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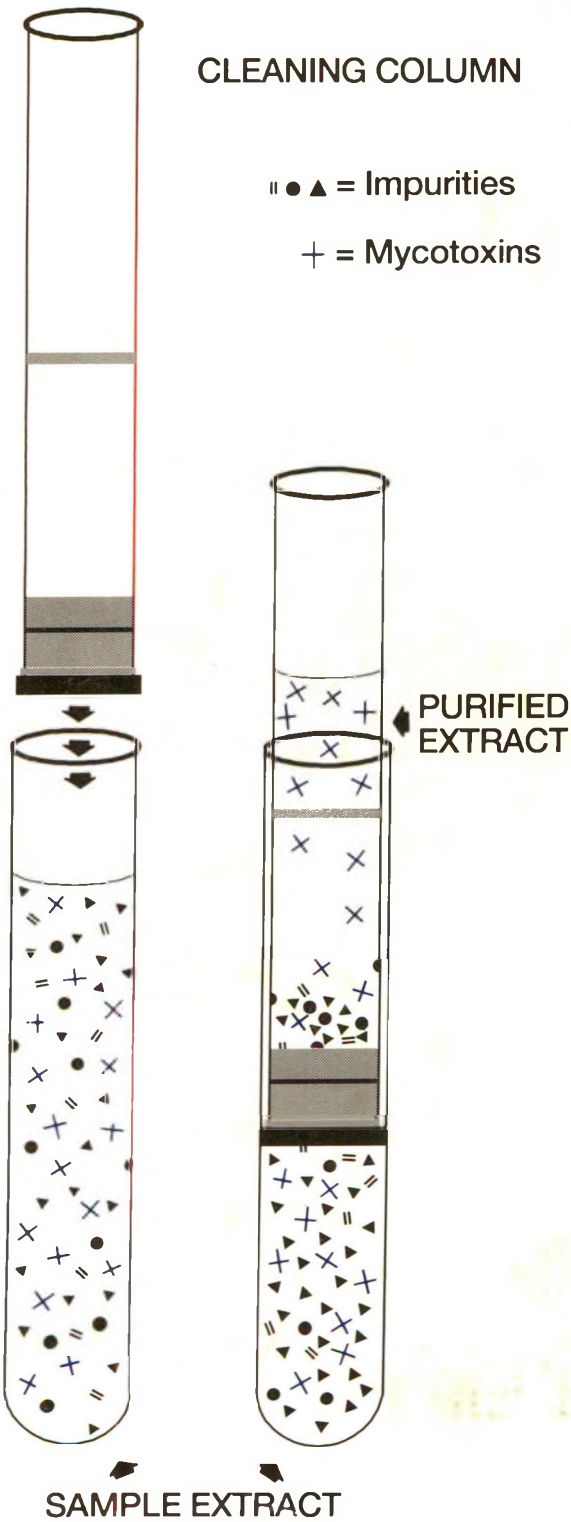

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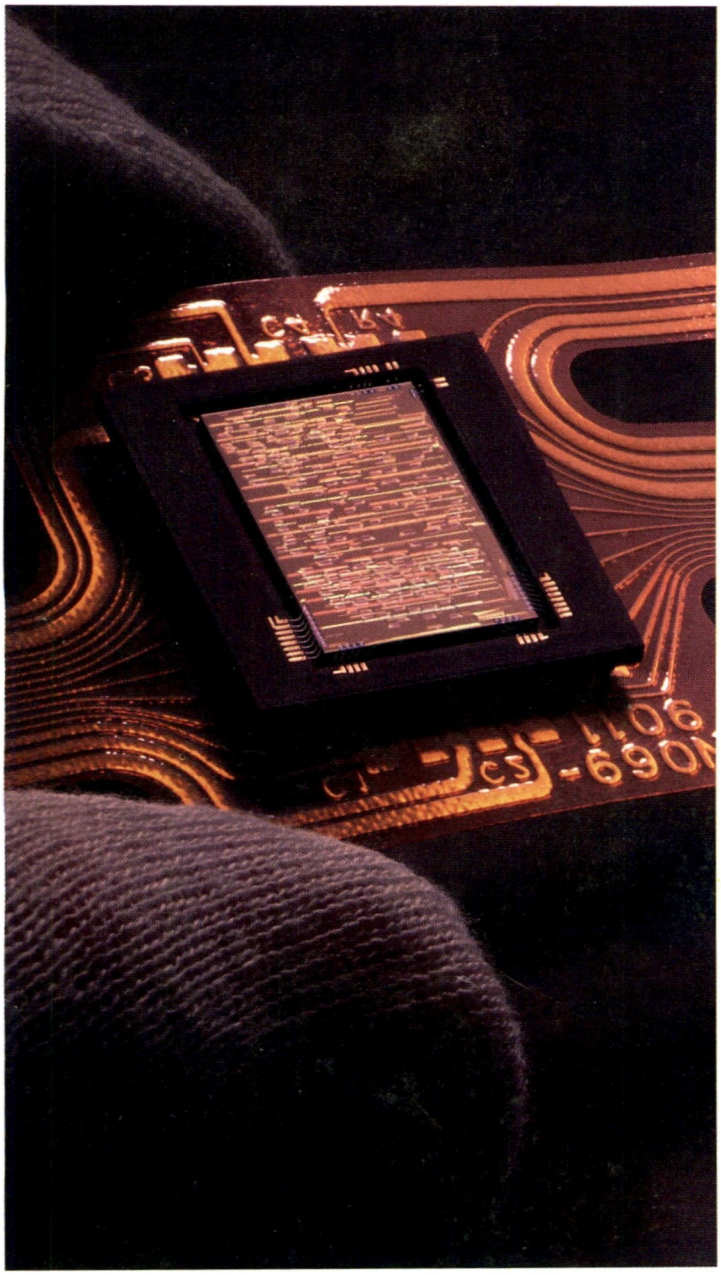

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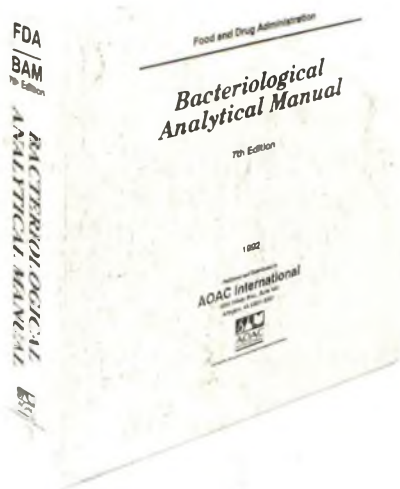

NEW EDITION!

This manual contains analytical methods to detect microorganisms and certain of their metabolic products. Although the methods are primarily for foods, the manual also contains methods for analysis of beverages and cosmetics. For use in regulatory and industry laboratories. Most chapters have been revised, expanded and updated.

Not all procedures in this manual have achieved official AOAC status through collaborative testing; but all represent the methodology currently in use in FDA laboratories.

CONTENTS

Food Sampling and Preparation of Sample Homogenate. Microscopic Examination of Foods. Aerobic Plate Count. *Escherichia coli* and the Coliform Bacteria. *Salmonella. Shigella. Campylobacter. Yersinia enterocolitica* and *Yersinia pseudotuberculosis. V. cholerae, V. parahaemolyticus, V. vulnificus* and Other *Vibrio* spp. *Lysteria monocytogenes.* Serodiagnosis of *Listeria monocytogenes. Staphylococcus aureus.* Staphylococcal Enterotoxins. *Bacillus cereus.* Diarrheagenic Enterotoxin. Clostridium perfringens. *Clostridium botulinum.* Yeasts, Molds and Mycotoxins. Parasitic Animals in Foods. Inhibitory Substances in Milk. Rapid HPLC Determination of Sulfamethazine in Milk. Examination of Canned Foods. Modification of Headspace Gas Analysis Methodology, using the SP4270 Integrator. Examination of Containers for Integrity. Microbiological Methods for Cosmetics. Identification of Foodborne Bacterial Pathogens by Gene Probes. Investigation of Food Implicated in Illness. Appendixes: Rapid Methods for Detecting Foodborne Pathogens. Most Probable Number Determination. Media and Reagents.

