64	1.85	
	20.00	18.45	2.88			19.19	3.55		
Butylate	0.80	0.57	0.24	0.11	$X = 0.769C - 0.034$	0.60	0.10	0.12	$X = 0.759C + 0.020$
	1.19	0.90	0.28		$s_R = 0.204X + 0.115$	0.98	0.10		$s_R = 0.182X - 0.026$
	3.18	2.51	0.38	0.68	$s_r = 0.203X - 0.028$	2.46	0.59	0.60	$s_r = 0.152X + 0.008$
	3.98	2.83	1.04			3.02	0.45		
	6.37	5.12	1.01	0.80		4.92	0.62	0.54	
	7.96	5.88	1.39			5.82	1.25		
Carboxin	3.00	3.36	0.82	0.70	$X = 0.870C + 0.674$	2.67	1.26	0.48	$X = 0.664C + 0.602$
	4.51	4.44	0.93		$s_R = 0.212X + 0.017$	3.45	1.54		$s_R = 0.386X + 0.287$
	12.00	10.30	0.85	3.23	$s_r = 0.242X - 0.223$	8.01	4.60	1.24	$s_r = 0.173X - 0.072$
	15.00	14.95	4.36			11.00	4.48		
	24.00	20.80	3.27	5.02		15.21	6.65	3.75	
	30.00	27.54	8.19			22.83	6.00		
Chlorpro-pham	2.00	2.22	0.43	0.27	$X = 0.944C + 0.322$	1.85	0.89	0.72	$X = 0.940C + 0.049$
	2.99	3.13	0.71		$s_R = 0.179X + 0.034$	3.07	0.61		$s_R = 0.144X + 0.510$
	7.98	7.48	0.29	1.79	$s_r = 0.211X - 0.287$	7.34	2.05	0.72	$s_r = 0.034X + 0.621$
	9.98	10.40	2.59			9.19	1.02		
	16.00	15.08	2.26	3.03		15.12	3.45	1.53	
	20.00	19.29	4.89			19.18	3.72		

continued

Table 3. Summary statistics and regression equations - continued

	Reagent Water					Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Cycloate	0.79	0.75	0.20	0.04	$X = 0.849C + 0.046$	0.65	0.25	0.10	$X = 0.859C - 0.018$
	1.19	0.99	0.19		$s_R = 0.185X + 0.044$	1.04	0.41		$s_R = 0.096X + 0.216$
	3.17	2.58	0.44	0.60	$s_r = 0.202X - 0.126$	2.62	0.47	0.23	$s_r = 0.071X + 0.039$
	3.96	3.58	0.91			3.37	0.47		
	6.34	5.28	0.94	0.95		5.49	0.61	0.51	
	7.92	7.14	1.37			6.85	0.90		
Diazinon	0.51	0.36	0.12	0.07	$X = 0.949C - 0.120$	0.43	0.14	0.08	$X = 0.906C - 0.034$
	0.77	0.60	0.14		$s_R = 0.160X + 0.058$	0.65	0.14		$s_R = 0.235X + 0.025$
	2.03	1.78	0.34	0.16	$s_r = 0.106X + 0.021$	1.85	0.47	0.43	$s_r = 0.183X - 0.013$
	2.54	2.40	0.42			2.18	0.48		
	4.06	3.70	0.57	0.60		3.74	0.84	0.62	
	5.08	4.61	0.97			4.55	1.36		
Dichlorvos	1.00	1.00	0.09	0.10	$X = 0.963C + 0.030$	1.02	0.09	0.11	$X = 0.883C + 0.132$
	1.50	1.45	0.10		$s_R = 0.181X - 0.119$	1.45	0.18		$s_R = 0.156X - 0.064$
	4.00	3.85	0.30	0.76	$s_r = 0.175X - 0.106$	3.61	0.53	0.22	$s_r = 0.076X + 0.008$
	5.00	4.99	1.08			4.66	0.36		
	8.00	7.68	1.18	1.25		7.09	1.11	0.79	
	10.00	9.58	1.88			9.05	1.68		
Diphenamid	1.00	1.04	0.27	0.17	$X = 0.916C + 0.138$	1.07	0.49	0.32	$X = 1.049C + 0.069$
	1.49	1.52	0.18		$s_R = 0.144X + 0.071$	1.75	0.71		$s_R = 0.159X + 0.351$
	3.98	3.68	0.24	0.96	$s_r = 0.204X - 0.080$	4.05	0.77	0.82	$s_r = 0.103X + 0.184$
	4.98	5.20	1.35			5.59	1.58		
	7.97	7.37	1.29	1.33		8.24	1.64	0.92	
	9.96	8.62	1.20			10.33	1.71		
Disulfoton	0.50	0.44	0.09	0.06	$X = 0.870C - 0.014$	0.39	0.13	0.07	$X = 0.814C - 0.042$
	0.76	0.61	0.11		$s_R = 0.192X - 0.004$	0.51	0.19		$s_R = 0.311X + 0.022$[e]
	2.02	1.65	0.24	0.36	$s_r = 0.190X - 0.044$	1.57	0.65	0.18	$s_r = 0.134X + 0.010$
	2.52	2.30	0.48			2.04	0.75		
	4.03	3.41	0.52	0.67		3.07	1.09	0.63	
	5.04	4.52	1.15			4.41	0.73		
Disulfoton sulfone	1.50	1.54	0.16	0.20	$X = 1.046C - 0.086$	1.37	0.61	0.28	$X = 0.908C + 0.008$
	2.25	2.14	0.19		$s_R = 0.243X - 0.272$	2.05	0.51		$s_R = 0.181X + 0.292$
	6.00	5.77	0.42	1.75	$s_r = 0.263X - 0.283$	5.38	1.19	0.59	$s_r = 0.114X + 0.079$
	7.50	8.34	2.47			7.18	1.48		
	12.00	11.79	1.97	3.14		10.37	2.63	1.78	
	15.00	16.55	5.08			13.82	2.95		
Disulfoton sulfoxide	3.00	2.98	0.31	0.31	$X = 0.933C + 0.183$	3.41	0.83	0.43	$X = 0.946C + 0.556$
	4.51	4.38	0.37		$s_R = 0.252X - 0.541$	4.80	0.84		$s_R = 0.094X + 0.496$[e]
	12.00	11.27	1.50	3.61	$s_r = 0.246X - 0.558$	11.59	1.95	1.86	$s_r = 0.096X + 0.063$[e]
	15.00	14.92	4.47			15.01	2.70		
	24.10	22.17	4.09	4.08		21.71	1.82	1.76	
	30.10	27.82	7.60			31.24	2.45		
EPTC	0.50	0.45	0.13	0.04	$X = 0.852C + 0.017$	0.51	0.12	0.07	$X = 0.813C + 0.096$
	0.76	0.65	0.11		$s_R = 0.165X + 0.038$	0.69	0.10		$s_R = 0.106X + 0.052$
	2.02	1.70	0.25	0.38	$s_r = 0.190X - 0.064$	1.70	0.24	0.14	$s_r = 0.064X + 0.026$
	2.52	2.26	0.53			2.19	0.20		
	4.03	3.50	0.56	0.55		3.37	0.47	0.30	
	5.04	4.20	0.83			4.23	0.61		
Ethoprop	0.50	0.47	0.10	0.09	$X = 0.908C + 0.026$	0.48	0.15	0.06	$X = 0.930C + 0.007$
	0.74	0.72	0.16		$s_R = 0.195X + 0.012$	0.68	0.14		$s_R = 0.157X + 0.058$
	1.98	1.72	0.42	0.19	$s_r = 0.122X + 0.011$	1.90	0.22	0.10	$s_r = 0.097X + 0.000$
	2.48	2.35	0.43			2.22	0.30		
	3.97	3.70	0.54	0.62		3.83	0.73	0.58	
	4.96	4.50	1.01			4.58	1.22		

continued

Table 3. Summary statistics and regression equations - continued

		Reagent Water				Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Fenamiphos	4.00	4.43	1.35	1.00	$X = 0.855C + 0.865$	3.38	0.86	0.70	$X = 0.898C - 0.190$
	6.01	5.74	1.14		$s_R = 0.166X + 0.515$	5.31	1.40		$s_R = 0.239X + 0.068$
	16.00	13.33	4.23	1.50	$s_r = 0.106X + 0.421$	14.23	3.97	2.83	$s_r = 0.156X + 0.041$
	20.00	18.26	2.65			16.80	2.95		
	32.00	30.52	4.38	4.85		27.87	6.98	4.53	
	40.00	34.85	6.68			37.97	9.85		
Fenarimol	1.00	1.17	0.34	0.29	$X = 0.933C + 0.194$	1.12	0.36	0.18	$X = 0.902C + 0.213$
	1.50	1.52	0.31		$s_R = 0.218X + 0.030$	1.59	0.54		$s_R = 0.134X + 0.237$
	4.00	3.53	0.40	1.18	$s_r = 0.251X - 0.043$	3.58	0.62	0.37	$s_r = 0.120X + 0.007$
	5.00	5.27	1.55			4.54	0.49		
	8.00	7.37	1.20	1.96		7.49	1.93	1.27	
	10.00	10.13	3.14			9.90	1.34		
Fluridone	3.00	3.35	1.20	0.70	$X = 0.971C + 0.489$	3.54	1.16	1.45	$X = 0.928C + 0.899$
	4.50	5.05	0.34		$s_R = 0.245X - 0.029$	5.53	2.79		$s_R = 0.216X + 0.659$
	12.00	11.15	1.64	3.07	$s_r = 0.262X - 0.402$	11.20	2.36	1.61	$s_r = 0.123X + 0.829$
	15.00	15.44	4.56			14.21	2.37		
	24.00	23.28	4.62	6.76		22.64	6.65	5.40	
	30.00	31.10	11.8			31.07	7.85		
Hexazinone	1.00	1.20	0.27	0.24	$X = 0.943C + 0.250$	1.13	0.35	0.13	$X = 0.881C + 0.254$
	1.51	1.67	0.33		$s_R = 0.217X - 0.020$	1.61	0.45		$s_R = 0.132X + 0.206$
	4.02	3.64	0.41	1.19	$s_r = 0.242X - 0.091$	3.65	0.73	0.45	$s_r = 0.134X - 0.055$
	5.02	5.27	1.53			4.62	0.50		
	8.03	7.43	1.13	1.74		7.29	1.73	1.16	
	10.00	10.47	2.97			9.46	1.16		
Merphos	1.66	0.76	0.27	0.30	$X = 0.499C - 0.036$	0.93	0.46	0.23	$X = 0.556C - 0.044$
	2.48	1.30	0.47		$s_R = 0.366X - 0.008$	1.21	0.48		$s_R = 0.212X + 0.243$
	6.62	3.08	1.14	0.44	$s_r = 0.150X + 0.132$	3.58	0.98	0.86	$s_r = 0.176X + 0.044$
	8.28	4.02	1.51			4.54	0.98		
	13.20	6.57	2.71	1.66		7.77	1.84	1.35	
	16.60	8.49	2.55			9.05	2.72		
Methyl paraoxon	1.99	2.20	0.47	0.61	$X = 0.977C + 0.270$	2.62	0.65	0.48	$X = 1.019C + 0.582$
	2.99	3.30	0.78		$s_R = 0.155X + 0.175$	3.65	0.60		$s_R = 0.204X + 0.031$
	7.97	7.35	1.35	0.88	$s_r = 0.071X + 0.402$	8.10	1.57	2.47	$s_r = 0.196X - 0.089$
	9.96	10.09	1.84			10.98	2.70		
	15.90	15.63	2.28	1.95		16.74	2.89	2.58	
	19.90	21.01	3.30			21.64	4.94		
Metolachlor	3.00	2.85	0.42	0.39	$X = 0.919C + 0.070$	3.46	0.84	0.37	$X = 0.939C + 0.657$
	4.51	4.17	0.60		$s_R = 0.124X + 0.073$	4.91	0.92		$s_R = 0.063X + 0.609$
	12.00	10.97	1.68	1.32	$s_r = 0.118X - 0.035$	12.27	1.14	1.17	$s_r = 0.079X + 0.045$
	15.00	13.84	1.60			14.29	1.73		
	24.00	22.71	2.33	3.11		22.04	1.63	1.97	
	30.00	27.28	3.94			30.22	3.14		
Metribuzin	0.60	0.66	0.15	0.07	$X = 0.920C + 0.097$	0.63	0.15	0.07	$X = 0.896C + 0.096$
	0.90	0.91	0.14		$s_R = 0.203X - 0.010$	0.92	0.16		$s_R = 0.103X + 0.079$
	2.40	2.16	0.19	0.54	$s_r = 0.229X - 0.104$	2.19	0.30	0.23	$s_r = 0.107X - 0.015$
	3.00	3.10	0.82			2.82	0.20		
	4.80	4.37	0.69	0.98		4.32	1.00	0.55	
	6.00	5.71	1.62			5.59	0.40		
Mevinphos	1.50	1.47	0.17	0.15	$X = 0.973C - 0.027$	1.51	0.42	0.32	$X = 0.927C + 0.053$
	2.25	2.06	0.23		$s_R = 0.137X - 0.045$	1.99	0.17		$s_R = 0.095X + 0.183$[e]
	6.00	5.73	0.32	1.15	$s_r = 0.171X - 0.142$	5.39	1.04	0.40	$s_r = 0.030X + 0.261$
	7.50	7.61	1.67			7.20	0.80		
	12.00	11.59	0.70	1.82		10.84	2.07	0.72	
	15.00	14.45	2.68			14.88	0.25		

continued

Table 3. Summary statistics and regression equations - continued

		Reagent Water				Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
MGK-264	2.36	2.02	0.58	0.62	$X = 0.937C - 0.144$	2.31	0.79	0.44	$X = 0.915C + 0.127$
	3.55	3.36	1.21		$s_R = 0.138X + 0.392$	3.34	1.07		$s_R = 0.137X + 0.532$
	9.46	8.26	0.96	1.04	$s_r = 0.134X + 0.241$	8.83	2.14	1.59	$s_r = 0.138X + 0.059$
	11.80	10.59	1.70			10.68	2.15		
	18.90	18.41	2.49	3.77		17.04	1.70	2.47	
	23.60	22.01	4.47			22.67	3.79		
Molinate	0.50	0.51	0.11	0.06	$X = 0.922C + 0.033$	0.51	0.23	0.08	$X = 0.839C + 0.086$
	0.75	0.69	0.14		$s_R = 0.172X + 0.018$	0.71	0.21		$s_R = 0.084X + 0.174$
	2.00	1.82	0.16	0.39	$s_r = 0.215X - 0.065$	1.77	0.31	0.16	$s_r = 0.056X + 0.044$
	2.50	2.47	0.53			2.23	0.27		
	4.00	3.60	0.48	0.84		3.35	0.53	0.25	
	5.00	4.76	1.23			4.30	0.67		
Naprop-amide	0.80	0.76	0.20	0.20	$X = 0.857C + 0.086$	0.64	0.36	0.18	$X = 0.868C - 0.075$
	1.19	1.12	0.32		$s_R = 0.130X + 0.119$	0.90	0.47		$s_R = 0.134X + 0.290$[e]
	3.18	2.69	0.32	0.62	$s_r = 0.122X + 0.088$	2.59	0.48	0.28	$s_r = 0.090X + 0.109$
	3.98	3.70	0.73			3.48	0.46		
	6.37	5.40	0.84	0.62		5.46	1.84	0.84	
	7.96	6.93	0.96			6.97	0.94		
Norflurazon	1.00	1.35	0.50	0.34	$X = 0.877C + 0.418$	1.10	0.35	0.19	$X = 0.867C + 0.226$
	1.50	1.63	0.35		$s_R = 0.227X + 0.103$	1.54	0.47		$s_R = 0.149X + 0.209$
	4.00	3.64	0.63	1.32	$s_r = 0.275X - 0.051$	3.50	0.87	0.43	$s_r = 0.109X + 0.040$
	5.00	4.79	1.66			4.45	0.61		
	8.00	7.48	1.29	1.99		6.93	1.50	1.02	
	10.00	9.97	2.95			9.73	1.41		
Pebulate	0.49	0.51	0.16	0.08	$X = 0.858C + 0.069$	0.51	0.20	0.06	$X = 0.790C + 0.114$
	0.74	0.64	0.21		$s_R = 0.166X + 0.092$	0.68	0.20		$s_R = 0.117X + 0.132$
	1.97	1.84	0.45	0.49	$s_r = 0.195X - 0.030$	1.64	0.25	0.19	$s_r = 0.077X + 0.018$
	2.46	2.22	0.55			2.03	0.44		
	3.94	3.40	0.42	0.52		3.24	0.51	0.24	
	4.92	4.28	0.79			4.14	0.68		
Prometon	0.50	0.47	0.13	0.05	$X = 0.969C - 0.014$	0.42	0.14	0.17	$X = 0.938C - 0.031$
	0.75	0.72	0.09		$s_R = 0.154X + 0.035$	0.71	0.15		$s_R = 0.118X + 0.085$
	2.00	1.79	0.17	0.45	$s_r = 0.215X - 0.072$	1.85	0.35	0.17	$s_r = 0.041X + 0.142$
	2.50	2.59	0.58			2.27	0.12		
	4.00	3.73	0.44	0.78		3.80	0.96	0.41	
	5.00	4.92	1.24			4.54	0.50		
Pronamide	2.00	2.44	0.74	0.30	$X = 0.913C + 0.586$	1.81	0.47	0.38	$X = 0.894C - 0.026$
	3.00	3.24	0.44		$s_R = 0.121X + 0.299$	2.54	0.85		$s_R = 0.115X + 0.352$
	8.00	7.56	0.82	1.38	$s_r = 0.190X - 0.248$	7.09	1.04	0.68	$s_r = 0.075X + 0.208$
	10.00	10.46	2.07			8.99	0.92		
	16.00	15.43	1.25	3.06		14.00	3.01	1.64	
	20.00	18.16	3.88			18.45	1.75		
Propazine	0.30	0.29	0.08	0.04	$X = 0.917C + 0.021$	0.31	0.06	0.06	$X = 0.891C + 0.043$
	0.45	0.44	0.08		$s_R = 0.159X + 0.024$	0.44	0.08		$s_R = 0.132X + 0.020$
	1.20	1.10	0.12	0.23	$s_r = 0.189X - 0.027$	1.09	0.18	0.14	$s_r = 0.092X + 0.021$
	1.50	1.49	0.33			1.42	0.11		
	2.40	2.19	0.31	0.42		2.14	0.45	0.24	
	3.00	2.67	0.60			2.74	0.34		
Simazine	0.49	0.48	0.07	0.14	$X = 0.928C + 0.041$	0.55	0.15	0.07	$X = 0.964C + 0.075$
	0.74	0.77	0.22		$s_R = 0.126X + 0.037$	0.78	0.16		$s_R = 0.078X + 0.102$
	1.97	1.80	0.25	0.25	$s_r = 0.089X + 0.081$	1.98	0.22	0.13	$s_r = 0.094X - 0.002$
	2.46	2.29	0.26			2.39	0.25		
	3.94	3.78	0.44	0.48		3.88	0.32	0.53	
	4.92	4.60	0.62			4.92	0.73		

continued

Table 3. Summary statistics and regression equations - continued

		Reagent Water				Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Simetryn	0.50	0.53	0.15	0.07	$X = 0.925C + 0.076$	0.56	0.10	0.07	$X = 0.897C + 0.114$
	0.74	0.77	0.12		$s_R = 0.163X + 0.032$	0.78	0.07		$s_R = 0.115X + 0.024$[e]
	1.98	1.81	0.10	0.40	$s_r = 0.212X - 0.063$	1.86	0.34	0.18	$s_r = 0.085X + 0.014$
	2.48	2.53	0.59			2.33	0.22		
	3.97	3.61	0.45	0.82		3.58	0.78	0.39	
	4.96	4.74	1.32			4.74	0.14		
Stirofos	4.00	3.97	1.09	0.63	$X = 0.914C + 0.286$	4.07	1.39	0.69	$X = 0.987C - 0.018$
	6.01	5.82	1.41		$s_R = 0.187X + 0.402$	5.59	1.38		$s_R = 0.163X + 0.633$
	16.00	13.36	5.31	2.19	$s_r = 0.187X - 0.317$	15.91	2.80	2.66	$s_r = 0.158X - 0.081$
	20.00	18.97	1.61			19.33	3.92		
	32.00	32.13	4.57	6.94		31.80	5.76	5.72	
	40.00	36.09	8.22			40.40	8.23		
Tebuthiuron	1.99	2.18	0.64	0.27	$X = 0.944C + 0.280$	2.44	0.46	0.27	$X = 0.881C + 0.647$
	2.99	3.07	0.65		$s_R = 0.163X + 0.207$	3.19	0.41		$s_R = 0.103X + 0.164$
	7.97	7.22	0.47	1.76	$s_r = 0.217X - 0.295$	7.49	0.94	0.71	$s_r = 0.081X + 0.043$
	9.96	10.20	2.35			9.57	0.90		
	15.90	15.17	2.04	3.19		14.40	2.36	1.43	
	19.90	19.54	5.46			18.83	1.76		
Terbacil	10.00	9.66	3.84	4.39	$X = 0.986C + 0.514$	10.04	0.63	2.17	$X = 0.939C + 0.977$
	15.00	17.30	6.58		$s_R = 0.124X + 3.170$[e]	16.00	2.37		$s_R = 0.239X - 1.520$
	40.00	38.44	11.2	4.84	$s_r = 0.018X + 4.136$	37.54	10.3	8.23	$s_r = 0.117X + 0.799$
	50.00	46.81	11.0			46.69	13.8		
	80.00	84.00	4.54	5.92		76.02	11.4	6.67	
	100.0	97.26	11.6			96.81	14.4		
Terbufos	3.00	2.10	0.47	0.28	$X = 0.885C - 0.547$	2.07	0.72	0.37	$X = 0.885C - 0.445$
	4.50	3.47	0.62		$s_R = 0.169X + 0.099$	3.90	0.52		$s_R = 0.250X + 0.070$
	12.00	9.85	2.24	0.82	$s_r = 0.105X - 0.029$	9.95	2.63	1.82	$s_r = 0.166X - 0.118$
	15.00	12.57	2.01			12.52	3.04		
	24.00	21.72	2.70	3.02		21.57	5.64	3.64	
	30.00	25.41	5.17			25.23	8.03		

continued

shown that a COD_w <0.5 indicates that the study has failed to establish a definitive linear relationship in the retained data set. For overall precision, s_R, only 8 of 90 precision equations had COD_w <0.50 (*see* footnote e, Table 3). For the 90 single-analyst precision regression equations, s_r, only the terbacil precision equation yielded a COD_w <0.50. These equations can be used effectively to derive performance-based quality control limits for this method.

To establish performance-based quality control limits, estimates of the mean recovery, overall precision, and single-analyst precision were calculated using a concentration value 10 to 15 times the estimated MDL using the regression equations reported. The precision estimates at this concentration level, expressed as both absolute and percent relative standard deviation, are presented as method performance parameters in the method. The average overall standard deviation (RSD_R) for all 45 pesticides in reagent water was 18.8%, and individually ranged from 12.8% for terbutryn to 36.4% for merphos, with only 2 analytes exceeding RSD_R of 25%. For finished drinking water, the average RSD_R was 19.2%, and ranged from 11.5% for mevinphos to 42.6% for carboxin. Seven compounds exceeded an RSD_R of 25% for finished drinking water: carboxin (42.6%), disulfoton (32.5%), fluridone (28.1%), merphos (25.6%), terbufos (25.8%), terbutryn (29.1%), and tricyclazole (25.1%). The average single-analyst standard deviation (RSD_r) for all 45 pesticides in reagent water was 16.8%; individual estimates ranged from 10.1% for terbacil to 26.4% for norflurazon. For finished drinking water, the average RSD_r was 12.0%, and individual values ranged from 5.8% for mevinphos to 20.4% for fluridone.

The precision estimates obtained from this study, as RSDs, were compared with those obtained from the collaborative study of EPA Method 508 (9). That collaborative study resulted in adoption of the method by AOAC as method **990.06**, which uses the same extraction procedures as well as the same GC column, temperature program, and carrier gas flow rates. The only difference is in GC detectors: Method **990.06** uses an electron capture detector; the present method uses a nitrogen-phosphorus detector. The average overall standard deviation of results for reagent water was similar for the 2 methods, with only slightly poorer precision for Method 507. The average RSD_R for reagent water was 18.8% for Method 507 and ranged from 12.8 to 36.4%. For method **990.06**, the average RSD_R for reagent water was 15.5% and ranged from 12.3 to 27.6%. However, the average

Table 3. Summary statistics and regression equations - continued

	Reagent Water					Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Terbutryn	0.60	0.60	0.10	0.10	$X = 0.934C + 0.028$	0.60	0.21	0.10	$X = 0.853C + 0.079$
	0.91	0.87	0.15		$s_R = 0.102X + 0.049$	0.84	0.28		$s_R = 0.260X + 0.055$
	2.42	2.20	0.30	0.26	$s_r = 0.109X + 0.018$	2.11	0.52	0.43	$s_r = 0.164X - 0.015$
	3.02	2.81	0.28			2.63	0.71		
	4.83	4.75	0.49	0.64		4.30	1.16	0.67	
	6.04	5.70	0.69			5.23	1.65		
Triademefon	0.50	0.57	0.12	0.11	$X = 0.937C + 0.090$	0.51	0.12	0.06	$X = 0.881C + 0.078$
	0.74	0.75	0.17		$s_R = 0.204X - 0.001$	0.75	0.13		$s_R = 0.134X + 0.041$
	1.98	1.82	0.19	0.43	$s_r = 0.199X - 0.022$	1.79	0.33	0.20	$s_r = 0.102X - 0.007$
	2.48	2.59	0.61			2.26	0.18		
	3.97	3.75	0.64	0.81		3.54	0.79	0.41	
	4.96	4.83	1.35			4.54	0.53		
Tricyclazole	5.00	5.72	0.47	0.91	$X = 0.841C + 1.284$	5.38	1.92	0.53	$X = 0.979C + 0.290$
	7.49	7.10	2.15		$s_R = 0.160X + 0.054$	7.29	2.22		$s_R = 0.215X + 0.710$
	20.00	17.26	0.92	3.84	$s_r = 0.197X - 0.341$	18.16	4.18	4.86	$s_r = 0.228X - 0.876$
	25.00	22.71	5.05			25.57	6.41		
	40.00	35.79	4.71	7.16		39.19	10.0	8.25	
	50.00	44.50	8.60			52.48	11.6		
Vernolate	0.50	0.43	0.14	0.07	$X = 0.798C + 0.029$	0.49	0.13	0.06	$X = 0.795C + 0.080$
	0.75	0.61	0.18		$s_R = 0.229X + 0.040$	0.64	0.12		$s_R = 0.120X + 0.061$
	2.00	1.66	0.20	0.40	$s_r = 0.231X - 0.049$	1.66	0.28	0.15	$s_r = 0.072X + 0.018$
	2.50	2.10	0.61			2.10	0.23		
	4.00	3.30	0.62	0.74		3.21	0.52	0.28	
	5.00	3.75	1.35			4.17	0.58		

[a] Spike concentration, μg/L.
[b] Mean recovery concentration, μg/L.
[c] Overall standard deviation, μg/L.
[d] Single-analyst standard deviation, μg/L.
[e] $COD_W < 0.50$. Do not use regression equation outside of study concentration range.
[f] $COD_W = 0$. Average precision is reported.

single-analyst standard deviations for reagent water were significantly different between the 2 methods. For Method 507, the average RSD_r for reagent water was 16.8% and ranged from 10.1 to 26.4%; whereas for method **990.06**, the comparable average RSD_r was 7.5% and ranged from 4.2 to 15.4%. The slight degradation of the average overall standard deviation and the significant degradation of the average single-analyst standard deviation for Method 507 must be related to the differences in the detectors because all other conditions were the same. Independent observations from the NPS participating laboratories (R. Maxey, USEPA, Bay St. Louis, MS, 1989, personal communication) also suggested that the instability of the nitrogen-phosphorus detector was the major factor related to precision.

Effect of Water Type

The data across water types were subjected to an analysis of variance test to determine the effect of water type on recovery and precision. Matrix effects due to water type were statistically significant for 5 compounds (carboxin, disulfoton, metolachlor, pronamide, and simazine). Many of the laboratories had clearly lower recoveries for carboxin and disulfoton in finished drinking water samples than in reagent water. Metolachlor and simazine were apparently recovered more effectively from finished drinking water than from reagent water, although most of the difference appeared to be anomalies in the study data set. The matrix effect observed for pronamide was due to unusually high recoveries in the reagent water matrix reported by the laboratories.

Conclusions and Recommendations

Method 507, which was developed for determination of 45 nitrogen- and phosphorus-containing pesticides in waters, was shown to be accurate and precise in a collaborative study involving 10 laboratories. Weighted linear regression equations presented for method recovery, overall precision, and single-analyst precision can be used to estimate method performance at any concentration value within the study range. The recovery, overall precision, and single-analyst precision values were comparable in reagent water and finished drinking water except for carboxin, disulfoton, metolachlor, pronamide, and simazine, which were found to have statistically significant matrix effects in finished drinking water.

The data are suitable for use in the development of performance-based quality control limits. It is recommended that users of this method routinely test a quality control sample prepared in reagent water and compare the results with performance-based acceptance limits derived from this study (Table **991.07C**).

Use of DEF, the breakdown product of merphos, was

unacceptable for quantitation of merphos in water samples.

On the basis of this interlaboratory method validation study, it is recommended that the method be adopted official first action.

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REFERENCES

(1) Engel, T. (1986) "Standardization of Methods for a National Pesticide Survey," U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH

(2) *Methods for the Determination of Organic Compounds in Drinking Water* (1988) EPA/600/4-88-039, U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH

(3) Proposed rules (1989) 40 CFR Parts 141, 142, 143, *Federal Register*, 22062

(4) Outler, E. C., & McCreery, J. H. (1982) "Interlaboratory Method Validation Study: Program Documentation," Battelle Columbus Laboratories, Columbus, OH

(5) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, p. 88

(6) *Annual Book of ASTM Standards* (1990) "Standard Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water," ASTM D2777-86, Vol. 11.01, Water (1), American Society for Testing and Materials, Philadelphia, PA

(7) Barnett, V., & Lewis, T. (1984) *Outliers in Statistical Data*, John Wiley and Sons, New York, NY

(8) Edgell, K. W., & Jenkins, E. L. (1989) "USEPA Method Study 39, Dibromoethane (EDB) and 1,2-Dibromo-3-chloropropane (DBCP) in Water by Microextraction and Gas Chromatography," EPA/600/4-89/015, U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH

(9) Lopez-Avila, V., Wesselman, R., & Edgell, K. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 276-286

Direct Aqueous Injection-Liquid Chromatography with Post-Column Derivatization for Determination of *N*-Methylcarbamoyloximes and *N*-Methylcarbamates in Finished Drinking Water: Collaborative Study

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An interlaboratory method validation study was conducted on EPA Method 531.1, Measurement of *N*-Methylcarbamoyloximes and *N*-Methylcarbamates in Water by Direct Aqueous Injection HPLC with Post Column Derivatization, to determine the precision and mean recovery for determination of 10 carbamate pesticide compounds in reagent water and in finished drinking waters. The study design was based on Youden's nonreplicate plan for collaborative tests of analytical methods. The waters were spiked with 10 carbamate pesticides at 6 concentration levels, as 3 Youden pairs. Eight laboratories analyzed the samples by direct aqueous injection, with separation by reverse-phase liquid chromatography and post-column hydrolysis of the carbamates and carbamoyloximes to methylamine, followed by reaction of the methylamine with *o*-phthalaldehyde and 2-mercaptoethanol using fluorescence detection. Results were analyzed using an EPA computer program, which measured precision and recovery for each of the 10 compounds and compared the performance of the method between water types. The method was acceptable for all analytes tested. After removal of a nonrepresentative data set for aldicarb sulfoxide, no matrix effects were observed; the statistics for the pooled drinking waters were not significantly different from the statistics for the reagent waters. The method has been adopted official first action by AOAC.

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The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Environmental Quality. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9-13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* **74**, January/February issue.

The widespread contamination of vulnerable groundwater supplies by aldicarb and other pesticides became a major concern to the U.S. Environmental Protection Agency (EPA) in the early 1980s. In the fall of 1983, EPA was charged by the Congress of the United States to monitor the drinking water supplies in the state of Florida and to assess the degree of contamination by aldicarb and related compounds. A liquid chromatographic procedure (LC), EPA Method 531, was developed to measure aldicarb and related compounds in this groundwater survey (1). As concerns over

possible groundwater contamination grew, the EPA initiated a National Pesticide Survey (NPS) to provide a comprehensive assessment of contamination in the 50 states. EPA Method 531 was modified for use in NPS to include additional analytes (NPS Method 5) (2).

The Safe Drinking Water Act of 1986 charged the EPA with the establishment of drinking water regulations for a number of previously unregulated compounds. NPS Method 5, now EPA Method 531.1 (3), was proposed for measuring the regulated contaminants aldicarb, aldicarb sulfoxide, aldicarb sulfone, and carbofuran, as well as the unregulated contaminants Baygon (propoxur), carbaryl, 3-hydroxycarbofuran, methiocarb, methomyl, and oxamyl in vulnerable water supplies (4).

The EPA Environmental Monitoring Systems Laboratory, Cincinnati, OH (EMSL-Cincinnati), is responsible for conducting interlaboratory method validation studies of analytical methods selected for the agency's operating programs. The objective of this study was to characterize the multilaboratory performance of Method 531.1 in terms of mean recovery, overall and single-analyst precision, and the effect of water type on recovery and precision. The study was conducted with the cooperation of 8 participating laboratories evaluated and selected by EMSL-Cincinnati. The Bionetics Corporation, as primary contractor to EMSL-Cincinnati, was responsible for the collection and characterization of the test waters, preparation and testing of the sample concentrates, writing user instructions, designing report forms, and screening of returned data. The raw data were statistically evaluated by EMSL-Cincinnati using a computer program (5) designed for these types of studies.

Collaborative Study

The study design was based on Youden's nonreplicate design for the collaborative evaluation of precision and recovery for analytical methods (6). In this design, 2 similar, yet different samples of the 10 compounds were analyzed in pairs to provide data for estimating single-operator precision rather than making these estimates from duplicate analyses of the same sample. For this study, 3 such Youden pairs were used, resulting in 6 concentration levels. The 8 collaborating laboratories were directed to analyze the spiked samples and report 1 value for all analytes in each sample. Analyses of the spiked reagent water evaluated the proficiency of the method on a sample free of interferences; analyses of the various finished drinking waters (provided by each participating laboratory) were intended to reveal the effects of matrix interferences on the method. All analyses were to be completed within 60 days from receipt of the samples.

The carbamate pesticides used in this study were obtained from the EPA Pesticide Repository, Research Triangle Park, NC. Spiking solutions, calibration solutions, and quality control samples were prepared in methanol and heat-sealed in ampoules, each containing approximately 1.5 mL solution. Prior to distribution, ampoule solutions were analyzed against standards freshly prepared from neat materials. At the completion of the study, the ampoules were analyzed again to verify the stability of the solutions over the time of the study. Each participating laboratory received 12 concentrates in ampoules (6 concentrations for each of 2 water matrixes), 2 calibration standard concentrates, 2 quality control samples with acceptance limits, a copy of the method, report forms, and a questionnaire. An external standard was used because the compound specified in the method was not commercially available for use as an internal standard at the time this study was conducted.

Preliminary analytical work by the primary contractor revealed decomposition of several carbamate pesticides in the presence of residual chlorine. Chlorinated, finished drinking water supplies would not be expected to contain all 10 carbamate pesticides covered in this method. To avoid loss of compounds, in accordance with the method the laboratories were required to dechlorinate their finished drinking water matrix with sodium thiosulfate prior to spiking. Also, the retention times of the analytes were not always reproducible among sample and calibration standard injections. These differences were traced to the amount of methanol introduced into the samples and standards during the spiking procedure. As a result, the participating laboratories were instructed to follow spiking procedures that ensured a constant methanol concentration in all samples and standards.

Treatment of Data

The returned data were grouped by water type, arranged as 6 subsets defined by the 6 different samples, and evaluated analyte by analyte using the EPA IMVS computer program. First, missing data points were replaced by values estimated by interpolation, and "less than" and "nondetect" values were converted to zero. Subsequent outlier tests were those suggested in the ASTM Standard Practice D2777-86 (7). Next, Youden's laboratory ranking procedure was used to reject laboratories that had a consistently higher or lower bias in their submitted data for a given analyte compared to the other laboratories. If a bias was determined, the 6 analyte values were rejected for that laboratory. This procedure was applied to each analyte data set, for each water type, at the 5% level of significance. Next, the zeroes and interpolated values were removed before any further analyses. Finally, Thompson's test for individual outliers (8) was applied to the data at the 5% significance level. If an individual data point was rejected on the basis of this test, it was removed from the subset and the test was repeated 1 more time using the remaining data in the subset.

Summary statistics were calculated for the mean recovery and overall method precision for each of the 6 concentration levels. Single-analyst precision was calculated for each of the 3 concentration pairs. The IMVS computer program used these summary statistics to calculate relationships between mean recovery and true concentration and between precision and mean recovery as linear regression equations using the weighted least squares technique (9). Coefficients of determination of the weighted regression equations (COD_w) were also calculated to evaluate the fit of the retained data sets. These weighted equations tend to degrade the calculated COD_w values, which in some cases resulted in very low or negative COD_w values. However, these regression equations can be used to estimate the mean method recovery, overall precision, and single-analyst precision at any value within the concentration range studied. IMVS also determined statistically significant matrix effects between water types for each of the 10 pesticides.

991.06 *N*-Methylcarbamoyloximes and *N*-Methylcarbamates in Finished Drinking Water

Liquid Chromatographic Method

First Action 1991

(Applicable to determination of 10 *N*-methylcarbamoyl-

Method Performance for **991.06**, *N*-methylcarbamoyloximes and *N*-methylcarbamates in finished drinking water[a]

Pesticide	Concn, μg/L[b]	Reagent Water				Finished Drinking Water			
		s_r	s_R	RSD_r, %	RSD_R, %	s_r	s_R	RSD_r, %	RSD_R, %
Aldicarb	10.0	0.32	0.58	3.4	6.1	0.46	1.00	4.4	9.7
Aldicarb sulfone	20.0	0.86	1.33	4.5	6.9	0.43	0.87	2.2	4.5
Aldicarb sulfoxide	20.0	0.89	1.35	4.5	6.9	0.52	0.85	2.7	4.4
Baygon (propoxur)	10.0	0.47	0.78	5.0	8.2	0.46	0.75	4.6	7.5
Carbaryl	20.0	0.79	1.35	4.1	6.9	0.93	1.35	4.8	6.9
Carbofuran	20.0	0.74	0.68	3.9	3.6	0.47	1.00	2.4	5.1
3-Hydroxycarbofuran	20.0	0.95	1.31	4.9	6.8	0.98	1.72	5.0	8.7
Methiocarb	50.0	2.07	3.93	4.4	8.4	1.69	3.08	3.5	6.4
Methomyl	5.0	0.33	0.37	6.7	7.5	0.14	0.20	2.8	4.0
Oxamyl	20.0	1.04	1.44	5.4	7.4	0.55	1.13	2.7	5.7
Average				4.7	6.9			3.5	6.3
Std dev.				0.91	1.34			1.07	1.92

[a] s_r and s_R = standard deviations for repeatability and reproducibility, respectively. RSD_r and RSD_R = corresponding relative standard deviations.

[b] Concentration value is 10–15 times estimated method detection limit (MDL).

oxime and *N*-methylcarbamate pesticides in finished drinking water)

Method Performance:

Collaborative study showed method acceptable for all analytes tested. No matrix effects were observed. In reagent water, RSD_R for 10 pesticides ranged from 3.6 to 8.4%. In finished drinking water, RSD_R for 10 pesticides ranged from 4.0 to 9.7%. *See* table of method performance data.

A. Principle

Water sample is filtered and measured volume (400 μL) is directly injected onto reverse-phase LC column. Analytes are separated by gradient elution chromatography. After elution from LC column, analytes are hydrolyzed with NaOH at 95°. Methylamine formed during hydrolysis is reacted with *o*-phthalaldehyde and 2-mercaptoethanol to form highly fluorescent derivative, which is detected by fluorescence detector. Estimated method detection limits range from 0.5 μg/L for methomyl to 4.0 μg/L for methiocarb; estimated method detection limits for 8 other compounds range from 1.0 to 2.0 μg/L.

B. Apparatus

(a) *Grab sample bottle.*—60 mL, borosilicate glass, screw-cap vials (Pierce No. 13075 meets these specifications) and caps with PTFE-faced silicone septa (Pierce No. 12722 meets these specifications). Before use, wash vials and septa with soap and water, followed by 3 tap water rinses and 3 deionized water rinses.

(b) *Balance.*—Analytical, capable of accurately weighing to nearest 0.0001 g.

(c) *Macrofilters.*—47 mm, 0.45 μm, nongridded, cellulose acetate filters for water phases; 47 mm, 0.5 μm, nongridded, PTFE filters for organic phases.

(d) *Microfilters.*—13 mm stainless steel filter holder and 13 mm diameter, 0.2 μm polyester filters (Nucleopore No. 180406 meets these requirements).

(e) *Hypodermic syringe.*—10 mL, glass, with Luer-Lok tip.

(f) *Syringe valve.*—3-way.

(g) *Syringe needle.*—7–10 cm long, 17 gauge, with blunt tip.

(h) *Microsyringes.*—Various sizes.

(i) *Solution storage bottles.*—Amber glass, 10–15 mL capacity with TFE-fluorocarbon-lined screw cap.

(j) *LC system.*—Capable of injecting 200–400 μL aliquots and performing binary linear gradients at constant flow rate. Data system is recommended for measuring peak areas. Primary column: 250 mm × 4.6 mm id stainless steel packed with 5 μm Beckman Ultrasphere ODS. Mobile phase linear gradient from methanol–water (15 + 85) to methanol in 32 min at 1.0 mL/min. Confirmation column: 250 mm × 4.6 mm id stainless steel packed with 5 μm Supelco LC-1. Mobile phase linear gradient from methanol–water (15 + 85) to methanol in 32 min at 1.0 mL/min.

(k) *Postcolumn reactor.*—Reactor constructed with PTFE tubing and equipped with pumps capable of mixing 0.5 mL/min OPA reaction solution, C(i), and 0.5 mL/min NaOH, C(f), into mobile phase. Reactor must contain mixing tees and two 1.0 mL delay coils, one thermostated at 95°.

(l) *Fluorescence detector.*—Capable of excitation at 230 nm and detection of emission energies >418 nm.

C. Reagents

(a) *Standard solutions.*—Use standards of test compounds with purity >96% to prepare stock solutions at 1.00 μg/μL in methanol. Commercially prepared stock standards may be used at any concentration if they are certified by manufacturer or independent source. Transfer stock standard solutions into TFE-fluorocarbon-sealed screw-cap vials. Store at room temperature protected from light. Replace stock standard solutions after 2 months, or sooner if comparison with laboratory control standards indicates degradation.

(b) *Instrument performance solution.*—Combine 20 μL 3-hydroxycarbofuran stock solution, (a), and 1.0 mL aldicarb sulfoxide stock solution, (a), in 10 mL volumetric flask and dilute to volume with methanol.

(c) *Reagent water.*—Water reasonably free of contamination that would prevent determination of analytes.

(d) *Water.*—LC grade.

(e) *Methanol.*—LC grade. Filter, B(c), and degas with helium before use.

(f) *Sodium hydroxide.*—0.05N. 2.0 g NaOH/1.0 L reagent water, (c). Filter, B(c), and degas with helium before use.

(g) *Mercaptoethanol-acetonitrile.*—(1 + 1). Mix 10.0 mL 2-mercaptoethanol and 10.0 mL CH_3CN. Store in borosilicate glass vial or bottle with PTFE-lined cap. (*Caution:* Strong odor. Store in hood.)

(h) *Sodium borate.*—0.05N. 19.1 g $Na_2B_4O_7 \cdot 10H_2O$/1.0 L reagent water, (c). Sodium borate dissolves completely at room temperature if prepared day before use.

(i) *OPA reaction solution.*—100 ± 10 mg *o*-phthalaldehyde (mp 55–58°)/10 mL CH_3OH, (e). Add to 1.0 L 0.05N $Na_2B_4O_7$ solution, (h). Mix, filter, B(c), and degas with helium. Add 100 μL 2-mercaptoethanol, (g), and mix. Prepare solution fresh daily.

(j) *Helium.*—For degassing solutions and solvents.

(k) *Monochloroacetic acid buffer.*—pH 3. Mix 156 mL 2.5M monochloroacetic acid and 100 mL 2.5M potassium acetate.

(l) *Sodium thiosulfate.*—$Na_2S_2O_3$. Granular, anhydrous. ACS grade.

(m) *Buffered reagent water.*—Mix 10 mL monochloroacetic acid buffer, (k), and 1 L reagent water, (c).

(n) *Internal standard solution.*—Prepare 4-bromo-3,5-dimethylphenyl *N*-methylcarbamate (BDMC) (purity >98%, Aldrich Chemical Co.) stock solution at 0.1 mg/mL in methanol.

D. Preparation of Sample Bottles

Add 1.8 mL monochloroacetic acid buffer, C(k), to sample bottle, B(a). If residual chlorine is expected, add 5 mg $Na_2S_2O_3$ to bottle before sample collection.

E. Sample Collection

Collect 60 mL grab samples in glass bottles by conventional sampling practices. Because bottles contain buffer and $Na_2S_2O_3$, do not prerinse with sample before collection. Add sample to bottle, seal, and shake vigorously 1 min. Refrigerate samples at 4° from time of collection until storage. Store at −10° until analyzed. Analyze samples within 28 days of collection.

F. Sample Preparation

Adjust pH of sample or standard to pH 3 ± 0.2 by adding 1.5 mL 2.5M monochloroacetic acid buffer, C(k), to 50 mL sample. This step should not be necessary if sample pH was adjusted during sample collection. Fill 50 mL volumetric flask to mark with sample. Add 5 μL internal standard stock solution, C(n), to the 50 mL of sample (final concentration 10 μg/L). Affix 3-way valve to 10 mL syringe. Place clean filter in filter holder, B(d), and affix filter holder and 7–10 cm syringe needle to syringe valve. Rinse needle and syringe with reagent water, C(c). Prewet filter by passing 5 mL reagent water through filter. Empty syringe and check for leaks. Draw 10 mL sample into syringe and expel through filter. Draw another 10 mL sample into syringe, expel through filter, and collect last 5 mL for analysis. Rinse syringe with reagent water. Discard filter. Inject 400 μL of collected sample into LC system under conditions in B(j).

G. Calibration of LC System

Table **991.06A** presents retention times observed using this method. Fig. **991.06** shows chromatographic separation of the pesticides studied. Initially, perform 5-level calibration using calibration standards prepared in buffered reagent water, C(m), within linear range of detector. Use calibration curve or ratio of response to concentration (calibration factor). If calibration factor values are constant over working range (<20% RSD), average calibration factor may be used for calculations. Verify calibration curve daily using 1 or 2 calibration standards. If response of any analyte varies >20% from average calibration factor for initial calibration, repeat analysis of single-level standard with fresh standard. Alternatively, prepare new calibration curve.

H. Quality Control

Minimum quality control requirements for this method include (*1*) initial demonstration of laboratory capability, (*2*) analysis of a sample blank with each set of extracted samples as continuing check on sample contamination, (*3*) analysis of a spiked reagent water sample as continuing check on method recovery, and (*4*) analyses of daily instrument QC standards to ensure acceptable instrument performance (*see* Table **991.06B**).

Demonstrate initial method performance by analyzing 4 reagent water samples spiked at concentration levels indicated in Table **991.06C** (ca 10 times estimated method detection limit). Calculate average percent recovery and standard deviation of percent recovery. For acceptable performance, relative standard deviation should be <20% and mean recovery should be within performance-based acceptance limits in Table **991.06C**. Demonstrate continuing check on method recovery by analyzing 1 reagent water sample spiked at con-

Table 991.06A. Relative Retention Times for Primary and Confirmation Columns and EDLs for 10 Carbamate Pesticides

Analyte	CAS No.	Retention Time, min		
		Primary[a]	Confirmation[b]	EDL[c]
Aldicarb	116-06-3	27.0	21.4	1.0
Aldicarb sulfone	1646-88-4	15.2	12.2	2.0
Aldicarb sulfoxide	1646-87-3	15.0	17.5	2.0
Baygon	114-26-1	29.6	23.4	1.0
Carbaryl	63-25-2	30.8	25.4	2.0
Carbofuran	1563-66-2	29.3	24.4	1.5
3-Hydroxycarbofuran	16655-82-6	23.3	19.0	2.0
Methiocarb	2032-65-7	34.9	28.6	4.0
Methomyl	16752-77-5	18.4	14.8	0.50
Oxamyl	23135-22-0	17.4	14.6	2.0

[a] Primary column, *see* B(j).
[b] Confirmation column, *see* B(j).
[c] Estimated method detection limit in μg/L.

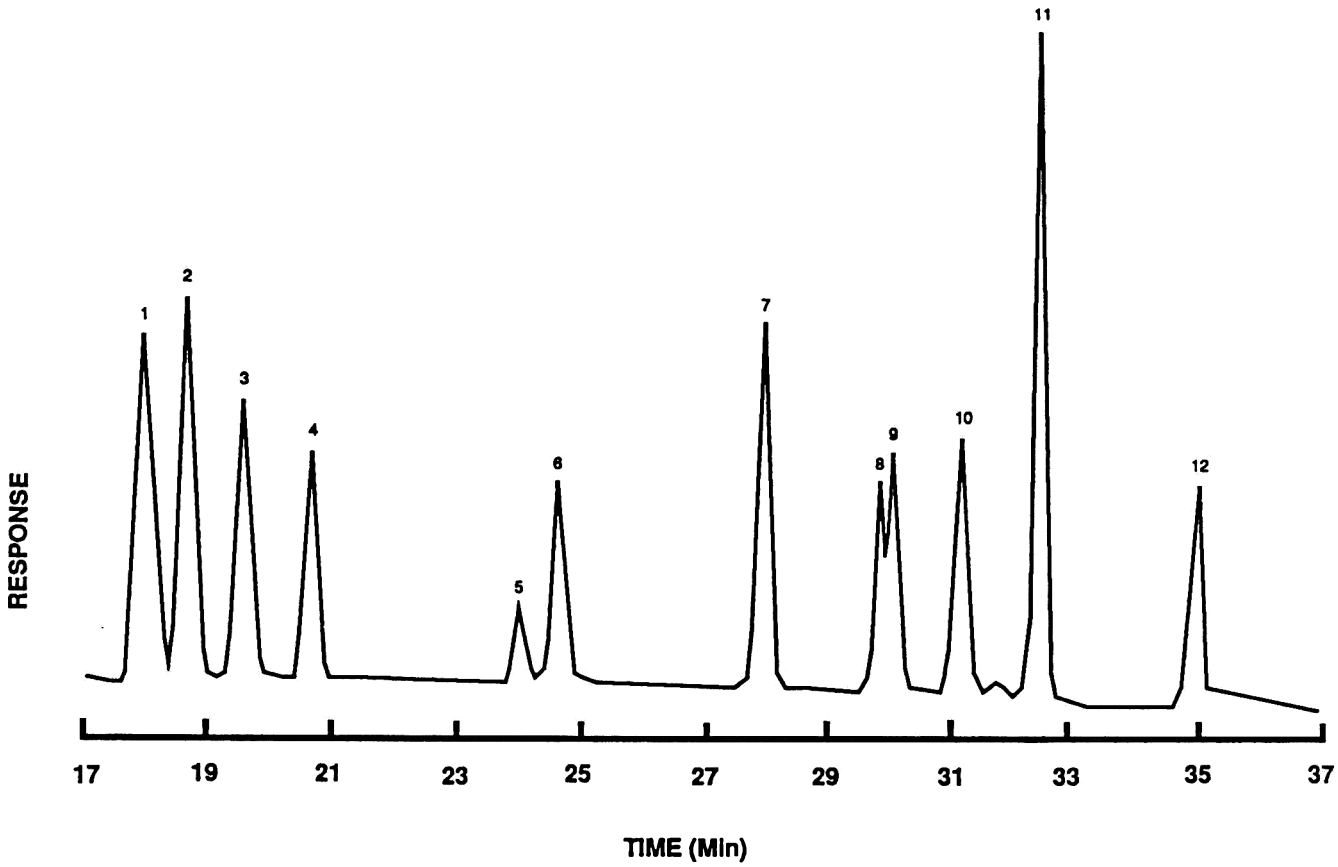

FIG. 991.06—Reconstructed liquid chromatogram of 10 carbamate pesticides separated on primary column, B(J). 1, aldicarb sulfoxide; 2, aldicarb sulfone; 3, oxamyl; 4, methomyl; 5, decomposition product of aldicarb sulfoxide; 6, 3-hydroxycarbofuran; 7, aldicarb; 8, carbofuran; 9, Baygon; 10, carbaryl; 11, decomposition product of carbaryl; 12, methiocarb.

centration levels in Table **991.06C** every 20 samples or one with each set of extracted samples, and compare recovery to performance-based acceptance limits in table.

Ref.: JAOAC **74,** March/April issue (1991).

Results and Discussion

Each study participant analyzed a quality control sample of known concentration after each 6 spiked samples and compared the recovery of each analyte to acceptance limits provided. The laboratories were instructed to take corrective action if analyte spike recoveries were outside 99% confidence limits derived from the method development project (3). Repeat analysis of the quality control sample was required to confirm corrective action. The final quality control data reported by each laboratory were reviewed, and all were within the control limits established for the study.

The 10 pesticides were not resolved under the conditions of the method by the participating laboratories. Minor adjustments to the mobile phase conditions were reported by all participants to effect adequate resolution of the compounds.

Table 991.06B. Instrument Quality Control Standard

Test	Analyte	Concn, μg/L	Requirements
Sensitivity	3-Hydroxy-carbofuran	2	Detection of analyte; S/N > 3
Chromatographic performance	Aldicarb sulfoxide	100	0.90 < PGF < 1.1[a]

[a] PGF = peak Gaussian factor = $(1.83 \times W^{1/2})/W^{1/10}$ where $W^{1/2}$ is the peak width at half height, and $W^{1/10}$ is the peak width at tenth height.

Rejection of Outliers

For the entire study, the EPA computer program rejected 14.4% of the 960 data points submitted. The percentage of rejected data was 8.2 for reagent water and 20.6 for finished drinking water (Table 1). The largest number of outliers, 21, was for carbofuran determinations; the least, 7, occurred for

Table 991.06C. Acceptance Limits for Analysis (as Percent of Mean Recovery) of Laboratory Quality Control Sample

Analyte	Concn Level[a]	Mean Recovery[b]	Overall Std Dev.[b]	Acceptance Limits,[c] %
Aldicarb	10.0	9.46	0.58	81.6–118
Aldicarb sulfone	20.0	19.3	1.33	79.3–121
Aldicarb sulfoxide	20.0	19.6	1.35	79.3–121
Baygon	10.0	9.52	0.78	75.4–124
Carbaryl	20.0	19.5	1.35	79.2–121
Carbofuran	20.0	19.1	0.68	89.3–111
3-Hydroxycarbofuran	20.0	19.2	1.31	79.5–120
Methiocarb	50.0	47.0	3.93	74.9–125
Methomyl	5.00	4.92	0.37	77.4–122
Oxamyl	20.0	19.4	1.44	77.7–122

[a] Concentration level ca 10 times the estimated method detection limit.

[b] Calculated from the mean recovery and overall standard deviation regression equations from the collaborative study.

[c] Acceptance limits are defined as the mean recovery ± 3 standard deviations, as percent.

Table 1. Total number of rejected data by compound by water type and in total

Compound	Reagent water[a]	Finished drinking water[a]	Total rejected data[b]
Aldicarb	8	7	15
Aldicarb sulfone	7	13	20
Aldicarb sulfoxide	6	12	18
Baygon	1	9	10
Carbaryl	6	9	15
Carbofuran	9	12	21
3-Hydroxycarbofuran	0	7	7
Methiocarb	0	13	13
Methomyl	2	9	11
Oxamyl	0	8	8
Total	39	99	138
(Percentage of total submitted data)	(8.2%)[c]	(20.6%)[c]	(14.4%)[d]

[a] Total submitted data per analyte by water type was 6 concentrations × 8 laboratories = 48.
[b] Total submitted data by compound was 6 concentrations × 8 laboratories × 2 water types = 96.
[c] Total submitted data by water type was 6 concentrations × 8 laboratories × 10 analytes = 480.
[d] Total submitted data for the study = 960.

Table 2. Total number of rejected data by laboratory by outlier test and in total

Laboratory code	Laboratory ranking test[a]	Thompson's outlier test[a]	Total rejected data[b]
01	0	10	10
02	54	0	54
03	24	8	32
04	18	1	19
06	12	1	13
07	0	3	3
09	6	0	6
10	0	1	1
Total	114	24	138

[a] Level of significance 0.05.
[b] Total submitted data by laboratory was 6 concentrations × 10 analytes × 2 water types = 120.

3-hydroxycarbofuran. Three compounds had 10 or fewer outliers, 6 compounds had between 11 and 20 outliers, and only 1 compound had over 20 outliers.

The number of rejected data points for each laboratory is presented in Table 2. The laboratory ranking test accounted for 82.6% of all rejected data. Of the 8 laboratories that submitted data, laboratories 2 and 3 accounted for more rejected data (62.3% of the total rejected) than did the remaining 6 laboratories combined. The laboratory ranking test detected a negative bias in the data submitted by laboratory 2 and a positive bias in the data submitted by laboratory 3. Laboratories 2 and 3 used C_8 columns instead of the C_{18} column recommended in the method and used by the other 6 laboratories. These 2 laboratories also used a ternary pumping system with the addition of acetonitrile by the third pump. The initial operating conditions were 15 + 85 + 0 (methanol–water–acetonitrile) programmed to final conditions of 0 + 30 + 70. The other 6 laboratories used binary pumping systems with initial conditions approximately 15 + 85 (methanol–water) programmed to final conditions of approximately 80 + 20. The data from laboratories 2 and 3 were retained because it was not clear whether their poorer data were method-related or laboratory-related. No significant difference was observed when the summary statistics were calculated both with and without these data. This verifies the effectiveness of the outlier routines incorporated in the EPA computer program to remove errant data points.

Method Recovery

The summary statistics calculated after removal of outliers are presented in Table 3. Where applicable, linear regression equations are presented to describe the mean recovery as a function of the spike concentration. Coefficients of determination (COD_w), calculated for these weighted regression equations, show that the equations fit the data sets very well; COD_w values for all 20 equations were above 0.98. These values confirm the acceptability of these equations for estimating the mean recovery at any concentration level within the range tested.

Precision

Because an external standard was used to quantify the analytes in this study, it is viewed that the precisions obtained would represent a conservative estimate of the performance of the method. It is anticipated that method precision would improve with use of the internal standard specified in the method and now commercially available.

The overall standard deviation (s_R) is the precision associated with measurements generated by the 8 laboratories, whereas the single-analyst standard deviation (s_r) is the precision associated with performance in an individual laboratory. The overall and single-analyst precisions were calculated using a value about 10 times the estimated method detection limit (EDL) in the precision regression equations presented in Table 3. Because the concentration ranges studied for the analytes were narrow (just under 1 order of magnitude), it was difficult to reliably correlate the expected increase in overall precision to increases in concentration levels. The correlations for 9 of the 20 overall precision regression equations were weak ($COD_w < 0.5$) and extrapolation of these relationships outside the study range is not recommended. The average overall precision for the 10 analytes in reagent water at about 10 times the EDL, expressed as RSD_R, was 6.9%, ranging from 3.6% for carbofuran to 8.4% for methiocarb. In finished drinking water, the average overall precision was 6.3%, ranging from 4.0% for methomyl to 9.7% for aldicarb. As seen from these statistics, there were no differences between the reagent water matrix and the various finished drinking water matrixes. The average single-analyst precision, expressed as RSD_r, for the 10 analytes in reagent water at about 10 times the EDL, was 4.7%, ranging from 3.4% for aldicarb to 6.7% for methomyl. In finished drinking water, the average single-analyst precision was 3.5%, ranging from 2.2% for aldicarb sulfone to 5.0% for 3-hydroxycarbofuran.

Effect of Water Type

The data across water types were subjected to an analysis of variance test using the EPA computer program to determine the effect of water type on recovery and precision. Only aldicarb sulfoxide was found to have method performance characteristics that are influenced by the sample matrix. The

Table 3. Summary statistics and regression equations for Method 531.1 collaborative study data sets

	Reagent Water					Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Aldicarb	3.24	3.24	0.33	0.37	$X = 0.926C + 0.202$	3.27	0.24	0.22	$X = 1.032C + 0.031$
	4.84	4.56	0.69		$s_R = 0.022X + 0.370$[f]	5.09	0.40		$s_R = 0.101X - 0.042$[f]
	9.70	9.26	0.91	0.15	$s_r = 0.32$[e]	10.87	2.10	0.51	$s_r = 0.040X + 0.046$
	12.90	12.37	0.57			13.84	1.73		
	19.40	18.12	0.12	0.44		19.08	0.43	1.04	
	27.40	25.30	0.72			26.77	1.90		
Aldicarb sulfone	6.44	6.71	0.63	0.58	$X = 0.942C + 0.446$	6.18	0.30	0.35	$X = 0.968C - 0.097$
	9.68	9.11	0.37		$s_R = 0.062X + 0.132$	9.24	0.53		$s_R = 0.039X + 0.119$[f]
	19.30	18.26	2.24	0.98	$s_r = 0.025X + 0.382$	18.42	1.34	0.38	$s_r = 0.008X + 0.276$
	25.80	25.07	1.11			24.55	1.14		
	38.60	37.10	2.04	1.43		36.75	1.70	0.79	
	54.80	52.83	3.56			54.60	1.57		
Aldicarb sulfoxide	6.40	6.58	0.66	0.39	$X = 0.941C + 0.876$[g]	5.65	0.77	0.22	$X = 0.952C + 0.460$[g]
	8.00	8.53	0.51		$s_R = 0.058X + 0.211$	8.18	0.60		$s_R = 0.021X + 0.440$
	19.20	17.99	2.42	1.18	$s_r = 0.040X + 0.103$	18.30	0.84	0.40	$s_r = 0.024X + 0.050$
	24.00	23.34	0.92			22.72	0.97		
	40.00	39.31	1.53	1.59		38.68	1.32	1.46	
	56.00	54.66	3.71			55.55	1.38		
Baygon	3.16	3.35	0.33	0.26	$X = 0.916C + 0.360$	3.20	0.16	0.20	$X = 0.994C + 0.101$
	4.76	4.47	0.74		$s_R = 0.058X + 0.230$	4.92	0.35		$s_R = 0.086X - 0.114$
	9.50	9.13	0.68	0.41	$s_r = 0.040X + 0.092$	9.55	0.68	0.24	$s_r = 0.046X - 0.005$
	12.70	12.02	0.95			13.06	0.23		
	19.00	17.62	0.99	1.12		18.93	2.80	1.48	
	27.00	25.65	1.62			26.26	1.79		
Carbaryl	6.38	6.66	0.58	0.62	$X = 0.949C + 0.542$	6.49	0.63	0.52	$X = 0.958C + 0.439$
	9.58	9.48	0.83		$s_R = 0.058X + 0.219$	9.82	0.28		$s_R = 0.068X + 0.015$
	19.20	18.73	1.32	0.63	$s_r = 0.016X + 0.480$	18.62	1.11	0.57	$s_r = 0.039X + 0.167$
	25.60	24.11	1.61			24.87	1.79		
	38.20	36.39	2.26	1.51		36.67	3.42	2.66	
	54.20	52.41	3.34			52.99	3.56		
Carbofuran	4.76	5.18	0.74	0.48	$X = 0.923C + 0.636$	4.87	0.49	0.37	$X = 0.970C + 0.220$
	7.16	6.90	0.29		$s_R = 0.006X + 0.564$[f]	7.03	0.31		$s_R = 0.042X + 0.178$
	14.30	13.58	0.33	0.41	$s_r = 0.022X + 0.322$	14.23	0.41	0.31	$s_r = 0.008X + 0.316$
	19.10	18.67	0.87			19.27	0.57		
	28.60	26.99	1.22	1.46		27.47	2.19	0.80	
	40.60	38.71	1.15			39.33	2.59		
3-Hydroxy-carbo-furan	6.36	6.59	0.79	0.84	$X = 0.940C + 0.438$	6.39	0.51	0.45	$X = 0.979C + 0.153$
	9.56	9.01	0.99		$s_R = 0.038X + 0.578$	9.51	0.91		$s_R = 0.085X + 0.045$[f]
	19.10	18.41	1.47	0.47	$s_r = 0.013X + 0.697$[f]	18.58	1.29	1.25	$s_r = 0.044X + 0.114$
	25.40	24.41	1.29			25.26	4.76		
	38.20	36.60	1.96	2.09		37.30	1.65	1.80	
	54.20	51.82	2.34			53.67	2.67		
Methiocarb	12.80	12.96	3.02	1.93	$X = 0.923C + 0.887$	13.00	0.76	0.61	$X = 0.958C + 0.474$
	19.20	17.94	2.35		$s_R = 0.035X + 2.286$	18.12	1.91		$s_R = 0.057X + 0.322$
	38.40	36.56	2.71	1.86	$s_r = 0.005X + 1.839$	38.08	3.21	1.29	$s_r = 0.034X + 0.046$
	51.40	48.35	4.50			49.53	3.97		
	77.00	72.44	4.92	2.58		74.25	2.70	3.52	
	109.0	101.9	7.70			105.2	4.56		

continued

Table 3. Summary statistics and regression equations - continued

		Reagent Water				Finished Drinking Water			
Analyte	C[a]	X[b]	s_R[c]	s_r[d]	Regr. Equations	X	s_R	s_r	Regr. Equations
Methomyl	1.60	1.61	0.21	0.17	X = 0.976C + 0.043	1.66	0.22	0.24	X = 0.988C + 0.000
	2.40	2.40	0.22		s_R = 0.048X + 0.133	2.67	0.34		s_R = 0.040X + 0.000
	4.80	4.53	0.60	0.41	s_r = 0.053X + 0.069	4.79	0.13	0.09	s_r = .14[e]
	6.40	6.42	0.40			6.59	1.03		
	9.60	9.41	0.17	0.61		9.31	0.45	0.09	
	13.60	13.49	0.93			13.63	0.22		
Oxamyl	6.40	6.84	0.88	1.02	X = 0.936C + 0.659	6.43	0.81	0.24	X = 0.998C + 0.045
	9.60	9.25	1.25		s_R = 0.038X + 0.699	9.65	0.80		s_R = 0.023X + 0.672[f]
	19.20	18.16	1.48	0.52	s_r = 1.04[e]	19.00	1.67	0.68	s_r = 0.025X + 0.048
	25.60	24.82	1.75			25.75	1.48		
	38.40	37.09	1.16	1.58		37.94	1.35	1.08	
	54.40	52.49	3.27			54.99	0.79		

[a] Spike concentration, μg/L.
[b] Mean recovery, μg/L.
[c] Overall standard deviation, μg/L.
[d] Single-analyst standard deviation, μg/L.
[e] Weighted linear regression equation had negative slope; average precision is reported.
[f] Coefficient of determination of weighted equation was weak (COD < 0.5).
[g] Lowest spike recovery (6.40 μg/L) not used for this regression (see text).

summary statistics for aldicarb sulfoxide in reagent water and in finished drinking water exhibited good overall method precision with RSD_R from 4 to 13% over the 6 concentration levels (Table 3). Recoveries for the 5 highest concentration levels are comparable between reagent water and finished drinking water. However, although the percent recovery in reagent water for the lowest test concentration (6.40 μg/L) was 102.9%, the percent recovery for the same concentration in finished drinking water was uncharacteristically low (88.3%). If these 2 data subsets are ignored and the linear regression equations are recalculated on the basis of the remaining 5 data points, the differences in the reagent water and finished drinking water equations are negligible. Because the difference was traceable to only 1 concentration level, the statistically significant matrix effect is not considered to be of practical importance. The mean recovery regression equations for both matrixes of aldicarb sulfoxide were revised to exclude the lowest spike recovery (*see* Table 3).

Conclusions and Recommendations

The method developed for 10 carbamate pesticides in finished drinking waters was shown to be accurate and precise in a collaborative study among 8 laboratories. Weighted linear regression equations for method recovery were shown to be a useful way to represent the performance of the method and can be used to estimate method recovery at any concentration value within the stated range. The regression equations for overall precision and single-analyst precision were shown to be less representative because of the narrow concentration ranges tested in this study and the highly variable nature of precision estimates and regressions based on such limited data. The recoveries, overall precisions, and single-analyst precisions were generally comparable whether obtained from reagent water or finished drinking water.

The method permits some latitude in the selection of chromatographic columns, mobile phases, and gradients. The data from 2 laboratories were retained for data treatment even though they used a C_8 column with a ternary gradient system rather than the C_{18} column and binary gradient system recommended in the method. These same 2 laboratories produced the poorest data as judged by the percentage of data rejected by outlier tests. It was not clear whether the large number of outlier data submitted by these 2 laboratories resulted from laboratory conditions or method deviations, although the study results clearly support the use of C_{18} columns. Therefore, it is recommended that C_{18} columns be used with this method.

It is recommended that the users of this method routinely analyze a quality control sample prepared in reagent water and compare the results with the performance-based acceptance limits derived from this collaborative study (Table **991.06C**).

On the basis of this interlaboratory method validation study, it is recommended that the method be adopted official first action.

Acknowledgments

The following collaborators participated in this study:

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REFERENCES

(1) Foerst, D. L., & Moye, H. A. (1984) "Aldicarb in Drinking Water via Direct Aqueous Injection HPLC with Post Column Derivatization," Proceedings of the 12th annual AWWA Water Quality Technology conference, American Water Works Association, Denver, CO
(2) Engel, T. (1987) National Pesticide Survey Method No. 5, "Measurement of N-Methylcarbamoyloximes and N-Methylcarbamates in Groundwater by HPLC with Post Column Derivatization," Battelle Columbus Laboratories, Columbus, OH
(3) *Methods for the Determination of Organic Compounds in Drinking Water*, (1988) EPA/600/4-88/039, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH
(4) Proposed rules (1989) 40 CFR Parts 141, 142, 143, *Federal Register*, 22062
(5) Outler, E. C., & McCreery, J. H. (1982) Interlaboratory Method Validation Study: Program Documentation, Battelle Columbus Laboratories, Columbus, OH
(6) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, p. 88
(7) *Annual Book of ASTM Standards* (1990) "Standard Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water," ASTM D2777-86, Vol. 11.01, Water (1), American Society for Testing and Materials, Philadelphia, PA
(8) Barnett, V., & Lewis, T. (1984) *Outliers in Statistical Data*, John Wiley and Sons, New York, NY
(9) Edgell, K. W., & Jenkins, E. L. (1989) "USEPA Method Study 39, 1,2-Dibromoethane (EDB) and 1,2-Dibromo-3-chloropropane (DBCP) in Water by Microextraction and Gas Chromatography," EPA/600/4-88/034, Environmental Monitoring Systems Laboratory, Cincinnati, OH

Liquid Chromatographic Determination of Glyphosate and Aminomethylphosphonic Acid (AMPA) in Environmental Water: Collaborative Study

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A new method for determination of glyphosate and aminomethylphosphonic acid (AMPA) residues in environmental water was collaboratively studied by 6 laboratories. The method is simpler and shorter than previous methods. A filtered volume of water is evaporated to dryness and the residue is dissolved in a buffered EDTA solution. Glyphosate and AMPA are determined by liquid chromatography with postcolumn reaction detection. The method was validated over the range 0.50–5000 ppb, although one of the collaborating laboratories could not reliably quantitate below 1.0 ppb. Statistical analysis of the results showed that typical reproducibility relative standard deviations (RSD_R) ranged from 11 to 20% for both glyphosate and AMPA, which compares very well with predicted values for this concentration range. Total variability (as measured by s_R) increased with increasing fortification level. The method has been adopted official first action by AOAC.

Glyphosate is the active ingredient in Roundup® and Rodeo® herbicides produced by Monsanto Company, St. Louis, MO, and is widely used in various applications for weed, turf, and vegetative control. Several methods have been developed for liquid chromatography of glyphosphate and its metabolite aminomethylphosphonic acid (AMPA), which use either precolumn or postcolumn derivatization. Precolumn procedures have focused on derivatization with 9-fluorenylmethyl chloroformate (FMOCCl) with fluorescence detection (1–3); however, other derivatizing agents, such as 1-fluoro-2,4-dinitrobenzene (4), have been used to form glyphosate and AMPA derivatives that can be detected in the UV-visible region. Postcolumn derivatization has been commonly used with *o*-phthalaldehyde–mercaptoethanol (OPA-MERC) (5, 6) or ninhydrin (7). A literature review in 1985 summarized these and several other methods (8).

A new method was developed for determination of glyphosate and AMPA in environmental waters. The water sample is evaporated and the residue is dissolved in an EDTA solution. Glyphosate and AMPA are separated and detected using liquid chromatography and a postcolumn derivatization reaction specific for primary amines (Figure 1). Glyphosate is oxidized with calcium hypochlorite in a postcolumn reaction coil to form glycine. Glycine is reacted with (OPA-MERC) reagent in a second coil to form a fluorophor, which is detected fluorometrically. AMPA is relatively unreactive toward hypochlorite and undergoes a similar reaction with (OPA-MERC) reagent.

The new method is more sensitive than previous methods and has the added benefits of being simpler and shorter (9). Since the method was developed to detect as little as 0.5 ppb glyphosate and AMPA in environmental water, it was of

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The report has been evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Environmental Quality. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9–13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* **74**, January/February issue.

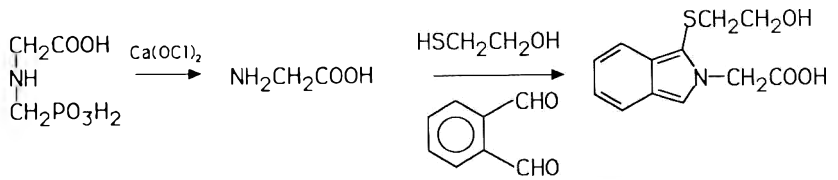

Figure 1. Postcolumn derivatization reaction: glyphosate is oxidized by calcium hypochlorite to a primary amine, which reacts with *o*-phthalaldehyde in presence of mercaptoethanol to form a fluorescent derivative.

interest to determine if the required sensitivity could be achieved. The present paper reports the results and evaluation of a collaborative study to determine the accuracy and precision of the method.

Collaborative Study

Each of the 6 collaborators was supplied with a total of 50 samples consisting of duplicate pairs of samples fortified at known concentrations, duplicate pairs of samples fortified at concentrations unknown to the participants, and check samples. The range of method validation was from 0.5 to 5000 ppb. Nine samples fortified at known concentrations were supplied in duplicate to cover the 5 orders of magnitude in the range studied. The known concentrations and the 10 check samples were supplied to allow each collaborator to validate the method and to demonstrate proper functioning of the method. The remaining 22 samples were 10 duplicates and 1 pair of samples fortified at slightly different levels. Among the duplicate samples fortified as unknown concentrations was one pair of unfortified samples used to identify false positives. All samples were fortified with equal amounts of glyphosate and AMPA.

Environmental water was collected from a lake in a wildlife refuge deemed to be relatively free of pesticide contamination by virtue of its location. The water was kept refrigerated until needed. Before the samples were prepared, the water was filtered through a glass-fiber filter to remove algae and other large particulates. Individual samples were fortified with concentrated aqueous standard solutions containing both glyphosphate and AMPA. After the standard was added, the sample was diluted to volume, the bottle cap was secured, the contents were shaken, and the container was placed in freezer storage. (Actual field samples should be frozen as soon as possible after collection to maintain sample integrity.)

Samples were sent to collaborators as frozen, premeasured 250 mL portions in 8 oz polyethylene bottles. Dry ice was shipped with the samples to ensure that they remained frozen in transit.

Each analyst received 5 sets of 10 frozen environmental water samples. The first set consisted of 2 check samples and 8 fortified samples for method validation. The second set included duplicate check samples, 4 fortified samples, and 2 duplicate blind samples. The remaining sets consisted of duplicate check samples, 2 fortified samples, and 3 duplicate blind samples. The check samples were filtered but otherwise untreated environmental water.

All fortified samples used for method validation were check samples fortified in advance at the stated concentrations by Monsanto. The blind samples were check samples fortified at Monsanto by the study director at levels that were unknown to all analysts, both at Monsanto and other laboratories. Standards of glyphosate and AMPA were also prepared at Monsanto and were provided to ensure that every laboratory used standards of the same concentration and quality. A 100 μg/mL composite standard was also supplied so that each laboratory could fortify locally supplied water samples to practice the method procedures prior to beginning the study.

991.08

Glyphosate and Aminomethylphosphonic Acid (AMPA) in Environmental Water

Liquid Chromatographic Method

First Action 1991

AOAC-American Water Works Association Method

(Applicable to determination of glyphosate and AMPA in ground water, drinking water, and surface water at 0.5–5000 ppb)

Method Performance:

Glyphosate, 0.50–5000 ppb

s_r = 0.037–722; s_R = 0.120–818; RSD_r = 2.11–16.6%; RSD_R = 4.62–49.8%

AMPA, 0.50–5000 ppb

s_r = 0.055–653; s_R = 0.055–653; RSD_r = 2.4–19.1%; RSD_R = 6.51–162%

A. Principle

Environmental water containing glyphosate and aminomethylphosphonic acid (AMPA) is evaporated to dryness using a rotary evaporator and the residue is dissolved in EDTA solution. Glyphosate and AMPA are separated and detected by liquid chromatography using a postcolumn reaction specific for primary amines. Glyphosate is oxidized in a postcolumn reactor coil at 50° with calcium hypochlorite to form glycine. Glycine is treated with *o*-phthalaldehyde (OPA) in the presence of mercaptoethanol (MERC) in a second coil to form a fluorophor, which is detected fluorometrically (λ excitation 340 nm, λ emission 425–455 nm). AMPA is relatively unreactive toward calcium hypochlorite but undergoes a similar reaction with OPA-MERC reagent to form another fluorophor, which is detected fluorometrically under same conditions as for glycine.

B. Apparatus

(a) *Liquid chromatograph.*—Pulse-free or pulse-dampened system that includes pump, injector, column heater, and integrator or data system.

(b) *Fluorescence detector.*—High sensitivity, capable of providing 340 nm excitation and 425–455 nm emission wavelengths. (Filter fluorometers such as Waters Associates Model 420 have demonstrated adequate sensitivity.) Signal-to-noise ratio for lowest calibration standard (0.025 μg/mL) should be >10:1.

(c) *Analytical column.*—Aminex A-9, 30 cm × 4.6 mm

with 11.5 μm particles. Operating temperature 50°. (Aminex A-9 prepared and packed by Bio-Rad Laboratories is the only column tested that gives adequate performance. Before packing into column, resin is converted to K^+ form and is packed using CH_3OH–0.005M KH_2PO_4 (4 + 96) adjusted to pH 2.1 with H_3PO_4.)

(d) *Guard column.*—C-18 bonded silica, 3.6 cm × 4.6 mm, with 5 μm particles.

(e) *Postcolumn reactor.*—Dual pump derivatization system that includes 2 reaction coils. Maintain 1 reaction coil at 40°. Flush pumps with water when not in use. Maintain mobile phase flow at reduced rate of 0.2–0.3 mL/min. When system is to be shut down, flush both column and pump with CH_3OH–H_2O (4 + 96).

(f) *Cellulose membrane filters.*—0.22 μm; 0.45 μm.

C. Reagents

(a) *Solvents.*—Reagent grade HCl, LC grade methanol, concentrated phosphoric acid, and water [17 megohm, filtered through 0.22 μm membrane filter, **B(f)**].

(b) *Potassium dihydrogen phosphate.*—KH_2PO_4. LC grade.

(c) *Calcium hypochlorite.*—$Ca(OCl)_2$. Certified, 70.9% available chlorine.

(d) *Disodium ethylenediaminetetraacetate dihydrate.*—Disodium EDTA dihydrate. Certified ACS grade.

(e) *EDTA solutions.*—(*1*) *0.03M EDTA solution.*—Dissolve 11.17 g disodium EDTA dihydrate in 1 L deionized H_2O using magnetic stirrer and filter through 0.22 μm membrane filter. (*2*) *0.001M EDTA solution.*—Dissolve 0.37 g disodium EDTA dihydrate in 1 L deionized H_2O using magnetic stirrer and filter through 0.22 μm membrane filter.

(f) *Glyphosate and AMPA fortification solutions.*—Stock solution: 100 μg each of glyphosate and AMPA/mL. Dissolve 0.1000 g *N*-(phosphonomethyl)glycine (glyphosate, 99%, Monsanto Co.) and 0.1000 g aminomethylphosphonic acid (AMPA, 99%, Monsanto Co.) in 1 L deionized H_2O. Prepare fortification solutions of 10.0 and 1.00 μg/mL by serial dilution of stock solution. Store refrigerated in polyethylene or polypropylene bottles. Stock solution is stable up to 1 year. Prepare fortification solutions every 6 months.

(g) *Glyphosate and AMPA calibration standards.*—Stock solution: 100 μg each of glyphosate and AMPA/mL. Dissolve 0.1000 g glyphosate and 0.1000 g AMPA in 1 L 0.001M EDTA solution. Prepare calibration solutions of 1.000, 0.500, 0.100, 0.50, and 0.025 μg/mL by serial dilution of stock solution with EDTA. Store refrigerated in polyethylene or polypropylene bottles. Stock solution is stable up to 1 year. Prepare calibration solutions every 6 months.

(h) *Mobile phase.*—0.005M KH_2PO_4. Dissolve 2.72 g KH_2PO_4 in 4 L CH_3OH–H_2O (4 + 96). Adjust solution to pH 2.1 with H_3PO_4, (a). Follow normal LC degassing procedures, and filter solution through 0.22 μm membrane filter.

(i) *Oxidative solution.*—In 500 mL volumetric flask, dissolve 0.5 g $Ca(OCl)_2$ in 500 mL deionized H_2O using magnetic stirrer at high speed for 45 min. In 1 L volumetric flask, dissolve 1.36 g KH_2PO_4, 11.6 g NaCl, and 0.4 g NaOH in 500 mL deionized H_2O. Add 10 mg $Ca(OCl)_2$ (10 mL of first solution) and dilute to volume with deionized water, mixing well. Filter solution through 0.22 μm membrane filter. Prepare fresh oxidative solution each day system is used.

(j) *o-Phthalaldehyde–mercaptoethanol solution.*—OPA-MERC solution. [Fluoraldehyde® Reagent Solution (Pierce Chemical Co.) is *o*-phthalaldehyde solution containing a stabilizing agent, Brij® 35, and mercaptoethanol in borate buffer.] Solution is stable up to 3 months with little loss of sensitivity when stored unopened and refrigerated at 4°. Once opened, use Fluoraldehyde reagent within 3 days. OPA-MERC solution prepared by the analyst or without a stabilizer should be prepared daily as follows: Dissolve 35 g boric acid in 950 mL deionized H_2O. Adjust pH to 10.4 with 45% KOH and filter solution through 0.22 μm membrane filter. To this borate buffer, add 3.0 mL 30% w/w Brij 35, 2.0 mL 2-mercaptoethanol, and 800 mg OPA dissolved in 10 mL CH_3OH.

D. Sample Preparation

Note: Samples should be frozen as soon as possible after collection.

Thaw 250 mL frozen water sample, shake thoroughly, and transfer ca half of sample to 500 mL round-bottom flask. If suspended material is visible, filter known volume of sample through 1 μm glass fiber filter. (*Note:* Method analyzes for glyphosate and AMPA in solution; any glyphosate or AMPA bound to suspended matter must be quantitated separately.)

For spiked samples, use analyte fortification solutions, **C(f)**, and proceed as for samples. Add 5 mL HCl to sample in flask and 5 mL to sample remaining in bottle. Concentrate sample on rotary-film evaporator by slowly increasing temperature of water bath from 20 to 60°. Before sample is evaporated to dryness, add remainder of sample to rotary-film evaporator and rinse bottle twice with ca 5 mL deionized H_2O, adding rinses to rotary-film evaporator. Concentrate sample to dryness and, if necessary, remove final traces of moisture with stream of dry nitrogen. Pipet 2.9 mL LC buffer [0.005M KH_2PO_4, **C(h)**] to rinse sides of flask and use long cotton swab to remove any solid residue from bottom. Leave swab in flask. Add 0.1 mL 0.03M EDTA solution, **C(e)** (*1*), and mix thoroughly, swirling mixture to rinse sides. Transfer solution to disposable syringe and filter through 0.45 μm membrane filter, **B(f)**. Sample is now ready for LC determination of glyphosate and AMPA.

If samples must be diluted to remain within standard concentration range, dilute with 0.001M EDTA solution.