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TECHNICAL COMMUNICATIONS

New Products

New Catalog of Plastic Laboratory Disposables

Globe Scientific has released its 1993/1994 catalog of disposable laboratory plasticware. Included in the extensive line are over 500 routine as well as hard-to-find plastic items. the Macrotube section lists tubes from 16 mL to 200 mL capacity in polystyrene, polypropylene, or TPX. The polyethylene transfer pipets range in size from the 1 mL Mini-Pipette to the 10 mL Ten-Pette. Multipurpose screwcap or snap cap containers are available in polystyrene, polypropylene, or polyethylene in sizes form ¼ oz. to 190 oz. Globe Scientific, Inc.
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Automated Solids Extractor

The Soxtherm is a highly automated Soxhlet extraction instrument capable of processing 6 samples simultaneously in less than 3 h, and is up to 5 times faster than traditional Soxhlet extraction. Programmed parameters control each of several timed steps of extraction for a completely automated system, and it pays for itself with savings in time, labor, and solvent. ABC Laboratories.
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Variable Pressure SEM Offers Simplified Operation, High Resolution Study of Natural State Specimens

The S-2460N variable pressure SEM from Hitachi permits observation of a virtually unlimited range of specimens without requiring special specimen preparation techniques such as drying or sputter coating for conductivity. It combines the ease of use and affordability of an optical light microscope with the high resolution of conventional SEMs, requiring no special expertise or training for operation; high resolution images are facilitated with the touch of a single button. The S-2460N's low vacuum mode makes the system ideal for the study of wet, oily, and nonconductive specimens in a wide range of application areas ranging from plant biology, food processing, and pharmaceutical to textiles, semiconductors, and cosmetic. Hitachi Scientific Instruments.
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Dataplate Digital Dry Block Heaters

Three new models of digital dry block heaters feature easy-to-use, simple digital controls, digital displays, and the ability to retain 2 temperatures in memory. All units have safety alarm, shorted heater drive protection, 99 h digital timer, and auto-off function. Temperature readout is to 0.1°C or F. Model 254 accepts 4 blocks, has a temperature range to 210.0°C and comes with a 6 ft. cable for remote operation. Models 251 and 252, which accept one and 2 blocks, respectively, have a temperature range to 125.0°C. PMC Industries, Inc.
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Mystaire Compact Waterweb Scrubbers

Mystaire Compact Waterweb scrubbers remove difficult and hard to scrub gases from 1 to 2000 CFM flow rates. Energy efficient and compact. Waterweb scrubbers are ideal for many applications, including laboratory hoods, gas cabinet venting, acid digestion, chemical reaction and process vessels, and odor control. Waterweb scrubbers feature Waterweb mesh to create continuous volume of liquid, eliminating large air paths and voids. Continuously flooded passages provide nonclogging, self-cleaning operation, important in particulate scrubbing. Compact Waterweb systems are available in component form or cart

mounted for portability. Closed loop system are also available. Heat Systems.
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An Alternative to Kjeldahl

The Model 7000N nitrogen analyzer offers an alternative to Kjeldahl analyses for water and wastewater. The Pyro-chemiluminescent detection method is the same technology used in ASTM water method D5176. By eliminating the need for acid digestion and resulting waste disposal problems, the nitrogen analyzer supplies a safe, clean analytical alternative from start to finish. The accuracy and precision of Pyro-chemiluminescent nitrogen determination is unrivaled by either wet chemistry or other instrumental methods. Antek Instruments, Inc.
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Fast, Affordable Microplate Washing

The Model 1250 automated plate/strip-well microplate washer adds affordability, speed, and convenience to tedious and time consuming EIA and ELISA incubation steps in microplate analysis. This system is ready to use, and comes complete with built-in vacuum pump, and wash, rinse, and waste bottles. The immunowash automated microplate washer features a positive displacement delivery system, 15 programmable settings, 8 and 12 port interchangeable manifolds, and standby row with automatic rinse functions. Bio Rad Laboratories.
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On-Line Monitoring of Fermentation Off-Gases

A new application sheet for the Quantum 3000 mass spectrometer from Asea Brown Boveri Process Analytics describes how accurate, on-line analysis of fermentation off-gases can improve processing speed and efficiency. Fermentation is widely used in research and development and the manufacture of a variety of products, including antibiotics, yeasts, enzymes, alcohols, fragrances, and genetically engineered materials. In contrast to slower dedicated analyzers located on each fermenter, this mass spectrometer is capable of monitoring multicomponents and multiple streams at speeds of 15–20 s/sample. It also detects process abnormalities as well as determines respiratory quotient and mass balance factors to maximize precise control of the fermentation process. ABB Process Analytics, Inc.
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On-the-Spot Gas Identification Software

The Foxboro Co. has announced a gas identification software package for use with its widely accepted MIRAN 1B line of portable ambient air analyzers. The new enhancement is a spectral matching software program that makes the MIRAN 1B2 and 1BX gas analyzers capable of identifying unknown gases. The spectral identification is made by a laptop computer comparison of the gas analyzer's sampled spectrum with an onboard computer library containing approximately 400 compounds. Spectral acquisition and matching is complete within 5 to 6 min. The software package graphically overlays the sampled spectrum with the 3 most likely matches. A second screen provides a list of other matching compounds in order of similarity. The Foxboro Co.
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Water Monitor from GAR

The LAN-I water monitor accurately measures the percent of water in liquid soaps, detergents, and similar products. With the monitor, the stream operator can adjust the water content to the top concentration permitted for the particular product being produced, thereby achieving the most economical level of operation. It uses infrared absorption to measure water specifically, independently of the other components in the stream. It is, thus, unaffected by changes in the relative concentrations of the other components and it is not affected by entrapped air. General Analysis Corp.
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Cartridge HPLC Columns Match Efficiency of Conventional Column Hardware

Supelco's newly designed HPLC cartridge column system offers the same high efficiency and leak-proof integrity as conventional HPLC columns. The system, comprised of polymer-to-steel connections, reusable end-fittings, an integrated guard system, and replaceable frit assemblies, provides all the benefits associated with cartridge systems in addition to offering opportunities for long-term process improvements in HPLC column manufacturing. Supelco offers the HPLC cartridge column system in most Supelcosil phases of 150 mm or 250 mm column lengths, 4.6 mm column id. Supelco, Inc.
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Single Use Disposable Reagent Generator and Dropper Dispenser

A single use disposable reagent generator and dropper dispenser is designed for extended storage and controlled delivery of freshly prepared reagents for test kit usage. This safe, easy-to-use reagent storage and delivery system is comprised of an outer plastic tube with dropper tip and 2 internal ampules containing separate product liquids or powders. The system is activated by squeezing the outer tube and crushing the

internal ampules. This allows the 2 solutions to mix and form an active reagent that can be delivered in droplet form when and where it is needed. Newton Industries, Inc.
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Enhanced Capabilities Announced for ChromJet and DataJet Integrators

Thermo Separation Products announced improved capabilities for its line of chromatography integrators. The ChromJet and DataJet integrators will now have maximum random access memory and the company's Labnet communications as standard features. The additional RAM now totals 512K, and will allow the ChromJet integrator to easily store all chromatograms from overnight runs. These stored chromatograms can then be reintegrated and replotted on the unit's high-resolution inkjet printer/plotter singly or in batches. Other reprocessing features include baseline subtraction, recalibration, and reporting. The integrator is completely self-contained, so an external disk drive or computer is not required. Thermo Separation Products.
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Column Thermostat Lowers HPLC Analysis Costs, Improves Resolution

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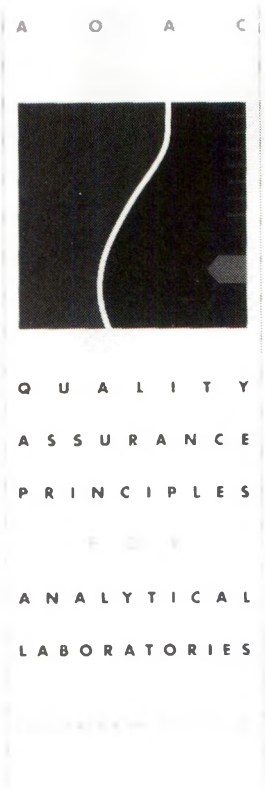

*E*ssential for any lab wanting to improve or initiate a quality assurance (QA) program. Material was extensively revised for this new edition which features new information and concepts developed since the handbook was last published. An important addition is a chapter on utilizing statistical applications and analytical control charting techniques. Appendixes, too, have been revised and one added on laboratory accreditation criteria – criteria which can be used for self-evaluation of lab QA programs and operations management procedures.

Each chapter offers recommendations for developing and operating a QA program. The book also provides solid justification for commitment of resources to a quality assurance program.

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Appendixes: Typical Contents of a Quality Manual for Testing Laboratories. Forms Used by U.S. Federal Agencies. Instrument Performance Checks. FDA Audit Measure Procedures. Proficiency and Check Sample Programs. Accreditation Criteria.