E. Determination

Stabilize instrument at mobile phase flow rate of 0.5 mL/min. Maintain column temperature at 50°. Flow rates for calcium hypochlorite solution and OPA-MERC solution are both nominally 0.5 mL/min, but can be adjusted to optimize relative peak response of glyphosate to AMPA. Increasing calcium hypochlorite flow rate decreases AMPA response relative to glyphosate response. Maintain hypochlorite reactor coil at 40°. Adjust detector attenuation so that a 200 μL injection of 1.000 μg/mL standard (0.200 μg) gives 75–95% full-scale recorder pen deflection for glyphosate peak. Analyze calibration standards (0.025–1.000 μg/mL) after every 2 samples to ensure accurate quantitation; place standards randomly with respect to concentration level. Prepare standard curves by using either peak area or peak height measurements for 5 standards. Perform linear least squares regression on response vs amount injected for standards within each set to generate standard calibration curve. Calculate amount of analyte in sample from standard calibration curve, using peak response to determine amount injected. Divide product of amount injected and concentration factor by sample size to yield concentration found. Root mean square of resulting standard curve should be ≥0.9900. If glyphosate or

AMPA response is greater than response for highest standard, dilute sample with 0.001M EDTA solution to achieve on-scale response. Typical chromatogram of fortified environmental water sample is shown in Fig. **991.08**.

F. Quality Control

Minimum quality control requirements for this method include (*1*) initial demonstration of method performance, (*2*) analysis of method blanks as continuing check on sample contamination, (*3*) analysis of spiked samples as continuing check on method recovery, and (*4*) verification of calibration by analysis of standards every 2 samples.

Ref.: JAOAC **74**, March/April issue (1991).

CAS-1071-83-6 (glyphosate)
CAS-1066-51-9 (aminomethylphosphonic acid)

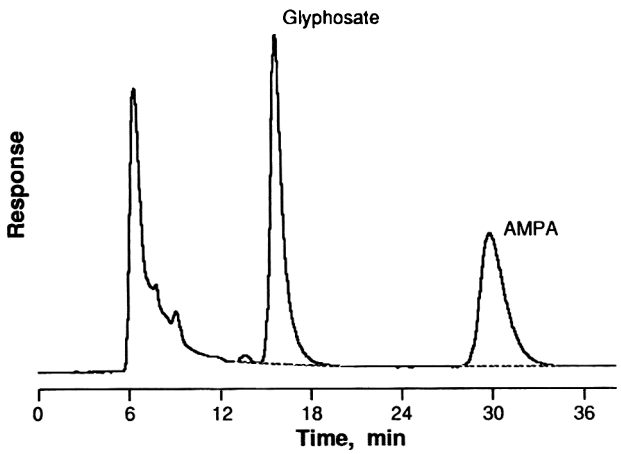

FIG. 991.08—Typical chromatogram for LC recovery of 10.0 ppb each of glyphosate and AMPA added to 250 mL environmental water. Final volume before injection 3.0 mL. Recoveries were 9.39 ppb glyphosate (93.9%); 9.35 ppb AMPA (93.5%).

Results and Discussion

The final results reported by the individual laboratories were statistically analyzed. Where results less than the lowest standard were reported, those values were used. Results reported as <0.5 ppb were treated for statistical purposes as missing data and therefore were not used during the statistical analysis. No individual values were excluded from analysis on the basis of an outlier test. Because all values do indeed come from the population group of reported results, excluding particular values as outliers would only serve to present a statistical profile of a nonrepresentative population.

Youden's ranking test (10) was applied to the results reported by the 6 collaborators. For this study, the results from the samples fortified at known and unknown concentration levels were kept separate for comparison as were glyphosate and AMPA results. Figure 2 shows the overall rank scores for each collaborator and analyte type. The axes are scores from the samples fortified at known and unknown concentration levels. For 6 collaborating laboratories and 9 levels of measurement, the upper and lower 5% critical limits for ranking scores are 18 and 45, respectively. Collaborators with scores outside this range have a significant systematic error. As seen in Figure 2, Collaborator 5 had ranking scores above the upper limit for the AMPA determinations on samples fortified at both known and unknown concentrations. Collaborator 3 had a score above the upper limit for the known fortification glyphosate samples. Based on ranking scores, the AMPA results from Collaborator 5 and the fortified AMPA results from Collaborator 3 could be considered significantly different from the others. However, because analysis of the total population of results was desired, no results were excluded from analysis. The ranking analysis does show that the majority of the results cluster midway between the critical limits and therefore have a small degree of systematic error.

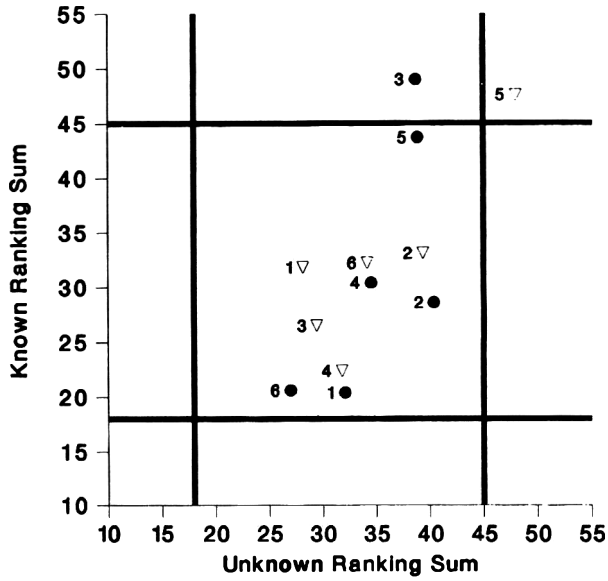

Figure 2. Ranking sums for results reported by collaborators (1–6) on samples fortified with known and unknown concentrations of glyphosate (●) and AMPA (▽). Horizontal and vertical lines at 18 and 45 designate upper and lower 5% critical ranking values.

Results obtained by the collaborators are shown in Table 1. The measures of precision for glyphosate and AMPA are summarized in Tables 2 and 3. These tables give the sample type, fortification level, mean recoveries, and intra- and interlaboratory standard deviations with their corresponding relative standard deviations. Comparison of the total between laboratory precision found in this study to the empirical precision relationship derived by Horwitz (11) from multiple analytical methods is shown in Figures 3 and 4. Method reproducibility as given by the relative standard deviation (RSD_R) is plotted against fortification levels, and the results obtained for this study are compared with the Horwitz curve. For this study, most points fall well below the curve, which indicates good method reproducibility. The exception to this is the 0.50 ppb fortification level, where the RSD_R is 50 and 162%, respectively, for glyphosate and AMPA fortified as an unknown concentration. The relative standard deviations may be high because Collaborator 3 reported very high recoveries for the 0.5 ppb fortification level. At the 0.80 ppb fortification level, although of similar magnitude to 0.5 ppb, the reproducibility relative standard deviations were 22 and 15% for glyphosate and AMPA, respectively, fortified at an unknown concentration. Relative standard deviations were also much lower at the 0.5 ppb fortification level for the known samples.

A total of 24 determinations on unknown check samples were performed and resulted in 8 false positives for glyphosate or AMPA. Collaborator 3 reported 4 of the 8 false

Table 1. Collaborators' results for recovery of glyphosate and AMPA added (ppb) to environmental water

Sample[a]	GLY and AMPA added, ppb	Lab. 1		Lab. 2		Lab. 3		Lab. 4		Lab. 5		Lab. 6	
		GLY	AMPA	GLY	AMPA	GLY	AMPA	GLY	AMPA	GLY	AMPA	GLY	AMPA
C01	0.00	<0.5	<0.5	<0.50	<0.50	0.6	<0.4	0.0	0.2	<0.25	<0.25	<0.50	<0.50
C02	0.00	<0.5	<0.5	<0.50	<0.50	0.4	<0.3	0.0	0.2	<0.25	<0.25	<0.50	<0.50
F03	0.50	0.59	0.38	0.48	0.48	0.74	0.44	0.56	0.36	<0.25	<2.5	0.51	<0.50
F04	1.00	0.96	0.85	0.96	0.96	1.4	0.94	1.1	0.83	<0.25	<2.5	0.94	0.88
F05	5.00	4.8	4.7	4.8	4.3	6.8	4.9	5.7	4.5	5.4	8.2	4.78	4.66
F06	10.0	9.8	8.9	8.8	7.8	12.2	8.6	11.0	8.8	11	12	8.99	9.03
F07	50.0	46	46	47	43	54.0	39.0	53.8	44.9	49	44	47.5	45.0
F08	100	94	102	101	95	110.0	86.0	94.7	81.6	120	130	88.5	84.8
F09	1000	792	912	1016	990	926.0	971.0	887	839	1000	1000	884	870
F10	5000	5160	5280	4830	4950	4320.0	4250.0	4455	4455	—[b]	—	4770	4600
C11	0.00	<0.5	<0.5	<0.50	<0.50	<0.46	<0.40	0.0	0.2	<0.25	4.2	<0.50	<0.50
C12	0.00	<0.5	<0.5	<0.50	<0.50	<0.46	<0.40	0.0	0.2	<0.25	4.6	<0.50	<0.50
F13	0.50	0.73	0.54	0.45	0.45	0.77	0.40	0.61	0.40	<0.25	<0.25	0.52	<0.50
F14	50.0	47	46	52	48	67.0	48.0	54.8	48.2	43	39	49.5	48.2
F15	500	468	468	540	471	542.0	460.0	487	494	620	490	452	441
F16	5000	3360	3360	4680	4560	5670.0	4520.0	4725	4928	6600	5300	5010	4950
T17	5.00	4.7	4.3	4.3	3.7	9.9	7.6	4.6	3.8	4.1	7.3	4.71	4.74
T18	5.00	4.7	4.1	4.5	3.8	8.5	7.8	4.5	3.7	4.4	7.6	4.59	4.70
T19	0.50	0.44	0.38	0.48	0.45	1.3	0.9	0.47	0.62	<0.25	5.7	0.50	0.50
T20	0.50	0.55	0.50	0.45	0.42	1.0	0.6	0.43	0.52	—	—	0.53	0.50
C21	0.00	<0.5	<0.5	<0.50	<0.50	<0.2	<0.2	0.0	0.2	<0.25	<0.25	<0.50	<0.50
C22	0.00	<0.5	<0.5	<0.50	<0.50	1.2	0.68	0.0	0.2	<0.25	<2.5	<0.50	<0.50
F23	1.00	1.07	1.02	0.90	0.78	1.2	0.74	1.15	0.82	<0.25	<2.5	0.86	0.87
F24	100	70	92	96	93	112.0	84.0	98.2	90.7	120	100	86.7	80.6
T25	100	97	89	101	97	95.0	91.0	98.1	83.2	90	60	92.5	86.0
T26	100	91	132	100	98	98.0	92.0	103.7	100.8	95	72	89.4	84.6
T27	20.0	24	24	20	21	22.0	19.0	20.6	18.2	24	21	18.2	18.5
T28	20.0	20	17	20	21	21.0	16.0	19.3	16.7	27	22	19.5	19.0
T29	0.80	0.43	0.55	0.90	0.78	0.82	0.79	0.79	0.85	<0.25	<2.5	0.69	0.71
T30	0.80	0.50	0.66	0.84	0.87	0.77	0.62	0.82	0.85	<0.25	<2.5	0.73	0.83
C31	0.00	<0.5	<0.5	<0.50	<0.50	—	—	0.1	0.3	<0.25	<2.5	<0.50	<0.50
C32	0.00	<0.5	<0.5	<0.50	<0.50	<0.2	<0.14	0.1	0.3	<0.25	<2.5	<0.50	<0.50
F33	5.00	5.16	4.80	5.9	4.7	6.0	4.7	5.5	4.7	—	—	4.89	4.90
F34	500	480	444	479	489	544.0	500.0	457	441	670	550	498	484
T35	40.0	36	32	40	36	36.0	36.0	38.8	35.8	26	24	38.4	37.2
T36	40.0	34	29	38	34	35.0	32.0	38.0	34.4	26	27	37.1	36.5
T37	5000	4920	4680	5130	4950	4727.0	4500.0	4719	4880	4800	5300	4820	4940
T38	5000	5040	5040	5190	5070	4478.0	4357.0	4563	4790	4900	5500	4710	4590
T39	0.00	<0.5	<0.5	0.48	0.48	1.24	0.99	0.4	0.7	<0.25	<2.5	<0.50	<0.50
T40	0.00	<0.5	<0.5	0.30	0.30	2.5	1.97	1.9	2.2	<2.5	<2.5	1.38	<0.50
C41	0.00	<0.5	<0.5	<0.50	<0.50	0.26	0.18	0.2	0.4	<0.25	<2.5	<0.50	<0.50
C42	0.00	<0.5	<0.5	<0.50	<0.50	<0.18	0.15	0.2	0.4	<0.25	<2.5	<0.50	<0.50
F43	10.0	9.5	11	9.9	9.1	12.8	7.8	10.5	9.3	12	18	9.39	9.35
F44	1000	912	828	1040	999	881.0	878.0	837	844	1300	1200	881	872
T45	800	780	780	789	792	778.0	690.0	691	673	1000	840	700	660
T46	800	660	720	840	807	660.0	657.0	684	681	1100	910	665	624
T47	400	372	336	426	408	375.0	356.0	341	329	540	420	369	350
T48	400	348	324	441	432	291.0	319.0	339	331	480	380	366	343
T49	4.80	4.1	3.7	4.0	3.6	4.9	3.8	5.2	4.1	4.6	12	4.35	4.17
T50	4.00	3.7	3.5	3.9	3.7	4.5	3.5	4.0	3.5	4.3	12	3.70	3.66

[a] C = check sample; F = known fortification; T = unknown fortification.
[b] — = sample lost during analysis.

positives and considering the high relative standard deviation (RSD_R) at low concentrations, Collaborator 3 may have had a low level interference. If laboratory 3 is excluded, the percentage of false positives falls to 20%.

Other deviations from the method were reported by the collaborators. Because of an interference, Collaborator 5 could not consistently achieve the sensitivity necessary to quantitate low levels of AMPA. Consequently, some of the data sets have a quantitation limit of 2.5 ppb.

Collaborator 1 adjusted the LC buffer to pH 2.0 instead of pH 2.1 as specified in the method.

Collaborator 2 used 55°C water baths instead of baths at 60°C as specified in the method.

Collaborator 5 deviated from the method in that they took up the residues in a total of 2.5 mL buffer instead of 3.0 mL as specified.

Several laboratories slightly changed the flow rates of the LC buffer, oxidative solution, or OPA-MERC solution as

Table 2. Measures of precision for collaborators' results for recovery of glyphosate added to environmental water[a]

Added, ppb	Mean recovery ppb	Mean recovery %	s_r	s_R	RSD_r, %	RSD_R, %
			Known concentrations			
0.50	0.60	120	0.049	0.120	8.17	20.0
1.00	1.05	105	0.080	0.173	7.62	16.5
5.00	5.43	109	0.451	0.655	8.31	12.1
10.0	10.5	105	0.506	1.38	4.82	13.1
50.0	50.9	102	4.43	6.39	8.70	12.6
100	99.3	99.3	7.19	15.1	7.24	15.2
500	520	104	28.1	70.2	5.40	13.5
1000	946	94.6	95.5	136	10.1	14.4
5000	4870	97.4	722	818	14.8	16.8
			Unknown concentrations			
0.50	0.62	124	0.103	0.309	16.6	49.8
0.80	0.73	91.3	0.037	0.160	5.07	21.9
5.00	5.29	106	0.420	1.94	7.94	36.7
20.0	21.3	107	1.56	2.60	7.32	12.2
40.0	35.3	88.3	0.966	4.86	2.74	13.8
100	95.9	95.9	3.05	4.69	3.18	4.89
400	391	97.8	30.9	71.9	7.90	18.4
800	779	97.4	59.3	147	7.61	18.9
5000	4830	96.6	102	223	2.11	4.62

[a] s_r and s_R are standard deviations for repeatability and reproducibility, respectively. RSD_r and RSD_R are relative standard deviations for repeatability and reproducibility, respectively.

Table 3. Measures of precision for collaborators' results for recovery of AMPA added to environmental water[a]

Added, ppb	Mean recovery ppb	Mean recovery %	s_r	s_R	RSD_r, %	RSD_R, %
			Known concentrations			
0.50	0.43	86.0	0.055	0.055	12.8	12.8
1.00	0.87	87.0	0.101	0.101	11.6	11.6
5.00	5.01	100	0.175	1.12	3.49	22.4
10.0	9.97	99.7	1.90	2.87	19.1	28.8
50.0	44.9	89.8	3.56	3.56	7.93	7.93
100	93.3	93.3	9.61	13.8	10.3	14.8
500	478	95.6	29.9	31.1	6.26	6.51
1000	934	93.4	68.2	109	7.30	11.7
5000	4650	93.0	653	653	14.0	14.0
			Unknown concentrations			
0.50	1.01	202	0.107	1.64	10.6	162
0.80	0.75	93.8	0.080	0.114	10.7	15.2
5.00	5.26	105	0.126	1.83	2.40	34.8
20.0	19.5	97.5	2.26	2.40	11.6	12.3
40.0	32.8	82.0	1.84	4.36	5.61	13.3
100	90.5	90.5	13.9	17.7	15.4	19.6
400	361	90.3	17.6	41.2	4.88	11.4
800	736	92.0	30.5	91.8	4.14	12.5
5000	4880	97.6	167	339	3.42	6.95

[a] See footnote a, Table 2.

suggested in the method to optimize peak response. None of these deviations were considered significant.

Conclusions

A new residue method for the determination of glyphosate and AMPA in environmental water was developed, which is simpler and shorter than previous methods. An interlaboratory study by 6 laboratories was conducted to validate the method and estimate its repeatability and reproducibility. The method was validated over the range 0.50–5000 ppb, although one of the collaborating laboratories could not reliably quantitate below 1.0 ppb. Statistical analysis of the results showed that typical total variability (RSD_R) ranged from 11 to 20% for both glyphosate and AMPA. This compares very well with values predicted from observations by Horwitz, which range from 16 to 50% in the 5000 to 0.5 ppb concentration range. Total variability (as measured by s_R) increased with increasing fortification level, which is typical for analytical methods that require sample cleanup. The average analytical recovery was above 82% for all concentrations.

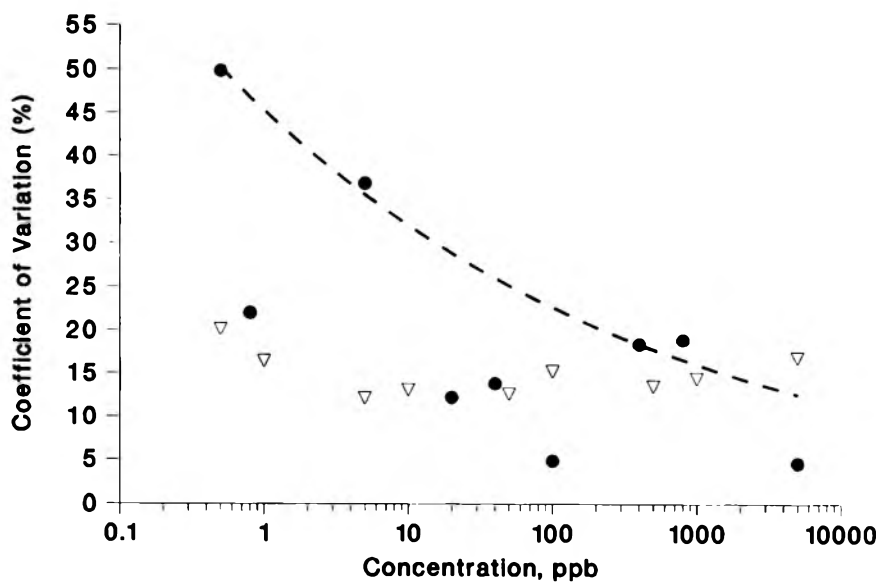

Figure 3. Comparison of between-laboratory precision data for samples fortified with known (▽) and unknown (●) concentrations of glyphosate with empirically derived Horwitz curve (-----).

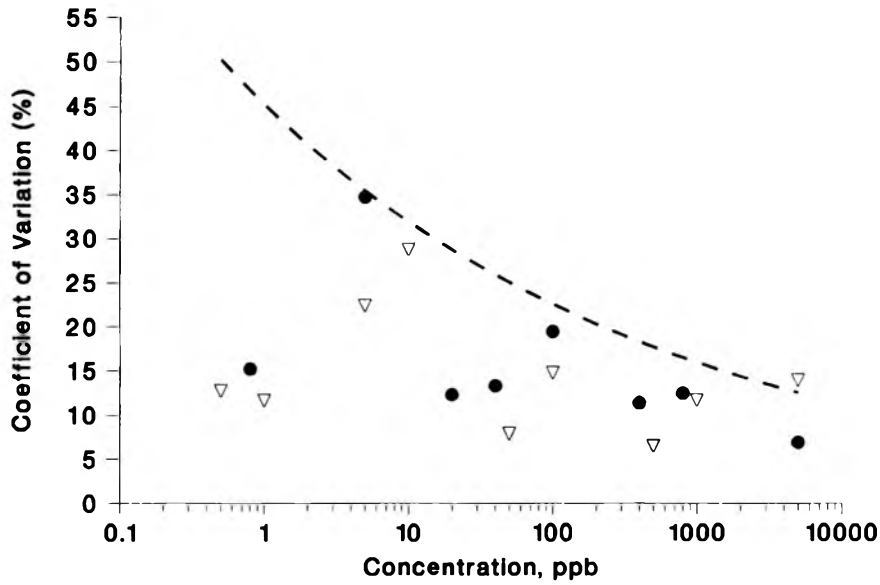

Figure 4. Comparison of between-laboratory precision data for samples fortified with known (▽) and unknown (●) concentrations of AMPA with empirically derived Horwitz curve (-----). RSD_R (CV) of samples fortified as unknown concentration at 0.50 ppb was 162% (not shown on graph).

Recommendation

The Associate Referee recommends that the liquid chromatographic method be adopted official first action as a new method for the determination of glyphosate and AMPA in environmental water.

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References

(1) Moye, H. A., & Boning, A. J., Jr (1979) *Anal. Lett.* **12**(B1), 25-35
(2) Moye, H. A., & St. John, P. A. (1980) *ACS Symp. Ser. No. 136*, Chap. 6, American Chemical Society, Washington, DC
(3) Miles, C. J., Wallace, L. R., & Moye, H. A. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 458-461
(4) Lundgren, L. N. (1986) *J. Agric. Food Chem.* **34**, 535-538
(5) Cowell, J. E. in (1980) J. Harvey, Jr, & G. Zwieg (Eds) *ACS Symp. Ser. No. 136*, Chap. 2, American Chemical Society, Washington, DC
(6) Moye, H. A., & St. John, P. A. (1980) *ACS Symp. Ser. No. 136*, Chap. 7, American Chemical Society, Washington, DC
(7) Rogers, P. E., Daniels, R. J., et al. in Newton, M., Howard, K. M., Kelpas, B. R., Danhaus, R., Lottman, C. M., Dubelman, S. (1984) *J. Agric. Food Chem.* **32**, 1144
(8) Bardalaye, P. C., Wheeler, W. B., & Moye, H. A. (1985) in *The Herbicide Glyphosate*, E. Grossbard & D. Atkinson (Eds), Butterworths, London, UK, pp. 263-285
(9) Cowell, J. E., Kunstman, J. L., Nord, P. J., Steinmetz, J. R., & Wilson, G. R. (1986) *J. Agric. Food Chem.* **34**, 955-960
(10) Youden, W. J. (1963) *Mater. Res. Std.*, 165-169
(11) Horwitz, W. (1982) *Anal. Chem.* **54**, 67-76A

FEEDS

Moisture Analysis in Forage by Near-Infrared Reflectance Spectroscopy: Collaborative Study of Calibration Methodology

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Fifteen collaborating laboratories analyzed 16 forage samples including 3 blind duplicate pairs for moisture by air-oven (AO) method 7.007 (14th Ed.; 930.15, 15th Ed.) and near-infrared reflectance spectroscopy (NIRS). Laboratories performed method 7.007 on 50 calibration samples and applied the NIRS calibration method independently. NIRS moisture equations were used to predict the 16 test samples, and the values were compared to those for method 7.007. Moisture concentration of the test samples ranged from approximately 6 to 16%. Within-laboratory repeatability (s_r) ranged from 0.10 to 0.18% and 0.16 to 0.39% for NIRS and method 7.007, respectively. Between-laboratory reproducibility (s_R) ranged from 0.22 to 0.57 and 0.29 to 0.57 for NIRS and method 7.007, respectively. Repeatability relative standard deviations (RSD_r) for the NIRS and AO methods ranged from 1.18 to 1.50% and 1.84 to 3.68%, respectively. The range in the average reproducibility relative standard deviations (RSD_R) for the NIRS and AO methods were 1.29–7.49% and 3.64–6.66%, respectively. The NIRS method demonstrated consistently lower within-laboratory RSD_r agreement and between-laboratory variabilities equal to method 7.007. Thereby, we demonstrated that NIRS can be used as a standard method for the determination of 6–16% moisture in forages. The method has been adopted official first action by AOAC.

The determination of the moisture content in forages and feeds is one of the most frequent and important analyses made on these materials because determination of nutritional quality requires that major constituents be expressed on a dry-matter basis. AOAC methods for moisture in feeds include drying under vacuum at 95–100°C (**934.01**) and air-oven (AO) drying at 135°C (**930.15**) (1), which were adopted on the basis of collaborative studies conducted by Grattan (2, 3). Since 1930 when Grattan completed his collaborative studies, no new method for moisture analysis in feeds has been adopted by AOAC.

Near-infrared reflectance spectroscopy (NIRS) has been extensively researched and widely accepted for the analysis of quality constituents of forages over the last 12 years. In 1988, the NIRS method of predictive determination of acid-detergent fiber and crude protein was adopted by AOAC. NIRS analysis has been used to determine moisture content in wheat (4), corn and grain sorghum (5), oilseeds (6), and corn silage (7), and proposed in the literature for routine moisture analysis of forages (8, 9) and cereal grains and concentrate rations (10). The success of NIRS for analyzing the moisture concentration of diverse agricultural samples is due to the strong absorbance of water in the NIR spectrum, regardless of sample type (8–10).

Early preliminary collaborative work by the National NIRS Forage Research Project reported by Windham et al. (9) showed that research laboratories could satisfactorily transfer and use the NIRS equation developed in the referee's laboratory to determine the moisture concentration of forages. Windham et al. (9) proposed that the next step in this work should be a collaborative study whereby all laboratories were instructed to perform AOAC method **7.007** (14th Ed.; **930.15**, 15th Ed.) (1) and apply the NIRS calibration method independently. The objectives of the present study were to clearly define the procedural details of NIRS calibration and to compare the performance of the 2 methods, NIRS and **7.007**.

Collaborative Study

Fifty NIRS calibration and 16 collaborative samples were sent to 15 collaborating laboratories including the Associate Referee's laboratory. The laboratories participating in the study were USDA-ARS, university, feed manufacturers, and commercial feed-testing laboratories. Bulk quantities of each sample were ground in a cyclone mill, mixed until completely homogeneous, and subdivided into 20 pairs in the Associate Referee's laboratory. Samples were subsequently packed in 6 oz Whirl-Pak bags (10 g per bag) and heat-sealed in Poly Kraft bags to ensure the moisture concentration did not change during the collaborative study. Collaborators were instructed to open one Poly Kraft bag at a time, pack the sample in the NIRS sample cell, reseal the bag, collect NIRS spectral data, and then determine moisture by method **7.007** on the same portion used for NIRS analysis.

To obtain best results by method **7.007**, collaborators were instructed to control time of drying; control time needed for the air oven to return to the desired temperature after opening; minimize exposure of dry matter residues to air; clean and properly lubricate seals and glass surfaces of the desiccator; use fresh and effective desiccant; and use a low desiccator loading rate.

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This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Feeds, Fertilizers, and Related Materials. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9–13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* **74**, January/February issue.

991.01 Moisture in Forage
Near-Infrared Reflectance Spectroscopy
First Action 1991

(Applicable to determination of 6–16% moisture in forages)

Method Performance:

s_r = 0.10–0.18; s_R = 0.22–0.57; RSD_r = 1.18–1.50%;

RSD_R = 1.29–7.49%

A. Apparatus

(a) *Wavelength-scanning monochromator.*—Instrument specifications: light source optics, 100 W tungsten halogen lamp; detector, lead sulfide; dynamic response, 4 optical density (OD); scan range, 1100–2500 nm; resolution, 0.79 nm; bandpass, 10 nm; linearity, 0.3 nm; wavelength accuracy, 0.5 nm; wavelength repeatability, 0.03 nm; stray light, 0.08% at 2500 nm and 0.01% at 1100 nm; and peak-to-peak noise, 0.0004 OD. Analysts are referred elsewhere [Landa, I. *Rev. Sci. Instrum.* **50,** 34–40 (1979)] for optical design and experiments conducted for instrument specifications. [Model 6100 or 6350 grating monochromator (NIRSystems, Silver Spring, MD 20910), or equivalent.]

(b) *Computer.*—Microvax-II or Microvax 200 series computers equipped with 4 to 10 megabytes of main memory; dual RX-50 double-density floppy disks; RD54 159 megabyte disk drive. Microvax VMS system software V5.1. PDP II series computers equipped with 64 k bytes of main memory; dual RX02 double-density floppy disks; RL01 5-megabyte or RL02 10-megabyte disk drive. PDP system software RT-11 V5.0 (Digital Equipment Corp., Nashua, NH 07061), or equivalent. IBM-AT or equivalent with 1 to 3 megabytes of main memory, 360 k or 1.2 megabyte double-density floppy disks, 20 to 140 megabyte disk drive. PC system software MS DOS V3.3.

(c) *Software.*—USDA Public Software. Software is described in detail in USDA Handbook No. 643 ("Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality." *U.S. Dep. Agric. Handb. 643* (revised with supplements), U.S. Government Printing Office, Washington, DC, 110 pp.). Infrasoft International Commercial Software (NIRSystems), or equivalent. Software consists of 14 programs written in FORTRAN IV to collect, store, and process NIRS data.

(d) *Mill.*—Tecator Cyclone mill with 1 mm screen meets specifications. Periodically change grinding ring to ensure consistency of particle size over time.

(e) *NIRS sample holder.*—Nylon holder, 2.5 cm diameter and 1 cm thick, with IR transmittance quartz window. Sample capacity 0.75–1.75 g. Sample is held in place with back made of rubber or foam core (NIRSystems), or equivalent.

(f) *Sample storage container.*—To maintain sample moisture concentration, store sample in tightly sealed container. Heat-sealed Poly-Kraft Bags-Mil-B-121 Type II Grade A, Class 1, were found to be adequate for this purpose (EDCO Supply Corp., Brooklyn, NY 11232, or equivalent).

B. Instrument Operation

(a) *Start-up.*—For best results, run instrument continuously. If instrument is cold, warm-up time should be >15 min and may require 1 h.

(b) *Monochromator diagnostic tests.*—(*1*) *Instrument noise.*—Scan ceramic reference to itself. Express deviations from zero as average deviation (bias); express root mean square (RMS) as log $(1/R)/10^6$, where R = reflectance. Bias indicates any systematic change in log (1/R) level of scans taken over time. All positive or all negative bias values indicate problem with instrument. RMS value can range from low of 10 to high of 50 without affecting analysis. Monochromators manufactured since 1983 should have average noise level below 30 RMS over 100 scans.

(*2*) *Wavelength accuracy.*—Use clear polystyrene petri dish to measure wavelength repeatability and accuracy. Place petri dish in light beam and pull out sample drawer to expose ceramic standard. Reference this scan to measurements without petri dish. Locate major peaks for polystyrene and compare with known locations at 1680.3, 2164.9, and 2304.2 nm. Repeatability standard deviation should be <0.05 nm, and deviation from known location should be <0.5 nm. Large values usually indicate mechanical problems in monochromator.

(c) *Maintenance.*—Whenever dirt accumulates, clean ceramic standard, all parts of drawer assembly, and windows above and below detector with vacuum, brush, or soft tissue. Detailed information on instrument operation is given in USDA Handbook No. 643 and in Landa [*see* **A (a)**].

C. Determination

Before NIRS data are collected, tare dishes for use with primary reference moisture method. Mix milled sample and place 4 random portions in 4 quadrants of NIRS cell to ensure that portions of 3–4 different subsamples are scanned. Continue to add random portions until NIRS sample holder is ⅔ to level full. Press back into holder until it is tight. As check, invert holder and make certain sample is firmly pressed against window. Consistency in sample handling and preparation is crucial to successful NIRS analysis.

Immediately, collect reflectance (R) measurements (log 1/R) of calibration set from 1100 to 2500 nm and record at 2-nm intervals. Immediately, empty entire NIRS sample from cell into prepared dishes and record weight. Dry 2 h at 135° and record weight. Calculate weight loss as moisture. This procedure minimizes, but does not eliminate, exposure of samples to atmospheric moisture. All manipulations (i.e., packing NIRS cells, transferring test portions to dishes, and performing gravimetric procedure) during such exposure should be performed with speed and care.

Enter primary reference moisture data from analysis of calibration samples with reflectance data of calibration samples by using program DATA.

Use program FILE to average duplicate reference moisture/reflectance data of NIRS samples. Repeat above steps until calibration samples have been analyzed. Then use program FILE to merge all files created into 1 calibration file.

D. Evaluation of Preliminary Calibration Set

Conduct preliminary NIRS calibration with program BEST (public software) or CAL (commercial software) to evaluate reference moisture/NIRS data, using only 1 mathematical treatment [1,10,10,2 in nanometers (public software) or 1,5,5,1 in data points (commercial software)] and 3 wavelengths or less than N/10 + 1 wavelengths, where N = number of samples in calibration file. Above mathematical treatments are denoted by shorthand expression (a, b, c, d),

where a = derivative function, b = segment length over which derivative was taken, c = segment length over which above function was smoothed, and d = segment length over which smooth function was subject to a second smooth.

Obtain printout of preliminary calibration process. Examine standard error of calibration (SEC) of first wavelength. If SEC for first wavelength is >0.9, laboratory reference method data for some samples are considered inaccurate. Examine differences (residuals) between NIRS moisture data and reference method data for samples with large *t*-values. Large positive or negative *t*-values represented by "*" on computer printout indicate that residual is 2.5 times the standard error of a difference (SED) between NIRS and reference method data. The significant SED occurs because moisture content of sample analyzed by reference method was not representative of that which was scanned or moisture concentration obtained from reference method was inaccurate. Reanalyze these samples by both methods. If some samples are reanalyzed, delete these samples from the original calibration file and enter reanalyzed samples to calibration file. Rerun preliminary calibration procedure to ensure that all *t*-starred samples have been eliminated from calibration population.

E. Generation of Equation

Perform calibration procedure and split calibration file into 2 subsets; calibration program provides option of splitting calibration file into 2 subsets, 1 file for equation generation and 1 for prediction as an aid in equation selection. Reserve every Ith sample (I = 3) in calibration file for prediction with first prediction sample in file position 3 or, as preferred, reserve a different Ith value (USDA) Handbook No. 643, pp. 96–103). Perform procedure with maximum of 3 wavelengths with following mathematical treatments of log (1/R) data.

Nanometers	Data points
1,20,10,2	1,10,5,1
1,30,10,2	1,15,10,1
1,40,10,2	1,20,5,1
2,20,20,2	2,10,10,2
2,30,20,2	2,15,10,2
2,40,20,2	2,20,10,2

Obtain output of calibration procedure containing 3 equations per mathematical treatment or 18 equations.

F. Selection of Equations Within Mathematical Treatments

Evaluate standard error of calibration (SEC) and R^2 for each equation. SEC will decrease progressively and R^2 will increase as wavelengths are added. Observe SEC to assure that equations have values in expected range (i.e., <0.9). Next, evaluate standard error of selection (SES) of each equation within each mathematical treatment using equation selection sample set. SES is indication of performance of equations on selection sample set. Unlike SEC, which must decrease with each additional term, SES decreases only until overfitting of equations in generation sample set becomes important and causes SES to increase. Find SEC in generation sample set for that equation where SES in selection sample set has reached either plateau or minimum. As a guide, values should be with 20% of each other. Select "best" equation(s) within a mathematical treatment with following set of guidelines:

(*1*) Equation should have lowest SES and fewest wavelengths.

(*2*) Equation should have wavelength from 1800 to 2000 nm.

(*3*) Wavelengths in equation should be >40 nm apart.

(*4*) No wavelengths should have *F*-statistic <10 for regression coefficients. Start with 1-term equation and stop evaluating equations after an equation contains coefficients with *F*-values <10.

Based on these guidelines, select 1 equation within each mathematical treatment.

G. Selection of Final "Best" Equation Among Mathematical Treatments

After "best" equation from each mathematical treatment has been selected, summarize results under following headings: mathematical treatment; wavelengths; SES; SEC; lowest *F*-test.

To select "best" single equation for use in NIRS analysis, choose equation with lowest SES. If 2 or more equations have SES values close in magnitude, reevaluate equation generation statistics for those equations. Choose equation that has a combination of fewest wavelengths, SEC similar to the SES, and highest *F*-statistic for the wavelength in region from 1800 to 2000 nm. When "best" equation has been selected, use calibration program to fit that mathematical treatment and wavelengths on all calibration samples (i.e., those combined for generation and selection) to derive final equation that will be used in NIRS analysis.

H. Equation Validation and Analysis

Final step in calibration is validation of the selected equation with samples not included in original calibration (generation and selection) population. This step is necessary to obtain an independent measure of equation accuracy expressed as standard error of prediction (SEP).

Conduct reference moisture method and NIRS scans on validation samples in duplicate as in C. Use program DATA to enter primary reference moisture data from analysis of validation samples with reflectance data of validation samples. Use program FILE to average duplicate reference moisture/reflectance data of NIRS samples. Create a separate prediction file for these samples by using program PRE (public software) or ANL (commercial software). Use program STAT to compare actual primary reference moisture data with predicted NIRS data. Compare SEP value for equation to value of SEC for equation. Values should be within 20% of each other. If not, evaluate SEP corrected for bias (SEPC). SEP(C) statistic indicates performance of equation corrected for difference (bias) between final NIRS equation and primary reference moisture method. Print difference (i.e., residuals) between reference moisture data and NIRS predicted data and determine samples with residuals 2.5 times the SED between reference moisture data and NIRS predicted data. Reanalyze these samples by both methods. If some samples are reanalyzed, delete these samples from the original file and enter new values via computer. If SEP is similar to SEC, final NIRS equation can be accepted for use.

Ref.: JAOAC **74,** March/April issue (1991).

Results and Discussion

The sample types used in the collaborative study are identified in Tables 1 and 2. The samples consisted of different cultivars of legume, tropical, and temperate forages, mixed

Table 1. Near-infrared reflectance spectroscopy (NIRS) moisture: calibration samples

Sample	No. of samples	Source
Alfalfa (*Medicago sativa*)	15	UT, MD, PA, WI
Orchardgrass (*Dactylis glomerata*)	3	MD, PA
Timothygrass (*Phleum pratense*)	2	PA
Ky-31 tall fescue (*Festuca arundinacea*)	4	MS, GA
Red Clover (*Trifolium pratense*)	1	WI
Birdsfoot trefoil (*Lotus corniculatus*)	1	WI
Grass/legume mix hay	7	MN, WI
Bermudagrass (*Cynodon dactylon*)	3	GA
Old world bluestem (*Bothriochloa caucasica*)	5	OK
Grass-hay	3	NE, WI, CO
Wheat forage (*Triticum aestivum*)	2	OK
Wheat straw	2	OK
Soybean stover (*Glycine max*)	1	MD
Corn cobs (*Zea mays*)	1	MD

Table 2. Collaborative samples for near-infrared reflectance spectroscopy and air-oven (7.007) moisture determination

Sample No.	Material	Source
1	Alfalfa-hay	UT
2	Grass/legume mixed hay	MN
3	Alfalfa-hay	OK
4	Tifton-44 bermudagrass	GA
5	Barley straw (*Hordeum vulgare*)	MD
6	Timothy grass/red clover hay	GA
7	Alfalfa-hay	CO
8	Sorghum silage (sorghum bicolor)	GA
9	Corn stover	MD
10	Orchardgrass (*Dactylis glomerata*)	PA
11	Ky-31 tall fescue	MS
12	Alfalfa/ryegrass-hay	WI
13	Grass-hay	MN
14	Blind duplicate of No. 6	GA
15	Blind duplicate of No. 7	CO
16	Blind duplicate of No. 8	GA

hays, and crop residues. Samples were preserved by either air oven, sun-cured, freeze-dried, and/or microwave.

NIRS calibration (Table 1) and collaborative samples (Table 2) ranged in moisture concentration from 5 to 16% and 7 to 15%, respectively. Repeatability was calculated from samples 6, 7, and 8, and 14, 15, and 16, because these were blind duplicates (Table 2).

NIRS moisture equations and calibration statistics for each collaborating laboratory are shown in Table 3. The mean moisture concentrations determined by method **7.007** for the 50 calibration samples were similar with the exception of data from Collaborator 8. The mean value from this laboratory was 1.25 percentage units lower than the pooled mean across other laboratories. For equation development, the first derivative segment and a wavelength gap of 40 or 30 nm for computing the derivative was common among 11 laboratories. The first derivative segment centered on 1844 nm was the primary explanatory wavelength followed by 1924 nm for Collaborators 1, 2, 4, and 5. For Collaborator 8, using the same mathematical treatment, 1854 nm was the primary explaining wavelength followed by 1922 nm. With a mathematical treatment of 1,40,10,2 and a center wavelength of 1844 and 1854, the actual NIRS signal regressed against water concentration is a combination of the signals from 1820 to 1868 and 1830 to 1878 nm, respectively. Similarly, with a center wavelength of 1924 and 1922 nm, the signals regressed span the regions from 1900 to 1948 nm and 1898 to 1946 nm, respectively. Therefore, 80 and 96% of the NIRS signals in these regions regressed against water concentration were common among collaborators 1, 2, 4, 5, and 8. Collaborators 9, 11, 13, and 14 also used a first derivative

Table 3. NIRS moisture calibration statistic for each collaborative laboratory

Coll.	Mean,[a] %	Mathematical treatment[b]	Wavelength, nm	SEC,[c] %	R^{2d}
1	8.65	1,40,10,2	1844,1664,1924	0.36	0.97
2	8.46	1,40,10,2	1844,1804,1924	0.27	0.93
3	8.57	2,20,20,2	2228,1804,1924	0.41	0.94
4	8.51	1,40,10,2	1924,1844	0.30	0.95
5	8.47	1,40,10,2	1844,1664,1924	0.29	0.96
6	8.70	2,30,20,2	1822,1712,1954	0.32	0.91
7	8.54	1,20,10,2	1854,1674,1934	0.32	0.95
8	7.43	1,40,10,2	1554,1854	0.53	0.89
9	8.92	1,30,10,2	1848,1928,2374	0.26	0.94
10	8.65	1,40,10,2	1922,2044,1662	0.34	0.95
11	8.98	1,30,10,2	1924,1742,1844	0.42	0.88
12	8.99	2,30,20,2	1950,2148,2214	0.37	0.85
13	8.62	1,30,10,2	1928,1848	0.42	0.87
14	8.38	1,30,10,2	1848,1928	0.40	0.86
15	9.11	2,40,20,2	1818,1658,1950	0.45	0.84

[a] Mean moisture of calibration set.
[b] Mathematical treatment = 1,40,10,2, where 1 = first derivative, 40 = wavelength gap for computing first derivative, 10 = smoothing interval for first smooth, 2 = smoothing interval for second smooth.
[c] Standard error of calibration.
[d] Fraction of explained variance.

Table 4. Collaborative results (%) for determination of moisture by air-oven reference method 7.007

Sample No.	Collaborator														
	1[a]	2	3	4	5	6	7	8[b]	9	10	11	12	13	14	15
1	8.47	8.45	8.94	8.61	8.02	8.08	8.95	7.27	8.17	9.07	9.50	9.26	9.07	8.60	9.48
2	9.76	8.98	8.84	9.16	8.56	9.28	9.05	7.78	8.58	9.60	9.64	9.55	8.86	9.11	9.69
3	7.35	7.36	6.91	7.16	6.81	7.83	7.24	5.86	7.37	8.02	8.05	8.16	7.56	7.17	8.79[c]
4	8.71	8.72	7.73	8.96	8.59	8.98	8.26	7.48	8.12	8.98	8.09	9.14	8.44	8.70	9.16
5	7.97	7.70	7.32	7.69	7.32	8.13	7.24	6.49	6.78	7.90	6.94	7.73	7.00	7.50	7.61
6[d]	9.30	8.74	8.26	8.74	8.59	8.56	8.49	7.88	8.04	8.93	9.14	8.94	8.12	8.84	8.90
7[e]	7.07	7.76	7.56	7.88	7.00	7.95	7.63	5.86	7.48	8.10	8.42	8.71	7.29	7.56	8.91
8[f]	15.38	14.61	15.93	15.83	15.93	16.05	15.87	14.37	15.83	15.19	15.45	15.57	14.68	16.70	14.42
9	7.55	7.58	7.14	7.82	7.21	7.89	7.22	6.10	6.85	7.49	7.34	7.72	7.58	7.32	7.80
10	8.12	7.89	7.66	8.26	7.55	8.09	7.72	6.53	7.47	7.96	8.45	8.26	8.05	7.83	8.36
11	9.12	8.82	8.84	8.48	8.64	8.98	8.64	7.01	8.86	9.85	10.08	9.49	9.91	8.87	8.66
12	9.39	8.90	8.89	8.78	8.74	9.22	8.79	7.33	8.82	8.82	9.57	10.17	9.08	9.00	9.71
13	8.64	8.09	7.95	8.31	8.10	8.45	8.10	6.99	8.04	8.35	9.23	8.97	8.38	8.32	8.88
14	8.84	8.49	8.03	8.69	8.66	9.06	8.52	7.78	8.10	8.65	8.58	9.25	8.35	8.83	8.84
15	8.39	7.65	7.06	7.09	7.35	8.36	7.20	6.13	7.42	7.52	8.68	8.56	7.98	7.45	8.70
16	15.20	15.38	15.91	15.99	16.17	15.91	15.27	14.61	15.99	15.66	15.98	16.55	15.84	16.81	13.23[c]

[a] Referee's laboratory.
[b] All data from Coll. 8 omitted from statistical summary on basis of Cochran test.
[c] Values omitted from statistical summary on basis of Grubbs test.
[d] Blind duplicate of sample 14.
[e] Blind duplicate of sample 15.
[f] Blind duplicate of sample 16.

segment but with a 30 nm wavelength gap for computing the first derivative. The center wavelength of 1848 nm was the primary wavelength followed by 1924 nm for Collaborators 9, 13, and 14. For Collaborator 11 using the same mathematical treatment 1844 and 1924 nm were the chosen center wavelengths. Using a 30 nm gap for computing the first derivative, the NIRS signals regressed against moisture concentration span a 36 nm region around the center wavelengths. Therefore, 89% of NIRS signals regressed against water concentration were common among Collaborators 9, 11, 13, and 14. Regardless of the mathematical treatment, all collaborators had 1 or 2 wavelengths in the 1800–2000 nm region as specified in the method. The primary and secondary wavelengths used are in close agreement with other NIRS moisture calibration studies (8–10). Murray and Williams (11) attributed this region to be important in moisture calibration because of the occurrence of a first overtone (1920–1950 nm) and third overtone (1820–1870 nm) OH stretch/deformation fundamental. The shift that occurred between primary and secondary wavelengths chosen by the collaborators occurred for several reasons: (*1*) interactions between sample and instrument; (*2*) minute differences in the internal geometry and optical components of the instruments; and (*3*) temperature changes that affect the position of absorbers (11). These changes may change the overall net absorption of the molecule and consequently the optimum wavelength for making the analytical measurement.

The error associated with the regression of moisture data with NIRS spectral data expressed as standard error of calibration (SEC) ranged from 0.27% for Collaborator 2 to 0.53% for Collaborator 8. The SEC values for all collaborators compare favorably with earlier reported NIRS air-oven moisture calibrations (8, 9).

A summary of all collaborative test results (including outliers) by sample material for method **7.007** and the NIRS method are reported in Tables 4 and 5, respectively. Collaborators are identified by number, and the same number represents the same collaborator for test results by both methods. The air-oven and NIRS test results were processed for outliers according to the Cochran and Grubbs tests (12). Of a total of 36 outlying values (18, Table 4; 19, Table 5), 32 were from Collaborator 8. The Cochran test for removal of laboratories confirmed that both air-oven and NIRS data from Collaborator 8 should be excluded from statistical analyses. Discussion of these results with Collaborator 8 revealed that the air oven was improperly calibrated for 135°C. The oven was calibrated with a thermometer laying on the oven shelf; as a result when the thermometer read 135°C the actual air-oven temperature was 129°C. This probably caused the low outlying values. The data were again cycled through the Cochran and Grubbs tests. Grubbs outliers occurred for Collaborator 15, 3; Collaborator 5, 1. These outliers are identified individually in Tables 4 and 5.

Validation statistics for NIRS moisture equations at each collaborating laboratory are listed in Table 6. The standard error of equation performance corrected for bias (SEPC) ranged from 0.24% (Collaborator 6) to 0.67% (Collaborator 12). These values, except that of Collaborator 12, are acceptable and similar to that reported by Windham et al. (8, 9) for NIRS moisture analysis of tropical and temperate grass, legume, silages, and silages and silage-based dairy cattle ration. If the SEP for validation is within 2 times the repeatability for the replicated primary reference method analysis, the final NIRS equation can be accepted for use and the SEP can be used as a reliable indication of the accuracy of the final equation (13). The pooled repeatability for moisture analysis of blind duplicates by reference method **7.007** was 0.29% (Table 7). Therefore, SEP are acceptable except that of Collaborator 12, who noted that many of the test samples were reanalyzed by both methods to eliminate or reduce the number of samples with large *t*-values. A large *t*-value indicates that the residual is greater than 2.5 times the standard error of difference between the NIRS and reference determinations. These significant residuals occurred because (*1*) the

Table 5. Collaborative results (%) for determination of moisture by near-infrared reflectance spectroscopy

Sample No.	Collaborator														
	1	2	3	4	5	6	7	8[a]	9	10	11	12	13	14	15
1	8.51	8.50	8.85	8.56	8.46	9.03	8.91	7.39	8.51	9.13	9.32	8.88	9.31	8.72	9.43
2	9.41	9.13	9.12	8.91	8.88	9.07	9.00	8.04	9.01	9.37	9.49	8.83	9.12	9.12	9.52
3	7.59	7.64	7.04	7.32	6.66	7.96	7.36	6.29	7.66	7.80	7.81	6.87	8.35	7.55	8.85
4	8.76	8.65	8.50	8.99	8.83	8.66	8.65	7.59	8.45	8.81	8.70	9.33	8.81	8.86	9.18
5	7.75	7.38	7.26	7.37	7.50	8.29	7.47	5.58	6.91	8.19	7.13	8.11	7.64	7.15	7.57
6[b]	9.31	9.31	8.78	9.16	9.05	9.18	8.91	7.70	8.67	8.68	8.16	8.48	8.77	9.17	9.21
7[c]	7.61	7.59	7.26	7.44	6.60	7.82	7.42	6.30	7.42	7.76	7.82	7.32	8.11	7.52	8.99[e]
8[d]	13.44	13.02	13.73	13.84	14.39	12.55	13.96	13.35	13.78	12.82	12.79	12.30	12.96	13.64	13.30
9	7.81	7.55	7.44	7.75	7.48	8.38	7.59	6.30	7.03	7.50	7.30	7.90	7.88	7.59	7.84
10	8.35	8.06	7.92	8.32	7.85	8.24	7.94	7.17	8.01	8.04	8.26	8.44	8.50	8.08	8.62
11	8.49	8.29	8.70	7.78	7.96	9.07	7.78	7.10	8.32	8.88	9.35	8.08	9.22	7.80	7.83
12	8.91	8.22	8.56	8.22	8.48	8.95	8.46	7.16	8.30	8.95	9.26	8.46	8.87	8.31	8.98
13	8.32	7.88	7.61	7.83	7.38	8.35	7.84	6.94	7.86	8.03	8.46	7.49	8.50	7.82	8.93
14	9.48	9.08	8.67	9.22	9.01	9.19	9.17	7.79	8.67	8.44	8.20	8.44	8.62	9.41	9.26
15	7.85	7.49	7.00	7.48	6.70	8.05	7.23	6.31	7.41	7.55	7.86	7.37	8.30	7.48	8.90
16	13.56	13.28	13.82	14.08	14.29	12.41	13.67	13.34	13.93	13.17	12.99	12.59	13.48	14.01	13.44

[a] All data from Coll. 8 omitted from statistical summary on basis of Cochran test.
[b] Blind duplicate of sample 14.
[c] Blind duplicate of sample 15.
[d] Blind duplicate of sample 16.
[e] Values omitted from statistical summary on basis of Grubbs test.

primary reference method data were inaccurate, or (*2*) the sample analyzed by NIRS was not representative of that analyzed by method **7.007**. Since the sample analyzed by NIRS was used with the reference method and the mean NIRS moisture value for Collaborator 12 is similar to the mean of other laboratories, the second reason may be eliminated as the probable cause of the high bias and SEP. In discussion with Collaborator 12 it was noted that a 1-h desiccation time was used with AO method **7.007**. It was also noted that the desiccant (Drierite) was not replaced with fresh desiccant during the study. By the time the test samples were analyzed and if the desiccation was not used for another purpose, 100 samples (i.e., calibration samples) would have been processed through the desiccator. It is therefore possible that the high bias and SEP was due to exhausted desiccant coupled to the 1-h desiccator time.

The moisture concentration of the sorghum silage test material determined by both methods has been omitted from the validation results. This sample was included in the test samples for the determination of incorrectness. Averaged across laboratories, the mean moisture concentration determined by method **7.007** was 15.71% compared to 13.40% determined by NIRS. All collaborators identified this sample as one with a significant *t* residual. The 2.31% unit greater moisture value by method **7.007** was caused by loss of volatiles as well as moisture at 135°C which resulted in over estimation of moisture. Moisture content of this material in the referee's laboratory by Karl Fischer analysis (8) was 13.7% and similar to that determined by NIRS. These data are in agreement with those of Windham et al. (8, 9), who previously reported that moisture calibration developed with samples not subject to volatile loss during drying at 135°C could be used to accurately predict the moisture concentration of fermented materials.

Table 6. Validation results for NIRS moisture equations within each collaborative laboratory[a]

Coll.	Mean,[b] %		Bias,[c] %	SEP,[d] %	SEP(C),[e] %
	AO	NIRS			
1	8.48	8.44	0.04	0.38	0.39
2	8.22	8.20	0.02	0.37	0.37
3	7.94	8.05	−0.11	0.37	0.36
4	8.26	8.17	0.09	0.38	0.39
5	7.94	7.92	0.02	0.42	0.43
6	8.57	8.59	−0.02	0.24	0.24
7	8.07	8.12	−0.05	0.39	0.39
9	7.86	8.02	−0.15	0.39	0.37
10	8.52	8.36	0.16	0.33	0.30
11	8.69	8.33	0.33	0.53	0.43
12	8.85	8.14	0.71	0.91	0.67
13	8.26	8.57	−0.31	0.57	0.50
14	8.22	8.18	0.05	0.45	0.45
15	8.82	8.79	0.03	0.35	0.35

[a] Sorghum silage test material omitted from validation results.
[b] Mean of moisture analysis by air-oven (AO) method and near-infrared reflectance spectroscopy (NIRS).
[c] Air-oven moisture minus NIRS.
[d] Standard error of performance.
[e] Standard error of performance corrected for bias.

Table 7. Statistical performance parameters for blind duplicate moisture analysis by near-infrared reflectance spectroscopy (NIRS) and air-oven (AO) reference method[a]

Material	Sample Nos.	Mean, %		s_r		RSD_r, %		r	
		NIRS	AO	NIRS	AO	NIRS	AO	NIRS	AO
Alfalfa-hay	7,15	7.57	7.81	0.11	0.29	1.50	3.68	0.32	0.80
Timothy/clover-hay	6,14	8.92	8.66	0.10	0.16	1.18	1.84	0.29	0.44
Sorghum silage	8,16	13.40	15.71	0.18	0.39	1.38	2.48	0.52	1.09

[a] s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation; r = repeatability value.

Table 8. Statistical performance parameters for moisture analysis by near-infrared reflectance spectroscopy (NIRS) and air-oven (AO) reference method[a]

Sample No.	s_R		RSD_R, %		R	
	NIRS	AO	NIRS	AO	NIRS	AO
1	0.37	0.49	1.29	5.63	0.32	1.38
2	0.22	0.41	2.45	4.43	0.63	1.14
3	0.57	0.44	7.49	5.84	1.59	1.22
4	0.24	0.43	2.74	5.02	0.67	1.21
5	0.41	0.41	5.47	5.41	1.16	1.13
6[b]	0.36	0.32	4.10	3.69	1.01	1.04
7[b]	0.30	0.52	3.93	6.66	0.83	1.46
8[b]	0.57	0.57	4.24	3.64	1.59	1.58
9	0.33	0.29	4.29	4.01	0.92	0.84
10	0.24	0.30	2.89	3.82	0.66	0.85
11	0.56	0.53	6.70	5.80	1.57	1.48
12	0.34	0.43	3.92	4.70	0.95	1.20
13	0.44	0.38	5.43	4.57	1.22	1.07

[a] s_R = reproducibility standard deviation; RSD_R = reproducibility relative standard deviation; R = Reproducibility value.
[b] Pooled blind duplicate data.

Within-laboratory (repeatability) agreement of test results for each material and each method is listed in Table 7. The statistical performance parameters for repeatability for each material were higher for the air-oven method. The change in repeatability over material is related to the moisture concentration of the test material. Test material low in moisture (i.e., samples 7, 15) and those high in moisture (i.e., 8, 16) are subject to change in moisture concentration during determination. As such, samples low in moisture would tend to gain moisture and those high in moisture lose moisture during sample preparation for determination. The variation in repeatability for each material by NIRS was much less than by the air-oven method. Pooled across materials (i.e., all data pooled) the s_r, RSD_r, and r value for NIRS and method **7.007** were 0.14, 1.40%, 0.39 and 0.29, 2.74%, and 0.82, respectively. The lower NIRS repeatability error agrees with data of Hruschka (14) who reported lower NIRS repeatability compared to data for Kjeldahl nitrogen analysis for determination of protein in wheat. It can be concluded that the within-laboratory difference between duplicates for NIRS is half that of method **7.007** when the tests are conducted by the procedures described in this study.

Reproducibility agreement of test results for each material and each method are listed in Table 8. s_R values ranged from 0.22 to 0.57% for NIRS and 0.29 to 0.61% for air-oven analysis. Pooled across all data s_R, RSD_R, and R value for NIRS and method **7.007** were 0.40, 4.22%, 1.11 and 0.43, 3.94%, and 1.21, respectively. In a previous collaborative study using laboratories 1, 2, 3, 4, 7, and 14, NIRS moisture equations were developed in the referee's laboratory and transferred (9) to collaborators. Collaborators received 20 test samples and were instructed to analyze the samples by NIRS and AO method **7.007.** In this case, Windham et al. (9) reported a pooled s_R for moisture analysis by NIRS and method **7.007** of 0.39 and 0.63%, respectively. The s_R data for the 2 NIRS methods (equation transfer vs laboratory calibration) are similar but between-laboratory error for the air-oven method was significantly lower in the current study. The decrease in AO s_R for the air-oven method (i.e., 0.63 vs 0.43%) is possibly because collaborators received a procedure on how to conduct method **7.007.** These s_R values are in agreement with those of Grattan (3) who reported between-laboratory error of 0.43 for method **7.007.** It can be concluded that the within-laboratory error for NIRS is equal to that for method **7.007** when the tests are conducted by the procedure described in this study.

Recommendation

The Associate Referee recommends that this description of the near-infrared reflectance spectroscopy method be adopted official first action as an improved method of moisture analysis in feeds.

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References

(1) *Official Methods of Analysis* (1984) 14th Ed.; (1990) 15th Ed., AOAC, Arlington, VA
(2) Grattan, G. E. (1930) *J. Assoc. Off. Agric. Chem.* **13,** 173–174
(3) Grattan, G. E. (1931) *J. Assoc. Off. Agric. Chem.* **14,** 152–154
(4) Law, D. P., & Tkachuk, R. (1977) *Cereal Chem.* **54,** 874–881
(5) Stermer, R. A., Pomeranz, Y., & McGintz, R. J. (1977) *Cereal Chem.* **54,** 354–351
(6) Robertson, J. A., & Barton, F. E., II (1984) *J. Am. Oil Chem. Soc.* **61,** 543–547
(7) Shenk, J. S., & Westerhaus, M. O. (1985) *Crop Sci.* **25,** 1120–1122
(8) Windham, W. R., Robertson, J. A., & Leffler, R. G. (1987) *Crop Sci.* **27,** 777–783
(9) Windham, W. R., Barton, F. E., II & Robertson, J. A. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 256–262

(10) Windham, W. R. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 255–258

(11) Murray, I., & Williams, P. C. (1987) in *Near-Infrared Technology in the Agricultural and Food Industries*, P. Williams & K. Norris (Eds), American Association Cereal Chemists, St. Paul, MN, pp. 17–31

(12) "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (1988) *J. Assoc. Off. Anal. Chem.* **71,** 161–172

(13) Windham, W. R., Mertens, D. R., & Barton, F. E., II (1989) in "Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality," *U.S. Dep. Agric. Handb. 643*, U.S. Government Printing Office, Washington, DC, pp. 96–103

(14) Hruschka, W. R. (1987) in *Near-Infrared Technology in the Agricultural and Food Industries*, P. Williams & K. Norris (Eds), American Association Cereal Chemists, St. Paul, MN, pp. 35–53

FERTILIZERS

Variance and Representativeness of the AOAC Sampling Procedure for Bagged Fertilizer

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The present paper evaluates both the variances and representativeness of the AOAC sampling procedure for bagged fertilizer (929.01a, 15th Ed., 1990) for concentrations of total nitrogen, available phosphoric acid, and soluble potash from 5% to 30% in blended, mixed fertilizers using materials in common use in 1983. The sampling variances determined did not differ significantly from sampling variances determined in earlier studies that are currently used by the Association of American Plant Food Control Officials (AAPFCO). The representativeness of the AOAC sampling procedure for bagged fertilizer was evaluated by testing the differences between AOAC bag sample means and rotary divider sample means (riffled down bags) of the blends (*a*) using AAPFCO investigational allowances (IAs) and (*b*) using *t*-tests. When AAPFCO IAs were used, there were only 2 bag means significantly below the rotary divider means. When t-tests were used, the bag means tested significantly lower than the rotary means for the 5, 10, 15, and 20% N blends and the 15% P_2O_5 blend. The bag means for the 5, 10, and 30% P_2O_5 blends were significantly above the rotary means. There were no significant differences between bag and rotary means for K_2O in any blends. Because *t*-tests are more sensitive than AAPFCO IAs, smaller differences are judged to be significant. Based on AAPFCO IAs, the AOAC sampling procedure for bagged fertilizer probably satisfactorily represents what is in the bag. However, significantly lower N and higher P_2O_5 bag means based on t-tests are cause for concern and should spur activity in determining the nature of this phenomenon. Interlaboratory variances were also determined and evaluated. These tended to verify those in current use by AAPFCO. The representativeness of the AOAC stream sampling procedure (929.01b) was also evaluated and found to be an acceptable method for determining the true analysis of a blend.

The Uniform State Fertilizer Bill of the Association of American Plant Food Control Officials (AAPFCO) states in Section 8(b), "The methods of sampling and analysis shall be those adopted by the Association of Official Analytical Chemists (AOAC)" (1). In Section 3(p) of the same document, the term "investigational allowance" is defined as "an allowance for variations inherent in the taking, preparation, and analysis of an official sample of fertilizer." An investigational allowance is used by a control official in judging whether an analytical result from an official fertilizer sample that is below the manufacturer's guarantee should be declared deficient. Current AAPFCO investigational allowances (IAs) were adopted in 1968 (2) based on published scientific studies by Miles and Quackenbush (3) and Quackenbush et al. (4). [For a detailed discussion of AAPFCO IAs, see Rund (5)]. These IAs are based on variances associated with the AOAC sampling procedure for bagged fertilizer (**929.01a**) (6), intralaboratory procedures (sample reduction and analytical), and interlaboratory analytical procedures. For these IAs to be applicable, the recommended AOAC procedures must be used for obtaining, preparing, and analyzing samples.

Because these IAs were adopted in 1968 based on studies conducted with fertilizers in use in 1955 and 1966, fertilizer industry and fertilizer regulatory officials have been interested in re-evaluating the variance associated with the AOAC sampling procedure for bagged fertilizer. In 1983, AAPFCO appointed a joint task force consisting of representatives from The Fertilizer Institute, AOAC, and AAPFCO to develop an experimental plan and conduct the study. The final plan was developed and the study conducted in 1983 (7). Progress reports have been published in 1985 (8) and 1986 (9).

The objectives of the study were (*a*) to determine the variance associated with the AOAC sampling procedure for bagged fertilizer over the range of nitrogen (N), phosphoric acid (P_2O_5), and soluble potash (K_2O) concentrations most commonly found in blended fertilizer and recommend changes to the AAPFCO IAs, if indicated, and (*b*) to find whether a diagonal core from a bag of blended granular materials (as described in the AOAC method) represents the mixture contained in the bag within the latitudes imposed by the AAPFCO IAs.

The following definitions adopted by the task force are critical to accomplishing the objectives of the study.

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Table 1. Analyses of stream samples from a 10-20-20 blend from preliminary blender test (av. of duplicates)

Cut No.	N, %	P_2O_5, %	K_2O, %
1	9.79	20.01	20.42
2	10.02	20.11	19.87
3	9.69	19.56	20.65
4	9.80	19.85	20.00
5	9.67	19.67	20.32
6	9.81	19.95	18.76
7	9.96	20.01	19.93
8	9.91	19.92	20.32
9	9.97	20.13	20.15
10	10.10	20.12	20.22
Mean	9.87	19.93	20.06
CV, %	1.4	1.0	2.6

Manufacturing variation.—Compositional differences (variation from guaranteed analysis or "target analysis") in a lot of fertilizer resulting from manufacturing processes such as weighing, segregation, quality of base materials, etc. This includes variation from top to bottom or middle to edge of a bulk pile, or variation from bag to bag.

Sampling variation.—The variation in composition of successive samples taken repetitively by the same tool and method, and, as much as possible, from the same path through the bag or pile.

METHOD

Blending Plant Selection

The fertilizer plant used in this study, the W. S. Clark & Sons Co., Tarboro, NC, had the following characteristics: 2-ton Burton horizontal mixer; horizontal drum dimensions: diameter, 59 in., length, 60 in.; drum rotational speed, 10 RPM. Materials are held in overhead storage bins located 10 ft above blender; after mixing, the blend is moved 9½ ft by a screw auger elevator and then 40 ft on a cup belt elevator to a location 3 ft above the bagging hopper. The bagging hopper has partitions 16 in. × 16 in.; bagger is a St. Regis Forced Flow Valve Pack.

Before the plant was selected, the blender was tested to determine variation among the N, P_2O_5, and K_2O analyses of 10 equal time-spaced stream cuts taken from a test mix in a preliminary experiment. The blender was considered satisfactory if none of the analyses varied more than 10% from the mean for a specific element. As shown in Table 1, the blender met this criterion.

Table 2. Sieve analyses of materials used

	Tyler sieve number				
	6	8	10	14	20
Material	Cumulative Retention, %				
Urea	1.0	32.5	85.0	98.0	99.6
DAP	5.9	36.2	85.2	99.0	99.7
TSP	0.8	28.5	71.4	93.8	99.0
MP	1.6	37.2	76.2	93.3	95.6
Filler	0.5	31.9	69.4	91.1	96.3
Mean	2.0	33.3	77.4	95.0	98.0
Max. diff. from mean	3.9	4.8	8.0	4.0	2.4

Table 3. Formulations of blends used with calculated true analyses

Material	Actual wt, lbs/2-ton batch	True analyses,[a] % N	P_2O_5	K_2O
Lot A: 5-15-30		5.01	14.99	30.55
DAP	1113			
TSP	192			
MP	2015			
Filler	681			
Lot B: 10-20-20		10.00	20.04	20.23
DAP	1739			
Urea	190			
MP	1334			
Filler	738			
Lot C: 15-30-15		15.01	30.06	15.19
DAP	2609			
Urea	286			
MP	1002			
Filler	105			
Lot D: 20-5-10		19.88	5.01	10.22
DAP	435			
Urea	1569			
MP	674			
Filler	1322			
Lot E: 30-10-5		29.83	10.02	5.09
DAP	870			
Urea	2270			
MP	336			
Filler	526			

[a] Calculated from actual weights and analyses of materials (see Table 4).

Fertilizer Materials Selection

The fertilizer materials used in the study were diammonium phosphate (DAP), triple superphosphate (TSP), muriate of potash (MP), urea, and filler made from limestone rock. Particle size distribution was determined for each material using Tyler 6, 8, 10, 14, and 20 mesh sieves; the cumulative retention percentage on each sieve for each material was determined (Table 2). The material was selected if its cumulative retention percentage on any sieve did not vary more than ±10 percentage points from the average of the cumulative retention percentages of all materials for that sieve. The maximum difference between the cumulative retention percentage of any 1 material and that of the average was 8.0 for the filler on sieve number 10 (Table 2). The materials used, therefore, met the stated criterion for particle size match.

Experimental Blend Formulations

Formulations of the 5 blends used in the study are shown in Table 3. Each material was sampled and analyzed (Table 4).

Table 4. Results of analyses of materials used in the study (av. of 6 laboratories)

Material	N, %	P_2O_5, %	K_2O, %
MP	—	—	60.67
TSP	—	45.17	—
Urea	45.68	—	—
DAP	18.02	46.11	—

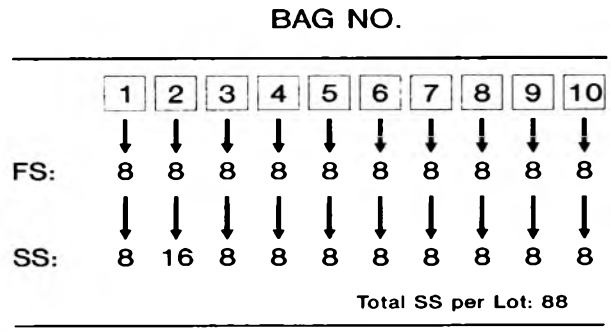

Figure 1. Scheme for splitting bags of each lot with rotary divider showing 8 first splits (FS)/bag, and then 16 subsplits (SS)/bag for bag number 2 and 8 SS/bag for the remaining 9 bags.

Each blend was made with 1 task force member recording the weights directly from the digital scale readout. The "true" analysis of each blend was calculated using the actual weights and material analysis (Table 3). The true analysis would be described as the "guaranteed analysis" by a fertilizer manufacturer. Two batches of each blend were manufactured. The first was used to purge the system; the second was sampled according to the design described below.

Sampling Design

As each experimental blend was discharged from the blender, 10 stream cuts were taken at equal time-spaced intervals with each cut being placed in a separate, marked container. The procedure and sampling cup used are described by the AOAC sampling procedure for bulk fertilizer (**929.01b**) (6).

Twenty 50-lb bags from each lot were randomly selected using random numbers generated by a computer. Bags were probed according to **929.01a** (6), except that each core was identified and kept separately for analyses. Of the 20 bags selected, every other bag was probed twice, following (as much as possible) the same path through the bag, and again each core was identified and kept separately for analyses. This resulted in 30 individual cores from each lot.

Bags that were probed twice from each of the lots were sent to the National Fertilizer Development Center, Tennessee Valley Authority (TVA), Muscle Shoals, AL, where they were reduced using a rotary divider. The rotary divider (Brinkman Retsch Type PT with a Type DR Vibratory Feeder) splits solid, granular material (up to 6 mm in size) into 8 equal portions.

The following procedure was used for reduction of the bags through the rotary divider (Figure 1).

First splits (FS).—Each bag from each lot was passed through the divider producing 8 equal splits of approximately 6.25 lb each. Each of the first split samples (or FS) from bag number 2 of each lot was weighed and an analysis of variance run on the data. According to Tukey's pair-wise comparison procedure, no differences among the slots were significant at the 0.01 level, except that slot 4 tended to have a higher

Table 5. Weights (g) of first splits (FS) from each lot by rotary divider slot

	Lot					
Slot	A	B	C	D	E	Mean[a]
1	2820	2846	2656	2850	2549	2744ab
2	2798	2863	2651	2877	2547	2747ab
3	2797	2825	2628	2847	2563	2732ab
4	2830	2854	2686	2876	2551	2759a
5	2784	2828	2659	2843	2530	2729ab
6	2790	2834	2656	2848	2517	2729ab
7	2783	2808	2645	2850	2540	2725b
8	2831	2840	2689	2848	2535	2749ab

[a] Only slots 4 and 7 were significantly different according to Tukey's pair-wise comparison procedure at $p = 0.01$ level.

Table 6. Weights[a] (g) of subsplits (SS) from each lot by rotary divider slot

	Lot					
Slot	A	B	C	D	E	Mean[b]
1	354	356	333	361	317	344
2	350	359	330	357	317	343
3	350	350	330	351	320	340
4	352	352	334	359	318	343
5	346	354	330	355	319	341
6	350	353	336	353	316	341
7	346	351	329	353	315	339
8	353	355	335	358	316	343

[a] Each weight is the mean of 2 SS.

[b] Slot means are not different according to Tukey's pair-wise comparison procedure at $p = 0.01$ level.

Table 7. Participating laboratories and samples analyzed

			Number of samples analyzed			
Code	Laboratory	Lot[a]	Material	Stream	Core	Rotary[b] slot
MD	Agrico Chemical Co.	A	4	10	30	1
KY	Univ. of Kentucky	B	4	10	30	6
AR	Arkansas Dept of Ag.	C	4	10	30	2
SC	Clemson Univ.	D	4	10	30	3
NC	North Carolina Dept of Ag.	E1	4	10	30	5
IN	Purdue Univ.	E2	4	10	30	4
AL	Tennessee Valley Authority	—	—	—	—	8
VA	Virginia Dept of Ag.	—	—	—	—	7

[a] All material, stream, and core samples from a specific lot were analyzed by the laboratory indicated. Material, stream, and core samples of lot E were split and each analyzed by the NC and IN laboratories. Some subsequent data for lot E are reported as the mean of the analyses from the 2 laboratories.

[b] Each lab analyzed 11 samples from each lot from the rotary slot indicated for a total of 55 samples/laboratory.

Table 8. Analysis of variance model for lot A for data from probed bags with duplicates on every 10th sample

Source of variation	Degrees of freedom	Mean square (MS)	Expected MS[a]
Bags	19	MS_b	$\sigma_d^2 + 1.1132\,\sigma_c^2 + 1.6364\,\sigma_b^2$
Cores (bags)	10	MS_c	$\sigma_d^2 + 1.0667\,\sigma_c^2$
Duplicates	3	MS_d	σ_d^2

[a] Where: σ_b^2 = expected bag variance component; σ_c^2 = expected core variance component; and σ_d^2 = expected duplicate variance component.

weight than slot 7 (Table 5). A difference of 34 g in ca 2700 g was considered tolerable for this study.

Subsplits (SS).—Two of the weighed FS samples from bag 2 were randomly selected using a random numbers table and each passed through the rotary divider, which produced 16, ca 0.78 lb, subsplits from 1 bag of each lot. Each of these subsplit samples (or SS) was weighed; an analysis of variance of the data showed that none of the differences among the slots was significant at the 0.01 level using Tukey's pair-wise comparison procedure (Table 6). These data, plus the FS data, show that the rotary divider is relatively free of bias.

The first split from slot 1 of bag 3 was passed through the divider and produced 8 SS/bag. The same procedure was followed for slot 2 from bag 4, for slot 3 from bag 5, for slot 4 from bag 6, for slot 5 from bag 7, for slot 6 from bag 8, for slot 7 from bag 9, for slot 8 from bag 10, and for slot 1 from bag 1. This procedure resulted in 88 SS from each lot or 11 SS/rotary divider slot/lot. Each SS from a specific divider slot from this second split was identified for analysis by a specific laboratory (Table 7). These SS are hereafter noted as *rotary divider samples.*

Each rotary divider sample (SS) was ground in its entirety with a Mikro-samplmill equipped with a screen with 1 mm openings. One ground sample from each bag was sieved to confirm that the ground material passed a U.S.A. No. 40 sieve. Each ground sample was placed on a sheet of 24 in. × 24 in. butcher paper and slowly rolled alternately from 4 directions until the sample was thoroughly mixed. The 440 ground and mixed samples (88/lot) were transferred to air-tight containers, properly labeled, and forwarded to participating laboratories (55/laboratory) for N, P_2O_5, and K_2O analyses.

Samples obtained from each lot included 4 from materials, 10 from stream cuts, 30 single cores from bags, and 88 from the rotary divider.

Chemical Analyses of Samples

Eight laboratories participated in the study (Table 7). All single core, stream cut, and rotary divider samples were ground in their entirety to pass a U.S.A. No. 40 sieve and thoroughly mixed in preparation for analyses. Total nitrogen, available phosphoric acid and soluble potash were run on each sample by an applicable AOAC method in use in the laboratory at the time. Replicates on every 10th sample were run several days apart.

Results and Discussion

Sampling Components of Variance

The first objective was to determine the sampling variance components associated with the AOAC sampling procedure for bagged fertilizer and compare them with those currently adopted by AAPFCO (5). Using the definition of sampling variation agreed upon by the task force, the study was designed to measure variation between 2 core samples taken as much as possible from the same path through the bag.

An analysis of variance model for the probed bags in which duplicate determinations were run on every 10th sample is shown in Table 8 for lot A. The term, σ_c^2, is the expected variance component of cores within a bag, which is of interest in accomplishing the first objective. It is calculated using the expected mean squares from the analysis of variance model as follows:

$$\sigma_c^2 = (MS_c - \sigma_d^2)/1.0667 = (MS_c - MS_d)/1.0667$$

The "between-cores-within-bag" [cores (bag)] variance components (σ_c^2) were calculated for N, P_2O_5, and K_2O at each concentration and are shown in Table 9 along with the "among-bags" (bag-to-bag) and "duplicates-within-laboratories" variance components.

In the core samples, most of the variability is from bag-to-bag, not core-to-core (Table 9). One reason may be that, in sampling, an attempt was made to sample the same material in the same location within a bag by the core sampler. The duplicates-within-laboratories variance component is much smaller, although we do not have a good estimate of this component because of few repeated measurements within the study.

A comparison of the sampling variance derived from this study with the official AAPFCO sampling variances is shown in Table 10. It should be noted that the AOAC sampling procedure for bagged fertilizer specifies that 10 cores are to be composited for an official sample; therefore, the sampling variances reported in Table 10 were calculated as $\sigma_c^2/10$.

Table 9. Variance component estimates for nitrogen, phosphorus, and potassium from core samples

		Variance components			
Lab.	Lot	Among bags (σ_b^2)	Between cores (bag) (σ_c^2)	Duplicates (lab.) (σ_d^2)	Means (%)
			Nitrogen		N
MD	A	0.03861	0.00999	0.000217	4.86
KY	B	0.08903	0.01197	0.00288	9.63
AR	C	0.2356	0.07647	0.00243	14.74
SC	D	1.0217	0.1333	0.03602	18.62
NC	E1	0.6133	0.4125	0.000025	29.52
IN	E2	0.6837	0.07071	0.219967	29.26
			Phosphorus		P_2O_5
SC	D	0.04012	0.02923	0.005633	5.23
NC	E1	−0.0185	0.2088	0.002425	10.31
IN	E2	0.02948	0.1372	0.000150	10.11
MD	A	0.1871	0.06111	0.00308	14.45
KY	B	0.3689	0.05103	0.01240	19.48
AR	C	0.2356	0.09526	0.15237	30.90
			Potassium		K_2O
NC	E1	0.1549	0.03100	0.0001250	5.31
IN	E2	0.1314	0.03626	0.003500	5.29
SC	D	−0.01169	0.1931	0.03703	10.24
AR	C	0.1859	0.1065	0.07628	15.62
KY	B	0.14773	−0.03525	0.1127	20.24
MD	A	0.01061	0.1437	0.01568	30.11

Table 10. AAPFCO sampling variances for N, P_2O_5, and K_2O and variances from present study

Nutrient concn,[a] %	Sampling variance: N AAPFCO	N Present	N F^b	P_2O_5 AAPFCO	P_2O_5 Present	P_2O_5 F	K_2O AAPFCO	K_2O Present	K_2O F
5	0.002	0.001	2.0	0.005	0.003	1.7	0.005	0.003	1.7
10	0.005	0.001	5.0**	0.006	0.018	3.0**	0.013	0.019	1.5
15	0.009	0.007	1.3	0.006	0.006	1.0	0.023	0.011	2.1
20	0.015	0.013	1.2	0.007	0.005	1.4	0.038	0.000	—
30	0.030	0.024	1.2	0.008	0.009	1.1	0.076	0.014	5.4**

[a] Nominal concentration.
[b] F = Larger MS/Smaller MS. Critical values: $F_{.05}$ (60, 10 df) = 2.62; $F_{.01}$ (60, 10 df) = 4.08; $F_{.05}$ (10, 60 df) = 1.99; $F_{.05}$ (10, 60 df) = 2.63 (df stands for degrees of freedom).
** Significant at 0.01% level.

The AAPFCO sampling variances were adopted from regression equations derived by Miles and Quackenbush (3) from data published earlier (10) from a study on sampling of pulverized fertilizer. The equations are found in column B, Table 2 of Miles and Quackenbush (3) and, specifically, are from the regression of the "net standard deviation," which was defined as the square root of the bag-to-bag variance component, against various concentrations of N, P_2O_5, and K_2O in mixed, pulverized fertilizers. In the present study, sampling variation was defined as "between-cores-within-bags" not "bag-to-bag" variation. We defined bag-to-bag variation as manufacturing variation.

The degrees of freedom associated with the net standard deviation are not stated by Miles and Quackenbush (3); however, R.C. Rund estimates that at least 60 observations were associated with each of the bag-to-bag net standard deviation estimates (R.C. Rund, personal communication Purdue University, West Lafayette, IN). The degrees of freedom used to determine the critical F values were 60 for the AAPFCO variances and 10 for those of the present study.

Table 11. Core, stream, and rotary divider sample means compared with true analysis (%)

Lot	True analysis	Sample means: Core	Stream	Rotary
		N		
A	5.01	4.86	5.05	5.16
B	10.00	9.63	10.00	9.93
C	15.01	14.74	14.81	14.93
D	19.88	18.62[a]	19.68	19.75
E	29.83	29.39	28.76[a]	30.12
		P_2O_5		
D	5.01	5.23	4.79	5.15
E	10.02	10.21	10.18	9.94
A	14.99	14.45	14.85	15.27
B	20.04	19.48	20.06	19.92
C	30.06	30.90[b]	30.41	30.03
		K_2O		
E	5.09	5.30	5.40	5.32
D	10.22	10.25	10.41	10.40
C	15.19	15.62	15.50	15.16
B	20.23	20.24	20.29	20.34
A	30.55	30.11	30.28	30.26

[a] Below true analysis by more than the AAPFCO IAs.
[b] Above true analysis by more than AAPFCO IAs.

Comparisons in Table 10 show significant differences for N and P_2O_5 at 10%, and K_2O at 20 and 30%. In 3 cases (10% N and 20 and 30% K_2O), sampling variances determined in the present study were smaller than AAPFCO variances. In 1 case (10% P_2O_5), the AAPFCO variance was smaller than that from the present study. Because the preponderance of comparisons indicates no difference between the AAPFCO sampling variances and those determined in the present study, the conclusion is that the sampling variances currently in use by AAPFCO are valid and there is no reason to recommend a change.

There is a valid concern about the different bases of the 2 variances. The AAPFCO sampling variance was defined as bag-to-bag while that of the present study is defined as core-to-core. The difference arises from our definition of sampling variance, which is based on the idea of measuring the varia-

Table 12. Core and rotary divider means and their comparisons for all blends of fertilizer studied (%)

Lot	Rotary	Core	Diff.	t^a
		N		
A	5.16	4.86	0.30	2.74*
B	9.93	9.63	0.30	3.83**
C	14.93	14.74	0.19	3.18**
D	19.75	18.62[b]	1.13	2.51*
E	30.12	29.39	0.73	1.73 NS
		P_2O_5		
D	5.15	5.23	−0.08	−3.61**
E	9.94	10.21	−0.27	−3.87**
A	15.27	14.45[b]	0.82	2.29*
B	19.92	19.48	0.44	0.68 NS
C	30.03	30.90[c]	−0.87	−3.78**
		K_2O		
E	5.32	5.30	0.02	0.79 NS
D	10.40	10.25	0.15	0.72 NS
C	15.16	15.62	−0.46	0.97 NS
B	20.34	20.24	0.10	1.68 NS
A	30.26	30.11	0.15	0.66 NS

[a] Tabular t's based on 10 degrees of freedom because of nonhomogeneity of variance between core and rotary divider data. This is a conservative approach. Actual degrees of freedom are probably larger.
[b] Core mean is below rotary mean by more than the AAPFCO IAs.
[c] Core mean is above rotary mean by more than the AAPFCO IAs.
* Significant at 0.05 level (2-sided test).
** Significant at 0.01 level (2-sided test).
NS = Nonsignificant at 0.05 level (2-sided test).