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SpectraBook Volume II, the second part of an award-winning spectral interpretation computer program, is now available from Savant Audiovisuals, Inc. This interactive software offers instruction for developing interpretative skills in infrared, proton nuclear magnetic resonance, C–13 NMR, and mass spectroscopy. The second volume, like the first, provides data and spectra on 50 organic compounds, especially selected to teach interpretation principles. Each volume contains 200 interpreted spectra in all. Savant Audiovisuals, Inc.
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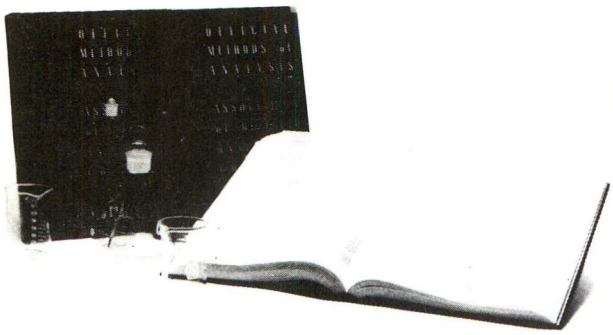

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- Spices & Other Condiments
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- Vitamins
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- Food Additives, Direct & Indirect
- Natural Poisons

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Appendixes: Standard Solutions and Certified Reference Materials, Laboratory Safety, Reference Tables. Subject and Method Number Indexes.

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Meetings

July 22–23, 1993: Fifth International Symposium on the Harmonization of Internal Quality Assurance Schemes for Analytical Laboratories, Washington, DC. Contact: George Heavner. AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

July 24–30, 1993: AOAC Short Courses, Washington, DC. Topics: QA, Microbiological QA, Lab Waste, How to Testify as Expert Witness, Statistics, Improving Technical Writing Skills, Lab Info Management Systems (A2LA), Accreditation vs Registration (A2LA). Contact: Carol Rouse, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

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

September 13–14, 1993: AOAC Central Europe Section Meeting, Prague, Czechoslovakia. Contact: Margreet Lauwaars, PO Box 153, 6720 AD, Bennekom, The Netherlands, telephone 31 8389 18725.

September 22–24, 1993: First International Food Data Base Conference Sidney, Australia. Contact: Professor Heather Greenfield, Department of Food Science and Technology, University of New South Wales, PO Box 1, Kensington 2033, Australia, telephone +61-2-697-4363.

September 26–October 1, 1993: 15th International Nutrition Conference, Adelaide, Australia. Contact: Congress Secretariat, CSIRO Division of Human Nutrition, PO Box 10041, Gouger St, Adelaide 5000, South Australia, Australia, telephone +61-8-224-1800.

October 4–9, 1993: AOAC Short Courses, Manhattan Beach, CA. Topics: QA, Microbiological QA, Statistics, Lab Waste, How to Testify as Expert Witness, Lead Abatement (A2LA), How to Design & Develop Quality Manual (A2LA), Achieving Accreditation (A2LA), Lab Occup. Safety & Health (A2LA), Lab Equipment Calibration (A2LA), NATA Lead Assessor (A2LA), Statistical Measurement Control (A2LA). Contact: Carol Rouse, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

November 3–5, 1993: AOAC Central Section Meeting, West Lafayette, IN. Contact: Hussein S. Ragheb, Purdue University, Department of Biochemistry, West Lafayette, IN 47904, telephone 317/494-1572.

February 2–3, 1994: AOAC Southeast USA Section Meeting, Atlanta, GA. Contact: Doug Hite, Technical Services, PO Box 40627 Melrose Station, Nashville, TN 37204, telephone 615/360-0317.

April 17–21, 1994: Sixth International Symposium on Biological and Environmental Reference Materials (BERM-6), Kona, HI. Contact: Wayne R. Wolf, U.S. Department of Agriculture, Nutrient Composition Laboratory, Beltsville, MD 20705, telephone 301/504-8927.

International Update

Over the last decade or so, the use of computers and computer-based systems has enveloped large segments of the analytical process. This trend has presented the analyst with new problems and challenges, according to A. Vilumara, Laboratorio Dr. Esteve, Barcelona, Spain.

Speaking at the third international symposium organized by AOAC Europe, "Good Automated Laboratory Practice Ensuring the Quality of Analytical Data," March 29–30, 1993, in Sitges, Spain, he explained that now, in addition to generating analytical data, analysts must also ensure the validity of their data. His paper gave an overview of the measures to take for doing this, from the receipt of the sample in the laboratory to the issue of the final report. Specific areas of data quality assurance were developed in detail by other symposium speakers.

When automatic or computing systems are employed in the analytical process, their use should be in accordance with the laboratory's standard operating procedures. Ensuring the quality of data necessitates certain measures and precautions when the data is being generated, captured, evaluated, secured, and stored.

At the data generation stage, the source and identity of the sample, the analyst and date of analysis, and the QC records, etc., should be properly documented and recorded.

Data capture may be manual or automatic. With automatically recorded systems, the time and date of entry of the data is important. Therefore, if corrections are required at a later stage, they will be both recognizable and traceable. Manual recording should be treated in a similar fashion and should be clearly legible.

When evaluating data and recording results, the laboratory may need to clarify what, when, and by whom the data may be corrected.

With the storage and security of data, only authorized persons should have access to the system and, in case of a system failure, a backup is required.

A paper presented by E. Ziegler, Max Plank Institut fur Kohlenforschung, Mulheim, Germany, outlined possible sources of corruption at the data capture stage, and a data system used at the Max Plank Institut fur Kohlenforschung was described. This chromatographic system consists of a microcomputer satellite for data acquisition and a multi-user host computer for data storage. COLACHROM software is used for evaluation.

"Reference Materials" for statistical software are under development at the Laboratory of the Government Chemist, UK, for validation of analytical instru-

mentation. These data sets, according to S. Ellison, Laboratory of the Government Chemist, Teddington, UK, have very carefully controlled properties, with which the accuracy of basic statistical procedures, such as calculations of means, standard deviations, and linear regression, can be checked. Using such sets, software can be tested, and analysts can determine if the best possible results are being obtained.

The validation of instrumentation was subjected to an in depth evaluation, both from the viewpoint of the manufacturer and user. Instrument manufacturers are conscious of the changing requirements of their customers. These requirements are largely driven by the constraints of GLP, ISO 9000, accreditation, etc. Manufacturers are adapting to meet this changing need, but as individual pieces of laboratory instrumentation are used for a variety of tasks, the manufacturer cannot supply a "validated instrument." Validation of the complete analytical process is, therefore, the responsibility of the user.

Two speakers illustrated the practical aspects of the symposium's theme by demonstrating how they approach the data quality issue in their respective work situations. C. Bachmann, Centre Hopitalier Universitaire Vaudois, Lausanne, Switzerland, provided an example that involved automatic analysis in a hospital environment in which "just in time" analysis and the interpretation of analytical data was emphasized. The day-to-day analytical variability of the hospital's automated system is incorporated into the analytical report enabling medical teams to interpret the data in a meaningful fashion and to accurately monitor both the patient's condition and response to treatment, he explained.

The second example provided by S. Piepponen, Technical Research Centre of Finland, Espoo, Finland, involved the ICP-AES automatic analysis of metals in hydrocarbon oil samples. This presentation centered around the QC of the automated system and the measures to take for ensuring the quality of data.

Next, Dr. Dirscherol of the European Communities, Division DG XII C-5, spoke about research and development programs aimed at improving the quality of measurement within the Community. Community research and development is carried out under the umbrella of the Framework Programs. The third program will terminate at the end of 1994, and the fourth program is scheduled to commence at the beginning of 1994 and to run for 4 years.

The measurement and testing part of the program is currently active in the biological, chemical, metrological, and physical fields. The measurement and testing program for 1994–1998 will be centered around: improving the competitive position of European industry; technically supporting the development and application of European Community policy (directives and regulations); undertaking prenormative research in support of European Community policy and measurements related to the needs of society in areas of health, safety, and trade; and providing an infrastructure that will ensure comparability and traceability in all areas of European Community measurement and testing.

The final paper gave a glimpse into the year 2000. In the future, according to W. Wegscheider, Technische Universitat Graz, Austria, analysis will be more biologically orientated, more automated, and limits of detection will decrease; improvements in "on-line" equipment and enhanced versatility will mean that much routine analysis currently carried out in a central facility will be performed on site; improved communications between the user of analytical results and the generator of analytical data, coupled to a more realistic approach to the importance of sampling and the purpose for which the analysis was requested, should lead to a reduction in sample throughput; and an increasing emphasis on traceability in measurement will place more relevancy on the purpose for which the measurement is being carried out.

AOAC Board Approves Formation of Technical Division on Reference Materials

The AOAC Board of Directors, at its March 23, 1993, meeting, approved a petition to form an AOAC Technical Division on Reference Materials. The Division evolved to address AOAC INTERNATIONAL's expanded mission statement "to promote quality measurements and method validation in the analytical sciences."

The issue of data reproducibility has been the previous focus of the AOAC Official Method Program. An expanded scope to make statements of accuracy on AOAC methods requires one very important extra step—independent verification of the accuracy of measurements generated by the method. Accuracy of an analytical method must be verified at three separate points:

— When the method is first developed and collaboratively validated. Can it generate the correct (accurate) number?

— When the method is implemented in a different laboratory (after becoming an AOAC Official Method). Can it generate the correct (accurate) number under different use conditions or by a different analyst?

— When the method is used routinely. Can it generate the correct (accurate) number each time it is used?

The petition outlined goals for the new division as follows:

- Promote policy changes to incorporate reference materials in AOAC methods.
- Identify and facilitate availability of reference materials needed.

■ Plan and conduct educational activities on use of reference materials in AOAC.

■ Provide pool/network/resource of experts in reference material areas.

Two specific proposed projects already under way include setting up a mechanism to provide appropriate reference materials for collaborative studies and routine use of validated methods for analytical measurements in response to the U.S. Nutrition Labeling and Education Act; and planning and organizing the international symposium series on Biological and Environmental Reference Materials (BERM). BERM-6 is planned for April 17–21, 1994, in Kona, HI.

Interim Executive Committee members, serving until annual elections during the AOAC INTERNATIONAL annual meeting in July, are:

Chairman: Wayne R. Wolf, Nutrient Composition Laboratory, U.S. Department of Agriculture, 10300 Baltimore Ave, Beltsville, MD 20705-2350; telephone +1 (301) 504-8927, fax +1 (301) 504-8314

Vice Chairman: James T. Tanner, Office of Special Nutritionals, U.S. Food and Drug Administration, 200 C St, SW, Washington, DC 20204; telephone +1 (202) 205-5364, fax +1 (202) 205-4594

Secretary: Jonathan DeVries, General Mills, Inc., 9000 Plymouth Ave, Minneapolis, MN 55427; telephone +1 (612) 540-2774, fax +1 (612) 540-7487

Treasurer: Sung Soo Lee, Kellogg Co., 235 Porter St, PO Box 3423, Battle Creek, MI 49016; telephone +1 (616) 961-2823, fax +1 (616) 961-3290

Member at Large: Norman Fraley, Armour Swift Eckrich, Express Analytic, 3131 Woodcreek Dr, Downers Grove, IL 60515; telephone +1 (708) 512-1013, fax +1 (708) 512-1062 or 512-1125

Member at Large: Philip Oles, Lancaster Laboratories, Inc., 2425 New Holland Pike, Lancaster, PA 17601; telephone +1 (717) 656-2301, fax +1 (717) 656-2681

Member at Large: Darryl Sullivan, Hazleton Laboratories, PO Box 7545, Madison, WI 53707; telephone +1 (608) 241-4471, fax +1 (608) 241-7227

Anyone interested in obtaining more information about the technical division may contact any of the executive committee or AOAC Technical Services.

New JAOAC Co-Editors Increase Journal's Specialization

To ensure the timeliness and quality of the reviews of papers submitted to the *Journal of AOAC International*, the AOAC Editorial Board recently voted to install two new section editors, increasing from six to eight the Journal's total number of co-editors. Appointed were George W. Latimer, Jr. Texas State Chemist, and George J. Jackson, from FDA's Office of Special Research Skills,

COMING IN THE NEXT ISSUE

CHEMICAL CONTAMINANTS MONITORING

■ Survey of Benzene in Foods, Using Headspace Concentration Technique and Capillary Gas Chromatography — T.P. McNeal, P.J. Nyman, G.W. Diachenko, and H.C. Hollifield

MICROBIOLOGICAL METHODS

■ Substrate Supporting Disc Method for Confirmed Detection of Total Coliforms and *E. coli* in all Foods: Collaborative Study — P.T. Feldsine, M.T. Falbo-Nelson, and D.L. Hustead

U.S. Food and Drug Administration Pesticide Program: Residues in Foods — 1992

Final Report: AOAC International Task Force on Methods for Nutrition Labeling to Official Methods Board

to the new sections on statistical analysis and food biological contaminants, respectively.

George Latimer, whose interest in statistics stems from chemometrics, has for many years served as a guest editor for Journal papers on method performance characteristics, statistical sampling approaches, and analytical use of linear regression, pattern recognition analysis, and diagnostic data evaluation. Latimer also serves as a member of the AAPFCO (Association of American Plant Food Control Officials) Board of Directors, is a member of the Magruder and AAFCO (American Association of Feed Control Officials) Check Sample Program Committees and a member of the AOAC Methods Committee on Feeds, Fertilizers, and Related Topics. He has also served as a AOAC General Referee for Feeds.

In addition to his interest in analytical methods, Latimer has concentrated in recent years on methods of sampling feeds and fertilizers and just finished a collaborative study that qualifies the Texas Tube as an AOAC Official Method for sampling fluid fertilizers.

George Jackson, trained as a microbiologist, has been active in scientific editing since working as a postdoctoral fellow and then faculty member in New York at Rockefeller University, where he served as an author and senior editor for a two-volume book on immunity to parasites. For 11 years, he was chief editor of the journal, *Experimental Parasitology*, and has been scientific editor of the sixth and seventh editions of *FDA's Bacteriological Analytical Manual*, published by AOAC.

At FDA's food center since 1972, Jackson became chief of the Food Microbiology Methods Development Branch (1981–1992), and is currently in the Office of Special Research Skills as assistant to the director. He is also on the U.S. delegation to the Codex Alimentarius Committee on Food Hygiene and has been a consultant for two United Nations Organizations (WHO and FAO). He is FDA's microbiology liaison with the International Organization for Standardization.

Overall, Latimer and Jackson will complement the work of the current six section editors: Rodney J. Noel (Agricultural Materials); Joseph Westheimer (Drugs, Cosmetics, Forensic Sciences); Albert E. Pohland (Food Chemical Contaminants); Joseph Sherma (Residues and Trace Elements); James F. Lawrence (Food Composition and Additives); and James D. MacNeil (Chemical Contaminants Monitoring). Section editors are responsible for the peer review of all manuscripts in their designated section.

Methods Adopted First Action

As directed by the Board of Directors, the Official Methods Board is responsible for consideration of methods for first action approval. The following methods were adopted first action at the Official Methods Board meeting January 28–30, 1993 in Orlando, FL, and became official at that time. These methods will be published in the fifth supplement (1994) to the 15th edition (1990) of *Official Methods of Analysis*.

Pesticide Formulations and Disinfectants

Phosphamidon in Technical and Formulated Products, Liquid Chromatographic Method

Bentazon in Pesticide Formulations, Liquid Chromatographic Method

Additives, Beverages, and Food Process Related Analytes

Nitrate in Baby Food, Spectrophotometric Method

Ethyl Carbamate (Urethane) in Distilled Spirits, Gas Chromatographic/Thermal Energy Analyzer Method

Commodity Foods and Products

L-Malic/Total Malic Acid Ratio in Apple Juice, Liquid Chromatographic, Enzymatic Methods

Microbiology and Extraneous Materials

Staphylococcal Enterotoxins in Foods, Polyvalent Enzyme Immunoassay Method (TECRA SET)

Salmonella from Cocoa and Chocolate, Motility Enrichment on Modified Semi-Solid Rappaport-Vassiliadis Medium

Salmonella in Foods, Colorimetric Monoclonal Enzyme Immunoassay Method (*Salmonella*-Tek)

Listeria in Dairy Products, Seafood, and Meats, DNA Hybridization Method (GENE-TRAK *Listeria*)

Clostridium perfringens from Seafood, Iron Milk Method

Bacteria Counts in Raw and Pasteurized Milk, Reflectance Colorimetric Method (Omnispec)

Listeria monocytogenes in Milk and Dairy Products, Selective Enrichment and Isolation Method (IDF-AOAC Method)

Somatic Cells in Milk, Optical Somatic Cell Counting Method (Fossomatic) (Modification of **978.26**)

Feeds, Fertilizers, and Related Topics

Nitrogen (Total) in Fertilizers, Combustion Method

Environmental Quality

1,2-Dibromoethane (EDB) and 1-2-Dibromo-3-Chloropropane (DBCP) in Water, Microextraction-Gas Chromatographic Method

Trace Elements in Waters and Wastewaters, Inductively Coupled Plasma-Mass Spectrometric Method

Books in Brief

Silica Gel and Bonded Phases: Their Production, Properties, and Use in LC. By Raymond P.W. Scott. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1993. 261 pp. Price: $64.95. ISBN 00471-93985-4.

Analytical techniques based on separation processes, such as chromatography and electrophoresis, are finding a growing range of applications in chemical, biochemical, and clinical laboratories. The aim of this series is to provide the analyst in these laboratories with well focused books covering individual techniques and important aspects of the techniques, so that they can be applied more efficiently and effectively to contemporary analytical problems. Silica gel is probably the most important single substance involved in modern chromatography. This book deals comprehensively with the subject of silica gel from the perspective of both the analyst and chromatographer. It aims to provide the reader with a basic understanding of the physical and chemical properties of silica gel and bonded phases and how those properties impact on the quality of the separations that are achieved by them.

Gas Mixtures: Preparation and Control. By Gary O. Nelson. Published by Lewis Publishers, 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 304 pp. Price: U.S.: $65.00, Outside U.S.: $78.00. ISBN 0-87371-298-6.