Table 13. Analysis of variance model for lot A of rotary divider data where 8 laboratories analyzed splits from 10 bags/blend

Source of variance	Degrees of freedom	Mean square (MS)	Expected MS[a]
Lab.	(l − 1) = 7	MS_l	$\sigma_m^2 + 1.1667\ \sigma_d^2 + 1.3542\ \sigma_{lb}^2 + 0.0774\ \sigma_b^2 + 12\ \sigma_l^2$
Bags	(b − 1) = 9	MS_b	$\sigma_m^2 + 1.0875\ \sigma_d^2 + 1.2327\ \sigma_{lb}^2 + 9.463\ \sigma_b^2$
Exp. error (lab. × bag)	(l − 1)(b − 1) = 63	MS_{lb}	$\sigma_m^2 + 1.0811\ \sigma_d^2 + 1.1758\ \sigma_{lb}^2$
Duplicates (lab. × bags)	8	MS_d	$\sigma_m^2 + 1.0417\ \sigma_d^2$
Measurement	8	MS_m	σ_m^2

[a] Where: σ_l^2 = expected laboratory variance component; σ_b^2 = expected bag variance component; σ_{lb}^2 = expected error mean square; σ_d^2 = expected duplicate variance component; σ_m^2 = expected measurement variance component.

tion associated with taking an official sample of bagged fertilizer. It is our opinion that the sampling variance based on the difference between cores within bags is a valid measure of the variation associated with the official AOAC sampling procedure for bagged fertilizer.

Representativeness of Core Samples

The second objective of the study was to determine whether a diagonal core from a bag taken according to the AOAC bag sampling procedure represents the mixture in the bag within the latitudes imposed by the AAPFCO IAs. We used 2 ways to evaluate the data relative to this second objective: (*a*) using AAPFCO IAs as the test criteria for the significance of the difference and (*b*) using *t*-tests with the average variance from the core and rotary divider analyses of variance as the test criteria.

In the first method, AAPFCO IAs were used to test the differences between the true analysis of each blend and the core means (Table 11) and between the rotary means and the core means (Table 12). Table 11 shows that only 1 core mean (18.62% N for lot D) was below the true analysis (19.88% N) by more than the AAPFCO IA. The difference is 1.26, and the IA for 19.88% N is 0.728. All other core means were within the AAPFCO IAs of the true analysis, which suggests that the AOAC bag sampling procedure secures a sample that represents what is in a bag under the experimental conditions of this study and the assumptions of the IAs.

The evaluation shown in Table 12 (see footnotes *b* and *c* of the table) of the differences between the rotary and core means using the AAPFCO IAs indicates that 2 core means (18.62% N for lot D and 14.45% P_2O_5 for lot A) were below the respective rotary means by more than the AAPFCO IAs. The IAs for 19.75% N and 15.27% P_2O_5 are 0.726 and 0.700, respectively. In making these comparisons, we assume that the rotary means represent the true composition in the bags. All other core means were within the AAPFCO IAs of the rotary means, which again suggests that the AOAC sampling procedure for bagged fertilizer secures a sample that represents what is in the bag—assuming that rotary samples are representative of the contents of the bag.

In the second method of evaluating differences between the core and rotary means, *t*-tests were used (Table 12). The variance used for making this comparison was obtained by averaging that obtained from the core analysis of variance and that obtained from the rotary divider analysis of variance. Core error was taken to be the "bag" plus the "duplicates" variance components. There was a question whether the core-to-core variance component should have also been included. It is a small component in this study because the same path was used in inserting the probe the second time; therefore, it is biased on the low side. This was omitted from the total; however, if included, the error would be slightly larger and tests slightly less sensitive. The rotary divider error was taken to be the "bag" mean square plus the "lab" mean square minus the "bag × lab" mean square.

The resulting difference would have variance components for bag, lab, bag × lab, duplicates, and measurement. In general, all variance components obtained from rotary divid-

Table 14. N, P_2O_5, and K_2O means (%) of rotary divider samples by laboratory and bag; and analysis of variance for lot A, 5-15-30

Lab.	*n*	N	P_2O_5	K_2O
AL	12	5.14	15.25	30.11
AR	12	5.15	15.32	30.23
IN	12	5.06	15.32	30.24
KY	12	5.35	15.27	30.60
MD	12	5.12	15.08	30.14
NC	12	5.15	15.19	30.45
SC	12	5.16	15.30	30.11
VA	12	5.14	15.42	30.20
Bag no.				
2	17	5.08	15.05	30.39
4	8	5.25	15.53	30.08
6	9	5.11	15.25	30.43
8	10	5.10	15.11	30.36
10	8	5.06	14.87	30.27
12	10	4.97	14.89	30.56
14	9	4.96	14.84	30.61
16	8	5.41	15.71	30.09
18	9	5.22	15.42	30.03
20	8	5.62	16.43	29.50
Grand means		5.16	15.27	30.26
		(96 observations)		

Analysis of variance

Source	Degrees of freedom	N	P_2O_5	K_2O
		Mean square[a]		
Lab.	7	0.081*	0.124*	0.368*
Bag	9	0.356**	2.05**	0.954**
Exp. error	63	0.0048	0.0499	0.1667
Dup. (lab. × bags)	8	0.0033	0.0114	0.0313
Measurement error	8	0.0088	0.0288	0.0365

[a] Lab. and bag sources tested by experimental error.
* Significant at 0.05 level.
** Significant at 0.01 level.

Table 15. N, P_2O_5, and K_2O means (%) of rotary divider samples by laboratory and bag; and analysis of variance for lot B, 10-20-20

Lab.	*n*	N	P_2O_5	K_2O
AL	12	9.92	19.90	20.30
AR	12	9.91	20.14	20.71
IN	12	10.01	19.86	20.59
KY	12	9.85	19.89	20.24
MD	12	9.91	19.88	20.05
NC	12	9.82	19.88	20.52
SC	12	10.09	19.81	20.25
VA	12	9.94	20.01	20.04
Bag no.				
2	17	9.83	19.85	20.45
4	8	10.09	20.16	20.41
6	9	9.77	19.79	20.35
8	10	9.66	19.58	20.57
10	8	10.11	20.03	20.40
12	9	9.74	19.36	20.22
14	9	9.78	19.47	20.23
16	8	10.34	20.70	20.03
18	9	10.09	20.13	20.29
20	9	10.10	20.35	20.28
Grand means		9.93	19.92	20.34
		(96 observations)		

Analysis of variance

Source	Degrees of freedom	N	P_2O_5	K_2O
		Mean square[a]		
Lab.	7	0.085**	0.132 NS	0.732**
Bag	9	0.446**	1.486**	0.166*
Exp. error	63	0.02200	0.1208	0.0675
Dup. (lab. × bag)	8	0.0167	0.0443	0.0867
Measurement error	8	0.0312	0.126	0.0283

[a] Lab. and bag sources tested by experimental error.
* Significant at 0.05 level.
** Significant at 0.01 level.
NS = Nonsignificant difference.

Table 16. N, P_2O_5, and K_2O means (%) of rotary divider samples by laboratory and bag; and analysis of variance for lot C, 15-30-15

Lab.	*n*	N	P_2O_5	K_2O
AL	12	15.00	29.99	15.20
AR	12	14.91	30.48	15.29
IN	12	15.16	30.04	15.42
KY	12	14.68	30.49	15.18
MD	12	15.01	30.08	15.01
NC	12	14.83	29.93	14.97
SC	12	14.92	29.61	15.06
VA	12	14.95	29.61	15.16
Bag no.				
2	17	14.81	30.39	14.84
4	9	14.90	29.94	15.25
6	8	14.93	29.84	15.23
8	8	15.00	30.10	15.14
10	10	15.10	30.08	14.87
12	8	14.76	29.77	15.48
14	9	14.80	29.71	15.83
16	9	14.99	30.33	15.04
18	9	15.08	30.20	14.99
20	10	15.09	29.64	15.26
Grand means		14.93	30.03	15.16
		(96 observations)		

Analysis of variance

Source	Degrees of freedom	N	P_2O_5	K_2O
		Mean square[a]		
Lab.	7	0.236**	1.339**	0.264**
Bag	9	0.161**	0.718**	0.846**
Exp. error	63	0.0264	0.0694	0.0427
Dup. (lab. × bag)	8	0.02362	0.2257	0.0331
Measurement error	8	0.00721	0.0416	0.0431

[a] Lab. and bag sources tested by experimental error.
** Significant at 0.01 level.

er data were much smaller than those obtained from core data. This results in error mean squares estimated from rotary divider data being about 10 times smaller than those estimated from core data. For this reason, a conservative number of degrees of freedom, 10, was used for finding the value of tabular *t* in the *t*-tests.

It is apparent using the *t*-tests that nitrogen determined in the core samples was consistently lower than nitrogen in the rotary divider samples (Table 12). The differences between means for the 5, 10, 15, and 20% N blends for the 2 groups were significant at the 0.05 or lower probability levels. There was a tendency for the difference to increase with level of nitrogen although this was not a consistent trend.

Differences in core and rotary divider means for phosphorus tended to be of mixed directions with the core means for 5, 10, and 30% P_2O_5 blends being significantly above the rotary means and the core mean for the 15% P_2O_5 blend being significantly below the rotary mean. There did not appear to be an obvious trend in the level of difference with level of phosphorus in the fertilizer.

In general, differences between core and rotary divider means for potassium were small and insignificant, indicating that the core samples were representative of potassium in the bags.

The *t*-tests are more sensitive than the test using IAs; therefore, small differences between the bag and rotary sample means were judged significant in more cases by the *t*-tests than by the IAs. These differences, while smaller than the IAs, indicate that something is consistently occurring to cause these discrepancies.

Baker et al. (11) studied possible mechanisms of sampler bias and concluded that sampling tubes (similar to the AOAC probe) used to take horizontal core samples tend to secure samples with an excess of finer particles. The idea is that when the slot in the probe is rotated up and the blended material flows into it, a higher proportion of fine particles flows into the slot than is in the mixture surrounding the probe. This could partially explain our data because there is a larger fraction of filler that passed the 10 mesh sieve than there is of the N materials, urea, and DAP. Thirty-one percent of the filler passed through the 10 mesh sieve; only about 15% of the DAP and urea was this fine (Table 2). Lot D (20-5-10) had the largest proportion of filler of any blend (33%) (Table 3) and the largest difference between the core and rotary means (1.13) (Table 12). Lot C had the smallest proportion of filler (3%) and the smallest difference between the core and rotary means (0.19).

If the probe selectively procured more fine filler and less of the coarser N materials, the percent N would be less than expected, as was observed with all the experimental blends.

Table 17. N, P_2O_5, and K_2O means (%) of rotary divider samples by laboratory and bag; and analysis of variance for lot D, 20-5-10

Lab.	*n*	N	P_2O_5	K_2O
AL	12	19.80	5.12	10.50
AR	12	19.84	5.22	10.51
IN	12	20.11	5.34	10.53
KY	12	19.44	5.48	10.53
MD	12	19.83	5.09	10.37
NC	12	19.73	5.09	10.29
SC	12	19.47	4.77	10.22
VA	12	19.75	5.07	10.26
Bag no.				
2	16	19.59	5.01	10.62
4	9	20.73	5.26	10.41
6	8	19.42	5.03	10.59
8	8	19.25	5.01	10.48
10	8	20.52	5.30	10.31
12	11	19.07	5.15	10.45
14	9	19.24	5.28	10.28
16	9	20.20	5.33	10.21
18	8	19.77	5.16	10.36
20	10	19.93	5.04	10.20
Grand means		19.75	5.15	10.40
		(96 observations)		

Analysis of variance

Source	Degrees of freedom	N	P_2O_5	K_2O
		Mean square[a]		
Lab.	7	0.551**	0.533**	0.211**
Bag	9	2.922**	0.164*	0.237**
Exp. error	63	0.0981	0.0638	0.0404
Dup. (lab. × bag)	8	0.0833	0.0102	0.0391
Measurement error	8	0.2661	0.0250	0.0414

[a] Lab. and bag sources tested by experimental error.
* Significant at 0.05 level.
** Significant at 0.01 level.

Table 18. N, P_2O_5, and K_2O means (%) of rotary divider samples by laboratory and bag; and analysis of variance for lot E, 30-10-5

Lab.	*n*	N	P_2O_5	K_2O
AL	12	30.08	9.96	5.36
AR	12	30.32	9.98	5.31
IN	12	30.11	10.02	5.31
KY	12	30.16	10.00	5.36
MD	12	30.23	9.86	5.22
NC	12	30.02	9.86	5.45
SC	12	29.64	9.72	5.20
VA	12	30.37	10.13	5.34
Bag no.				
2	18	30.16	9.98	5.15
4	9	30.75	9.75	5.12
6	9	30.15	9.91	5.32
8	8	29.97	9.86	5.37
10	8	30.13	9.97	5.38
12	9	29.70	9.83	5.57
14	9	29.44	9.89	5.62
16	9	30.64	10.12	5.18
18	9	30.24	10.12	5.36
20	8	29.89	9.94	5.32
Grand means		30.12	9.94	5.32
		(96 observations)		

Analysis of variance

Source	Degrees of freedom	N	P_2O_5	K_2O
		Mean square[a]		
Lab.	7	0.617**	0.187**	0.076**
Bag	9	1.492**	0.121*	0.278**
Exp. error	63	0.113	0.043	0.022
Dup. (lab. × bag)	8	0.220	0.014	0.0021
Measurement error	8	0.007	0.024	0.022

[a] Lab. and bag sources tested by experimental error.
* Significant at 0.05 level.
** Significant at 0.01 level.

While the data do not confirm this theory, it is consistent with what Baker et al. (11) found.

In summary, the significantly lower N values in the core samples are of concern and indicate a possible problem with the AOAC sampling procedure for bagged fertilizer. However, the relative size of the differences and the fact that the core means were generally not significantly below the true analysis when tested by the AAPFCO IAs allows us to conclude that the AOAC sampling procedure for bagged fertilizer does procure samples representative of what is in the bag. Additional studies are required to fully elucidate the phenomenon observed in the core samples.

Interlaboratory Component of Variance

Determination of this component of variance was not part of the original objective; however, design of the experiment allowed for its determination.

The 10 bags from each lot that were double-cored were reduced to 11 sets of 8 equal portions by the rotary divider procedure described earlier. Eight different laboratories analyzed all the samples from a specific slot from the rotary divider (Table 7). The analysis of variance model for the data is shown in Table 13.

The rotary divider data presented as laboratory and bag means of the N, P_2O_5, and K_2O analyses for each of the lots and their analysis of variance are shown in Tables 14–18. Most of the variability is because of the lab and bag sources. Differences among labs and bags are significant when tested against experimental error for all lots except phosphorus in lot B (Table 15).

The interlaboratory component of variance was calculated for each concentration of N, P_2O_5, and K_2O using the expected mean square formulae of Table 13 and the mean squares from the analysis of variance in Tables 14–18. These calculated components, along with those in current use by AAPFCO, are shown in Table 19.

AAPFCO interlaboratory variances that were adopted from data reported by Quackenbush et al. (4) involved 23 laboratories; therefore, we used 22 degrees of freedom in the *F* tests shown in Table 19. There were 6 *F* ratios that were significant and, in each case, the AAPFCO variance was significantly larger than that of the present study. The interlaboratory variance from the present study was not significantly larger than that of AAPFCO for any concentration except for 5% K_2O, where the AAPFCO variance was zero.

The data in Table 19 tend to confirm that AAPFCO interlaboratory variances are appropriate for current laboratory conditions. The significant differences found are noted; however, the consensus is to recommend no change in AAPFCO interlaboratory variances.

Table 19. AAPFCO interlaboratory variances and variances from present study

Nutrient concn,[a] %	Interlaboratory variance[b]								
	N			P_2O_5			K_2O		
	AAPFCO	Present	*F*	AAPFCO	Present	*F*	AAPFCO	Present	*F*
5	0.023	0.006	3.83*	0.043	0.038	1.13	0.000	0.0054	-0-
10	0.028	0.005	5.60*	0.044	0.012	3.67*	0.036	0.014	2.57
15	0.032	0.017	1.88	0.045	0.004	11.25**	0.074	0.018	4.11*
20	0.037	0.035	1.06	0.045	0.000	-0-	0.113	0.055	2.05
30	0.045	0.041	1.10	0.047	0.106	2.26	0.190	0.015	12.66**

[a] Nominal concentration.
[b] *F* = Larger MS/smaller MS. Critical *F* values: $F_{.05}$ (22, 7 df) = 3.43; $F_{.01}$ (22, 7 df) = 6.12; $F_{.05}$ (7, 22 df) = 2.46; $F_{.01}$ (7, 22 df) = 3.59 (df stands for degrees of freedom).
* Significant at 0.05% level.
** Significant at 0.01% level.

Table 20. Confidence limits on the true mean nutrient levels estimated from stream samples

Lot	Mean[a]	99% Confidence limits[b]
	Nitrogen, %	
A	5.05	±0.108
B	10.00	±0.177
C	14.81	±0.144**
D	19.68	±0.759
E	28.76	±0.900**
	Phosphorus (P_2O_5), %	
D	4.79	±0.251
E	10.18	±0.286
A	14.85	±0.120**
B	20.06	±0.300
C	30.41	±0.459
	Potassium (K_2O), %	
E	5.40	±0.306
D	10.41	±0.422
C	15.50	±0.525
B	20.20	±0.337
A	30.28	±0.237**

[a] Stream sample means.
[b] **99% Confidence limits do not include true analysis (see Table 3).

Representativeness of Stream Sample Means

Evaluation of the representativeness of the AOAC stream sampling procedures was not an original objective; however, the data allow valid comparisons of the true analysis and stream sample means. Two methods were used in the evaluation: (*a*) AAPFCO IAs as the test criteria for the significance of the differences, and (*b*) confidence limits on the true analyses.

The first method of comparison, in which AAPFCO IAs were used to test the differences between the true analyses and the stream means, revealed that only 1 mean was below the true analysis by more than the IA (28.76% N for lot E; see Table 11, footnote *a*). Except for this N mean, the stream samples appeared to represent the true or "guaranteed analysis" when AAPFCO IAs are used as the test criteria.

The second method of comparison was with 99% confidence limits on the true mean nutrient levels estimated from the stream samples (Table 20). Confidence intervals for lots C and E do not include what would be the true nitrogen (N) analysis, and those for lot A do not include what would be the true phosphorus (P_2O_5) and potassium (K_2O) analysis. All other confidence intervals include (or exceed) guaranteed nutrient levels. While this comparison yields 3 more incidences of significance than the first, it is our conclusion that the AOAC stream sampling method is adequate in representing the true analysis of a blend.

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References

(1) *Official Publication No. 42* (1989) Assn American Plant Food Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 25-36
(2) *Official Publication No. 21* (1968) Assn American Fertilizer Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 99-100
(3) Miles, S.R., & Quackenbush, F.W. (1955) *J. Assoc. Off. Agric. Chem.* **38**, 108-130
(4) Quackenbush, F.W., Rund, R.C., & Miles, S.R. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 915-943
(5) Rund, R.C. (1975) in *Official Publication No. 28*, Assn American Plant Food Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 67-75
(6) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs. **2.001(a)** & **2.001(b)**; 15th Ed. (1990), secs **929.01(a)** & **929.01(b)**
(7) *Official Publication No. 37* (1983) Assn American Plant Food Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 187-193
(8) *Official Publication No. 38* (1985) Assn American Plant Food Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 128-136
(9) *Official Publication No. 39* (1986) Assn American Plant Food Control Officials, Inc., University of Kentucky, Lexington, KY, pp. 175-176
(10) Miles, S.R., & Quackenbush, F.W. (1950) *J. Assoc. Off. Agric. Chem.* **33**, 424-437
(11) Baker, W.L., Gehrke, C.W., & Krause, G.F. (1967) *J. Assoc. Off. Anal. Chem.* **50**, 407-448

FOOD ADULTERATION

Detection of Orange Juice Adulteration with Beet Medium Invert Sugar Using Anion-Exchange Liquid Chromatography with Pulsed Amperometric Detection

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Carbohydrate analysis of 5 beet medium invert sugar (BMIS) samples and 10 pure orange juices was carried out using anion-exchange chromatography with a pulsed amperometric detector. This analysis revealed the presence of several oligosaccharides in BMIS that were in either low concentration or nonexistent in the orange juice samples. These oligosaccharides may be naturally present in sugar beets or synthesized during the acid and/or enzyme catalyzed hydrolysis of sucrose during the production of BMIS. BMIS was intentionally added to pure orange juice at levels of 5, 10, 15, and 20%. Subsequent liquid chromatographic (LC) analysis of these intentionally adulterated samples revealed that detection of 5% BMIS in orange juice was possible.

Fruit juice adulteration has progressed from the addition of sugar and water to the addition of adulterants made specifically to mask detection by regulatory agencies. Over the years, processors have used several methods of adulterating orange juice. The simplest form of adulteration is the overdilution of orange juice from concentrate. This form of adulteration can be detected by determining the Brix value of the orange juice (1). If this value is lower than the minimum of 11.8° Brix set by the industry, dilution with water has occurred.

Blending inexpensive fruit juices with orange juice has also occurred. The first attempt at detecting this type of adulteration was by Tillmans and Kiesgen (2), who developed the formal index to quantitate free amino acids. Other researchers have attempted to detect adulteration by monitoring changes in the concentration of various amino acids (for a review, see Ref. 3). Analysis of flavanone glycosides by liquid chromatography (LC) has been successfully used to detect the presence of grapefruit juice in orange juice (4).

Another major adulteration problem in the orange juice industry is addition of orange pulpwash to orange juice. A method was developed (5) to detect this type of adulteration using the difference in visible and ultraviolet absorption and fluorescence excitation and emission characteristics of orange juice and orange pulpwash.

Detection of orange juice adulteration has been attempted by the analysis of trace compounds such as phenolics (6); chloramine-T (7); lipids (8); minerals (9); vitamins, sugars, and nicotinic acid (10); and various organic acids (11). A microbiological assay was developed (12) to determine fruit content in orange juice products; however, this method has achieved limited success in the detection of adulterants in orange juice.

Analysis of $^{13}C/^{12}C$ isotope ratio has proven to be invaluable for detection of sugar cane and corn-derived syrups in orange juice (13, 14), but the detection of sugar beet syrups has proven to be much more difficult. Although progress has been made in this area by analysis of $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios (15, 16), climatic variations in $^{18}O/^{16}O$ isotope ratios and difficulty in routinely determining $^{2}H/^{1}H$ isotope ratio limits the widespread use of these methods.

Orange juice adulteration with beet medium invert sugar (BMIS) is perceived to be a serious problem facing the citrus industry because, at present, there is no method widely recognized for detection of BMIS in orange juice. Adulteration of orange juice with only 5 to 20% BMIS is of economic concern to the citrus industry because the market value of orange juice is more than $1 billion (U.S.) annually.

The present paper describes a method using liquid chromatography for detection of BMIS adulteration of orange juice at levels as low as 5%.

Experimental

Sample Preparation

Orange juice and BMIS samples were obtained as follows: 3 orange juice samples (**a**–**c**) (gift of Florida Department of Citrus); 7 orange juice samples (**d**) (gift of Allan Brause); 1 BMIS sample (**e**) (gift of Alberta Sugar Co.); and 4 BMIS samples (**f**–**i**) (gift of Florida Department of Citrus).

(**a**) *Reconstituted commercial orange juice concentrate.*—Sample A, 11.73° Brix, 0.72% acid.

(**b**) *Reconstituted orange juice concentrate.*—(Valencia) prepared in Florida Department of Citrus pilot plant, 11.71° Brix, 0.78% acid.

(**c**) *Reconstituted commercial orange juice concentrate.*—Sample B, 11.74° Brix, 0.78% acid.

(**d**) *Reconstituted orange juice concentrates.*—Seven samples 11.8 °Brix, 0.78% acid.

(**e**) *Beet medium invert sugar (BMIS).*—(Alberta Sugar Co.) 24.5% H_2O.

(**f**) *BMIS.*—(Amalgamated Sugar Co., Kansas) 23.6% H_2O.

(**g**) *BMIS.*—(Holly Sugar Corp., Texas) 23.2% H_2O.

(**h**) *BMIS.*—(Holly Sugar Corp., Colorado) 22.5% H_2O.

(**i**) *BMIS.*—(Michigan Sugar Corp., Michigan) 25.1% H_2O.

(**j**) *Orange juice (sample A)-BMIS.*—(95 + 5) (BMIS from Amalgamated Sugar Co.) 11.69° Brix, 0.68% acid.

(**k**) *Orange juice-BMIS.*—(90 + 10) 11.68° Brix, 0.64% acid.

(**l**) *Orange juice-BMIS.*—(85 + 15) 11.68° Brix, 0.61% acid.

(**m**) *Orange juice-BMIS.*—(80 + 20) 11.72° Brix, 0.58% acid.

Orange juice samples were first passed through fiberglass

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wool to remove the majority of pulp. One g BMIS or 5 g of an orange juice sample was passed through a C-18 Sep Pak cartridge (Waters Associates) and 3 cm^3 AG 501-X8 mixed-bed resin, 20–50 mesh (Bio-Rad Laboratories). Samples were passed through 3 cm^3 AG 1-X4 anion exchange resin, 100–200 mesh (Bio-Rad Laboratories), to remove organic acids. Monosaccharides were removed from the BMIS and orange juice by a procedure from Whistler and Durso (17) that was modified. Samples were stirred with 4 g activated charcoal, 50–200 mesh (Fisher Scientific Co.) for 17 h at 4°C. After mixing, samples were placed on a 3 cm diameter column containing 4 g activated charcoal–celite (50 + 50, w/w) (Fisher Scientific Co.). Approximately 99% of monosaccharides were removed from the column by washing with 1 L of 0.1% (v/v) ethanol adjusted to a flow rate of 10 mL/min by vacuum aspiration. Remaining oligosaccharides were eluted from the column with 500 mL of a 60°C solution of 50% (v/v) ethanol at the same flow rate. The column effluent was frozen at −70°C and dried at 30°C in a freeze dryer (Lab Con Co., Freeze Dry 5). Ten mL deionized water was added to the lyophilized BMIS and orange juice samples. Samples were stored at −20°C until required for analysis. All samples were passed through a 0.2 μm nylon-66 filter (Rainin Instrument Co., Woburn, MA) to remove particulate matter. Ninhydrin and Bradford tests were carried out on samples to ensure that no amino acids or proteins were present.

LC Analysis of Oligosaccharides in Orange Juice and BMIS

Resulting samples were analyzed on a Dionex Bio LC 4000 gradient liquid chromatograph containing 2 Dionex 10 μm Carbo Pac PA1 pellicular anion exchange columns (4 × 250 mm) connected in series. A 100 μL sample loop was used for analysis of orange juice samples, while a 50 μL sample loop was used for BMIS samples. Flow rate was set at 0.70 mL/min. Carbohydrates were detected by a pulsed amperometric detector (PAD) with a gold electrode and triple pulsed amperometry at a sensitivity of 10K. The electrode was maintained at the following potentials and durations: E_1 = 0.05 V (t_1 = 120 ms); E_2 = 0.80 V (t_2 = 120 ms); E_3 = −0.60 V (t_3 = 420 ms).

A postcolumn delivery system of 300 mM sodium hydroxide (NaOH) at a flow rate of 0.80 mL/min was used to prevent baseline drift. The following gradient elution was used to achieve separation of the oligosaccharides: 100 mM NaOH for 4 min; after 20 min, the mobile phase was 100 mM NaOH and 3 mM sodium acetate (NaOAc); after 50 min, the mobile phase was 100 mM NaOH and 100 mM NaOAc. This eluant was held for 10 min before a 300 mM NaOH wash was used to remove acetate ions from columns. Following the wash step, columns were re-equilibrated with 100 mM NaOH in preparation for the next injection. Carbohydrates eluting from the columns were plotted by a Spectra Physics model 4290 integrator.

Results and Discussion

The major carbohydrates present in orange juice are glucose, fructose, and sucrose in an approximate ratio of 1:1:2 (18). More than 95% of the carbohydrate in sugar beets is sucrose (19). Controlled acid and/or enzyme catalyzed hydrolysis of sucrose derived from sugar beets can be used to produce a BMIS with a similar glucose–fructose–sucrose ratio (i.e., 50% hydrolyzed). The majority of naturally occurring carbohydrates exist in the pyranose conformation (for a review, see Ref. 20). Carbohydrates, such as sucrose, which contain a furanose ring, are more susceptible to acid hydrolysis than oligosaccharides that contain pyranose moieties (21, 22). Therefore, acid hydrolysis of sucrose from sugar beets will leave most of the naturally occurring oligosaccharides intact.

Invertase catalyzed hydrolysis of sucrose results in the production of several minor oligosaccharides. Many researchers have observed the invertase catalyzed formation of oligosaccharides via transglucosylation/transfructosylation reactions in a number of natural foods (23–26). The origin of the oligosaccharides in BMIS may arise from a combination of the action of hydrolase enzymes in the native plant and the action of commercially available invertase in the production of BMIS.

The main commercial sources of invertase that could be used to hydrolyze sucrose for production of BMIS are from yeast, *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (27). These enzymes, plus the invertases naturally present in sugar beets, all exhibit fructosidase activity (28); while invertase isolated from oranges exhibits glucosidase activity (29). The oligosaccharides formed during the natural hydrolysis of sucrose in oranges would be glucose-linked because the only hydrolase enzymes found in oranges exhibit glucosidase activity. Oligosaccharides in BMIS would be fructose-linked because the invertases naturally present in sugar beets and the invertases that could be used for BMIS production are fructosidases. It is also possible that these oligosaccharides could be formed during the acid hydrolysis of beet sugar during the production of BMIS (30).

Initial carbohydrate analysis of orange juice or BMIS by LC indicated that the high concentration of monosaccharides present in these 2 products saturate the column's active sites, obscuring further separation of the oligosaccharides present in these products. A purification step was introduced employing charcoal/celite chromatography followed by elution with suitable solvents to remove approximately 99% of monosaccharides present in both orange juice and BMIS with no removal of oligosaccharides.

Figure 1 is an LC chromatogram showing the carbohydrates present in pure orange juice (sample A) following removal of monosaccharides with charcoal/celite. Small amounts of glucose and fructose were still present, with elution times of 14 and 16 min, respectively, while sucrose eluted at approximately 20 min. The other carbohydrates in orange juice were present in low concentrations as shown in this chromatogram. Analysis of 9 other pure orange juice samples yielded virtually identical chromatograms.

The carbohydrate elution pattern of BMIS supplied by Amalgamated Sugar Co. (after charcoal/celite chromatography) is shown in Figure 2. The chromatogram of BMIS was different from orange juice in number and concentration of oligosaccharides. Analysis of 4 other BMIS samples yielded chromatograms identical to that shown in Figure 2, except for slight variations in oligosaccharide concentration. The 4 oligosaccharide peaks that eluted between 65 and 71 min appear to be fingerpri4nts for BMIS.

Figures 3 and 4 are chromatograms of orange juice (sample A) prepared with 20% and 5% BMIS (Amalgamated Sugar Co.), respectively. This BMIS contained the lowest levels of oligosaccharides of the BMIS analyzed. These chromatograms indicated that adulteration was clearly distinguishable by the presence of characteristic BMIS oligosaccharides that eluted in the 65 to 71 min region. These figures

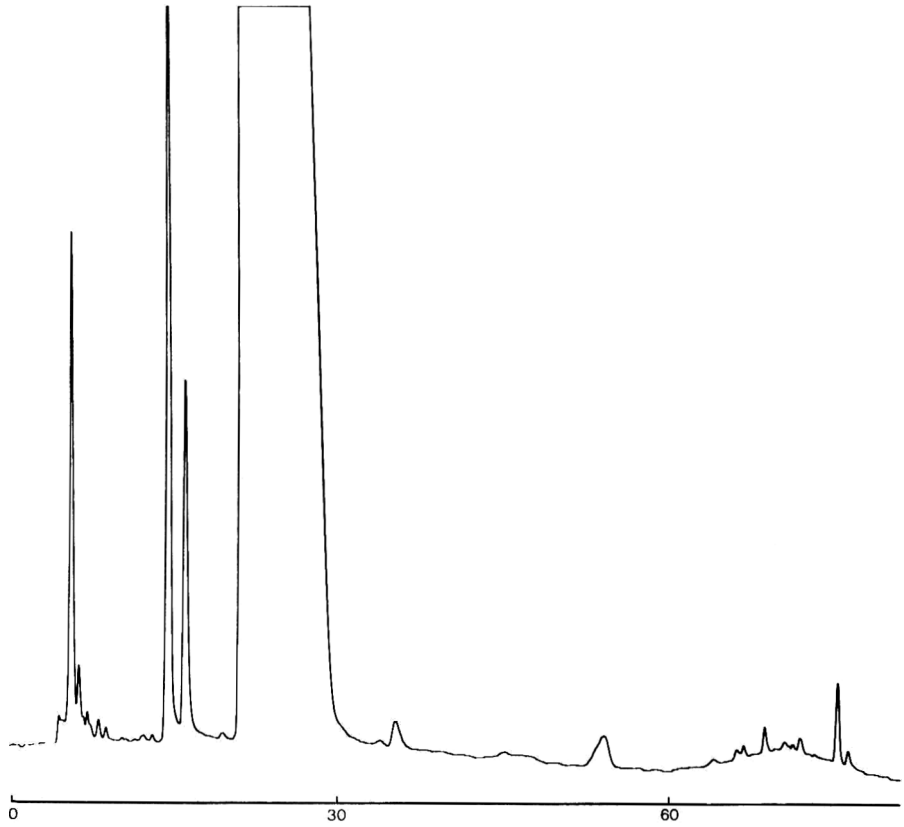

Figure 1. LC chromatogram of the carbohydrates in pure orange juice sample A.

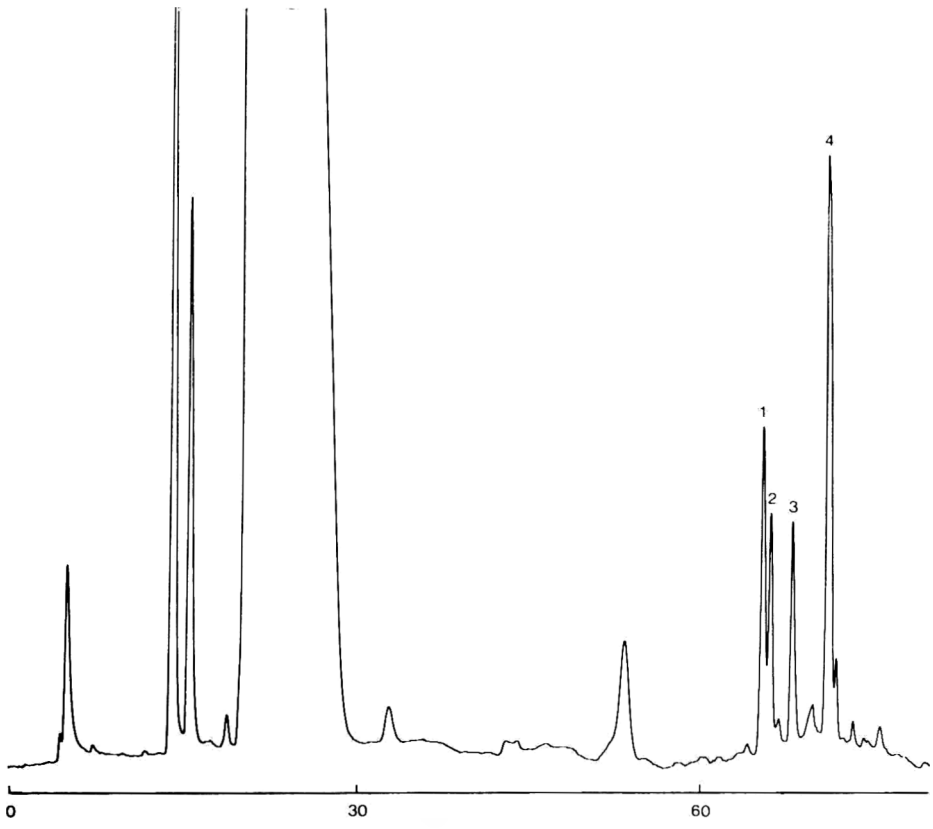

Figure 2. LC chromatogram of carbohydrates in BMIS (Amalgamated Sugar Co.). The 4 fingerprint oligosaccharides elute between 65 and 71 min.

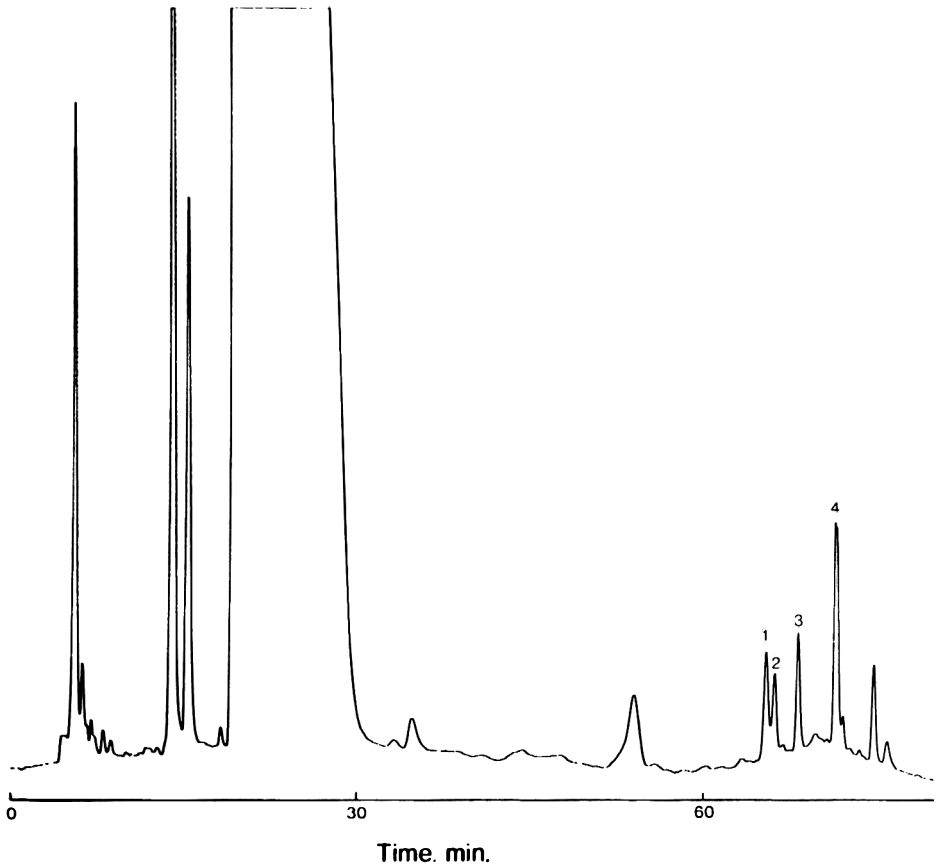

Figure 3. LC chromatogram of orange juice sample A intentionally adulterated with 20% BMIS (Amalgamated Sugar Co.).

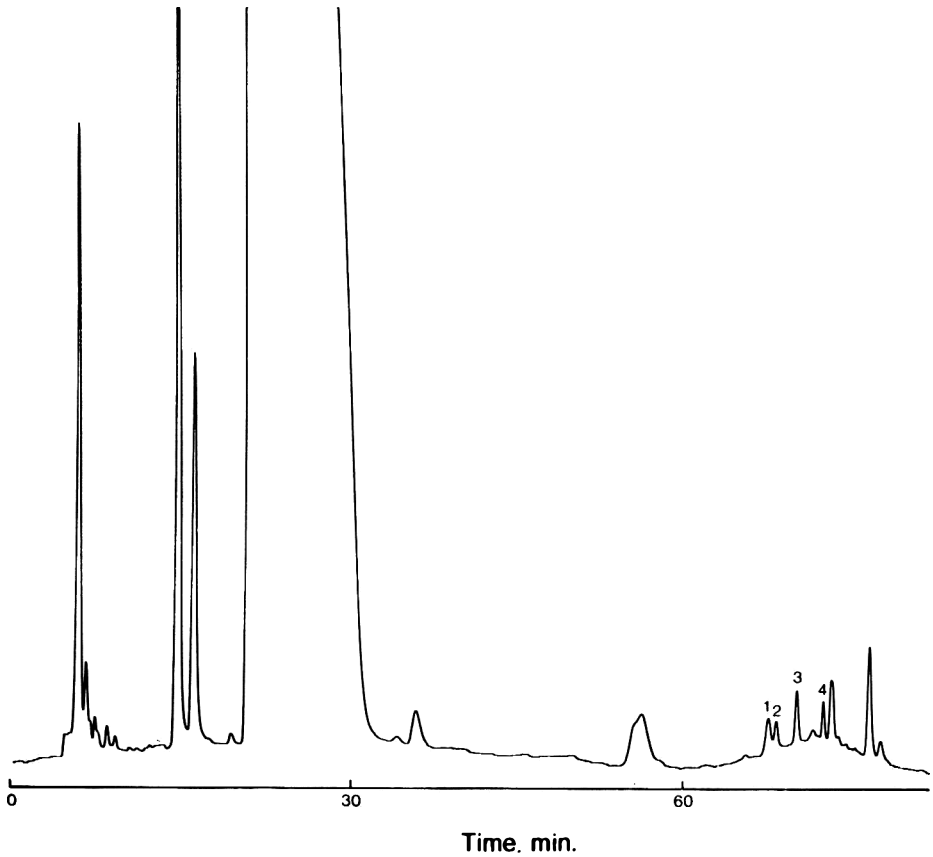

Figure 4. LC chromatogram of orange juice sample A intentionally adulterated with 5% BMIS (Amalgamated Sugar Co.).

also indicate that there is a relationship between the extent of adulteration and the size of the peaks in this region of the chromatogram. The area under the specific peaks may be used (if sufficient baseline data on both orange juice and BMIS are known) to determine the percent adulteration of orange juice with BMIS. Levels as low as 1% BMIS addition to orange juice would appear to be detectable.

The structures of the oligosaccharides eluting between 65 and 71 min in BMIS and orange juice have not yet been elucidated. Several di- and trisaccharide standards have been analyzed using our methodology. From standard injections of dextrose polymers (DP2 to DP7), retention times of the fingerprint oligosaccharides are less than DP4. Because the hydrolase enzymes in oranges and sugar beets exhibit different specificity, it is likely that the oligosaccharides eluting between 65 and 71 min in the BMIS chromatogram are different from the oligosaccharides eluting in the same region of the orange juice chromatogram. The possibility also exists that there is a low level of fructosidase activity in oranges that could account for the synthesis of oligosaccharides identical to those found in BMIS.

Although only 10 orange juice samples and 5 beet medium invert samples have been analyzed, results are promising. The need to analyze a number of pure orange juice samples is recognized to ensure that the oligosaccharide patterns are similar to those that we have analyzed to date. We are currently isolating the fingerprint oligosaccharides present in BMIS for structural identification. In addition, we are also in the process of corroborating data obtained by LC with capillary gas chromatographic analysis of oligosaccharides in BMIS and orange juice.

Conclusion

A major adulteration problem facing the orange juice industry today is the addition of BMIS. Detection of this form of adulteration has, so far, proved elusive with the methods available. Analysis of trace carbohydrates in BMIS and orange juice by LC indicate that there are several oligosaccharides unique to BMIS or present in much greater concentration than in orange juice. Trace carbohydrates in BMIS may be naturally present in sugar beets or may be formed during production of BMIS. Preliminary analysis of these trace carbohydrates indicates that adulteration of orange juice with BMIS at levels as low as 5% can be detected. The extension of this type of analysis for detection of BMIS in other pure fruit juices should also be possible.

Acknowledgments

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References

(1) Stevens, J. W., & Baier, W. E. (1939) *Ind. Eng. Chem. Anal.* **11**, 447-449
(2) Tillmans, J., & Kiesgen, J. (1927) *Z. Unter. Lebensm.* **53**, 131-137
(3) Petrus, D. R., & Vandercook, C. E. (1980) in *Citrus Nutrition and Quality*, S. Nagy & J. A. Attaway (Eds.), *ACS Symposium Series No. 143*, American Chemical Society, Washington DC, pp. 395-421
(4) Fisher, J. F., & Wheaton, T. A. (1976) *J. Agric. Food Chem.* **24**, 898-899
(5) Petrus, D. R., & Attaway, J. A. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1317-1331
(6) Davis, W. B. (1947) *Anal. Chem.* **19**, 476-478
(7) Safina, G., & Trifiro, E. (1957) *Conserve. Deriv. Agrumari.* **6**, 12-14
(8) Nagy, S. (1977) in *Citrus Science and Technology*, Vol. 1, S. Nagy, P. E. Shaw, & M. K. Veldhuis (Eds.), Avi, Westport, CT, Chapter 7
(9) Morgan, R. H. (1963) *Food Process Packag.* **32**, 163-167
(10) Sawyer, R. (1963) *J. Sci. Food Agric.* **14**, 302-312
(11) Primo Yufera, E., Munarriz, J. M., Casas, A., & Miralles, M. C. (1967) *Rev. Agroquim. Tecnol. Aliment.* **7**, 244-249
(12) Vandercook, C. E., Smolensky, D. C., & Nakamura, L. K. (1976) *J. Food Sci.* **41**, 709-710
(13) Nissenbaum, A., Lifshitz, A., & Stepak, Y. (1974) *Lebensm. Wiss. Technol.* **7**, 152-154
(14) Doner, L. W., & Bills, D. D. (1982) *J. Assoc. Off. Anal. Chem.* **65**(3), 608-610
(15) Doner, L. W., Ajie, H. O., Sternberg, L. O., Milburn, J. M., DeNiro, M. J., & Hicks, K. B. (1987) *J. Agric. Food Chem.* **35**, 610-612
(16) Bricout, J., & Koziet, J. (1987) *J. Agric. Food Chem.* **35**, 758-760
(17) Whistler, R. L., & Durso, D. F. (1950) *J. Am. Chem. Soc.* **72**, 677-679
(18) Curl, A. L., & Veldhuis, M. K. (1948) *Fruit Prod. J.* **27**, 342-361
(19) Hardinge, M. G., Swarner, J. B., & Crooks, H. (1965) *J. Am. Diet. Ass.* **46**, 197-204
(20) Govil, G., & Hosur, R. V. (1982) in *Conformation of Biological Molecules*, NMR Vol. 20, Springer-Verlag, Berlin, pp. 144-154
(21) Aspinall, G. O., & Telfer, R. G. J. (1955) *J. Chem. Soc.* 1106-1110
(22) Pazur, J. H., & Gordon, A. L. (1953) *J. Amer. Chem. Soc.* **75**, 3458-3460
(23) Low, N. H., Va Vong, K., & Sporns, P. (1986) *J. Apic. Res.* **25**(3), 178-181
(24) Chiba, S., Asada-Komatsu, Y., & Kawashima, K. (1984) *Agric. Biol. Chem.* **48**, 1173-1178
(25) Chiba, S., Kimura, A., Kobori, T., & Saitoh, K. (1985) *Depun Kagaku* **32**, 213-216
(26) White, J. W., Jr, & Maher, J. (1953) *J. Am. Chem. Soc.* **75**, 1259-1260
(27) Beuchat, L. R. (1987) in *Food and Beverage Mycology*, 2nd. Ed. AVI, Van Nostrand Reinhold Co., New York, pp. 474-483
(28) Masuda, H., Takahashi, T., & Sugawara, S. (1987) *Agric. Biol. Chem.* **51**(9), 2309-2314
(29) Huleme, A. C. (1970) in *The Biochemistry of Fruits and Their Products*, Vol. 1, Academic Press, New York
(30) Belitz, H. D., & Grosch, W. (1987) in *Food Chemistry*, Springer-Verlag, Berlin, pp. 201-256

FOOD COMPOSITION

Conductometric and Colorimetric Determination of Volatile Acidity of Vinegars by Flow-Injection Analysis

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Recent methods for determination of the volatile acidity of vinegars are relatively slow (about 40 min) and involve techniques subject to a variety of errors (ca 2.5%). The present paper describes a method that provides results in a shorter time (ca 2 min, including dilution), with a smaller relative error rate (ca 1%). Conductometric analysis consists of the injection of the sample in a deionized water stream that then flows past a PTFE membrane separator. Acetic acid diffuses through the membrane to another deionized water stream that passes through a conductivity cell. Colorimetric analysis also consists of sample injection into a deionized water stream that passes through the same PTFE membrane separator. However, the acetic acid diffuses into a bromocresol purple solution stream at pH 7. This solution passes through a flow cell in a spectrophotometer set at 540 nm. Before injection, samples were treated with hydrogen peroxide to ensure complete oxidation of sulfite to sulfate. Results of the proposed method were also compared with another similar method. At a 95% confidence level, the statistical *t*-test indicates no significant difference between them. Typical estimates of the relative standard deviations obtained with the new methods are ca 1%. Analyses were performed with red and white wine vinegars.

Since its introduction in 1974–1975 in a classic paper (1, 2), flow-injection analysis (FIA) has been a valuable means of automating analyses and increasing sample output in most analytical laboratories. Besides being a method that permits many analyses in a short period of time, for analysis of volatile acidity of vinegars in particular, FIA systems provide a substantial increase in the precision of results when compared with traditional methods. Generally, those methods have many steps, which increases the rate of error.

Baadenhuijsen and Seuren-Jacobs (3) used gas diffusion in FIA in a determination of carbon dioxide in plasma using a gas-permeable membrane. Gas-permeable membranes in FIA systems are now widely used to transfer certain compounds from a donor (sample) stream to an acceptor (detector) stream. The membrane transport process in a flow-through unit and its dependence on characteristic membrane parameters were investigated by Van der Linden (4), both from the theoretical and the practical points of view. Some volatile compounds (e.g., carbon dioxide, ammonia, and acetic acid) have been studied using different types of gas-diffusion membranes.

Gas diffusion is a very selective technique because few species are sufficiently volatile at room temperature. Some compounds (e.g., carbon dioxide, sulfur dioxide, HCN, HF, HCl, and acetic acid) can be measured by this technique, depending on the pH of the donor stream. It is also interesting to note that these species will rarely be present in the sample at the same time. However, some samples, such as beverages, present high amounts of carbon dioxide, which can interfere in the determination of relatively small amounts of sulfur dioxide. In such cases, a more selective, colored reagent is preferable to an acid–base indicator.

In the analysis of volatile acidity of vinegars, the only species in the sample that will permeate the microporous Teflon® membrane are acetic acid, carbon dioxide, and sulfur dioxide. Carbon dioxide does not interfere because of the low pH of the sample; sulfur dioxide can be eliminated by the use of hydrogen peroxide.

Vinegar is a product in which 100 g contains 5 to 15.5 g anhydrous acetic acid produced by acetic fermentation of liquids containing alcohol (5). Vinegar is mainly used by consumers for acidification of salads and vegetables and for seasoning meat and fish; the food industry uses vinegar to preserve and season food at the same time. Nunheimer and Fabian (6) studied the relationship between dissociation constants of several acids and the inhibition of microorganisms in foodstuffs. They found that, when compared with citric acid, lactic acid, malic acid, and tartaric acid, acetic acid is a stronger growth inhibitor of microorganisms at a higher pH than other acids. Szakall (7) found that vinegar has a specific inhibitory effect on the growth of microorganisms, as compared to diluted acetic acid, whose effect is a function of acid concentration only. The reason for this phenomenon is not yet known.

Besides acetic acid and alcohol, vinegar contains secondary constituents that contribute to its smell, taste, and preserving qualities. These constituents have their origin in the raw material, in added nutrients, and in the water used for dilution.

Vinegar can be analyzed for 2 different reasons: (*a*) for process control using routine methods, and (*b*) for a comprehensive knowledge of its chemical constituents using special methods. In vinegars, it is most important to measure volatile acidity, fixed acidity, and total acidity.

METHOD

Samples are treated separately with a few drops of hydrogen peroxide and then analyzed in a flow-injection system. The diffused acetic acid changes the conductivity [in microsiemens (μS)] of a deionized water stream (conductometric method) or the color of the bromocresol purple (BCP) indicator solution stream (colorimetric method). Absorbances are read in a 10 mm flow cell at 540 nm.

Apparatus

(**a**) *Peristaltic pump.*—Ismatec GJ04 mp 13 at a flow rate of 1.26 mL/min.

(**b**) *Sampling valve system.*—Microvolume 2-position sampling valve fabricated in our laboratory, made of graphitic Teflon.

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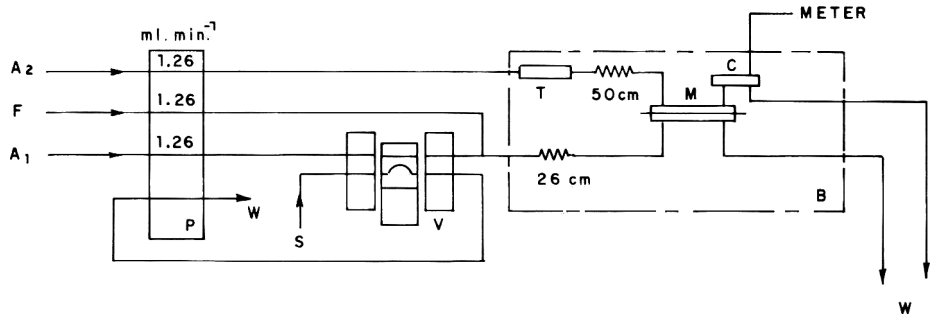

Figure 1. Conductometric flow-injection manifold. T = ion-exchange resin column, P = peristaltic pump, S = sample inlet, V = sampling valve system, B = water bath, M = diffusion cell, C = conductance flow cell, W = waste, A_1 and A_2 = deionized water streams, and F = 0.05M sulfuric acid solution.

(c) Diffusion cell.—Gas-diffusion unit similar to models described in Refs. 4 and 8. Each block, made of acrylic resin, had a shallow groove 0.1 mm deep, 3 mm wide, and 5 cm long. Commercial PTFE (Teflon) microporous tape was placed between the 2 pieces; the unit was secured with 6 screws.

(d) *Conductance flow cell.*—Stainless-steel flow cell (as described in Ref. 8) for conductance measurement. Estimated volume is 60 μL. The cell was covered with epoxy resin to isolate it from the water bath in which it was immersed.

(e) *Conductivity meter.*—Micronal, model B331 connected to a chart recorder.

(f) *Spectrophotometer.*—Zeiss PM2D, equipped with 10 mm flow cell (volume 50 μL) connected to a chart recorder.

Reagents

Prepare all reagents from analytical reagent quality chemicals unless otherwise specified.

(a) *Acetic acid standards.*—Concentrated acetic acid diluted with boiled deionized water to produce solutions 0.2 to 0.6% in acetic acid.

(b) *Bromocresol purple (BCP) solution.*—(1×10^4M). Dissolve 0.27 g BCP in 10 mL ethanol; complete volume to 500 mL with boiled deionized water. Take 50 mL of this solution and dilute with boiled deionized water to 500 mL to produce working solution.

Analytical System

Schematic flow diagrams for conductometric and colorimetric determinations of volatile acidity of vinegars are shown in Figures 1 and 2.

Conductometric analysis.—Combine injected sample (S), previously treated with hydrogen peroxide, with deionized water carrier stream (A_1) pumped at a flow rate of 1.26 mL/min. Add 0.05M sulfuric acid solution (F) and mix in a 26 cm long coil. After mixing, acetic acid diffuses through the Teflon membrane separator (M) to another deionized water stream (A_2) that passes through the conductivity cell. This deionized water stream (A_2) passes, initially, through a column containing an ion-exchange resin to guarantee water free of ions. Immerse conductivity cell, diffusion cell, and resin column in a constant-temperature water bath to avoid temperature changes during analysis.

Colorimetric analysis.—Combine injected sample (S), treated in the same way, with deionized water stream (A_1) pumped at a flow rate of 1.26 mL/min. After injection, acetic acid diffuses through the Teflon membrane separator (M) to the bromocresol purple solution stream (I) at pH 7, passing through a flow cell in the spectrophotometer with absorbance measured at a wavelength of 540 nm. To avoid CO_2 interference (from atmosphere), flask with BCP solution should be protected with a tube containing solid $CaCl_2/NaOH/CaCl_2$.

Preparation of Samples

Add 3 to 5 drops 3.5M hydrogen peroxide to 10 mL vinegar in a 100 mL volumetric flask and complete volume with boiled deionized water. Solutions will contain ca 0.38 to 0.48 g acetic acid/100 mL solution.

Results and Discussion

Tables 1 and 2 show results obtained with FIA methods and with the Jaulmes method (9), which is similar to the AOAC method (10). To statistically compare results, the Student's *t*-test was used (11).

Calibration curves are not linear (Figures 3 and 4); however, volatile acidity can be evaluated by graphical interpolation with acceptable precision. Examination of calibration data show that the experimental curve fits the equation $y = C + Bx + Ax^2$, where y = peak height, x = volatile acidity and A, B, and C = adjustable parameters. Student's *t*-test values in Tables 1 and 2 show that there is no statistical difference between results at a 95% confidence level.

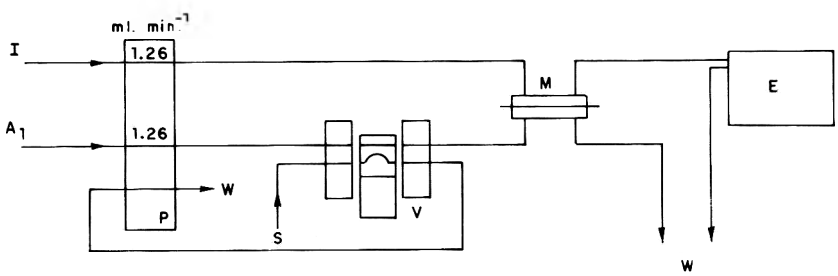

Figure 2. Colorimetric flow-injection manifold. P = peristaltic pump, S = sample inlet, V = sampling valve system, M = diffusion cell, E = spectrophotometer, W = waste, A_1 = deionized water stream, I = BCP solution stream.

Table 1. Volatile acidity of wine vinegars (g acetic acid/100 mL vinegar) using conductometric FIA system and Jaulmes method (9) (%)

Sample	FIA[a]	t_d	FIA[b]	t_e	Jaulmes
1(R)	4.25	0.32	—	—	4.23
2(W)	4.41	0.16	4.41	0.16	4.40
3(R)	4.31	0.47	4.31	0.47	4.34
4(W)	4.52	0.00	4.57	0.79	4.52
5(R)	4.46	1.11	4.62	2.21	4.53
6(R)	4.46	1.90	4.67	1.42	4.58

[a] FIA method (Figure 3) with Teflon sampling loop, 0.9 mm id; volume, 62 μL.

[b] FIA method with polyethylene sampling loop, 0.8 mm id; volume, 120 μL.

Note: (R) = red wine vinegar, (W) = white wine vinegar. Estimates of standard deviations are ±0.04 for FIA method and ±0.08 for Jaulmes. t_d and t_e are calculated Student t values; tabulated t value for the degree of freedom (ν) 4 is 2.776 ($\alpha = 0.05$); $\nu = n_1 + n_2 - 2$ and $n_1 = n_2 = 3$ in this case.

Table 2. Volatile acidity of wine vinegars (g acetic acid/100 mL vinegar) using colorimetric FIA system and Jaulmes method (9) (%)

Sample	FIA[a]	t_d	FIA[b]	t_c	Jaulmes
1(R)	4.20	0.47	4.22	0.16	4.23
2(W)	4.44	0.63	4.43	0.47	4.40
3(R)	4.31	0.47	4.33	0.16	4.34
4(W)	4.52	0.00	4.53	0.16	4.52
5(R)	4.52	0.16	4.52	0.16	4.53
6(R)	4.57	0.16	4.55	0.47	4.58

[a] FIA method (Figure 4) with polyethylene sampling loop, 1.6 mm id; volume, 240 μL.

[b] FIA method with tygon sampling loop, 1.14 mm id; volume, 46 μL.

Note: (R) = red wine vinegar, (W) = white wine vinegar. Estimates of standard deviations are ±0.04 for FIA method and ±0.08 for Jaulmes. t_d and t_e are calculated Student t values; tabulated t value for the degree of freedom (ν) 4 is 2.776 ($\alpha = 0.05$); $\nu = n_1 + n_2 - 2$ and $n_1 = n_2 = 3$ in this case.

Loops

We tested many loops of different materials, volumes, and diameters to try to resolve a problem of retention of acetic acid in the walls of the loop. This phenomenon was responsible for the increase of peak height in the descending curve. It was established, empirically, that either a polyethylene loop (volume 120 μL, 0.8 mm id) or a Teflon loop (volume 62 μL, 0.9 mm id) can be used in the conductometric method without interference from this phenomenon.

For the colorimetric method, a polyethylene loop (volume 240 μL, 1.6 mm id) and a tygon loop (volume 46 μL, 1.14 mm id) presented the best results.

Figure 5 shows results from a faulty loop. For standards of the same acid concentration, the signal was greater for the decreasing order of injections.

The nonlinearity of the calibration curves is probably the result of different factors. In the conductometric method, because acetic acid is a weak electrolyte, the relationship between conductivity and concentration is not linear. A similar effect occurs in the colorimetric method that is within the limits of the validity of Beer's law. In both cases, the relationship between the diffusion rate of acetic acid through the membrane and concentration is not linear.

Because a complete study of the materials, sizes, and diameters of the loops would be an exhaustive work, an empirical selection was made. However, special attention must be paid to the choice of the size and material of the loops in initiating either method.

Comparison with Other Methods

Traditional methods, including those of Jaulmes and Cazenave-Ferré, have 2 principal steps: distillation and titration. In general, the sampling rate is 1 sample/h. According to data obtained from vinegar manufacturers, a relative error rate of 2.5% is considered acceptable.

The advantages of FIA are evident if one compares the sampling rate (60 samples/h) and the relative error rate (1.0%) of the proposed methods with those of traditional

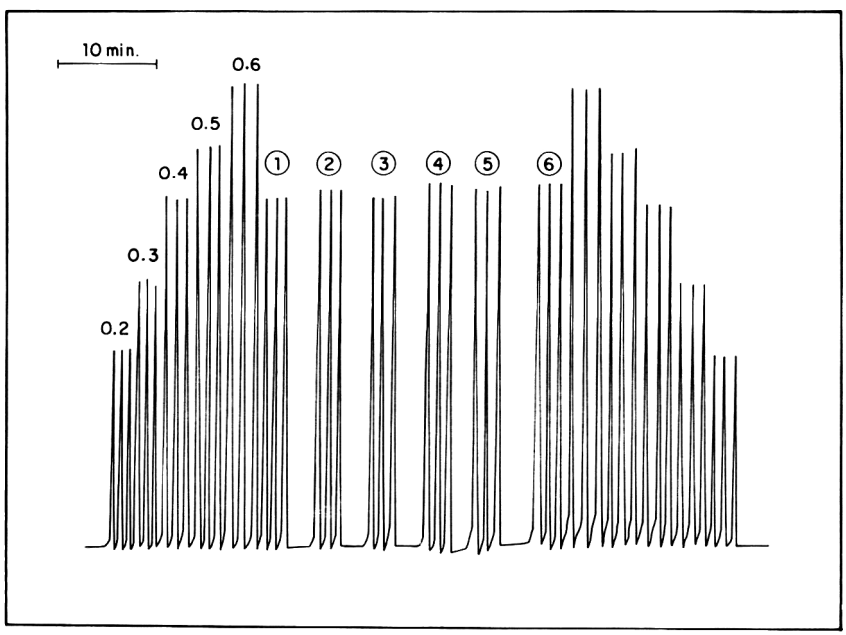

Figure 3. Calibration and sample runs for volatile acidity determination (conductometric system). Left to right: triplicate signals for acetic acid standards (0.2, 0.3, 0.4, 0.5, and 0.6% g acetic acid/100 mL solution). Triplicate signals for vinegars and standards in reverse order. Teflon loop (62 μL, 0.9 mm id).

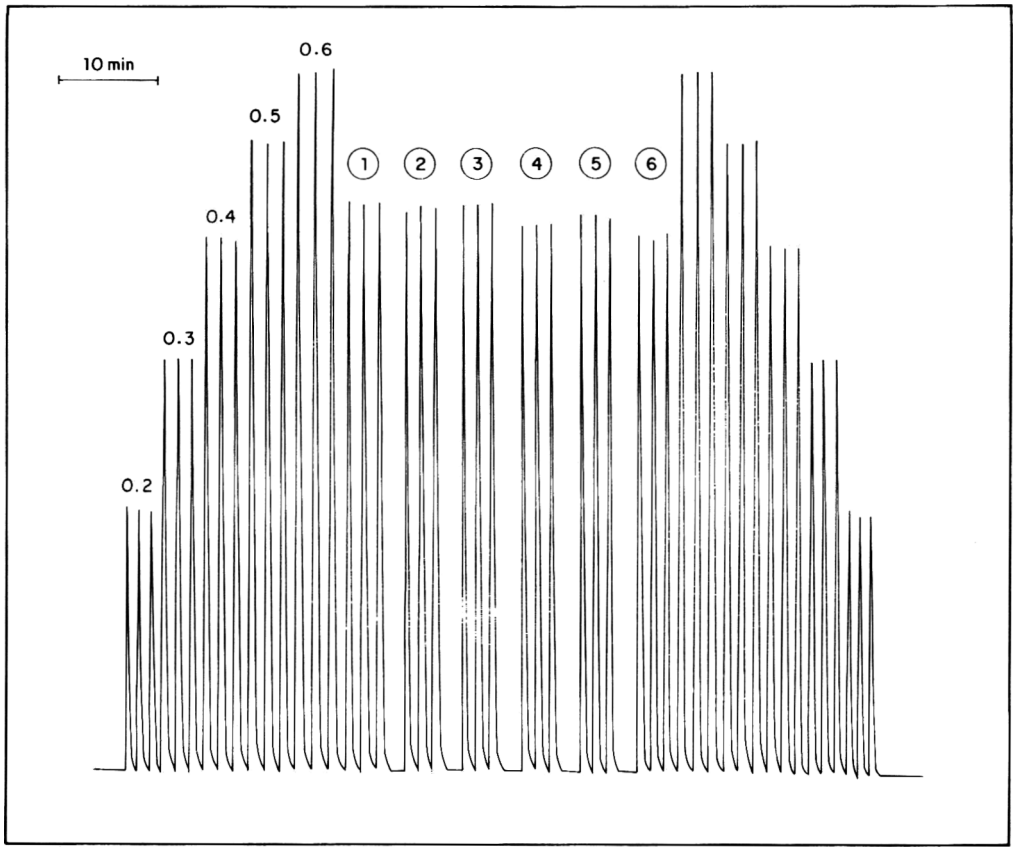

Figure 4. Calibration and sample runs for volatile acidity determination (colorimetric system). Left to right: triplicate signals for acetic acid standards (0.2, 0.3, 0.4, 0.5, and 0.6% g acetic acid/100 mL solution). Triplicate signals for vinegars and standards in reverse order. Polyethylene loop (240 μL; 1.6 mm id).

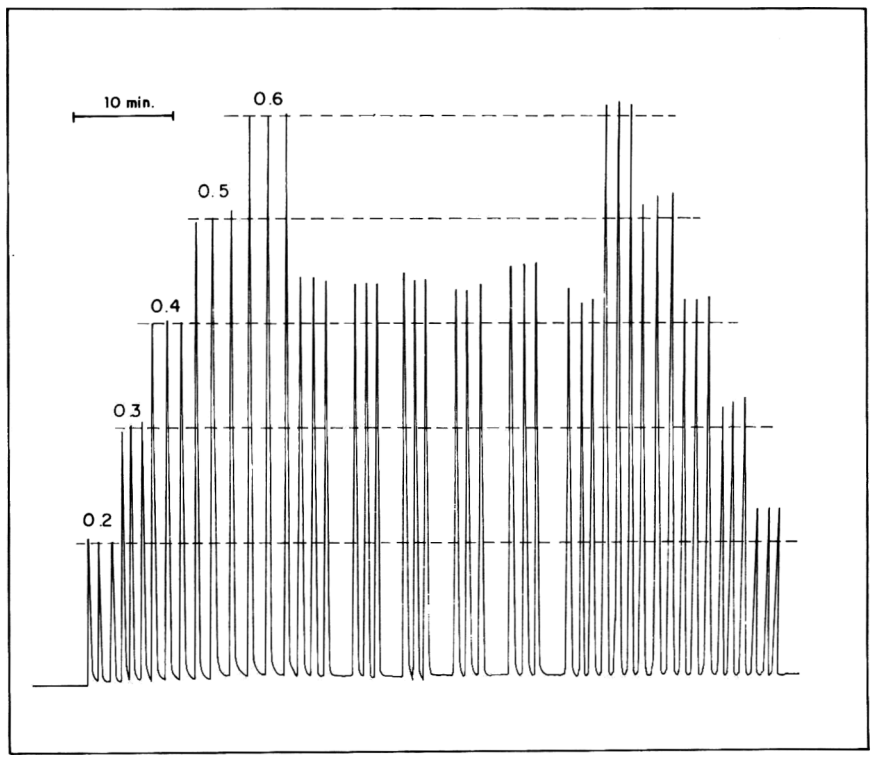

Figure 5. Runs for volatile acidity determination using faulty loop in the colorimetric system (conductometric method, with faulty loop, presents similar behavior). Left to right: triplicate signals for acetic acid standards (0.2, 0.3, 0.4, 0.5, and 0.6% g acetic acid/100 mL solution). Triplicate signals for 6 vinegars; triplicate signals for standards in reverse order. Polyethylene loop (27.5 μL; 0.9 mm, id).

methods. Although the instrumentation for the traditional methods is less expensive than that for FIA, the additional cost is not great and can easily be justified by the faster sampling rate.

In the present study, analyses were carried out in an apparatus with a steam-boiler, which permitted a sampling rate of 4 samples/h. However, the apparatus that enabled standard deviations similar to those obtained with FIA methods is much more expensive than the FIA systems proposed.

A comparison between the 2 FIA methods proposed shows no significant difference in the precision of the results. However, the colorimetric method is simpler because it needs no water bath and there is no confluence of strong acid (sulfuric acid). On the other hand, the conductometric method permits a more rapid sampling rate (about 70/h) than the colorimetric (about 40–45/h).

Acknowledgments

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REFERENCES

(1) Stewart, K.K. (1981) *Talanta* **28**, 789–797
(2) Ruzicka, J., & Hansen, E.H. (1988) *Chemical Analysis*, Vol. 62, J.D. Winefordner (Ed.), John Wiley & Sons, New York
(3) Baadenhuijsen, H., & Seuren-Jacobs, H.E.H. (1979) *Clin. Chem* **25**, 443
(4) Van der Linden, W.E. (1983) *Anal. Chim. Acta* **151**, 359–369
(5) Ebner, H. (1983) in *Biotechnology*, Vol 5, H.J. Rehm & G. Reed (Eds.) Verlag Chemie, Weiheim
(6) Nunheimer, T.D., & Fabian, F.W. (1940) *Am. J. Public. Health* **30**, 1040–1049
(7) Szakall, A. (1952) *Z. Lebensm. Unters. Forsch.* **95**, 100–107
(8) Pasquini, C., & Faria, L.C. (1987) *Anal. Chim. Acta* **193**, 19–27
(9) Ribereau-Gayon, J., Peynaud, E., Sudraud, P., & Ribereau-Gayon, P. (1976) *Sciences et Techniques du Vin*, Vol. 1, Dudot, Paris, p. 89
(10) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec **930.35L**
(11) Eckschlager, K. (1972) *Errors, Measurement and Results in Chemical Analysis*, Van Nostrand Reinhold Company, New York, NY, pp. 107–112

Determination of Total Dietary Fiber in Japanese Foods

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Total dietary fiber was determined in Japanese foods by the Prosky-AOAC method. To accomplish the analyses of unsuitable samples, we introduced a few minor modifications to the versions for (I) seaweed and fruits, (II) cereals, and (III) fish and meats. These modified methods were used together with the standard method to obtain results with reasonably good relative standard deviation for 231 foods and 21 groups of mixed foods. In this study, dietary fiber was defined so as not to exclude the nondigestible polysaccharide portions of animal foods. A method was proposed which could estimate more accurately the fiber components of animal foods by measuring the "nondigestible protein" of the fiber sample by the Biuret colorimetric method, instead of the Kjeldahl method, to avoid deducting the values for aminopolysaccharides. In Japanese diets, the amount of fiber obtained from animal foods was less than 5% of the total intake of dietary fiber.

The method of determination of total dietary fiber (TDF) by the enzymatic-gravimetric method of Prosky et al. has been adopted officially by AOAC (1, 2). When applied to the analysis of a number of Japanese foods, this method proved to be inadequate, mainly because rice and related cereals were consumed in large amounts as an important source of dietary fiber. Thus, more accurate measurements were desired for the correct estimation of the amount of intake of dietary fiber for Japanese.

On the other hand, the definition by H. C. Trowell in 1985, namely, that dietary fiber contains "the sum of the polysaccharides and lignin which are resistant to the digestion," is generally interpreted to mean dietary fiber of plant origin (3), and to exclude fibrous components of animal origin. During our analyses, we became aware of the fact that Japanese foods include many kinds of fish and shellfish including shrimp, lobsters, crabs, and squid, together with other seafood such as fish paste products and algae. Some of them are considered a good source of chitin. This situation is reflected in the proposed definition by Japanese authors (4, 5) of dietary fiber: "The whole of nondigestible components in the food which is resistant against human digestive enzymes." In

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this report, we showed the results of our study of the determination of the amount of aminopolysaccharides (e.g., chitin, chitoacids, and chondroitin sulfates) in Japanese seafoods which had been deducted from the amount of TDF as a part of nondigestible protein.

Recently, the concept was proposed that water-soluble and insoluble dietary fiber should be determined separately, so that those data can be evaluated in terms of their physiological significance (6). Reported results in pioneering attempts of this kind indicate clearly that an improvement in the accuracy and precision of the assay method for total dietary fiber will provide an impetus for the determination of soluble and insoluble dietary fiber. Some inconsistency (e.g., IDF > TDF) has been reported in part of the data. Our results in this study indicated that a few modifications for different sample groups in the standard method for the determination of TDF would increase the ruggedness of the methods. We therefore introduced a few minor modifications into the Prosky method (1, 2) and used them to considerably reduce the relative standard deviations (RSDs) and to improve the applicability of the standard method to a much wider variety of Japanese foods.

Collaborative Study

The collaborators and 55 laboratories that participated in this study were all members of Japan Association of Prefectural and Municipal Public Health Institutes (N. Kunita, president).

Each sample was analyzed in at least 3 laboratories and more than 2 results were reported by each laboratory. Each laboratory received up to 24 dried, defatted, and powdered samples of known materials. The purchase, defatting, and powdering of sample foods were carried out at 8 key laboratories (of the senior author, M. Doguchi and 6 collaborators) located throughout Japan.

Sample Preparation

Only the edible portion of each sample was analyzed; the percentage of discarded portion followed precisely the standard nutrition table of Japanese foods (7). The wet samples were first freeze-dried with a Labconco VAC-STOP tray dryer, or equivalent, and cut or crushed roughly to large pieces; then, unless clearly indicated otherwise, petroleum ether (25 mL/g) was added and mixed well, the mixture was kept at room temperature for 30 min. The upper layer was decanted off to minimize the loss of any floating insoluble components, and the extraction was repeated 3 more times. After the last extraction, the solvent was filtered off through a glass filter and the residue was air-dried. After the weight loss was measured, the sample was ground to ≤0.5 mm by a Wiley-type grinding mill and filtered through a standard 0.5 mm sieve (Japanese industrial standard). The ground sample was stored in an appropriate container which could be sealed so as to be semi-airtight and delivered to the laboratory where the analysis was carried out. When there was some difficulty in dispersing the sample to uniform wetness in the 0.05 M sodium phosphate buffer (pH 6.0), or when the sample showed a tendency to stick to the glass wall rather than to disperse into the buffer, we found it absolutely necessary to carry out a second extraction of the residual fat. After the sample was weighed accurately, this second extraction was performed in the same way as the first extraction (repeated 3–4 times). We compared a number of samples and confirmed that the value of TDF obtained was substantially larger if we omitted the second extraction.

Every sample analyzed was an individual food obtained at a retail store. Neither locational or seasonal differences nor variations between plant strains were explored. Those foods consumed in smaller average amounts per capita per day in the latest national nutrition survey (2–11 items) were combined within each group of similar foods according to the relative amounts consumed, and mixed thoroughly after freeze-drying and powdering. The numbers of foods mixed were chosen to cover major amounts of consumption in each food group when taken together with other items in Table 1. The TDF for those mixed samples are listed at the end of Table 1.

Analytical Methods

We determined only one TDF value with every duplicate run of the enzyme reaction, obtaining both the amounts of nondigestible protein (NDP) and ash for each TDF assay, so that we could observe the role of variation of NDP measurement in the RSDs of TDF values. The modified versions of the AOAC official method are described in Results and Discussion.

Biuret colorimetry for NDP was carried out by using the method of Gornall et al. (9). Bovine serum albumin was used as the standard protein at 550 nm absorption. For the solubilization of BSA and samples, 10% NaOH was used. In some samples (e.g., raw egg), the colorimetry had to be carried out immediately after the solubilization; otherwise the value of NDP decreased gradually.

Calculations

Statistical outliers for all the data of each food sample were rejected first by the Grubb equation (10) with 5% probability of error. Next, the intralaboratory average values were calculated for every laboratory for every food sample. Again, the candidates for outlier within each food were listed for the intralaboratory averages with 5% probability of error. The listed candidates for outlier were examined as to whether they were true or apparent outliers. If the RSD for all the initial data was below 20% for the food, the outlier candidate for the intralaboratory average was deemed to be within the ordinary variation; e.g., in the case of "Okara," (i.e., soybean components separated from the protein fraction called "Tofu"), the intralaboratory averages of TDF for the freeze-dried sample were 55.7, 58.5, and 55.7. The candidate for outlier was 58.5. The overall RSD was 3.2% for the measured data of 56.2 and 55.2 in Lab. A; 58.6, 58.4, and 58.5 in Lab. B; and 57.3, 53.4, and 56.5 in Lab. C. The candidate was deemed to be within the ordinary variation, and it was concluded that the other 2 values coincided by chance.

Reagents

The enzymes used are specified in Table 2. The adverse side reactions of the enzymes used were monitored by using the indicated substrates (β-glucan and citrus pectin, Sigma Chemical Co.) or cellulose (Asahi Kasei Co.) and running them through the standard (Method I) or modified (Method II, III) procedures together with the original AOAC method. The results showed that Method III gave the best recoveries of soluble dietary fiber like β-glucan or pectin (data not shown).