Gas Mixtures: Preparation and Control provides practical suggestions and calculations for producing multicomponent test gas atmospheres. General topics addressed include sorbent evaluation, methods development, dosimeter testing, instrument calibration, atmospheric simulation, and gas analysis. Learn the tricks of the trade for producing gas mixtures over a wide range of concentrations using even the most difficult-to-handle materials.

Reagent Chemicals, Eighth Edition. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 815 pp. Price: $149.95. ISBN 0-8412-2502-8.

Reagent Chemicals is the only resource of its kind that provides specifications and analytical procedures to assure the quality of your chemicals. ACS specified reagent chemicals are the choice of most organizations such as APHA, ASTM, FCC, SEMI, USP, etc., and many regulatory agencies, such as the EPA. ACS reagent specifications for solvents using analytical methods on EPA protocols are the only specifications that will meet the strict criteria for use in environmental analysis. Improved and expanded, the eighth edition of this authoritative reference now comes in a user-friendly format that makes look-up quick and easy.

EPA-Speak: The Interpharm Glossary of EPA Acronyms and Regulatory Terms. Edited by Dean E. Snyder. Published by Interpharm Press, 1358 Busch Parkway, Buffalo Grove, IL 60089, 1993. 631 pp. Price: 141.00.

This new reference is an invaluable guide to all terms, phrases, and terminology defined in EPA regulations together with other technical and general terms used in the world of environmental affairs. It includes definitions and explanations of more than 3500 environmental terms. Each EPA term contains its citation to the *Code of Federal Regulations*, providing instant look-up capability. Official EPA definitions are used in those terms that are defined in the CFR. The Clean Air Act, Clean Water Act, Safe Drinking Water Act, Superfund, and TSCA regulations are all covered. End-of-text appendixes include an extensive list of acronyms and abbreviations related to the environment.

Chromatography in Biotechnology. Edited by Csaba Horvath and Leslie S. Ettre. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1993. 180 pp. Price: $49.95. ISBN 0-8412-2669-5.

This book provides the most up-to-date compilation of significant research on preparative liquid chromatography used for the separation of biomolecules and proteins. It presents recent advances in LC techniques for the isolation and purification of bioproducts in the laboratory and manufacturing plant. It discusses novel approaches to the preparative/process chromatography of complex carbohydrate and glycoconjugates. It also describes recent advances in column materials.

CRC Handbook of Organic Analytical Reagents, Second Edition. By Keihei Ueno, Toshiaki Imamura, and K.L. Cheng. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 534 pp. Price: U.S.: $159.00, Outside U.S.: $189.00. ISBN 0-8493-4287-2.

The Handbook of Organic Analytical Regents, Second Edition, is an indispensable source book of physico-chemical properties, preparation, and analytical applications of the most commonly used organic reagents. Updated from the first edition, this volume includes data on 40 new reagents, such as ultra-high sensitive azo dyes, fluorescent calcium indicators, and chromogenic crown ethers and porphyrin reagents, a new Reagent Index listing reagents according to the elements to be assayed, and completed updated references. Each entry contains information on synonyms, sources of methods of synthesis, analytical applications, complexation reactions and the properties of complexes, purification and purity of the reagent, and other reagents with a related structure.

Cumulative Author Index

CHEMICAL CONTAMINANTS MONITORING

Total Organochlorine Content of Fish from the Great Lakes

W. HARVEY NEWSOME, PAUL ANDREWS, and HENRY B.S. CONACHER
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Residues of polychlorinated biphenyls (PCBs) and organochlorine pesticides were determined in several species of commercial fish from the Great Lakes and compared to the total organic chlorine determined by neutron activation analysis. The mean organochlorine contents ranged from 44 to 138 ppm (lipid basis) and were 5 to 72 times higher than the contents of PCBs and organochlorine pesticides. Marine fish also contained a large proportion of unidentified organic chlorine. The unknown material in the Great Lakes fish was found to chromatograph with the high molecular weight lipid fraction by gel permeation chromatography.

Several reports indicated that the total organic chlorine in fish, water, and sediment exceeded the total known polychlorinated biphenyls (PCBs), chlorinated pesticides (OCs) such as DDTs, chlordane, and toxaphene, and other organochlorine compounds. Thus, Lunde and coworkers (1–3) and Hakansson et al. (4) determined by neutron activation analysis (NAA) that the total organic chlorine in oil from marine fish was ≥10 times the content of PCBs or DDTs determined by gas chromatography (GC). Ofstad and Martinsen (5) extended these findings to seals from Norwegian coastal waters. Norstrom et al. (6) reported that some samples of herring gull eggs from Lake Ontario contained 2–4 times as much organic chlorine as could be accounted for by known compounds. Marine fish from Japan were found to contain an average of 21 ppm organic chlorine on a lipid basis, 83–98% of which was unidentifiable as known compounds (7).

Surface water from various locations in Sweden has also been found to contain organic chlorine that could not be attributed to industrial effluents (8). Asplund and Grimvall (9) summarized data indicating that surface water from areas remote from industrial activity contains considerable organic chlorine. These authors relate the organic chlorine to the occurrence of humic matter, and suggest that the unknown material may be of natural origin. Whether the organic chlorine in fish lipid arises from that present in water remains to be demonstrated, but it could arise via a mechanism similar to that described for the production of chloroveratrols from high molecular weight chlorinated lignin (10). By this mechanism, high molecular weight chlorinated lignin is degraded microbially to chlorinated guaiacols, which are in turn O-methylated to veratroles, which are then accumulated in fish tissue.

The present study was initiated to determine the mass balance of organic chlorine in the edible portion of fish from the Great Lakes and thereby obtain an indication of the potential for human exposure to these unidentified compounds.

Received May 1, 1992. Accepted November 12, 1992.

Experimental

Materials

All solvents were glass-distilled and were tested for contaminants by GC with electron capture detection after a 300-fold concentration. Florisil and sodium sulfate were purified and activated according to the procedure of Mes (11). Pesticide standards were gifts from the U.S. Environmental Protection Agency. Chlorinated benzenes and PCB congeners were purchased either from Ultra Scientific (Hope, RI) or from Wellington Laboratories (Guelph, ON, Canada).

The standard reference materials (SRMs) used for NAA were purchased from the U.S. National Institute of Standards and Technology (NIST).

Sampling

Fish samples were obtained from commercial processing plants by the Inspection Branch, Fisheries and Oceans Canada. They consisted of fillets from 2–10 fish and were stored frozen at −20°C before compositing. Composites were prepared by partially thawing the fillet, removing any skin, and mincing enough subsample in a screw-type meat grinder to provide approximately 500 g tissue. The species, number, and lake of origin of the samples were as follows: bass, 2 (Erie); bullhead, 4 (Ontario); carp, 2 (Ontario); eel, 3 (Ontario); herring, 8 (Superior); menominee, 7 (Superior); perch, 7 (Erie), 4 (Ontario); pickerel, 2 (Erie); smelt, 2 (Erie); trout, 2 (Erie), 1 (Ontario), 15 (Superior); whitefish, 2 (Erie), 6 (Superior). To compare fish from a relatively contaminated source, 6 samples of carp were obtained from a fishery closed to commercial operation. Marine fish were purchased from a local fish market.

Extraction

Minced fish fillet (20 ± 0.5 g) was placed in a 250 mL centrifuge bottle and extracted by mixing 3 min in a Polytron ho-

mogenizer with 150 mL acetone–hexane (2 + 1). The solids were sedimented by centrifugation and the supernatant was decanted into a 250 mL round-bottom flask. The solids were re-extracted by mixing with 75 mL hexane on the Polytron. After centrifugation, the supernatants were combined and the volume was reduced to ca 25 mL on a rotary evaporator. The concentrate was transferred to a 250 mL separatory funnel with 100 mL hexane, and the organic layer was partitioned against 50 mL deionized water. The hexane layer was dried by passage through a bed of anhydrous Na_2SO_4 in a 75 mm glass funnel, reduced to ca 2 mL on a rotary evaporator, and transferred to a graduated centrifuge tube. The solution was diluted to 10.0 mL with hexane, and a 1.0 mL aliquot was taken for determination of extractable lipid.

Extractable lipid was determined by letting the aliquot evaporate in a tared 5 mL vial in the fume hood until constant weight was attained, usually 24 h. After the weight was recorded, 2.0 mL isooctane was added, and the vial was capped with a PTFE-lined silicon rubber septum pending chlorine determination by NAA.

Fractionation

Lipid was removed from extracts by gel permeation chromatography on an ABC Autoprep (Analytical Bio-Chemistry Laboratories Inc., Columbia, MO) fitted with a 25 × 600 mm column containing 60 g Bio-Beads SX-3, 200–400 mesh. Mobile phase consisting of cyclohexane–dichloromethane (1 + 1) was supplied to the column at a flow of 5 mL/min. The sample, containing ≤500 mg lipid, was fortified with 0.5 mL ^{13}C internal standard (9.6 ng of each compound) and added to the column in 5 mL mobile phase. The initial 120 mL mobile phase was discarded and the next 250 mL was collected; 2 mg methyl myristate was added as a keeper and the solvent was removed on a rotary evaporator.