Table 1. Results of determination of total dietary fiber in Japanese food[a]

No.	Food	Systematic No. of Japanese standard nutrition table	Total dietary fiber (wet%)	RSD (%)	Total dietary fiber (dry%)	Assay method	No. of assay	No. of lab.
1	Oatmeal	1-4	7.46	27.1	8.25	3	24	8
2	Barley milled & pressed		5.26	10.3	5.93	3	7	3
3	Barley, milled & cut	1-6-c	5.25	36.7	5.83	13	12	4
4	Soft flour	1-10-a	2.12	10.9	2.40	3	16	6
5	White bread	1-13-a	2.55	24.0	4.41	1	48	16
6	Bread-type rolls	1-14-a	1.99	8.0	3.11	3	6	3
7	Rye bread	1-17	5.21	12.7	8.38	3	8	3
8	Fiber bread		3.96	14.5	6.01	3	6	3
9	Raisin bread	1-18	3.35	25.8	5.02	3	8	3
10	Soft rolls	1-19	1.83	5.2	2.64	3	4	3
11	Udon noodle, raw, wet	1-21-a	1.45	15.2	2.29	3	15	6
12	Udon noodle, boiled	1-21-b	0.99	7.1	3.63	3	8	3
13	Udon noodle, raw, dry	1-23-a	2.09	11.4	2.38	1	29	10
14	Somen-Hiyamugi noodle, raw, dry	1-24-a	2.08	29.5	2.37	1	9	3
15	Chinese LaoMien noodle raw, wet	1-26-a	1.48	14.4	2.22	3	12	6
16	Chinese LaoMien noodle boiled	1-26-b	1.08	12.6	2.92	3	15	6
17	Chinese LaoMien noodle steamed	1-27	0.97	14.6	2.71	3	15	6
18	Chinese cooked noodle flying-dried	1-31-a	2.43	25.9	2.87	1	11	4
19	Chinese cooked noodle hot air-dried	1-31-c	2.08	15.6	2.39	1	42	14
20	Macaroni & spaghetti, dry	1-34-a	2.72	22.2	3.04	1	53	18
21	Wheat germ	1-38	11.12	8.0	11.47	13	12	5
22	Bread crumbs	1-40	3.36	6.7	3.67	3	15	6
23	Brown rice grain	1-41-a	2.92	5.3	3.41	3	7	3
24	Half-milled rice, yield 95–96%	1-41-b	2.27	35.3	2.60	3	6	3
25	Under-milled rice, yield 93–94%	1-41-c	1.73	18.3	1.85	3	7	3
26	Well-milled rice	1-41-d	0.72	48.2	0.81	1	64	22
27	Mochi glutinous rice cake	1-47	0.33	15.3	0.59	13	5	3
28	Sekihan glutinous rice & azuki bean	1-48	1.56	68.8	3.01	3	6	3
29	Rice bran	1-57	22.20	2.3	31.56	1	6	3
30	Soba buckwheat noodle, boiled	1-61-b	1.62	3.0	5.12	1	7	3
31	Soba buckwheat noodle, raw, dry	1-62-a	4.74	19.6	5.28	1	45	15
32	Popcorn, popped	1-67	9.71	13.5	10.05	123	12	5
33	Cornflakes	1-68	2.89	8.5	2.96	13	10	3
34	Devil's tongue, block type	2-3	1.67	4.6	86.13	2	24	8
35	Devil's tongue, noodle type	2-4	3.62	2.6	94.57	2	6	3
36	Sweet potatoes, raw tuber	2-5-a	2.32	14.9	7.23	1	54	18
37	Satoimo dasheen, raw	2-8-a	2.20	7.3	14.11	1	9	3
38	Potatoes, raw tuber	2-11-a	1.35	10.0	6.15	1	56	19
39	Potato chips, fried	2-13	3.38	6.4	5.40	1	8	3
40	Corn starch	2-14-h	0.30	111.5	0.34	3	8	3
41	Yam tuber, Ichoimo, raw	2-16	1.43	10.3	4.71	1	15	7
42	Nagaimo Chinese Yam, raw	2-18-a	0.87	3.3	5.32	1	8	3
43	Brown sugar lump	3-1	0.17	127.4	0.17	1	10	6
44	Honey	3-16	<0.05	—	<0.06	—	3	1
45	Milk chocolate	4-77-b	4.02	13.5	4.05	1	16	6
46	Cashew nuts, roasted	6-4	3.98	20.2	8.53	1	12	4
47	Ginkgo, nuts, raw	6-7-a	0.62	21.2	1.52	1	9	5
48	Chestnuts, raw	6-8-a	3.71	9.7	9.90	1	7	3
49	Chestnuts, roasted	6-8-d	7.02	2.4	12.47	3	9	3
50	Sesame seeds, dried	6-12-a	15.37	13.5	24.60	1	13	5
51	Sesame seeds, roasted	6-12-b	11.58	6.7	25.98	1	10	4
52	Peanuts, dried	6-25-a	7.66	10.1	8.43	1	7	3
53	Peanuts, roasted	6-25-b	8.68	17.9	14.56	1	12	5
54	Peanut butter	6-25-d	5.88	13.5	10.47	1	11	4
55	Azuki beans	7-1-a	15.97	4.4	18.52	1	9	3
56	Kidney beans, whole, dry	7-4-a	19.76	1.4	23.18	1	7	3
57	Uzuramame kidney beans, cooked	7-5	6.86	2.4	13.99	1	8	3
58	Garden peas, whole, boiled & frozen	12-10-b	5.21	4.7	21.93	13	7	3
59	Broad beans, whole, dry	7-11	19.53	3.6	22.08	1	8	3
60	Otafukumame broad beans, cooked	7-13	5.63	3.7	15.22	1	7	3
61	Soybeans, dry	7-15-a	15.03	7.7	19.40	1	6	3

Table 1. (Continued)

No.	Food	Systematic No. of Japanese standard nutrition table	Total dietary fiber (wet%)	RSD (%)	Total dietary fiber (dry%)	Assay method	No. of assay	No. of lab.
62	Soybeans, boiled	7-15-b	7.11	20.0	21.76	1	12	5
63	Defatted soy beans, whole	7-18-a	15.96	6.8	17.51	1	149	51
64	Soybeans, roasted & ground	7-19	17.14	3.8	17.45	1	8	3
65	Soybean curd (Tofu), Momen medium	7-21-a	0.62	44.1	6.45	1	11	3
66	Soybean curd (Tofu), Kinukosi fine	7-21-b	0.35	19.1	3.10	1	9	3
67	Soybean curd baked (Yaki-tofu)	7-23	0.91	43.7	8.60	1	6	3
68	Soybean curd product steamed (Namaage)	7-24	1.71	96.2	3.58	1	6	3
69	Soybean curd product fried (Aburaage)	7-25	1.60	10.7	5.19	1	7	4
70	Ganmodoki	7-26	2.37	37.8	8.99	1	7	3
71	Soybean curd dried (Kori-dofu)	7-27	7.35	69.1	11.41	1	6	3
72	Itohiki-natto	7-29	9.60	27.1	24.84	1	6	3
73	Miso, sweet type	7-32-a	4.28	21.6	8.30	1	6	3
74	Miso, dark yellow type	7-32-c	6.42	7.7	11.58	1	9	3
75	Okara	7-38	9.42	3.2	58.24	1	8	3
76	Soy milk, reconstituted	7-39-b	0.26	25.1	2.64	1	9	3
77	Horse mackerel, raw, uncooked	8-4-a	1.34	74.3	5.15	4	15	7
78	Salmon, raw	8-77	0.30	173.0	1.16	4	24	8
79	Mackerel, raw, uncooked	8-84-a	0.41	56.1	1.94	4	20	6
80	Pacific saury, raw, uncooked	8-95-a	0.52	57.4	2.53	4	8	3
81	Tunas, bluefin, lean meat, raw	8-150-a	0.31	95.0	1.38	4	6	3
82	Oysters, raw, uncooked	8-179-a	0.26	41.6	2.44	14	7	3
83	Squid, raw, uncooked	8-206-a	0.57	19.6	2.69	4	8	3
84	Shrimp, boiled & dried, whole	8-222-a	3.89	28.9	4.65	4	10	3
85	Shrimp, boiled & dried, w/o shell	8-222-b	2.05	42.3	2.56	4	11	5
86	Fish paste, Kamaboko, steamed	8-246	0.56	29.5	2.33	4	8	3
87	Fish paste, Kamaboko, broiled	8-249	0.19	34.2	0.98	4	10	6
88	Fish paste, Chikuwa, broiled	8-250	0.46	36.2	1.60	4	7	3
89	Fish paste, Satsuma-age, fried	8-256	0.26	15.0	0.90	4	10	3
90	Beef chuck loin, total edible	9-6-d	0.58	65.7	3.05	4	6	3
91	Chicken, thigh, broiler	9-49-b	0.12	47.8	0.70	4	21	6
92	Chicken, thigh, broiler, flesh only	9-50-b	0.21	110.4	1.21	4	11	3
93	Swine, inside ham, separable lean	9-72	0.14	156.1	0.71	4	8	3
94	Ham, mixed press	9-86-f	0.36	74.3	1.21	4	8	3
95	Sausage, mixed	9-87-h	1.01	65.1	4.19	4	8	3
96	Chicken, whole egg, fresh	10-5-a	0.13	15.0	0.84	4	10	3
97	Ordinary liquid milk	11-2	0.22	25.6	2.37	4	10	3
98	Milk beverage, coffee flavored	11-5-a	0.07	88.9	0.62	1	12	6
99	Yogurt, whole milk, unsweetened	11-9-a	0.10	82.9	0.98	4	8	3
100	Lactic acid bacteria beverage	11-10-b	0.00	—	0.00	4	6	3
101	Skim milk powder, domestic	11-17-a	0.85	19.0	0.89	4	12	7
102	Process cheese	11-23	0.58	46.6	1.90	4	6	3
103	Asparagus, green, raw	12-4-a	1.68	3.6	23.00	1	24	8
104	Asparagus, green, boiled, canned	12-5	1.26	3.8	18.61	1	5	3
105	Kidney beans, pods, immature, raw	12-6-a	2.36	7.6	36.39	1	6	3
106	Green soybeans, immature, raw	12-8-a	5.44	13.3	16.63	1	25	8
107	Garden peas pods, immature, raw	12-9-a	2.04	3.4	20.14	1	23	8
108	Green peas, canned	12-11	7.74	4.6	21.22	1	8	3
109	Osaka-shirona, leaves, raw	12-12-a	1.51	4.4	28.38	1	9	3
110	Okra pods, immature, raw	12-14-a	4.59	4.1	38.54	1	8	3
111	Turnip root, raw	12-16-a	1.30	3.2	27.17	1	6	3
112	Turnip root, salted	12-16-c	1.77	1.2	20.59	1	6	3
113	Pumpkin, raw	12-17-a	2.99	6.3	11.04	1	9	3
114	Cauliflower, raw	12-20-a	1.71	4.5	28.74	1	7	3
115	White ground shavings, dried	12-21-a	25.84	1.5	38.78	1	10	4
116	Chrysanthemum, edible flowers, raw	12-22-a	2.99	1.7	28.69	1	8	3
117	Cabbage, raw head	12-24-a	1.42	6.4	25.11	1	12	12
118	Cucumber, raw fruit, whole	12-25-a	0.85	4.3	21.64	1	9	3
119	Nukazuke cucumber pickles, fruit	12-25-c	1.41	13.3	10.89	1	9	3
120	Pot herb mustard, raw leaves	12-27-a	2.01	2.0	36.96	1	7	3
121	Edible burdock, boiled root	12-31-b	3.58	3.0	50.98	13	11	4

Table 1. (Continued)

No.	Food	Systematic No. of Japanese standard nutrition table	Total dietary fiber (wet%)	RSD (%)	Total dietary fiber (dry%)	Assay method	No. of assay	No. of lab.
122	Komatsuna, leaves, raw	12-32-a	1.73	16.0	28.42	1	6	3
123	Garland chrysanthemum, leaves, raw	12-39-a	1.39	5.0	32.55	12	7	3
124	Ginger tuber, raw	12-41-a	1.83	3.7	32.38	13	8	3
125	Sugukina, pickles	12-48	3.78	4.0	26.35	13	9	3
126	Water dropwort, raw leaves	12-49-a	2.18	4.1	30.62	1	24	3
127	Celery, stalk	12-50	1.93	6.4	26.08	12	7	8
128	Royal fern, fresh & boiled	12-51-b	3.45	6.6	53.54	1	7	3
129	Japanese radish immature greens	12-54	1.18	8.0	21.58	1	7	3
130	Japanese radish greens	12-55-a	2.74	7.0	28.83	1	8	3
131	Japanese radish	12-56-a	1.34	12.2	26.29	1	36	12
132	Japanese radish, cut & dried	12-57	17.89	1.8	23.04	1	9	3
133	Japanese radish, pickles	12-58	3.79	2.6	27.92	1	4	3
134	Broad leaved mustard, leaves	12-65	2.42	1.9	29.42	1	7	3
135	Bamboo shoots, boiled	12-67-b	2.27	5.7	37.56	1	7	3
136	Onion, raw	12-70-a	1.50	3.3	15.26	1	9	3
137	Head lettuce, butter head type	12-72	1.14	7.5	23.98	1	9	3
138	Head lettuce, crisp head type	12-73	0.96	2.8	24.92	1	9	3
139	Chingen tsuai, leaves, raw	12-74-a	1.01	4.1	24.44	1	7	3
140	Wax gourd, fruit raw	12-81-a	0.84	5.2	28.18	1	7	3
141	Sweet corn, boiled	12-83-b	2.01	7.1	7.42	13	8	3
142	Tomato, raw	12-85	0.79	13.0	17.53	1	35	12
143	Tomato, juice, canned	12-86-b	0.52	9.3	9.83	1	6	3
144	Egg plant, raw	12-87-a	1.66	4.9	27.88	12	8	3
145	Rape, flower cluster, raw	12-91-a	2.67	1.6	28.04	1	4	2
146	Chinese chive, leaves raw	12-93-a	1.92	4.7	30.39	1	7	3
147	Carrot, raw root	12-94-a	2.55	10.0	25.11	1	24	8
148	Welshonion, leaf & leaf sheath	12-96	1.89	1.9	27.28	1	8	3
149	Nozawana, pickles, seasoned	12-99	1.69	0.4	23.71	1	6	3
150	Chinese cabbage, head, raw	12-101-a	1.09	1.9	23.98	1	9	3
151	Chinese cabbage, head, salted	12-101-b	2.03	3.7	23.93	1	9	3
152	East Indian lotus, root raw	12-103-a	1.11	7.2	10.62	1	7	3
153	Parsley	12-104	3.14	3.5	31.61	1	24	8
154	Sweet pepper, raw fruit	12-108-a	1.97	1.4	35.35	1	9	3
155	Hirosimana, leaves, raw	12-110-a	1.34	5.9	28.42	1	11	4
156	Japanese butterbur petiole, raw	12-111-a	0.98	3.1	27.70	1	6	3
157	Japanese butterbur petiole, boiled	12-111-b	1.41	4.1	85.42	13	9	3
158	Swiss chard leaves, raw	12-113-a	2.88	5.2	39.36	1	6	3
159	Broccoli, head, raw	12-114-a	2.68	2.4	28.00	1	6	3
160	Spinach, raw leaves	12-117-a	2.50	5.4	28.31	1	9	3
161	Mytsuba, leafstalk & leaves, green	12-123-a	2.26	2.3	31.81	1	9	3
162	Myoga, bract & flower	12-124	1.41	6.7	44.49	13	7	3
163	Brussels sprouts, head, raw	12-125-a	4.52	4.0	34.74	1	6	3
164	Soybean sprouts, raw	12-126-a	1.56	10.0	23.83	1	8	3
165	Mungbean sprouts	12-127-a	1.20	5.2	21.01	1	8	3
166	Blackgram sprouts	12-128-a	1.39	15.3	22.27	1	6	3
167	Lily scale, raw	12-131-a	6.97	2.8	21.46	1	6	3
168	Scallion, sweetened pickles	12-136	8.91	9.5	41.39	1	6	3
169	Shallot	12-139-a	2.17	9.5	30.96	1	10	4
170	Bracken, fresh, boiled	12-142-b	3.95	24.2	62.93	1	6	3
171	Apricot, dried	13-3	8.29	2.9	11.34	1	7	3
172	Strawberries, raw fruit	13-6	1.52	6.7	18.18	1	7	3
173	Strawberry jam	13-7	0.76	21.5	1.14	1	17	6
174	Figs, raw fruit	13-8	1.46	5.0	11.57	1	8	3
175	Satsuma mandarins, common	13-17-b	1.05	5.1	10.16	1	9	3
176	Satsuma mandarin fruit juice	13-19-a	0.27	108.4	3.47	2	6	3
177	Japanese persimmons, raw fruit	13-26-a	1.60	6.0	10.02	1	8	3
178	Japanese persimmons, dried fruit	13-27	10.80	4.1	20.25	1	8	3
179	Kiwi fruit, raw fruit	13-31	2.65	12.3	16.19	2	8	3
180	Grapefruit, raw w/o membrane	13-37	0.73	15.2	7.83	2	24	8
181	Watermelon, raw fruit	13-45	0.22	13.6	2.14	1	7	3
182	Japanese plum	13-47	0.77	7.2	8.21	1	7	3

Table 1. (Continued)

No.	Food	Systematic No. of Japanese standard nutrition table	Total dietary fiber (wet%)	RSD (%)	Total dietary fiber (dry%)	Assay method	No. of assay	No. of lab.
183	Japanese pears	13-52-a	1.07	9.7	9.25	12	9	3
184	European pears w/o skin	13-52-c	1.74	2.7	12.92	1	6	3
185	Pineapple, raw fruit	13-58	0.92	14.3	6.45	12	9	3
186	Pineapple, canned	13-60	1.00	2.4	5.66	12	8	3
187	Bananas, raw fruit	13-64	1.48	5.2	7.02	1	9	3
188	Bananas, dried	13-65	6.47	6.5	25.96	1	9	3
189	Grapes,raw fruit	13-70	0.39	4.2	2.06	1	7	3
190	Raisins	13-71	4.60	6.2	20.27	12	11	4
191	Hybrid melon (Arms), raw fruit	13-80-a	0.41	10.7	4.05	13	7	3
192	Muskmelon, raw fruit	13-80-a	0.96	6.0	9.07	1	5	3
193	Peaches, canned with syrup	13-83-a	1.47	3.2	7.81	13	8	3
194	Apples, raw fruit w/o skin	13-88	1.63	6.3	11.90	1	22	10
195	Apple juice, single strength	13-89-a	0.08	21.4	0.63	1	7	3
196	Apple drink, 30% apple juice	13-89-e	0.01	100.1	0.09	1	8	3
197	Enokitake fungi, raw	14-1-a	2.87	7.4	26.87	1	9	3
198	Jew's-ear, brack, dried	14-3-a	74.18	1.6	86.32	1	10	4
199	Shiitake fungi, uncooked raw	14-6-a	4.54	3.6	43.20	1	10	10
200	Shiitake, uncooked dry	14-7-a	43.41	2.1	47.43	1	9	3
201	Honshimeji fungi, raw	14-9	2.30	5.6	28.02	1	24	8
202	Shimeji fungi, raw	14-9	3.09	3.8	30.62	1	6	3
203	Nameko fungi, raw, uncooked	14-11-a	1.80	4.7	34.26	1	9	3
204	Common mushroom, raw, uncooked	14-18-a	1.55	16.0	16.78	1	6	3
205	Common mushroom, boiled	14-18-b	2.23	2.6	28.33	1	10	3
206	Green laver, dried	15-2	38.62	6.5	44.09	12	10	4
207	Purple laver, dried	15-3	29.68	4.1	31.07	12	28	10
208	Makonbu kelp, dried	15-15	28.58	5.2	31.38	12	47	16
209	Konbu kelp, salted, dry	15-20	14.61	3.7	19.64	2	9	3
210	Agar-agar, dry	15-26	81.29	1.7	97.31	12	9	3
211	Hijiki algae, boiled & dried	15-28	54.94	2.8	62.53	1	44	15
212	Mozuku algae, raw, desalted	15-33-b	0.65	19.1	62.29	1	7	3
213	Wakame algae, raw	15-34	9.90	31.4	24.65	2	8	3
214	Wakame, dried	15-35-a	37.95	9.8	43.73	2	57	19
215	Coffee, instant	16-30-c	14.18	60.1	14.80	1	7	3
216	Coffee drink, canned	16-31	0.08	74.1	0.93	13	14	6
217	Consomme, dried	17-1	0.61	21.8	0.71	13	10	6
218	Soy-sauce, Koikuti thicker type	17-3-a	0.81	40.1	2.73	1	13	6
219	Worcester sauces, common	17-5-a	0.39	48.1	1.10	1	14	6
220	Worcester sauces, thick type	17-5-c	1.36	15.3	4.01	1	9	3
221	Tomato ketchup	17-6-a	1.01	51.4	19.72	1	6	3
222	Mayonnaise, whole egg type	17-10-a	0.07	82.6	1.32	1	13	6
223	Curry roux	17-16-b	4.34	16.9	6.02	3	14	6
224	Dip sauce for staked beef	17	1.16	12.4	3.25	1	15	6
225	Sprinkling mix for rice with tea	17	2.03	15.0	2.07	1	7	3
226	Miso soup, precooked & dried	18	6.19	3.2	6.68	1	6	6
227	Gyo-za, frozen	18-3	2.10	8.3	8.46	1	16	3
228	Coroquettes, frozen, potato type	18-6-b	1.37	6.8	4.17	1	15	6
229	Shumai, frozen	18-8	1.33	34.7	3.22	14	7	3
230	Hamburg, frozen	18-9	1.22	4.4	3.36	4	6	3
231	Meat balls, frozen	18-13	1.59	19.9	5.59	4	21	6
232	Processed rice products, mixed	1	0.34	30.1	0.43	3	11	3
233	1:1 mixture of hard & medium flour	1	2.44	6.0	2.76	3	15	6
234	Starch noodle, potato starch, mixed	2	1.11	17.1	1.28	1	6	3
235	Bun with minced beef, bean, jam, etc.	4	1.16	17.5	3.45	3	16	6
236	Potatc tips & other snacks, mixed	4	3.44	9.6	5.00	3	7	3
237	Cookies, mixed	4	1.60	9.6	2.00	3	6	3
238	Japanese crackers, mixed	4	1.37	18.3	1.43	13	6	3
239	Sponge-cake & other cakes, mixed	4	0.80	19.2	1.39	13	12	5
240	Japanese cakes & sweet pastes, mixed	4	3.47	35.6	5.41	3	14	6
241	Buns & desert rolls, mixed	4	2.20	16.3	3.65	3	6	3
242	Cooked soy beans, Yuba, mixed	7	5.65	6.1	10.45	1	7	3
243	Cooked green peas, Adzuki beans etc.	7	4.25	17.7	5.96	1	6	3

Table 1. (Continued)

No.	Food	Systematic No. of Japanese standard nutrition table	Total dietary fiber (wet%)	RSD (%)	Total dietary fiber (dry%)	Assay method	No. of assay	No. of lab.
244	Salted or boiled & dried fishes	8	1.27	36.3	2.88	4	23	6
245	Fishes boiled down in soy sauce	8	1.44	15.2	2.94	4	23	6
246	Garlic bulb, sweet pepper, etc., mixed	12	8.64	10.8	34.36	1	6	3
247	Komatsuna, turnip leaves, Perilla	12	1.78	2.7	27.78	1	9	3
248	Pickled vegetables, mixed	12	3.32	11.9	21.89	12	12	5
249	Orange marmalade & apricot jam	13	1.38	7.0	42.54	2	12	6
250	Hiratake & other fungi, mixed	14	3.22	3.5	30.89	1	7	3
251	Shaved dry kelp & other algae, mixed	15	18.34	3.0	26.83	2	8	4
252	Corn soup powder, hash roux, etc.	17	4.98	6.0	5.21	4	8	3

[a] The first numeral of each systematic number (5) indicates the following food group. 1: cereals, 2: potatoes and starches, 3: sugars and sweeteners, 4: confectioneries, 6: nuts and seeds, 7: pulses, 8: fishes and shellfishes, 9: meats, 10: eggs, 11: milks, 12: vegetables, 13: fruits, 14: fungi, 15: algae, 16: beverages, 17: seasonings and spices, 18: prepared foods.

The number in "assay method" corresponds to the method in Table 2. Two or 3 numerals in "assay method" indicate that more than one method was used for the analyses. "Number of assay" and "number of laboratories" were counted after the statistical rejection of outliers. The RSDs for intralaboratory variation were always smaller than the RSD shown.

Table 2. Reagents used and their conditions in modified versions of official AOAC method

	Method I	Method II	Method III	Method IV[a]
Scope of application	Other samples than those indicated in Methods II–IV	Seaweed that tends to be solid gel. Fruits that contain much organic acids	Cereals that contain low TDF	Animal foods with low TDF and high protein
Samples, g	1	0.5	3	3
Termamyl (Novo 120L)	0.1 mL (120K Novo U/mL) 90°C × 15 min	0.1 mL 90°C × 15 min	0.3 mL 90°C × 1 h	0.3 mL 90°C × 1 h
Protease (Sigma P5380)	5 mg (7–15 U/mg) 60°C × 1 h	5 mg 60°C × 1 h	15 mg 60°C × 1.5 h	15 mg 60°C × 16 h
Pepsin (Merck 7185)	—	—	—	15 mg (1 m Anson U/mg) pH 1.5, 40°C × 16 h
Amyloglucosidase (Boehringer 208469 or Sigma A9913)	25 mg (6 U/mg) or 0.3 mL (1.2–3K U/mL) 60°C × 0.5 h	25 mg or 0.3 mL 60°C × 0.5 h	25 mg or 0.3 mL 60°C × 1 h	25 mg or 0.3 mL 60°C × 0.5 h
Special note	—	—	Secondary defatting for all samples	Secondary defatting for all samples

[a] Method IV-2: The same conditions were used as in Method IV except nondigestible protein was measured by the Biuret method.

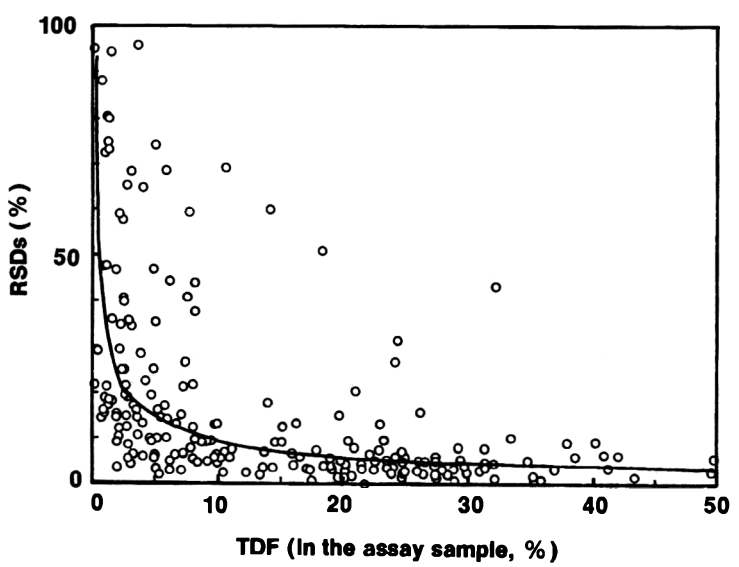

Figure 1. Relative standard deviation of total dietary fiber value in samples containing different levels of dietary fiber. The average value for 247 samples was 15.7% (analysis sample) TDF; equation for regression curve, $Y = 36.4X^{-0.594}$.

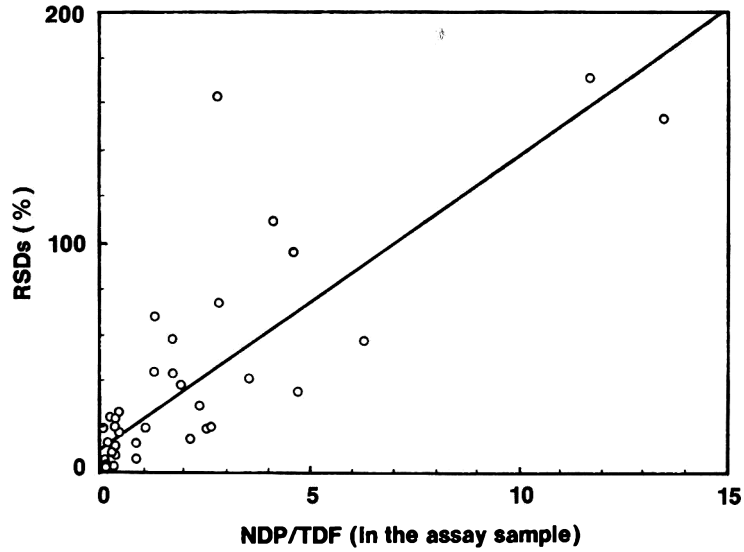

Figure 2. Relative standard deviation of total dietary fiber value in samples containing different levels of nondigestible protein. The correlation coefficient was 0.832 for 50 samples; equation for regression line, Y = 9.87 + 12.79X. Samples which contained >5% NDP were analyzed. To make clear the favorable effect of pepsin treatment, this figure contains the data rejected for Table 1, and also the data obtained without pepsin treatment.

Results and Discussion

The minor modifications introduced into the Prosky method are summarized in Table 2. All procedures other than those described in Table 2 were carried out according to the published versions (1), except for the method of calculation and the method of ethanol precipitation. That is to say, only one TDF value was determined from every duplicate run of enzyme reactions, and overnight standing after the addition of ethanol was recommended.

Method II was introduced because of the need to reduce the viscosity of seaweed samples in the acid conditions of the amyloglucosidase reaction and to reduce acidification of the starting buffer (pH 6.0) by organic acids contained in the citrus samples and other fruits. Method III was introduced to increase the accuracy of data by increasing the absolute amount of dietary fiber precipitate weighed at the last stage of enzyme reactions. The rationale of the increased sample in this method is shown in Figure 1. The lower applicable limit of the enzymatic-gravimetric method seems to be about 2% TDF concentration in the analytical sample (see Guidelines for Interlaboratory Collaborative Study Procedure (8)).

Table 3. Effect of pepsin treatment on dietary fiber value[a]

Food	Dietary fiber, av. ± SD, % (N) Method IV w/o pepsin	Method IV	Difference (%) (% vs Method IV)
Mackerel	0.37 ± 0.24 (10)	0.44 ± 0.25 (10)	−0.07 (−16.1%)
t value	0.599		
Av. NDP	16.37	9.73	
Squid	1.35 ± 0.90 (7)	1.17 ± 0.50 (7)	0.18 (15.7%)
t value	0.471		
Av. NDP	6.30	4.09	
Chikuwa	0.76 ± 0.30 (9)	0.67 ± 0.30 (9)	0.09 (12.6%)
t value	0.600		
Av. NDP	3.58	1.39	

[a] No statistically significant difference was observed by the addition of pepsin treatment.

Method IV was introduced to overcome both a low content of dietary fiber as in Method III and a high amount of NDP. The logic of this modification is based on the fact that the relation between the RSDs of the TDF values and the NDP per TDF value (see Figure 2) in samples of relatively high NDP content (>5%) clearly shows that the amount of NDP must be considerably reduced to achieve low RSDs. Actually, some of the NDP obtained in Figure 2 were not nondigestible but seemed to be digestible if we applied some other proteases such as pepsin (see NDP values in Table 3), or if we had a longer digestion period. We also found that the RSD for NDP values was much higher than the RSD for TDF values in almost all food samples. Method IV was not sufficient for many animal foods without the pepsin treatment; if we omitted the pepsin treatment, we usually obtained substantially larger RSDs. The NDP was measured by the Biuret method in Method IV-2. We limited the use of Method IV-2 to determination of the amount of underestimate of TDF in animal foods, with the results shown in Table 4.

The results of determination of TDF of Japanese foods are shown in Table 1. The results of Table 4 are not included in Table 1. It can be seen that Prosky's method is excellent for vegetables and very good for cereals and fruits when performed carefully, but the application to animal food was relatively difficult. The amounts of TDF obtained by Methods IV and IV-2 are compared in Table 4. It is shown that in some animal foods the value of TDF is underestimated if NDP is measured by the Kjeldahl method. The RSDs obtained throughout this study by the Kjeldahl method are summarized in Table 5. The relation between RSDs and the content of TDF in the assay sample shown in Figure 1 indicates that when the assay sample contains less than about 2% TDF, the RSD is expected to be more than 30%, and those samples appear to be outside the lower limit of the applicable range of this method (8). In other words, if we get a higher RSD in a sample which contains more than 2% TDF, there should be some specific reason for the variation of data. Many participants in this collaboration had no previous experience in TDF analysis. The value of 14.5% average RSD

Table 4. Results of total dietary fiber determination with nondigestible protein by Kjeldahl method and Biuret method

Food	TDF (wet%) Kjeldahl	Biuret	Relative S.D. %		No. of analyses	
Mackerel, raw	0.41		56.1		20	
		0.93		41.2		22
Tunas, lean, raw	0.31		95.0		6	
		0.67		36.2		6
Squid, raw	0.57		19.6		8	
		0.52		34.8		4
Shrimp, whole, boiled & dried, w/o shell	2.05		42.3		11	
		5.50		34.4		11
Fish paste, Kamaboko, steamed	0.56		29.5		8	
		0.65		12.9		5
Fish paste, Kamaboko, broiled	0.19		34.2		10	
		0.29		89.9		12
Fish paste, Chikuwa, broiled	0.46		36.2		7	
		0.64		19.4		7
Fish paste, Satsuma-age, fried	0.26		15.0		10	
		0.42		7.8		10
Beef chuck loin total edible	0.58		65.7		6	
		0.82		32.7		6
Chicken, thigh, broiler	0.12		47.8		21	
		1.02		78.0		14
Chicken, thigh, broiler flesh only	0.21		110.4		11	
		0.52		38.2		11
Ham, mixed press	0.36		74.3		8	
		0.56		29.0		8
Sausage, mixed	1.01		65.1		8	
		1.23		49.3		8
Chicken egg, whole, fresh	0.13		15.0		10	
		0.39		29.4		10
Ordinary liquid milk	0.22		25.6		10	
		0.28		32.8		10
Yogurt, whole milk, unsweetened	0.10		82.9		8	
		0.15		81.1		8
Lactic acid bacteria beverage	0		114.0		6	
		0		184.7		6
Cheese, processed	0.58		46.6		6	
		0.89		30.9		6
Meat balls, frozen	1.54		16.4		20	
		1.84		9.9		8
Sea foods, mixed, cooked, Tsukudani	1.44		15.2		23	
		2.28		24.0		10
Corn soup powder & other roux, mixed	4.98		6.0		8	
		5.17		3.2		5

Table 5. Relative standard deviation of the TDF assay using Methods I–IV

	Av. TDF (wet%)	No. of samples	Av. RSD (%)	No. of analyses	No. of rejected data
All measurements	4.81	252	19.3	3428	78
Without animal foods and microbial foods	5.32	220	14.5		

(repeatability and reproducibility mixed) in Table 5 seems to be low enough when we make allowance for TDF contents as low as 5.32%. The reported average values for 9 unknown samples were 17.6% RSD for samples containing an average TDF of 19.1% (1, 2).

For confirmation of comparable results by a variant (Method III) of the official method (Method I) when applied to the same sample, we carried out pilot experiments and found that the 2 methods were comparable. The results by the 2 methods for various samples are shown in Table 6. It

Table 6. Comparison of Method I with Method III[a]

No.	Food	Dietary fiber, av. ± SD, % (N) Method I (N)	Method III (N)	III-I	III-I/I (%)
(14)	Somen-Hiyamugi noodle	2.10 ± 0.62 (9)	2.28 ± 0.18 (31)	0.18	8.57
(26)	Well-milled rice	0.72 ± 0.34 (64)	0.82 ± 0.27 (34)	0.11	15.5
3	Barley, milled & cut	5.82 ± 0.69 (4)	4.97 ± 2.32 (8)	−0.85	−14.59
21	Wheat germ	11.07 ± 0.97 (10)	11.36 ± 0.40 (2)	0.29	2.62
27	Mochi, glutinous rice cake	0.40 (1)	0.31 ± 0.03 (4)	−0.09	−22.50
32	Popcorn, popped	10.27 ± 1.17 (7)	10.19 ± 0.04 (2)	−0.08	−0.78
33	Corn flakes	2.89 ± 0.30 (7)	2.90 ± 0.00 (3)	0.01	0.35
58	Peas, whole, boiled	5.37 ± 0.21 (4)	5.00 ± 0.00 (3)	−0.37	−6.89
121	Edible burdock root, boiled	3.53 ± 0.07 (8)	3.72 ± 0.03 (3)	0.19	5.38
124	Ginger tuber, raw	1.84 ± 0.02 (2)	1.83 ± 0.08 (6)	−0.01	−0.54
125	Sugukina, pickles	3.81 ± 0.16 (3)	3.60 ± 0.10 (3)	−0.21	−5.51
141	Sweet corn, boiled	2.08 ± 0.00 (3)	1.96 ± 0.17 (5)	−0.12	−5.77
157	Japanese butterbur petiole	1.42 ± 0.07 (6)	1.39 ± 0.02 (3)	−0.03	−2.11
162	Myoga, bract & flower	1.35 ± 0.05 (4)	1.48 ± 0.09 (3)	0.13	9.63
191	Hybrid melon (Ams), raw fruit	0.43 ± 0.02 (4)	0.38 ± 0.06 (3)	−0.05	−11.63
193	Peaches, canned with syrup	1.43 ± 0.06 (2)	1.50 ± 0.02 (6)	0.07	4.90
216	Coffee, drink, canned	0.07 ± 0.06 (10)	0.12 ± 0.02 (2)	0.05	71.43
217	Consomme, dried	0.76 ± 0.36 (10)	0.67 ± 0.02 (2)	−0.09	−11.80
238	Japanese crackers, mixed	1.65 ± 0.07 (2)	1.23 ± 0.15 (4)	−0.42	−25.45
239	Sponge cake & cakes mixed	1.06 ± 0.04 (2)	0.75 ± 0.10 (10)	−0.31	−29.25
Av		2.90 0.28 (19)	2.82 0.21 (20)	−0.08 (−2.8% vs 2.90)	−0.92

[a] No. corresponds to that of Table 1. Nos. 14 and 26 were results of pilot experiments for comparing the 2 methods. N ≤ 3 means analyses by a single lab.

No statistically significant difference was observed between the 2 results (t values were 1.478 and 1.584 for No. 14 and No. 26, respectively).

was also confirmed that the introduction of pepsin treatment did not affect the final result obtained, shown in Table 3.

To reduce the variation of TDF data, we found it important to overcome the following factors. The conditions which tend to increase variations of data were (*a*) incomplete digestion of proteins, (*b*) too low amounts of TDF, and (*c*) incomplete enzyme reaction (by incomplete hydration of sample with a residual fat; some enzyme inhibitory components of sample, etc.). The factors which tend to increase or decrease the value of TDF were (*a*) rate of discard of the food to obtain the edible portion, (*b*) incomplete extraction of the fat from the sample, and (*c*) incomplete digestion of starch (by low temperature, improper pH, incomplete mixing through solid gel formation, protected susceptibility to the amylase by incomplete heat denaturation of cell structures or the molecular structure, etc.).

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References

(1) Prosky, L., Asp, N., Furda, I., Devries, J. W., Schweizer, T. F., & Harland, B. F. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 677–679
(2) Deutsch, M. J. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 259
(3) Dreher, M. L. (1987) in *Handbook of Dietary Fiber,* Marcel Dekker, New York & Basel, pp. 9–10
(4) Kiriyama, S. (1980) *Chem. Biol.* **18,** 95–105
(5) Innami, S. (1982) in *Dietary Fiber,* Kiriyama, S., & Innami, S. (Eds.) Daiichi Shuppan, Tokyo, pp. 7–10
(6) Prosky, L., Asp, N., Schweizer, T., Devries, J. W., & Furda, I. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 1017–1023
(7) Resources Council, Science & Technology Agency, Japan (1982) *Standard Tables of Food Composition in Japan* (4th revised ed.)
(8) Guidelines for Interlaboratory Collaborative Study Procedure to Validate Characteristics of a Method of Analysis (1984) *J. Assoc. Off. Anal. Chem.* **67,** 433–440
(9) Gornall, A. G., Bardawill, C. S., & David, M. M. (1949) *J. Biol. Chem.* **177,** 751–755
(10) Grubb, F. E. (1950) *Ann. Math. Stat.* **21,** 27–58

METALS AND OTHER ELEMENTS

Development and Validation of a Method for Determining Elements in Solid Waste Using Microwave Digestion

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A microwave-assisted method for preparing samples for determination of elements in solid waste has been developed (draft EPA Method 3051). Validation of the sample preparation method was performed through a collaborative study to determine its precision and accuracy. Fifteen independent laboratories digested 4 National Institute of Standards and Technology (NIST) standard reference materials (SRMs) and 1 solvent recovery waste in duplicate. Digestates were analyzed for 19 elements using inductively coupled plasma (ICP) emission spectroscopy. The precision and bias of the method were evaluated. When compared with an open vessel hot-plate digestion method (SW-846 Method 3050), the microwave method produced similar analytical results with better overall precision. Bias for the 1 sample that allowed this determination was found to be excellent.

The techniques typically used to prepare Resource Conservation and Recovery Act (RCRA) wastes for analysis for metals and other elements can require several hours or even several days to complete. These techniques also often involve use of acid digestions and thermal decomposition steps, which may result in analyte losses, incomplete recoveries, and/or sample contamination. These limitations are well known to the analytical community and to end users of data produced by these methods, including the U.S. Environmental Protection Agency (EPA), state agencies, and industry. The inefficiency of these techniques reduces laboratory sample throughput, drives up the cost of analytical testing, and impedes decision-making. Given these concerns, EPA is interested in developing cost-effective sample preparation techniques for metals and other elements in environmental and process waste samples. Once developed, these techniques can then be written as methods for inclusion in *Test Methods for Evaluating Solid Waste SW-846* and made available to the user community (1). One of these new methods uses microwave-assisted sample digestion.

Microwave-assisted sample digestion is now receiving considerable attention and use in the laboratory. The procedure generally involves placing a sample in an acid solution in a closed vessel equipped with a pressure relief valve. The vessel is then subjected to microwave energy in a modified microwave oven. The conditions of high pressure generated in the container, coupled with the rapid heating of the sample via direct microwave energization of the acid molecules, can result in significantly reduced preparation time—from several hours in a conventional convection oven, hot plate, or steam bath, to several minutes in the microwave oven (2).

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Many studies have been conducted in the area of microwave-assisted digestion. The field has evolved since the days when analysts used commercially available microwave ovens and open vessels (3), venting via an acid trap (4), or a stream of CO_2 gas (5), and the use of a partially evacuated desiccator that was removed and vented in a fume hood at several stages during the digestion (6). Workers now are able to digest an ever-growing number of matrixes ranging from biological materials (7–13), to food (14–17), to geological materials (18–20) using sophisticated laboratory ovens and specially designed Teflon® digestion vessels.

Preparation of oils and soils can be performed with a number of microwave-based methods or techniques, depending on the degree of dissolution desired. Parameters varied include digestion time, microwave power, and acids or combinations of acids used. Sediments have been digested using a mixture of aqua-regia/HF (21), HNO_3/7:3 HCl:HF (20), $HNO_3/HClO_4$/HF (22), and sewage sludge by HNO_3 (23).

Microwave decomposition procedures take advantage of direct energy absorption by acid and water. They also rely on the equilibrium between microwave energy converted to heat, heat loss by the digestion vessels, and equipment configurations affecting both energy processes (24). Establishing the temperature and pressure conditions used in the test must also be based on several fundamental reactions involving nitric acid decomposition relevant to organic and geological compounds. Reaction rates are controlled by the temperature of nitric acid. There are minimum temperatures required for nitric acid to efficiently oxidize particular organic compounds, whether it is heated by conventional methods or through the microwave heating mechanism (24–27). There are also examples of total decomposition of geological materials using microwave energy that suggest appropriate target temperatures (28). A balance of each of these parameters was used to predict appropriate test conditions. These theoretically appropriate conditions were subjected to evaluation and then to validation.

Using the research cited above and temperature and pressure profile studies conducted by the National Institute of Standards and Technology (NIST), we were able to determine standardized microwave oven preparation conditions for oils and soils and establish a draft EPA method (Method 3051) (29). The method uses concentrated nitric acid as the digestion medium. The intent is not to completely solubilize all elements in the sample; rather, it is to solubilize those elements most likely to become environmentally available, providing a "leach" digestion and not a "total" solubilization. Previous work has been reported on evaluation of this draft method at the Research Triangle Institute (RTI) using NIST standard reference material (SRM) representative of oils and soils (30). It was reported that this method should be

a suitable alternative to SW-846 Method 3050, with a substantial time/cost savings. Based on these results, a collaborative study of the method was conducted for final validation of Method 3051.

The present paper reports on results of the collaborative study for validation of the microwave method. Four NIST SRMs and 1 solvent recovery waste were digested by 15 laboratories using the draft microwave method (Method 3051) and SW-846 Method 3050. Analysis of these digests was carried out using inductively coupled plasma (ICP) emission spectroscopy.

Experimental

Microwave Oven

The MDS-81D microwave system (CEM Corporation, Indian Trail, NC) was used for this study. The oven resembles a standard microwave oven, but is equipped with additional features to facilitate sample preparation. The unit has a Teflon-coated microwave cavity and a variable-speed corrosion-resistant exhaust system. The main element of the system consists of a precise variable microwave power system. Other elements include a rotating turntable, Teflon vessels with caps, a patented pressure relief valve, and a capping system. The Teflon sample vessels and caps are designed to withstand pressures up to 100 psi and temperatures up to 200°C.

In developing the method, temperature measurements in the microwave field were made using an optical fiber measurement system (Model 750, Luxtron Corp.). Pressure measurements were accomplished by bringing the pressure from the vessel out of the microwave cavity to a pressure transducer. Both measurements were electronically recorded against time (2, 24).

Collaborative Study Materials

The collaborative study was carried out using the following materials: NIST SRM 2704, Buffalo River sediment; NIST SRM 4355, Peruvian soil; NIST SRM 1085, wear metals in oil; NIST SRM 1634b, trace elements in fuel oil; and solvent recovery waste. To simulate a contaminated soil, a 1-to-1 mixture of SRM 1634b and SRM 2704 was prepared and analyzed.

Microwave Test Method

Reproducing conditions of the test is the key element in transferring this procedure and obtaining consistent results. The method relies on coupling microwave energy from a similar field strength to an identical quantity of acid. If the field strength is different, or if the mass of acid is changed, the energy transfer and resulting temperature profile will be different. Conditions also depend on a balance between the heat gained and lost from the digestion vessels. This is determined by the applied microwave energy field and the heat loss characteristics of a particular type of vessel (24). A microwave calibration procedure is described in SW-846 Method 3051. This calibration method must be used to transform a given instrument's partial power settings to applied microwave power, in watts (24).

If the temperature profile can be reproduced under different conditions, the chemical reactions should be the same and produce identical results. In fact, the validation study used 2 different sets of conditions to produce the same results. Therefore, 2 sets of conditions are specified in the method—one for 2 vessels and one for 6 vessels. These are considered equivalent. The conditions produced in these alternative method configurations are shown in Figure 1.

In the procedure, a 0.1 to 0.5 g of solid sample is placed in a 120 mL vessel (≤0.25 g, if organic material) with 10 mL HNO_3; then 2 or 6 vessel groups are placed in the microwave with accompanying vapor trap vessels. We applied microwave power at 344 and 574 watts, respectively, to the 2 vessel group and the 6 vessel group for 10 min. The microwave energy heats the acid in each vessel to 170°C in ca 5 min.; it is then sustained at 170–180°C for the balance of 10 min.

The leveling of temperature is due to heat loss of the vessel that reaches a thermal equilibrium with the applied power. It is the balance of this applied microwave energy and heat loss that is primarily responsible for the temperature profile (24).

Pressure in the vessel is a combination of the partial pressures of nitric acid, the water contained in the acid and sample, and gaseous digestion products produced in the decomposition reaction. For solid samples containing small quantities of carbon, pressure contributed from the reaction is low. Pressure does not control the reaction, but is a result of temperature on the vessel contents and on reaction products. Pressure does not have a significant effect on the reaction

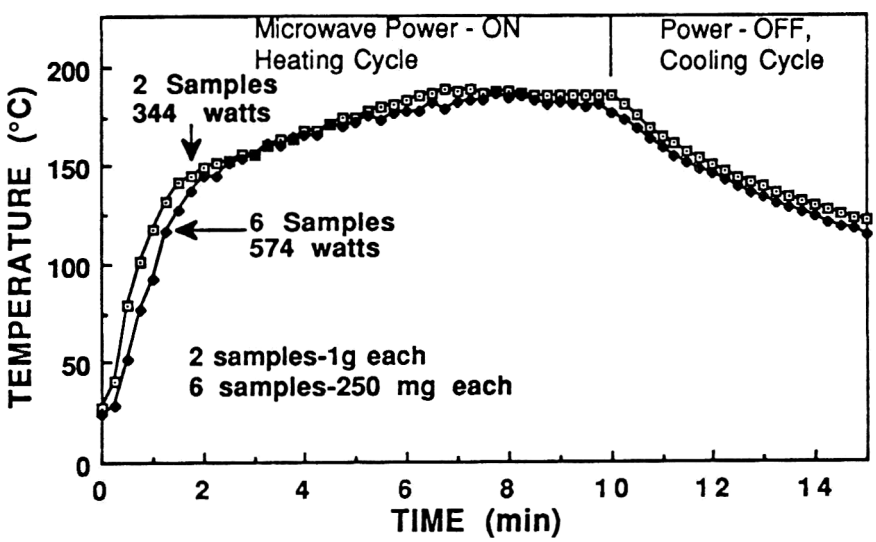

Figure 1. Temperature profiles of samples in 10 mL nitric acid for microwave-assisted sample preparation (draft Method 3051).

rates, but it must be kept to less than the maximum pressure rating for the vessel. Pressures in closed microwave vessels cannot be predicted from temperature data because vessels continuously lose heat and different vessels produce different pressure conditions (24).

Vapor trap vessels reduce the likelihood of analyte loss when decomposing samples produce significant gas. This second vessel traps hot acid vapor and any aerosol expelled when the pressure relief valve of the first vessel opens. A PFA Teflon tube connects the digestion vessel to the second vessel with a double-ported cap. A second port on the catch vessel is connected to the center well of the carousel to capture potential venting from this overflow vessel. The acid and any sample condensed in the second vessel is washed back into the sample digestion vessel at the end of the microwave procedure. Vessel contents are filtered into an acid-cleaned 50 mL volumetric flask following digestion.

Collaborative Study Design

The objective of the collaborative study was to validate the draft microwave method. This involved determination of the precision of the method within a single laboratory and also the total or between-laboratory precision of the method. In addition, bias of the method was evaluated for those samples for which known or accepted compositional values are available.

A total of 15 laboratories participated in the study. Each laboratory was sent aliquots of the 4 NIST SRMs and the solvent waste, along with instructions and a copy of the draft method. Each laboratory was instructed to digest 2 replicates—1 replicate to be digested under "2 vessel" conditions (344 watts, 10 min) and the other replicate to be digested under "6 vessel" conditions (574 watts, 10 min). In addition, each laboratory was to perform duplicate digestions using SW-846 Method 3050, an open vessel hot-plate acid digestion. All digests were forwarded to RTI where ICP analyses were performed.

Results and Discussion

ICP analyses were performed on the digests for the following 19 elements: silver, aluminum, boron, barium, beryllium, calcium, cadmium, cobalt, chromium, copper, iron, magnesium, manganese, molybdenum, nickel, lead, strontium, vanadium, and zinc. ICP results for the microwave method (now proposed as EPA Method 3051) and for Method 3050 are shown in Tables 1 and 2, respectively. Buffalo River sediment, Peruvian soil, the 1-to-1 mixture of Buffalo River sediment/trace elements in fuel oil, and the solvent recovery waste were analyzed for all 19 elements. SRM 1085, wear metals in oil, was analyzed for the 9 elements that are certified by NIST. The number of observations varies from the ideal of 30 because of exclusion of outliers and nondigested samples.

A comparison of results for the microwave Method 3051 and SW-846 Method 3050 is included in Tables 3 through 7 for Buffalo River sediment, Peruvian soil, the 1-to-1 mixture of Buffalo River sediment/trace elements in fuel oil, wear metals in oil, and the solvent recovery waste. The precision is expressed as percent relative standard deviation (RSD) and is calculated as follows:

$$\text{RSD, \%} = \frac{\text{sd}}{\text{mean}} \times 100\%$$

The F test was applied to compare standard deviations and the t test was applied to compare means at the 95% confidence limit. The percent difference is calculated as follows:

$$\text{Difference, \%} = \frac{M_1 - M_2}{M_2} \times 100\%$$

Table 1. ICP analyses of microwave Method 3051 digests (μg/g)

	Sample[a]				
Element	1 Mean ± SD (*n*)[b]	2 Mean ± SD (*n*)	3 Mean ± SD (*n*)	4 Mean ± SD (*n*)	5 Mean ± SD (*n*)
Ag	<4.0	<4.0	234 ± 36 (22)	<4.0	<4.0
Al	1.18 ± 0.14% (30)	1.92 ± 0.22% (30)	295 ± 31 (28)	0.685 ± 0.145% (21)	0.148 ± 0.027% (28)
B	34.6 ± 9.3 (30)	35.5 ± 7.5 (30)		20.7 ± 7.6 (22)	37.4 ± 7.0 (25)
Ba	77.7 ± 5.9 (30)	135 ± 11 (30)		43.5 ± 7.9 (22)	538 ± 110 (28)
Be	0.562 ± 0.068 (30)	0.493 ± 0.069 (30)		0.297 ± 0.064 (23)	<0.25
Ca	2.00 ± 0.38% (30)	1.09 ± 0.28% (30)		1.13 ± 0.26% (23)	0.219 ± 0.093% (28)
Cd	3.19 ± 0.61 (29)	0.901 ± 0.227 (27)		1.50 ± 0.22 (21)	4.90 ± 1.04 (27)
Co	10.7 ± 1.5 (30)	10.4 ± 1.2 (30)		5.89 ± 1.27 (23)	21.9 ± 5.0 (26)
Cr	81.7 ± 5.3 (30)	13.8 ± 1.2 (28)	293 ± 27 (30)	43.1 ± 4.9 (21)	161 ± 24 (26)
Cu	80.3 ± 6.9 (30)	53.4 ± 5.7 (30)	289 ± 24 (30)	41.4 ± 5.0 (23)	208 ± 33 (26)
Fe	2.96 ± 0.21% (30)	2.50 ± 0.39% (30)	311 ± 35 (27)	1.58 ± 0.18% (19)	0.316 ± 0.052% (26)
Mg	0.810 ± 0.047% (30)	0.705 ± 0.041% (30)	270 ± 29 (28)	0.410 ± 0.064% (23)	0.038 ± 0.009% (26)
Mn	460 ± 26 (30)	541 ± 29 (30)		238 ± 30 (21)	31.7 ± 5.0 (26)
Mo	<2.5	<2.5	238 ± 30 (30)	<2.5	19.1 ± 3.4 (28)
Ni	36.4 ± 2.5 (27)	9.59 ± 1.10 (28)	293 ± 25 (30)	30.5 ± 5.1 (23)	50.9 ± 10.0 (26)
Pb	143 ± 9 (30)	121 ± 8 (30)	279 ± 22 (30)	74.5 ± 8.9 (20)	437 ± 70 (26)
Sr	33.0 ± 2.0 (30)	81.0 ± 7.0 (30)		17.5 ± 2.0 (19)	71.1 ± 13.5 (26)
V	21.0 ± 2.5 (30)	61.2 ± 5.8 (30)		34.2 ± 6.5 (23)	9.92 ± 1.76 (26)
Zn	383 ± 26 (30)	366 ± 27 (30)		195 ± 30 (23)	748 ± 108 (28)

[a] Sample: 1 = NIST 2704, Buffalo River sediment; 2 = NIST 4355, Peruvian soil; 3 = NIST 1085, wear metals in oil; 4 = 1:1 mixture of 2704 and 1634b, trace elements in fuel oil; 5 = solvent recovery waste.

[b] *n* = number of observations.

Table 2. ICP analyses of Method 3050 digests (μg/g)

Element	Sample[a] 1 Mean ± SD (n)[b]	2 Mean ± SD (n)	3 Mean ± SD (n)	4 Mean ± SD (n)	5 Mean ± SD (n)
Ag	<4.0	<4.0	172 ± 52 (24)	<4.0	<4.0
Al	1.31 ± 0.37% (24)	2.55 ± 0.69% (27)	277 ± 26 (26)	0.638 ± 0.170% (18)	0.141 ± 0.030% (27)
B	55.4 ± 25.8 (27)	56.8 ± 21.7 (27)		23.8 ± 7.8 (19)	39.6 ± 10.8 (27)
Ba	85.3 ± 17.9 (27)	160 ± 28 (27)		45.8 ± 8.6 (19)	513 ± 132 (27)
Be	0.682 ± 0.209 (25)	0.608 ± 0.115 (27)		0.335 ± 0.097 (20)	<0.25
Ca	1.83 ± 0.20% (27)	1.02 ± 0.12% (27)		0.932 ± 0.075% (20)	0.190 ± 0.077% (27)
Cd	3.32 ± 0.44 (27)	1.03 ± 0.20 (23)		1.67 ± 0.20 (21)	4.96 ± 0.86 (27)
Co	11.1 ± 2.8 (27)	13.7 ± 2.8 (27)		5.89 ± 1.43 (20)	21.6 ± 8.3 (27)
Cr	83.3 ± 14.0 (27)	17.1 ± 2.4 (25)	274 ± 34 (26)	42.3 ± 6.0 (18)	157 ± 35 (27)
Cu	83.2 ± 11.0 (24)	57.0 ± 9.0 (27)	274 ± 24 (26)	39.0 ± 5.4 (18)	206 ± 35 (25)
Fe	3.06 ± 0.31% (27)	2.98 ± 0.34% (25)	288 ± 26 (24)	1.52 ± 0.20% (20)	0.345 ± 0.074% (23)
Mg	0.850 ± 0.120% (27)	0.785 ± 0.097% (27)	244 ± 25 (25)	0.419 ± 0.050% (20)	0.038 ± 0.007% (25)
Mn	472 ± 57 (27)	606 ± 76 (27)		238 ± 23 (20)	31.5 ± 5.0 (25)
Mo	2.95 ± 0.90 (23)	<2.5	258 ± 29 (26)	<2.5	20.0 ± 4.2 (27)
Ni	37.7 ± 5.2 (27)	9.93 ± 1.46 (24)	282 ± 26 (26)	31.4 ± 5.6 (19)	51.2 ± 11.7 (25)
Pb	147 ± 17 (27)	131 ± 14 (27)	279 ± 20 (26)	76.4 ± 8.2 (18)	463 ± 83 (25)
Sr	35.0 ± 7.0 (26)	98.9 ± 19.0 (27)		17.7 ± 3.3 (20)	71.0 ± 16.8 (27)
V	24.2 ± 7.2 (25)	81.4 ± 17.3 (27)		37.4 ± 10.2 (19)	9.73 ± 1.86 (26)
Zn	393 ± 61 (27)	401 ± 49 (27)		207 ± 24 (20)	747 ± 120 (27)

[a] Sample: 1 = NIST 2704, Buffalo River sediment; 2 = NIST 4355, Peruvian soil; 3 = NIST 1085, wear metals in oil; 4 = 1:1 mixture of 2704 and 1634b, trace elements in fuel oil; 5 = solvent recovery waste.
[b] n = number of observations.

where M_1 = the mean of Method 3051 and M_2 = the mean of Method 3050.

Because HCl and H_2O_2 are used in addition to HNO_3 for Method 3050, higher recoveries for Method 3050 would be expected. This is generally true; however, differences between the 2 methods are slight. Recoveries are very similar for SRM 2704 (Buffalo River sediment) (Table 3), with the exception of boron. The large difference in boron recovery is probably directly attributable to leaching from the borosilicate glassware used for Method 3050. Recovery differences range from a low of 2% for chromium and zinc to a high of 18% for beryllium. A comparison of method precision for Buffalo River sediment, as expressed in percent RSD, shows the microwave method to be more precise than Method 3050 for 15 out of 17 elements (Table 3). The 2 exceptions are calcium and cadmium. For all 17 elements, based on the *F* test, standard deviations were significantly different at the 95% confidence limit.

For SRM 4355 (Peruvian soil) (Table 4), differences in recoveries between the 2 methods are more pronounced, with percent differences generally ranging from 5 to 30% lower for the microwave method. Even so, with the exception of boron

Table 3. Comparison of microwave Method 3051 and Method 3050 based on ICP analyses of SRM 2704, Buffalo River sediment (μg/g)

Element	3051 Mean ± SD	(n)	RSD, %	3050 Mean ± SD	(n)	RSD, %	Difference, %	SD equiv. at 95% confidence limit
Ag	<4.0			<4.0				
Al (%)	1.18 ± 0.14	(30)	12	1.31 ± 0.37	(24)	28	−10	No
B	34.6 ± 9.3	(30)	27	55.4 ± 25.8	(27)	46	−38	No
Ba	77.7 ± 5.9	(30)	8	85.3 ± 17.9	(27)	21	−9	No
Be	0.562 ± 0.068	(30)	12	0.682 ± 0.209	(25)	31	−18	No
Ca (%)	2.00 ± 0.38	(30)	19	1.83 ± 0.20	(27)	11	+9	No
Cd	3.19 ± 0.61	(29)	19	3.32 ± 0.44	(27)	13	−4	No
Co	10.7 ± 1.5	(30)	14	11.1 ± 2.8	(27)	25	−4	No
Cr	81.7 ± 5.3	(30)	7	83.3 ± 14.0	(27)	17	−2	No
Cu	80.3 ± 6.9	(30)	9	83.2 ± 11.0	(24)	13	−3	No
Fe (%)	2.96 ± 0.21	(30)	7	3.06 ± 0.31	(27)	10	−3	No
Mg (%)	0.810 ± 0.047	(30)	6	0.850 ± 0.120	(27)	14	−5	No
Mn	460 ± 26	(30)	6	472 ± 57	(27)	12	−2	No
Mo	<2.5			2.95 ± 0.90	(23)			
Ni	36.4 ± 2.5	(27)	7	37.7 ± 5.2	(27)	14	−3	No
Pb	143 ± 9	(30)	7	147 ± 17	(27)	11	−3	No
Sr	33.0 ± 2.0	(30)	6	35.0 ± 7.0	(26)	20	−6	No
V	21.0 ± 2.5	(30)	12	24.2 ± 7.2	(25)	30	−13	No
Zn	383 ± 26	(30)	7	393 ± 61	(27)	15	−2	No

n = number of observations.

Table 4. Comparison of microwave Method 3051 and Method 3050 based on ICP analyses of SRM 4355, Peruvian soil (μg/g)

	3051			3050					
Element	Mean ± SD	(*n*)	RSD, %	Mean ± SD	(*n*)	RSD, %	Difference, %	SD equiv. at 95% confidence limit	Mean equiv. at 95% confidence limit
Ag	<4.0	—	—	<4.0	—	—	—		
Al (%)	1.92 ± 0.22	(30)	12	2.55 ± 0.69	(27)	27	−25	No	
B	35.5 ± 7.5	(30)	21	56.8 ± 21.7	(27)	38	−38	No	
Ba	135 ± 11	(30)	8	160 ± 28	(27)	18	−16	No	
Be	0.493 ± 0.069	(30)	14	0.608 ± 0.115	(27)	19	−19	No	
Ca (%)	1.09 ± 0.28	(30)	25	1.02 ± 0.12	(27)	12	+7	No	
Cd	0.901 ± 0.227	(27)	25	1.03 ± 0.20	(23)	20	−12	Yes	No
Co	10.4 ± 1.2	(30)	12	13.7 ± 2.8	(27)	21	−24	No	
Cr	13.8 ± 1.2	(28)	8	17.1 ± 2.4	(25)	14	−19	No	
Cu	53.4 ± 5.7	(30)	11	57.0 ± 9.0	(27)	16	−6	No	
Fe (%)	2.50 ± 0.39	(30)	16	2.98 ± 0.34	(25)	11	−16	Yes	No
Mg (%)	0.705 ± 0.041	(30)	6	0.785 ± 0.097	(27)	12	−10	No	
Mn	541 ± 29.1	(30)	5	606 ± 76	(27)	12	−11	No	
Mo	<2.5	—	—	<2.5	—	—	—		
Ni	9.59 ± 1.10	(28)	11	9.93 ± 1.46	(24)	15	−3	Yes	Yes
Pb	121 ± 8	(30)	7	131 ± 14	(27)	11	−8	No	
Sr	81.0 ± 7.0	(30)	9	98.9 ± 19.0	(27)	19	−18	No	
V	61.2 ± 5.9	(30)	10	81.4 ± 17.3	(27)	21	−25	No	
Zn	366 ± 27	(30)	7	401 ± 49	(27)	12	−9	No	

n = number of observations.

again, differences are all 25% or less. In preliminary studies, it was determined that Peruvian soil is highly reactive under microwave conditions. This could account for lower recoveries if the solution was lost through venting during excessive pressure buildup in the microwave vessel. The microwave method exhibits better precision for 14 out of 17 elements. Standard deviations were significantly different with the exception of cadmium, iron, and nickel. Using the *t* test to compare means, of these 3 elements, only nickel was equivalent at the 95% confidence limit.

Recoveries for the 1-to-1 mixture of Buffalo River sediment/trace elements in fuel oil (Table 5) are quite similar. There is excellent agreement with Method 3050. With the exception of calcium, percent differences ranged from 0 to 13%. For this nonhomogeneous material chosen to simulate an oily waste, precisions for the 2 methods were generally similar. Standard deviations were statistically equivalent at the 95-percent confidence limit for 13 out of 17 elements. Of these 13 elements, 12 had equivalent means at the 95-percent confidence limit.

Recoveries for SRM 1085 (wear metals in oil) are generally higher for the microwave method than for Method 3050

Table 5. Comparison of microwave Method 3051 and Method 3050 based on ICP analyses of 1:1 mixture of Buffalo River sediment and trace elements in fuel oil (μg/g)

	3051			3050					
Element	Mean ± SD	(*n*)	RSD, %	Mean ± SD	(*n*)	RSD, %	Difference, %	SD equiv. at 95% confidence limit	Mean equiv. at 95% confidence limit
Ag	<4.0	—	—	<4.0	—	—	—		
Al (%)	0.685 ± 0.145	(21)	21	0.638 ± 0.170	(18)	27	+7	Yes	Yes
B	20.7 ± 7.7	(22)	37	23.8 ± 7.8	(19)	33	−13	Yes	Yes
Ba	43.5 ± 7.9	(22)	18	45.8 ± 8.6	(19)	19	−5	Yes	Yes
Be	0.297 ± 0.064	(23)	22	0.335 ± 0.097	(20)	29	−11	No	
Ca (%)	1.13 ± 0.26	(23)	23	0.932 ± 0.075	(20)	8	+21	No	
Cd	1.50 ± 0.22	(21)	15	1.67 ± 0.20	(21)	12	−6	Yes	No
Co	5.89 ± 1.27	(23)	22	5.89 ± 1.43	(20)	24	0	Yes	Yes
Cr	43.1 ± 4.9	(21)	11	42.3 ± 6.0	(18)	14	+2	Yes	Yes
Cu	41.4 ± 5.0	(23)	12	39.0 ± 5.4	(18)	14	+6	Yes	Yes
Fe (%)	1.58 ± 0.18	(19)	11	1.52 ± 0.20	(20)	13	+4	Yes	Yes
Mg (%)	0.410 ± 0.064	(23)	16	0.419 ± 0.050	(20)	12	−2	Yes	Yes
Mn	238 ± 30	(21)	13	238 ± 23	(20)	10	0	Yes	Yes
Mo	<2.5	—	—	<2.5	—	—	—		
Ni	30.5 ± 5.1	(23)	17	31.4 ± 5.6	(19)	18	−3	Yes	Yes
Pb	74.5 ± 8.9	(20)	12	76.4 ± 8.2	(18)	11	−2	Yes	Yes
Sr	17.5 ± 2.1	(19)	12	17.7 ± 3.3	(20)	19	−1	No	
V	34.2 ± 6.5	(23)	19	37.4 ± 10.2	(19)	27	−8	No	
Zn	195 ± 30	(23)	15	207 ± 24	(20)	12	−6	Yes	Yes

n = number of observations.

Table 6. Comparison of microwave Method 3051 and Method 3050 based on ICP analyses of SRM 1085, wear metals in oil (μg/g)

	3051			3050					
Element	Mean ± SD	(*n*)	RSD, %	Mean ± SD	(*n*)	RSD, %	Difference, %	SD equiv. at 95% confidence limit	Mean equiv. at 95% confidence limit
Ag	234 ± 36	(22)	15	172 ± 52	(24)	30	+36	No	
Al	295 ± 31	(28)	10	277 ± 26	(26)	9	+6	Yes	No
Cr	293 ± 27	(30)	9	274 ± 34	(26)	12	+7	Yes	No
Cu	289 ± 24	(30)	8	274 ± 24	(26)	9	+5	Yes	No
Fe	311 ± 35	(27)	11	288 ± 26	(24)	9	+8	Yes	No
Mg	270 ± 29	(28)	11	244 ± 25	(25)	10	+11	Yes	No
Mo	238 ± 30	(30)	13	258 ± 29	(26)	11	−8	Yes	No
Ni	293 ± 25	(30)	8	282 ± 26	(26)	9	+4	Yes	Yes
Pb	279 ± 22	(30)	8	279 ± 21	(26)	7	0	Yes	Yes

n = number of observations.

Table 7. Comparison of microwave Method 3051 and Method 3050 based on ICP analyses of solvent recovery waste (μg/g)

	3051			3050					
Element	Mean ± SD	(*n*)	RSD, %	Mean ± SD	(*n*)	RSD, %	Difference, %	SD equiv. at 95% confidence limit	Mean equiv. at 95% confidence limit
Ag	<4.0	—	—	<4.0	—	—	—		
Al (%)	0.148 ± 0.027	(28)	18	0.141 ± 0.030	(27)	21	+5	Yes	Yes
B	37.4 ± 7.0	(25)	19	39.6 ± 10.8	(27)	27	−6	No	
Ba	538 ± 110	(28)	20	513 ± 132	(27)	26	+5	Yes	Yes
Be	<0.25	—	—	<0.25	—	—	—		
Ca (%)	0.219 ± 0.093	(28)	42	0.190 ± 0.077	(27)	40	+15	Yes	Yes
Cd	4.90 ± 1.04	(27)	21	4.96 ± 0.86	(27)	17	−1	Yes	Yes
Co	21.9 ± 5.0	(26)	23	21.6 ± 8.3	(27)	38	+1	No	
Cr	161 ± 24	(26)	15	157 ± 34.7	(27)	22	+2	No	
Cu	208 ± 33	(26)	16	206 ± 35.1	(25)	17	+1	Yes	Yes
Fe (%)	0.316 ± 0.052	(26)	16	0.345 ± 0.074	(23)	21	−8	No	
Mg (%)	0.038 ± 0.009	(26)	24	0.038 ± 0.007	(25)	18	0	Yes	Yes
Mn	31.7 ± 5.1	(26)	16	31.5 ± 5.0	(25)	16	+1	Yes	Yes
Mo	19.1 ± 3.4	(28)	18	20.0 ± 4.2	(27)	21	−4	Yes	Yes
Ni	50.9 ± 10.0	(26)	20	51.2 ± 11.7	(25)	23	−1	Yes	Yes
Pb	437 ± 70	(26)	16	463 ± 83	(25)	18	−6	Yes	Yes
Sr	71.1 ± 13.5	(26)	19	71.0 ± 16.8	(27)	24	0	Yes	Yes
V	9.92 ± 1.76	(26)	18	9.73 ± 1.86	(26)	19	+2	Yes	Yes
Zn	748 ± 108	(28)	14	747 ± 120	(27)	16	0	Yes	Yes

n = number of observations.

Table 8. ICP analysis of SRM 1085, wear metals in oil, using microwave Method 3051 (μg/g)

Element	Mean ± SD	RSD, %	NIST value	Bias, %
Ag	234 ± 35.9	15	291[a]	−20
Al	295 ± 31.1	10	296	0
Cr	293 ± 26.6	9	298	−2
Cu	289 ± 23.8	8	295	−2
Fe	311 ± 34.7	11	300	+4
Mg	270 ± 29.1	11	297	−9
Mo	238 ± 30.3	13	292	−18
Ni	293 ± 25.0	8	303	−3
Pb	279 ± 22.1	8	305[a]	−8

[a] Not certified.

(Table 6). With the exception of silver, recoveries were from 0 to 11% higher. Lower recoveries for silver would be expected for Method 3050 because of precipitation by hydrochloric acid. Except for silver, the precision is excellent for both methods. Standard deviations were equivalent for 8 out of 9 elements. It should be noted that Method 3050 is not generally used for oils; SW-846 Method 3040 is normally applied.

For the solvent recovery waste, which was chosen to represent a "real world" sample, recoveries are again quite similar (Table 7). With the exception of silver, which precipitates in the presence of hydrochloric acid, and calcium, recovery differences were from 0 to 8%. Microwave method precision is superior for 13 out of 17 elements; although overall, it is generally similar to that obtained by Method 3050 for this nonhomogeneous material. For the 2 methods, standard deviations were equivalent for 13 out of 17 elements, and all 13 elements had statistically equivalent means at the 95% confidence limit.

Because the microwave digestion method was designed to provide a "leach" digestion and not a "total" solubilization, it was only feasible to determine bias for SRM 1085 (wear metals in oil), where the elements are readily extracted from the matrix into solution. Table 8 presents a comparison of elemental levels obtained using the microwave method with NIST-certified values. Of 9 certified elements, 7 exhibit excellent recovery with 0 to 9% bias. Silver and molybdenum are low, but have generally provided poor recoveries in our laboratory regardless of method.

Conclusion

Evaluation of the draft microwave digestion method through a collaborative study indicates that this method should prove a suitable alternative for SW-846 Method 3050 with a substantial time/cost savings.

Comparison of draft Method 3051 with Method 3050 reveals similar analytical results with overall better precision. Bias for the 1 sample that allowed this determination was found to be excellent.

In addition to equal or better precision and increased sample throughput, another advantage of microwave Method 3051 over Method 3050 is an assured uniformity of experimental conditions. The equipment is identical and parameters such as digestion time, microwave power, and volume of acid are strictly controlled.

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References

(1) *Test Methods for Evaluating Solid Waste* (1986) 3rd Ed., U.S. EPA, Washington, DC
(2) Kingston, H. M., & Jassie, L. B. (1986) *Anal. Chem.* **58**, 2534-2541
(3) Abu-Samra, A., Morris, J. S., & Koirtyohann, S. R. (1975) *Anal. Chem.* **47**, 1475-1477
(4) Barrett, P., Davidowski, L. J., Jr, Penaro, K. W., & Copeland, T. R. (1978) *Anal. Chem.* **7**, 1021-1023
(5) Matthes, S. A., Farrell, R. F., & Mackie, A. J. (1983) *A Microwave System for the Acid Dissolution of Metals and Mineral Samples*, Technical Project Report No. 120, U.S. Bureau of Mines
(6) Copeland, T. R. (1985) *Methods for Determining As, Be, Cd, Cr, Ni, and Pb in Waste Oils*, Interim Report on Task 6, U.S. EPA, Contract No. 68-01-7075
(7) Blust, R., Vander-Linden, A., & Decleir, W. (1985) *Atom. Spectrosc.* **6**, 163-165
(8) White, R. T., & Douthit, G. E. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 766-769
(9) Burguera, M., Burguera, J. L., & Alarcon, O. M. (1986) *Anal. Chim. Acta* **179**, 351-357
(10) Aysola, P., Anderson, P., & Lanford, C. H. (1987) *Anal. Chem.* **59**, 1582-1583
(11) De la Guardia, M., Salvador, A., Burguera, J. L., & Burguera, M. J. (1988) *J. Flow Inj. Anal.* **5**, 121-131
(12) Burguera, M., Burguera, J. L., & Alarcon, O. M. (1988) *Anal. Chim. Acta* **214**, 421-427
(13) Stripp, R. A., & Bogen, D. C. (1989) *J. Anal. Toxicol.* **13**, 57-59
(14) Tsukada, S., Demura, R., & Yamamoto, I. (1985) *ESKGA* **31**, 37-41
(15) Demura, R., Tsukada, S., & Yamamoto, I. (1985) *ESKGA* **31**, 405-409
(16) Schelkoph, G. M., & Milne, D. B. (1988) *Anal. Chem.* **60**, 2060-2062
(17) Nikdel, S., & Temelli, C. M. (1987) *J. Microchem.* **36**, 240-244
(18) Smith, F., Cousins, B., Bozic, J., & Flora, W. (1985) *Anal. Chim. Acta* **177**, 243-245
(19) Fischer, L. B. (1986) *Anal. Chem.* **58**, 261-263
(20) Lamothe, P. J., Fries, T. L., & Consul, J. J. (1986) *Anal. Chem.* **58**, 1881-1886
(21) Nadkarni, R. A. (1984) *Anal. Chem.* **56**, 2233-2237
(22) Papp, C.S.E., & Fisher, L. B. (1987) *Analyst* **112**, 337-338
(23) Morales, A. (1989) *J. Anal. Atom. Spectrosc.* **4**, 329-332
(24) Kingston, H. M., & Jassie, L. B. (1988) "Monitoring and Predicting Parameters," in *Microwave Sample Preparation: Theory and Practice*, H. M. Kingston & L. B. Jassie (Eds.), American Chemical Society, Washington, DC, pp. 93-154
(25) Kingston, H. M., & Jassie, L. B. (1988) *J. Res. Natl. Bur. Stds.* **93**, 269-274
(26) Pratt, K. W., Kingston, H. M., MacCrehan, W. A., & Koch, W. F. (1988) *Anal. Chem.* **60**, 2024-2027
(27) Wurfels, M., Jackwerth, E., & Stoeppler, M. (1988) *Fresenius, Z. Anal. Chem.* **330**, 160-161
(28) NIST Reports of Analysis for Standard Reference Materials, unpublished work
(29) Kingston, H. M. (1988) *EPA IAG #DWI-393254-01-0*, January 1-March 31, Quarterly Report
(30) Binstock, D. A., Grohse, P. M., Gaskill, A., Jr, Luk, K. K., Swift, P. L., Kingston, H. M., & Sellers, C. (1990) "Validation of Methods for Determining Elements in Solid Waste by Microwave Digestion," *Waste Testing and Quality Assurance*, 2nd Vol., ASTM STP 1062, D. Friedman (Ed.), American Society for Testing and Materials, Philadelphia

PACKAGING MATERIALS

Determination of Benzene in Polypropylene Food-Packaging Materials and Food-Contact Paraffin Waxes

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An analytical procedure was developed for determination of benzene in polypropylene food packaging and was adapted for determination of benzene in commercial paraffin waxes intended for food-contact use. The polymer was dissolved in hexadecane at 150°C. The wax was melted in an 80°C oven. A simple helium-sparging apparatus was used to remove the volatile chemical from the polymer or wax. The contaminant was collected in methanol, distilled water was added, and the resulting solution was analyzed by headspace gas chromatography. The instrument was equipped with a 30 m fused silica open tubular capillary column and a photoionization detector. Average recoveries of benzene from polymer and paraffin wax at low parts-per-billion concentrations were 63 and 70%, respectively. Limits of detection and quantitation for analysis of polypropylene were 8 and 17 ppb, respectively; the limit of quantitation for analysis of paraffin wax was 2 ppb. In several commercial polypropylene products examined, benzene levels ranged from none detected to 426 ppb. In 3 commercial waxes examined, concentrations of 16–73 ppb benzene were determined. The presence of benzene was confirmed by gas chromatography/mass spectrometry.

Some commercial food-grade polypropylene has been found to contain small amounts of benzene, according to reports made available through industry (personal communication, Jerome H. Heckman, Keller and Heckman, Washington, DC, 1986). Low levels of this contaminant can be present in the hexane used as a polymerization solvent. Polymer manufactured by a new fast-acting catalyst system had the highest concentrations; levels in the low parts-per-million range were found in some lots. When no hexane or fast-acting catalyst was used, benzene concentrations were in the low parts-per-billion range.

Polypropylene is used to manufacture food-packaging materials, such as syrup bottles, yogurt cups, and salad tubs. Because of its high melt temperature, some microwavable containers are also produced from polypropylene. With direct contact and extended storage times, chemicals from packaging materials can migrate into foods; therefore, any items held in benzene-containing polypropylene containers could become contaminated. Because the amount of migration is related to concentration of the migrant in the polymer, it is important to determine levels of benzene.

In the work reported by industry (personal communication, Jerome H. Heckman, Keller and Heckman, Washington, DC, 1986), benzene was separated from polymer by vacuum distillation and purge-and-trap procedures and quantitated by gas chromatography. A potential problem with both techniques is that the polypropylene is not dissolved. As observed with styrene in polystyrene, vinyl chloride in polyvinylchloride, and many other monomer/polymer systems (1), residual levels of benzene could remain trapped in the undissolved plastic, yielding low results and making quantitation of low parts-per-billion levels very difficult.

Residual levels of benzene have been determined previously in styrene-containing polymers (2). The polymers were dissolved, and the resultant solutions were analyzed by headspace gas chromatography (HS-GC). Similarly, a method was developed in which polypropylene is dissolved before analysis for trace levels of benzene. The contaminant is removed from the resulting solution by sparging with helium, collected in methanol, and determined by HS-GC. (A similar purge-and-trap method has been used to determine vinyl chloride residues in polyvinyl chloride food packaging (3). Benzene levels as low as 8 ppb can be detected in the polypropylene.

Several commercial food packages were examined by this method, including syrup bottles, yogurt cups, salad and dessert tubs, bottle caps, and cookie trays. For positive identification, analytical results were confirmed by mass spectrometry (MS).

The method was subsequently modified for determination of benzene in paraffin wax. As a result of a manufacturing malfunction, commercial wax from a major producer inadvertently became contaminated with minute amounts of benzene. Because some of the product was intended for food-contact use, a method was needed to determine benzene in the wax. In the modified method, paraffin wax is melted, and benzene is removed by sparging with helium, collected in methanol, and determined by HS-GC. Analytical results were confirmed by MS. Production lots representing 3 commercial waxes were monitored to assure that the problem had been remedied.

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Experimental

Apparatus

(a) *Screw-cap bottles.*—1 oz narrow-mouth clear glass (35 ± 0.5 mL), with screw caps and Teflon-faced septa (Alltech Associates, Inc., Deerfield, IL 60015, Cat. Nos. 9529, 95301, and 95302).

(b) *Crimp-top bottles.*—40 and 100 mL clear glass (Alltech Associates, Inc.). Fermpress tools H 207 and HO 207 (Perkin-Elmer Corp., Norwalk, CT 06859, Cat. Nos. B003-8134 and B003-8135) were used to cap and uncap bottles, respectively.

(c) *Headspace sample vials.*—23 mL clear glass (Perkin-Elmer Corp., Cat. No. 0105-0129).

(d) *Septa.*—Aluminum-faced with crimp-top seals and spacers (Perkin-Elmer Corp., Cat. No. B010-4243).

(e) *Tubing.*—Stainless steel, od 1/16 in. (1.6 mm), id 0.04 in. (1.0 mm) (Alltech Associates, Inc., Cat. No. 30181).

(f) *Gas chromatograph.*—Perkin-Elmer Model Sigma

2000 equipped with automated headspace sampler Model HS-100, subambient accessory for the column oven (Perkin-Elmer Corp.), and H-nu photoionization detector (HNU Systems, Inc., Newton Highlands, MA 02161). Operating conditions of headspace sampler: temperature 60°C; pressurization time 0.5 min with helium pressure 75 kPa; injection time 0.05 min; withdrawal time 0.2 min; equilibration time 0.5 min; and transfer line 90°C. GC operating conditions: column 25°C (or 30°C) for 10 min, increasing at 30°C/min to 150°C for 5 min; detector 200°C; helium carrier flow rate 6 mL/min; and helium make-up gas flow rate 30 mL/min. (The subambient accessory was used to maintain the column at 25 or 30°C.) A 10.2 eV lamp was installed in the detector. Under these conditions (column 25°C), benzene elutes in 6.8 min. Instrument background signal was automatically zeroed at start of each analysis. Data were collected by an IBM 9000 computer with chromatography software package (IBM Corp., Boca Raton, FL 33432).

(g) *GC column.*—30 m DB-1 Megabore fused silica open tubular (FSOT) capillary (0.53 mm id) (J & W Scientific, Inc., Rancho Cordova, CA 95670).

(h) *Gas chromatograph-mass spectrometer.*—Finnigan Model 9500 gas chromatograph interfaced with glass jet separator to Finnigan MAT 3300 quadrupole mass spectrometer, equipped with Finnigan INCOS 2300 data system (Finnigan MAT, San Jose, CA 95134). GC column: nickel, 2 ft (61 cm) × 1/8 in. (3.2 mm) od, packed with SE-30 on 80/100 mesh Chromosorb W-HP. GC operating conditions: injection port 180°C; column 95°C; separator 200°C; helium carrier gas. Operate mass spectrometer in multiple ion detection (MID) mode with electron ionization. Monitor ions at *m/z* 78, 77, 52, 51, and 50. MS parameters: electron energy 70 eV; filament current 0.49 mA; preamplifier 10 A/V; multiplier 1400 V; scan time 1.1 s. Inject headspace sample with solvent divert on; after 3 min, turn divert off and begin acquisition of MID data.

Reagents

(a) *Benzene.*—Certified 99 mol/% pure, thiophene-free (Fisher Scientific Co., Pittsburgh, PA 15238, Cat. No. B414-1).

(b) *Methanol.*—For purge-and-trap analysis (American Burdick & Jackson, Muskegon, MI 49442, Cat. No. 232-1). Analyze by headspace sampling technique to assure absence of interferences at retention time of benzene.

(c) *Hexadecane.*—99% (Aldrich Chemical Company, Inc., Milwaukee, WI 53233, Cat. No. H670-3). Analyze blank to determine suitability of particular solvent lot; solvent should not contain more than 3–5 ppb benzene.

(d) *Water.*—Deionized, distilled, obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA 01730). Test under conditions of analysis to assure absence of substances with approximate retention time of benzene.

(e) *Silicone bath fluid.*—High thermal stability and boiling point over 360°C (Thomas Scientific, Swedesboro, NJ 08085, Cat. No. 6428).

(f) *Polypropylene pellets.*—Exxon 1024 homopolymer (Sweetheart Plastics Inc., Wilmington, MA 01887).

Preparation of Standards

Stock standard solution.—Accurately weigh 1 oz narrow-mouth bottle, screw cap, and septum. Pipet 25 mL methanol into bottle, cap, and reweigh. Using 50 μL syringe, add 30 μL benzene to methanol by quickly uncapping bottle and injecting standard. (This procedure is used to avoid puncturing septum, which would shorten lifetime of standard.) Immediately recap bottle, and thoroughly mix solution by shaking. Reweigh bottle, and calculate benzene concentration (ca 1300 ppm). Prepare fresh stock solution every month. Stock solution may be stored at room temperature.

Working standard solution.—Dilute stock standard solution with methanol to prepare working standard solution of ca 130 ppm benzene. Pipet 1 part stock standard solution into 1 oz narrow-mouth bottle containing 9 parts methanol. Quickly cap bottle and thoroughly mix solution by shaking. Prepare fresh working standard every month. Working standard may be stored at room temperature.

From above solutions of stock standard and working standard, prepare headspace standards for quantitation of benzene. Pipet 10 mL methanol–water (5 + 5) into 23 mL sample vial. Using 10 μL syringe, inject predetermined volume of stock standard or working standard solution into measured solvent and cap vial. Thoroughly mix solution by shaking. Prepare headspace standards daily and use for only one injection.

Separation of Benzene from Polypropylene

Cut polypropylene containers into pieces ca 1/4 × 1 in. (6 × 25 mm) to fit into crimp-top bottles. Accurately weigh 10 g polymer and place in 100 mL bottle. Add 70 mL hexadecane and cap bottle, using aluminum-faced septa. Place mixture in 150°C oven for 3 h to dissolve polymer. Remove polymer solution from oven and place in oil bath heated to 150°C (Figure 1). (Prepare oil bath by filling 400 mL beaker with silicone bath fluid. Place beaker on hot plate with magnetic stirrer and heat oil to 150°C, adjusting temperature with variable transformer.) Connect polymer solution to helium source and to trapping solvent with 1/16 in. (1.6 mm) od stainless steel tubing. To condense and trap volatiles purged from polymer, add 5 mL methanol to each of 2 headspace sample vials. Seal vials with aluminum-faced septa and connect them in series. Place vials in ice bath. Sparge polymer solution for 1 h with helium delivered at ca 50 mL/min flow rate. Pass purge gas with extracted chemicals through cooled methanol. After sparging, disconnect vials containing trapping solvent. Uncap vials, and add 5 mL deionized, distilled water. Reseal vials. Analyze resulting solutions by HS-GC.

Separation of Benzene from Paraffin Wax

With metal spatula, break paraffin wax into pieces that will fit into crimp-top bottle. Weigh ca 10 g and place in 40 mL bottle. Cap, using crimp-top seals and aluminum-faced septa. Place bottle in 80°C oven for 1 h to melt wax. Remove bottle from oven and place in oil bath heated to 80°C (Figure 1). Sparge contents of bottle and continue with the method as described in the previous section.

Determination of Benzene Levels

Equilibrate solutions resulting from extraction of benzene from polypropylene or paraffin wax for 30 min in automated headspace sampler heated to 60°C. After injection, maintain column temperature at 25°C (or 30°C) for 10 min. When determining benzene in polypropylene, raise column temperature rapidly to 150°C and maintain for 5 min to remove late-eluting components.

To calculate benzene levels in polypropylene containers or paraffin wax, prepare calibration curve daily from external headspace standards. Analyze at least 3 standards over the

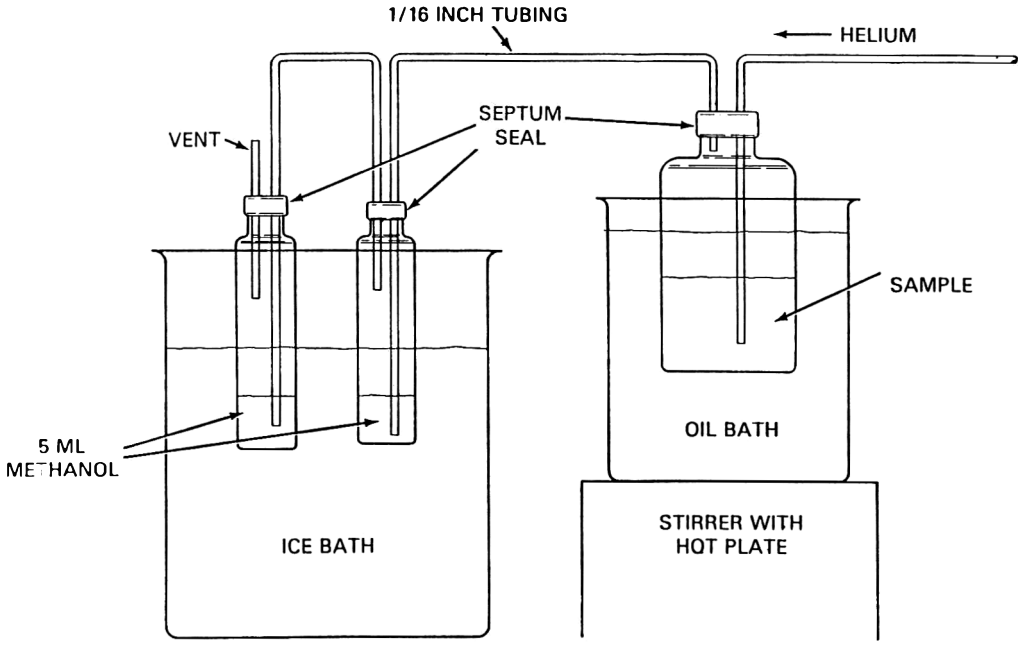

Figure 1. Diagram of purge-and-trap apparatus.

concentration range of benzene suspected to be present in polymer containers or wax. Add together heights of peaks obtained for both trapping solutions to determine total amount of benzene. For determinations of benzene in polypropylene, analyze hexadecane blank; if a peak is found at the retention time of benzene, subtract its height from total peak height obtained for polymer.

Recovery Studies

The percentage of benzene recovered from the polymer was determined by analyzing spiked test portions of polypropylene pellets. Studies were conducted at concentrations of ca 130, 52, and 20 ppb, with 3 determinations at each level. (Because of instrument malfunction, only 2 results are reported for the 20 and 130 ppb levels.) The polymer was accurately weighed and placed in a crimp-top bottle. Hexadecane was added, and the solution was fortified with the prepared benzene standards. Unfortified polymer was analyzed with each group of spiked test portions, and the height of the chromatographic peak found at the retention time of benzene was subtracted from the heights of the spike peaks. Corrected results were quantitated by comparison to the peak heights of standards prepared in methanol–water (5 + 5) with the same quantity of benzene as the fortified test portions.

The percentage of benzene recovered from the wax was estimated by analyzing 2 spiked test portions of commercial paraffin wax. The wax with the lowest benzene concentration was accurately weighed and placed in crimp-top bottles. Test portions were spiked with the prepared benzene standards at levels of ca 500 and 100 ppb. The amount of benzene purged from the fortified wax was determined from the calibration curve. The benzene concentration in the unspiked wax was subtracted, and the result compared to the spiking level to determine the percentage of benzene recovered.

Infrared Analysis of Commercial Polypropylene Packaging

A number of commercial polypropylene food-contact materials were analyzed. The products, which were examined after food contact, included syrup bottles, yogurt cups, salad and dessert tubs, bottle caps, cookie trays, and catsup containers. Infrared (IR) spectra were obtained to identify the polymeric composition of the packages as follows: pieces 1/4 in. (6 mm) square were cut from the containers. Each section was placed between 2 sheets of smooth aluminum foil and hot-pressed into a thin (1–2 mil) film with a Rucker PHI Model SP-215C heated bench press. A Perkin-Elmer Model 1430 ratio recording IR spectrophotometer was used to obtain the spectrum from each film. The qualitative make-up of each polymer product was determined from comparisons with standard spectra.

GC/MS Analysis of Commercial Polypropylene Packaging and Commercial Paraffin Wax

Benzene was determined in samples of 3 commercial paraffin waxes. The presence of benzene in commercial polypropylene materials described in the previous section and in commercial waxes was confirmed by electron ionization MID GC-MS. The methanol–water (5 + 5) solutions resulting from the extraction of benzene from the polymer and paraffin wax, along with standards and a methanol–water blank, were examined. After the headspace sample vials were heated in an oven at 80°C for 15 min, 2 mL headspace was manually injected into the instrument. Five ions present in the mass spectrum of benzene were monitored. Their retention times and relative abundances in test portions were compared to those in the standards.

Results and Discussion

Method Development

For determination of benzene in polypropylene food-contact materials, a method was developed in which the polymer was dissolved to enable more efficient extraction of the analyte from the matrix. A benzene-free solvent was sought that would dissolve the polymer in a relatively short time and at a reasonable temperature. Hexadecane met these criteria. At 150°C, 10 g polypropylene dissolved in 70 mL hexadecane in 2–3 h. The weight of polymer to be analyzed (10 g) was chosen on the basis of a compromise between sensitivity and

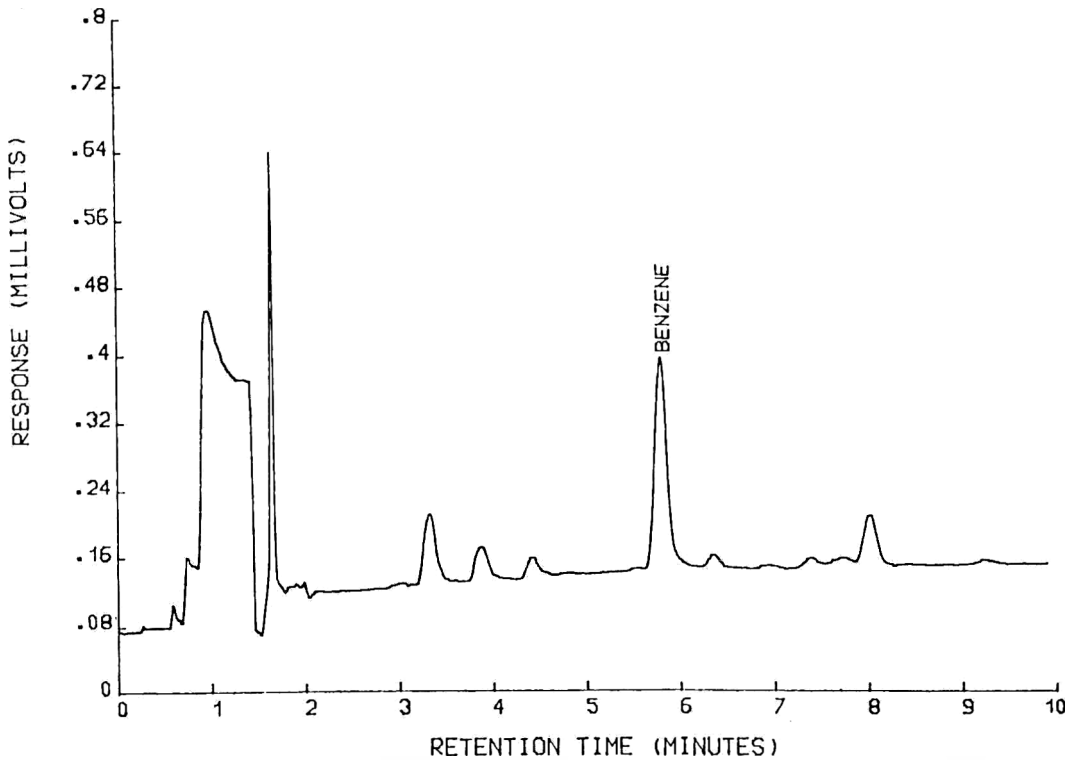

Figure 2. Headspace gas chromatogram of 20 ppb benzene standard, prepared in methanol–water (5 + 5). Column temperature was 30°C.

dissolution time. The polymer–solvent mixtures were held in the oven for 3 h to assure that all packaging pieces completely dissolved.

After the polypropylene was dissolved, the benzene concentration could be determined by HS-GC. However, direct headspace analysis of the polymer solutions resulted in a high detection limit (about 500 ppb) because the high solubility of benzene in hexadecane led to a low concentration in the headspace. To reduce the detection limit, a purge-and-trap method was developed to transfer the benzene from the polymer solution into a solvent in which it is less soluble, and to concentrate it in a small volume of solution.

A simple purge-and-trap apparatus was constructed (Figure 1). To prevent solidification, the polymer solution was placed in an oil bath heated to 150°C. The polymer solution and the methanol used as the trapping solvent were contained in bottles and vials sealed with aluminum-faced septa. (Teflon linings are typically used to prevent adsorption of volatiles into rubber septa; however, Teflon has been reported to adsorb low levels of benzene (4).) All connections were made with 1/16 in. (1.6 mm) stainless steel tubing. The tubing was pushed through the septa to form gas-tight connections. (The connections were tested to ensure that no leakage occurred.) A small piece of septum material was removed when the steel tubing was forced through. To prevent these plugs from blocking the connections, small holes were drilled in the sides of the tubing about 1/4 in. (6.4 mm) from both ends. The tubing connected to the polymer solution occasionally became blocked. This difficulty was overcome by using wide-bore tubing of 0.04 in. (1.0 mm) id and by closely monitoring the sparging operation. After each analysis, the tubing was rinsed with hot hexadecane followed by ethyl ether.

To trap the benzene that was sparged from the polypropylene, a solvent was needed that was not contaminated with benzene, that did not contain any interferences, and in which benzene was soluble. Methanol manufactured for purge-and-trap analysis was the solvent of choice. Two traps of 5 mL each were used to collect the purged benzene; small amounts of the chemical were found in the second volume of methanol. Deionized, distilled water was added to the collection vials after sparging to lower the solubility of benzene and thus increase the sensitivity of the method.

The resulting solutions were analyzed by HS-GC. The injection conditions were chosen on the basis of a compromise between detection limit and resolution, i.e., greater pressurization and longer injection times resulted in higher sensitivity but poor separation. A Megabore FSOT capillary column was used for separations. These columns have a larger capacity than smaller bore capillaries, and good resolution was obtained with headspace injections. A column temperature of 30°C was used for initial analyses. Because of interferences present in some of the polymer packaging products, the temperature was decreased to 25°C to improve resolution. To clean the column of any late-eluting compounds, the temperature was increased to 150°C at the end of each analysis.

A flame ionization detector (FID) was connected to the gas chromatograph for the initial method development work. The methanol–water (5 + 5) solvent yielded a large tailing peak that interfered with the measurement of benzene. To eliminate the solvent background, a photoionization detector was installed with a 10.2 eV lamp, which eliminated the detector response for water and methanol. This detector is also more sensitive than the FID for determination of aromatic hydrocarbons.

The GC system was equilibrated each day before analyses by injecting the headspace from 1 μL benzene in sample vial, followed by 3 headspace injections of 500 ppb benzene standards. Because the peak heights were still somewhat inconsistent, benzene standards were injected before each analysis of

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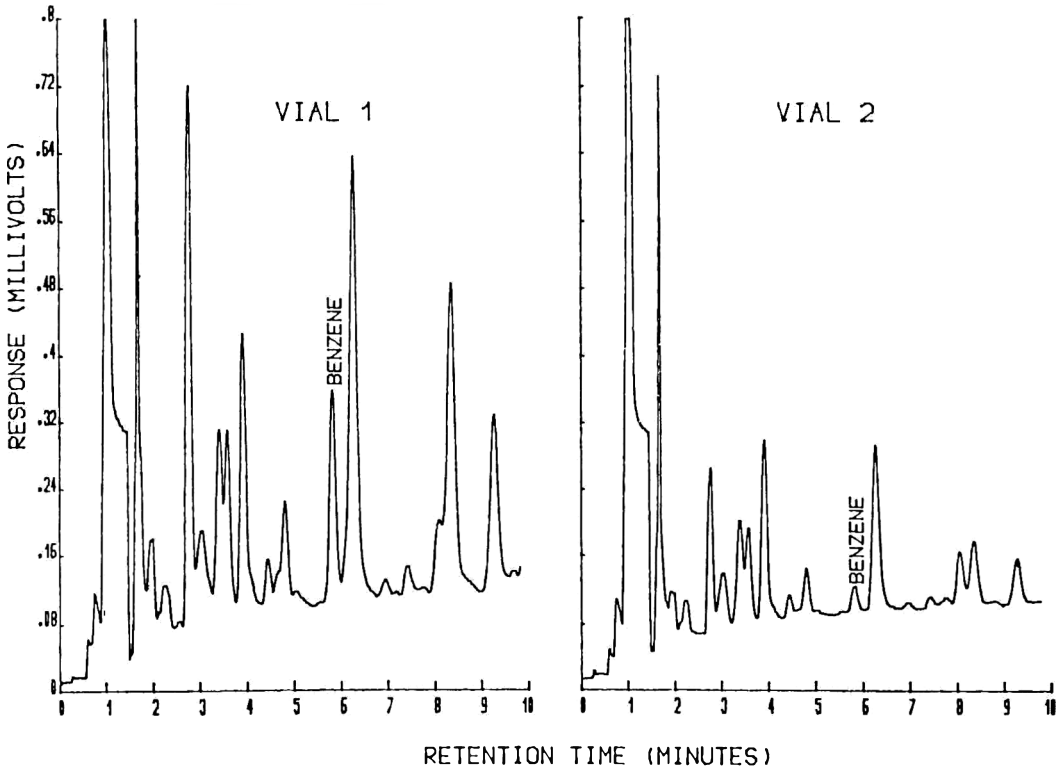

Figure 3. Headspace gas chromatograms from analysis of polypropylene pellets spiked with 20 ppb benzene. Column temperature was 30°C. Chromatograms labeled vial 1 and vial 2 represent analyses of first and second volumes of trapping solution, respectively.

packaging to enable more accurate quantitation. To calculate the total concentration of benzene, the heights of the peaks obtained for both trapping vials were summed.

Evaluation of Method

A headspace gas chromatogram of 20 ppb benzene in methanol–water (5 + 5) is shown in Figure 2. The retention time of benzene is 5.8 min with a column temperature of 30°C. (When the column is cooled to 25°C, benzene elutes in 6.8 min.) The other peaks represent contaminants present in the water. Analyses of methanol–water blanks showed no peaks at the retention time of benzene. Blanks of hexadecane that were analyzed by the method described above had levels of 3–5 ppb benzene. The calibration curves, prepared from external headspace standards, were linear over the concentration range examined–13 ppb to 1 ppm benzene.

The limit of detection (LOD) has been defined as the lowest concentration that is statistically different from an analytical blank. In accordance with the American Chemical Society (ACS) recommendation that this value be calculated as the blank signal plus 3 times its variability (5, 6), the LOD for determination of benzene in polypropylene by this method is 8 ppb. The concentration above which numerical results may be determined with a specified degree of confidence is the limit of quantitation (LOQ). The ACS suggests that this value be calculated as the blank signal plus 10 times its variability, which corresponds to an uncertainty of ±30% at the 99% confidence level (5, 6). The LOQ for determination of benzene in polypropylene by this method is 17 ppb.

Recovery studies were carried out at spiking levels of 130, 52, and 20 ppb benzene. Chromatograms from analysis of polypropylene pellets fortified with 20 ppb benzene are shown in Figure 3. As stated previously, a small peak is present at the retention time of benzene in the chromatogram for the second trapping vial. (Twenty-five percent or less of the benzene was in the second trapping vial. The percentage varied with the concentration of benzene.) The other peaks represent additional volatile compounds found in the polymer or reagent blank. Unfortified test portions of the polypropylene pellets had an average of 15 ppb benzene. The results of the evaluation are summarized in Table 1. Benzene recoveries from polypropylene ranged from 54 to 72%, with averages of 70% (coefficient of variation (CV) = 2.8%) at 130 ppb, 64% (CV = 5.5%) at 52 ppb, and 55% (CV = 1.4%) at 20 ppb. Benzene recoveries became somewhat lower as fortification concentrations decreased.

Benzene Levels in Polypropylene

A number of commercial polypropylene food-packaging materials were examined for benzene by the HS-GC method described here. (IR spectra confirmed that the containers were manufactured from polypropylene.) Chromatograms from the analysis of a whipped topping container are displayed in Figure 4. The heights of the peaks at the retention time of benzene correspond to a total concentration of ca 8 ppb.

Table 1. Recovery of benzene from spiked polypropylene pellets

Benzene added, ppb	Rec., %[a]
20	54, 56
52	69, 58, 64
130	72, 68

[a] Each value represents a single determination.

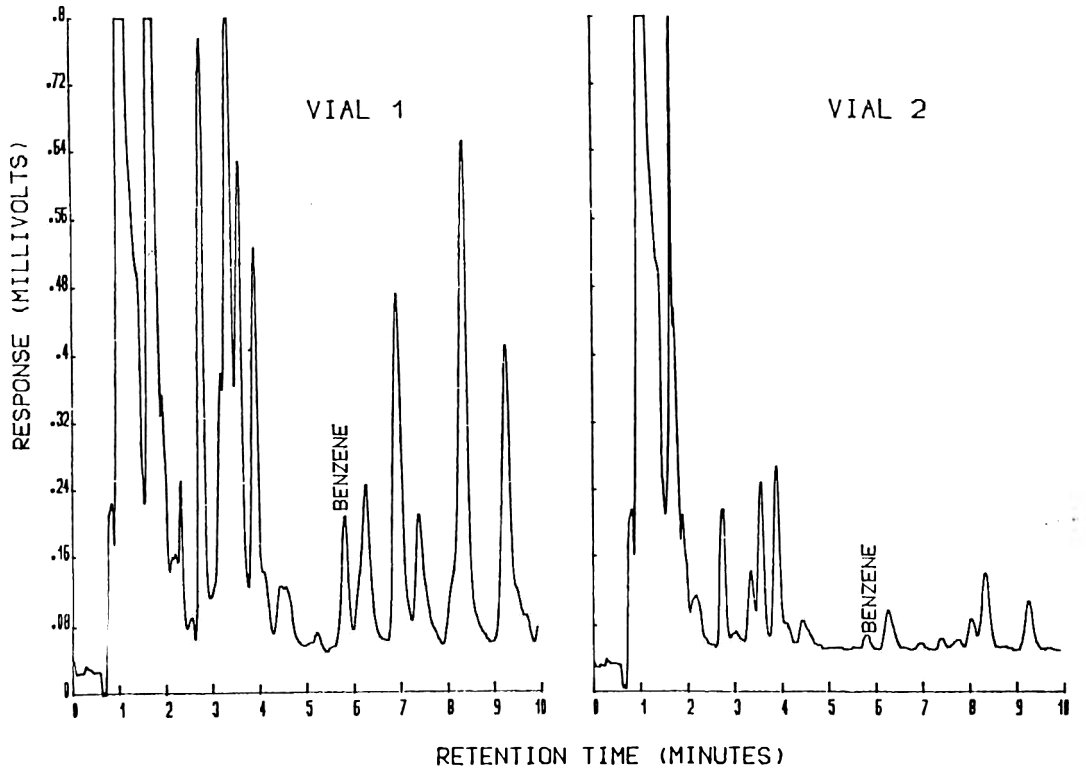

Figure 4. Headspace gas chromatograms from analysis of whipped topping container. Column temperature was 30°C. Chromatograms labeled vial 1 and vial 2 represent analyses of first and second volumes of trapping solution, respectively. The apparent benzene concentration is 8 ppb.

The number of extraneous peaks in the chromatograms varied between packaging products because of the different additives and manufacturing processes used, and/or because of the different components absorbed from the foods stored in the packaging. In all cases, the benzene peak was sufficiently resolved from the neighboring peaks to allow accurate peak height measurements and, hence, quantitation. Analyses of several blanks of hexadecane found 3–5 ppb benzene. (The peak height value was subtracted from values obtained for the unknowns before benzene concentrations were calculated.) Calibration curves were obtained on each day by analyzing 3 external headspace standards.

Apparent levels of benzene found in polypropylene packages are summarized in Table 2 (results are not corrected for recovery). Concentrations ranged from none detected to 515 ppb. The average level of benzene found in the packaging products was 133 ppb. Yogurt cups and dessert tubs appeared to contain the highest levels.

To confirm the identity of benzene, 8 of the packages were re-examined by MID GC-MS, as indicated in Table 2. Fresh test portions were analyzed. Ions at m/z 78, 77, 52, 51, and 50 were monitored. Retention time and relative abundance of the ions were determined by analyzing benzene standards; the concentrations of the standards were comparable to levels found in the polymer packaging by HS-GC. Blanks of methanol–water (5 + 5) were interspersed between standards and test portions to ensure the absence of carry-over from the previous injection.

Based on agreement of retention time and relative abundance for the ions monitored in the test portions compared with the same data for the standards, the identity of benzene was confirmed in the 8 polypropylene containers. Benzene concentration ranges for the packages were estimated by comparing responses observed for m/z 78 from known amounts of standard with responses recorded from the test portions. These approximate benzene levels were similar to those obtained by HS-GC for 5 of the food-contact materials. However, for the salad tubs previously determined to contain 515 and 385 ppb benzene, the MID MS data indicated lower concentrations (less than 100 ppb). The third salad tub, with 92.2 ppb benzene, was estimated to contain less than 50 ppb by GC/MS. Thus, because some polypropylene food packaging may contain a component(s) that elutes at the same time as benzene, the identity and concentration of benzene should be confirmed by MS.

Table 2. Benzene levels found in polypropylene food packaging

Product	Benzene level, ppb[a]
Syrup bottle A, B, C, D	(17), 23, ND, ND
Syrup bottle E, F, G,	ND, 52, 46
Whipped topping tub A, B, C	ND, (8), 60*
Yogurt cup A, B	426,* 256*
Dessert tub A, B	188, 370
Salad tub A, B, C	515,** 385,** 92**
Bottle caps A, B, C	63,* 165, 112
Catsup bottle	(15)
Cookie tray	111*

[a] Values in parentheses = concentrations >LOD (8 ppb) and <LOQ (17 ppb). Each value represents a single determination. ND = None detected.

* = Identity and concentration confirmed by MS.

** = MS analysis indicated lower concentrations (<100, <100, <50 ppb, respectively).

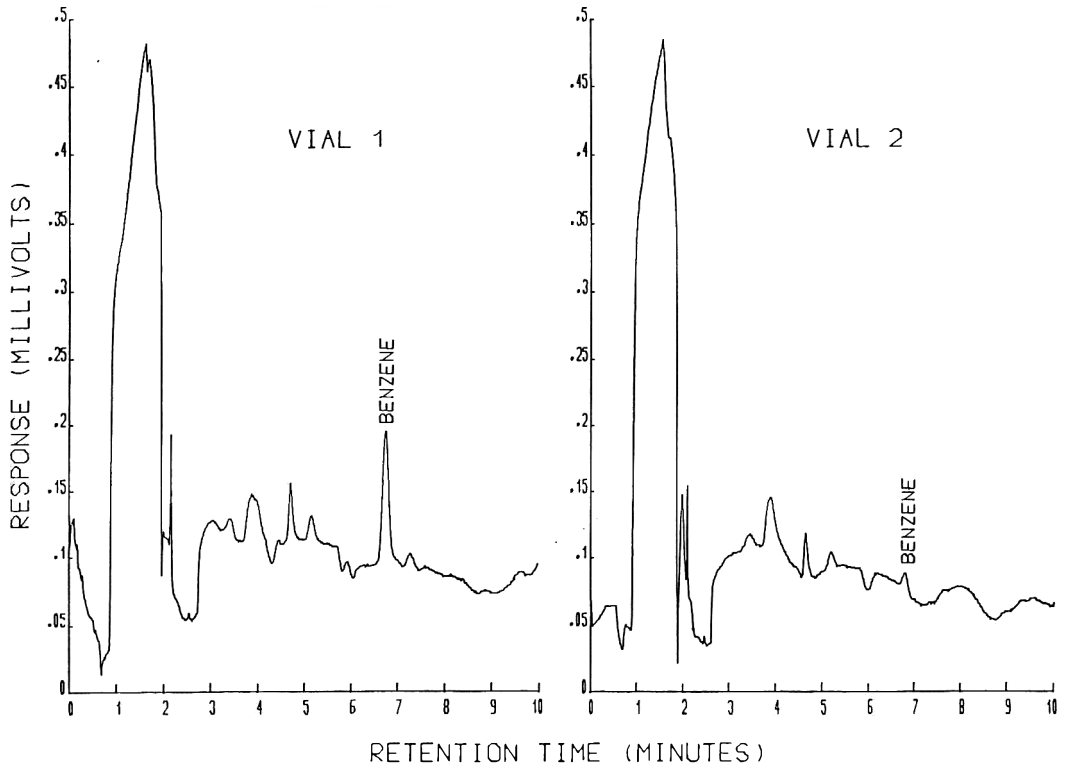

Figure 5. Headspace gas chromatograms from analysis of paraffin wax. Chromatograms labeled vial 1 and vial 2 represent analyses of first and second volumes of trapping solution, respectively. The apparent benzene concentration is 16 ppb.

Modification of Method for Paraffin Wax

The method developed for quantitation of benzene in polypropylene was modified for its determination in paraffin wax. Because of the low melting point of wax, a solvent did not have to be added to dissolve it. When placed in an 80°C oven, test portions melted in 30–60 min. After 1 h, the melted wax was taken out of the oven, and the benzene was removed from the wax by using the simple purge-and-trap apparatus shown in Figure 1. To prevent solidification, the wax was placed in an oil bath heated to 80°C. The test portions and the methanol used as the trapping solvent were in bottles and vials sealed with aluminum-faced septa. After the wax was sparged with helium for 1 h, vials containing the trapping solvent were disconnected, and deionized, distilled water was added. The 1/16 in. (1.6 mm) od tubing connected to the paraffin wax in the sparging set-up became blocked when cooled to room temperature after analysis; therefore, the tubing was rinsed with chloroform between determinations.

The solutions containing methanol–water (5 + 5) and purged volatiles were analyzed by HS-GC as described previously. The chromatographic column was conditioned each day by injecting standards containing high levels of benzene. Standards were also injected before analysis of each wax. To calculate the total concentration of benzene, the heights of the peaks obtained for both trapping vials were summed.

By defining the limit of quantitation as that concentration yielding a peak height 10 times the level of the background noise (5), 2 ppb benzene can be determined. This limit varied with the age of the detector lamp; with a new lamp, lower concentrations of benzene could be determined.

Recovery studies were carried out with fortifications at 101 and 504 ppb benzene. Because of the limited supply of paraffin wax, only 1 determination was conducted at each concentration. The amounts of benzene recovered were 67 and 73%, respectively. Recoveries are similar to those obtained for determination of benzene in polypropylene.

Commercial paraffin waxes were examined for benzene by the HS-GC method developed. Figure 5 shows chromatograms from 1 of the analyses. After peak heights obtained for both trapping vials at the retention time of benzene were added together, the wax was calculated to contain 16 ppb of the contaminant. Only a few other peaks appear in the chromatograms: These peaks are minor and do not appear close to the retention time of the component of interest.

Apparent levels of benzene found in the 3 paraffin waxes are summarized in Table 3. Concentrations ranged from 16 to 73 ppb, with levels of 61, 18 (av.), and 69 (av.) ppb for the 3 waxes, respectively. MID GC-MS was used to confirm the identity of benzene, as described previously. Ions at m/z 78, 77, 52, 51, and 50 were monitored. The retention time and relative abundance of the ions were determined by analyzing benzene standards at comparable concentrations. These data agreed with those obtained for the test portions; thus, the presence of benzene was confirmed in the 3 paraffin waxes that were examined.

Table 3. Benzene found in food-contact paraffin waxes

Wax	Benzene found, ppb[a]
1	61
2	16, 20
3	65, 73

[a] Each value represents a single determination.

Conclusions

This method, which combines a purge-and-trap procedure and HS-GC, can determine low parts-per-billion levels of benzene in polypropylene food packaging. The method was used to analyze a number of items, including syrup bottles, yogurt cups, salad and dessert tubs, bottle caps, and cookie trays. Benzene concentrations ranged from none detected to 426 ppb.

The method was modified to determine low parts-per-billion levels of benzene in paraffin waxes. Three commercial paraffin waxes were analyzed, and concentrations of 16–73 ppb were found in the test portions examined.

The method could possibly be adapted to determine benzene in other materials with the limitation that they be in liquid form.

References

(1) Withey, J.R. (1976) *Environ. Health Perspect.* **17**, 125–133
(2) Varner, S.L., & Breder, C.V. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 516–520
(3) Dennison, J.L., Breder, C.V., McNeal, T., Snyder, R.C., Roach, J.A., & Sphon, J.A. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 813–819
(4) Keeley, D.F., Hoffpauir, M.A., & Meriwether, J.R. (1986) *Anal. Chem.* **58**, 1258
(5) ACS Committee on Environmental Improvement (1983) *Anal. Chem.* **55**, 2210–2218
(6) ACS Committee on Environmental Improvement (1980) *Anal. Chem.* **52**, 2242–2249

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Dioxins and Furans in Foods and Biological Tissues: Review and Update

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Determination of trace residues of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs and PCDFs) in various matrixes is carried out by a limited number of laboratories in the United States, Canada, and other countries. Current methods for analysis of foods and biological tissues include a combination of preparation, extraction, cleanup, isolation, determination, and identity confirmation procedures. Soxhlet, liquid/liquid, solid-phase, and column extraction procedures are used as well as treatment with acid or base before solvent extraction. Cleanup and isolation steps include sulfuric acid partitioning; adsorption chromatography on Florisil, silica gel, or alumina; gel permeation chromatography; multistage column chromatography on sulfuric acid silica and alkali silica; carbon column chromatography; and liquid chromatography fractionation with size exclusion, normal-phase, and reverse-phase columns. Activated carbon and multistage chromatographic columns are widely used in cleanup schemes. Isomer-specific identification and quantitation of PCDD and PCDF congeners at parts-per-trillion levels or lower are carried out by high resolution (capillary) gas chromatography (HRGC) and multiple ion detection mass spectrometry. In addition to chemical methods, bioassay procedures have been recommended (e.g., use of monoclonal antibodies, for immunoassay determination of PCDDs and PCDFs).

The formation and environmental distribution of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are of great interest because of the toxicity and persistence of some PCDD and PCDF congeners. Also, recent reports have indicated that the food chain is the major source of human exposure to these compounds (1, 2). Because quantitation at extremely low levels is required, a variety of combinations of multistage chromatographic cleanup procedures, capillary high resolution gas chromatography (HRGC), and high or low resolution mass spectrometry (HRMS, LRMS) or tandem mass spectrometry (MS/MS) have been developed to confirm the presence of sub-parts-per-trillion (sub-ppt) as well as ppt levels of various congeners in a variety of matrixes.

A variety of procedures for determination of PCDDs and PCDFs in foods and biological tissues involve a series of complex extraction and cleanup procedures before quantitation and confirmation by GC/MS (3-7). GC/MS systems with multiple ion detection (MID) are generally used for analyte quantitation and confirmation. A survey conducted by the Ontario Ministry of the Environment (8) showed that a number of different extraction, cleanup, and GC/MS techniques were being used to examine various environmental materials. Many cleanup methods were based on combinations of acidic and basic silica and alumina column chromatography. The use of carbon column chromatography was increasing. Most laboratories reported that cleaned up extracts were analyzed by HRGC/LRMS. Their principal quality control procedure was adding stable isotope-labeled standards to test samples before extraction and determining the percentage of recovery of the standards after analysis.

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Rappe (9) noted the following requirements for generating good analytical data: (*1*) representative sampling, (*2*) high selectivity, (*3*) high specificity, (*4*) high sensitivity, (*5*) reliable quantification, (*6*) good reproducibility, and (*7*) confirmation. He pointed out that, although many complex cleanup systems are used to extract PCDDs and PCDFs, very few studies have compared the different systems. With suitable cleanup, the level of quantitation of PCDDs and PCDFs depends on the MS technique used and the level of chlorination. Quantitation levels for octachlorinated congeners are generally 10 times those for the tetra- and pentachlorinated congeners. Rappe observed that a low resolution mass spectrometer operating with single ion monitoring (SIM) and electron ionization (EI) has a quantitation level in the range of 1-10 pg/injection. Quantitation levels of 0.05-2 pg/injection can be obtained with high resolution mass spectrometers operating in the EI mode. According to Rappe, congener identity confirmation requires that (*1*) isomer specificity must be demonstrated initially and verified daily; (*2*) GC retention time must be within ±3 s of the retention time of the $^{13}C_{12}$-labeled congener, which normally elutes before the $^{12}C_{12}$ (unlabeled) compound; (*3*) all isomers must elute within a defined elution window; (*4*) the signal-to-noise ratio must exceed 3; (*5*) the relative intensities of ions in the chlorine cluster must be within ±10% of the theoretical values; (*6*) fragment ions must display the correct chlorine cluster ratios; (*7*) HRMS data should confirm the empirical formula; and (*8*) known interferences (artifacts) should be eliminated.

Hass and Friesen (10) reviewed methods for determination of PCDDs in 1979. They concluded that the most sensitive and specific methods required efficient cleanup, determination by capillary HRGC, and identification by negative ion chemical ionization (NICI) as well as EI-MS. Crummett (11), who outlined the status of analytical systems for determination of PCDDs and PCDFs in 1983, observed that many extraction-cleanup and GC/MS systems were available. Selection of specific methodology depended on the matrix to be analyzed and the sensitivity, specificity, and degree of certainty required. Available techniques could be combined in an almost infinite number of ways to produce methods that seem to be reliable. Because generally acceptable validation studies were seldom carried out, quality assurance programs were needed.

Afghan et al. (12) described advances in ultratrace determination of PCDDs and PCDFs in various matrixes. Several extraction methods and cleanup procedures included the following techniques: gel permeation chromatography (GPC), liquid-liquid extraction with Na_3PO_4, liquid-liquid extraction with H_2SO_4, basic alumina column chromatography, and carbon column chromatography. Noting that EI at 70 eV was used most widely in conventional MS analyses, the

authors suggested that H_2-NICI would provide enhanced sensitivity for penta- to octachlorinated PCDDs.

Because a variety of MS techniques have been used for determination of PCDDs and PCDFs, Clement et al. (13) organized a "round robin" study to determine the feasibility of using HRGC/MS/MS with reduced cleanup for analysis of environmental materials (e.g., fish and sediments that contain many coextractants). Fish extracts were subjected to 3 degrees of cleanup. Minimal cleanup involved only lipid removal (passage through a column of 44% H_2SO_4 on silica). Intermediate cleanup involved lipid removal plus cleanup with a dual-column system (H_2SO_4-silica/NaOH-silica and $AgNO_3$-silica/alumina). Full cleanup included use of reverse-phase (C-18) liquid chromatography (LC) after intermediate cleanup. The authors reported that the level of cleanup needed depends on the matrix, the specific PCDD/PCDF congeners determined, and the instrumentation used. Some extracts contained such a high level of chemical interference that even highly selective systems such as HRGC/MS/MS required more than minimal cleanup. Another "round robin" (14) comparing HRGC/MS/MS with reduced cleanup to HRGC/LRMS and HRGC/HRMS showed that GC/MS/MS with minimal pretreatment was suitable for analysis of environmental materials such as fish or soil. However, the procedure should be validated by comparative MS analysis of selected, fully cleaned up test portions.

High sensitivity EI sources developed recently for magnetic instruments permit improved sensitivity, made possible by lowering the emission current (<1 mA) and ionization energy (ca 28–40 eV) and increasing the trap current (>500 μA) in the ion source. These modifications produce a lower level of helium ionization, which leads to an increase in the signal-to-noise ratio. Alexander et al. (15) installed a new, high-sensitivity, EI-only ion source (VG Analytical, Ltd.) in VG ZAB-2F, 70/70E, and 70S high resolution mass spectrometers used to measure PCDDs and PCDFs in human serum and plasma. Levels of 20 fg on column (signal-to-noise ratio >3:1) were routinely determined using the new source, and the linearity range extended into the lower femtogram levels. Source operation included use of 500 μA trap current and 28–36 eV ionization energy.

Charles et al. (16) showed that the sensitivity of HRGC/MS/MS for determination of PCDDs and PCDFs was enhanced at increased collision gas pressures and at lower collision energies, with collision energy optimal in the range of 20–40 eV. McCurvin et al. (17) also reported greatly enhanced sensitivity with HRGC/MS/MS (Finnigan MAT TSQ-70 triple quadrupole mass spectrometer) vs HRGC/LRMS (Finnigan MAT 4500 quadrupole mass spectrometer). In addition, McCurvin et al. (18) compared the capabilities of GC/LRMS, GC/HRMS, and GC/MS/MS for PCDD/PCDF determinations. HRMS achieved the lowest limits of detection (5:1 signal-to-noise peak) at 200–400 fg. MS/MS achieved comparable detection limits, while LRMS quadrupole systems displayed detection limits that were 1–2 orders of magnitude higher.

Because analytical methodology for trace residues of PCDDs and PCDFs is not standardized, current analytical schemes used for analysis of foods and tissues were reviewed. In 1988, inquiries were mailed to 55 laboratories concerning their procedures for determination of PCDDs and PCDFs in foods and biological tissues. Responses as well as selected reports published in recent years were reviewed to document extraction, cleanup, and quantitation techniques and to record analytical schemes used for various food and biological matrixes.

Discussion

Responses were received from 18 laboratories in the United States, Canada, Europe, and Japan that were analyzing foods and/or tissues for PCDD/PCDF residues. The various extraction, cleanup, quantitation, and confirmation techniques used in these laboratories and reported in the recent literature by other laboratories are shown in Table 1. The number of laboratories using each of these techniques is shown in Table 2. A binary solvent system, usually hexane–methylene chloride, cyclohexane–methylene chloride, or hexane–acetone, was most frequently used for extraction.

Table 1. Extraction, cleanup, and quantitation techniques[a]

Extraction techniques
1. Soxhlet extraction
2. Moderate heating or reflux with alkali followed by solvent extraction
3. Room temperature digestion with HCl followed by solvent extraction
4. Room temperature digestion with alkali followed by solvent extraction
5. Solvent extraction
6. Solid-phase (column) extraction with solvent
7. Steam distillation extraction
8. Supercritical fluid (CO_2) extraction
Preliminary cleanup techniques
a. Liquid-liquid partitioning
b. Adsorption chromatography on Florisil
c. Adsorption chromatography on silica gel
d. Adsorption chromatography on alumina
e. Gel permeation chromatography (GPC) (Bio-Beads S-X3, etc.)
f. Size-exclusion liquid chromatography (LC)
g. Bulk lipid removal by H_2SO_4 oxidation (column or liquid partitioning)
h. Alkali or trisodium phosphate wash
i. Multistage column chromatography (H_2SO_4-silica/alkali-silica, etc.)
j. Silver nitrate-silica column cleanup
k. Alkali-silica column cleanup
Isolation techniques
A. Carbon column chromatography
B. Multistage column chromatography
C. Adsorption chromatography on Florisil
D. Adsorption chromatography on alumina
E. Normal phase LC
F. C-8 reverse-phase LC
G. C-18 reverse-phase LC
H. GC cleanup
Quantitation/confirmation techniques
I. HRGC/EC
II. HRGC/LRMS
III. HRGC/HRMS
IV. HRGC/mass selective detector
V. HRGC/MS/MS
VI. HRGC/EC and 2 capillary columns of differing polarity

[a] Variation in numbering systems for the sets of techniques within this table allows identification of each technique under the column labeled "Scheme" in Table 3.

Table 2. Laboratories using various extraction, cleanup, and quantitation/identity-confirmation techniques

Technique	No. of laboratories
Extraction	
Steam distillation extraction into hexane	1
Extraction with organic solvents	
Acetonitrile	1
Benzene or toluene	3
Diethyl ether	1
Methylene chloride	1
Hexane or pentane	9
Binary solvent systems	20
Multiple solvent systems	5
Acid or base treatment before solvent extraction	
Concentrated HCl (room temperature)	5
Strong alkali with heat	4
Strong alkali at room temperature	3
Cleanup	
Sulfuric acid extraction	23
Adsorption chromatography	
Alumina	32
Silica	8
Florisil	7
Acid silica	18
Basic silica	18
Silver nitrate silica	6
Carbon column	29
GPC	8
Reverse-phase LC	6
Normal-phase LC	2
Preparative gas chromatography	1
Quantitation/identity-confirmation	
HRGC/LRMS	24
HRGC/HRMS	13
HRGC/mass selective detector	3
HRGC/MS/MS	3
HRGC/EC	4
HRGC/EC (with 2 capillary columns of differing polarity)	1

Treatment with acid or base followed by solvent extraction and solid-phase (column) extraction was also used as an initial treatment. Preparation of the test portion by using hot ethanolic alkali, which has been long employed in the determination of PCDDs and PCDFs, decomposes these compounds (19-21). The higher the degree of chlorination of PCDDs or PCDFs, the greater the degradation. 2,3,7,8-TCDD, however, is stable in hot alkali. Jasinski (Food and Drug Administration, Detroit, MI, personal communication, 1988) observed that alkali digestion at room temperature decomposes PCDFs (lower chlorinated congeners are more stable than higher chlorinated congeners), although PCDDs are stable.

Cleanup techniques include sulfuric acid partitioning and column chromatography with various adsorbents. GPC, reverse-phase LC, and carbon column chromatography can be automated. Multistage chromatographic columns with acid- and alkali-silica and activated carbon columns are widely used in various cleanup schemes that are modifications of the procedures proposed by Smith et al. (22) and/or Lamparski et al. (23).

Niemann et al. (24) described a procedure for determination of ppt residues of 2,3,7,8-TCDD in fish that involved test portion digestion in base, extraction with hexane, sulfuric acid wash of the hexane extract, 3 LC cleanups using size exclusion, C-8, and C-18 LC columns to remove interfering coextractive substances, and quantitation by capillary GC with electron capture detection. GC/MS with MID that monitored 12 ions including the fragment ion cluster resulting from loss of COCl was used to confirm the presence of 2,3,7,8-TCDD in the extract. Fehringer et al. (25) modified the methods of Niemann et al. (24) and Lamparski et al. (23) and used the combined method to survey fish from the Great Lakes and selected Michigan rivers for 2,3,7,8-TCDD residues. Thompson et al. (26) used 2 LC fractionations for determination of 2,3,7,8-TCDD in fish tissue. Twenty gram portions of homogenized tissue were digested overnight at room temperature with concentrated hydrochloric acid; digests were extracted with hexane, and extracts were cleaned up on a sulfuric acid-silicic acid column. The extracts were then fractionated by normal-phase silica LC and then by normal-phase alumina LC before analysis by GC/MS.

Sherry and Tse (27) carried out an investigation of extraction-cleanup procedures for determination of PCDDs in fish, designed to improve efficiency without compromising performance. Solid-phase extraction was as efficient as liquid-phase acidic (HCl) extraction, while using less equipment and glassware and permitting 16 extractions a day/lone operator vs about 6 for liquid-phase extraction. Elimination of acid/base treatments or basic alumina chromatography compromised analytical performance. Use of a multilayer cleanup column (sulfuric acid-silica/potassium hydroxide-silica/silver nitrate-silica) for single step removal of several interference types was less time-consuming than liquid-phase acid/base treatments and allowed extract processing without emulsions. A method consisting of the following procedures was developed: solid-phase (neutral column) extraction, GPC multilayer column chromatography, basic alumina chromatography, and determination by HRGC/LRMS. The method was evaluated by analyses of a variety of fish. Results indicated acceptable performance with $^{13}C_{12}$-TCDD recoveries of 75% or greater (mean recovery for 10 fish determinations, 101% at the 50 pg/g level).

LC and GPC have not been used as much as standard column chromatographic techniques for analyte cleanup before quantitation by capillary column HRMS or LRMS. Lindstrom and Rappe (28) used a typical method for determining low or sub-ppt levels of PCDDs and PCDFs in milk. The extraction-cleanup procedure, a modification of the method of Smith et al. (22), involves a solvent extraction with ethanol-ether-hexane (2 + 1 + 2), chromatography on a potassium silicate-silica gel column, cleanup on an activated carbon-glass fiber column, chromatography on a basic silica-acidic silica column followed by chromatography on an acidic aluminum oxide adsorbent, and then quantitation of the PCDD and PCDF fraction by capillary HRGC/HRMS with SIM. Interlaboratory recovery and validation studies were performed and reported by these authors. Nam et al. (29) investigated the use of supercritical fluid extraction (SFE) with carbon dioxide as a solvent near its critical point for rapid extraction and determination of TCDD and other xenobiotics from biological tissues.

Table 3 outlines current analytical schemes. Limits of quantitation are generally in the range of 1-10 pg/g. Current methods for determination of 2,3,7,8-TCDD, 2,3,7,8-TCDF, and their most biologically active 2,3,7,8-substituted congeners are isomer specific as verified with available PCDD and PCDF standards.

Table 3. Analytical schemes for determination of PCDDs and PCDFs

Matrix	Analyte	Scheme[a]	Approx. limit of detection, pg/g	Reference no.
Fish, tissue	PCDDs, PCDFs	6-k-k-A-B-D-II	1–5	(22)
Fish, adipose, etc.	PCDDs, PCDFs	3-i-j-d-G-E-II-III	0.2–21	(23, 30)
Fish	2,3,7,8-TCDD	4-g-h-f-F-G-I-II	3	(24, 31)
Fish	2,3,7,8-TCDDs; PCDDs	2-h-g-d-A-III	1–5[b] 20–100[c]	(32)
Fish	PCDDs, PCDFs	1-i-A-III	1,[b] 2–40[c]	(33)
Fish	PCDDs, PCDFs	1-g-h-d-A-III	low	(34)
Fish, tissue	PCDDs	5(or 6)-e-A-C-D-II	low	(35, 36)
Fish	PCDDs	3-e-h-g-d-A-I-II	low	(37)
Fish, tissue	PCDDs	3-e-h-g-d-A-IV-II-III	low	(38)
Fish	2,3,7,8-TCDD	5-g-j-(D-A-D)[d]-B-IV-III	low	(39, 40)
Fish	PCDDs, PCDFs	1-g-h-i-D-A-III	low	(41)
Fish	2,3,7,8-TCDD	3-g-E-E-II	low	(26)
Fish	PCDDs	6-e-i-D-A-II	low	(27)
Chicken, pork, fish, adipose	PCDDs, PCDFs	5-g-b-(G)-V-III	2–4, 10–20 (OCDD)	(42, 43)
Fruits and vegetables	PCDDs, PCDFs	5-e-c-k-A-B-D-III	<1	(44)
Fish and meat	PCDDs, PCDFs	6-e-c-k-A-B-D-III	<1 (fat basis)	
Food	PCDDs, PCDFs	5-e-C-D-A-II	0.5	(45)
Freeze-dried vegetation	PCDDs, PCDFs	6-A-B-C-B-III	<1	(46)
Food, human tissue	PCDDs, PCDFs	4-i-j-C-D-II	low	(47, 48)
Food, human tissue	PCDDs, PCDFs	2-g-a-D-II	10	(49)
Food, milk, tissue	PCDDs, PCDFs	5-g-b-A-V-III	1–10	(50–53)
Human tissue	PCDDs, PCDFs	2-g-h-c-d-H-A-II	low	(54, 55)
Food	PCDDs, PCDFs	2-g(or 5)-h-c-d-H-A-H-II	low	
Biological tissue	PCDDs, PCDFs	1-g-g-A-D-VI-II	—	(56)
Biological tissue	PCDDs, PCDFs	5-g-B-A-D-II	low	(57)
Biological tissue	TCDD	8-g-A-II	low	(29)
Wildlife tissue	PCDDs	5-g-b-d-f-G-II	ng/g and sub-ng/g	(58)
Wildlife tissue	PCDDs, PCDFs	1-g-g-A-D-I-II	10	(59)
Animal tissue	2,3,7,8-TCDD	7-G-IV	5	(60)
Eggs, biological tissue	PCDDs, PCDFs	5-6-B-A-II	low	(61)
Blubber, Arctic	PCDDs, PCDFs	e-C-D-A-II	<1	(62)
Human tissue	PCDFs	2(or 5)-e-g-A-C-II	<1	(63)[e]
Human adipose tissue	2,3,7,8-TCDD	5-g-k-c-A-B-D-III	3–5	(64, 65)
Human serum	2,3,7,8-TCDD	6-k-c-A-B-D-III	1.25 ppq (200 g test portion)	
Human adipose tissue	2,3,7,8-TCDD, 2,3,7,8-TCDF	2-g-c-d-A-III	low	(66)[f]
Human adipose tissue	PCDDs, PCDFs	5-g-g-d-A-II-III	low	(67)
Human adipose tissue	PCDDs, PCDFs	5-g-A-III	ca 1	(68)
Human serum	PCDDs, PCDFs	6-k-A-B-D-III	0.003–0.02	
Human adipose tissue; liver	PCDDs, PCDFs	g-i-k-III	<1	(69)
Animal tissue	PCDDs, PCDFs	6-d-A-III	0.05	(70)
Human milk	PCDDs, PCDFs	5-e-C-D-A-II	1	(71)
Human milk	PCDDs, PCDFs	e-d-c-e-D-D-III	<1	(72)
Human milk	PCDDs, PCDFs	5-g-i-j-G-IV-III	<1	(73)
Human milk	PCDDs, PCDFs	5-k-A-D-B-D-II	low	(74)
Human milk	PCDDs, PCDFs	5-A-D-V	1–10 (fat basis)	(75)
Human milk	PCDDs, PCDFs	5-A-D-III	0.1–1 (fat basis)	(76)
Human and cow's milk	PCDDs, PCDFs	5-k-c-A-B-D-III	<1 (fat basis)	(77)
Human and cow's milk	PCDDs, PCDFs	5-k-A-i-D-II-III	<1	(28, 78–80)
Cow's milk	2,3,7,8-TCDD, 2,3,7,8-TCDF	5-g-g-i-b-k-d-F-G-I-II	<0.1	(81)
Cow's milk	PCDDs, PCDFs	5-g-k-k-d-A-III	<0.01	(82)

[a] Refer to Table 1 for technique.
[b] TCDD/TCDF.
[c] PCDD/PCDF other than TCDD/TCDF.
[d] Semi-automated.
[e] Also Masuda, Y., Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan, personal communication, 1988.
[f] Also Tong, H., University of Nebraska, Lincoln, personal communication, 1988.

Either HRGC/HRMS or HRGC/LRMS is generally used to quantitate PCDDs and PCDFs and confirm their presence in extracts. General criteria for HRGC/LRMS isomer-specific analysis are listed in Table 4. U.S. Environmental Protection Agency (EPA) criteria for HRGC/HRMS confirmation are shown in Table 5. Supplemental

Table 4. Criteria for HRGC/LRMS isomer-specific analysis

General criteria
1. Efficient extraction and cleanup to assure adequate removal of interfering coextractives and recovery of analyte.
2. LC retention windows for specific congeners that also provide good recovery of analytes.
3. Correct HRGC retention time compared with that of corresponding reference standard. The 2,3,7,8-substituted dioxins/furans must be separated from their non-2,3,7,8-substituted isomers.
4. Selective ion monitoring; correct nominal mass of monitored ions.
5. Signal-to-noise ratio ≥2.5.
6. Chlorine isotope ratios within 20% (10% for 2,3,7,8-TCDD) of correct values for at least 3 members of molecular ion cluster.
7. Coincidental maxima of 3 or more selective ion scans of individual members of molecular ion cluster.
8. Presence of MS fragment ion from loss of COX from parent ion.
U.S. Food and Drug Administration criteria for 2,3,7,8-TCDD
1. Demonstration that cleanup is sufficient so that no other TCDD isomer or interfering coextractive coelutes (HRGC) with 2,3,7,8-TCDD.
2. HRGC/EI-MS monitoring of 12 ions at *m/z* 257, 259, 261, 305, 307, 320, 321, 322, 324, 326, 332, and 334 (the last 2 ions from the molecular cluster of the $^{13}C_{12}$-2,3,7,8-TCDD internal standard).
3. Abundance of ions at *m/z* 320, 324, 257, and 259 relative to the base peak at *m/z* 322 should be ±10% of those abundances observed with standards for the same amount of 2,3,7,8-TCDD injected.
4. Relative abundances of *m/z* 257 and 259 are ca 50% of base peak.
5. No interferences should be observed on ions at *m/z* 305, 307, and 321.
6. The ion at *m/z* 321 should display normal isotope abundance relative to *m/z* 320.

criteria for TCDD confirmation include loss of COCl. However, fragment ions at *m/z* 257 and 259 cease to be detectable at low femtogram levels, as would be the case in analysis of serum for TCDD. Commercially available ^{13}C- or ^{37}Cl-labeled PCDDs and PCDFs are used as internal standards.

Quality assurance/quality control plans are important elements of any analytical program. However, most reports of PCDD and PCDF data do not describe data validation and/or quality control procedures. EPA quality assurance/quality control requirements for analytical data are outlined in Table 6. Both recovery analytes and internal standards for MS analysis are required in addition to data from analysis of method blanks, fortified matrixes, duplicate test portions, and reference environmental test samples. In addition, periodic interlaboratory studies must be carried out to check data comparability. These studies generally involve distribution of blind, coded, environmental reference test samples.

Analysis of Milk and Other Foods

The U.S. Food and Drug Administration recently developed an analytical method capable of determining 2,3,7,8-TCDD and 2,3,7,8-TCDF in whole milk at parts-per-quadrillion (ppq) levels (81). This effort was undertaken to carry out a domestic survey following Canadian reports of low levels of 2,3,7,8-TCDD and 2,3,7,8-TCDF in milk packaged in polyethylene-coated bleached paperboard cartons. The cleanup steps involve extraction of 1 L test portions of milk plus internal standard with acetone–hexane (6.25 + 1), hexane elution from a sulfuric acid–silica gel column followed by shaking with concentrated sulfuric acid (to defat

Table 5. U.S. EPA analytical criteria for HRGC/HRMS confirmation of 2,3,7,8-TCDD and other CDDs/CDFs (32)

Criteria for 2,3,7,8-TCDD
1. Correct HRGC/HRMS retention time of 2,3,7,8-TCDD (±3 s) on a 2,3,7,8-TCDD isomer-specific column relative to the labeled internal standard. The 2,3,7,8-TCDD isomer must be separated from all other isomers. GC peak resolution should not exceed 35% valley for TCDD isomers of equal concentrations eluting before and after 2,3,7,8-TCDD.
2. Correct chlorine isotope ratio of molecular ion, *m/z* 320/322 (0.67–0.87).
3. Correct HRGC/HRMS multiple ion monitoring response for 2,3,7,8-TCDD masses and $^{13}C_{12}$- or $^{37}Cl_4$-TCDD mass (simultaneous response, ±3 s, for elemental compositions *m/z* 320, 322, and 334 or 328).
4. Response of *m/z* 320/322 must be greater than 2.5 times the noise level.
Supplemental criteria
a. COCl loss indicative of TCDD structure.
b. HRGC/HRMS peak matching analysis of *m/z* 320/322 in real time to confirm exact masses that correspond to TCDD elemental compositions.
Criteria for other CDDs and CDFs
1. HRGC/HRMS retention time of specific CDD/CDF isomer available relative to the labeled internal standard.
2. HRGC/HRMS retention time window of respective CDD or CDF series of isomers based on reference extract.
3. Chlorine isotope ratio of molecular ions of respective CDD or CDF isomers within ±20% of theoretical values (penta-1.54, hexa-1.23, hepta-1.03, octa-0.88).
Supplemental criteria
a. HRGC/HRMS peak matching analysis of molecular ions in real time to confirm exact masses corresponding to elemental compositions of respective CDDs or CDFs.
b. Comparison of test sample analysis with that of reference which contains all tetra- through octa-CDDs and CDFs.
c. HRGC/HRMS analysis to confirm absence of specific chlorinated diphenyl ethers at appropriate retention times.

Table 6. U.S. EPA quality assurance/quality control requirements for analytical data (32)[a]

Criteria	Requirements
Method efficiency achieved for $^{13}C_{12}$-2,3,7,8-TCDD	50–120%
Method efficiency achieved for $^{13}C_{12}$-OCDD	40–120%
Analytical criteria used for confirmation of 2,3,7,8-TCDD and other CDDs and CDFs	Satisfies specified criteria
Accuracy and precision for 2,3,7,8-TCDD and other specified CDDs and CDFs in laboratory-fortified sample or control	50–150% at >6 × MDL may have greater variability
Method blank and matrix blank free of CDD and CDF contamination at target minimum limits of detection	See below

Compounds		Target minimum LOD for fish, pg/g/isomer
2,3,7,8-TCDD	2,3,7,8-TCDF	1–5
21 TCDD isomers	37 TCDF isomers	1–5
14 Penta-CDDs	28 Penta-CDFs	20–40
10 Hexa-CDDs	16-Hexa-CDFs	30–60
2 Hepta-CDDs	4 Hepta-CDFs	40–80
OCDD	OCDF	50–100

[a] Each set of data must satisfy the criteria shown above.

the extract), chromatography on a multistage (sulfuric acid–silicic acid/sodium hydroxide–silicic acid/silicic acid) column mounted over a Florisil column, chromatography on a cesium hydroxide–silicic acid/sulfuric acid–silicic acid multistage column over an acid alumina column, and finally C-8 and C-18 LC. Analytes were determined by HRGC/EI-LRMS with MID (3 molecular ions, 2 COCl fragment ions, and 2 ^{13}C-2,3,7,8-TCDD or 2,3,7,8-TCDF internal standard molecular ions). For 2,3,7,8-TCDD, quantitation was accomplished by using ion chromatograms for m/z 320 (analyte) and 332 (internal standard) and for m/z 322 (analyte) and 334 (internal standard). The mean of the 2 intensity values was then calculated. Similarly, 2,3,7,8-TCDF quantitation was carried out for m/z 304 and 306 (analyte) and m/z 316 and 318 (internal standard), and the mean of the 2 values obtained was calculated and corrected for the response of the reagent blank extract. Ion chromatograms obtained for quantitation were also used to calculate analyte recoveries. This method was able to quantitate and confirm the presence of ≥20 ppq 2,3,7,8-TCDD in milk.

The New Zealand Department of Health also conducted a survey of PCDDs and PCDFs in retail milk supplies in early 1989 (82). They based their analytical method on the procedures of Smith et al. (22), Ryan and Pilon (83), and Stanley et al. (84). A test portion of fixed volume was fortified with ^{13}C-labeled standards and extracted with acetone–hexane (2 + 1) and hexane. The extract was defatted by partitioning with concentrated sulfuric acid (83) and then was purified by a series of chromatographic columns of silica and alumina (22) and carbon on Celite (84). The resulting extract was dissolved in 10 μL of toluene containing octachloronaphthalene standard. Quantitation was carried out by capillary (30 m DB-5) GC on a high resolution mass spectrometer. Two ions in the molecular ion cluster were monitored for each congener. The limits of quantitation of various PCDD and PCDF congeners was <10 ppq. Levels as low as 15 ppq 2,3,7,8-TCDD were reported in retail whole milk.

DeJong et al. (75) described a method for isolation of PCDDs and PCDFs in human milk, using Carbopack C graphitized carbon and alumina column chromatography. Analysis was performed by HRGC/MS/MS (VG 70SQ tandem hybrid mass spectometer with EBQQ configuration). Multiple reaction monitoring was used to detect the 2 most abundant ions of the molecular ion cluster of both labeled and unlabeled PCDDs/PCDFs. Detection limits were in the range of 1–10 pg/g on a fat basis. (The sensitivity of the MS/MS mode was reported to be about 5-fold less than that of HRMS.) Liem et al. (76) investigated the use of Carbosphere activated carbon vs Carbopack C for analysis of milk and found that Carbosphere was more effective, removing larger amounts of fat and improving the recovery of PCDDs and PCDFs. Mean recoveries of labeled internal standards added to cow's milk ranged from 72 ± 28% to 102 ± 22% for tetra- to octachlorinated congeners at a level of about 25 pg/g milk fat. Detection limits were in the range of 0.1–1 pg/g fat.

Beck et al. (44) examined a variety of foods (meat, milk, dairy products, eggs, fish, vegetables, vegetable oil, and fruit) collected in West Berlin for residues of PCDDs and PCDFs. Animal materials other than cow's milk were ground with sea sand and sodium sulfate. Fat was extracted by column elution with hexane–acetone (2 + 1). Fat in cow's milk was extracted with hexane–ether (1 + 1) and sodium oxalate. Plant materials (except vegetable oil) were extracted and purified according to the method of Specht and Tillkes (85). This method specifies extraction with acetone followed by GPC (Bio-Beads S-X3, Bio-Rad Laboratories, Richmond, CA) with ethyl acetate–cyclohexane (1 + 1), column chromatography on deactivated silica gel, and then activated carbon column fractionation according to the method of Smith et al. (22). Because of low concentrations of the congeners in foods (except cow's milk), GPC was added as an additional cleanup step before chromatography with silica, potassium silicate, activated carbon, silica–sulfuric acid, cesium silicate, and alumina as described by Smith et al. (22). Individual PCDDs and PCDFs were found in animal materials at levels ranging from 0.01 to 98 ppt (fat weight basis). Concentrations of 0.2 ppt 2,3,7,8-TCDD (fat weight basis) were reported in cow's milk and eggs, whereas 3, 5, and 23 ppt 2,3,7,8-TCDD (fat weight basis) were reported in redfish, herring, and cod, respectively.

Furst et al. (45) analyzed 107 foods collected in Germany for PCDD and PCDF residues. A variety of foods of animal and plant origin were examined after solvent extraction and cleanup involving GPC on Bio-Beads S-X3 followed by adsorption column chromatography on Florisil, acid alumina, and Carbopack C. Foods of plant origin had very low levels of PCDDs and PCDFs near the detection limit (0.5 pg/g), whereas those of animal origin had typical patterns of PCDDs and PCDFs with TCDD toxic equivalents as high as 17 pg/g.

Ryan et al. (42) used a gas chromatograph/tandem mass spectrometer (Taga 6000, Sciex, Inc., Thornhill, Ontario, Canada) to screen for all possible TCDDs and TCDFs in chicken and pork fat extracts that were cleaned up by a multistep procedure (86) previously used for analysis of fish. Health and Welfare Canada evaluated the use of MS/MS in analysis of foods and environmental materials (42) subjected to simplified workups. MS/MS provides additional separation, i.e., improved selectivity. Thus, the GC step, which uses short capillary columns and is very rapid compared with conventional capillary GC/MS methods, need not separate the PCDDs and PCDFs from all other components.

Analysis of Biological Tissues

A number of cleanup procedures have been reported for human serum and tissues (47–49, 63–69). Cleanup and analysis generally included use of carbon chromatography and HRGC/HRMS. Patterson et al. (64, 65) reported cleanup procedures for determination of 2,3,7,8-TCDD in human adipose tissue and human serum that employed the highly specific cleanup procedures of Smith et al. (22). Limits of detection for 2,3,7,8-TCDD in adipose tissue and serum were 1 pg/g and 1.25 pg/kg, respectively; the procedures were shown to be unaffected by a number of potentially interfering compounds. Patterson et al. (87) also compared 3 extraction procedures for determination of PCDDs and PCDFs in human serum.

Chang et al. (61) described a method for determination of PCDDs/PCDFs in biological materials. The method involved solvent (acetonitrile) extraction of tissue homogenate, solid-phase extraction of the extract with a disposable C-18 bonded silica column (blood plasma is passed through the C-18 column directly after adding formic acid and degassing), column cleanup with a dual-column system consisting of a carbon column and a guard column containing potassium silicate and acid silicate, and analysis by HRGC/LRMS.

Recoveries of $^{13}C_{12}$-labeled surrogates from biological tissues fortified at 10 pg/g ranged from 65 to 85%.

Lapeza et al. (88) described an automated apparatus for extraction and enrichment of 2,3,7,8-TCDD in human adipose tissue. The apparatus was reported to reduce the analyst's time by 50% compared to the method that is entirely manual. A new automated cleanup apparatus was recently developed that improves the versatility and ease of using the unit and reduces from 20 to 4 h the time required for the initial extraction, cleanup, and adsorption onto activated carbon (89). Cramer et al. (90) evaluated an analytical method designed for PCDDs and PCDFs in human adipose tissues (91, 92) that was applied to determination of polybrominated dioxins and furans in these tissues.

Lindstrom and Rappe (70) developed a procedure for HRGC/HRMS determination of PCDDs and PCDFs in biological tissues that met the following criteria: (*a*) minimize the analyst's time when large numbers of tissues are analyzed; (*b*) provide for a total method detection level (TMDL) in the range of 0.1–1 pg/g; and (*c*) generate data with a high level of accuracy and precision. The first criterion was met by using disposable glassware, an integrated extraction/enrichment procedure, and autoinjection HRGC/HRMS together with computerized quantitation, which further reduced the time required. A TMDL in the range of 0.1–1 pg/g was achieved with the use of an HRGC/HRMS SIM technique that provided a detection limit of 0.05–0.5 pg (signal-to-noise ratio = 3:1) with a test portion of 1–10 g (an amount equivalent to 1/10 of the test portion was injected). The third criterion was met by reducing manual manipulations in the cleanup procedure on the basis of analytical properties and reagents (22). HRGC was performed using a polar capillary column (Supelco SP-2330) for separation of all isomers, or a nonpolar column (SPB-5 or CP-Sil 8CB) when total separation was not required.

Patterson et al. (93) identified several major areas of potential analytical contamination to be avoided by laboratories performing analyses of biological materials. It was found that white chemical wipes and certain floor cleaning solutions as well as cigarette smoke and ash contain PCDDs and PCDFs and cause laboratory contamination and analytical interferences that prevent accurate quantitation of native PCDDs and PCDFs in biological tissue extracts.

Stanley et al. (94) presented a summary of quality assurance/quality control efforts including data quality criteria for a study of PCDDs and PCDFs in human adipose tissue. 2,3,7,8-TCDD results were verified by using both a 60 m DB-5 GC column with HRMS at >3000 resolution and a 60 m SP-2330 column with HRMS at >10 000 resolution.

Bioassays

Analysis by bioassay requires fewer cleanup steps than analysis by GC/MS, offers the potential of reduced cost and time for detecting minute amounts of dioxins and furans in food or tissue extracts, and provides a quick screening procedure for these compounds. In vitro cytosolic receptor-binding bioassays approximate potential in vivo toxicities of dioxins, furans, and related compounds present in various matrixes (95–98). Casterline et al. (97) applied the aryl hydrocarbon hydroxylase (AHH) induction bioassay to extracts of freshwater fish to determine the presence of ppt levels of PCDDs, PCDFs, and polychlorinated biphenyls (PCBs) in the fish. Their results suggested that fish extracts could be examined for AHH inducers before chemical analysis, although it was recognized that the sensitivity was not as good as that obtained with traditional chemical methods and that the bioassays could not identify congeners. Safe et al. (99) compared the effects of chemical structure on toxic activity. Rats given 27 PCDDs, PCDFs, PCBs, and polybrominated dibenzo-*p*-dioxins were used to validate the usefulness of the in vitro AHH induction assay for quantitative estimation of the potential toxicity of these compounds. The assay, which is rapid and relatively inexpensive, uses the established cultured rat hepatoma H-4-II E cells, which are capable of detecting 20–50 pg of 2,3,7,8-TCDD equivalents/plate.

Other in vivo and in vitro systems have also been proposed for assay of PCDDs and PCDFs (100). The use of immunoassays (101, 102) has also been suggested for field-portable screening. Helder and Seinen (103) described a bioassay based on mortability in rainbow trout eleutheroembryos after exposure to PCDDs and PCDFs. Gierthy and Crane (104) described studies that tested the specificity and sensitivity of the TCDD-induced flat-cell effect in the XBF/3T3 culture system and its application as a bioassay for determination of PCDDs and related compounds in fish and other environmental materials. 2,3,7,8-TCDD at a minimal concentration of 10^{-11}M induced a morphological change (appearance of flat cobblestone-like cells instead of the multilayered fusiform, high-density control cells) and reversible inhibition of post-confluent cell proliferation in the XBF-3T3 system (105). When this flat-cell assay was applied to determination of PCDD activity in fish, simple organic extracts caused cell death. However, crude extracts of fish that contained either zero or measurable levels of TCDD, when treated with concentrated sulfuric acid followed by neutralization, gave flat-cell assay results consistent with GC/MS analysis.

Kennel et al. (106, 107) produced monoclonal antibodies for use in developing a solid-phase radioimmunoassay (RIA) for PCDDs in environmental materials. However, these monoclonal antibodies failed to recognize free PCDD in solution (they recognized only dioxin–protein conjugates) and thus were unsuitable for screening environmental materials. Stanker et al. (108) described the production and use of monoclonal antibodies in the development of a competitive enzyme-linked immunosorbent assay (ELISA) for evaluation of the antibodies' ability to distinguish among various PCDDs, PCDFs, and related compounds. A set of 5 antidioxin antibodies were found to discriminate among various congeners as well as recognize tetra- and penta-CDDs and CDFs substituted in at least 3 of the 4 lateral (2,3,7,8) ring positions. Chlorine substitution on both rings may be necessary for antibody binding; chlorines in the 1,2,3,7, and 8 positions were preferred. The 2,7-dichlorodibenzo-*p*-dioxin was recognized, and it is presumed that the 2,3,7-trichlorodibenzo-*p*-dioxin, which was not tested, would also have been recognized. One of the antidioxin antibodies (DD-1) recognized 1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin (HCDD) and 1,2,3,7,8,9-HCDD. With this antibody, the competitive ELISA is specific for the most toxic congeners of PCDD and PCDF and is sensitive in the high-ppt range. The assay takes only a few hours to complete and is amenable to automation. It is estimated that one analyst can perform 15–20 assays/day with a competitive ELISA.

Sherry et al. (109) evaluated the performance of an RIA for detection of PCDDs in fish after minimal or extensive cleanup. Fish tissue was extracted with HCl–toluene (1 + 1), and the extracts were fortified with unlabeled 2,3,7,8-

TCDD. (The fish contained no detectable TCDD, levels of penta- and heptachlorodioxin below the RIA's detection limit for those homologs, or octachlorodioxin, which did not cross-react significantly with the antibody.) Combinations of cleanup steps were investigated, including GPC, trisodium phosphate wash, sulfuric acid wash, and basic alumina and carbon column chromatography. Size of the test portion analyzed as well as degree of cleanup affected assay performance; Triton solubilization was superior to DMSO solubilization. The smallest amount of 2,3,7,8-TCDD detected with the assay was 60–70 pg/g, which required a 300 mg test portion, Triton solubilization, and all the cleanup steps under study.

Conclusions

A wide variety of extraction, cleanup, and isolation techniques are used for isomer-specific determination of PCDD and PCDF residues in foods and biological tissues. A series of complex extraction-cleanup steps are frequently required to eliminate or drastically reduce coextractives that interfere with quantitation and identity confirmation of the PCDDs and PCDFs. Current cleanup procedures are generally modifications of procedures proposed by Smith et al. (22) and/or Lamparski et al. (23). Activated carbon and multistage chromatographic columns with acid- and alkali–silica are widely used in various cleanup schemes. Both HRGC/HRMS and HRGC/LRMS are used for quantitation and identity confirmation of PCDD and PCDF congeners. GC/MS/MS is used in some laboratories after minimal pretreatment. Bioassays that require minimal cleanup can reduce cost and time for screening many test samples. Because analytical methods for determination of PCDDs and PCDFs are not standardized, it is necessary to conduct periodic interlaboratory evaluation studies as well as validation studies of new methods and ongoing quality assurance programs along with routine use of individual procedures.

References

(1) Beck, H., Eckart, K., Mathar, W., & Wittkowski, R. (1988) *Lebensmittelchem. Gerichtl. Chem.* **42**, 101–105
(2) Hattemer-Frey, H.A., & Travis, C.C. (1989) *Chemosphere* **18**, 643–649
(3) Esposito, M.P., Tiernan, T.O, & Dryden, F.E. (1980) *Dioxins*, Publ. No. EPA-600/2-80-197, Environmental Protection Agency, Cincinnati, OH
(4) National Research Council Canada (1981) *Polychlorinated Dibenzo-p-dioxins: Limitations to the Current Analytical Techniques*, NRCC Publication No. 1857, Ottawa, Ontario, Canada
(5) Ontario Ministry of Environment (1985) *Polychlorinated Dibenzo-p-dioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs)*, Scientific Criteria Documents for Standard Development No. 4-84, Ontario Ministry of Environment, Rexdale, Ontario, Canada
(6) Stanley, J.S., Going, J.E., Redford, D.P., Kutz, F.W., & Young, A.L. (1985) in *Chlorinated Dioxins & Dibenzofurans in the Total Environment II*, L.H. Keith, C. Rappe, & G. Choudhary (Eds.), Butterworth Publishers, Stoneham, MA, pp. 181–195
(7) Donnelly, J.R., Sovocool, G.W., Tondeur, Y., Billets, S., & Mitchum, R.K. (1986) in *Chlorinated Dioxins and Dibenzofurans in Perspective*, C. Rappe, G. Choudhary, & L. Keith (Eds.), Lewis Publishers, Chelsea, MI, pp. 381–398
(8) Clement, R.E., & Lennox, S.A. (1986) *Chemosphere* **15**, 1941–1946
(9) Rappe, C. (1989) *Chemosphere* **18**, 17–21
(10) Hass, J.R., & Friesen, M.D. (1979) *Ann. N.Y. Acad. Sci.* **320**, 28–42
(11) Crummett, W.B. (1983) *Chemosphere* **12**, 429–446
(12) Afghan, B.K., Carron, J., Goulden, P.D., Lawrence, J., Leger, D., Onuska, F., Sherry, J., & Wilkinson, R. (1987) *Can. J. Chem.* **65**, 1086–1097
(13) Clement, R.E., Bobbie, B., & Taguchi, V. (1986) *Chemosphere* **15**, 1147–1156
(14) Bobbie, B.A., Clement, R.E., & Taguchi, V.Y. (1989) *Chemosphere* **18**, 155–162
(15) Alexander, L.R., Maggio, V.L., Gill, J.B., Green, V.E., Turner, W.E., & Patterson, D.G. (1989) *Chemosphere* **19**, 241–248
(16) Charles, M.J., Green, B., Tondeur, Y., & Hass, J.R. (1989) *Chemosphere* **19**, 51–57
(17) McCurvin, D.M.A., Schellenberg, D.H., Clement, R.E., & Taguchi, V.Y. (1989) *Chemosphere* **19**, 201–204
(18) McCurvin, D.M.A., Clement, R.E., Taguchi, V.Y., Reiner, E.J., Schellenberg, D.H., & Bobbie, B.A. (1989) *Chemosphere* **19**, 205–212
(19) Firestone, D. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 354–356
(20) Lamparski, L.L., Mahle, N.H., & Shadoff, L.A. (1978) *J. Agric. Food Chem.* **26**, 1113–1116
(21) Ryan, J.J., Lizotte, R., Panopio, L.G., & Lau, B. P.-Y. (1989) *Chemosphere*, **18**, 149–154
(22) Smith, L.M., Stalling, D.L., & Johnson, J.R. (1984) *Anal. Chem.* **56**, 1830–1842
(23) Lamparski, L.L., Nestrick, T.J., & Stehl, R.H. (1979) *Anal. Chem.* **51**, 1453–1458
(24) Niemann, R.A., Brumley, W.C., Firestone, D., & Sphon, J.A. (1983) *Anal. Chem.* **55**, 1497–1504
(25) Fehringer, N.V., Walters, S.M., Kozara, R.J., & Schneider, L.F. (1985) *J. Agric. Food Chem.* **33**, 626–630
(26) Thompson, T.S., Schellenberg, D.H., Naikwadi, K.P., & Karasek, F.W. (1989) *Chemosphere* **19**, 45–50
(27) Sherry, J.P., & Tse, H. (1990) *Chemosphere* **20**, 865–872
(28) Lindstrom, G., & Rappe, C. (1988) *Chemosphere* **17**, 921–935
(29) Nam, K.S., Kapila, S., Yanders, A.F., & Puri, R.K. (1990) *Chemosphere* **20**, 873–880
(30) Lamparski, L.L., Nestrick, T.J., & Crummett, W.B. (1990) *Compound-Specific HRGC-LRMS Determination of Halogenated Dibenzo-p-dioxins and Dibenzofurans in Environmental and Biological Matrices* in IARC (International Agency for Research on Cancer) monograph *Environmental Carcinogens–Methods and Exposure Measurement, Vol. 2, Polychlorinated Dibenzodioxins, Dibenzofurans and Biphenyls*, C. Rappe (Ed.), WHO Publications Centre, USA, Albany, NY, in press
(31) Niemann, R.A (1986) *J. Assoc. Off. Anal. Chem.* **69**, 976–980
(32) U.S. EPA National Dioxin Study (1986) *Analytical Procedures and Quality Assurance Plan for the Analysis of 2,3,7,8-TCDD in Tier 3-7 Samples of the U.S. Environmental Protection Agency National Dioxin Study*, Publ. No. USEPA/600/3-85/019, May 1986, section II
(33) U.S. EPA National Dioxin Study (1987) *Phase II Analytical Procedures and Quality Assurance Plan for the Determination of PCDD/PCDF in Fish*, Environmental Research Laboratory, Duluth, MN, Sept. 1, 1987
(34) Tondeur, Y. (1987) *Analytical Procedure and Quality Assurance for Multimedia Analysis of Polychlorinated Dibenzo-p-dioxins and Polychlorinated Dibenzofurans by High Resolution Gas Chromatography/High Resolution Mass Spectrometry*, USEPA Method 8290, Environmental Monitoring Systems Laboratory, Las Vegas, NV, June 1987
(35) Norstrom, R.J., Simon, M., & Mulvihill, M.J. (1986) *Int. J. Environ. Anal. Chem.* **23**, 267–287
(36) Norstrom, R.J., & Simon, M. (1990) *Determination of Specific Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Biological Matrices by Gel-permeation/Carbon Chromatography and Gas Chromatography Mass Spectrometry* (to be published as Method 4 in forthcoming IARC monograph *Environmental Carcinogens–Methods and Exposure Measurement*, Vol. 2, *Polychlorinated Dibenzodioxins, Dibenzofurans and Biphenyls*, C. Rappe (Ed.), WHO Publications Centre USA, Albany, NY, in press
(37) Lawrence, J., Onuska, F., Wilkinson, R., & Afghan, B.K. (1986) *Chemosphere* **15**, 1085–1090
(38) Afghan, B.K., Carron, J., Goulden, P.D., Lawrence, J., Leger, D., Onuska, F., Sherry, J., & Wilkinson, R. (1987) *Can. J. Chem.* **65**, 1086–1097
(39) O'Keefe, P. (1987) *New York State Health Department*

Protocols for the Analysis of 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Fish and Sediments, Report of Analytical Procedures in Use in State of New York Department of Health Laboratories, Albany, NY, April 1987

(40) O'Keefe, P.W., Smith, R.M., Hilker, D.R., Aldous, K.M., & Gilday, W. (1985) in *Chlorinated Dioxins and Dibenzofurans in the Total Environment II*, L.H. Keith, C. Rappe, & G. Choudhary (Eds.), Butterworth Publishers, Stoneham, MA, pp. 139–152

(41) Tondeur, Y., Beckert, W.F., Billets, S., & Mitchum, R.K. (1989) *Chemosphere* **18**, 119–131

(42) Ryan, J.J., Lizotte, R., Sakuma, T., & Tori, B. (1985) *J. Agric. Food Chem.* **33**, 1021–1026

(43) Sakuma, T., Gurprasad, N., Tanner, S.D., Ngo, A., Davidson, W.R., McLeod, H.A., Lau, B.P.-Y., & Ryan, J.J. (1985) in *Chlorinated Dioxins and Dibenzofurans in the Total Environment II*, L.H. Keith, C. Rappe, & G. Choudhary (Eds.), Butterworth Publishers, Stoneham, MA, pp. 139–152

(44) Beck, H., Eckart, K., Mathar, W., & Wittkowski, R. (1989) *Chemosphere* **18**, 417–424

(45) Furst, P., Furst, C., & Groebel, W. (1990) *Chemosphere* **20**, 787–792

(46) Startin, J.R., Rose, M., & Offen, C (1989) *Chemosphere* **19**, 531–534

(47) Ogaki, J., Takayama, K., Miyata, H., & Kashimoto, T. (1987) *Chemosphere* **16**, 2047–2056

(48) Miyata, H., Takayama, K., Ogaki, J., Kashimoto, T., & Fukushima, S. (1987) *Chemosphere* **16**, 1817–1822

(49) Takizawa, Y., & Muto, H. (1987) *Chemosphere* **16**, 1971–1975

(50) Ryan, J.J., Lizotte, R., Panopio, L.G., Lau, B.P.-Y. Lewis, D., & Sun, W.-F. (1986) *Analysis of Biological Samples for Chlorinated Dibenzo-p-dioxins (PCDDs) and Chlorinated Dibenzofurans (PCDFs)*, Report of Procedures Used in the Food Research Division, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada, December 1986

(51) Ryan, J.J., Lizotte, R., & Lau, B.P.-Y. (1985) *Chemosphere* **14**, 697–706

(52) Ryan, J.J., Lizotte, R., & Lewis, D. (1987) *Chemosphere* **16**, 1989–1996

(53) Ryan, J.J., Schechter, A., Sun, W.-F., Lizotte, R. (1986) in *Chlorinated Dioxins and Dibenzofurans in Perspective*, C. Rappe, G. Choudhary, & L.H. Keith (Eds.), Lewis Publishers, Inc., Chelsea, MI, pp. 3–16

(54) Ono, M., Wakimoto, T., Tatsukawa, R., & Masuda, Y. (1986) *Chemosphere* **15**, 1629–1634

(55) Ono, M., Kashima, Y., Wakimoto, T., & Tatsukawa, R. (1987) *Chemosphere* **16**, 1823–1828

(56) Tarhanen, J., Paasivirta, J., & Soikkeli, J. (1986) *Chemosphere* **15**, 2109–2110

(57) Orazio, C., Meadows, J., Kapila, S., Palmer, C., & Yanders, A.F. (1989) *Chemosphere* **18**, 69–76

(58) Morita, M., Yusuhara, A., Seki, H., & Ohi, G. (1987) *Chemosphere* **16**, 1749–1752

(59) Paasivirta, J., Tarhanen, J., & Soikkeli, J. (1986) *Chemosphere* **15**, 1429–1433

(60) Zwickenpflug, W., & Richter, E. (1988) *Chemosphere* **17**, 647–650

(61) Chang, R.R., Jarman, W.M., King, C.C., Esperanza, C.C., & Stephens, R.D. (1990) *Chemosphere* **20**, 881–886

(62) Oehme, M., Furst, P., Kruger, C., Meemken, H.A., & Groebel, W. (1988) *Chemosphere* **17**, 1291–1300

(63) Kuroki, H., Haraguchi, K., & Masuda, Y. (1987) *Chemosphere* **16**, 2039–2046

(64) Patterson, D.G., Holler, J.S., Lapeza, C.R., Alexander, L.R., Groce, D.F., O'Conner, R.C., Smith, S.J., Little, J.A., & Needham, L.L. (1986) *Anal. Chem.* **58**, 705–713

(65) Patterson, D.G., Hampton, L., Lapeza, C.R., Belser, W.T., & Green, V. (1987) *Anal. Chem.* **59**, 2000–2005

(66) Weerasinghe, N.C.A., Schechter, A.J., Pan, J.C., Lapp, R.L., Giblin, D.E., Meehan, J.L., Hardell, L., & Gross, M.L. (1986) *Chemosphere* **15**, 1787–1794

(67) Stanley, J.S., Ayling, R.E., Bauer, K.M., McGrath, M.J., Sack, T.M., & Thornburg, K.R. (1986) *Analysis for Polychlorinated Dibenzo-p-dioxins (PCDD) and Dibenzofurans (PCDF) in Human Adipose Tissue: Method Development Study*, Publ. No. USEPA 560/5-86-020, October 1986

(68) Nygren, M., Hansson, M., Sjostrom, M., Rappe, C., Kahn, P., Gochfeld, M., Velez, H., Ghent-Guenther, T., & Wilson, W.P. (1988) *Development and Validation of a Method for Determination of PCDDs and PCDFs in Human Blood Plasma. A Multivariate Comparison of Blood and Adipose Tissue Levels Between Viet Nam Veterans and Matched Controls*, New Jersey Agricultural Experiment Station Publ. No. D-01405-8-88, New Brunswick, NJ

(69) Thoma, H., Mucke, W., & Kretschmer, E. (1989) *Chemosphere* **18**, 491–498

(70) Lindstrom, G., & Rappe, C. (1990) *Chemosphere* **20**, 851–856

(71) Furst, P., Meemken, H.-A., Kruger, C., & Groebel, W. (1987) *Chemosphere* **16**, 1983–1988

(72) Noren, K. (1988) *Chemosphere* **17**, 39–49

(73) Van den Berg, M., van den Wielen, F.W.M., & Olie, K. (1986) *Chemosphere* **15**, 693–706

(74) Hayward, D.G., Charles, J.M., Voss deBettancourt, C., Stephens, S.E., Stephens, R.D., Papanek, P.J., Lance, L.L., & Ward, C. (1989) *Chemosphere* **18**, 455–468

(75) DeJong, A.P.J.M., Liem, A.K.D., den Boer, A.C., van der Heeft, E., Marsman, J.A., van de Werken, G., & Wegman, R.C.C. (1989) *Chemosphere* **19**, 59–66

(76) Liem, A.D.K., de Jong, A.P.J.M., Marsman, J.A., den Boer, A.C., Groenemeijer, G.S., den Hartog, R.S., de Korte, G.A.L., Hoogerbrugge, R., Kootstra, P.R., & van't Klooster, H.A. (1990) *Chemosphere* **20**, 843–850

(77) Beck, H., Eckhart, K., Kellert, M., Mathar, W., Ruhl, C.-S., & Wittkowski, R. (1987) *Chemosphere* **16**, 1977–1982

(78) Lindstrom, G., & Rappe, C. (1988) *Analytical Method for Analysis of Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Milk*. Report of method developed in Department of Organic Chemistry, University of Umea, Sweden

(79) Nygren, M., Rappe, C., Lindstrom, G., Hansson, M., Bergqvist, P.-A., Marklund, S., Domellof, L., Hardell, L., & Olsson, M. (1986) in *Chlorinated Dioxins and Dibenzofurans in Perspective*, C. Rappe, G. Choudhary, & L.H. Keith (Eds.), Lewis Publishers, Inc., Chelsea, MI, pp. 17–34

(80) Rappe, C., Nygren, M., & Lindstrom, G. (1987) *Environ. Sci. Technol.* **21**, 964–970

(81) Brown, P.J., Sittig, R.A., Syvertson, C.N., & Smith, P.V. (1989) *Determination of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-Tetrachlorodibenzofuran (TCDF) in Milk by Gas Chromatography-Mass Spectrometry*, poster presentation (PLP 29) at the Ninth International Symposium on Chlorinated Dioxins and Related Compounds, Toronto, Canada, September 17–22, 1989; (1990) *Chemosphere* in press

(82) New Zealand Department of Health (1989) *A Survey of Some New Zealand Retail Milk Supplies for the Presence of Dioxin, March-April 1989*, Wellington, New Zealand, May 12, 1989

(83) Ryan, J.J., & Pilon, J.C. (1980) *J. Chromatogr.* **197**, 171–180

(84) Stanley, J.S., Boggess, K.E., Onstot, J., & Sack, T.M. (1986) *Chemosphere* **15**, 1605–1612

(85) Specht, W., & Tillkes, M. (1980) *Fresenius Z. Anal. Chem.* **301**, 300–307

(86) Ryan, J.J., Pilon, J.C., Conacher, H.B.S., & Firestone, D. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 700–707

(87) Patterson, D.G., Furst, P., Alexander, L.R., Isaacs, S.G., Turner, W.E., & Needham, L.L. (1989) *Chemosphere* **19**, 89–96

(88) Lapeza, C.R., Patterson, D.G., & Liddle, J.A. (1986) *Anal. Chem.* **58**, 713–716

(89) Isaacs, S.G., Turner, W.E., & Patterson, D.G. (1990) *An Automated Sample Cleanup Apparatus Used in the Procedure for Polychlorinated Dibenzo-p-dioxins (PCDDs), Dibenzofurans CDFs), Ortho-substituted (Non-planer) and Ortho-substituted (Planer) Biphenyls (PCBs) in Serum and Adipose Tissue*, paper presented at the 10th International Meeting Dioxin 90, Bayreuth, Germany, September 10–14, 1990

(90) Cramer, P.H., Ayling, R.E., Thornburg, K.R., Stanley, J.S., Remmers, J.C., Breen, J.J., & Schwemberger, J. (1990) *Chemosphere* **20**, 821–827

(91) U.S. Environmental Protection Agency, Office of Toxic Substances (1986) *Broad Scan Analysis of the FY82 National Human Adipose Tissue Survey Specimens: Volume IV; PCDDs and PCDFs*, EPA-560/5-86-038

(92) U.S. Environmental Protection Agency, Office of Toxic Substances (1986) *Analysis of PCDDs and PCDFs in Human Adipose Tissue: Method Evaluation Study*, EPA-560/5-86-020
(93) Patterson, D.G., Turner, W.E., Isaacs, S.G., & Alexander, L.R. (1990) *Chemosphere* **20**, 829-836
(94) Stanley, J.S., Ayling, R.E., Remmers, J.C., Conomos, M.G., Breen, J.J., & Kang, H.K. (1990) *Chemosphere* **20**, 837-842
(95) Bradlaw, J.A., Garthoff, L.H., Hurley, N.E., & Firestone, D. (1980) *Food Cosmet. Toxicol.* **18**, 627-635
(96) Bradlaw, J.A., & Casterline, J.L. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 904-916
(97) Casterline, J.L., Bradlaw, J.A., Puma, B.J., & Ku, Y. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1136-1139
(98) Sawyer, T.W., Vatcher, A.D., & Safe, S. (1984) *Chemosphere* **13**, 695-701
(99) Safe, S., Zacharewski, T., Safe, L., Harris, M., Yao, C., & Holcomb, M. (1989) *Chemosphere* **18**, 941-946
(100) Matsumura, F., Brewster, D.W., Bombick, D.W., & Madhukar, B.V. (1986) in *Chlorinated Dioxins and Dibenzofurans in Perspective*, C. Rappe, G. Choudhary, & L.H. Keith (Eds.), Lewis Publishers, Chelsea, MI, pp. 243-268
(101) Vanderlaan, M., Watkins, B., & Stanker, L. (1987) *Environ. Sci. Technol.* **22**, 249-254
(102) Vanderlaan, M., Stanker, L.H., Watkins, B.E., Petrovic, P., & Gorbach, S. (1988) *Environ. Toxicol. Chem.* **1**, 859-870
(103) Helder, Th., & Seinen, W. (1985) *Chemosphere* **14**, 183-193
(104) Gierthy, J.F., & Crane, D. (1986) in *Chlorinated Dioxins and Dibenzofurans in Perspective*, C. Rappe, G. Choudhary, & L.H. Keith (Eds.), Lewis Publishers, Chelsea, MI, pp. 269-284
(105) Gierthy, J.F., & Crane, D. (1984) *Toxicol. Appl. Pharmacol.* **74**, 91-93
(106) Kennel, S.J., Jason, C., Albro, P.W., Mason, G., & Safe, S.H. (1986) *Toxicol. Appl. Pharmacol.* **82**, 256-263
(107) Kennel, S.J., Mason, G., & Safe., S. (1986) *Chemosphere* **15**, 2007-2010
(108) Stanker, L.H., Watkins, B., Rogers, N., & Vanderlaan, M. (1987) *Toxicology* **45**, 229-243
(109) Sherry, J., ApSimon, J., Collier, L., Wilkinson, R., Albro, B., & Afghan, B. (1989) *Chemosphere* **19**, 255-261

Determination of Nabam Fungicide in Crops by Liquid Chromatography with Postcolumn Reaction Detection

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The ethylenebisdithiocarbamate (EBDC) fungicide, nabam, was determined in several crop matrixes using liquid chromatography with postcolumn reaction detection. After separation by micellar liquid chromatography, nabam (EBDC sodium salt) was acid hydrolyzed to ethylenediamine and fluorogenically labeled with *o*-phthalaldehyde-mercaptoethanol (OPA-MERC). Standard curves were linear from the detection limit of ca 1 ng to 1000 ng. Nabam was recovered in high yield (89 ± 7.7%) over a range of concentrations (0.1 to 20 ppm) from fortified samples of papaya, lettuce, cucumber, spinach, and applesauce using a simple extraction method. Efforts to convert the more popular EBDC fungicides, maneb and mancozeb, to nabam are discussed.