The sample was further fractionated on a 1.0 × 19 cm chromatography column dry-packed with 6.5 g Florisil deactivated with 0.8% water. The sample was added to the column in 2 mL hexane, and the first fraction, which contained all of the PCBs and most of the organochlorine pesticides, was eluted with 60 mL 20% dichloromethane in hexane. The second fraction, consisting of 60 mL 60% dichloromethane in hexane, removed dieldrin and the remainder of the heptachlor epoxide.

Determination of PCB and OC

Thirty-nine PCB congeners (as listed in ref. 12) were determined and summed to give a total PCB figure. The OCs consisted of tetra-, penta-, and hexachlorobenzenes, ΣHCH, Σchlordane, DDTs, Σnonachlor, octachlorostyrene, heptachlor, heptachlor epoxide, dieldrin, mirex, and toxaphene. The PCBs and OCs in the first fraction eluted from the Florisil column were determined by capillary column chromatography with mass selective detection using a Hewlett-Packard model 5890 gas chromatograph and model 5971A mass selective detector as described in an accompanying paper (12). Dieldrin and that portion of the heptachlor epoxide appearing in the second fraction were determined by gas chromatography with electron capture detection (12). Toxaphene was determined by gas chromatography and high resolution electron impact mass spectrometry (12).

Table 1. Chlorine content of standard reference materials analyzed by NAA

Material	Mean Cl (ppm) ± SD		
	This work	No. replicates	Certified value
Milk powder[a]	10890 ± 190	12	10900 ± 100
Bovine liver[b]	2750 ± 120	12	2800 ± 100
Citrus leaves[c]	406 ± 12	10	414

[a] NIST-SRM-1549.
[b] NIST-SRM-1577a.
[c] NIST-SRM-1572.

Determination of Total Organic Chlorine

Aliquots of 750 µL of sample extract were transferred to 1.2 mL polyethylene vials by a calibrated Eppendorf pipet. These vials were heat-sealed and kept in 7 mL polyethylene vials for irradiation. The 1.2 mL polyethylene vials were acid-washed before use with Ultrex nitric acid and dried. Comparator standards consisted of KCl solutions containing 0.1–10 µg chlorine per vial. All samples and comparator standards were prepared to be identical in all respects and irradiated 30 min in an integral flux of 5×10^{11} n cm^{-2} s^{-1} at the Dalhousie University SLOWPOKE-2 reactor. The sample and comparator standard vials were carefully opened in a fume hood of the radiochemistry laboratory, and 500 µL portions of the irradiated aliquots were transferred to fresh 1.2 mL polyethylene vials. These vials were accurately weighed before and after the addition of 500 µL portions of the irradiated aliquots, and were counted for 30 min after a decay period of 5 min. The 1642 keV photopeak of ^{38}Cl was used to quantitate the chlorine content of the samples.

Counting was performed by an Aptec 25 cm^3 active volume hyperpure Ge detector (full-width at half-maximum of 2.08 keV at 1332 keV photopeak of ^{60}Co) connected to a Link high-count rate pulse processor coupled to a Nuclear Data ND-66 analyzer.

The absolute detection limit of the NAA procedure for chlorine measurement under the experimental conditions of irradiation, decay, and counting (30:5:30 min) is 30 ng.

Quality Assurance

Every sixth sample analyzed for organochlorine pesticides and PCBs consisted of 500 mg corn oil spiked with ca 20 ppb of each of the PCB congeners and chlorinated hydrocarbons.

The precision and accuracy of chlorine measurements by the NAA method were checked by analyzing replicate samples of the chlorine comparator standards and control standard reference materials at regular intervals during the course of the project. The chlorine content of the SRMs determined in this study is shown in Table 1. The blank organic chlorine value and reproducibility of the extraction and NAA procedures were determined by analysis of blind samples inserted at regular inter-

Table 2. Total organic chlorine (NAA Cl), PCBs, chlorinated pesticides (OC), and lipid in Great Lakes fish

Species	Lipid, %	Total OC, ppm[a]	Total PCB, ppm[a]	NAA Cl, ppm
Bass (n = 2)	0.9 ± 0.1	5.0 ± 2.0	43.8 ± 13.1	138 ± 43
Bullhead (n = 4)	1.3 ± 0.1	2.2 ± 1.5	5.3 ± 4.4	87.1 ± 52.6
Carp–O (n = 2)[b]	9.0 ± 7.1	0.6 ± 0	1.4 ± 0.2	73.4 ± 31.9
Carp–C (n = 6)[c]	12.5 ± 5.3	10.9 ± 3.5	24.1 ± 9.6	73.6 ± 26.7
Eel (n = 3)	13.3 ± 1.3	4.8 ± 3	85.9 ± 3.2	44.3 ± 13.8
Herring (n = 8)	6.6 ± 2.5	8.8 ± 2.1	1.4 ± 0.9	93.4 ± 32.3
Menominee (n = 7)	1.9 ± 1.4	2.9 ± 2.1	2.9 ± 3.0	96.5 ± 39.4
Perch (n = 11)	0.9 ± 0.3	2.2 ± 1.4	4.0 ± 2.5	130 ± 38
Pickerel (n = 2)	1.9 ± 1.4	4.7 ± 4.5	4.7 ± 0.7	81.1 ± 23.0
Smelt (n = 2)	4.9 ± 0.9	1.2 ± 1.2	1.5 ± 0.1	108 ± 8
Trout (n = 18)	19.7 ± 5.6	7.0 ± 3.4	3.1 ± 1.9	98.1 ± 14.2
Whitefish (n = 8)	8.0 ± 4.9	5.4 ± 2.6	2.7 ± 2.8	109 ± 59

[a] Mean, µg/g lipid ± SD.
[b] Open fishery.
[c] Closed fishery.

vals with each batch of fish extracts. Blank chlorine values were determined by extracting a 20 g sample of corn oil and carrying out a lipid determination and subsequent NAA for total chlorine. The repeatability was established by re-extracting the same sample of fish and subjecting the extract to NAA and lipid determinations. Accuracy was determined by spiking a duplicate aliquot of the corn oil blank with an isooctane solution of hexachlorobenzene at levels ranging from 25 to 195 ppm.

Results and Discussion

The mean blank corn oil value for organic chlorine determined by NAA was 4.4 ± 2.4 ppm. A vial blank was eliminated by irradiating an aliquot of the sample and then transferring it to a second vial for counting. The mean recovery of chlorine from corn oil samples spiked with 25–195 ppm hexachlorobenzene was 86.0 ± 6.0%. Data from fish extracts are uncorrected for this value. Repeatability is indicated by a mean value of 86.1 ± 8.1 ppm for the same sample of fish extracted on 6 different runs and subjected to NAA and lipid determination.

Table 3. Total organic chlorine (NAA Cl), PCBs, chlorinated pesticides (OC), and lipid in marine fish

Species[a]	Lipid, %	Total OC, ppm[b]	Total PCB, ppm[b]	NAA Cl, ppm[b]
Sole	2.18	0.11	0.03	74.5
Cod	0.69	0.54	0.24	111
Haddock	0.75	0.26	0.16	56.4
Red snapper	0.97	0.07	0.03	165
Boston bluefish	1.09	0.16	0.27	200
Shark	0.78	1.24	10.3	172
Turbot	17.2	0.27	0.33	17.3

[a] A single sample of each species was analyzed.
[b] µg/g lipid.

The total organic chlorine values and the total of the PCBs and OCs determined in the 11 species of Great Lakes fish analyzed are summarized in Table 2. On a lipid basis, bass, perch, smelt, and whitefish contained the highest levels of total chlorine and eel the lowest. Carp from the closed fishery contained markedly higher amounts of total PCBs and OCs than carp from the open source, although the total organic chlorine level was similar for both. In comparison to the Great Lakes fish, marine samples, with the exception of turbot, contained somewhat higher levels of total organic chlorine, as shown by the data in Table 3. These latter species, with the exception of shark, had lower levels of PCBs and OCs than the Great Lakes fish. The total chlorine levels found in the Great Lakes samples are 2–7 times as high as those reported for marine fish sampled off Japan (7), although whole fish were analyzed in the latter case. The present data more closely resemble the data for total

Table 4. Total organic chlorine (NAA Cl) compared to total PCB and chlorinated pesticides (OC) chlorine

Species	PCB + OC Cl, ppm[a]	NAA Cl, ppm[a]	Unaccounted, %
Bass	28.4	138	79.5
Bullhead	4.1	87.1	95.3
Carp–O[b]	1.1	73.4	98.5
Carp–C[c]	19.3	73.6	73.8
Eel	6.2	44.3	86.0
Herring	6.7	93.4	92.8
Menominee	3.5	96.5	96.4
Perch	3.6	130	97.2
Pickerel	5.8	81.1	92.8
Smelt	1.5	108	98.6
Trout	6.4	98.1	93.2
Whitefish	5.0	109	95.4

[a] µg/g lipid.
[b] Open fishery.
[c] Closed fishery.