Ethylenebisdithiocarbamate (EBDC) fungicides, such as mancozeb and maneb, are the most widely used fungicides because they are effective on a wide range of fungi and have low toxicity. In addition, the incidence of resistance in major crops is low. Although these compounds have relatively low toxicity, ethylenethiourea (ETU), a manufacturing and environmental by-product and metabolite, is carcinogenic. This has led the U.S. Environmental Protection Agency (EPA) to put EBDC fungicides under special review.

Most EBDCs are complex metal polymers of ethylenebisdithiocarbamic acid or nabam (the sodium salt). Current methods for EBDC residue analysis use acid hydrolysis to evolve carbon disulfide, which is either distilled or trapped and quantitatively measured by titration (1), by colorimetry (2), or by gas chromatography with flame photometric detection (3). This last method has significantly improved EBDC analysis by increasing sensitivity as well as increasing sample throughput. These methods lack specificity because naturally occurring carbon disulfide and EBDC breakdown products, such as dialkyl dithiocarbamate and thiuram disulfide, are potential interferences.

Dithiocarbamates have been determined by liquid chromatography (LC) using a micellar mobile phase (4); some EBDC fungicides have been determined by reverse phase LC with UV detection (272 nm) after ion-pair methylation (5). The latter method used the technique described by Pflugmacher and Ebing (6) whereby polymeric metal salts, like mancozeb and maneb, are extracted from plant surfaces and converted to their monomer, nabam, by an alkaline solution of EDTA. It was subsequently found that addition of L-cysteine to the EDTA solution yielded better recoveries of zineb, maneb, and mancozeb, presumably by preventing reaction between nabam and sample co-extractives (7). In all cases, sample homogenization was not possible because of resulting low analyte recovery.

Reports that EBDC metal complexes, such as maneb and mancozeb, could be converted to nabam by EDTA lead us to approach EBDC analysis as described in Figure 1. Micellar liquid chromatography was used to separate nabam from co-extractives followed by postcolumn acid hydrolysis to form ethylenediamine, which is then fluorogenically labeled with *o*-phthalaldehyde-mercaptoethanol (OPA-MERC). Homogenized extracts of several crops were fortified over a wide range of concentrations and analyzed for nabam.

Experimental

LC Apparatus

(a) *Gradient pump.*—With eluant degas module, Model GPM and EDM II, respectively (Dionex, Sunnyvale, CA 94086).

(b) *Injector.*—2000 psi limit with 50 μL loop (Dionex).

(c) *Analytical column.*—4 × 150 mm, 5 μm Ion Pac NS1 (Dionex).

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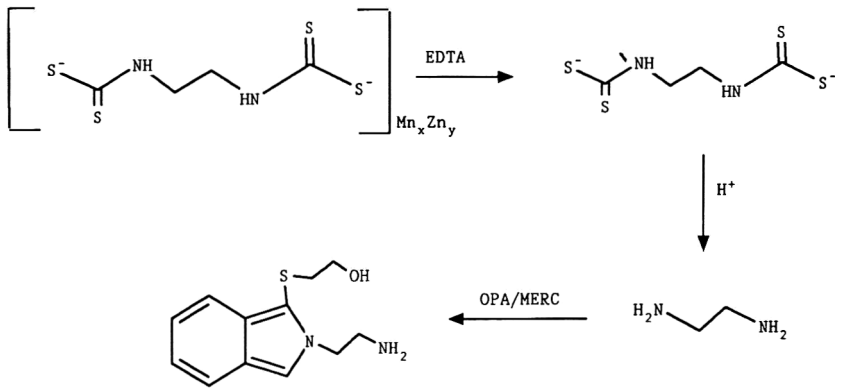

Figure 1. Analytical scheme for EBDC fungicides.

(d) *Reagent pumps.*—Two mini-pumps for addition of postcolumn reagents delivered at 0.1 to 1.0 mL/min and pressures <1000 psi, Model RP-1 (Dionex).

(e) *Reaction coils.*—Two 5.2 m × 0.5 mm id × 1.6 mm od (ca 1 mL) TFE Teflon® coils woven as described by Engelhardt and Neue (8) and connected to reagent pumps by 3-way manifolds (Dionex P/N 024313).

(f) *Water bath.*—Maintained at 65°C, 6 L Versa-Bath (Fisher Scientific, Pittsburgh, PA 15219).

(g) *Fluorometer.*—Model 980 (Ex 235; Em >418) (Applied Bio Systems, Ramsey, NJ 07446) or Model 121 (Ex 356; Em 450) (Gilson Medical Electronics, Middleton, WI 53562).

(h) *Integrator.*—PC Integrator (PE Nelson, Cupertino, CA 95014).

(See apparatus configuration in Figure 2.)

Reagents

(a) *Water.*—Obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(b) *Acetonitrile.*—Optima grade (Fisher, Fair Lawn, NJ 07410).

(c) *Phosphate buffer.*—Dissolve 2.72 g potassium phosphate monobasic (Fisher, ACS grade) in 1 L water (20 mM) and adjust pH to 7.8 with KOH.

(d) *Cetylpyridinium/phosphate buffer.*—Dissolve 358 mg cetylpyridinium chloride monohydrate (Aldrich Chem. Co., Milwaukee, WI 53233) in 1 L phosphate buffer to produce 1 mM solution.

(e) *Mobile phase.*—Combine cetylpyridinium/phosphate buffer and acetonitrile (78 + 22) (both degassed) using gradient pump controller and deliver at 0.4 mL/min.

(f) *Acid hydrolysis reagent.*—Combine 2.8 mL concentrated sulfuric acid (Fisher, ACS grade) in 1 L water (0.1N H_2SO_4) and filter (0.45 μm; Durapore, Millipore). Deliver at 0.67 mL/min.

(g) *Fluorogenic labeling reagent.*—Dissolve 38 g sodium borate (Fisher, reagent grade) in 1 L water and combine with 44 mg *o*-phthalaldehyde (Aldrich) dissolved in 5 mL methanol. Add 1 mL mercaptoethanol (Aldrich), filter (0.45 μm), and deliver at 0.35 mL/min.

(h) *EDTA-MERC solution.*—Dissolve 9.25 g disodium EDTA (reagent grade, Baker, Phillipsburg, PA 08865) in 100 mL water (0.25M) and adjust pH to 9.25 with NaOH. Add 1 mL mercaptoethanol (1%, v/v)(*Caution:* Stench).

(i) *Standard solutions.*—Dissolve nabam (78%; Crescent Chem Co., Inc., Hauppauge, NY 11788), maneb (98%; DuPont, Wilmington, DE 19898), or mancozeb (80.3%; Rohm & Haas, Philadelphia, PA 19114) in EDTA-MERC solution to yield 1 mg/mL. Dilute to appropriate concentration in EDTA-MERC.

General Apparatus

(a) *Homogenizer.*—Tissumizer (Techmar Co., Cincinnati, OH 45222).

(b) *Centrifuge bottle.*—Teflon 50 mL capacity (Nalge Co., Rochester, NY 14602).

(c) *Centrifuge.*—Capable of 1500 g (Model RC2-B, Sorvell, Newtown, CT 06470).

(d) *Filter.*—PVDF Acrodisc, 0.45 μm pore size, 25 mm disc (Gelman Sciences, Ann Arbor, MI 48106).

Sample Extraction

Crops (papaya, applesauce, cucumber, lettuce, and spin-

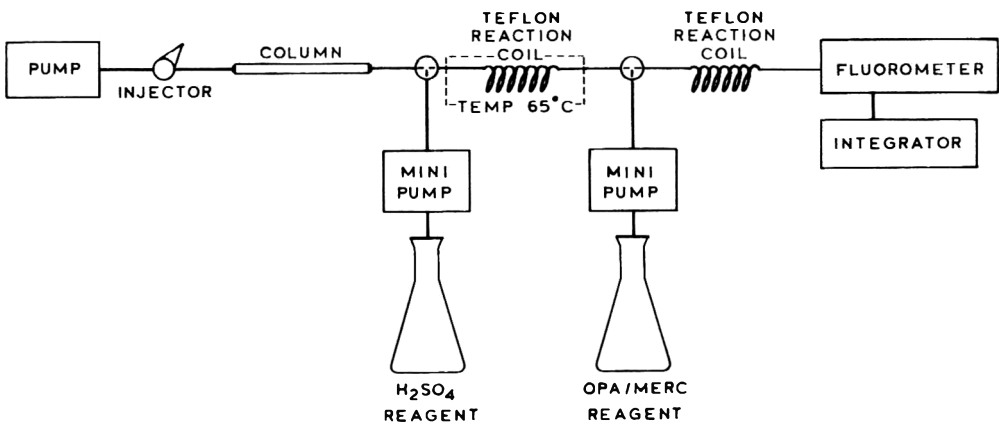

Figure 2. LC postcolumn reaction detection system.

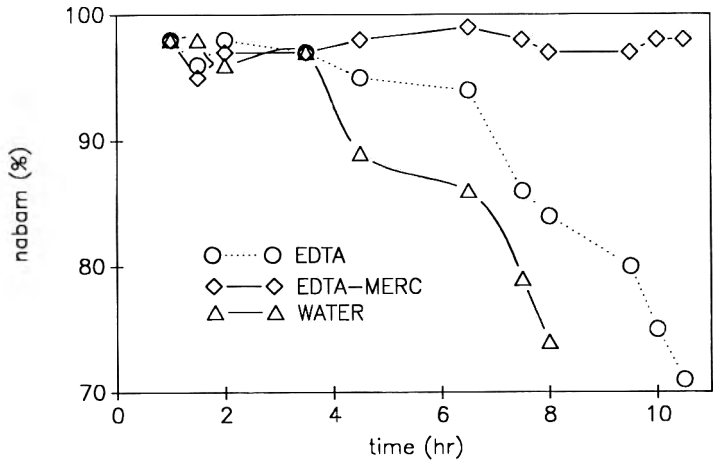

Figure 3. Stability of nabam in aqueous solutions [0.25M EDTA, pH 9.25, and MERC (1%, v/v)].

ach) obtained from local grocery stores were chopped into small pieces (ca 0.5–1 cm) and 50 g homogenized in 50 mL EDTA–MERC for 1 min. After fortification, samples were centrifuged at 1500 g and filtered (0.45 μm) before injection on the LC system. Some samples were fortified before homogenization.

Calculations

$$\text{Residue, } \mu g/g = (PH_{sam}/PH_{std} \times (\mu g/mL \text{ std}) \times (\text{total volume/g sample wet wt})$$

where standard (std) is not corrected for purity, and total volume equals milliliters EDTA–MERC added plus milliliters sample water.

Results and Discussion

Postcolumn reactions were optimized using flow injection analysis (no column) by fixing one reagent flow rate and varying the other. During this procedure, it was found that borate concentration in the OPA–MERC reagent had to be doubled (0.10M) from the concentration used in the base hydrolysis reaction for carbamate analysis (0.05M). This allowed the pH of the second reaction to approach optimum conditions for isoindole formation after acid hydrolysis.

Separations were based on those developed by Kirkbright and Mullins (4), using micellar liquid chromatography. We substituted cetylpyridinium chloride (CPC) for the cetyltrimethylammonium bromide (CTAB) because of a high fluorescence background attributed to reaction of OPA–MERC with CTAB contaminants. We used CPC concentration (1 mM) above the critical micelle concentration of 0.12 mM (9) to achieve micellar conditions. A phosphate buffer adjusted to pH 7.8 was used to control on-column acid hydrolysis. The capacity factor of nabam varied linearly as the percentage of acetonitrile changed.

Initial separations were performed on a C_{18} silica column contained in stainless steel. Peak losses that were observed at low concentrations of nabam were attributed to reaction with iron, although addition of EDTA and diethylenetriaminopentaacetic acid (DETPA) (Aldrich) had no significant effect. Use of a macroporous copolymer resin in a plastic column (Dionex, IonPac NS1) eliminated this problem. To control iron in our LC system, we used all metal-free components, except for fluorometer flow cell connections. Standard curves for nabam were linear from the detection limit of ca 1 ng to more than 1000 ng.

Nabam oxidized readily in aqueous solution to the disulfide (10), which had a different retention time and postcolumn response from nabam. No significant change in nabam concentration was noted for ca 4 h in distilled water or EDTA (Figure 3). Addition of mercaptoethanol (1%, v/v) to the EDTA stabilized nabam for more than 48 h. Gustafsson and Fahlgren (7) reported that L-cysteine stabilized nabam in the presence of crop co-extractives.

High yield of nabam from fortified crops was reproducible over a wide range of concentrations (Table 1). Recoveries averaged 89% with a relative standard deviation of 7.7%. These crops were chosen because EBDC is commonly used as a fungicide on them (11). Fortification of crop before and after homogenization had no significant effect on recovery; data in Table 1 are from crops fortified after homogenization. We estimated method detection limits ($S/N = 3$) of 0.05 ppm with a 50 g crop sample (equivalent to about 3.5 ng nabam injected; see Figure 4), although this varied with crop type and LC system conditions.

After several hundred crop sample injections, the peak shape of nabam deteriorated, probably because of co-extrac-

Table 1. Recoveries of nabam in fortified crops

Crop	Fortification conc. (μg/g)	*n*	Av. ± SD
Papaya	20[a]	2	89 ± 3.0
	2.5[a]	2	90 ± 1.3
	2.0[a]	2	94 ± 1.5
	1.0	2	86 ± 6.4
	0.2	2	88 ± 1.5
	0.1	2	79 ± 5.7
Applesauce	1.0	3	94 ± 6.8
	0.2	2	84 ± 6.6
	0.1	2	79 ± 5.7
Cucumber	1.0	4	90 ± 1.2
	0.2	2	89 ± 13
	0.1	2	88 ± 4.7
Lettuce	1.0	3	97 ± 7.9
	0.2	2	92 ± 8.3
	0.1	2	86 ± 1.3
Spinach	1.0	4	93 ± 3.9
	0.1	3	81 ± 8.8

[a] Based on 2.5 g sample in 2.5 mL EDTA–MERC solution.

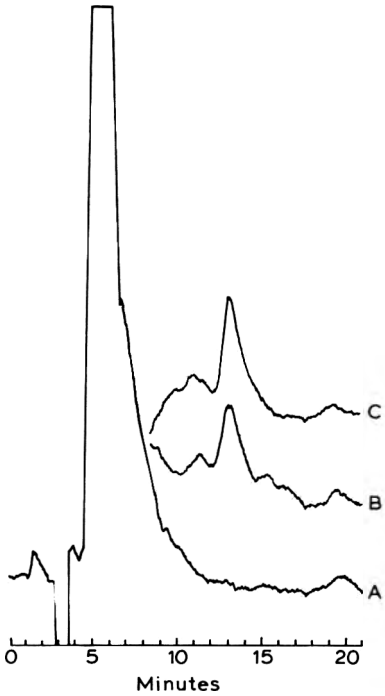

Figure 4. Chromatograms of nabam (ca 13 min) in (A) control applesauce, (B) fortified (0.1 ppm) applesauce, and (C) 0.15 μg/mL (7.5 ng) nabam standard.

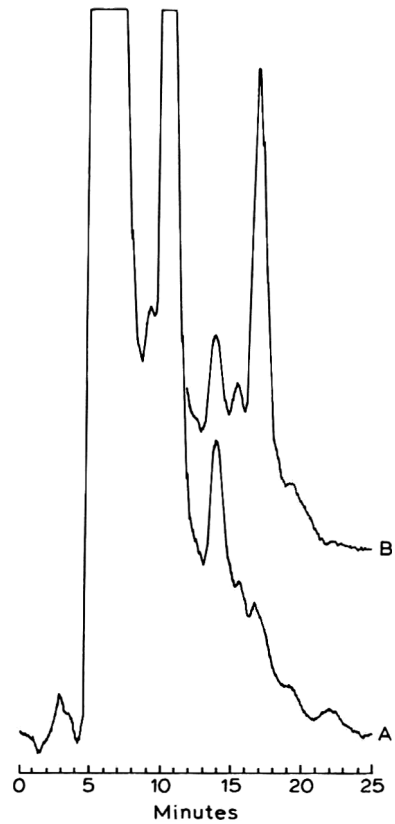

Figure 5. Chromatograms of nabam in fortified (0.1 ppm) papaya using (A) ABI fluorometer and (B) Gilson fluorometer.

tives. Reversing the column flow direction restored acceptable peak shape. Using a precolumn with a regular regeneration schedule would extend analytical column life. We found that certain crops, such as papaya and lettuce, had an interference peak that eluted just after the nabam peak. Observation of this interference peak was dependent on the fluorometer used (Figure 5). Apparently, the fluorescent interference was observed only when excited at 350 nm with a quartz-halogen lamp and not when excited at 235 nm with a deuterium lamp.

Efforts to recover mancozeb or maneb as nabam from solution or from fortified crops were unsuccessful. Recoveries reached only ca 2% using the extraction scheme described as successful by others (6, 7, 12). This included omitting homogenization, which, reportedly, severely lowers recoveries. It is likely that UV absorption at 285 nm (6, 12) measures products other than nabam and that the methylation reaction included in the extraction step (7) is responsible for the differences observed.

Several experiments were done to improve conversion of metal polymers to nabam and to explain low yields. The possibility that nabam was released but rapidly oxidized was minimized by addition of excess thiol (MERC and dithiothreitol), which would be preferentially oxidized. Also, addition of Mn^{2+} or Zn^{2+} (or both) as well as addition of mancozeb and maneb to solutions of nabam had no significant effect on nabam response after exposure for several hours. Fresh standards of mancozeb obtained from the manufacturer (Rohm and Haas) eliminated the possibility that the metal polymers were irreversibly aged. Metal chelators, such as dithiocarbamic acid, 1,2-ethanedithiol and DETPA, did not improve conversion to nabam, although they are more effective chelators than EDTA.

The fact that addition of EDTA to aqueous solutions of maneb and mancozeb caused dissolution suggested that these polymers were simplified by breaking into smaller units with this treatment. Increasing mobile phase strength (more acetonitrile) to search for other EBDC fragments in EDTA–MERC/mancozeb solutions resulted in a peak with a capacity factor of 2.6 using 35% acetonitrile. Unfortunately, this peak disappeared when diluted with EDTA–MERC below 50 mg/L. The analyte represented by this peak may interact with the stationary phase or not be quantitatively converted to ethylenediamine in the postcolumn reactor.

The present method demonstrates quantitative recovery of nabam from fortified and homogenized crops as low as 0.1 ppm with minimal sample cleanup. The fact that mancozeb and maneb were not recovered from fortified crops, suggests the need for further investigations of alternative methods or procedures for quantitative recoveries. However, even if mancozeb and maneb could be converted to nabam, this method would not directly measure parent fungicides. Nevertheless, it can eliminate many potential interferences in EBDC analysis by using the selectivity of both micellar separation and postcolumn reaction detection.

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References

(1) Lowen, W.K., & Pease, H.C. (1964) in *Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives,*

Vol. 3, G. Zweig (Ed.), Academic Press, New York, pp. 69–77
(2) Thier, H.P., & Zeumer, H. (1987) *Manual of Pesticide Residue Analysis*, Vol. 1, Method S15, VCH Publishers, New York, pp. 353–360
(3) Westberg, G.L. (1989) *Method No. ETU-89AM-001*, Morse Laboratories, Inc., Sacramento, CA
(4) Kirkbright, G.F., & Mullins, F.G.P. (1984) *Analyst* **109**, 493–496
(5) Gustafsson, K.H., & Thompson, R.A. (1981) *J. Agric. Food Chem.* **29**, 729–732
(6) Pflugmacher, J., & Ebing, W. (1980) *Z. Lebensm. Unters. Forsch.* **170**, 349–354
(7) Gustafsson, K.H., & Fahlgren, C.H. (1983) *J. Agric. Food Chem.* **31**, 461–463
(8) Engelhardt, H., & Neue, U.D. (1982) *Chromatographia* **15**(7), 403–408
(9) Cline-Love, L.J., Habarta, J.G., & Dorsey, J.G. (1984) *Anal. Chem.* **56**, 1132A–1146A
(10) Thorn, G.D., & Ludwig, R.A. (1962) *The Dithiocarbamates and Related Compounds*, Elsevier, Amsterdam
(11) *Pesticide & Toxic Chemical News* (1989) Food Chemical News, Inc., Vol. **17**, 52, November 1, pp. 8–11
(12) Hylin, J.W., Kawano, Y., & Chang, W. (1978) *Bull. Environ. Contam. Toxicol.* **20**, 820–845

Determining Organohalides in Animal Fats Using Gel Permeation Chromatographic Cleanup: Repeatability Study

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Evaluation of a previously published gel permeation chromatographic (GPC) procedure was undertaken to determine whether it can be used for additional organochlorine pesticides. After repeatability studies of many pesticides, the following compounds were approved for inclusion in the U.S. Department of Agriculture Domestic Residue Monitoring Program: coumaphos-S, stirophos, chlorpyrifos, ronnel, carbophenothion, chlorfenvinphos, phosalone, kepone, captan, linuron, and endosulfan I and II. Recoveries ranged from 54% for captan to 123% for ronnel. Ranges of CVs varied from 0–9.5% for carbophenothion to 7.1–47.7% for kepone. Although the minimum acceptable recovery of 50% was attained for all 12 pesticides, the anticipated CV of 20% was waived to include chlorpyrifos, endosulfan I and II, and kepone. For a multiresidue procedure involving approximately 40 compounds, these results were within the acceptable criteria.

The U.S. Department of Agriculture Food Safety and Inspection Service (USDA/FSIS) is responsible for the wholesomeness of the nation's meat and poultry products and has a long history of monitoring pesticides in the food chain. In the fall of 1986, a study was undertaken to include additional pesticides in the current USDA gel permeation chromatographic (GPC) procedure for chlorinated hydrocarbons (1). Compounds were added and deleted as statistical data were generated and evaluated. This procedure was collaboratively studied by Ault and Spurgeon in 1984 (2) for aldrin, alpha BHC, lindane, *cis*- and *trans*-chlordane, oxychlordane, dieldrin, *o,p*-DDT, *p,p*-DDT, *p,p*-DDE, *p,p*-TDE, endrin, heptachlor epoxide, HCB, methoxychlor, mirex, and toxaphene. The method was adopted official first action for all the compounds listed except toxaphene.

In August 1987, a report to U.S. Congressman Morris Udall from Leon Panetta (Chairman, Subcommittee on Domestic Marketing, Consumer Relations and Nutrition) focused on the use of oncogenic pesticides in the United States (3). Two of the pesticides implicated in this study were linuron and captan. Based on this report, the Chemistry Division of USDA included these 2 compounds (and others) in the repeatability study.

A report in February 1989 by the Natural Resources Defense Council of a 2-year study of pesticides in children's food, further substantiated the need to monitor other pesticides (i.e., captan) in food (4).

Fat from 4 species (beef, pork, poultry, and sheep) was used in this repeatability study. Recovery of 50% or greater at the minimum proficiency level (see below) without modification of the methodology was the main stipulation for acceptance. CVs of 20% or less were anticipated but not required.

Compounds that were included in the trial but did not meet these criteria were: dichlorvos, crufomate, trichlorfon, diazinon, dioxathion, and dichlofenthion.

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METHOD

Apparatus and Reagents

(a) *Gel permeation chromatographic system (GPC).*—AutoPrep gel permeation chromatograph (Analytical Bio-Chemistry Laboratories, Inc., PO Box 1097, Columbia, MO 65205) or equivalent.

(b) *Solvent concentration apparatus.*—Auto Vap (Analytical Bio-Chemistry Laboratories, Inc.) or equivalent. Parameters: dump time: 32 min; keeper time: 0.01 s; evaporation time: 17 min; cool dry time: 30 s; diluent time: 5 s; mixing time: 10 s; transfer time: 2 s; rinse time: 15 s; wash time: 3.50 min; evaporation temperature: 58°C; cleaning temperature: 45°C; vacuum: 300 torr.

(c) *Gas chromatograph (GC).*—Electron capture detector (ECD) (^{63}Ni) operated as in AOAC official method **29.008** (14th Ed., **970.52H**, 15 Ed.) (5). Conditions: capillary column: DB-608, fused silica, 30 m 0.53 mm id; detector: ^{63}Ni electron capture/FPD (1:1 split, optional); injector temperature: 230°C; detector temperature: 325°C; carrier gas: ultrapure He with flow rate of 20 mL/min; makeup gas: argon/methane with flow rate of 30 mL/min; splitless mode: valve

on for 1.5 min. Glass column effluent splitter. Oven temperature program: initial temperature, 170°C; hold for 10 min; increase temperature at rate of 3°C/min; final temperature, 260°C.

(d) *Solvents.*—Methylene chloride (CH_2Cl_2), cyclohexane (C_6H_{12}) and isooctane. Nanograde quality.

(e) *Gel column.*—Prepared with Bio Beads SX-3, 200–400 mesh according to *USDA Chemistry Laboratory Guidebook*, Procedure 5.003C (1).

(f) *Pesticide standards.*—U.S. Environmental Protection Agency, Research Triangle Park, NC 27711. Purity range: 95–99%.

GPC Calibration Procedure

Adjust flow rate through the SX-3 gel column to 5 mL/min. Determine the correct "dump" and "collect" times for desired residues. Fractionate volume of eluant from 150 to 320 mL to ensure residue collection. Evaporate fractions, reconstitute in isooctane, and determine collection volume by GLC-EC.

Determination

Weigh 2 g rendered fat into 10 mL volumetric flask. Fortify "blank" fat at this point with appropriate levels of pesticides. Add 0.2 ppm aldrin and dilute to 10 mL with CH_2C_{12}–C_6H_{12} (1 + 1); mix thoroughly. Centrifuge or filter if particulate matter is visible. Load sample loops on GPC with 8 mL sample. A 5 mL aliquot equivalent to 1 g of sample is accepted into sample loop. Process through GPC/AutoVap using dump/collect times from calibration procedure. Adjust final volume for GC quantitation as required. Usually, the final volume is 5 mL and injection volume is 3 μL for a sample weight of 0.6 mg injected into the GC.

Calculations

For this study, all results were calculated using aldrin as the internal standard. The following formula was used to quantitate each pesticide.

$$\text{ppm} = \text{conc std } (\mu\text{g/mL}) \times \frac{\text{peak size sample}}{\text{peak size std}} \times \frac{\mu\text{L std}}{\mu\text{L sample}} \times \frac{\text{diln vol}}{1\text{ g sample}}$$

Official sample results should be corrected for percent recovery of fortified sample (fat) carried through the entire procedure.

Table 1. Average recovery, recovery range, and range of CVs for each pesticide for all species at all levels

Pesticide	Av. rec., %	Rec. range, %	CV range
Ronnel	106	87–123	1.6–12.7
Chlorpyrifos	80	62–100	4.0–29.0
Chlorfenvinphos	77	55–100	2.4–11.1
Stirophos	92	72–111	1.8–14.9
Carbophenothion	96	75–111	0–9.5
Coumaphos-S	98	88–110	2.7–16.7
Linuron	69	57–83	3.6–12.6
Endosulfan I	93	86–103	8.8–22.2
Captan	75	54–95	5.1–12.9
Kepone	68	58–81	7.1–47.7
Endosulfan II	89	76–101	4.4–26.2
Phosalone	105	93–111	5.0–15.2

Repeatability Study

Samples of rendered fat from each of 4 species (beef, pork, poultry, and sheep) were fortified at 4 levels: zero, minimum proficiency level, one-half minimum proficiency level, and twice minimum proficiency level. (Minimum proficiency level is the smallest amount of analyte expected to be identified

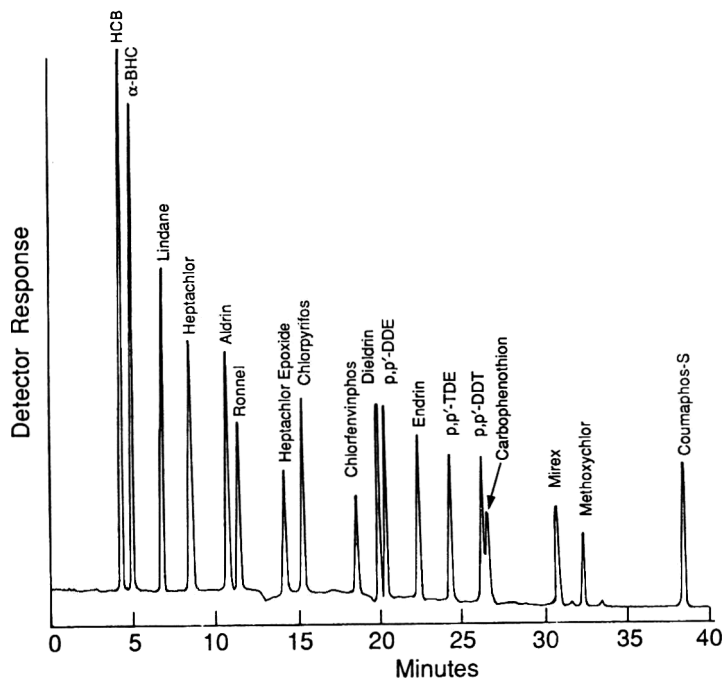

Figure 1. Chromatogram of mixed pesticide standard of chlorinated hydrocarbons and chlorinated organophosphates (0.2–0.6 μg/g).

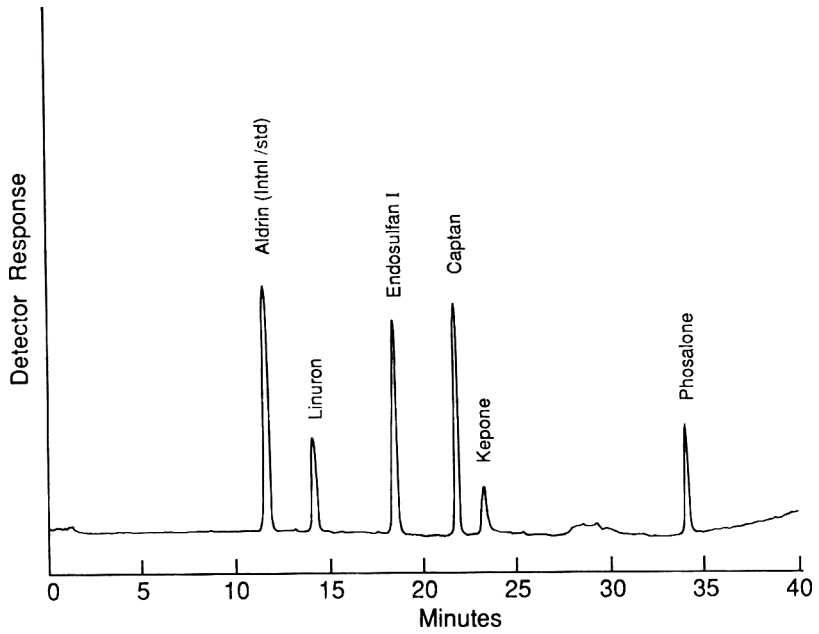

Figure 2. Chromatogram of additional pesticides included in repeatability study (0.2–0.6 μg/g).

and quantified, and upon which ongoing analytical capability will be based.) The fat was rendered at 80°C for 1–4 h in a forced-air oven.

Animal fats for this study were "official" samples submitted for analysis and determined to be "pesticide-free." A single analysis was performed at 3 levels on each species on 3 different days by the current GPC procedure using a megabore column and electron capture detection. Average recovery for 3 days, the standard deviation, and coefficient of variation for each pesticide at each level are shown in Appendix A, Tables 1-A to 20-A.

Results and Discussion

The objective of this study was to include as many additional pesticides as possible in our current GPC methodology without changing parameters. Many previously considered compounds, such as dichlofenthion, dioxathion, and crufomate, were deleted because they could not be recovered without modification of existing conditions.

Although average recoveries for the 12 pesticides (Table 1) were acceptable (>50%), CVs for some compounds covered a wide range [e.g., kepone (Tables 14-A, 17-A, and 19-A)]. Chlorpyrifos, on the other hand, showed good repeatability in beef, pork, and sheep fat, but very poor repeatability in poultry fat.

Figure 1 shows the separation of 18 pesticides by GLC with electron capture detection using a DB-608 megabore column. The compounds include HCB, BHC, lindane, heptachlor, aldrin (internal standard), ronnel, chlorpyrifos, heptachlor epoxide, chlorfenvinphos, dieldrin, *p,p*-DDE, endrin, *p,p*-TDE, *p,p*-DDT, carbophenothion, mirex, methoxychlor, and coumaphos-S. Chromatography of linuron, endosulfan I, captan, kepone, and phosalone under the same conditions are shown in Figure 2. (Results for these pesticides are shown in Tables 13-A to 20-A.)

Results of this study were evaluated and accepted by the USDA Chemistry Division, Washington, DC. These 12 compounds are now part of the more than 40 pesticides included in the USDA Residue Monitoring Program.

Acknowledgments

The authors thank Louis Gamache and Larry Menke of the USDA Western Laboratory for their analytical assistance and Richard Raskop for his typing and proofreading skills.

References

(1) *USDA Chemistry Laboratory Guidebook* (1987 Revision) Chlorinated Pesticide Method, sec **5.003**, U.S. Dept of Agriculture, Food Safety and Inspection Service
(2) Ault, J.A., & Spurgeon, T.E. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 284–289
(3) Panetta, L. (August 3, 1987) Letter (Report) to Congressman Morris Udall, Washington, DC
(4) Sewall, B., & Whyatt, R. (1989) *Intolerable Risk: Pesticides in Our Children's Food*, Natural Resources Defense Council, New York
(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec **29.008**; 15th Ed. (1990), sec **970.52H**

Appendix A. Pesticide Results by Type of Sample

Table 1-A. Recovery of ronnel and chlorpyrifos from fortified beef fat

	Ronnel			Chlorpyrifos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.12	0.20	0.36	0.077	0.13	0.25
Av. rec., %	123	102	89	77	67	63
Std dev.	0.015	0.015	0.0058	0.0090	0.0058	0.010
CV, %	12.7	7.6	1.6	11.7	4.4	4.0

Table 2-A. Recovery of chlorfenvinphos and stirophos from fortified beef fat

	Chlorfenvinphos			Stirophos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.059	0.11	0.24	0.080	0.14	0.31
Av. rec., %	59	55	59	80	72	77
Std dev.	0.0056	0.0042	0.0058	0.0051	0.021	0.012
CV, %	9.4	3.8	2.4	6.4	14.9	3.7

Table 3-A. Recovery of carbophenothion and coumaphos-S from fortified beef fat

	Carbophenothion			Coumaphos-S		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.076	0.15	0.35	0.093	0.18	0.36
Av. rec., %	76	75	86	93	88	90
Std dev.	0.0046	0.010	0.012	0.016	0.012	0.020
CV, %	6.0	6.7	3.3	16.7	6.4	5.6

Table 4-A. Recovery of ronnel and chlorpyrifos from fortified pork fat

	Ronnel			Chlorpyrifos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.11	0.20	0.35	0.068	0.12	0.25
Av. rec., %	113	98	87	68	62	63
Std dev.	0.012	0.015	0.0058	0.0096	0.0058	0.017
CV, %	10.5	7.6	1.6	13.8	4.8	6.9

Table 5-A. Recovery of chlorfenvinphos and stirophos from fortified pork fat

	Chlorfenvinphos			Stirophos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.064	0.11	0.24	0.079	0.16	0.32
Av. rec., %	64	57	61	79	80	81
Std dev.	0.0068	0.0058	0.0058	0.0061	0.010	0.0058
CV, %	10.6	5.2	2.4	7.7	6.3	1.8

Table 6-A. Recovery of carbophenothion and coumaphos-S from fortified pork fat

	Carbophenothion			Coumaphos-S		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.082	0.18	0.36	0.089	0.18	0.37
Av. rec., %	82	88	90	89	88	92
Std dev.	0.0078	0.015	0.010	0.0093	0.0058	0.012
CV, %	9.5	8.5	2.8	10.4	3.2	3.1

Table 7-A. Recovery of ronnel and chlorpyrifos from fortified poultry fat

	Ronnel			Chlorpyrifos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.11	0.22	0.42	0.087	0.18	0.35
Av. rec., %	113	108	104	87	90	88
Std dev.	0.0058	0.0058	0.025	0.025	0.050	0.066
CV, %	5.1	2.7	6.0	29.0	27.8	18.7

Table 8-A. Recovery of chlorfenvinphos and stirophos from fortified poultry fat

	Chlorfenvinphos			Stirophos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.10	0.20	0.38	0.10	0.22	0.40
Av. rec., %	100	100	96	100	110	101
Std dev.	0.010	0.020	0.035	0.010	0.017	0.021
CV, %	10.0	10.0	9.2	10.0	7.9	5.2

Table 9-A. Recovery of carbophenothion and coumaphos-S from fortified poultry fat

	Carbophenothion			Coumaphos-S		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.11	0.21	0.44	0.11	0.21	0.41
Av. rec., %	110	103	109	110	105	102
Std dev.	0	0.0058	0.035	0.010	0.017	0.042
CV, %	0	2.8	8.0	9.1	8.3	10.2

Table 10-A. Recovery of ronnel and chlorpyrifos from fortified sheep fat

	Ronnel			Chlorpyrifos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.12	0.22	0.42	0.10	0.20	0.37
Av. rec., %	117	108	106	100	98	93
Std dev.	0.0058	0.0058	0.032	0.010	0.012	0.020
CV, %	4.9	2.7	7.6	10.0	5.9	5.4

Table 11-A. Recovery of chlorfenvinphos and stirophos from fortified sheep fat

	Chlorfenvinphos			Stirophos		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.090	0.18	0.36	0.11	0.21	0.44
Av. rec., %	90	90	91	110	107	111
Std dev.	0.010	0.020	0.021	0.010	0.0058	0.032
CV, %	11.1	11.1	5.7	9.1	2.7	7.3

Table 12-A. Recovery of carbophenothion and coumaphos-S from fortified sheep fat

	Carbophenothion			Coumaphos-S		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.11	0.21	0.44	0.11	0.21	0.43
Av. rec., %	110	107	111	107	107	108
Std dev.	0.010	0.0058	0.032	0.0058	0.0058	0.038
CV, %	9.1	2.7	7.3	5.4	2.7	8.7

Table 13-A. Recovery of linuron and endosulfan I from fortified beef fat

	Linuron			Endosulfan I		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.25	0.50	1.0	0.10	0.20	0.40
Av. rec., ppm	0.21	0.34	0.57	0.090	0.17	0.35
Av. rec., %	82	67	57	90	86	87
Std dev.	0.0098	0.018	0.021	0.016	0.027	0.070
CV, %	4.8	5.3	3.6	17.5	15.5	20.2

Table 14-A. Recovery of captan and kepone from fortified beef fat

	Captan			Kepone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.025	0.050	0.10	0.10	0.20	0.40
Av. rec., ppm	0.019	0.045	0.095	0.073	0.14	0.23
Av. rec., %	76	91	95	73	70	58
Std dev.	0.0010	0.0059	0.0085	0.0058	0.010	0.040
CV, %	5.3	12.9	9.0	7.9	7.1	17.3

Table 15-A. Recovery of endosulfan II and phosalone from fortified beef fat

	Endosulfan II			Phosalone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.081	0.16	0.31	0.099	0.21	0.43
Av. rec., %	81	80	76	99	104	107
Std dev.	0.0036	0.024	0.048	0.015	0.031	0.036
CV, %	4.4	15.1	15.6	15.2	14.8	8.3

Table 16-A. Recovery of linuron and endosulfan I from fortified pork fat

	Linuron			Endosulfan I		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.25	0.50	1.0	0.10	0.20	0.40
Av. rec., ppm	0.21	0.34	0.58	0.096	0.19	0.35
Av. rec., %	83	69	58	96	93	88
Std dev.	0.026	0.026	0.051	0.014	0.041	0.061
CV, %	12.6	7.6	8.7	15.0	22.2	17.4

Table 17-A. Recovery of captan and kepone from fortified pork fat

	Captan			Kepone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.010	0.020	0.040	0.10	0.20	0.40
Av. rec., ppm	0.0072	0.012	0.022	0.081	0.12	0.23
Av. rec., %	72	60	54	81	58	58
Std dev.	0.00066	0.00092	0.0011	0.014	0.021	0.076
CV, %	9.1	7.8	5.1	16.7	17.8	32.4

Table 18-A. Recovery of endosulphan II and phosalone from fortified pork fat

	Endosulfan II			Phosalone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.093	0.18	0.35	0.11	0.19	0.44
Av. rec., %	93	88	88	106	93	111
Std dev.	0.014	0.037	0.093	0.0078	0.011	0.044
CV, %	14.7	21.3	26.2	7.4	5.0	9.9

Table 19-A. Recovery of endosulfan I and kepone from fortified poultry fat

	Endosulfan I			Kepone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.10	0.18	0.41	0.074	0.15	0.25
Av. rec., %	101	92	103	74	77	63
Std dev.	0.0089	0.016	0.069	0.023	0.070	0.12
CV, %	8.8	8.9	16.7	30.6	45.7	47.7

Table 20-A. Recovery of endosulfan II and phosalone from fortified poultry fat

	Endosulfan II			Phosalone		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Added, ppm	0.10	0.20	0.40	0.10	0.20	0.40
Av. rec., ppm	0.10	0.20	0.39	0.10	0.22	0.44
Av. rec., %	100	101	98	103	109	110
Std dev.	0.011	0.045	0.10	0.0052	0.018	0.043
CV, %	11.4	22.1	26.2	5.1	8.8	9.8

Multiresidue Screen for Organophosphorus Insecticides Using Gel Permeation Chromatography—Silica Gel Cleanup

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A multiresidue screen for quantitative determination of 43 organophosphorus insecticides in 5 g of plant and animal tissues is described. The organophosphorus insecticides are extracted with methanol–dichloromethane (10 + 90, v/v) and cleaned up using automated gel permeation chromatography with hexane–ethyl acetate (60 + 40) eluant and in-line silica gel minicolumns. Concentrated extracts are analyzed by gas chromatography with flame photometric detection. The method recovers 43 organophosphorus insecticides in the range of 72 to 115%. Analysis of fortified bovine liver (n = 5) shows an average 95.9 ± 4.8% recovery at the 0.05 μg/g level and 93 ± 3.8% at the 0.5 μg/g level. Analysis of fortified bovine rumen content (n = 5) shows an average 98 ± 4.2% recovery at the 0.1 μg/g level and 98.7 ± 2.8% at the 1 μg/g level. Method detection limits ranged from 0.01 to 0.05 μg/g for the compounds studied using a nominal 5 gram sample.

Previous work has demonstrated the utility of a multiresidue approach to pesticide residue analysis (1, 2). Current organophosphorus (OP) insecticide screen methodology usually requires extraction with a polar solvent, followed by liquid-liquid partition. The sample is concentrated by evaporation, often with a solvent exchange, before gas chromatographic (GC) analysis. Liquid-liquid partition is labor intensive and the subsequent cleanup steps required for complex sample matrixes must be useful for target compounds across a wide polarity range. This may require multiple fractionation and analysis of the same sample.

Extraction solvents such as acetone (3, 4), ethyl acetate (5), acetonitrile (6), and toluene/hexane (7) have been used for OP analysis. These solvents either inadequately extract the more polar OPs such as methamidophos (8) or extract large amounts of water. This water may be removed by subsequent liquid-liquid partition (3), solid phase extraction (9) or by salting out with NaCl or Na_2SO_4 (10). Single or multiple extractions with more polar solvent mixtures can improve the extraction efficiency of polar OP compounds (3); however, dissolved water remains and must be removed by some means.

In pesticide residue screening, the cleanup step is often eliminated to allow for rapid analysis (10). For complex matrix samples, this can have negative effects on analytical quality. Removal of matrix from sample extracts enables more consistent and reproducible injections during analysis, as well as minimizing matrix effects on column behavior and detector response. Reducing the number of extraneous peaks on a chromatogram decreases the necessity for confirming analyses. With the injection of cleaner samples, the lifetime of expensive columns is extended and instrument downtime is minimized.

Common multiresidue approaches to the removal of coextractives have met with limited success when applied to a wide range of OP compounds. Liquid-liquid partitioning with immiscible solvents such as petroleum ether–acetonitrile (11, 12) is labor intensive (9), requires multiple back extractions, and is inadequate for recovery of some OP compounds. Sweep co-distillation can successfully cleanup samples; however, decomposition of some OP compounds has been observed (13). Adsorbent column chromatography has been applied to OP analysis with varying success. Florisil has been shown to retain some OP insecticides (14), and will oxidize OP insecticides with thio-ether groups. Neutral alumina can degrade some OP compounds (15). Magnesia-charcoal columns have been shown to retain acephate (16). Silica has been used successfully for OP analysis, but requires a wide range of solvent polarities to elute all OP insecticides (4, 17). Solid phase extraction minicolumns have been used successfully to cleanup extracts before OP analysis (9) and concomitant miniaturization of analysis can be desirable for small samples or increased production efficiency.

Gel permeation chromatography (GPC) has been effectively applied to cleanup OP extracts. Solvent systems such as cyclohexane–methylene chloride (18), hexane–methylene chloride (19) and ethyl acetate–cyclohexane (4) have all been used. Cyclohexane in the mobile phase adds viscosity

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and thus diminishes chromatographic resolution. Methylene chloride is a recognized workplace hazard with expensive waste disposal. The evaporation step following GPC cleanup is impaired with the use of low volatility solvents (cyclohexane) or solvents incompatible with many GC analyses (dichloromethane).

The present paper describes a multiresidue OP method for the complex matrixes of the various types of samples used in veterinary diagnostic toxicology investigations. These may include feed and environmental samples as well as postmortem tissues and ingesta. The method uses extraction with methanol–dichloromethane (10 + 90, v/v), followed by gel permeation chromatography cleanup and analysis by gas chromatography with flame photometric detection. The protocol was tested for 43 organophosphorus insecticides by means of a recovery study from pooled bovine liver and pooled bovine ingesta also known as rumen content. As a sample matrix, rumen content can be described as a water saturated, partially digested, grain and vegetation mixture.

METHOD

Equipment and Apparatus

(a) *Gas chromatographs.*—(*1*) (Model 5890, Hewlett-Packard) equipped with flame photometric detector (FPD); phosphorous filter (525 nm); autosampler (Model 7673, Hewlett Packard); 30 m × 0.53 mm × 1.0 μm DB-17 capillary column (J & W Scientific); glass insert with 0.5 cm loosely packed silanized glass wool; He carrier gas flow, 15 psi (12 mL/min); detector flows: H_2 at 40 psi, air at 60 psi; temperature program: 60°C for 0.5 min, 30°C/min to 160°C, 5°C/min to 280°C, 280°C for 4.2 min; run time: 32 min; split/splitless injection, valve off at 0.5 min; injector temperature: 240°C; detector temperature: 280°C; volume of injection: 2.0 μL. (*2*) (Model Sigma 2000, Perkin Elmer) equipped with nitrogen phosphorous detector (NPD); autosampler (Model AS-2000, Perkin Elmer). 30 m × 0.25 mm × 0.25 μm DB-5 capillary column (J & W Scientific); glass insert with 0.5 cm loosely packed silanized glass wool; carrier gas flow: helium at 20 psi (2.5 mL/min); detector gas flows: H_2 at 4 psi (2 mL/min), air at 5 psi (120 mL/min); temperature program: 60°C for 0.5 min, 30°C/min to 160°C, 5°C/min to 290°C, 290°C for 5 min. Run time 34.8 min; split/splitless injection; valve off at 0.5 min; injector temperature: 240°C; detector temperature: 300°C; volume of injection: 2 μL.

(b) *Centrifuge.*—IEC Centra-7R refrigerated centrifuge (International Equipment Company, USA).

(c) *Data output and processing system.*—Chromatography data system (Turbochrom data system with 900 series interface, Perkin Elmer–Nelson); integrator (Model LCI-100, Perkin Elmer).

(d) *Gel permeation chromatograph.*—(Model 1002A Autoprep GPC, ABC Laboratories) with 5 mL sample loop. Additional fittings included: 4-way switching valve to remove column from solvent path (Rainin Instrument Co.); 0.5 cm Luer-lock to solid phase extraction column fitting (Bio-Rad Laboratories); low pressure 1/4–28 tube couplings, flangeless PFTE fittings for 1/8 in. and 1/16 in. tubing, 1/16 in. od × 0.01 mm id PFTE tubing (Rainin Instrument Co.). GPC column: 25 mm id × 300 mm packed with 60 g S-X3 Biobeads, 200–400 mesh (Bio-Rad Laboratories); mobile phase, hexane–ethyl acetate (60 + 40); flow rate, 5 mL/min; 19 min dump cycle, 29 min collect cycle, 4 min wash cycle.

(e) *Temperature controlled nitrogen gas evaporator.*—(N-Evap Analytical Evaporator, Organomation Assoc. Inc., Berlin, MA).

(f) *Screw cap test tubes.*—10 mL and 50 mL, with PTFE lined caps (Fisher Scientific).

(g) *French square homogenization vessels.*—250 mL, with Teflon® lined caps (Fisher Scientific).

(h) *Silica gel solid phase extraction columns.*—6 mL, 500 mg disposable (Bakerbond SPE, J. T. Baker, Inc.).

(i) *Tissue homogenizer.*—(Polytron Model PT 10/35, Brinkman Instruments, Inc.).

(j) *Analytical paper filter disks.*—1/2 in., No. 740-E (Schleicher & Schuell Inc., Keene, NH).

(k) *Single-use syringe with Luer tip.*—6 mL (Monoject, Division of Sherwood Medical, St. Louis, MO).

Reagents

(a) *Methylene chloride, hexane, ethyl acetate, acetone, toluene.*—Pesticide grade (Fisher Scientific).

(b) *Sodium sulfate.*—ACS reagent grade (Fisher Scientific), washed with ethyl acetate and oven dried at 120°C.

Preparation of Standard Solution

Dissolve 25 mg neat organophosphorus insecticide in 25 mL toluene to make 1000 μg/mL standard solution. Some compounds may need to be dissolved in methanol before dilution in toluene. Subsequent dilutions are made using ethyl acetate. OP compounds are divided into 4 mixes for analysis on the standard column. An initial standard solution of each of the 4 mixes is made at 50 μg/mL. This is then diluted to 0.5 μg/mL with ethyl acetate for analysis (Table 1).

Extraction

Thoroughly mix sample to obtain a representative subsam-

Table 1. Organophosphorus insecticide standard mixes and CAS registry numbers

Mix A	Mix B
Coumaphos [56-72-4]	Carbophenothion [786-19-6]
DEF [78-48-8]	Dicrotophos [141-66-2]
Diazinon [333-41-5]	Dimethoate [60-51-5]
Ethion [563-12-2]	Disulfoton [298-04-4]
Fonofos [944-22-9]	Isofenphos [25311-71-1]
Methidathion [950-37-8]	Malathion [121-75-5]
Methyl parathion [298-00-0]	Merphos [150-50-5]
Mevinphos [7786-34-7]	Naled [300-76-5]
Parathion [56-38-2]	Phosmet [732-11-6]
Phorate [298-02-2]	Tetrachlorvinphos [961-11-5]
Phosalone [2310-17-0]	
Terbufos [13071-79-9]	
Mix C	**Mix D**
Azinphos-methyl [86-50-0]	Acephate [30560-19-1]
Chlorfenvinphos [470-90-6]	Chlorpyrifos [2921-88-2]
Dichlorvos [62-73-7]	Crotoxyphos [7700-17-6]
Demeton-O [298-03-3]	Crufomate [299-86-5]
Demeton-P [126-75-0]	Ethoprop [13194-48-4]
Dioxathion [78-34-2]	Fensulfothion [115-90-2]
EPN [2104-64-5]	Methamidophos [10265-92-6]
Fenamiphos [22224-92-6]	Monocrotophos [919-44-8]
Fenthion [55-39-9]	Propetamphos [31218-83-4]
Phosphamidon [13171-21-6]	Profenophos [41198-08-7]
	Ronnel [299-84-3]
	Triazophos [24017-47-8]

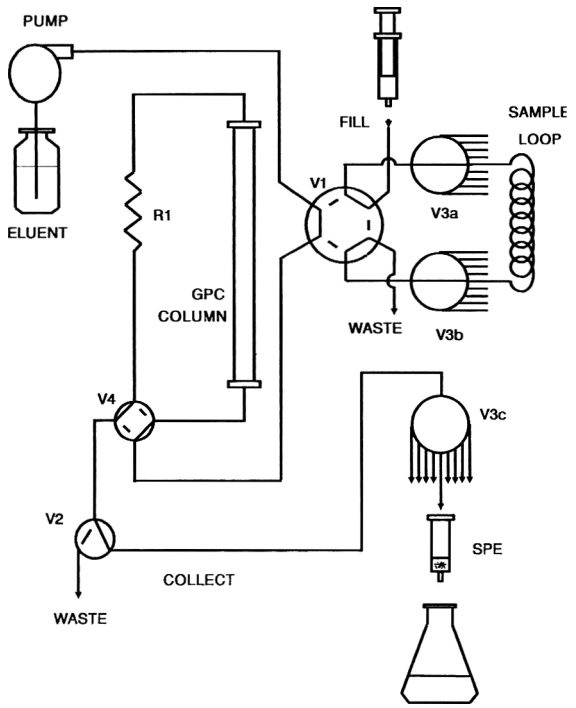

Figure 1. Schematic of GPC-SPE automatic cleanup system. V1 = 6-way manual load/run valve. V2 = 3-way solenoid dump/collect valve. V3 = 24-way, 3-gang solenoid sample selector valve. V4 = 4-way manual eluent bypass valve. R1 = 0.010 in. id capillary restrictor. SPE = silica gel solid phase column.

ple. Fracture crop samples by Stein milling with liquid nitrogen. Mix tissue samples in Waring blender or chop finely if there is insufficient sample for blending. Weigh 5 g frozen sample into homogenizing vessel. Add 40 g sodium sulfate and 100 mL methanol–dichloromethane (10 + 90, v/v). Homogenize 2 min at high speed. Crops may be shaken vigorously for 30 min on mechanical shaker as needed. Centrifuge cloudy extracts at 1200 rpm for 5 min. If water is still present in the extract as a top layer, add 50 g additional sodium sulfate and shake vigorously for 2 min. Pipet a 40 mL aliquot of extract into a 50 mL test tube, adding 3 drops of 5% decanol in acetone. Carefully evaporate to dryness using nitrogen evaporator at 40°C. Add 10 mL GPC solvent [hexane–ethyl acetate (60 + 40)] and sonicate 2 min to redissolve residue. Filter samples through 0.45 μm PTFE filters as they are loaded onto the GPC.

Gel Permeation Chromatographic (GPC) Cleanup

Sample extracts are cleaned by GPC and silica gel SPE minicolumns attached to the GPC outlet (Figure 1). Prerinse silica gel SPE columns with 5 mL GPC solvent and attach to output from GPC. Load 5 mL extract into GPC. Run GPC program, collecting in 250 mL flasks. Remove GPC column from solvent path using 4-way switching valve. Set GPC to "load." Change GPC solvent to 20% acetone in ethyl acetate. Elute SPE columns with 15 mL of 20% acetone in ethyl acetate at 5 mL/min, running GPC as follows: dump, 0; collect, 3; wash, 0. Combine with GPC eluate. Add 3 drops of 5% decanol in acetone. Evaporate to 10 mL with stream of nitrogen at 40°C. Transfer to 15 mL Kuderna-Danish receiving flask with 5 mL ethyl acetate. Continue concentration to 0.5 mL.

Gas Chromatographic (GC) Determination

Determine residues by GC, using GC/FPD with DB-17 megabore column. Inject 2 μL analytical standard (0.5 μg/

Table 2. Retention times (RT) in minutes of organophosphorus insecticides

Compound	Alternate name	RT 1[b]	RT 2[c]
	Mix A		
Mevinphos	Phosdrin	7.21	5.9
Phorate	Thimet	10.9	9.3
Terbufos	Counter	11.03	10.51
Diazinon	Spectracide	12.33	10.9
Fonofos	Dyfonate	12.93	10.7
Methyl parathion		15.48	12.5
Parathion	Parathion ethyl	16.52	15.15
DEF	Butifos	19.22	17.3
Methidathion	Supracide	20.58	16.2
Ethion	Bladan	22.15	19.3
Phosalone	Zolone	27.11	23.6
Coumaphos	Co-Ral	30.3	26.3
	Mix B		
Naled	Dibrom	11.1	8.6
Dicrotophos	Bidrin	12.12	8.8
Disulfoton	Di-Syston	12.95	11.1
Dimethoate	Cygon	13.74	9.8
Merphos	Folex	15.88	
Malathion	Sumitox	16.58	13.8
Isofenphos	Oftanol	17.83	15.7
(Merphos) as DEF	Butifos	19.22	17.3
Tetrachlorvinphos	Gardona	19.82	16.62
Carbophenothion	Trithion	23.03	20
Phosmet	Imidan	27.51	22.2
	Mix C		
Dichlorvos	DDVP	5.07	4.4
Demeton-O	Systox-O	9.24	8.1
Demeton-S	Systox-S	11.06	9.71
Dioxathion[a]	Delnav	13.36	10.5
Phosphamidon "E"	Dimecron "E"	13.89	12.2
Phosphamidon "Z"	Dimecron "Z"	15.34	12.2
Fenthion	Baytex	17.48	14.2
Chlorfenvinphos "E"	Supona "E"	18.39	15.3
Chlorfenvinphos "Z"	Supona "Z"	18.39	15.6
Fenamiphos	Nemacur	20.22	17.19
EPN	Santox	25.93	22.4
Azinphos-Methyl	Guthion	29.4	23.7
Dioxathion	Delnav		26.6
	Mix D		
Methamidophos	Monitor	5.74	5.0
Acephate	Orthene	8.29	6.2
Ethoprop	Mocap	9.86	8.35
Propetamphos	Safrotin	12.26	10.5
Monocrotophos	Azodrin	12.85	9.3
Ronnel	Fenchlorfos	15.05	13.0
Chlorpyrifos	Dursban/Lorsban	16.25	15.2
Crufomate	Ruelene	17.69	14.6
Crotoxyphos	Ciodrin	19.57	16.05
Profenophos	Curacron	20.13	17.38
Fensulfothion	Dasanit	23.19	18.89
Triazophos	Hostathion	24.52	19.72

[a] Thermal breakdown peak.
[b] Standard conditions: 30 m DB-17 megabore (FPD-P).
[c] Standard confirmation conditions: 30 m DB-5 0.25 mm id 20 psi flow (NPD).

Table 3. Fortified matrix mean percent recoveries ($n = 5$) and coefficients of variation of organophosphorus insecticides from rumen content and liver by the tissue/crop procedure

	Rumen content				Liver			
	0.1 μg/g		1.0 μg/g		0.05 μg/g		0.5 μg/g	
	Mean Recovery	%CV	Mean Recovery	%CV	Mean Recovery	%CV	Mean Recovery	%CV
Acephate	95	1	92	1	76	4	74	3
Azinphos-Methyl	113	7	112	7	103	10	86	2
Carbophenothion	84	4	102	2	85	5	91	2
Chlorfenvinphos	108	3	102	2	94	3	88	2
Chlorpyrifos	99	3	98	1	105	2	97	3
Coumaphos	114	1	111	1	110	5	111	4
Crotoxyphos	105	1	107	1	110	3	102	3
Crufomate	103	2	102	1	105	2	100	2
DEF	100	2	100	2	103	3	102	4
Demeton	81	8	78	3	86	4	72	4
Diazinon	95	3	95	4	88	3	88	5
Dichlorvos	102	9	81	2	91	4	74	6
Dicrotophos	92	4	106	2	93	4	90	6
Dimethoate	88	3	101	2	92	3	93	2
Dioxathion	88	8	98	6	87	6	102	15
Disulfoton	90	4	97	6	94	10	109	7
EPN	101	3	107	3	90	2	84	3
Ethion	115	12	99	3	100	13	93	5
Ethoprop	84	4	91	2	95	1	93	4
Fenamiphos	111	3	101	2	108	2	87	3
Fensulfothion	105	2	104	1	107	6	100	2
Fenthion	103	10	95	2	83	5	81	3
Fonofos	95	3	93	5	85	4	85	4
Isofenphos	93	4	95	2	95	4	92	1
Malathion	92	5	100	2	97	5	94	1
Merphos	110	7	107	8	114	7	115	2
Methamidophos	85	6	78	1	91	5	80	5
Methidathion	107	5	100	3	103	4	101	4
Methyl parathion	100	3	99	3	91	5	94	4
Mevinphos	94	6	93	5	83	3	83	5
Monocrotophos	99	2	101	1	86	12	93	2
Naled	91	3	97	7	95	13	87	9
Parathion	99	2	99	2	103	6	97	4
Phorate	89	4	86	5	81	2	78	5
Phosalone	92	8	106	2	95	8	106	4
Phosmet	103	2	101	6	103	0	107	3
Phosphamidon	113	5	114	2	91	3	97	3
Profenophos	101	2	103	1	108	2	100	3
Propetamphos	89	3	90	1	96	4	89	3
Ronnel	93	2	97	1	103	1	96	4
Terbufos	90	4	90	5	84	3	84	4
Tetrachlorvinphos	101	5	109	3	102	5	101	1
Triazophos	102	3	103	1	111	10	100	3

mL) and samples (2 g/mL). Residues are confirmed using GC/NPD with DB-5 capillary column. Quantitate using external calibration based on injections of 1 ng of OP insecticides.

Validation Study

Pooled liver and pooled rumen content were fortified at 2 levels with each OP standard mix. Liver was fortified at 0.5 and 0.05 mg/kg; rumen content at 1.0 and 0.1 mg/kg. Five replicates of each fortification level for both matrixes were prepared. This scheme was repeated for each of the 4 standard mixes.

Results and Discussion

Results for the 43 organophosphorus insecticides studied are presented in Tables 2 and 3. The method quantitatively extracts the polar OP insecticides (i.e., methamidophos) and removes matrix coextractives without loss of the more volatile OP insecticides (i.e., dichlorvos). Extraction with methanol–dichloromethane (10 + 90, v/v), combined with GPC/silica gel cleanup resulted in good precision and accuracy for both a high water content, highly pigmented matrix (bovine rumen content) and a matrix of high fat content (bovine liver).

Use of 10% methanol in dichloromethane as an extraction

solvent solves the problems associated with the use of a polar extraction solvent. A mixture of methanol–dichloromethane (10 + 90) has sufficient polarity to extract highly polar compounds such as acephate, monocrotophos, and methamidophos, yet is immiscible with water. Any water in the solvent extract is removed with sodium sulfate.

A small amount of water in the tissue/crop extract after extraction is not a problem if the sample is shaken before removal of the aliquot. Tissue and crop extracts do not usually require coarse filtration; the aliquot is easily withdrawn from the homogenization vessel after momentary settling. If required, the 40 mL aliquot can be filtered under vacuum through an empty 6 mL column with a 0.5 in. filter paper disc placed at the bottom and rinsed with 5 mL extraction solvent.

One limitation of this method is the small amount of sample (5 g) analyzed. A 10 g sample lowers recovery of the polar OP insecticides to less than 70% in samples with greater than 50% water content. It is, therefore, important to fully mix samples before subsampling to obtain representative results. This liability can be overcome by increasing the scale of the extraction step.

Preliminary studies showed that high (120–160%) recoveries resulted from injection of concentrated sample extracts (>1 g/mL) without the final silica gel cleanup because of matrix effects on the chromatography. These influences can be reduced by using toluene as the injection solvent. These results indicate that all extracts >0.5 g/mL concentration and all tissue extracts should undergo at least silica gel cleanup. If GPC cleanup is omitted to reduce analysis time, the silica gel column alone gives a moderate cleanup. If the silica gel column is the only cleanup, the sample should be added to the column in 1 mL hexane, washed with an additional 9 mL hexane (discarded) and eluted with 15 mL of 20% acetone in ethyl acetate. Poor recovery of chlorpyrifos and ronnel may result from this abbreviated cleanup. This modified cleanup was not used in the validation study.

A challenging aspect of this work was the development of a GPC solvent system that could elute highly retained compounds (especially azinphos methyl) with a minimum of solvent, while providing satisfactory resolution of the pesticides from the lipid coextractives. The reduced GPC elution solvent volume and its higher volatility allows nitrogen evaporation, avoiding the variable recoveries associated with rotary evaporation. A high percent of ethyl acetate is necessary to elute azinphos methyl in a reasonably small volume.

Gel permeation chromatography is excellent for removal of lipids and other large molecules from sample extracts. When coupled in series with a silica gel minicolumn to remove polar matrix coextractives, a clean extract can be obtained. A filtered liver extract representing 1 g sample has a dry mass of 19.2 mg before cleanup. This extract following cleanup has a dry mass of 1.2 mg. The remaining small, relatively nonpolar interferences typically cause the fewest chromatographic difficulties. Figure 2 shows a typical GC-FPD chromatogram for analysis of a 0.1 μg/g fortification of bovine rumen content, with a minimum of matrix peaks present.

The data suggest that the evaporation steps in this procedure result in minimal analyte loss. This was accomplished by the use of volatile solvents throughout analysis, a stream of nitrogen at 40°C for evaporation, and 5% decanol in acetone as a "keeper." Volatile OP insecticides are all well recovered with low coefficients of variation. When comparing percent recoveries for several compounds fortified at the 0.1 μg/g level in bovine rumen content, the more volatile compounds dichlorvos (Vp^{20} = 20 000 mPa at 20°, 102% recovery), naled (Vp^{20} = 260 mPa, 91%), phorate (Vp^{20} = 110 mPa, 89%), and ethoprop (Vp^{26} = 46.5 mPa, 84%) produced comparable recoveries to compounds of intermediate volatility such as methyl parathion (Vp^{20} = 1.3 mPa, 100% recovery), parathion (Vp^{20} = 5 mPa, 99%), chlorpyrifos (Vp^{25} = 2.5 mPa, 99%), and malathion (Vp^{30} = 5.3 mPa, 92%) (20).

Chromatographic conditions were chosen to maximize resolution between peaks, while minimizing total run time (32 min). Routine sample analysis has shown that the chromatographic conditions chosen also minimize the need for confirming analyses. An autosampler enables this long run time to be practical for analysis of many samples. Additionally, in the present study, the FPD was superior in performance to the NPD as a detector for analysis of OP insecticides. FPD background interference was significantly less than that of the NPD with better day-to-day stability. Some polar OP insecticides, such as acephate and methamidophos, do not chromatograph well on the nonpolar DB-5 confirmatory column. These are typically confirmed on a more polar column, such as a DB-1701.

The diversity of physical properties of OP insecticides studied presented some challenges in developing a comprehensive multiresidue screen. For example, dioxathion undergoes thermal degradation in the injection port. The intact compound does not chromatograph under standard conditions, although it does under confirmation conditions. In the present study, we found that, when samples were not sufficiently cleaned up, there could be a resultant variability in the breakdown of dioxathion. Demeton was shown to be unstable, with recoveries dropping from 90 to 25% in 72 h at room temperature for both matrixes. This degradation causes difficulties on long analyses, or on re-analysis of extracts. Naled and ethion coeluted with matrix interferences on the DB-17 column. Naled results are based on confirmational analysis, while the background level was subtracted for ethion results. This results in the greater coefficient of variation for ethion fortifications at low levels. Merphos was shown to oxidize to DEF (Butifos) during sample cleanup. Quantitation is based on the DEF level, with a factor based on the ratio of the 2 molecular weights used to adjust the amount found. The merphos standard also undergoes this oxidation, and must be routinely monitored and replaced. The presence of DEF in the sample extract indicates the presence of either DEF or merphos in the sample. These compounds must be confirmed by a different cleanup scheme. The 2 isomers of mevinphos are resolved on the DB-17, but not the DB-5 confirmation columns. The "E" isomer was used for fortification quantitation on the primary analysis, while the combined "E" and "Z" isomers were used for confirming analysis. Both isomers are used for quantitation of mevinphos levels in samples.

In summary, this multiresidue screen has the ability to extract, cleanup, and determine 43 organophosphorus insecticides in liver tissue and rumen content. The method performed well for high fat content and for extremely pigmented, high water content matrixes, with good fortification recoveries and coefficients of variation for all 43 OP insecticides. The procedure is especially suited to situations in which sample size is limited, or in which a large number of difficult samples must be analyzed, by providing good cleanup with minimal preparation time. The method has been used

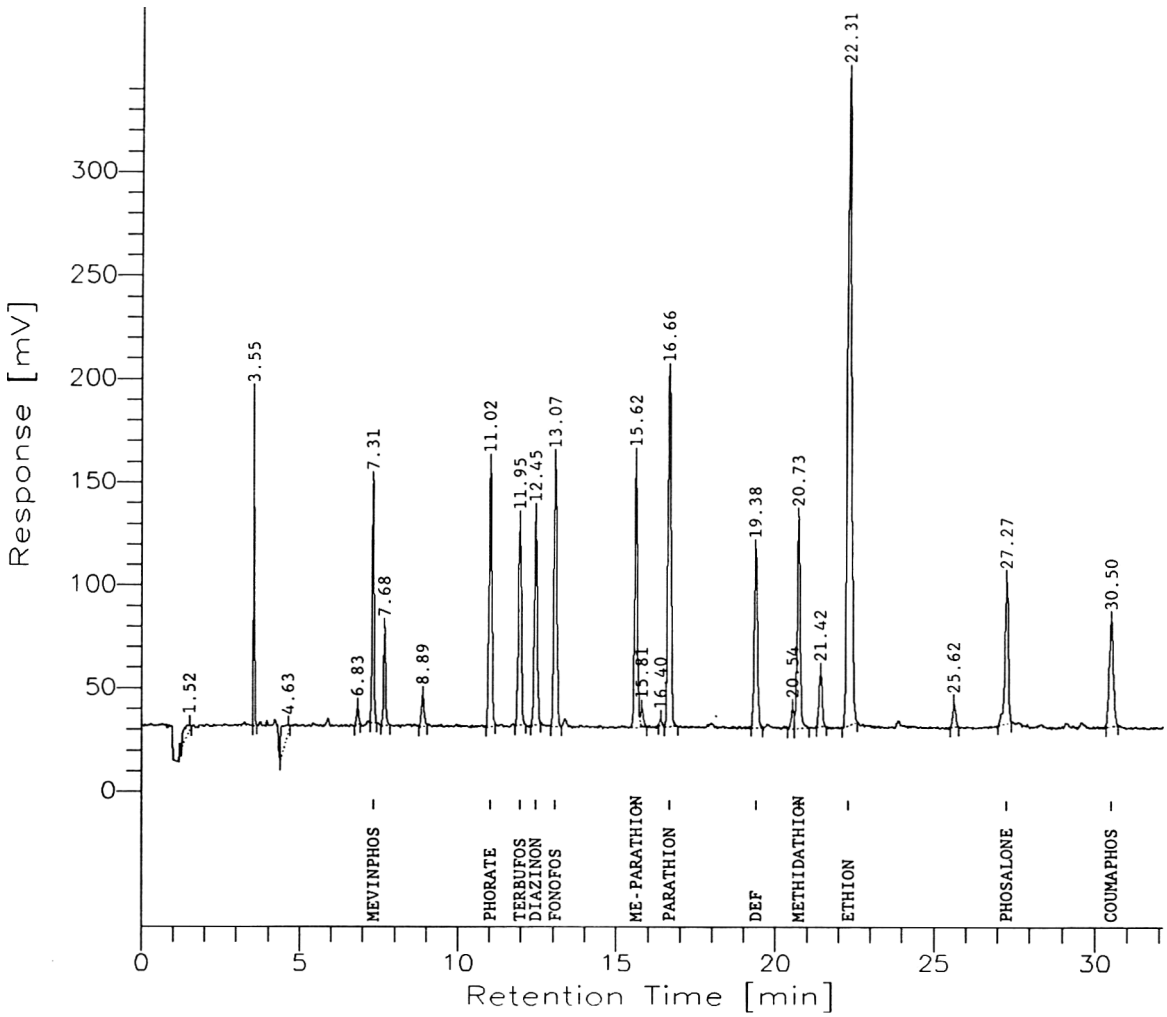

Figure 2. GC/FPD chromatogram of bovine rumen content extract, fortified at 0.1 ppm with standard mix A. 2 μL of 2 g/mL sample extract injected using 30 m DB-17 megabore column.

in routine sample analysis with good spike recoveries from a variety of matrixes that have included a variety of animal feeds and tissue types. While methylene chloride has been eliminated from GPC cleanup, it remains in the extraction solvent. Work is in progress to develop a satisfactory nonchlorinated extraction solvent mixture and cleanup for the analysis of organophosphorus, organochlorine and organonitrogen pesticides in a combined multiresidue protocol.

REFERENCES

(1) Seiber, J.N., Glotfelty, D.E., Lucas, A.D., McChesney, M.M., Sagebiel, J.C., & Wehner, T.A. (1990) *Arch. Environ. Contam. Toxicol.* **19**, 583–592
(2) Ripley, B.D. (1987) *Pestic. Sci. Biotechnol., Proc. Int. Cong. Pestic. Chem. 6th 1986*, pp. 357–360
(3) Luke, M.A., Froberg, J.E., & Masumoto, H.T. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 1020–1026
(4) Specht, W., Tillkes, M. (1985) *Fresenius Z Anal. Chem.* **322**, 443–455
(5) Roos, A.H., Van Munsteren, A.J., Nab, F.M., & Tuinstra, L.G.M. Th. (1987) *Analytica Chimica Acta* **196**, 95–102
(6) Storherr, R.W., Ott, P., & Watts, R.R. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 513–516
(7) Johansson, C.E. (1978) *Pestic. Sci.* **9**, 313–322
(8) Luke, M.A., Froberg, J.E., Doose, G.M., Masumoto, H.T. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1187–1195
(9) Hopper, M.L. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 731–734
(10) California Department of Food and Agriculture Pesticide Residue Program (1988) *Multi-Residue Screens*, January 27, 1988
(11) *Pesticide Analytical Manual* (1988) Vol. I, Food and Drug Administration, Washington, DC, secs 231, 232
(12) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec **970.52**
(13) Luke, B.G., & Richards, J.C. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 902–904
(14) Beckman, H., & Garber, D. (1969) *J. Assoc. Off. Anal. Chem.* **52**, 286–293
(15) Ambrus, A., & Thier, H.-P. (1986) *Pure & Appl. Chem.* **58**(7), 1035–1062
(16) Blaha, J.J., & Jackson, P.J. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1095–1099
(17) Lores, E.M., Moore, J.C., & Moody, P. (1987) *Chemosphere* **16**, 1065–1069
(18) Ault, J.A., Schofield, C.M., Johnson, L.D., & Waltz, R.H. (1979) *J. Agric. Food Chem.* **27**, 825–828
(19) Hopper, M.L. (1982) *J. Agric. Food Chem.* **30**, 1038–1041
(20) *The Agrochemicals Handbook* (1988) 2nd Ed., Update 2, The Royal Society of Chemistry, Nottingham, England

SEAFOOD PRODUCTS

Gas Chromatographic Method for Determination of Dimethylamine, Trimethylamine, and Trimethylamine Oxide in Fish-Meat Frankfurters

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A method is described for analysis of minced fish-meat and surimi-meat frankfurters for dimethylamine (DMA), trimethylamine (TMA), and trimethylamine oxide (TMAO) using a headspace-gas chromatographic technique. After simple acid extraction and addition of NaOH, the headspace was directly injected into a gas chromatograph by a gas-tight syringe. DMA and TMA were separated on a Chromosorb 103 column and detected by a flame ionization detector. TMAO was measured as TMA after Zn reduction. Repeatability of the method for DMA, TMA, and TMAO was 6.6, 1.0, and 18.8 ppm, respectively. The method was applicable to Alaska pollock-meat and Atlantic menhaden-meat frankfurters, unwashed, and washed mince and surimi.

Based on advances in food technology, use of minced fish (mechanically separated flesh) and surimi (a washed form of mince, comprised primarily of stabilized myofibriller protein) has been proposed as a partial substitute for meat in heretofore formulated all-meat products (1-3). Use of fish in nitrite-cured products raises concerns regarding formation of the potent carcinogen, *N*-nitrosodimethylamine (NDMA), because fish generally contains more dimethylamine (DMA) than meat (4, 5). The presence of NDMA at the low ppb level, particularly in salt-dried fish and other seafoods not exposed directly to nitrite, has been noted in several reviews (6, 7). Deterioration of fresh fish through microbial spoilage is accompanied by a parallel decomposition of trimethylamine oxide (TMAO) to trimethylamine (TMA) to the extent that the latter compound is used as an indicator of freshness (8).

In fish of the gadoid family, TMAO principally forms DMA and formaldehyde by endogenous enzymes (9), with the maximum formation below the freezing point of fish (−5 to −10°C) (10) and under refrigeration conditions in the absence of oxygen (11). One member of the gadoid family, Alaska pollock, is the preferred raw material for shellfish analogues made from surimi. While DMA can be nitrosated directly to form NDMA, both TMAO and TMA have also been shown to form NDMA (12-16). Under certain conditions, NDMA forms more readily from TMAO than TMA (17). Therefore, any investigation of NDMA in frankfurters containing meat in combination with fish mince or surimi requires accurate measurement of all 3 compounds—TMAO, TMA, and DMA.

For reasons of specificity, gas chromatography (GC) has been the common means for direct analysis of DMA and TMA, despite problems associated with its use. Analysis of volatile amines by GC has been hampered by loss of sample response, the ghosting phenomena, and badly tailed peaks because of adsorptive effects between the aliphatic amines and the chromatographic support or adsorbent (18, 19). Most efforts to overcome these difficulties have involved deactivating the column packing material by adding a strongly basic material such as KOH or by adding ammonia to the carrier gas with noticeable improvement. Lack of published statistical data on the quantitative aspects of measuring low ppm of methylamines in seafood samples, except for a few fortification-recovery studies, also suggests problems in using GC for amine analysis. Lundstrom and Racicot describe an apparently successful method for determining both DMA and TMA in seafood (20). Despite this and other investigations on different sample preparations and GC column packings and detectors, there remains a need for a simple, specific, accurate method for methylamines as recently discussed (21).

The present paper describes an improved method applicable to fish-meat frankfurters containing Alaska pollock and Atlantic menhaden unwashed and washed mince and surimi.

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METHOD

Reagents

(a) *Hydrochloride salts of DMA and TMA.*—(Aldrich Chemical Co.) Dry to constant weight in a vacuum oven at 70°C.

(b) *Hydrochloric acid (HCl).*— Concentrated, 2.0 and 0.5N (J.T. Baker).

(c) *Sodium hydroxide (NaOH).*—15N (J.T. Baker).

(d) *Zinc powder.*—(Fisher Scientific) Purify 12 g by stirring for 1 min with 30 mL of 2N HCl, then transfer to a Buchner funnel and wash with 30 mL water, 20 mL ethanol and 20 mL acetone. Dry in a vacuum oven at 165°C for 1 h, then place in a suitable container.

(e) *Amine standard solution.*— Prepare 1 mg/mL individual standard solutions from dried crystals of DMA-HCl and TMA-HCl with 0.5N HCl. Prepare a combined working standard by taking 100 μL of each amine solution and adding it to 25 mL volumetric flask and diluting to volume with 0.5N HCl. Final concentration of DMA and TMA is 4 μg/mL, equivalent to 10 ppm in a 10 g sample.

(f) *Fish-meat samples.*—Frankfurters in which 15% or 50% of the meat was substituted with fish were prepared by the National Marine Fisheries Service and shipped to Eastern Regional Research Center as described previously (22). Samples of fish were either the raw ingredients for the frankfurters or ones purchased at local retail stores.