Table 5. Distribution of organic chlorine by molecular weight[a]

Species	Lipid, mg		Chlorine, %	
	High MW	Low MW	High MW	Low MW
Eel	389	3.5	85.0	15.0
Carp[b]	399	1.8	78.8	11.2
Herring	338	3.0	91.2	8.8
Bullhead	147	1.9	96.8	3.2
Perch	104	3.5	94.9	5.1
Whitefish	387	3.8	98.3	1.7
Pickerel	256	4.0	92.5	7.5
Smelt	412	5.0	97.1	2.9
Menominee	103	2.4	91.2	8.8
Bass	173	4.6	90.6	9.4

[a] "High and low MW" refer to the excluded and non-excluded fractions respectively, from the GPC column.
[b] Closed fishery.

chlorine found in marine fish oils (1) and in the crude lipid extracts of herring gull eggs (10). The accountability of organic chlorine is presented in Table 4, in which the total PCB and OC data were calculated as total chlorine and compared to the NAA chlorine values. The percentage of chlorine that cannot be reconciled by the sum of the compounds determined is relatively constant among the various species, even though the total chlorine varies by a factor of 3. Carp from the contaminated area contained the lowest percentage of unidentified organic chlorine. These data are similar to those of Watanabe et al. (7), in which 83–98% could not be accounted for by PCBs, DDTs, and HCH.

To further characterize the unknown organic chlorine, an extract from 1 sample of each species was subjected to GPC followed by NAA of the concentrated high and low molecular weight fractions. The distribution of chlorine is shown in Table 5. Most of the organic chlorine was found to reside in the high molecular weight fraction, and the proportion in this fraction was relatively constant from species to species. This proportion also agrees well with the percentage of unaccounted chlorine determined from the sum of the PCB and OC and the NAA data (Table 4). Further studies are in progress to determine the exact nature of the unknown chlorinated material.

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CHEMICAL CONTAMINANTS MONITORING

Organochlorine Pesticides and Polychlorinated Biphenyl Congeners in Commercial Fish from the Great Lakes

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Fillets from 11 species of commercial fish from the Great Lakes were analyzed for residues of 39 polychlorinated biphenyl (PCB) congeners and 24 other organochlorine compounds. Eel and trout contained the highest amount of PCB (753 and 633 ppb wet weight, respectively) and other organochlorines (607 and 1404 ppb, respectively); perch and carp contained the lowest residues. The penta- and hexachlorinated PCBs were the major congeners in all species except whitefish, in which the tetrachlorinated congeners predominated. Toxaphene was the most abundant organochlorine pesticide in trout; *p,p'*-DDE was the major component of this class in eel. Residue concentrations in commercial carp were compared with residues present in carp from a fishery closed to commercial operation. Although both PCB and organochlorine pesticide levels in carp were among the lowest for all commercial fish samples, levels from the contaminated area were among the highest.

The multitude of organochlorine compounds found in fish from the Great Lakes has been described to some extent in the literature. Schmidt et al. (1) summarized the results of the U.S. national contaminant monitoring program for freshwater fish from 1976 to 1984. These data were obtained from homogenates of whole fish and included composites of several species from the Great Lakes. Among the compounds determined were polychlorinated biphenyls (PCBs), which were reported as Aroclor 1248, 1254, or 1260, and several organochlorine pesticides, including DDTs, dieldrin, heptachlor, chlordane, HCHs, toxaphene, and mirex. In addition, DeVault et al. (2) have described temporal trends in concentrations of PCBs, DDTs, and dieldrin in composites of whole lake trout from Lakes Michigan, Huron, and Superior, and Borgmann and Whittle (3) published monitoring data showing the trends from 1977 to 1988 in concentrations of total PCBs, mirex, dieldrin, chlordane, and DDE in whole salmon from Lake Ontario. Rasmussen et al. (4) have also published Ontario Ministry of Natural Resources data on PCB levels in several of the Great Lakes. Thorough studies have been conducted of the PCB congener distribution in whole fish or muscle of trout from Lake Ontario (5) and in fillets of several species from Lakes Michigan and Superior and various rivers in Wisconsin (6).

There are indications that the concentrations of several of the compounds are declining in Great Lakes fish (1–3), with the possible exception of toxaphene (1). Whereas several of these compounds previously exceeded the International Joint Commission objectives (2), most are now below these limits, and it is likely that the consumer of Great Lakes fish today would be exposed to these contaminants to a lesser extent. The amount of chlorinated contaminant is considerably less in the muscle than in whole fish (5), and few data are available on the PCB congeners or chlorinated pesticides in commercial fish as offered for sale from fisheries on the Great Lakes. For these reasons, the present study was initiated to determine the potential for exposure to these compounds that might be expected from the consumption of these fish.

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Experimental

Materials

Glass-distilled solvents were purchased from Caledon Laboratories, Georgetown, Ontario. Analytical standards of chlorinated pesticides were obtained from the U.S. Environmental Protection Agency. Chlorinated benzenes and most PCB congeners were purchased from Ultra Scientific (Hope, RI). Congeners No. 87, 170, and 191 were obtained from Wellington Laboratories (Guelph, Ontario). [^{13}C] PCB internal standards used to quantitate the PCB congeners and organochlorine pesticides were congeners No. 15, 28, 52, 101, 138, 180, and 194 (Cambridge Isotope Laboratories, Woburn, MA), as numbered by Ballschmitter and Zell (7).

Instrumentation

Analytical columns were 0.25 mm × 30 m fused silica with a 0.25 µm film thickness of DB-5 and were coupled to 0.53 mm × 1 m retention gaps. Helium was used as the carrier gas at an inlet pressure of 20 KPa for mass selective detection and 83 KPa for electron capture detection.

PCB congeners and most chlorinated pesticides were determined on a Hewlett-Packard 5890 chromatograph with a cold

Table 1. Lipid, organochlorine pesticides, and PCBs in Great Lakes fish

Species	Lipid, %	Total OC[a]	Total PCB[a]
Bass (n = 2)	0.9 ± 0.1	45.0 ± 13.6	395 ± 80
Bullhead (n = 4)	1.3 ± 0.1	30.1 ± 23.0	69.9 ± 59.1
Carp–O (n = 2)[b]	9.0 ± 7.1	56.9 ± 47.0	14.6 ± 8.8
Carp–C (n = 6)[c]	12.5 ± 5.3	1307 ± 588	2715 ± 1794
Eel (n = 3)	13.3 ± 1.3	607 ± 457	753 ± 362
Herring (n = 8)	6.6 ± 2.5	559 ± 205	94.2 ± 81.4
Menominee (n = 7)	1.9 ± 0.8	43.5 ± 15.7	41.2 ± 24.3
Perch (n = 11)	0.9 ± 0.3	20.9 ± 17.4	33.6 ± 16.1
Pickerel (n = 2)	1.9 ± 1.4	57.7 ± 16.7	94.4 ± 80.9
Smelt (n = 2)	4.9 ± 0.9	59.7 ± 0.7	71.2 ± 6.7
Trout (n = 18)	19.7 ± 5.6	1404 ± 811	633 ± 504
Whitefish (n = 8)	8.0 ± 4.9	491 ± 458	226 ± 221

[a] ng/g wet weight ± SD.
[b] Open fishery.
[c] Closed fishery.

on-column injector and model 5971A mass selective detector. A 2 µ portion of sample extract was injected with a model 7673 autosampler. Samples were injected with the column at 70°C; after a 2 min delay, the column was programmed at 8°C/min to 230°C; and then after a further 4 min delay, it was programmed to 270°C at 5°C/min. The temperature was held at 270°C for 11 min and then cooled for the subsequent injection.

The mass selective detector was operated with a transfer line temperature of 280°C and was tuned on autotune with perfluorotributylamine. The electron multiplier was set to 400 V above tune voltage. Stable isotope internal standards of PCBs representing each congener group except the nonachlorinated PCBs were used to quantitate congeners. The nonachlorinated congeners were quantitated with the octachlorinated internal standard. Chlorinated pesticides were also determined with either the ^{13}C dichlorinated PCB (No. 15) or the ^{13}C hexachlorinated PCB (No. 138) internal standards. The chlorinated benzenes HCH, heptachlor, and aldrin were determined with congener No. 15 and the remainder with No. 138. Compounds were quantitated by using a linear 3-level calibration curve consisting of 10, 20, and 40 ng of each analyte/mL. The minimum quantitation limit was determined by the accuracy of the integrator on the data system and was estimated to be 5 ng/mL. This limit corresponded to 0.025 ng/g of fish tissue.