Apparatus

(a) *Tissumizer.*—Tekmar model SDT18/10 with a model SDT100EN shaft or equivalent.

(b) *Vortex mixer.*—Lab-Line Instruments Super-Mixer or equivalent.

(c) *Centrifuge tubes.*—Oak Ridge style, 50 mL polypropylene (Sorvall Instruments, Dupont Co.) or equivalent.

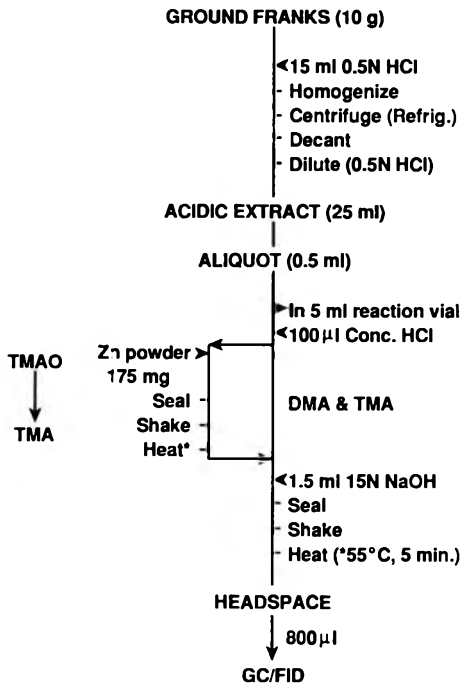

Figure 1. Schematic of amine method.

(d) *Refrigerated centrifuge.*—Sorvall RC-5B with an SA-600 rotor or equivalent.

(e) *Water bath.*—Exacta-Heat constant temperature bath or equivalent.

(f) *Reaction vials.*—Kontes microflex 5 mL vials, No. 749000-0005 or equivalent.

(g) *Gas-tight syringe.*—(Precision Sampling Corp.) 1 mL syringe.

(h) *Gas chromatograph.*—Hewlett-Packard model 5880A gas chromatograph equipped with a flame ionization detector and a controller-electronic integrator terminal level four. A 3.3 m × 2 mm id glass column packed with Chromosorb 103 was preconditioned at 250°C overnight with helium flow. Operating conditions: oven, 125°C isothermal; injector, 150°C; detector, 250°C; helium, 20 cc/min; hydrogen, 30 cc/min; air, 200 cc/min.

Procedure

Figure 1 is a schematic of the method.

Sample preparation.—Grind fish-meat frankfurters or fish twice through a 1/8-in. plate before analysis. Mix thoroughly. Weigh 10 g comminuted fish-meat or fish sample into a centrifuge tube. Add 15 mL of 0.5N HCl to tube and homogenize sample for 7 min with Tissumizer power setting at 70. After homogenization, centrifuge sample for 45 min at 15 000 rpm and 0°C. Decant liquid from tube into 25 mL volumetric flask and dilute to volume with 0.5N HCl. This is the sample solution.

Amine determination.—Pipette 0.5 mL DMA/TMA standard solution into a 5 mL vial containing 100 µL concentrated HCl, then add 1.5 mL of 15N NaOH. Immediately cap vial and agitate on vortex mixer for 1 min. Heat for 5 min in 55°C water bath. Inject, on column, 800 µL of the vial's headspace gases into GC. (*Caution*: do not permit any liquid contained in vial to be injected into column). Repeat standard determinations until response is reproducible—that is, at least 4 injections in a row that yield peak areas within 10% of each other. An average of the usable injections is used in the calculations.

Pipette 0.5 mL sample solution into a 5 mL vial containing 100 µL concentrated HCl and determine DMA and TMA as above.

For TMAO, pipette 0.5 mL sample solution into a 5 mL vial containing 100 µL concentrated HCl. Add a spatula tip amount (ca 175 mg) of powdered zinc, cap, agitate, and heat as above. Add 1.5 mL 15N NaOH, cap, agitate, heat, and inject headspace sample as above.

Calculation.—Concentrations of DMA and TMA are each calculated as follows:

$$Cx = \frac{Ax}{As} * Cs$$

where Cx is the concentration of amine in the liquid phase of the sample; Cs, the concentration of amine in the liquid phase of the standard; and Ax and As are the corresponding peak areas. TMAO is calculated by subtracting the TMA value from total TMA after reduction, then multiplying the result by 1.28 to express the concentration based on the oxide. All values in the paper are discussed in terms of amount of amine present in the sample, not in the liquid or vapor phase.

Statistical analysis.—The general linear models (GLM) procedure of the Statistical Analysis System PC software (version 6.04, SAS Institute, Inc., Box 8000, SAS Circle, Cary, NC 27512) was used to analyze results. Results were interpreted according to the methods of Snedecor and Cochran (23).

Results and Discussion

The method initially evaluated was as described by Lundstrom and Racicot (20); it had been successfully used to measure DMA and TMA in a wide variety of fish and seafood products. Under the same conditions, including use of nitrogen phosphorus specific detector (NPD), we could not obtain repeatable responses for a standard concentration of DMA in benzene. The high degree of variability precluded application of this method. Substitution of benzene by 2-propanol as used by Zeisel et al. (24), also produced erratic results. Our change to the more commonly available flame ionization detector (FID) improved repeatability of the DMA response in organic solvents. To lessen problems associated with repeated injections of extraction solvents containing amines and other sample components, we considered a headspace method. Concentrations of amines in the combination fish-meat frankfurters were sufficiently high so that the greater sensitivity of the NPD vs FID was not a factor. This meant that a less rigorous isolation/cleanup procedure might be employed. Miller and coworkers developed an equilibrium vapor analysis method in which an aliquot of the headspace was removed from a sealed screw-cap vial after addition of NaOH and heating (25). Using this approach, we employed a 5 mL reaction vial with Teflon-coated septa and a gas-tight syringe that permitted analysis of the products containing fish without loss of volatile amines.

In addition to the porous polymer, Chromosorb 103, selected for this study, a few other packings were evaluated. One, also a porous polymer, HayeSep B was recommended for separation of C_1 and C_2 amines and ammonia (26). A 3.3 m (10 ft) × 2 mm id glass column packed with 60–80 mesh HayeSep B was operated isothermally at 125°C. Helium carrier flow rate was 20 cc/min. Under these recommended conditions, DMA and TMA had long retention times of >7 min, did not produce sharp peaks, and produced erratic peak areas upon repeated injection of amine standard.

Changing operating conditions failed to improve the per-

Table 1. Recovery of dimethylamine and trimethylamine in fish-meat frankfurters

Amine added, ppm	Av. Recovery, % DMA	Av. Recovery, % TMA
5	100.2 ± 4.7	94.2 ± 6.9
50	92.1 ± 3.0	92.6 ± 5.5
500	88.1 ± 5.2	91.7 ± 3.1

$n = 4$.

formance of this column. We also tested another packing material, 4% Carbowax 20M/0.8% on KOH, on Carbopack B (27), specifically designed for analysis of volatile amines. After packing and conditioning as recommended, both DMA and TMA were resolved and separated under 4 min with sharp peaks. Unfortunately, amine levels below 5 ppm gave unpredictable results, with the DMA peak often disappearing. Because we needed to measure lower concentrations of this amine, this packing was not suitable. Despite successful application of Chromosorb 103 to determine DMA and TMA in fish and fish products (20, 28), Chromosorb 103 was not entirely problem-free. The columns packed with this material had to be conditioned daily with several injections of amine standards before peak areas reached a plateau and were reproducible. After conditioning, the column could be used the entire day.

Typically, the Chromosorb 103 column could be used for injection of at least 2000 samples and standards. Then, a sudden, unexplained deterioration of the column could be observed in which the DMA peak became very small or disappeared. Conditioning by injection of *n*-nonylamine to reduce the active sites did not improve column performance with respect to DMA repeatability. On occasion, new columns conditioned overnight at 250°C with carrier gas flow exhibited similar behavior and necessitated repacking. We found Chromosorb 103 to be the best column packing available for our use.

With the GC operating conditions described in the *Experimental* section, monomethylamine, DMA, and TMA were well resolved with retention times of 1.4, 2.3, and 2.8 min, respectively. Only the latter 2 volatile amines were quantitated in the samples tested. A peak for acetone, the solvent used to clean the syringe, was occasionally observed at 3.2 min.

The GC chromatographic detector linearity response to DMA and TMA was calculated from the means of triplicate determinations from standards ranging from 1 to 1000 ppm. This would cover anticipated concentrations in the product type tested. Calibration curves were plotted as amine concentration in ppm vs peak area. For $y = ax + b$, DMA had a slope of 0.00777 ppm/unit area and intercept of +9.52 ppm, $r^2 = 0.997$; TMA had $a = 0.00443$ ppm/unit area, $b = 4.01$, $r^2 = 0.996$. Both correlations were highly significant ($P < 0.001$).

To measure TMAO as TMA and keep the method simple, ca 175 mg Zn powder was added to the sample extract containing 100 μL conc. HCl. The acid was needed for the Zn to work effectively. In fish-meat frankfurters fortified with 10, 100, and 1000 ppm TMAO, 100% was converted by Zn to TMA.

Overall recovery studies were performed in duplicate on DMA and TMA where fortification levels, added to samples before analysis, were representative of concentrations found in this type of cured product.

Results of recovery studies are shown in Table 1. These recoveries and standard deviations show that the method was satisfactory for this type of analysis.

Figure 2 shows a chromatogram of (A) a standard containing the equivalent of 10 ppm DMA and TMA; curve (B) is from a frankfurter sample containing 50% unwashed Alaska pollock mince. Because the peak areas were measured, no attempt was made to attenuate the signal to keep peaks on scale. Curve (C) is the corresponding washed mince sample. While not indicated in the figure, these 2 samples also contained 1112 and 207 ppm TMAO, respectively. Concentrations of DMA were higher than TMA in all Alaska pollock samples and for most sample frankfurters containing Atlantic menhaden samples. No interfering peaks were observed in either the fish-meat frankfurters after processing and broiling or in raw fish. This was also true for the TMAO reduced samples, although 2 noninterfering peaks whose retention times corresponded to methanol and ethanol were sometimes noted.

To date, this method has been used to analyze more than 250 samples of minced fish (unwashed and washed) and surimi-meat frankfurters, in which the meat has been substituted at the 15 and 50% levels. The fish source was comprised of Alaska pollock (high amine) and Atlantic menhaden (low amine). This accounts for the wide range of concentrations of DMA, TMA, and TMAO shown in Table 2. Repeatabilities and coefficients of variation were estimated from the error term of the ANOVA. The CV for DMA is acceptable for our uses, given the known difficulty in accurately measuring this amine. The minimum detectable level was 0.1 ppm for DMA and 0.05 ppm for TMA. This method was also applied to all-meat frankfurters and the minced fish and surimi used in the preparation of the frankfurters. Limited analysis indicated that this approach may be effective for analysis of other fish species and seafoods.

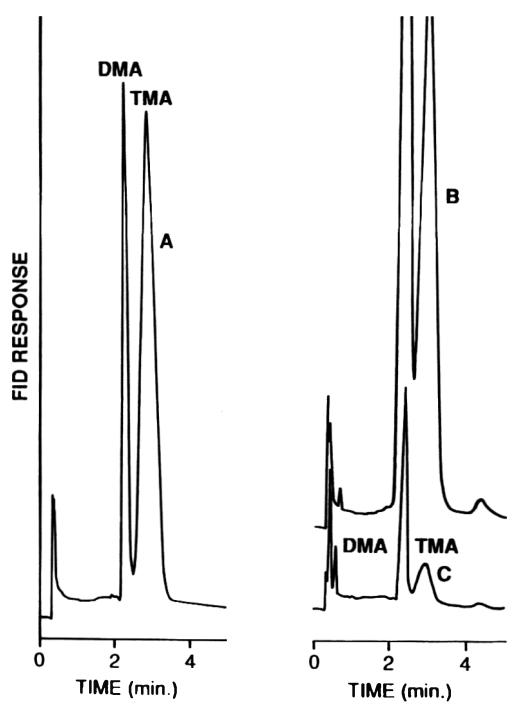

Figure 2. Chromatograms of DMA and TMA: (A) standards (10 ppm), frankfurter sample containing 50% Alaska pollock; (B) unwashed mince (190.5 and 11.4 ppm); (C) washed mince (4.5 and 0.9 ppm, respectively).

Table 2. Amine ranges and repeatabilities in fish-meat frankfurters

Amine	Range	Repeatability, ppm	CV, %
DMA	ND[a]–711.7	6.6	20.5
TMA	ND–120.8	1.0	16.0
TMAO	ND–1785.7	18.8	12.7

[a] ND = not detectable.
n = 255 samples in duplicate.

Conclusion

The direct equilibrium headspace GC method described is rapid and easy to perform requiring no organic solvents directly in the analysis and a reasonable array of reagents and equipment readily available in the analytical laboratory. As a result, other preparation and sampling techniques that cause column deterioration are avoided. The method is applicable for routine analysis of methylamines in fish-meat samples and fish.

The authors recommend that this method be evaluated by others as an alternative to other proposed procedures.

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References

(1) Agnello, R. J. (1983) *Marine Fish. Rev.* **7**(9), 21–26
(2) Lanier, T. C. (1985) *38th Ann. Reciprocal Meat Conf. Proc.* **38**, 129–134
(3) Rasekh, J. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 91–95
(4) Kamamura, T., Sakai, K., Miyazawa, F., Wada, H., Ito, Y., & Tanimura, A. (1971) *J. Food Hyg. Soc. Japan* **12**, 192–197
(5) Singer, G. M., & Lijinski, W. (1976) *J. Agric. Food Chem.* **24**, 550–555
(6) Sen, N. P. (1974) in *Toxic Constituents of Animal Foodstuffs*, I. E. Liener (Ed.), Academic Press Inc., New York, pp. 152–154
(7) Hotchkiss, J. H. (1987) in *Advances in Food Research*, Vol. 31, C. O. Chichester, E. M. Mrak, & B. S. Schweigert (Eds.) Academic Press Inc., New York, pp. 53–115
(8) Dyer, W. J. (1959) *J. Assoc. Off. Anal. Chem.* **42**, 292–294
(9) Amano, K., & Yamada, K. (1964) *Bull. Jap. Soc. Sci. Fish.* **30**, 430–435
(10) Castell, C. H., Smith, B., & Neal, W. (1971) *J. Fish Res. Bd. Can.* **28**, 1–5
(11) Lundstrom, R. C., Correia, F. F., & Wilhelm, K. A. (1982) *J. Food Biochem.* **6**, 229–241
(12) Ender, F., Harve, G. N., Madsen, R., Ceh, L., & Helgebostad, A. (1967) *Z. Tierphysiol. Tierenache Futtermittelk.* **22**, 181–189
(13) Fiddler, W., Pensabene, J. W., Doerr, R. C., & Wasserman, A. E. (1972) *Nature* (London) **236**, 309
(14) Lijinsky, W., Keefer, L., Conrad, E., & Van de Bogart, R. (1972) *J. Natl. Cancer Inst.* **49**, 1239–1249
(15) Lijinsky, W., & Singer, G. M. (1974) in *N-Nitroso Compounds in the Environment*, P. Bogovski, & E. A. Walker (Eds.) International Agency for Res. on Cancer, Sci. Publ. No. 9, Lyon, France, pp. 111–114
(16) Scanlan, R. A., Lohsen, S. M., Bills, D. D., & Libbey, L. M. (1974) *J. Agric. Food Chem.* **22**, 149–151
(17) Oshima, H., & Kawabata, T. (1978) in *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, L. Griciute, & R. E. Lyle (Eds.), Int'l. Agency for Res. on Cancer, Sci. Publ. No. 19, Lyon, France, pp. 143–153
(18) DiCorcia, A., & Samperi, R. (1974) *Anal. Chem.* **46**, 977–981
(19) *Amine Analysis* (1979) GC Bulletin 737E, Supelco, Inc.
(20) Lundstrom, R. C., & Racicot, L. D. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1158–1163
(21) Perez Martin, R. I., Franco, J. M., Molist, P., & Gallardo, J. M. (1987) *Intl. J. Food Sci. & Tech.* **22**, 509–514
(22) Pensabene, J. W., & Fiddler, W. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 839–843
(23) Snedecor, G. W., & Cochran, W. G. (1979) *Statistical Analysis*, 6th Ed., Iowa State Univ. Press, Ames, IA
(24) Zeisel, S. H., Gettner, S., & Youssef, M. (1989) *Fd. Chem. Toxicol.* **27**, 31–34
(25) Miller, A., Lee, J. S., & Libbey, L. M. (1972) *J. Agric. Food Chem.* **20**, 709–711
(26) *Chromatographic Products Catalog* (1989) Alltech Assoc., Inc., pp. 96–97
(27) *Chromatographic Products Catalog, No. 28* (1990) Supelco, Inc. p. 38
(28) Gruger, E. H. (1972) *J. Agr. Food Chem.* **20**, 781–785

SEAFOOD TOXINS

A Study of Ten Toxins Associated with Paralytic Shellfish Poison Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

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Ten paralytic shellfish toxins [saxitoxin, neosaxitoxin, B-1, B-2, gonyautoxin 1, 2, and 3 (i.e., GTX-1, GTX-2, and GTX-3), C-1, C-2, and C-3] were oxidized at room temperature under mildly basic conditions with hydrogen peroxide or periodic acid. The products were then analyzed by liquid chromatography (LC). The *N*-1-hydroxylated toxins (neosaxitoxin, B-2, GTX-1, and C-3) formed fluorescent products after periodate oxidation at ca pH 8.7, but did not form fluorescent derivatives with peroxide oxidation. The non-*N*-1-hydroxylated toxins (saxitoxin, B-1, GTX-2, GTX-3, C-1, and C-2) formed highly fluorescent derivatives with both peroxide and periodate oxidations. Individual toxins produced mainly single fluorescent peaks by reverse-phase LC. However, all GTX toxins eluted with the same retention time. Also, C-1 and C-2 eluted together, as did neosaxitoxin and B-2. The non-*N*-1-hydroxylated toxins could be detected in quantities as low as 20–50 pg/injection, while the *N*-1-hydroxy analogues could be detected at levels as low as 100–500 pg/injection. UV absorption and fluorescence emission spectra were similar for the oxidation products of all toxins examined (max. 333 ± 2 nm absorption, 389 ± 4 nm fluorescence emission).

Toxins associated with paralytic shellfish poison (PSP) are among the most acutely toxic substances known (1–3). The structures of 12 of these toxins are shown in Figure 1 and Table 1. Contamination of shellfish with PSP is now recognized as a worldwide problem (4). The most common method for PSP toxin determination has been the mouse bioassay (5). However, this requires a regular supply of mice of the proper size along with facilities to maintain them. In recent years, there has been a move away from mammalian bioassays; several European countries are considering banning them altogether. Alternative methods for PSP determination have been investigated. These include immunoassay (6), colorimetry (7, 8), and fluorescence (9, 10). The immunoassay technique has been developed only for saxitoxin. However, research on immunoassay procedures for other toxins is underway and should lead to a simple, fast approach for screening shellfish.

The best method up to now for monitoring PSP toxins is a combination of liquid chromatography (LC) with on-line postcolumn oxidation and fluorescence detection (4, 11). The original peroxide oxidation procedure (9, 10) was found to be unsuitable for N-1-hydroxy analogues and, thus, periodate was used, which enabled detection of all PSP analogues. When the LC method was evaluated, it was found to correlate well with the mouse bioassay method (12, 13) at high PSP concentrations; however, correlation was poor near the regulatory limit of 80 μg/100 g (14). Unfortunately, it is the only method available that is capable of separating and quantitating the major PSP toxins. The setup and operation of the equipment is not simple. It requires a lengthy start-up time (days or weeks) for optimization and considerable daily maintenance. The method is best suited to continuous routine monitoring of PSP toxins and not to determination on an irregular basis.

We have been investigating alternative LC methods for determination of PSP toxins. The most promising to date has involved prechromatographic oxidation of the toxins followed by reverse-phase LC separation and fluorescence detection of the resulting products. One of the main advantages of this approach is that a postcolumn reaction system is not required because oxidation reactions are carried out before chromatographic analysis. Secondly, the method uses a silica-based C_{18} column yielding better separation efficiencies than the PRP-1 column recommended in the postcolumn method. Also, the sensitivity of the prechromatographic approach is significantly better. Results of our investigation are reported here.

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Experimental

Apparatus

The LC system consisted of 2 Beckman pumps (Model 110B) with gradient controller (Model 421A) and an Altex injection port (Model 210A) with a 20 μL loop. The column used was a Supelcosil LC-18 (15 cm × 4.6 mm id, 5 μm), while a Varian Model 2070 dual monochromator (ex 330 nm, em 410 nm) spectrofluorometer or a Jasco Model 820-FP dual monochromator (ex 333 nm, em 390 nm) detector was used for monitoring the LC effluent. PSP oxidation products were eluted using a gradient of 0–3.5% (v/v) acetonitrile in 0.02M KH_2PO_4/Na_2HPO_4 (pH 6.8) over 11 min. Other mobile phases studied were: tetrabutylammonium hydrogen sulfate (0.005M) in 0.02M phosphate buffer (pH 6.3) with an acetonitrile gradient of 0–5% (v/v) from 3–18 min, and sodium heptane sulfonate (0.005M) in 0.02M phosphate buffer (pH 6.9) with an acetonitrile gradient of 0–12.5% (v/v) from 3–18 min.

Reagents

All solvents and reagents were analytical or LC grade materials. Water was doubly deionized. Small quantities of purified standards were obtained from cultures of dinoflagellates produced at the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, D.C. following procedures described elsewhere (15). Shellfish (clams, mussels, oysters, and whelks) were purchased at local retail outlets.

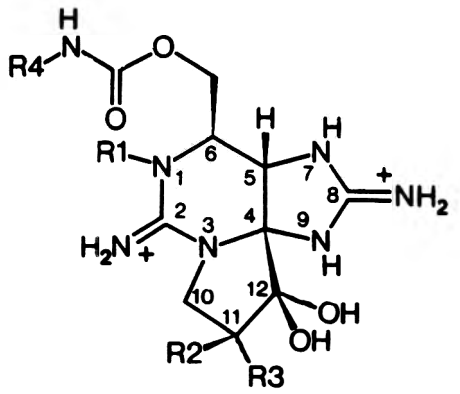

Figure 1. Structures of PSP toxins.

Table 1. Structures of 12 PSP toxins

Substituents				
R1	R2	R3	R4	Name
H	H	H	H	Saxitoxin (SAX)
H	H	H	SO_3	B-1
H	H	OSO_3	H	Gonyautoxin 2 (GTX-2)
H	H	OSO_3	SO_3	C-1
H	OSO_3	H	H	Gonyautoxin 3 (GTX-3)
H	OSO_3	H	SO_3	C-2
OH	H	H	H	Neosaxitoxin (NEO)
OH	H	H	SO_3	B-2
OH	H	OSO_3	H	Gonyautoxin 1 (GTX-1)
OH	H	OSO_3	SO_3	C-3
OH	OSO_3	H	H	Gonyautoxin 4 (GTX-4)
OH	OSO_3	H	SO_3	C-4

Sample Cleanup

A 1 mL aliquot of shellfish extract prepared according to the mouse bioassay procedure (5) was passed through a 3 mL SPE C_{18} cartridge (Supelco, U.S.A.) that had been conditioned with 6 mL methanol followed by 6 mL 0.1N HCl. The effluent (containing the toxins) was collected. Following this, 2 mL H_2O were passed through the cartridge to elute the remaining toxins. The effluent was collected and combined with the first portion and an aliquot of the solution used for the oxidation reactions.

Peroxide Oxidation

All reagents and solutions were measured using autopipets (Eppendorf) with disposable plastic tips. A 5 μL volume of 10% (w/v) aqueous solution of H_2O_2 was added to 20 μL of aqueous sample solution in a 0.5 mL plastic microcentrifuge tube and mixed (vortex). To this was then added 50 μL of 1N NaOH. The solution was mixed again and allowed to react at room temperature (20°C) for 2 min. A 4 μL volume of concentrated acetic acid was added and the solution mixed well (vortex). A 20 μL aliquot was then injected into the LC system.

Periodate Oxidation

A 70 μL volume of a 1 + 1 (v/v) mixture of 0.01M periodic acid and 0.05M phosphate buffer, pH 12, (prepared daily) was added to 20 μL of aqueous sample in a 0.5 mL plastic microcentrifuge tube and mixed well. The mixture was permitted to react at room temperature for 3 min after which time 2 μL of concentrated acetic acid was added. The solution was mixed again and a 20 μL aliquot injected into the LC system. The solution remains stable for about 1 h, after which a slow decrease in peak heights occurs.

Results and Discussion

Because of the extremely limited supply of purified, characterized toxins available for this work (in some cases, only micrograms), all reactions were carried out in microliter volumes using nanograms or less of material at a time. This severely limited our attempts to spectroscopically characterize the oxidation products of the toxins. However, in spite of these limitations, the results of the study have shed new light on the oxidation of the PSP toxins and provided the basis for a simplified method for monitoring PSP in shellfish.

PSP Toxin Oxidation

Before we conducted this study, only the oxidation product of saxitoxin had been extensively characterized. Wong et al. (16), using alkaline hydrogen peroxide, oxidized saxitoxin to a highly fluorescent purine, the identity of which was found to be 8-amino-6-hydroxymethyl-2-iminopurine-3-(2H)-propionic acid (9). The oxidation involved a hydrolysis of the carbamoyl moiety, a cleavage of the bond between carbons 4 and 12, and an aromatization of the remaining rings to form the purine nucleus.

We have found, using prechromatographic oxidation with H_2O_2, that saxitoxin and the 5 other non-*N*-1-hydroxy toxins yield highly fluorescent derivatives with almost identical UV absorption and fluorescence emission spectra; however, these are chromatographically separable into 4 groups based on their substituents.

For example, C-1 and C-2 eluted first as a single peak, then gonyautoxin 2 and 3 [i.e., GTX-2 and GTX-3] together, followed by B-1, then saxitoxin. The *N*-1-hydroxy toxins produced little or no response with H_2O_2 oxidation. However, all toxins produced fluorescent derivatives with periodate oxidation. It appears that the periodate oxidation products of the nonhydroxy toxins are the same as those obtained with peroxide based on fluorescence spectra and retention times. Table 2 lists the UV absorbance and fluorescence emission maxima obtained for the periodate oxidation products for selected toxins. Both types of spectra exhibited broad maxima and were qualitatively similar.

Figure 2 shows reverse-phase chromatograms obtained from a standard mixture of 12 PSP toxins after (A) peroxide and (B) periodate oxidations and a chromatogram (C) of the same solution obtained using the ion-pair LC-postcolumn oxidation method (4). The last chromatogram (C) represents the separation of the toxins and not their oxidation products. The quantities injected for chromatograms (A) and (B) were

Table 2. UV absorption and fluorescence emission maxima of oxidation products of PSP toxins

Compound	Absorption[a] max., nm	Fluorescence[b] emission max., nm
Saxitoxin	335	386
Neosaxitoxin	333	392
B-1	333	391
B-2	331	385
C-2	335	393

[a] Values obtained with Hewlett-Packard Model 1040A diode array detector. LC conditions as described in text.

[b] Values obtained at 330 nm excitation with Jasco Model 820-FP fluorescence detector with emission scanning capability. LC conditions as described in text.

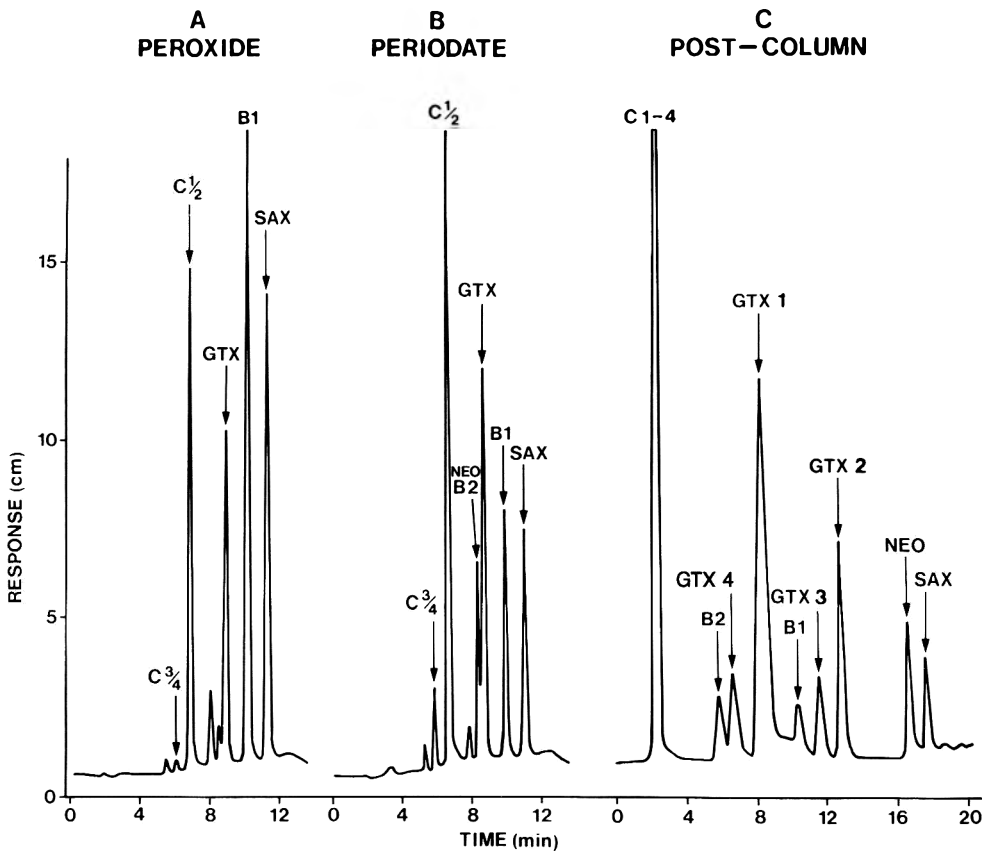

Figure 2. Chromatograms of a PSP toxin standard solution. Prechromatographic oxidation (A) with peroxide and (B) with periodate. (C) Postchromatographic oxidation with periodate. See text for reverse-phase chromatography conditions for (A) and (B) (acetonitrile–phosphate buffer mobile phase). For (C), ion-pair chromatography is as described in Ref. (4). Quantities of saxitoxin (SAX) injected: (A) 0.6 ng, (B) 0.5 ng, (C) 3.7 ng. Varian Model 2070 detector used. In chromatograms (A) and (B), GTX = gonyautoxins 1–4.

6 to 7 times less than that for chromatogram (C). This particular solution also contained C-4 and GTX-4, which were not studied individually because they were not available in pure form.

We observed that oxidation of saxitoxin by either peroxide or periodate yielded 2 well-separated fluorescent peaks, the later eluting one being predominant. By restricting the reaction time to 2–3 min, the latter accounted for more than 95% of the total yield. The smaller peak eluted with the same retention time [ca 9 min; see Figures 2(A) and (B)] as neosaxitoxin in the reverse-phase system, but was separable when heptane sulfonate was added to the mobile phase. The other toxins examined similarly yielded single fluorescent products (>95% of the total yield) by either the peroxide or periodate oxidations under the conditions described in the *Experimental.*

Peroxide oxidation only yields fluorescent products with the non-N-1-hydroxy containing toxins (except for a weak response to GTX-1). Thus, for a routine screening method, peroxide oxidation is not satisfactory because it would not detect neosaxitoxin, B-2, GTX-1, GTX-4, C-3, or C-4 that may be present in the extracts; and, therefore, it could seriously underestimate the toxicity of the samples. Peroxide oxidation has been used to determine saxitoxin in mussels by LC (17). However, as a routine screening approach, it cannot be recommended particularly from a regulatory and health point of view.

Periodate oxidation produced fluorescent derivatives with all toxins studied. The fluorescence response of the *N*-1-hydroxy containing toxins was 5 to 10 times less sensitive than their nonhydroxy analogues. Table 3 lists the approximate sensitivities (peak height, cm/ng) obtained for the 2 prechromatographic oxidation reactions with a comparison to values we obtained by the LC postcolumn oxidation meth-

Table 3. Approximate sensitivities for PSP toxins by pre- and postchromatographic oxidation

	Sensitivity (peak height),[a] cm/ng		
	Prechromatographic		Postchromatographic
Toxin	Peroxide	Periodate	Periodate
Saxitoxin	154	90 (117)[b]	4.0
Neosaxitoxin	<0.1	2.5 (24)	1.1
B-2	<0.1	5 (13)	0.4
B-1	141	89	1.0
GTX-3	105	118	13.3
GTX-2	111	157	7.0
GTX-1	1.4	7	1.6
C-3	<0.1	7	ND[c]
C-2	52	82	ND[c]
C-1	74	31	ND[c]

[a] Baseline noise ca 1 mm (peak to peak). Values obtained with Jasco Model 820-FP fluorescence detector.

[b] Values in parentheses obtained with heptane sulfonate in the mobile phase.

[c] Not determined.

od using the Jasco detector. As shown in the table, the prechromatographic oxidation method is more sensitive by factors of 5 up to about 140. With prechromatographic oxidation, as little as 20–50 pg of nonhydroxylated toxins, and 100–500 pg of the hydroxy analogues could be detected (3:1, signal:noise ratio). Reaction conditions described in the *Experimental* represent the best conditions found after extensive testing of times, temperatures, and reagent compositions.

The reason for the great difference in response between the pre- and postchromatographic periodate oxidations is not known, although better column efficiency with the C_{18} column is partially responsible. The sulfonate ion-pairing agents in the mobile phase do not seem to have had a negative influence on the postchromatographic oxidation because no effect was observed when they were added to the prechromatographic oxidation reactions. The Jasco detector was found to be about 4–10 times more sensitive than the Varian detector for both pre- and postchromatographic oxidations. Also, the optimum excitation and emission wavelengths were slightly different for the 2 detectors.

With the original column used for the reverse-phase LC studies, we found that the oxidation products of neosaxitoxin and B-2 chromatographed satisfactorily (as shown in Figure 2). However, with new C_{18} columns, chromatograms were less satisfactory. The addition of the ion-pair agents improved chromatographic efficiency. Also, the addition of ammonium formate to the mobile phase appears to improve the peak shape of these 2 compounds. Further chromatographic studies are in progress.

Figure 3 compares chromatograms obtained with ion-pairing systems for oxidation products of a mixture of 7 toxins. The C toxins were not included in these results but separate analyses showed that their products eluted unretained when heptane sulfonate was used as ion-pairing agents but much later than B-1 with the tetrabutylammonium ion-pairing system.

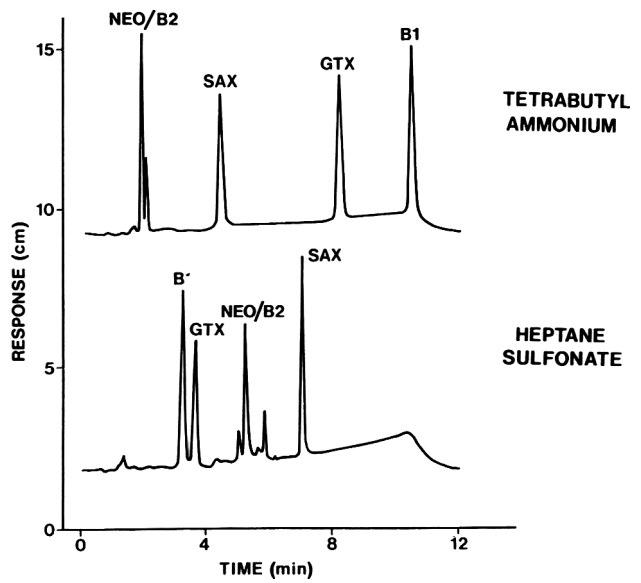

Figure 3. Chromatograms of a mixture of prechromatographic oxidation products of saxitoxin (SAX), neosaxitoxin (NEO), gonyautoxins 1–3 (GTX), B-1, and B-2 obtained with periodate. Upper chromatogram obtained with 0.005M tetrabutylammonium hydrogen sulfate in the mobile phase; lower chromatogram contained 0.005M heptane sulfonate. Varian Model 2070 detector used.

In no system could the oxidation products of all individual toxins be resolved. The separations observed in Figures 2 and 3 indicate that the oxidation products of the C toxins are more acidic than those of GTX 1–3 or saxitoxin (as are their parent toxins). The order of elution of the oxidation products with heptane sulfonate in the mobile phase was C, B-1, GTX, and saxitoxin, while with tetrabutylammonium ion, the order was reversed, as might be expected. However, in all systems, the products of neosaxitoxin and B-2 eluted together, indicating that the sulfocarbamoyl moiety in B-2 is destroyed during oxidation and that, probably, they yield the same oxidation product. Evidence for this is found in the results of the ion-pair studies. If the sulfocarbamoyl group remained intact, the oxidation product of B-2 should have behaved chromatographically like that of B-1 in terms of relative elution order (i.e., it should have eluted before the saxitoxin product in the heptane sulfonate system and after it in the tetrabutylammonium system). This was not the case. The B-2 product behaved exactly like the product of neosaxitoxin, which is of similar basicity to saxitoxin but more polar because of the *N*-1-hydroxy substituent. In all systems, the oxidation product of neosaxitoxin eluted before saxitoxin.

The apparent sulfocarbamoyl destruction was found to occur only with the B-2 oxidation product because the products of B-1 and the C toxins were separated from the products of their respective carbamates, saxitoxin and the GTX toxins, indicating that the sulfocarbamoyl group was still present. Also, the observed elution orders indicate that the sulfocarbamoyl groups are intact in these products.

The effect of the *N*-1-hydroxy substituent on the chromatographic characteristics of the products was not consistent. The oxidation products of saxitoxin and C-1 were easily separated from their *N*-1-hydroxy analogues, neosaxitoxin and C-3, which eluted earlier as would be expected. However, the product of GTX-1 was not separated from that of GTX-2 in any of the chromatography systems evaluated.

The oxidation products of the epimer pairs, C-1 and C-2, as well as GTX-2 and GTX-3 were not separable. It is likely that they are no longer epimeric after oxidation but probably become enantiomers, which would require a chiral stationary phase for separation. The compounds C-4 and GTX-4 were not available in pure form and, thus, were not studied individually. These compounds were assumed to behave like their respective epimers, C-3 and GTX-1.

Application to Shellfish

The prechromatographic oxidation method was applied to the analysis of shellfish extracts containing spiked or naturally incurred residues of PSP toxins. The SPE cleanup procedure, instrumental, and reaction conditions were optimized by carrying out numerous experiments while changing different parameters. Figure 4 shows typical chromatograms obtained by the prechromatographic periodate oxidation for a blank and a naturally contaminated mussel sample (Gulf of St. Lawrence, Canada) containing ca 10 μg/g total PSP toxins. The periodate oxidation was used as an initial screen for all toxins. Positive samples were then analyzed using the peroxide reaction. The epimers, C-1/C-2, were quantitated together using the peroxide reaction for a total estimate employing average response factors from the values in Table 3. The GTX-2/GTX-3 epimers were quantitated in the presence of their *N*-1-hydroxy analogues also by using peroxide oxidation, which gave only a weak response for GTX-1. GTX-1 was calculated by difference using the results of the

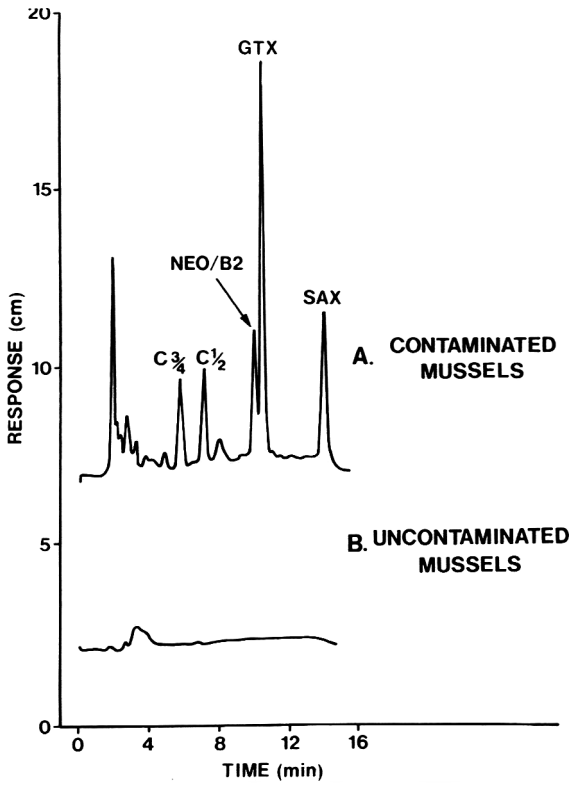

Figure 4. Chromatograms of extracts of (A) contaminated (ca 10 μg/g total PSP) and (B) uncontaminated mussels. Conditions as described in text except that the slope of the gradient was slightly lowered. Varian Model 2070 detector used. GTX = gonyautoxins 1-3.

periodate reaction. The oxidation product of C-4 was assumed to elute with that of C-3 and that of GTX-4 was assumed to elute with the oxidation product of GTX-1. They were quantitated as C-3 or GTX-1.

Because neosaxitoxin did not separate from B-2 with the chromatography conditions employed, it could not be quantitated with certainty. However, B-2, particularly after the acid extraction procedure, is not often found in shellfish as has been reported in the Northeast United States (14), for example. Thus, any peak appearing at that retention time should normally be considered as being only neosaxitoxin, because neosaxitoxin is far more toxic than B-2. Also, it has been suggested (15) that, for a truer estimate of human oral potency, all sulfocarbamoyl toxins should be hydrolyzed in acid to their respective carbamates. Thus, all C toxins should be converted to their GTX analogues and B-1 and B-2 converted to saxitoxin and neosaxitoxin, respectively. In this case, the prechromatographic oxidation method with the LC conditions employed would be satisfactory because the problem of neosaxitoxin/B-2 quantitation would be eliminated.

One advantage of the prechromatographic oxidation method is that the C toxins can be quantitated along with the others. Also, they and B-1 can be confirmed by acid hydrolysis and conversion to their carbamate analogues (GTX toxins and saxitoxin, respectively). In addition, the fact that the *N*-1-hydroxylated toxins do not respond to the peroxide oxidation can serve as a confirmation of their identity when they are observed in the periodate reaction mixture.

The detection limit (3:1 signal:noise ratio) in samples was estimated to be about 10–15 ng/g each for non-*N*-1-hydroxylated toxins and 50–100 ng/g for *N*-1-hydroxy analogues.

Table 4. Comparison of precolumn oxidation LC method to the mouse bioassay for PSP toxins

Sample	Total PSP found, μg/g	
	LC Method[a]	Mouse bioassay
Mussels	0.51	0.50
Mussels	0.85	0.98
Mussels	1.82	1.60
Mussels	<0.06	<0.42
Clams	2.22	2.80
Clams	1.42	2.30
Oysters	<0.06	<0.42
Whelks	0.07	<0.42

[a] Total toxicity based on concentration of individual toxins times the toxicity factor for each, expressed as μg/g saxitoxin (4).

Solid phase extraction cleanup of extracts before oxidation and analysis led to cleaner chromatograms and decreased overall contamination of the LC column, thus extending its lifetime. Recoveries through SPE cartridges were checked using standard solutions and were greater than 90%. For routine work, standards were not normally passed through the SPE cleanup.

The oxidation reactions produced linear results for all toxins studied over a 25- to 50-fold range. The absolute ranges for each toxin differed and depended on their individual relative responses and oxidation conditions employed.

The same-day repeatability coefficient of variation (CV) of replicate analyses (n = 3) for an extract of a naturally contaminated sample containing saxitoxin (2.6 μg/g), GTX-2/GTX-3 (3.4 μg/g), and C-1/C-2 (5.3 μg/g) were 4.5, 3.8, and 9.8%, respectively for the peroxide reaction, and 4.3, 4.5, and 5.9%, respectively, for the periodate reaction. These values are quite good considering the fact that the reactions involved only nanograms of toxins and microliter volumes.

To evaluate effects of the sample matrix on the oxidation reaction, limited (due to the lack of standard material) recovery studies were carried out by spiking extracts of blank mussels after the SPE C_{18} cleanup and before the oxidation reaction. Recoveries through the oxidation procedure varied from 74–122% for the non-*N*-1-hydroxy containing toxins using the peroxide reaction and, with the exception of GTX-1, from 77–140% for all toxins with the periodate reaction at individual concentrations ranging from 0.2–3.8 μg/g in the sample. GTX-1 recovery was only 21% with the periodate reaction and attempts to resolve this problem are in progress. There was some indication of sample matrix effects in the periodate reaction leading to increased responses of some toxins. For example, saxitoxin, B-2, and GTX-3 showed ca 140% recoveries at spiking levels around 1 μg/g. This effect is also being studied.

Table 4 compares results obtained by the method described herein with results from the mouse bioassay procedure for several shellfish samples. As the results show, agreement is reasonably good, although only results of a few samples have been compared.

As described, the method has potential for rapid screening of samples for PSP contamination. However, in order to be used for regulatory purposes, work is required to generate more data on the quantitative aspects. Such research is limited by the lack of pure standards.

In spite of this, we are continuing to assess the technique on a long-term basis, including a comparison to the postcolumn

LC method (4) and the mouse bioassay (5). Chromatographic studies on oxidation reactions have produced new information on the identity of the reaction products and should be most useful in the development of a simplified quantitative chromatographic screening procedure for PSP toxins.

References

(1) Evans, M. H. (1964) *Br. J. Pharmacol. Chemother.* **22**, 478–485
(2) Kao, C. Y., & Nishiyama, A. (1965) *J. Physiol.* **180**, 50–66
(3) Moczydlowski, E., Miller, C., Hall, S., & Strichartz, G. (1984) *Biophys J.* **45**, 286a
(4) Sullivan, J. J., & Wekell, M. M. (1987) in *Seafood Quality Determination*, D. E. Kramer & J. Liston (Eds.) Elsevier, New York, pp. 357–371
(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **18.086–18.092**, 15th Ed. (1990), secs **959.08G**
(6) Renz, V., & Terplan, G. (1988) *Arch. Lebensmittelhyg.* **39**, 30–33
(7) Gershey, R. M., Nevé, R. A., Musgrave, D. L., & Reichardt, P. B. (1977) *J. Fish Res. Bd. Can.* **34**, 559–563
(8) McFarren, E. F., Schantz, E. J., Campbell, J. E., & Lewis, K. H. (1958) *J. Assoc. Off. Agric. Chem.* **41**, 167–168
(9) Bates, H. A., & Rappoport, H. (1975) *J. Agric. Food Chem.* **23**, 237–239
(10) Bates, H. A., Kostriken, R., & Rappoport, H. (1978) *J. Agric. Food Chem.* **26**, 252–254
(11) Sullivan, J. J., & Iwaoka, W. T. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 297–303
(12) Sullivan, J. J., Wekell, M. M., & Kentala, L. L. (1985) *J. Food Sci.* **50**, 26–29
(13) Sullivan, J. J., Jonas-Davies, J., & Kentala, L. L. (1985) *Toxic Dinoflagellates*, D. M. Anderson, A. W. White, & D. G. Baden (Eds.) Elsevier, New York, pp. 275–280
(14) Salter, J. E., Timperi, R. J., Henningan, L. J., Sefton, L., & Reece, H. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 670–673
(15) Hall, S., & Reichardt, P. B. (1984) *ACS Symposium Series, No. 262, Seafood Toxins*, E. P. Ragelis (Ed), American Chemical Society, Washington, DC, pp. 113–123
(16) Wong, J. L., Brown, M. S., Matsumoto, R., Oesterlin, R., & Rappoport, H. (1971) *J. Amer. Chem. Soc.* **93**, 4633–4634
(17) Preun, J., Kolloch, M., & Kutscher, U. (1988) *Deutsche Lebens. Rundschau* **84**, 114–116

TECHNICAL COMMUNICATIONS

Development of a Rapid Equine Serological Test (REST) by Modified Agar-Gel Immunodiffusion

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A rapid equine serological test (REST) has been developed for detection of horse meat in a wide variety of raw meat products. The test is an adaptation of previously developed field screening immunodiffusion tests for beef, poultry, pork, and sheep detection. Results show that the REST test was specific, sensitive, and accurate in the analysis of 101 samples.

In 1981, large quantities of boxed boneless beef entering the United States were suspected of containing undeclared horse and kangaroo tissues. Positive identification of the presence of these tissues was resolved and confirmed in U.S. Dept of Agriculture (USDA) Food Safety Inspection laboratories by traditional ring precipitin (1, 2) and agar-gel immunodiffusion (3, 4) methods. To protect against future entry of fraudulently labeled products, the overnight rapid bovine identification test (ORBIT) (5) was developed as the first on-site screening test for species identification. Initially, the test was available only to USDA inspectors at import stations. Use of the ORBIT test saved time and expense in sample analysis, reagent consumption, and in eliminating the need to ship all samples to the laboratory for species determination. Now that the ORBIT test kit is produced commercially, it is available to USDA inspectors in domestic meat processing plants, as well as to private industry and foreign governments monitoring import and export products.

The ORBIT test employs agar-gel immunodiffusion plates with a printed template for placement of stable freeze-dried reagent paper discs and sample discs saturated in meat tissue fluids. Within 18–24 h at room temperature, fusion of a sample immunoprecipitin line with a reference band formed in the agar between beef antigen and antibody discs indicates presence of beef in the meat product. Adaptations of this basic procedure produced the poultry rapid overnight field identification test (PROFIT) (6), porcine rapid identification method (PRIME) (7), and serological ovine field test (SOFT) (8) for identifying poultry, pork, and sheep, respectively, in raw whole, ground, or emulsified meat products. Accuracy of the basic procedure was substantiated in a collaborative study (9). The method was subsequently adopted final action by AOAC (10). The present paper reports further modification of this basic procedure for horse detection.

Experimental

Reagent Modifications

Prepare reference antiequine antibody discs by impregnating blank filter paper discs (BBL No. 31039, Becton, Dickinson and Co., Box 243, Cockeysville, MD 21030) with 40 μL undiluted sheep antiequine albumin serum (Environmental Diagnostics, Inc., Box 908, Burlington, NC 27215). Prepare equine reference antigen discs by impregnating additional blank paper discs with 40 μL horse serum albumin Fraction V (No. A-9888, Sigma) at 0.1% concentration in phosphate buffered saline, pH 7.2. Let both sets of discs absorb reagents and freeze-dry overnight as previously described (5). Prepare immunodiffusion plates as previously described (5) with the following modifications for agar preparation: substitute Oxoid purified agar (Code L-28) for Difco purified agar, which is no longer in production. For 1 L agar, add 10 g agar to 976.66 mL buffered saline and dissolve until clear on a hot plate. Substitute Orange G stain (No. O 7252, Sigma) for Lanaperl fast pink dye. Prepare a 5% stock solution of Orange G stain in phosphate buffered saline, pH 7.2, clarify and sterilize through successive 0.45 μm and 0.22 μm Millipore filters. Add 13.34 mL stock stain solution to 986.66 mL clear agar on the hot plate, mix, and filter stained agar under vacuum through a single layer of Miracloth (Calbiochem-Behring, 10933 N. Torrey Pines Road, La Jolla, CA 92037) before sterilization in autoclave. Final Orange G stain concentration in agar is 1:1500, which distinguishes REST from ORBIT, PROFIT, PRIME, and SOFT plates of other colors. Cool sterile dyed agar to 60°C. From a sterile stock 5% (aqueous) merthiolate solution, add sufficient quantity to effect a final concentration of 1:10 000.

Reaction Characteristics

Specificity for the REST test was determined by reacting blank paper sample discs saturated in homologous (equine) and heterologous species meat tissue fluids against reference antibody discs. Sensitivity, as applied to ground meat mixtures, was assessed by testing prepared sample composites of known amounts of horse adulterant tissue added to ground red meat base tissue. Three replicates were tested at each adulterant level (1–22% by weight). Presence of a visible sample immunoprecipitin band that completely fused with the reference band was held as evidence of detection at a given percentage level of adulterant.

Shelf Stability

Longevity of reagent discs was evaluated by storing some prepared sheep antiequine antibody discs and equine reference antigen discs in vials with screw caps under conditions of room temperature and refrigeration (4°C). These discs were tested periodically to note any loss of immunoprecipitin band intensity.

Sample Analysis

To determine the accuracy and reliability of the REST

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Table 1. Results of REST pretrial and laboratory trial on meat product samples

Product	Species composition[a]	Number of samples	Number of samples with equine	REST positive samples
Prelaboratory trial				
Frank emulsion	bovine	2	0	0
Bologna emulsion	bovine	1	0	0
Gyro	bovine, ovine	1	0	0
Sausage	pig, (bovine)	1	0	0
Sausage	bovine, ovine, pig	2	0	0
Ground beef	bovine	5	0	0
Ground beef	bovine, (chicken)	3	0	0
Ground beef	bovine, (equine)	3	3	3
Ground horse	equine	1	1	1
Whole horse	equine	35	35	35
Whole beef	bovine	1	0	0
Whole lamb	ovine	1	0	0
Whole pork	pig	1	0	0
Whole chicken	chicken	1	0	0
Whole kangaroo	kangaroo	2	0	0
Total		60	39	39
Laboratory trial				
Frank emulsion	bovine	2	0	0
Bologna emulsion	bovine	1	0	0
Gyro	bovine, ovine	1	0	0
Sausage	pig, (bovine)	1	0	0
Ground beef	bovine	4	0	0
Ground beef	bovine, (chicken)	3	0	0
Ground beef	bovine, (equine)	3	3	3
Ground horse	equine	1	1	1
Whole horse	equine	21	21	21
Whole beef	bovine	1	0	0
Whole lamb	ovine	1	0	0
Whole pork	pig	1	0	0
Whole chicken	chicken	1	0	0
Total		41	25	25

[a] Identity of species in all samples was confirmed by Ouchterlony agar-gel immunodiffusion technique (3) using antispecies sera and extracts of authentic reference tissue. Species given in parentheses represent known adulterant tissue present in the test samples.

test, 60 meat samples (Table 1) of a wide variety were first analyzed using AOAC method **987.06** (10) with reagent modifications for equine detection described above. Forty-one additional unknown samples (Table 1) were then analyzed in a blind in-house laboratory trial. Species origin of all 101 samples was confirmed by Ouchterlony agar-gel immunodiffusion technique (3).

Results and Discussion

In specificity determinations using reference antibody discs prepared with sheep antiequine albumin, the following REST test reactions occurred for whole and ground tissue samples of known species origin: horse (+), mule (+), donkey (+), bovine (−), pig (−), sheep (−), deer (−), chicken (−), turkey (−), and red kangaroo (−). The cross reactions of mule and donkey with horse were entirely expected because of the known close relationship of serum proteins in these 3 tissues (11). Because products containing mule or donkey tissue would be very unusual, we see no reason why this should impose limitations on use of the REST test as a screening procedure.

Sensitivity determinations showed that adulterant horse was detectable at the 3% level in either beef or pork bases (data not shown). As expected, immunoprecipitin bands were weak at the endpoints.

Tests of shelf stability revealed that REST reagent antigen and antibody discs stored for 1 year at 4°C produced immunoprecipitin bands of intensity equal to that of freshly prepared discs. Reagent discs stored at room temperature for 4 months lost considerable reactivity. Decreased band intensity was primarily attributable to decline in antibody discs. Therefore, we recommend that reference reagent discs be stored in the refrigerator for maximum shelf life.

Results of the pretrial and blind laboratory trial are shown in Table 1. Of 101 total samples analyzed, 64 contained equine proteins and gave positive reactions. Thirty-seven samples, devoid of equine proteins, gave negative reactions. No false positive or false negative reactions occurred, indicating complete accuracy and reliability of the procedure. However, because the REST test is considered a screening procedure, it is recommended that positive results be confirmed with traditional Ouchterlony immunodiffusion techniques (3) or by isoelectric focusing (12), especially when legal action for violative samples is being considered.

The ORBIT (beef), PROFIT (poultry), PRIME (pork), and SOFT (sheep) tests are commercially produced at this time. However, no commercial production of the REST test is planned at present. This is because, in the United States, horse meat is not a common product for human consumption and there are few domestic horse slaughter plants. However, sporadic incidents of horse adulteration or substitution do occur domestically. Commercial production of REST kits might be of greater interest for use in countries such as

France, Belgium, Italy, or Japan where horse meat is more commonly consumed.

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REFERENCES

(1) Boyden, A. (1963) *Syst. Zool.* **12,** 1-7
(2) Warnecke, M. O., & Saffle, R. L. (1968) *J. Food Sci.* **33,** 131-135
(3) Fugate, H. G., & Penn, S. R. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 1152-1156
(4) Swart, K. S., & Wilks, C. R. (1982) *Aust. Vet. J.* **59,** 21-22
(5) Mageau, R. P., Cutrufelli, M. E., Schwab, B, & Johnston, R. W. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 949-954
(6) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 483-487
(7) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 444-445
(8) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 60-61
(9) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 230-233
(10) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec **987.06**; "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* **74,** 235-238
(11) Boyden, A. (1942) *Physiol. Zool.* **15,** 109-145
(12) Hamilton, W. D. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 119-122

Joint AOAC-AOCS-AACC-IUPAC Mycotoxin Committee

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Other Members: R. Bernetti (AACC); H. Casper (AOAC); J. C. Henderson (Secretary) (AOCS); D. L. Park (IUPAC); A. E. Pohland (IUPAC); O. L. Shotwell (AACC); R. D. Stubblefield (AOCS); S. N. Tanner (AACC); M. W. Trucksess (AOAC); A. E. Waltking (AOCS)

Member organizations of the Joint Mycotoxin Committee presented the following reports at the Committee's annual meeting in New Orleans, LA, September 11, 1990.

AOAC

Peter M. Scott reviewed the AOAC General Referee report on mycotoxins. Activities of the Associate Referees were highlighted, with the following recommendations: adopt as official first action the interim official first action immunoaffinity column method for aflatoxins in corn, peanuts, and peanut butter; adopt as official first action the liquid chromatographic (LC) method for ochratoxin A as quantitative for barley and corn and qualitative for pork kidney; discontinue topic on sterigmatocystin; continue study on all other topics.

Fumonisins has been added as a new topic, with Ronald D. Plattner (USDA, Peoria, IL) as the Associate Referee. Plattner reviewed some of the conclusions of the recent conference on fumonisins and *Fusarium moniliforme* held in Ames, IA, September 6-7, 1990. A lot of work was reported at this conference—field cases in swine, horses, and poultry were discussed—and yet no questions were answered. The need for large quantities of fumonisins for further testing and for use as standards was also discussed.

AOCS

James C. Henderson reported that AOCS Mycotoxin Committee activities during 1989-90 were primarily devoted to review of proposed methods to be added to the AOCS *Book of Methods and Recommended Practices*. The AOAC method for ELISA screening for aflatoxin B_1 in cottonseed products and mixed feeds, written in AOCS format, was not approved by committee members. Their reasons for rejecting the method included (*1*) complaints about erratic performance from the field, (*2*) failures in the hands of competent researchers in the laboratory, and (*3*) rejection ot the statistics to support the collaborative study. A representative of the company that manufactured the ELISA screening kit stated that a new kit has been developed to replace the old one.

The Smalley Aflatoxin Committee report of John McKinney stated that coefficients of variation were still comparable to those of previous years. McKinney also drew attention to the increasing use of the immunoaffinity column in conjunction with LC. A new immunochemical methods program for the Smalley Series will be introduced for the 1990-91 program.

Future activities on aflatoxin methodology should be more focussed on sampling, both for the primary sample and for the final portion taken for analysis.

AACC

AACC representative Steve Tanner highlighted the following topics of the AACC Mycotoxin Committee. The aflatoxin problem in the 1988-89 corn crop in Iowa resulted in a task force summary entitled "Aflatoxin Strategies for the Future." Blending was recommended to reduce aflatoxin in corn and to avoid penalties to operators. A method for deoxynivalenol in wheat has been adopted for the AACC method book. Fumonisins need further study. Test kits will not be incorporated into the method book until further experience is obtained in the field. FGIS approved 6 kits separately from AACC. A subcommittee on sampling has been formed; recommendations on this topic are to be presented at the next annual meeting.

IUPAC

A progress report by Douglas Park on current projects of the IUPAC Commission on Food Chemistry pertaining to the Mycotoxins Working Group included a collaborative study of a method for ochratoxin A in barley, corn, and swine kidney (joint with AOAC); spectroanalytical parameters for *Fusarium* toxins; the check sample program; survey of data on worldwide incidence of ochratoxin A; detoxification of

some mycotoxins other than aflatoxins; and a collaborative study on an LC method for fumonisins in cereals and mixed feeds. New projects include: an evaluation of immunoaffinity columns for aflatoxin M_1 in milk; 1990–91 check sample program; guidelines for immunochemical methods; development and evaluation of a method for ochratoxin A in blood; symposia; and books on food safety.

IDF and Other Business

Peter Scott reported that IDF Group E33-Mycotoxins is preparing the final version of Provisional IDF Standard 111 for determination of aflatoxin M_1 in milk and dried milk. The group has prepared a draft of an immunoaffinity column method for aflatoxin M_1; discussed rapid methods for aflatoxin M_1; and noted that Codex Alimentarius was reviewing limits for aflatoxin B_1 in feedstuffs for dairy cattle. The next meeting of the group will be in Milan, Italy, March 12, 1991.

There was a general discussion on test kits for mycotoxins including the observations that too many collaborative studies are being requested and that AOAC approval should not be used as a selling point for test kits.

Douglas Park reviewed the problems of aflatoxin contamination in cottonseed and corn in the southern U.S. and northern Mexico, and discussed developments in regulation of decontamination by ammoniation.

Future conferences related to mycotoxins brought to the attention of the Joint Mycotoxin Committee are: Symposium/Workshop on Food Contamination—Mycotoxins and Phycotoxins, November 4–15, 1990, Cairo, Egypt; Joint meeting of SRIEG 51 and NC 129 and Symposium on Fumonisins, April 22–25, 1991, Raleigh, North Carolina; International Symposium on Nitrogen-Containing Mycotoxins, June 4–7, 1991, Pushchino, USSR; International Meeting on Mycotoxins, Nephropathy and Urinary Tract Tumors, June 6–8, 1991, Varna, Bulgaria; Gordon Research Conference on Mycotoxins and Phycotoxins, June 24–28, 1991, Plymouth, New Hampshire; 105th AOAC Annual International Meeting, August 12–15, 1991, Phoenix, Arizona; and the 8th International IUPAC Symposium on Mycotoxins and Phycotoxins to be held in Mexico in 1992.

REGULATORY ROUNDTABLE ON MICROBIOLOGICAL STANDARDS FOR FOODS—REALITIES AND FALLACIES

103rd Annual International Meeting of AOAC, September 1989

One of the major attractions and certainly one of the highlights for microbiologists at the 1989 AOAC annual meeting in St. Louis, MO, was the presentation of the Regulatory Roundtable on "Microbiological Criteria for Food—Realities and Fallacies." A panel of experts representing government and industry in the United States, Canada, and Europe was assembled to provide a comprehensive overview of the international concerns regarding microbiological criteria for foods. Members of the panel were asked to address their concerns about specific matrixes and analytes within the context of 3 areas: microbial indicators of food safety and quality, traditional concepts, and new trends; pathogenic microorganisms in food, established species, and emerging etiological agents; and principles and application of Hazard Analysis of Critical Control Points (HACCP).

Kenneth Helrich served as moderator and welcomed a full house of attendees to the 5th Annual AOAC Regulatory Roundtable. He reviewed the purpose of the roundtables as forums for discussing regulatory problems by encouraging interaction among speakers and by getting audience participation, particularly when the floor was opened for discussion following the formal presentations.

Helrich then called on Michael H. Brodsky for some introductory remarks. Brodsky outlined the premise he followed in developing this roundtable. As the Chief of Environmental Bacteriology for the Ontario Ministry of Health, Laboratory Services Branch in Toronto since 1982, Brodsky became acutely aware that laboratory data are often perceived as omnipotent, when, in fact, the data might be of questionable significance. In this regard, laboratories are frequently forced to play 'magic number games' or 'lotteries' for the interpretation of analytical results, particularly when dealing with indicator organisms. These problems transcended municipal, provincial, state, federal, and international boundaries. The purpose of assembling this panel for the regulatory roundtable was, thus, to present a global review of the realities and fallacies associated with the application of microbiological criteria for food and to critically assess how best to ensure food safety and quality.

The first speaker was John E. Kvenberg, Program Manager, Division of Microbiology, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Washington, DC. Kvenberg reviewed FDA's historical role in attempting to promulgate microbiological standards for food and industry's opposition to end-point criteria. In 1987, the National Advisory Committee on Food Safety was established. As a member of the subcommittee on seafood, Kvenberg focused his presentation on microbiological criteria to the seafood industry, using cooked, ready-to-eat crabmeat and shrimp as models. The committee conceded that sampling programs for either specific pathogens or indicator organisms were of limited value unless HACCP-based programs were also operational. The committee has suggested that the implementation of HACCP programs is the responsibility of industry, whereas the role of government is to register food processing plants that are in compliance with HACCP requirements and to monitor their performance. Kvenberg emphasized the need for more government/industry cooperation to develop and promote HACCP education programs.

The second speaker was Bruce Brown, Chief, Evaluation Division, Bureau of Microbial Hazards, Health and Welfare Canada, Ottawa, Ontario. Brown compared the viability of microbiological criteria for end products with HACCP programs. He emphasized that regulated acceptability criteria must include methodology as well as appropriate sampling plans. He suggested that although indicator organisms may be useful for monitoring processing parameters, they cannot be substituted for specific pathogen analysis. Such analyses must be food-specific because foods vary in their bacterial growth-promoting capabilities; however, the application of such analytical protocols to end products is not cost-effective. Brown agreed with Kvenberg that HACCP-based programs were the only reliable means to ensure food quality and safety. To illustrate how Health and Welfare Canada has accepted the HACCP concept, Brown concluded his remarks by reporting that Canada has regulated and defined refrigeration temperatures ($\leq 4°C$). This regulation ensures that food handling liability is shared by anyone and everyone who is responsible for the products from production and processing to retail sales.

David A. A. Mossel was the third distinguished speaker. Mossel is Research Professor Emeritus, Chairman of Medical, Food, and Water Microbiology, The Netherlands Government University at Utrecht. Mossel gave a European perspective on the use of microbial reference values for foods. In his opinion, which is shared by many of his colleagues, the term microbial standard implies a specific numerical value, and should be replaced by the concept of reference values or ranges. Such ranges should be developed by ecologically justified surveys of valid food specimens capable of supporting microbial survival and growth, and should be incorporated into 3-class sampling plans specifically designed for each individual commodity. Mossel added that not only should microbiological criteria be limited to ecologically justifiable agents, but also certain traditional indicator organisms should be deleted from our repertoire, including that "rag bag" of ill-defined, misapplied group of organisms known as "coliforms." Mossel showed data summaries illustrating a significant relationship between the incidence of *Salmonella* sp. in processed, ready-to-eat foods and the "Thermotropic Enterobacteriaceae" count (incubation at 42°C). Using some literary licence, I believe Mossel's comments in this regard could be summarized as: "The microbiologist who lives by the coliform will die by the coliform." In his concluding comments, Mossel stressed the poor precision of quantitative microbiological methods and the need for constant performance monitoring, with specific reference to culture media.

H. Michael Wehr, Administrator, Laboratory Services Division, Oregon Dept of Agriculture, Salem, OR, was the fourth speaker of the first session. Wehr focused his remarks on state regulatory perspectives and on probable recommendations of the National Advisory Committee on Microbiological Criteria for Foods. A member of the National Advisory Committee, Wehr indicated that the criteria will be relat-

ed to food safety, rather than food quality. Resolving food spoilage problems and, hence, food quality is a proper role for the food industry, rather than state or federal regulatory agencies. In addition, 2- or 3-class sampling plans, recommended by the International Commission on Microbiological Specifications for Foods (ICMSF), will be utilized. Should they adopt Committee recommendations, state and local governments will have to implement these statistically based sampling plans. Compromises, however, may have to be adopted. The National Advisory Committee recognizes the limitations of even the best sampling plans for ensuring microbiological product safety and will recommend an integration of inspection activities with HACCP systems. As a result, states will have to assist firms with the development and implementation of appropriate HACCP systems and integrate their inspection approach to encompass HACCP checkpoints, including increasing their level of monitoring relevant documentation and records. The net effect will be a restriction on product sampling and a reduction in analytical needs. Wehr concluded his comments by stating that in his opinion these changes in traditional approaches to assessing food safety are a positive move, but will require closer liaison among state, federal, and industrial microbiologists for resolution.

The second session was opened by Jerry Carosella, Deputy Director, Microbiology Division, Food Safety and Inspection Service, U.S. Dept of Agriculture, Washington DC. Carosella presented an overview of the working group discussions on meat and poultry of the National Advisory Committee. This group is drafting microbiological criteria for cooked, ready-to-eat meat and poultry products, which require refrigeration and have Aw and pH values of ≤ 0.93 and ≤ 4.6, respectively. The committee has suggested that the risk of microbial pathogens can be minimized by controlling the parameters that inhibit the growth of *Clostridium botulinum* and *Listeria monocytogenes*. However, HACCP programs are mandatory to ensure proper processing/holding parameters and, hence, food safety. In this regard, critical temperature control of the foods in question by manufacturers, retailers, and consumers is paramount. Furthermore, built-in temperature indicators should be included to allow consumers to identify temperature-abused products. Packaging systems, i.e., moisture barriers, and other approaches to extending shelf life have also been applied to minimize microbial growth and the spread of pathogens. In the committee's opinion, meat and poultry products can be placed in 3 general categories based on hazard potential and application of heat treatment: (*1*) assembled and cooked; (*2*) cooked, then assembled; (*3*) cooked foods to which raw ingredients are added. Each food type and process, however, must have a risk assessment before a proper HACCP program can be implemented. To assist in the development of HACCP programs, the National Advisory Committee has created an HACCP working group, which has identified 7 HACCP principles. Carosella concluded by briefly discussing these principles:

(*1*) Assess hazards and risks associated with ingredients and final preparation;
(*2*) Determine critical control points;
(*3*) Define conditions to be met at each control point;
(*4*) Develop procedures to monitor critical control points;
(*5*) Describe corrective action to be taken when a deviation is identified;
(*6*) Develop a record and documentation system; and
(*7*) Implement a process to verify the performance of HACCP procedures.

Robert Moir, Chief, Food Borne Pathogen Unit Agri-Food Safety Division, Food Production and Inspection Branch, Agriculture Canada, discussed microbiological criteria for food from the perspective of minimizing the risk. The concept of zero risk in today's food products is not viable from a microbiological standpoint. Given the great multitude of foods produced, the high speed technology the food industry uses to meet public demands, and the ubiquitousness of various foodborne pathogens, it is not realistic or practical to expect food products to be free of such organisms. The aim of both industry and regulatory agencies must be to minimize the risk to the public and to control any problems that do arise. The adoption of guidelines for the food industry for some food products can help indicate potential problems.

The concept of microbiological criteria for dairy products was presented by Richard Holley, Corporate Quality Assurance Section, Technical Services Dept, John Labatt Co., Ltd. Dairy products can be categorized into 5 basic types related to physio-chemical characteristics and, hence, their ability to support the growth of microorganisms, or into 2 broad subcategories based on relative shelf life. In Holley's opinion, it is, therefore, unreasonable to apply the same microbiological criteria to all dairy products. We emphasized the misapplication of the standard plate count and coliform count as indicators of post-pasteurization contamination. These groups of indicators may have some merit as indexes of processing problems; however, the application of HACCP programs would be more appropriate for monitoring processing parameters and plant sanitation. Holley also suggested that many of the problems of post-pasteurization contamination are related to outdated processing equipment and the post-treatment commingling of microbiologically contaminated raw materials, e.g., fruit and nuts, with pasteurized products, which might occur with the production of certain types of ice cream. In addition, Holley reflected on his observations that most filling rooms are not refrigerated, and many filling machines used in North America are archaic compared to the technology developed and implemented in Europe. He concluded that although there is no acceptable microbial indicator for dairy product safety, eliminating post-pasteurization contamination, e.g., by the use of aseptic filling techniques and the implementation of HACCP protocols, would guarantee product safety more reliably than microbiological assessment and monitoring.

The final speaker on the program was William H. Sperber, Manager, Microbiology and Food Safety, Research and Development Laboratories, Pillsbury Co., Minneapolis, MN. Sperber entitled his presentation, "Use of the HACCP System to Assure Food Safety." It was appropriate for Sperber to conclude the formal presentations in view of Pillsbury's pioneering efforts to develop the HACCP concept. The implementation of HACCP systems within the Pillsbury Co. has greatly reduced the requirements for end-product testing, thereby diminishing the need for microbiological analysis. There is still a need within HACCP-based programs, however, to establish and monitor in-process microbiological criteria. Modern HACCP programs specify certain cleaning and sanitation procedures as critical control points. In addition, microbiological criteria may still be applicable to sensitive ingredients and raw materials that should be analyzed before they are incorporated into the processing procedures.

Such criteria must be ecologically based. Sperber concluded his remarks by noting the evolution of the HACCP concept. Originally there were 3 levels of concern and currently there are 7.

A question and answer session followed the formal presentations.

Brodsky concluded the roundtable by summarizing the salient points, thanking the panel for their participation, and thanking the audience for supporting the program. In addition, he noted the increased awareness and stature of microbiology within AOAC and encouraged the microbiologists in the audience to spread the word to their colleagues to support AOAC-sponsored microbiology programs, particularly during the AOAC Analytical Week and during the AOAC annual meetings.

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Comments from Health and Welfare Canada

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Regulatory criteria for foods exist. Some of them have found their way into regulations and have become standards, or a weaker version of a standard that we in Canada call a guideline. I do not intend to present arguments in support of microbiological criteria as they may be used in regulations in Canada. Instead, I will present concerns of the Health Protection Branch of Health and Welfare Canada with specific aspects of microbiological criteria for foods relative to particular matrixes and analytes from the following areas:

(*1*) microbiological indicators (e.g., ACC, coliforms, fecal coliforms);

(*2*) established foodborne pathogens (e.g., *Salmonella*, *Bacillus cereus*, *Staphylococcus aureus*);

(*3*) recently emerged or emerging foodborne pathogens (e.g., *Listeria monocytogenes*, *Escherichia coli* 0157:H7); and

(*4*) HACCP principles and application.

Microbiological indicators figured predominantly in early regulations that remain in existence today, and unless amended, will continue to be in force. Methods for their determination were among the first dependable and relatively rapid methods developed in food microbiology. The isolation and identification of specific pathogens has always been laborious and time-consuming, and still is in many instances. Microbiological indicators have played an important role in the improvement of the microbiological quality of foods, specially processed foods; however, their limitations as regulatory standards in view of improved methods for the isolation and identification of specific foodborne pathogens will seriously impede their use in future regulatory standards.

I would be remiss if I failed to say that they continue to be applicable to the assessment or validation of thermal and other types of processing designed to appreciably or totally reduce the microbiological population of foods. Although it is wise for a processor to assume that his raw products are contaminated with a host of foodborne pathogens, studies have shown that this is not always the case. For a processor to determine which pathogens exist in all his raw products and at what level would be exceedingly costly and probably of limited value. Generally microbiological indicators are composed of a broad spectrum of organisms, e.g., aerobic colony count, coliforms, enterobacteriaceae, and are easier to isolate and measure. If it has been established that destruction of such groups of organisms is analogous to the destruction of the pathogens that may exist in a food, their use in criteria and even in regulatory standards for processed foods can be supported.

The situation with respect to specific known foodborne pathogens is not as clear. It would be simple to demand that known foodborne pathogens should be absent from processed foods, but it would not be realistic. As we well know, not all foodborne pathogens affect all persons in the same manner, nor are the effective doses the same. As we have come to know, even low numbers of *Salmonella* (1–3 cells/g) can result in illness, whereas fairly high numbers of *S. aureus* (10^5) are required before significant concentrations of enterotoxin are produced to induce illness. Within the limits of knowledge concerning the effect of the various foodborne pathogens, criteria have been developed.

The criteria are influenced by the nature of the food and what, if any, microbiocidal treatment it would normally receive before consumption. The criteria with respect to foodborne pathogens in raw foods must be more critical than those for foods that are normally fully cooked before consumption. Even this is an over-simplification. The microbiological state, the ability of the food to support growth, and the manner in which it is maintained before consumption must also be taken into consideration. We now know that pathogenic psycrotrophs grow at normal refrigeration temperatures and even mild temperature abuse can considerably increase the hazard, not only for the psycrotrophs but also for the mesophilic bacteria.

General regulatory prohibition of foodborne pathogens in all food is neither practical nor desirable. Regulations and hence criteria must be considered on a food-by-food, organism-by-organism basis.

Regulations providing for either the absence or some maximum acceptable level of a foodborne pathogen are virtually end-product regulations requiring sampling and analysis to demonstrate compliance or violation. End-product analysis is not cost-effective and certainly will not effectively guarantee compliance.

Regulatory criteria with respect to specific foodborne pathogens provide the food industry with a target that must be achieved.

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The situation with newly emerged or emerging pathogens is even more difficult for regulators. In the case of *Listeria monocytogenes* and *E. coli* 0157:H7, infective dosages (necessary information for the formation of regulatory criteria) have not been ascertained. However, that does not mean that their presence in foods, especially those that will be consumed with little or no thermal processing (cooking) before consumption, is safe. In Canada, guidelines in the form of policy statements have been issued for *Listeria monocytogenes* that require it to be absent from foods from which epidemiological evidence exists or in which the organism can be expected to grow, or foods that are maintained under conditions favorable to growth and are consumed with little or no cooking. Although the methods for the isolation and identification of these pathogens have improved considerably, they are still time-consuming and lack sufficient precision to permit determining levels.

Regulatory microbiological criteria require the support of specific sampling plans. The number of samples and the size of the analytical unit are integral in determining the tolerance that may be permitted. In Canada, 2 types of sampling plans are used, 2-class and 3-class. The former includes those organisms the presence of which in the food is prohibited, e.g., *Salmonella* in milk powder. A 3-class plan is used for those organisms that are tolerated at some level in food, e.g., *S. aureus* in cheese. The degree of hazard as described in the various cases by ICMSF is used in determining the sample size, acceptable criteria, etc.

Using HACCP principles in the assessment and control of their processes or food service is the only reliable method for the food industry to ensure that their products are free of the hazards presented to the consumer by foodborne pathogens. Health and Welfare Canada supports and encourages the application of these principles in the food industry. Inspections are carried out on the basis of good manufacturing practices devised by a combined effort of both the private and public sectors. Regulatory standards or guidelines serve as the target that the industry must attain. End-product analyses cannot assure that products will be produced free of any particular pathogen.

Health and Welfare Canada publishes both the methods and regulatory criteria used in the assessment of the microbiological quality of foods. If a regulatory standard exists, there is an official method that is an integral part of the regulation. Official methods contain a detailed description of the method, a sampling plan, and acceptance criteria. Other methods used in the assessment of the microbiological quality of foods for which there is no regulatory standard are termed "Health Protection Branch methods," they also contain a description of the method, sampling plan, and acceptance criteria. Methods in development to be used in support of the Food and Drug Act are designated "laboratory procedures." The sampling plans and acceptance criteria have been designed for compliance use. The industry is advised to use other plans that would give them the assurance that their product will pass our assessment. Many a processor has found to his dismay that a product sampled and analyzed in accordance with our methods and found to be in compliance fails when sampled and analyzed by our inspection services—an understandable situation for marginal products. The proper application of HACCP principles can give the highest assurance of compliance.

Before ending this short discourse on the concerns of Health and Welfare Canada with respect to microbiological criteria of foods, I would like to address the subject of liability. In the past, regulatory liability was primarily directed toward the manufacturer of a food; little or no attention was directed toward the treatment afforded the product after it was out of direct control of the processor. Concern over the safety of refrigerated foods, especially those for which extended refrigerated shelf-life was proposed, has directed attention to the handling all along the distribution chain, right into the consumer's home. If, for instance, temperature abuse is deemed the cause of a product's noncompliance, the persons or concern responsible for the product at the point of the abuse, may be the subject of regulatory activity.

Comments from Oregon Department of Agriculture

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Most states and other nonfederal U.S. regulatory agencies have really had little in the way of formal microbiological criteria, and certainly little that is in rule or statute. With the exception of dairy products, any criteria that exist are usually in the form of guidelines, are usually internal (that is, in-house), and concern such test areas as aerobic plate count, coliforms, and *E. coli*. They usually cover products such as delicatessen items and sensitive meat products, and are typically end-point standards. They generally have little in the way of scientific data to back them up and seldom employ statistical sampling plans. In most cases, they are based on a perceived need to provide some sort of consumer protection and reflect the relatively limited level of analytical resources available to states and, in some cases, limited technical expertise in designing and implementing programs. This sounds somewhat negative, but the problems are real. State and local programs, however, in spite of their shortcomings, do indeed help by giving us some measure and control over sensitive microbiological products and by creating an awareness on the part of state and local manufacturers and retailers about the need to maintain microbiological control of their products.

From surveys of state agencies conducted in the recent past, I have found a significant amount of interest in working toward a meaningful series of food microbiological criteria and developing an appropriate program. This is particularly true today in light of the growing concern both with microbiological safety of food and with new microbiologically sensitive product types such as freshly prepared refrigerated foods. I believe there is an interest in what is accomplished by the National Advisory Committee on the Microbiology of Foods; however, this interest will be tempered with the reality of what the states can effectively do in view of their limited budgets and the large number of establishments under their jurisdiction. States and local governments are, I believe, truly interested in receiving some technically sound information to improve their programs. The challenge of the National Advisory Committee is to provide this kind of assistance in a form usable by state and local governments.

I would like to discuss what I think will result from the National Advisory Committees efforts in terms of basic philosophy and approach, how it will impact state and local governments, and what I believe will be some of the challenges we will have to meet.

First, the thrust of the committee will be one of safety rather than quality. This approach as such is not subject to argument. It is really a "Mom and apple pie" situation, and no one is going to disagree. It is and must be the prime factor to consider. The committee's criteria will be built around this philosophy.

I am a firm believer, however, that regulatory agencies have 2 roles to play in this area. Most important is food safety. Of secondary importance is excessive rates of product loss due to spoilage, which is in essence a form of consumer fraud. The National Advisory Committee has gone clearly on record as saying that this area of adverse product quality, as it results in excessive rates of product loss, is not an appropriate role for agencies to play. It is the proper role of the industry to monitor and control this factor—the marketplace through consumer purchasing and returns will control this area. This viewpoint may be somewhat naive and even perhaps a bit self-serving. In any case, the committee will not generate much activity on this topic. This is unfortunate, I believe, because some state and local agencies as well as consumer interest groups will continue to see the need for some sort of role in this area, and there will continue to be a scientific gap in this respect.

Second, the thrust will be on 2- or 3-class sampling plans for product sampling. Essentially the same statistical sampling approach is recommended by the International Commission on Microbiological Specifications for Foods (ICMSF). Fundamentally, this is clearly the proper approach to use. State and local governments would be wise to improve their approach to sampling to bring it more into line with proper statistical methods.

However, and this is important, the ICMSF approach is very resource-intensive. Usually, the number of subsamples taken is at least 5 per product type and can routinely climb to 10 or more. This can present a problem for states, particularly those with limited resources and a large number of establishments for which they are responsible.

For example, Oregon, which is a small state, has more than 2000 retail grocery stores. Assuming that only half of them sell regular ground beef, which is an appropriate product to consider, a once-a-year sampling with the ICMSF approach at a minimal level yields 10 000 samples annually. Our routine food microbiology work allows for the analysis of approximately 2400 samples per year for all product types, so the problem is serious.

Let us hope that the committee will address this issue and find some compromise resolution. A possible resolution would be to use M, the microbiological value above which a product is unacceptable, as a single "random grab" sample criterion, with appropriate resampling by the ICMSF approach when a problem is identified.

The third and last area that I want to mention is the use of the Hazard Analysis and Critical Control Point (HACCP) approach. The National Advisory Committee is clearly going to recommend the HACCP approach as the single way of handling microbiologically sensitive food products. HACCP is basically applied across all product areas and across all establishment types (manufacturing to retailing) and is an approach that will have some fundamental and far-reaching changes for state and local governments as well as for the industry it serves. Currently, HACCP in its truest form really is applied only to low-acid canned foods. Applying HACCP to all products and all establishments will clearly change the way we are doing business. Specifically, it will modify the way we inspect establishments, including warehouses and retail outlets, the way we monitor record keeping, and the way we sample products for analysis. It will probably also affect the frequency of conducting certain types of tests.

For many years, I have been a strong advocate of integrating inspectional activities with analytical programs. Employing end-point standards, except for certain products, and

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then dealing almost solely with pathogens, has not normally proved effective in relating microbiological analysis to product safety or excessive rates of product spoilage. In a sense, the HACCP system provides the ultimate in inter-relating inspectional work with product analysis. What states will then have to do is to: (*1*) assist firms with the development and implementation of their HACCP system; (*2*) integrate their inspectional approach to encompass the HACCP checkpoints; (*3*) increase their level of monitoring firms' record keeping; and (*4*) modify their product sampling and analytical needs.

This will most certainly require a significant educational program for state and local inspectors in HACCP, because few of them are sufficiently knowledgeable to utilize the HACCP approach. I believe it is the intent of the National Advisory Committee to have USDA, FDA, and the National Marine Fisheries Service, in conjunction with national commodity trade and technical associations, design model HACCP programs for use by industry as well as by state and local agencies as they move toward full HACCP implementation.

The committee has also strongly endorsed the development and implementation of HACCP educational programs. Although this effort will be directed to all users, it will be of particular importance for state and local governments, smaller in-state or regional processors, and institutional and retail establishments. It is the intent that the HACCP approach be completely integrated from processing to distribution to retail sale to institutional and home food service.

These 3 changes, the almost exclusive emphasis on product safety, the use of HACCP, and the use of statistical sampling, along with other changes in the system, will have a profound affect on how state and local governments do business in the food microbiology area. The net change will certainly be positive, but some real challenges will have to be met. I strongly believe that 5 or 10 years from now, we will see a different operational world from what it is today. It will most certainly be interesting to see how well it develops.

Microbiological Criteria: Minimizing the Risk

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In this day and age, the ideal concept of food products having zero risk with respect to microbiological hazards is not viable. Given the ever-increasing population of the planet, together with the ever-increasing need for food products, industry must adapt to meet these needs with newer and faster technologies that will assure the necessary throughput of food products. In view of the ubiquity of various foodborne pathogens, whether salmonellae, *Listeria*, staphylococci, or *Escherichia coli*, it is neither feasible nor practical to assure consumers that the various food products that they purchase are free of all types of microbiological hazard.

At Agriculture Canada, we have recognized and accepted this fact, and the main business statement of the Agri-Food Safety Division within our Food Production and Inspection Branch states, in part, that we will ensure the use of programs and principles that minimize the risk to consumers from chemical, physical, and biological hazards in agri-food products.

Now let us consider the feasibility of setting microbiological criteria for foods. When one speaks about such criteria, one usually separates them into two possible categories, namely, guidelines and regulatory standards. For the purpose of this discussion, guidelines are exactly what the word implies: an established range of values based on previous experience and data that serve as a guide to the industry with respect to good manufacturing practices. Regulatory standards are another set of criteria that normally fall under the jurisdiction of a federal or provincial act and that, when applied to a given foodstuff, will be used to deem whether that foodstuff is or is not acceptable with respect to microbiological quality.

In most cases, the use of microbiological regulatory standards in food products is not desirable; it is, in fact, a hindrance to the food industry and can also create false impressions among the consuming public. Because of the dynamic population and diversity of bacteria that may inhabit, coinhabit, or contaminate foodstuffs, any attempt to generalize microbiological conditions with a blanket statement is virtually impossible. For instance, to say that 10^6 bacteria on a total plate count is too much or that 10^3 *Staphylococcus aureus* in a given foodstuff is too much is difficult to defend scientifically because of the myriad conditions present in the various food products and the factors that influence the growth of such bacteria. Certain bacteria at levels of 10^3 per g in a given food product can definitely be much more dangerous than levels of 10^8 bacteria in another product; thus, the cataloguing of food products into an acceptable/nonacceptable compartment is not very realistic. I believe that such a standard was applied to ground beef about 1980 in Oregon. I understand that it was not a workable solution and that the program was dropped after a short time.

One area in which regulatory standards certainly may be useful is for those food products in which the existence of even one given species of bacterium may be considered completely unacceptable. An example is the presence of *Salmonella* in a ready-to-eat meat product, or perhaps in the very near future, the presence of *Listeria* in a ready-to-eat meat product. In such cases, the fact that these bacteria may survive and even grow in such products that do not undergo any further heat processing before they are consumed may be enough to warrant such regulatory action. When such products are found through testing procedures, whether at the retail level or the producer level, action should be taken and the product either removed from commerce or treated in another manner to render such bacteria innocuous.

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When we are implementing this type of action, the next obvious question is, how do we compare our results? It is fairly obvious that zero *Salmonella* in a frankfurter based on a 1 g sample may not be the same as zero *Salmonella* based on a 25 g sample. This conclusion leads us into the various sampling schemes, including statistically based sampling plans as well as sample size, methods of collection, packaging, storage and transportation, and analytical methodology to be used in the evaluation of the various bacteria. Thus comparison of results, whether between laboratories, between state and federal agencies, or between provincial and federal agencies can be a very difficult, if not impossible task unless all such parameters are standardized.

In the great majority of cases, I find that the establishment of microbiological guidelines can be readily accepted and that they provide some measure of control both for the industry and for regulatory agencies. Over the last 7–8 years, Agriculture Canada, through data accumulation and evaluation of a great many meat products, has published guidelines in our manual of procedures for meat hygiene. The data were basically derived from the establishment and evaluation of microbial profiles on many raw and cooked meat products. These profiles consisted of a total aerobic colony count, a coliform and fecal coliform count, a *Staphylococcus aureus* count (coagulase positive), and the presence or absence of salmonellae. These guidelines, based on a 5 subsample set, include cooked, cured, and raw meat products and are intended only to give an indication to the industry and to ourselves whether a product has been time/temperature abused or improperly processed, and is, as a result, potentially hazardous to consumers or prone to early shelf spoilage. To date, we have found that use of such guidelines has been very helpful by assisting various segments of the food industry to establish quality control programs in order to stay within these parameters. In addition, on many occasions the use of the guidelines has led us to conduct investigative work at a given establishment where parameters were exceeded and there were obvious problems at some point in the processing.

In my opinion, it is clearly not valid for government agencies to establish standards on any type of raw meat product. On the other hand, I think it is quite acceptable for industry to have their own microbial guidelines on the various raw meat products they purchase. For instance, if a firm wants to produce beef patties and the deboned beef received from the supplier has a total count of 10^7 or greater, it is highly unlikely that those patties will have much of a shelf life. It is an integral part of industry's ongoing quality control systems that criteria should be set in some cases for the evaluation of incoming product. The efforts of regulatory agencies must focus on ready-to-eat food products, and we must bear in mind that proposed regulatory standards should be very thoroughly evaluated before they are put into place. Regardless of the "apparent" new problems associated with some foodborne pathogens, it is important that we not institute measures which, in the very near future, could be seen to have been arrived at without scientific validity.

Microbiological Reference Values for Foods: A European Perspective

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Microbiological criteria for food products and meals serve to gauge the results obtained upon monitoring samples from manufacturing plants or catering units which strictly adhere to good manufacturing practices (GMP). Hazardous practices thus already having been eliminated, the aim of monitoring is to detect and, above all, immediately correct accidental failures in processing or preparation. An essential element of this system of monitoring is that the number of criteria used is kept to a minimum by carefully selecting the criteria of major ecological significance. In view of the sporadic and erratic distribution of pathogenic organisms in foods from well-run manufacturing plants and food service operations, criteria for disease agents are used only occasionally. Where they are used, their selection and detection methodology require intensive and expert scrutiny, including (*I*) careful designation and precise definition of the relevant agents of disease, toxin formers, or preformed toxins; (*II*) prescription of very carefully validated and standardized methods, usually including a resuscitation step; and (*III*) mathematically correct interpretation of failure to detect particular infectious or toxinogenic organisms. On the other hand, "marker" organisms ("indicator" and "index" organisms, according to Ingram) are frequently used. These should also be carefully selected by ecological surveys on each specific food product; in particular, their value for reliably revealing deficiencies in hygiene or handling should be assessed. The applicability of the entire group of Enterobacteriaceae (or particular parts of that taxon), *Enterococcus* species, *Staphylococcus aureus*, and streptococci of the "mitis-salivarius" group are critically examined. Numerical reference values for microbiological criteria are derived by mathematical treatment from data obtained in surveys of products produced under GMP previously validated by safety analysis. Target values thus derived will include tolerance limits and policies recommended in case high results are obtained. Consequently, "3-class sampling plans" (according to ICMSF) are to be used in most instances.

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Principles

The Purpose and Applicability of Microbiological Criteria

Probably no aspect of food microbiology has been more amply (and often emotionally) discussed than microbiological

criteria for foods. Such controversy is unnecessary, however, provided the purpose served by criteria is accurately defined. Their main aim is to gauge the results obtained from the monitoring of particular recently manufactured food samples that originated from well-operated production and distribution facilities and are being examined to allow prompt rectification of any accidental process failures which may occur. In addition, microbiological monitoring of food may serve to assess whether consignments of food or purchased food ingredients that have been manufactured according to the best possible practices have also been distributed in such a way that they reach the point of sale in a condition which assures wholesomeness and good quality (1). Provided that the product is stored under the conditions specified on the package and only until the "best before" date has expired, the food will reach the consumer in good condition.

It may be of equal importance to dwell for a minute on what monitoring cannot achieve, and hence for what purpose criteria are not intended. It used to be thought that microbiological inspection of *samples* of food or raw materials which gave reassuring results would ensure that the *consignment* from which the samples were taken was safe and of the expected shelf life. This is unfortunately scientific heresy (2–4). An act of inspection can never result in quality assurance, as a matter of principle. Furthermore, simple Poisson statistics supported by identification of the stratified way in which microorganisms are usually distributed in foods (5) show that sample monitoring tests which give negative results cannot at all ensure that the entire production batch from which the sample was taken has reached the acceptable safety and quality level.

Many reputable food manufacturers have already drawn up microbiological guidelines, also called "limits" or "reference ranges" (3). These may be mandatory or purely advisory. The United Nations, through the Food and Agriculture Organization and the World Health Organization, issues comprehensive codes of practice for the manufacture of foods, namely, the *Codex Alimentarius*. These allow preventive assurance of safety and quality (6). Whenever monitoring forms part of such codes of practice, the Codex Advisory Group has chosen the following terms (7, 8):

Guidelines are reference levels of microorganisms at points during or after production, distribution, or storage, which are devised usually by a manufacturer or distributor and sometimes by government agencies, in order to check that the system is functioning properly. End-product *specifications* are issued by regulatory authorities or distributors as part of the purchase conditions. Microbiological *standards* are specifications that are legally enforceable once they are adopted by individual countries. Standards consist of limits for numbers of pathogens, their toxins, or their metabolic products, and they include detailed sampling plans.

There is, unfortunately, much disagreement about the need for microbiological standards. Some countries, for example, Canada, The Netherlands, and Hungary, have drawn up standards for many foods; others, such as the United Kingdom, consider them less practicable. It is not the authors' aim to enter into arguments about the necessity for legally enforceable standards. In the following sections, the subject will be discussed from the scientific point of view only. For this purpose, the concepts *criterion* (of qualitative nature) and *reference values* (numerical limits) will be used (9–12). When required, guidelines, end-product specifications, and standards can be derived from such data.

The General Assessment Procedure

As in medicine, reference values should be determined empirically. More particularly, they should be established by 5 essential sequential steps, including:

(*i*) selecting target organisms;

(*ii*) choosing and very carefully standardizing a method to be used for their enumeration;

(*iii*) carrying out surveys of samples taken from production lines while strictly adhering to validated good manufacturing practices (GMP);

(*iv*) deriving acceptable numerical values from survey data guided by holistic quantitative risk analysis (8, 13);

(*v*) establishing a policy for dealing with consignments which fail the target values (3).

Selection of Criteria

General Principles

The number of microbiological criteria to be used for the estimation of safety and quality of foods must be strictly limited for at least 2 reasons.

First, reducing the number of tests to be carried out enables more samples to be examined. This markedly increases the accuracy of the results, particularly since, as indicated before, contaminating microbial populations are usually heterogeneously distributed (5).

Second, the use of criteria which stem from an imaginary microbiological problem (14) does little to improve the credibility of the food microbiologist (15). Only a few tests are of particular value in most cases. These must be responsive to need and therefore must be based on ecological considerations: every type of food has its characteristic associated flora, including pathogens and spoilage agents (16). Hence, continued adherence to the "shopping list"—plate count and coliforms—is not warranted.

Criteria for Pathogens

Pathogens may multiply in food and become a hazard. Even if they do not proliferate, they are undesirable because of the risk of contaminating, and possibly multiplying in, foods which were initially of good quality (17). Numbers of pathogens must therefore be kept to a minimum in the interest of the consumer's health.

When pathogens may occur naturally in a food while ecological factors favor their growth, the ecology of such foods must be modified to ensure consumer protection. This can be achieved by "elimination" [preferably by substantially reducing colony forming units (cfu)] through processing, changing the composition, or improved conditions of storage. The success of such measures can often be checked by the use of properly chosen "marker" organisms, as to be discussed in the following section.

Monitoring Spoilage Risks

The chances of microbial spoilage of foods can always be estimated by storage tests. When onset of spoilage is rapid, however, it should rather be assessed by determining the cfu numbers of the specific component of the spoilage association and gauging the results against previously determined "models." The "model" is the fate of the spoilage association as affected by the intrinsic properties of the food and its usual mode of storage (16). Clearly such tests must provide results very rapidly in the case of highly perishable foods.

Microbiological Essentials

The species, genera, or other groups of organisms to which criteria apply should be described in taxonomically valid terms. Vague and invalid expressions such as "pathogenic organisms," "coliforms," "enterobacteria," "enteric organisms," "diphtheroids," or "cocci" should be avoided.

Criteria for disease agents should be considered and formulated with particular care. Precautions to be taken include:

(*i*) Careful designation and precise definition of the relevant agents of disease, toxin formers, or preformed toxins;

(*ii*) Prescription of very carefully validated and standardized methods, usually including a resuscitation step;

(*iii*) Mathematically correct interpretation of failure to detect particular infectious or toxinogenic organisms.

The Use of Marker Organisms: Concepts and Misconceptions

Historical Introduction

Early history.—The examination of potable water for the group of bacteria that later became known as "coli-aerogenes bacteria of fecal origin," or in taxonomic terms *Escherichia coli*, was independently introduced by Schardinger (18) in Austria in 1892 and by Smith (19) in the United States in 1895. This test was designed to replace testing for specific pathogenic Enterobacteriaceae, particularly *Salmonella typhi*, as this presented insurmountable difficulties at that time. To this day, the monitoring of water and shellfish has been based—rightly or wrongly—on the detection of *E. coli* as a surrogate marker for enteric pathogens.

In addition, bacteria of the so-called coli-aerogenes (or coliform) group, defined as the lactose-positive organisms in the family Enterobacteriaceae, have been used as marker organisms in the examination of pasteurized milk and ice cream since about 1920 (20, 21). Their use originated from the so-called Wilson triad (1) (Table 1) based on the premise that isolating such organisms from heat-treated foods points to inadequate processing, i.e., (*i*) failure to "eliminate" all bacteria of this group initially present; (*ii*) failure of packaging such that recontamination is prevented; or (*iii*) failure to store and distribute under conditions which ensure that proliferation of the extremely low numbers of survivors is arrested (22–24).

The present situation.—Nowadays, direct tests for many enteric pathogens are available. They are indispensable in epidemiological investigations, but less so in day-to-day food inspection. On the other hand, suitably designed tests for marker organisms always have a place in routine monitoring of a great many foods for the following reasons (25):

(*i*) Failure to detect disease agents, e.g., pathogenic Enterobacteriaceae, is of limited significance because (*a*) they are often unevenly distributed in the food (5, 26) and (*b*) the detection techniques have several shortcomings. This applies even to the intensively studied genus *Salmonella*, and more so to other pathogenic bacteria transmitted by foods, e.g., *Shigella* (27). Also, in a recent intra-European evaluation of methods used for the detection of *Listeria monocytogenes* in foods (28), serious deficiencies in currently used methods were demonstrated.

(*ii*) Tests for certain pathogens, e.g., enteropathogenic viruses (Table 2) and helminths, cannot yet be carried out by a nonspecialist laboratory. Failure to make such an examination or to seek marker organisms may lead to the release of dangerously contaminated foods.

Table 1. The Wilson triad underlying processing for safety

1. Reduction of unwanted organisms to the highest achievable extent by:
   (i) keeping initial colonization of raw materials at a minimum by adherence to hygienic practices and avoidance of temperature abuse;
   (ii) increasing microbial lethality of processing to the highest level, compatible with preservation of nutritive value and sensory attributes.
2. Avoiding recontamination of treated commodities which would nullify the effect of the treatment referred to under 1 by:
   (a) processing after hermetic packaging; or
   (b) aseptic packaging;
   in both instances relying on validated measures of prevention.
3. Where intrinsic antimicrobial protection is absent, distribution and storage of the processed, packaged commodities under conditions arresting (or at least markedly delaying) proliferation:
   (a) of the infinitesimally low numbers of viable organisms that survive processing step 1; and
   (b) of the sporadic contaminant introduced during step 2.

(*iii*) Even if enteric pathogens are indeed not present in a sample of a food consignment, the result is of significance only with respect to the specific batch that has been sampled. However, if the absence of suitable marker organisms can repeatedly be demonstrated in a series of samples from a processing line, it is almost certain that the food is never dangerously contaminated. This information is of value to public health authorities, manufacturers, and consumers alike.

Ecologically valid semantics and strategies introduced in the 1970s.—Since the 1930s the suitability of both types of marker organism initially chosen (*E. coli* and the coli-aerogenes group) has frequently been questioned (22). Much of the dispute came from the lack of a clear definition of terms and the failure to pay sufficient attention to ecological principles. In an attempt to resolve these disputes a distinction has been made between *index* and *indicator* organisms within the groups named markers (25) or model organisms (29). The

Table 2. Viruses of concern in foods: cardinal properties of viruses associated with enteritis

Taxogroup	Diameter, nm	Tissue culture[a]	Identification or detection when TC -ve	Transmission demonstrated[b]
DNA-containing group				
Adenovirus types 40 and 41	65–80	+	pf, cpe[c]	s
Norwalk group[d]	27–32	−	em, imm	f
Winter vomiting disease group	25–26	−	em	f
Small, round featureless viruses[d]	20–25	−	em	f
RNA-containing group				
Rotavirus	60–80	+	em, imm, gp	f
Coxsackie	22–30	+	em, imm, gp	f
Hepatitis A and non A–non B	27	+	em, imm, gp	f
Reovirus	65–75	+		s

[a] + = possible; − = not possible.
[b] f = food; s = sewage.
[c] pf = macroscopic plaque formation; cpe = cytopathogenic effect; em = electron microscopy; imm = immunological techniques; gp = gene probe detection method available.
[d] Core nucleic acid type not definitely established.

term *index organisms* has been introduced for markers whose presence in numbers above certain limits indicates the possible presence of ecologically similar *pathogens*, e.g., *E. coli*, in the examination of potable water. Ingram suggested the use of the term *indicator organisms* for those markers whose presence above certain numbers indicates inadequate processing for safety, such as the coli-aerogenes group used in the monitoring of milk and ice cream.

A positive test for indicator organisms in a food processed for safety indicates, as demonstrated previously, that the Wilson triad has not been heeded, but does not necessarily (in fact, only occasionally) mean that pathogenic organisms are present in the food (30, 31). The detection of an index organism in a food, however, provides evidence that a related pathogen may also be present, if not in the inspected consignment, then in a previous or later one.

Clearly, a given marker can function both as an index and as an indicator organism, even in the same food. For instance, the presence of significant numbers of colony-forming units of *E. coli* in precooked meats, poultry, or seafood indicates (*i*) inadequate processing for safety, thereby fulfilling an indicator function, and (*ii*) the potential presence of enteric pathogens, i.e., functioning as an index for organisms such as *Salmonella* or *Shigella*.

The following marker organisms or appropriate combinations of them (32) have been used since before 1950 to provide useful information for the industry and government agencies alike: the Enterobacteriaceae group, *E. coli*, and to a lesser extent Lancefield group D streptococci (*Enterococcus* spp.) (33, 34), and the coli-aerogenes group. However, detection techniques for the last group have to be very carefully standardized because of the widely varying definitions of this group. For monitoring such commodities as fermented meat products with particular microbial community structures, additional marker organisms, including *Staphylococcus aureus*, may be useful. For the monitoring of the food environment, including tableware in catering establishments, the "mitis-salivarius" group of streptococci qualifies.

To avoid any misconceptions in these practices, it is essential to always adhere to Ingram's maxim, which is summarized in Table 3.

Rationale behind the use of Enterobacteriaceae as indicators.—A considerable advance in the use of marker organisms arose from the replacement of coli-aerogenes bacteria by the entire Enterobacteriaceae group. This was first suggested by Seeliger (35) in 1952 for the inspection of products like milk and ice cream processed for safety by heat. Somewhat later Henriksen (36), Habs and Langeloh (37), Kretzchmar (38), and Bonde (39) introduced this group for monitoring water that had been chlorinated, i.e., also processed for safety, but by a chemical process. The detection of *any* member of the Enterobacteriaceae in such products is equally important, since all types of this group are supposed to be eliminated by the treatment methods mentioned. Their presence in significant numbers is an indication of process failure in the sense of the Wilson triad and thus potentially hazardous to the consumer.

Such considerations also apply to the coli-aerogenes bacteria. Nevertheless, testing only for these lactose-positive members of the Enterobacteriaceae is not advisable for at least 3 reasons:

(*i*) As indicated previously, the coli-aerogenes or coliform bacteria are taxonomically a rather ill-defined group (40–42). In general, all Gram-negative bacteria capable of growing on media containing bile salts and of producing acid from lactose are included in the coliform count. This leads to (*a*) including all sorts of entirely unrelated bacteria in coli-aerogenes counts, depending on the medium used, incubation temperature, criteria chosen in reading the results, etc. (43–48); and (*b*) sometimes wrongly excluding organisms because of their unusual colony type or aberrant biochemical behavior (49, 50). This obviously results in considerable discrepancies between data obtained by different techniques (51–53) and can cause difficulties when results from different laboratories are compared (54). So-called "fecal coliforms" are even less precisely defined (55).

(*ii*) A test for lactose-positive *Enterobacteriaceae* alone can lead to falsely reassuring results in situations where lactose-negative organisms are dominant. A good illustration of this is the outbreak of diarrhea caused by soft cheese contaminated by the enteropathogenic strain of *E. coli*, type 0 124 (56). This mutant happened to be a very slow fermenter of lactose. Coli-aerogenes counts on the suspect cheese were therefore about 10^3/g, which would have been just in the intermediate acceptance region (57, 58). However, the total Enterobacteriaceae count was approximately 10^7/g, a value certainly resulting in immediate refusal (59).

(*iii*) As illustrated in Table 4, the sensitivity of the test is often reduced because it is limited to the lactose-positive types (60). The exact proportion of total Enterobacteriaceae to that of lactose-positive coli-aerogenes varies greatly with the genera of organisms present (see Table 5) and with the microbial quality of a food. This ratio represents an example

Table 3. Use of marker organisms in the microbiological monitoring of foods, originating from operations adhering to validated good manufacturing and distribution practices (GMDP): concepts and misconceptions

Ingram (25) introduced the distinction between:
- Indicator organisms = markers whose presence in given numbers indicates failure of adherence to GMDP
- Index organisms = markers whose presence in numbers exceeding given numerical values indicates the possible presence of ecologically similar pathogens

Indicators consequently should *never* be considered as surrogate markers for the presence of pathogenic organisms in foods.

Index organisms may not be considered valid as surrogate markers for food pathogens *unless* a correlation has been firmly established between their presence and that of well-defined pathogens or at least a marker threshold level has been established, below which contamination with the pathogen under study is unlikely at a given p-level (Frank, J. F., et al. (1990) *J. Food Prot.* **53** 928–932).

Table 4. Recovery of Enterobacteriaceae vs. "coliforms" from perishable foods not infrequently involved in foodborne enterobacterioses[a]

Food	No. samples examined	Colony-forming units (cfu) per mL or g	
		Coliforms	Enterobacteriaceae
Fruit ice cream	7	1.8	1.8
Vanilla ice cream	28	1.1	1.8
Meat salad	49	2.9	3.1
Egg salad	10	2.1	2.9
Fish and vegetable salads	10	2.5	3.3
Total	104		
Mean counts		2.1	2.6

[a] From Mossel et al. (60).

Table 5. Review of genera of Enterobacteriaceae with special reference to their enteric pathogenicity and detection by "coliform" tests

Genus	Predominant origin[a]	Detected by "coliform" tests[b]	Pathogenicity[c]
Arizona	F	D	P
Citrobacter	N	+	N
Edwardsiella	F	N	(P)p
Enterobacter	N	+	N
Erwinia	N	N	N
Escherichia	F	+	p
Hafnia	B	N	N
Klebsiella	B	+	p
Proteus	B	N	p
Providencia	N	N	p
Salmonella	F	N	P
Serratia	N	N	N
Shigella	F	N	P
Yersinia	F	N	P(p)

[a] F = fecal origin; N = not of fecal origin; B = both.
[b] + = most strains; D = variable; N = virtually no strains.
[c] P = most types enteropathogenic to humans; p = occasional species enteropathogenic; N = virtually no pathogenic species.

of what has been termed the *ecology determinant* or ϵ factor. It is defined as the ratio of one group of organisms of ecological significance to a related, similar group; in this instance the proportion of Enterobacteriacea cfu to the coli-aerogenes cfu, termed ϵ_{CA} (61). In the case of milk and dairy products, the ϵ_{CA}-factor may be approximately 1 because of the selection of the lactose-positive types by the high level of lactose in these products. Generally, however, the ϵ_{CA}-factor is far greater than 1, reaching median values of up to 10^4 and much higher maxima (62).

To summarize, there is never anything to be gained by using coli-aerogenes bacteria instead of the better-defined and generally more numerous Enterobacteriaceae as indicator organisms.

Misconceptions about the Use of Enterobacteriaceae as Index Organisms

As explained in the second part of Table 3, organisms should never be considered as surrogate markers (index organisms) for ecologically and physiologically related pathogens, unless a correlation has been established between cfu numbers of both groups (63–68). There is no doubt that in particular instances (30, 69, 70), such a relationship has been demonstrated to exist. A rather convincing example is given in Table 6.

Nonetheless, as illustrated by our data summarized in Table 7, in products stored for some time at refrigeration

Table 6. Distribution of Enterobacteriaceae and *Salmonella* spp. in environmental samples taken from a milk drying plant[a]

Enterobacteriaceae, cfu per g^{-1}	*Salmonella* +ve in 50 g, %
≤2	0.5
2–100	0.9
100–500	8.7
>500	9.0

[a] Data from Cox (1978), quoted by Stadhouders et al. (71).

Table 7. Effect of refrigerated storage and comminution of fresh meat on differences in Enterobacteriaceae colony counts at 37°C vs. 42°C

	$\log_{10}$ cfu Ent 37°C − $\log_{10}$ cfu Ent 42°C
At the slaughterhouse:	
Carcass meat (n = 10)	0.1 ± 0.0
At retail level:	
Consumer-size cuts (n = 10)	0.6 ± 0.2
Minced meat (n = 10)	1.8 ± 0.2

temperatures, psychrotrophic Enterobacteriaceae will markedly increase in numbers, whereas the thermotrophic types (including most pathogens) will not. In this way no correlation whatsoever can be expected between cfu of "all" Enterobacteriaceae, currently determined at 30°C, and the presence of, for example, *Salmonella* species.

If the use of Enterobacteriaceae as indices for the presence of *Salmonella* is to hold any promise, the Enterobacteriaceae colony count has to be carried out at ca 42°C. The level of correlation that can be expected when this procedure is followed is illustrated by Tables 8–10, in which use is made of the ecology determinant factor introduced earlier; in this instance this is ϵ_s, and is the ratio of Enterobacteriaceae cfu to *Salmonella* cfu.

Standardization of Laboratory Techniques

Essentials of Microbiological-Analytical Practice

Obviously, the methods used to determine reference values should be the same as those later adopted to assess compliance with the reference values. For this purpose, methods should be carefully selected, modified, or adapted if necessary, and finally tested. Examination of reference samples, i.e., carefully spiked specimens (72–74), is indispensable for validating procedures. Returns must be very carefully analyzed to ensure that any aberrant data obtained by participants do not result from failure to carry out correctly the procedure under review.

Once methods have been found adequate, they should be described in full to avoid discrepancies in results between

Table 8. ϵ_S factors in raw minced beef and pork

$\log_{10}$ cfu Enterobacteriaceae			MPN, g^{-1}	$\log_{10}$ ϵ_S factor		
30°C	37°C	42°C	*Salmonella*[a]	30°C	37°C	42°C
			Minced Beef			
5.7	3.9	3.1	0.04	7.1	5.3	4.5
5.4	4.6	4.2	0.23	7.8	6.6	4.9
5.8	5.6	4.3	0.09	7.2	7.1	5.3
6.5	6.5	4.7	0.23	7.1	7.1	5.3
6.7	6.1	4.8	0.23	7.8	6.7	5.4
Av.				7.4	6.6	5.1
			Minced Pork			
3.6	3.2	2.7	0.04	5.0	4.6	4.1
4.2	4.2	3.2	0.09	5.3	5.3	4.1
5.3	4.4	3.4	0.23	5.9	5.0	4.1
6.2	5.9	4.3	0.23	6.8	6.5	5.3
Av.				5.8	5.4	4.4

[a] Aliquots of 10, 1, and 0.1 g examined in triplicate by the Rappaport–Vassiliadis enrichment procedure at 43°C (79).

Table 9. ϵ_S factors in raw chicken meat

$\log_{10}$ cfu Enterobacteriaceae			MPN, g^{-1}	$\log_{10}$ ϵ_S factor		
30°C	37°C	42°C	*Salmonella*	30°C	37°C	42°C
3.0	2.0	2.0	0.23	3.6	2.6	2.6
3.0	3.0	2.3	0.09	4.4	4.4	3.4
4.7	4.0	2.8	0.04	5.8	5.1	4.2
4.7	4.0	2.8	0.09	5.7	5.0	3.8
4.3	3.3	2.9	0.23	4.9	3.9	3.5
6.1	5.0	3.0	0.21	6.7	5.7	3.7
4.7	4.4	4.4	0.23	5.3	5.0	5.0
Av.				5.2	4.5	3.7

Table 10. ϵ_S factors in egg yolk powder before processing in hot room

$\log_{10}$ cfu Enterobacteriaceae			MPN, g^{-1}	$\log_{10}$ ϵ_S factor		
30°C	37°C	42°C	*Salmonella*	30°C	37°C	42°C
3.9	3.9	3.3	0.09	4.6	4.1	3.6
3.6	3.6	3.5	0.09	4.5	4.0	4.5
4.5	4.4	3.9	0.23	5.5	5.4	5.0
Av.				4.9	4.5	4.4

laboratories. Even then, the successful use of a method requires more than studying the described procedure and applying it in accept-or-reject situations. In addition, gathering experience with new methods is required; this can be gained only from repeated practical studies under the supervision of experienced microbiologists.

Irrespective of the purpose of any particular examination and the procedure to be followed, attention has to be paid to some fundamentally important analytical steps. These have recently been reviewed in detail elsewhere (75). Three of these analytical essentials have not received as much attention as they deserve: (*i*) the exact definition of the taxogroups of organisms to be included in detection techniques (27) as emphasized above; (*ii*) resuscitation of sublethally stressed microbial populations (76, 77); and (*iii*) the need for continuous monitoring of the quality of all culture media used, whether prepared in-house or purchased from commercial suppliers (78, 79).

Resuscitation—An Indispensable Step

It has been well-established that most nonsporing bacteria as well as spores incur sublethal injury when exposed to heating, drying, freezing, or low pH. This results in various structural and physiological deficiencies, including an inability to develop in selective media that would otherwise support growth. There is abundant evidence that sublethally injured Gram-negative and Gram-positive bacterial cells maintain their pathogenic properties and virulence (80–89). Consequently, in order to include such relevant debilitated cells in colony counts or presence-absence (P-A) tests, a repair treatment called *resuscitation* is required in the examination of food products processed for safety, in which survivors will inevitably be injured.

Details of conditions for adequate repair of particular taxogroups injured by some sublethal treatments are yet to be determined (62). This determination should be made by constructing destruction-repair curves, schematically illustrated in Figure 1 (76, 90). Experimental data obtained by this procedure indicate that it is very difficult, if not impossible, to develop selective differential media that allow cells which have been sublethally injured by all types of conditions encountered in food processing (heating, freezing, drying, low pH) to repair lesions adequately (28, 62, 91–95).

Ignoring the need for resuscitation of bacteria occurring in processed foods may result in very misleading, falsely low colony or MPN counts. It not only leads to *underestimation* of health and spoilage risks, but in addition, very *inconsistent*

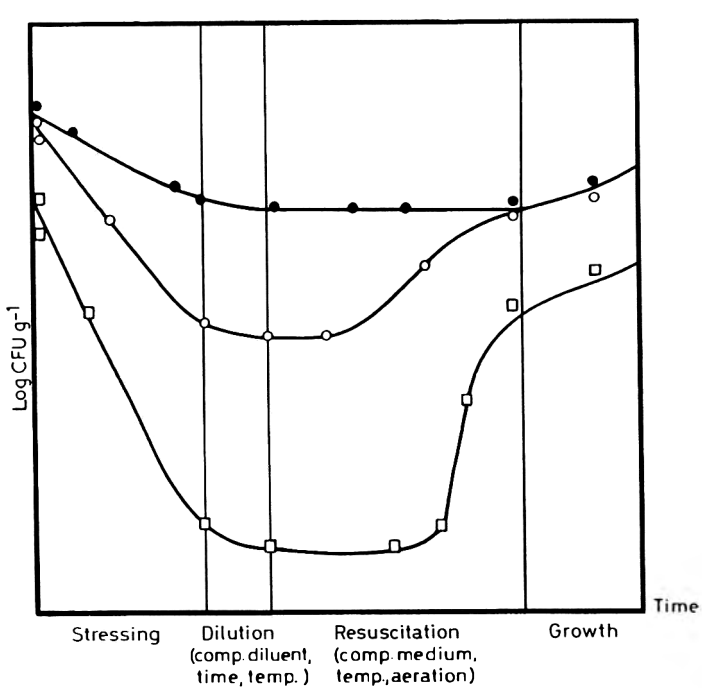

Figure 1. Destruction-repair (DR) curve. Fate of organisms occurring in foods, during stressing, release from the food matrix, maceration, dilution, resuscitation, and ultimately plating onto selective media of varying performance. Closed circles: optimal nonselective medium; open circles: optimal selective medium; open squares: suboptimal selective medium.

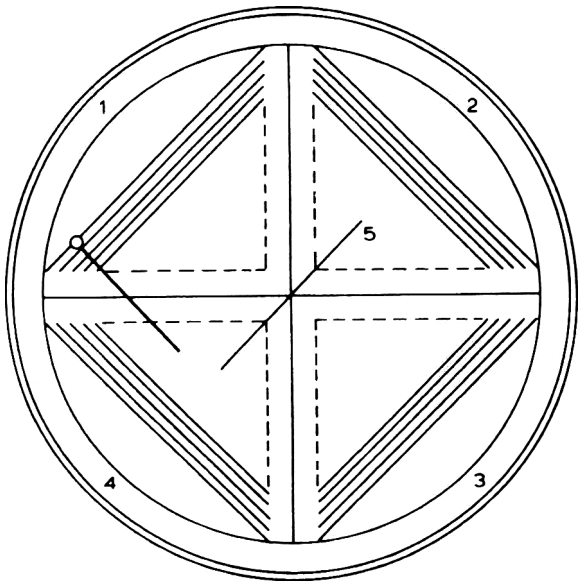

Figure 2. Principle of ecometric evaluation of culture media.

data are obtained as a result of the fortuitous (though very incomplete) repair that occurs during customary preparation of macerates and dilutions. Disputes caused in this way between buyers and sellers, or the industry and government agencies, could severely damage the credibility of the food microbiological profession (8, 76, 90).

When previously no resuscitation, or only an inadequate resuscitation step, had been included in the analytical procedure, its introduction will inevitably lead to higher results. Following the principle of mandatory twinning of methods with reference values explained before, the limit-setting procedure has to be scrupulously adapted to the true values of microbiological colonization which will be obtained upon adoption of adequate resuscitation procedures (96, 97).

Quality Assurance of Culture Media

The productivity, selectivity, and (where appropriate) the diagnostic properties of a medium vary with (*i*) the accurate choice of selective or elective components such as selective inhibitory agents, 5-carbon sugars, etc., and (*ii*) the degree to which the instructions for preparing the medium are closely followed. It would therefore be unwise to rely on the consistent functioning of commercially available selective media, even if these media were obtained from the most trustworthy manufacturers (17, 27). Variations between batches are almost unavoidable and the size and impact of the variation should be monitored by the bacteriologist using them.

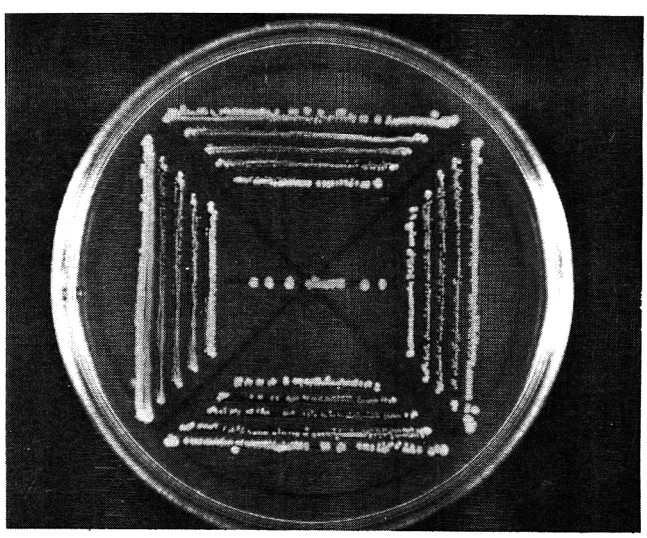

Figure 3. Reading of agar plates, inoculated by the ecometric technique of Figure 2 after incubation. As a result of the combination of medium and test strain, growth has occurred on the final streak in the central area; hence absolute growth index (AGI) = 5.

Various methods have been suggested for this purpose (98, 99). One method is particularly suitable for extensive monitoring of large numbers of different selective-differential solid media. Plates are inoculated with 1 μl portions of 18 h stationary phase cultures of test strains containing about 10^7 cfu/mL by sequential dilution. This is achieved by streaking 21 successive lines, as illustrated in Figure 2, while delivering ever-decreasing numbers of colony-forming units on succeeding lines, as in spiral plating. The procedure results in isolated colonies on the final line, as illustrated in Figure 3.

As always, media under test are inoculated with (*i*) a selection of organisms that should grow freely on the medium, and (*ii*) a group of bacteria and molds that should be totally or at least markedly inhibited. Because this method assesses, in a very simple way, a medium's ability to favor the development of one group of organisms while resisting colonization by other groups, it is called the ecometric procedure (100).

Results of ecometric testing are expressed in the absolute growth index (AGI), i.e., the highest numbered line (or quadrant) where good growth is observed. Absolute growth indices correlate rather well with the much more elaborate colony counting procedures, as illustrated by the data collected in Figure 4.

For all practical purposes, AGIs are converted to relative growth indices (RGIs), which are the ratios of the AGIs obtained on the medium under test to the AGIs obtained on a nonselective control medium. When challenged with 3 target strains of increasing sensitivity to the inhibitory system and 3 test strains with increasing resistance that should not grow on the medium, an "ideal" selective medium should have an RGI-profile of 1.1.1.0.0.0. This cannot always be achieved because of the phenotypic and sometimes genetic flux observed in bacteria (101). However, selective media that function quite well are available for all organisms of concern (17, 27, 99).

In addition to the intrinsic shortcomings of ready-to-use media, unfortunately errors in the composition or preparation occur frequently. These errors can likewise be easily detected by the use of the ecometric technique (102).

The performance of liquid media can be monitored by adding agar at a level of 15 g/L and carrying out ecometric testing on the solidified systems. An attractive alternative is to use Pasteur's dilution-to-extinction method as currently applied in most probable number (MPN) testing (28, 103, 104).

Precautions to be Taken When Examining Foods for Pathogenic Agents

Specifications for food products often include the requirement that "pathogenic agents should be absent." Such a specification, however, is inaccurate, arbitrary, and illogical.

Qualification

To begin with, the statement is equivocal because it does

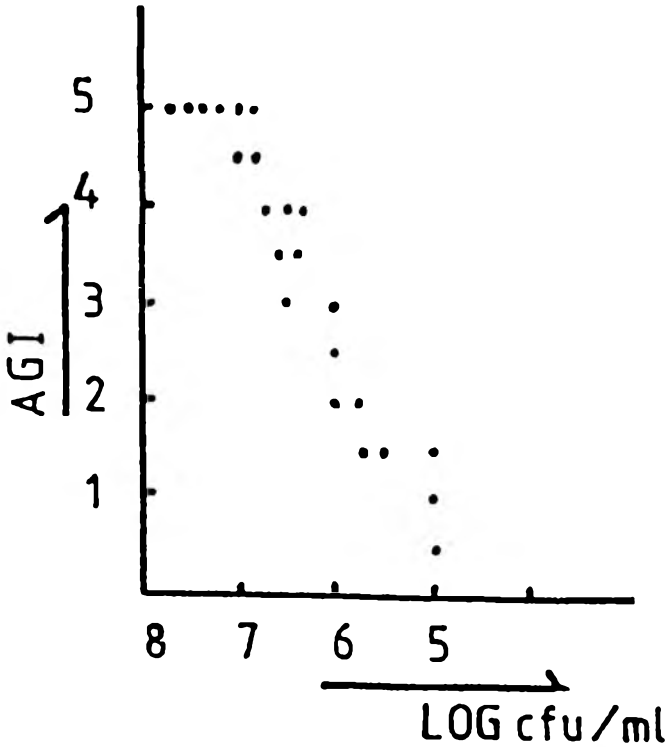

Figure 4. Relation between the number of cfu in the inoculum used in ecometric evaluation of media (log_{10} cfu · ml^{-1}) and the reading of absolute growth index (AGI).

not define the pathogens concerned. Certainly, it cannot mean absence of all agents of disease which can be transmitted by food, because there are at least 35 such organisms (Table 11). Even if the statement refers to the 10 or so major pathogens, it still makes no sense because they are not normally a hazard in all foods.

Because of this, *Salmonella* spp., *Campylobacter jejuni*, *S. aureus*, and (in Latin countries especially) *Clostridium perfringens* are generally included. The recent alarm about food-transmitted listeriosis (105) suggests that *L. monocytogenes* should be added to the list.

Will *Clostridium botulinum* and its toxins soon also be targets? A dairy product containing underprocessed hazelnut paste caused an outbreak of botulism in the United Kingdom (106, 107) and the psychrotrophic types constitute a potential hazard in meals distributed under refrigeration. The same also applies to *Yersinia enterocolitica* (108). And what about the replicating pathogenic agent (prion) that shares characteristics with Creutzfeldt-Jakob disease, kuru, Gerstmann-Sträussler syndrome, scrapie, and bovine spongiform encephalopathy (109–111)? The data presented in Table 11 provide some guidance in this respect.

Quantification

The second problem is how "absence" is to be interpreted. Calculations by Foster (112), summarized in Table 12, illustrate what a negative result for a test on food samples means in terms of absence of pathogens in the whole batch. Therefore, as emphasized by Bray et al. (113), unless an accurate definition of a sampling plan is given, a requirement defined as "absence" is meaningless.

In addition, the term "absence" should be replaced by "failure to detect." Even the methods available for the demonstration of the presence in food products of the genus

Table 11. Epidemiological guidance for selection of pathogenic target organisms in the microbiological monitoring of foods

Pathogen	Major food source
Salmonella spp.	Raw foods of animal origin and heated foods recontaminated by these raw foods
Campylobacter spp.	
Enterovirulent *E. coli* & particular *Yers. enterocolitica* types	
Shigella spp.	
Vibrio spp.	Fecally contaminated handled foods
Aeromonas hydrophila	Raw seafood
Plesiomonas shigelloides	
Brucella melitensis	Cheese made from raw milk
Listeria monocytogenes	Vegetables, soft cheeses, recontaminated processed meat and poultry products, seafood
Staph. aureus	Cream, pastries, cured meat products
Strept. pyogenes	Handled foods contaminated off human respiratory tract
Clostridium botulinum	Heat preserved foods, novel foods insufficiently monitored during processing
Clostridium perfringens, *Bacillus cereus*, and a few related species	Large masses of heated foods which have been temperature-abused
Coxiella burnetii	Cheese made from raw milk
Small round structured viruses, Hepatitis A and E viruses, Enteroviruses 40/41	Fecally contaminated handled foods
Trichinella spiralis	Raw meats
Taenia spp.	
Diphyllobothrium latum	Raw fish
Anisakis marina	
Capillaria philippinensis	
Echinococcus spp.	Fecally contaminated foods of animal and vegetable origin
Ascaris spp.	
Toxoplasma gondii	Raw meats
Entamoeba histolytica	Fecally contaminated foods of vegetable origin
Cryptosporidium parvum	

Salmonella, a pathogen that has been studied intensively for 40 years, are far from perfect, as demonstrated by our data in Tables 13 and 14 (74, 78, 79, 114–117). Methods for the detection of *B. cereus* (118) and *S. aureus* are more developed (94, 119). On the other hand, detection methods for almost all other bacterial pathogens transmitted by food are hardly beyond the primary development stage (17–27). Methods for analytical food virology are almost nonexistent.

Table 12. Reliability of negative results obtained in the examination of foods processed for safety for enteric pathogens[a]

Total g sample material examined and found negative	Upper limit of 5% one-sided confidence interval, cfu/10^3 g
1	3,000
2	1,500
10	300
20	150
50	60
100	30
1,000	3

[a] From Foster (112).

Table 13. Recovery expressed as "titer" (= negative logarithm of the highest decimal dilution confirmed +ve) of *Salmonella* serotypes in various enrichment media and at 2 temperatures[a]

Serotypes	37°C			43°C		
	TSB	R100	LICNR	TSB	R40	MK
Brandenburg	9	9	9	9	8	5•
Derby	9	9	9	8	ND	6
Dublin	9	10	8	9	5•	5•
Eastbourne	9	9	9	9	9	4•
Hadar	9	9	8	9	8	7
Heidelberg	9	8	8	9	8	5•
Indiana	9	8	9	8	7	4•
London	9	9	8	9	8	3•
London L+	9	9	9	9	ND	5•
Panama	9	9	9	9	8	5•
Petra L+	8	9	9	9	5•	3•
Typhimurium 1	8	5•	8	8	3•	<3•
Typhimurium 2	8	9	9	9	5•	4•
Typhimurium 3	8	8	8	8	7	3•

[a] • Titers ≤ 5 indicate no growth in the medium (103).
TSB = buffered glucose tryptone soya peptone broth (Oxoid)
R100 = medium of Rappaport–Vasilliadis, formula 100
R40 = ditto, formula 40 (*J. Appl. Bacteriol.* **54**, 69–76 (1983))
LICNR = lysine iron cystine neutral red medium of Morgan Jones (1982). In *Isolation and Identification Methods for Food Poisoning Organisms*, Corry, J. E. L., and Skinner, F. A. (Eds), Academic Press, London, pp. 83–90.
MK = Muller-Kauffmann broth (Oxoid)
ND = not determined

To summarize, when specifications require that food samples be tested for pathogens, careful and expert selection of organisms and exact standardization of methodology are necessary. Even so, negative results must be interpreted with great care.

Assessment of Numerical Values for Organisms of Concern

As emphasized above, reference values for particular organisms should not be set arbitrarily, but should be derived from careful experimental surveys borrowed from internal medicine (3).

Table 14. Effect of intrinsic colonization of various foods on the recovery of added *Salmonella*,[a] using the Rappaport–Vasilliadis enrichment procedure[b]

Type of food	No. of assays	Positive for *Salmonella*	
		No.	%
Control	80	74	93
Egg yolk	20	19	95
Meat, poultry, egg salads	20	19	95
Cream pastries	20	18	90
Shrimp, cooked/frozen	10	8	80
Filet americain	20	14	70
Minced meat	20	11	55

[a] *Salmonella*, 0.2 g test capsule containing ca 25 cfu *S. typhimurium*, g^{-1} carrier substance.
[b] R40 (see Table 13); incubation at 43°C, secondary to overnight pre-enrichment.

The Surveys Proper

These surveys should be carried out on adequate numbers of samples taken from food industries or catering establishments *which are known to employ* GMD (120–130). As a first step, the relevant details of the manufacturing processes must be checked, with special attention to critical sites, segments, and practices. This involves visual inspection, instrumental measurements, and bacteriological spot tests. Where necessary, deficiencies are to be corrected and the effectiveness of such changes is to be confirmed *before* samples are taken.

Examination of samples thus obtained should rely on validated methods, as explained above. Sometimes time-temperature challenging should be applied, taking into account the natural condition and mode of distribution of the relevant

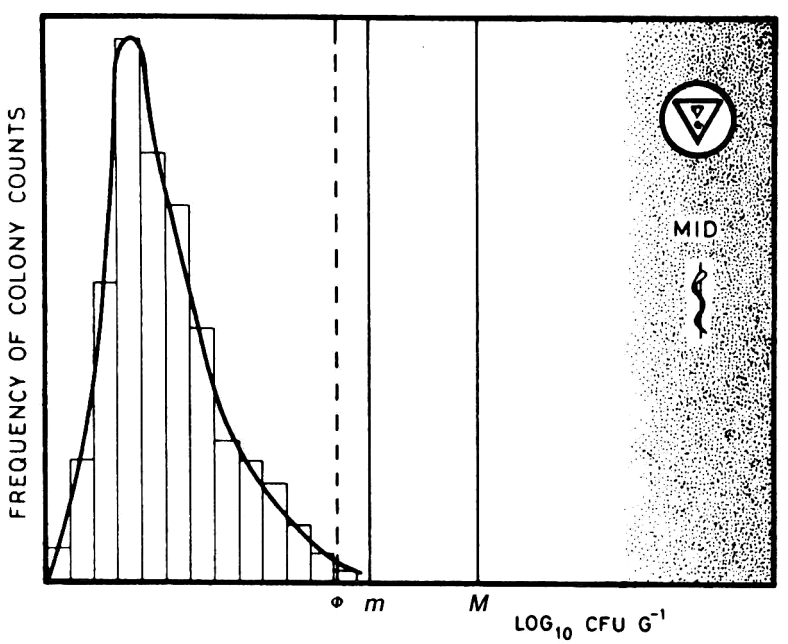

Figure 5. Distribution plot of the results of a bacteriological survey of ca 100 samples of a particular food, all drawn from manufacturing operations previously inspected for adherence to GMP and found to comply. ϕ, 95th percentile; *m*, target value; *M*, maximum cfu count, i.e., not to be exceeded; MID, minimal infectious dose of microorganism whose distribution was determined.

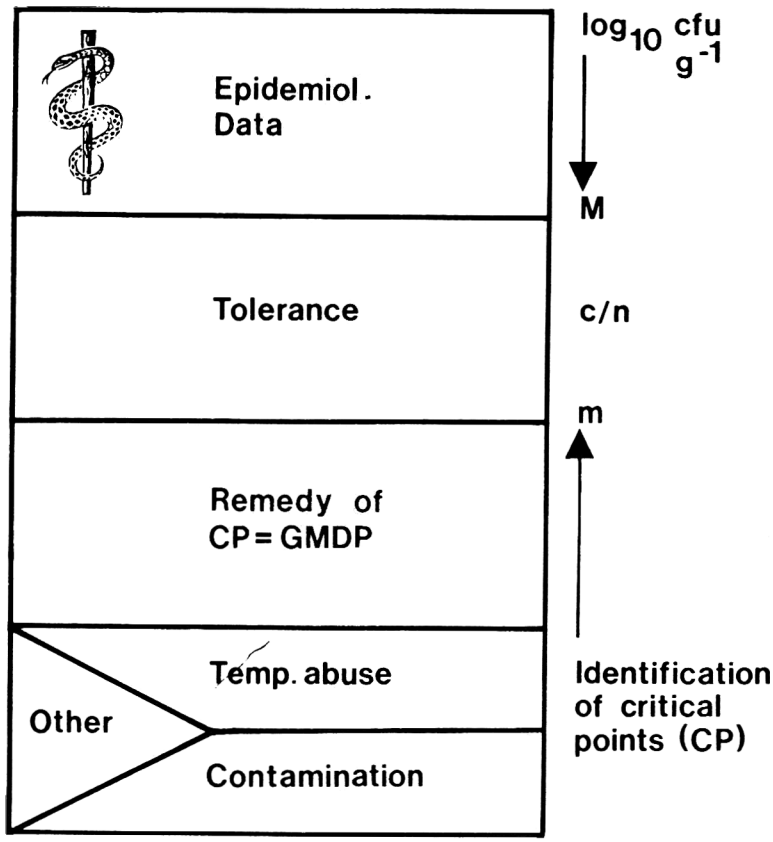

Figure 6. Decision tree for "target" or reference values.

organisms in the food under study. The results of the final examination are plotted as a cfu-frequency distribution (Figure 5). This procedure determines the lowest technologically attainable and maintainable colonization levels (131); whether they ensure adequate consumer protection is yet to be validated.

Treatment of Data

For nonpathogenic organisms, the target value *m* is situated somewhat above the 95th percentile of the distribution curve. The value *M* is the limit of acceptable quality (113, 132), defined as the maximum count ever expected under conditions of validated GMD. Obviously *M* remains far below levels of colonization at which illness upon ingestion or spoilage is unavoidable, i.e., the progressively shaded area in Figure 5. If too many samples exceed *m*, or one sample shows a count above *M*, the manufacturing and distribution practices must be considered deficient, despite the fact that management of the critical points had previously appeared satisfactory. In this case, technological improvements are required. The effectiveness of such improvements must be checked by assessment and evaluation of the prevailing cfu-frequency distribution relying on the strategy outlined in Figure 6.

The area between *m* and *M* in Figure 5 is the range of concern. As indicated above, no more than a certain fraction of samples (c/n) should fall into this area. The acceptable size of this range is determined by (*i*) the limits within which the food processing system can be controlled; (*ii*) the anticipated effect of post-process conditions (transport, storage, and distribution) on the microbial flora; (*iii*) the effect of normal kitchen preparation on the microbial community structure; (*iv*) the effect on the most sensitive groups of consumers likely to eat the product; (*v*) the degree of stratification of colonization in a food; and (*vi*) the confidence limit of the microbiological examination method used, including the sampling and dilution method, the diluent, and the method of enumeration, which may be very wide, as shown by Table 15. Hence M/m may vary from as little as 3 to as much as 10^2. A good average is about 10. Tolerances are usually expressed as c/n = 0.2.

Inspection procedures relying on the above approach are generally termed 3-class acceptance-or-rejection procedures.

The Fallacy of Zero Tolerance

At first sight it seems unjustified, if not irresponsible, to treat target values for pathogens in the same way as those for marker and spoilage organisms. From a closer view, it can be seen that this is unavoidable.

Reference values for pathogens are generally expressed in terms of their absence (preferably, "failure to detect") in, for example, 25 g. As can be seen from Table 12, it is impossible to be certain of the "absence" of a pathogen in a consignment of food, and just as impossible to prove it! Nondetection of *Salmonella* in *x* samples of *y* (112) means that no salmonellae could be demonstrated in *xy* g, or that the established numbers of colony-forming units of this pathogen are below

Table 15. Confidence limits for analytical techniques used in food microbiology[a]

Method of examination	Interval ($\log_{10}$) at 2σ-level
Rigorously standardized colony counts	0.2–0.3
Routine colony counts	0.5–1.0
MPN determinations	2–4

[a] Data from Pierson et al. (51), Silliker et al. (52), Mossel et al. (133), and van Netten et al. (28).

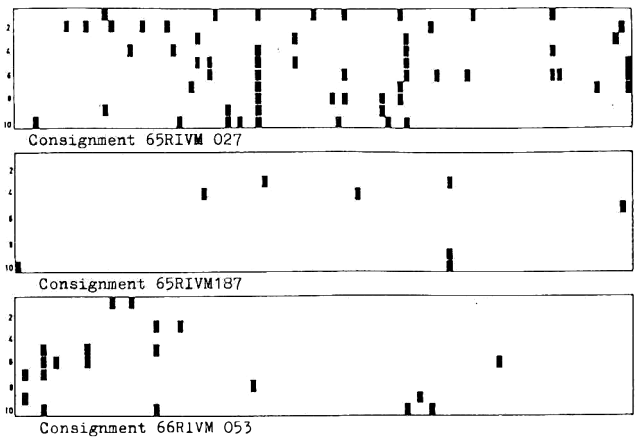

Figure 7. Analytical impact of stratified contamination of foods. Black squares represent aliquots found positive for target organism when *total* consignment would have been examined for that purpose. Aliquots of all white areas would therefore have been found negative. Experimental data from van Schothorst et al. (134).

z/kg, where $z = xy^{-1}$. The data in Table 12 illustrate that if fifty 20 g samples of a product have been examined and no pathogen has been found, it is still possible for 3 cfu/kg of these bacteria to be present in the consignment. As a matter of fact, the situation is even worse because of the severe inhomogeneity of the contamination of many foods, indicated in Figure 7.

Therefore, numerical limits must also apply to levels of pathogens in food products, whether this is perceived as unethical or not! There is no objection, however, to maintaining the concept of a 2-class acceptance-or-rejection procedure for pathogens, provided no confusion arises about the significance of negative test results leading to approval of consignments, as summarized in Figure 8.

Deciding the Fate of Batches of Food Products Which Fail Reference Values

Once reference values have been responsibly laid down and are being used in practice, the bacteriologist is then faced with the question of what to advise if samples occasionally fail to meet them. "Excessive counts" can either be more than c/n samples exceeding m but not M, or any sample exceeding M. The failure may involve pathogens or may be a high colony-count of markers or spoilage organisms.

Such decisions should not be taken lightly. In advising on what to do with the consignment from which the sample was taken, the microbiologist must be familiar with all the relevant technological, analytical, and microbiological information. Factors to be considered include (*i*) the severity of the failure, i.e., the character of the organisms encountered in excessive numbers, and the extent to which the limits are exceeded, and (*ii*) the commercial value of the commodity and the availability of alternative consignments. For example, if excessive numbers of harmless spoiler organisms are detected, it may be possible to shorten the normal shelf-life by ordering that the food be eaten within a shorter period of time. Alternatively, the food could be stored frozen instead of chilled. In other cases, e.g., when a large amount of a dried food fails to meet requirements, the most defective units could be removed or the entire consignment could be reprocessed. Another option would be to use the food (with appropriate precautions!) for animal feed. Where serious health risks have been detected, however, a consignment might have to be destroyed.

Obviously, taking such decisions should not be left to the microbiologist alone. It requires consultations with technologists and other commodity specialists, statisticians, and government and/or industrial administrators (135). Only in this way can the interests of both the consumer and producer (136) be fairly assessed. Experience has shown that where this policy has found its way into practice, it has greatly contributed to the credibility of the food microbiology profession (3).

Retrospect

The approach to the elaboration and use of reference values presented here has been used since about 1950 by the authors and some 10 senior colleagues in monitoring-for-acceptance of meat, poultry, seafood, and dairy products, freshly prepared meals, refrigerated meals of extended shelf life, drinking water, and the food and catering environments.

Where the recommended practices were faithfully followed, the previously encountered endless and, worse, fruitless debates about "standards" in general and acceptability of given consignments or food premises in particular vanished as if by magic.

Acknowledgment

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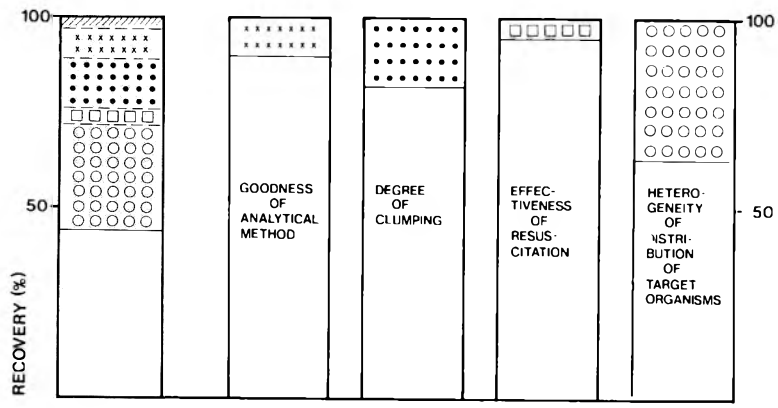

Figure 8. Theoretical histogram showing relationship between recovery of target microorganism from food sample as affected by analytical-procedural factors, illustrating discrepancy between "true" incidence and experimentally assessed level of colonization.

References

(1) Mossel, D. A. A. (1989) *Int. J. Food Microbiol.* **9,** 271-294
(2) Wilson, G. S. (1973) in *The Microbiological Safety of Food*, Hobbs, B. C., & Christian, J. H. B. (Eds), Academic Press, London, pp. xi-xii
(3) Mossel, D. A. A. (1980) in *Food and Health*, Birch, G. G., & Parker, K. J. (Eds), Applied Science Publishers, London, pp. 146-150
(4) Law, B. J., Urias, B. A., Lertzman, J., Robson, D., & Romance, C. E. (1989) *J. Clin. Microbiol.* **27,** 1560-1566
(5) Habraken, C. J. M., Mossel, D. A. A., & van den Reek, S. (1986) *Neth. Milk Dairy J.* **40,** 99-116
(6) Silliker, J. H., Baird-Parker, A. C., Bryan, F. L., Christian, J. H. B., Roberts, T. A., & Tompkin, R. B. (Eds) (1988) *Microorganisms in Foods. 4. Application of the Hazard Analysis Critical Control Point (HAACP) System to Ensure Microbiological Safety and Quality*, Blackwell, Oxford
(7) Garrett, E. S. (1988) *Food Technol.* **42**(3), 90-93
(8) Mossel, D. A. A., Jaisli, F. K., Visser, I. J. F., & Smeenk, I. (1989) in *Food Science: Basic Research for Technological Progress*, J. P. Roozen, F. M. Rombouts, & A. G. J. Voragen (Eds), Pudoc, Wageningen, The Netherlands, pp. 29-58
(9) Scully, R. E., McNeely, B. U., & Galdabini, J. J. (1978) *N. Engl. J. Med.* **298,** 34-45
(10) Harris, E. K. (1981) *Progr. Clin. Pathol.* **8,** 45-66
(11) Rifkind, B. M., & Segal, P. (1983) *J. Am. Med. Assoc.* **250,** 1869-1872
(12) Silver, H. J. (1984) *J. Am. Med. Assoc.* **252,** 826
(13) Lave, L. B. (Ed.) (1987) *Risk Assessment and Management*, Plenum Press, New York
(14) Briskey, E. J. (1976) *Food Technol.* **30**(5), 38-45
(15) Buyens, H. (1971) *Quart. Bull. Assoc. Food Drug Off. U.S.* **35,** 276-284
(16) Mossel, D. A. A. (1983) In *Food Microbiology: Advances and Prospects*, Roberts, T. A., & Skinner, F. A. (Eds), Academic Press, London, pp. 1-45
(17) Mossel, D. A. A. (1987) *Microbiol. Aliments. Nutr.* **5,** 267-282
(18) Schardinger, F. (1892) *Wien. Klin. Wochenschr.* **5,** 403-405; 421-423
(19) Smith, T. (1895) *Am. J. Med. Sci. New Series* **110,** 283-302
(20) McCrady, M. H., & Langevin, E. (1932) *J. Dairy Sci.* **15,** 321-329
(21) Swenarton, J. C. (1927) *J. Bacteriol.* **13,** 419-429
(22) Mossel, D. A. A. (1967) *J. Assoc. Off. Anal. Chem.* **50,** 91-104
(23) Hunter, A. C. (1939) *Food Res.* **4,** 531-538
(24) Mercuri, A. J., Cox, N. A., Carson, M. O., & Tanner, D. A. (1978) *J. Food Prot.* **41,** 427-428
(25) Mossel, D. A. A. (1982) *Antonie van Leeuwenhoek J. Microbiol. Serol.* **48,** 609-611
(26) Mettler, A. E. (1989) *J. Soc. Dairy Technol.* **42,** 48-55
(27) Mossel, D. A. A. (1987) In *Application of Biotechnology to the Rapid Diagnosis of Infectious Diseases*, Ballows, A. J. (Ed.), Royal Society Medical Congress & Symposium Series nr. 113, London, pp. 45-73
(28) Van Netten, P., Perales, I., Van de Moosdijk, A., Curtis, G. D. W., & Mossel, D. A. A. (1989) *Int. J. Food Microbiol.* **9,** 299-316
(29) Havelaar, A. H., & Pot-Hogeboom, W. M. (1988) *Water Sci. Technol.* **20,** 399-407
(30) Banks, J. G., & Board, R. G. (1983) *J. Hyg.* **90,** 213-223
(31) Smulders, F. J. M., Barendsen, P., Logtestijn, J. G. van, Mossel, D. A. A., & Van der Marel, G. M. (1986) *J. Food Technol.* **21,** 419-436
(32) Grabow, W. O. K., Gaus-Muller, V., Prozesky, O. W., & Deinhardt, F. (1983) *Appl. Environ. Microbiol.* **46,** 619-624
(33) Mundt, J. O. (1976) *J. Milk Food Technol.* **39,** 413-416
(34) Berg, G., & Berman, D. (1980) *Appl. Environ. Microbiol.* **39,** 361-368
(35) Seeliger, H. P. R. (1952) *Milchwissenschaft* **7,** 389-394
(36) Henriksen, S. D. (1955) *Acta Pathol. Microbiol. Scand.* **36,** 87-95
(37) Habs, H., & Langeloh, H. (1958) *Arch. Hyg. Bakteriol.* **142,** 401-414
(38) Kretzchmar, W. (1959) *Z. Gesamte Hyg. Ihre Grenzgeb.* **5,** 73-91
(39) Bonde, G. J. (1966) *Health Lab. Sci.* **3,** 124-128
(40) Clark, J. A., Burger, C. A., & Sabatinios, L. E. (1982) *Can. J. Microbiol.* **28,** 1002-1013
(41) Sayler, G. S., Nelson, J. D., Justice, A., & Colwell, R. R. (1975) *Appl. Microbiol.* **30,** 625-638
(42) Gavini, F., Leclerc, H., & Mossel, D. A. A. (1985) *Syst. Appl. Microbiol.* **6,** 312-318
(43) Hopton, J. W. (1958) *J. Gen. Microbiol.* **19,** 507-516
(44) Lupo, L., Strickland, E., Dufour, A., & Cabelli, V. (1977) *Health Lab. Sci.* **14,** 117-121
(45) Austin, B., Hussung, D., Weiner, R. M., & Colwell, R. R. (1981) *J. Appl. Bacteriol.* **51,** 101-112
(46) Evans, T. M., LeChevalier, M. W., Waarvick, C. E., & Siedler, R. J. (1981) *Appl. Environ. Microbiol.* **41,** 657-663
(47) Standridge, J. H., & Delfino, J. J. (1982) *Appl. Environ. Microbiol.* **44,** 1001-1003
(48) LeChevalier, M. W., Cameron, S. C., & McFeters, G. A. (1983) *Appl. Environ. Microbiol.* **45,** 1126-1128
(49) Lindberg, R. B. (1960) *Ann. Inst. Pasteur Lille* **11,** 77-82
(50) Jones, G. A., Gibson, D. L., & Cheng, K. J. (1967) *Can. J. Publ. Health* **58,** 257-264
(51) Pierson, C. J., Emswiler, B. S., & Kotula, A. W. (1978) *J. Food Prot.* **41,** 263-266
(52) Silliker, J. H., Gabis, D. A., & May, A. (1979) *J. Food Prot.* **42,** 638-644
(53) Peterkin, P. I., Conley, D., Foster, R., Lachapelle, G., Milling, M., Purvis, U., Sharpe, A. N., & Malcolm, S. (1989) *Food Microbiol.* **6,** 79-84
(54) Peterz, M., & Norberg, P. (1986) *Int. J. Food Microbiol.* **3,** 161-166
(55) Paille, D., Hackney, C., Reily, L., Cole, M., & Kilgen, M. (1987) *J. Food Prot.* **50,** 545-549
(56) Marier, R., Wells, J. G., Swanson, R. C., Callahan, W., & Mehlman, I. J. (1973) *Lancet* **ii,** 1367-1378
(57) Elliot, J. A. (1978) *J. Dairy Sci.* **61,** 1192-1195
(58) Collins-Thompson, D. L., Erdman, I. E., Milling, M. E., Burgener, D. M., Purvis, V. T., Loit, A., & Coulter, R. M. (1977) *J. Food Prot.* **40,** 411-414
(59) Mossel, D. A. A. (1974) *Lancet* **i,** 173
(60) Mossel, D. A. A., Visser, M., & Cornelissen, A. M. R. (1963) *J. Appl. Bacteriol.* **26,** 444-452
(61) Drion, E. F., & Mossel, D. A. A. (1977) *J. Hyg.* **78,** 301-324
(62) Van Netten, P., Perales, L, & Mossel, D. A. A. (1988) *Lett. Appl. Microbiol.* **7,** 17-21
(63) Miskimin, D. K., Berkowitz, K. A., Solberg, M., Riha, W. E., Franke, W. C., Buchanan, R. L., & O'Leary, V. (1976) *J. Food Sci.* **41,** 1001-1006
(64) Goldmintz, D., Hale, M. B., Sidwell, V. D., & Willis, B. D. (1978) *Develop. Industrial Microbiol.* **19,** 367-376
(65) Splittstoesser, D. F. (1983) *Food Technol.* **37**(6), 105-106
(66) Aroujo, R. M., Arribas, R. M., Lucena, F., & Pares, R. (1989) *J. Appl. Bacteriol.* **67,** 213-217
(67) Cook, D. W., & Ruple, A. D. (1989) *J. Food Prot.* **52,** 343-349
(68) Herbold, K., Flehmig, B., & Botzenhart, K. (1989) *Appl. Environ. Microbiol.* **55,** 2949-2953
(69) Grunnet, K., & Nielsen, B. B. (1969) *Appl. Microbiol.* **18,** 985-990
(70) Van Schothorst, M., & Oosterom, J. (1984) *Antonie van Leeuwenhoek J. Microbiol. Serol.* **50,** 1-6
(71) Stadhouders, J., Hup, G., & Hassing, F. (1982) *Neth. Milk Dairy J.* **36,** 231-260
(72) Beckers, H. J., Van Leusden, F. M., Meijssen, M. J. M., & Kampelmacher, E. H. (1985) *J. Appl. Bacteriol.* **59,** 507-512
(73) Peterz, M., & Norberg, P. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 1510-1513
(74) Van de Moosdijk, A., Mossel, D. A. A., & Van Netten, P. (1989) *J. Appl. Bacteriol.* **67,** xviii
(75) Mossel, D. A. A. (1991) *J. Assoc. Off. Anal. Chem.* **74,** 1-13
(76) Mossel, D. A. A., & Van Netten, P. (1984) In *The Revival of Injured Microbes*, M. H. E. Andrew & A. D. Russell (Eds), Academic Press, London, pp. 329-369
(77) Ray, B. (Ed.) (1989) *Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water, and Feeds*, CRC Press, Inc., Boca Raton, FL
(78) Mossel, D. A. A., Bonants-Van Laarhoven, T. M. G., Ligtenberg-Merkus, A. M. Th., & Werdler, M. E. B. (1983) *J. Appl. Bacteriol.* **54,** 313-327
(79) Mossel, D. A. A., Van Netten, P., & Van der Zee, H. (1983)

In *Les Bacilles a Gram Negatif d'Interet Medical et en Sante Publique: Taxonomie-Identification-Applications*, Leclerc, H. (Ed.), Colloques INSERM, Paris, **114,** 629–663
(80) Simon, E. M., Stahl, K. L., & Wilson, J. B. (1963) *Appl. Microbiol.* **11,** 371–376
(81) Sorrels, K. M., Speck, M. L., & Warren, J. A. (1970) *Appl. Microbiol.* **19,** 39–43
(82) Fung, D. Y. C., & Van der Bosch, L. L. (1975) *J. Milk Food Technol.* **38,** 212–218
(83) Heckly, R. J., & Blank, H. (1980) *Appl. Environ. Microbiol.* **39,** 541–543
(84) Smith, J. L., Bencivengo, M. M., & Buchanan, R. L. (1984) *J. Food Safety* **6,** 203–209
(85) LeChevalier, M. W., Singh, A., Schiemann, D. A., & McFeters, G. A. (1985) *Appl. Environ. Microbiol.* **50,** 412–419
(86) Singh, A., Yeager, R., & McFeters, G. A. (1986) *Appl. Environ. Microbiol.* **52,** 832–837
(87) LeChevalier, M. W., Schiemann, D. A., & McFeters, G. A. (1987) *Appl. Environ. Microbiol.* **53,** 1358–1364
(88) Singh, A., & McFeters, G. A. (1987) *Appl. Environ. Microbiol.* **53,** 1768–1774
(89) Walsh, S. M., & Bissonnette, G. K. (1987) *Can. J. Microbiol.* **33,** 1091–1096
(90) Van Netten, P., Zee, H. van der, & Mossel, D. A. A. (1984) *J. Appl. Bacteriol.* **57,** 169–173
(91) Dallmier, A. W., & Martin, S. E. (1988) *Appl. Environ. Microbiol.* **54,** 581–582
(92) Goldon, D. A., Beuchat, L. R., & Brackett, R. E. (1988) *Appl. Environ. Microbiol.* **54,** 1451–1456
(93) Northolt, M. D., Stadhouders, J., & Van Asseldonk, W. (1985) *Neth. Milk Dairy J.* **39,** 49–55
(94) Van Netten, P., Ven, A. van de, Perales, I., & Mossel, D. A. A. (1988) *Int. J. Food Microbiol.* **6,** 187–198
(95) Lammerding, A. M., & Doyle, M. P. (1989) *Int. J. Food Microbiol.* **9,** 249–268
(96) Read, R. B. (1979) *J. Food Prot.* **42,** 368–369
(97) Andrews, W. H., & Ray, B. (1989) In *Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds*, Ray, B. (Ed.), CRC Press, Boca Raton, FL, pp. 217–223
(98) Baird, R. M., Barnes, L. M., Corry, J. E. L., Curtis, G. D. W., & Mackey, B. M. (Ed.) (1985) *Int. J. Food Microbiol.* **2,** 1–136
(99) Baird, R. M., Corry, J. E. L., & Curtis, G. D. W. (Eds) (1987) *Int. J. Food Microbiol.* **5,** 187–299
(100) Mossel, D. A. A. (1971) In *Effects of Sterilization on Components in Nutrient Media*, Miscellaneous Paper Series nr. 9, Agricultural University, Wageningen, The Netherlands, pp. 29–39
(101) Dijkmann, K. E. (1982) In *Quality Assurance and Quality Control of Microbiological Culture Media*, Corry, J. E. L. (Ed.), G. I. T. Verlag, Darmstadt, Germany, pp. 175–180
(102) Corry, J. E. L., Leclerc, H., Mossel, D. A. A., Skovgaard, N., Terplan, G., & Van Netten, P. (1986) *Int. J. Food Microbiol.* **3,** 109–120
(103) Mossel, D. A. A., Harrewijn, G. A., & Nesselrooij-Van Zadelhof, C. F. M. (1974) *Health Lab. Sci.* **11,** 260–267
(104) Richard, N. (1982) In *Quality Assurance and Quality Control of Microbiological Culture Media*, Corry, J. E. L. (Ed.), G. I. T. Verlag, Darmstadt, Germany, pp. 51–57
(105) Kampelmacher, E. H., & Mossel, D. A. A. (1989) *Culture* **10**(1), 1–4
(106) Beecham, L. (1989) *Br. Med. J.* **298,** 1669
(107) Critchley, E. M. R., Hayes, P. J., & Isaacs, P. E. T. (1989) *Lancet* **ii,** 849
(108) Mossel, D. A. A., & Thomas, G. (1988) *Microbiol. Aliments Nutr.* **6,** 289–309
(109) Burdon, D. W. (1989) *J. Med. Microbiol.* **29,** 145–157
(110) Hope, J., Reekie, L. J. D., Hunter, N., Multraup, G., Beyreuther, K., White, H., Scott, A. C., Stack, M. J., Dawson, M., & Wells, G. A. H. (1988) *Nature* **336,** 390–392
(111) Collinge, J., Owen, F., Lofthouse, R., Shah, T., Harding, A. E., Poulter, M., Bourghey, A. M., & Crow, T. J. (1988) *Lancet* **ii,** 15–17
(112) Foster, E. M. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 259–266
(113) Bray, D. F., Lyon, D. A., & Burr, I. W. (1973) *Technometrics* **15,** 575–585
(114) Beckers, H. J., Van Leusden, F. M., & Peters, R. (1986) *J. Food Safety* **8,** 1–9
(115) Perales, I., & Audicana, A. (1989) *J. Food Prot.* **52,** 316–319
(116) Morinigo, M. A., Martinez-Manzanares, E., Munoz, A., Cornax, R., Romero, P., & Borrego, J. J. (1989) *J. Appl. Bacteriol.* **66,** 353–360
(117) Peterz, M., Wiberg, C., & Norberg, P. (1989) *J. Appl. Bacteriol.* **66,** 523–528
(118) Bouwer-Hertzberger, S. A., & Mossel, D. A. A. (1982) In *Isolation and Identification Methods for Food Poisoning Organisms*, Corry, J. E. L., Roberts, D., & Skinner, F. A. (Eds), Academic Press, London, pp. 165–172
(119) Isigidi, B. K., Devriese, L. A., Croegaert, T., & Van Hoof, J. (1989) *J. Appl. Bacteriol.* **66,** 379–384
(120) Leininger, H. V., Shelton, L. R., & Lewis, K. H. (1971) *Food Technol.* **25,** 224–226
(121) Surkiewicz, B. F., Johnston, R. W., Elliott, R. P., & Simmons, E. R. (1972) *Appl. Microbiol.* **23,** 515–520
(122) Surkiewicz, B. F., Harris, M. E., Elliott, R. P., Macaluso, J. F., & Strand, M. M. (1975) *Appl. Microbiol.* **29,** 331–334
(123) Surkiewicz, B. F., Johnston, R. W., & Carosella, J. M. (1976) *J. Milk Food Technol.* **39,** 7–9
(124) Surkiewicz, B. F., Campbell, B. F., & Harris, M. E. (1979) *J. Food Prot.* **42,** 46–48
(125) Splittstoesser, D. F., & Corlett, D. A. (1980) *J. Food Prot.* **43,** 717–719
(126) Duran, A. P., Wentz, B. A., Lanier, J. M., McClure, F. D., Schwab, A. H., Swartzentruber, A., Barnard, R. J., & Read, R. B. (1983) *J. Food Prot.* **46,** 974–977
(127) Wentz, B. A., Duran, A. P., Swartzentruber, A., Schwab, A. H., McClure, F. D., Archer, D., & Read, R. B. (1983) *J. Food Prot.* **48,** 44–49
(128) Schwab, A. H., Wentz, B. A., Jagow, J. A., Swartzentruber, A., Duran, A. P., Lanier, J. M., Barnard, R. J., & Read, R. B. (1985) *J. Food Prot.* **48,** 70–75
(129) Warburton, D. W., Peterkin, P. I., & Weiss, K. F. (1986) *J. Food Prot.* **49,** 229–230, 232
(130) Warburton, D. W., Weiss, K. F., Purvis, U., & Hill, R. W. (1987) *Can. Inst. Food Sci. Technol.* **21,** 84–89
(131) Pace, P. J. (1975) *J. Milk Food Technol.* **38,** 347–353
(132) Roberts, T. A., Bryan, F. L., Christian, J. H. B., Kilsby, D., Olson, J. C., & Silliker, J. H. (Eds) (1986) *Micro-organisms in Food: Sampling for Microbiological Analysis—Principles and Specific Applications*, Blackwell, Oxford
(133) Mossel, D. A. A., Veldman, A., & Eeldrink, I. (1980) *J. Appl. Bacteriol.* **49,** 405–419
(134) Van Schothorst, M., Mossel, D. A. A., & Kampelmacher, E. H. (1966) *Zentralbl. Veterinaermed.* **13,** 273–285
(135) Springer, J., & McClure, F. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 246–250
(136) Mossel, D. A. A., & Drion, E. F. (1979) in *Food Microbiology and Technology*, B. Jarvis, J. H. B. Christian, & H. D. Michener (Eds), Medicina Viva, Parma, pp. 417–430

Use of the HACCP System to Assure Food Safety

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Occasions such as the Regulatory Roundtable permit us to discuss complex issues from the perspective of the industry, the regulatory agencies, and academia. Let us hope we all will be better prepared to develop the policies and regulations that will affect the food industry.

Despite any differences in opinions that may have been expressed at the Roundtable, let there be no doubt about one fact—it is our common duty and intent to assure the safety of our food supply. Different approaches have been proposed to assure food safety. One common proposal in the past was the use of microbiological criteria or standards for finished products to indicate the absence of hazards.

We in the industry feel that such end-point regulation is not in our common best interest. It is time-consuming, costly, and relatively ineffective in assuring product safety. We feel, rather, that systems of food safety that do not rely upon end-point standards can be established to achieve this goal.

One such system is the Hazard Analysis and Critical Control Point (HACCP) system of food safety. This system, originated by The Pillsbury Co., was first presented publicly at the 1971 Conference for Food Protection. It is a proactive system of food safety that focuses attention on the use of hazard-free ingredients and process controls to assure the safety of the finished product. Safety cannot be "tested into" the finished product. It is much more effective to "design" safety into the process.

This design process is one of the central themes in "TQC" or "continuous improvement" programs that are being developed in the United States to duplicate the Japanese success at quality control. We should remember that HACCP was at work in the food industry 15 years before it became fashionable to adopt "Japanese" styles of business management. HACCP is now an idea whose time has come—and it works.

The HACCP system is relatively simple; it follows a logical sequence of events. Today it is viewed as consisting of 7 steps:

(*1*) An analysis of all the hazards potentially present in a food all the way from growth and harvesting of commodities, through production, distribution, and consumer use of the finished product. It is much more extensive than a "rehash of GMPs," as some may suggest.

(*2*) Determination of the Critical Control Points for each identified hazard. We need to recognize that HACCP systems are evolutionary; they themselves are subject to "continuous improvement." For example, we have learned in recent years that cleaning and sanitation procedures are Critical Control Points for biological hazards such as *Listeria*.

(*3*) Establishment of limits or specifications for each Critical Control Point.

(*4*) Monitoring of each Critical Control Point, including log books that must be signed by all responsible parties.

(*5*) Establishment of corrective actions that must be taken if a Critical Control Point deviates from requirements.

(*6*) Implementation of a record keeping system to document that the HACCP system is functioning according to design. This step may have important regulatory significance in the future as the regulatory agencies move toward adoption of the HACCP approach.

(*7*) Verification of the effectiveness of the HACCP system.

We in industry know that HACCP systems can be implemented to assure product safety, and we would strongly discourage any attempt to enact end-point microbiological criteria or standards for foods. It is much more effective to concentrate our attention on ingredient and process controls.

The table below lists 5 biological hazards that may be present in ingredients. One typical example of a food involved is given in each case. All progressive food companies have sensitive ingredient lists and proactively determine that their ingredients are free of these hazards. Ideally, the sensitive ingredient testing should be done by the supplier. Eventually suppliers themselves will implement and maintain their own HACCP programs.

Pathogen	Ingredient example
Salmonella spp.	dried eggs
Staphylococcus aureus	cheese (fermented)
Aflatoxin	corn meal
Bacillus cereus	cooked rice
Listeria monocytogenes	refrigerated cooked meats

Our general disdain for end-product microbiological criteria does not mean that we are automatically opposed to the use of all microbiological criteria. In addition to sensitive ingredient clearance, 3 criteria can be used to indicate the overall quality of ingredients, namely, total counts, coliforms, and *Escherichia coli*. Often these can be incorporated into a 3-class sampling plan, into which is built an automatic decision-making apparatus (which is another topic by itself).

Microbiological testing can also be employed very effectively to monitor in-process controls. The below table lists some general examples of controls that can be used in manufacturing facilities. Not all of these criteria would be used in all facilities; rather, one or more would be used as required, depending on the food being produced. For example, in fruit juice production, total counts will be a useful criterion. In a chicken-deboning operation, the most useful criterion may be

1. Monitor equipment sanitation
   Coliforms
   Total counts
2. Monitor handling procedures
   Staphylococcus aureus
   E. coli
3. Evaluate system control
   Staphylococcus aureus
   Total counts
4. Monitor environment for specific hazards
   Listeria monocytogenes

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the number of *Staphylococcus aureus* introduced by human handling. In an ice cream plant, the most important criterion would be the assurance of a production environment free of *Listeria monocytogenes*. The ice cream industry has learned through extensive experience that indicator organisms such as coliforms do not correlate with the presence of *L. monocytogenes*. There are many other uses of such in-process criteria which can be developed for specific production facilities.

The title of this Roundtable was "Microbiological Standards for Foods: Realities and Fallacies." In the view of the food industry, the reality is that microbiological criteria can be usefully applied to ingredient and process control. Attempts to establish microbiological standards for finished products are usually fallacies.

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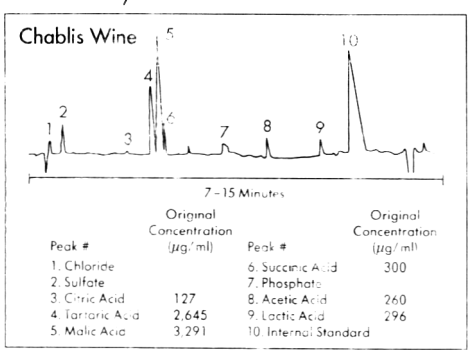

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