The following PCB congeners were determined, in order of elution, using the respective target and qualifier ions: trichlorinated (No. 28, 33, 37; m/z 256, 258), tetrachlorinated (No. 52, 49, 41, 40, 44, 74, 66, 60; *m/z* 292, 290), pentachlorinated (No. 90, 99, 87, 110, 118, 105; *m/z* 326, 328), hexachlorinated (No. 136, 151, 153, 141, 137, 138, 129, 128, 156, 157; *m/z* 360, 362), heptachlorinated (No. 187, 183, 185, 180, 193, 191, 170, 189; *m/z* 394, 396), octachlorinated (No. 201, 203, 194; *m/z* 430, 428); and nonchlorinated (No. 206, *m/z* 464, 462). The following organochlorine compounds were determined in the second injection: 1,2,3,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene (*m/z* 216, 214), pentachlorobenzene (*m/z* 250, 248), α-HCH, β-HCH, γ-HCH, δ-HCH (*m/z* 181, 183), hexachlorobenzene (*m/z* 284, 286), heptachlor (*m/z* 272, 274), aldrin (*m/z* 263, 265), octachlorostyrene (*m/z* 308, 310), heptachlor epoxide (*m/z* 353, 355), oxychlordane (*m/z* 387, 389), α-chlordane, γ-chlordane (*m/z* 375, 373), *trans*-nonachlor (*m/z* 409, 407), *p,p*-DDE (*m/z* 246, 248), DDD (*m/z* 235, 237), *o,p*-DDT, *p,p*-DDT (*m/z* 235, 237), *cis*-nonachlor (*m/z* 409, 407), and mirex (*m/z* 272, 274).

Dieldrin and a portion of the heptachlor epoxide were determined on an identical gas chromatograph fitted with an autosampler and a ^{63}Ni electron capture detector. The sample was injected with the column at 70°C, and after 2 min was programmed at 10°C/min to 230°C. After 12 min at 230°C, the column was cooled before the next injection. The minimum quantitation limit, defined as 10 × signal:noise, was 9 pg/g for heptachlor epoxide and 17 pg/g for dieldrin.

Toxaphene was quantitated by selected ion high resolution mass spectrometry monitoring of the dichloro-tropyllium ion, which is characteristic of most of the chlorinated bornanes present in the toxaphene mixture (8). The analytical column was interfaced to a Kratos Concept 1 mass spectrometer running at 8000 ppm resolution, and after injection of the sample, was programmed at 30°C/min to 200°C, and then after 5 min elapsed time, at 16°C/min to 280°C. Toxaphene was quantitated by averaging the responses at *m/z* 158.9769 and 160.0739. The internal standards consisted of [^{13}C] PCB No. 138, 180, and 194, which spanned the time window for elution of toxaphene, and were monitored at *m/z* 230.0092 and *m/z* 263.9702. The response factor for toxaphene was determined by summing the areas of the peaks in the toxaphene standard and dividing by the sum of the areas of the internal standards. The average response factor for various amounts of toxaphene was determined by injecting toxaphene standards at 2.5, 5.0, and 10.0 µg/mL and was used to quantitate the unknown samples.

Sample Preparation

Sampling, extraction, and purification were described previously in detail (9). Briefly, fillets from commercial fish processing plants were composited, extracted with acetone-hexane (2 + 1), and, after the addition of [^{13}C] PCB internal standards, defatted by gel permeation chromatography. The fraction containing the PCBs and chlorinated pesticides was further purified by chromatography on Florisil. Two fractions were collected: the first, eluting with 20% dichloromethane in hexane, contained the PCBs and most of the organochlorine pesticides. The second was obtained by elution with 60% dichloromethane in hexane and contained dieldrin and the remainder of the heptachlor epoxide.

The solvent was removed from the respective fractions on a rotary evaporator, and the residue was taken up in 0.5 mL hexane for analysis. Three injections of the first fraction were made: one each for PCB and for organochlorine pesticide determinations by mass selective detection and a third for toxaphene determination by high resolution mass spectrometry. Dieldrin and heptachlor epoxide in the second fraction

Table 2. Chlorinated hydrocarbons in Great Lakes fish (ng/g wet wt)

Species/compound	Bass	Bullhead	Carp–O[a]	Carp–C[b]	Eel	Herring	Menominee	Perch	Pickerel	Smelt	Trout	Whitefish
Σ TeCBz	ND[c]	ND	ND	0.33	0.88	0.06	ND	ND	ND	0.04	0.11	0.2
PnCBz	0.03	ND	0.23	1.10	1.54	0.16	0.02	ND	0.05	0.17	0.46	0.25
HCBz	0.63	0.22	1.28	9.27	9.04	2.94	0.48	0.27	0.89	1.50	9.05	3.85
Σ HCH	0.54	0.42	2.91	5.49	7.80	8.39	2.82	0.28	0.96	0.68	23.4	22.8
Heptachlor	0.96	0.02	ND	ND	ND	ND	0.08	0.06	0.13	ND	ND	ND
HEO	0.56	0.67	1.42	8.79	15.0	3.57	0.77	0.20	0.76	1.79	14.8	11.2
Dieldrin	2.71	0.24	3.72	40.2	30.6	11.7	1.77	0.98	3.17	6.62	41.2	25.0
OCS	1.27	0.21	1.28	58.2	9.58	0.28	ND	0.15	0.88	0.15	0.36	0.49
Σ Chlordane	6.60	2.54	3.97	44.9	28.2	25.8	0.94	1.06	6.85	6.96	33.0	16.1
Oxychlordane	0.76	ND	ND	4.79	5.92	1.68	0.05	0.06	0.59	0.27	23.9	4.50
Σ Nonachlor	11.1	5.40	3.89	52.8	46.5	20.8	2.97	2.26	7.52	8.73	115	28.6
p,p'-DDE	17.1	10.1	20.4	653	166	14.4	6.26	3.49	4.67	9.04	148	48.3
TDE	ND	ND	ND	40.0	ND	0.89	0.25	0.24	ND	10.4	6.90	4.74
Σ DDT	2.40	4.86	9.42	124	95.1	9.81	1.61	1.12	3.11	6.15	50.3	36.1
Mirex	0.38	3.63	5.24	120	56.8	0.44	0.17	0.82	0.18	0.20	2.38	0.82
Toxaphene	ND	ND	3.18	144	133	458	25.3	9.90	28.0	7.36	936	288

[a] Open fishery.
[b] Closed fishery.
[c] Not detected.

were determined by gas chromatography with electron capture detection.

Recoveries of the chlorinated hydrocarbons from the gel permeation and Florisil columns averaged 91.1% (SD 6.5); that for the PCB congeners averaged 99.3% (SD 3.8).

Results and Discussion

The present sampling, although limited to small numbers in some species, revealed that trout and eel contained the highest concentration of total organochlorine pesticides and PCBs (Table 1) relative to bullhead, menominee, perch, pickerel, and smelt. The total PCB residues found in bass, eel, trout, and whitefish exceeded the International Joint Commission objectives (10). The mean total PCB residues were below the Health and Welfare Canada guideline of 2.0 ppm in the edible portion of fish for all species from commercial fisheries. The carp from the closed fishery contained markedly higher PCB residues than the commercial fish, and the mean did exceed the 2 ppm guideline. The average PCB values in trout are approximately one-fourth of those reported for whole trout from Lake Ontario in 1988 (3) or Lake Huron in 1982 (2) and are similar to those found in this species from Lake Superior in 1981 (1) or 1982 (2). The average PCB values for the noncommercial carp fillets are also similar to those reported for whole carp in Lake St. Clair or Lake Erie in the U.S. National Pesticide Monitoring Program (1); the values for whitefish are approximately double those in the U.S. study of fish in these lakes.

Trout fillets contained the highest concentration of chlorinated pesticides, with toxaphene as the most abundant member (Table 2). Relatively large amounts of organochlorine pesticides were also found in eel and herring. The major contributor found in herring was also toxaphene. In eel the most abundant members were toxaphene and DDE, which were present in similar proportions. Carp fillets from the contaminated fishery contained markedly higher concentrations of all compounds than those in carp from the commercial fishery, and contained the highest concentrations of mirex and octachlorostyrene of all fish analyzed. The amount of toxaphene varied widely with species; it was undetectable in bass or bullhead and present in small amounts in menominee, perch, and smelt. The amount of toxaphene in trout or whitefish fillets was approximately one-half that previously found in whole fish for those species in Lake Superior (1). The relationship of smelt and trout in the food chain is paralleled by the relative amounts of all contaminants in both. Dieldrin, oxychlordane, and DDT levels in trout that were predominantly from Lake Superior are comparable

Table 3. Percentage distribution of PCB homologues in Great Lakes fish

Species	Cl_3	Cl_4	Cl_5	Cl_6	Cl_7	Cl_8	Cl_9
Bass	0.2	6.4	27.8	41.9	18.6	4.7	0.3
Bullhead	0.1	19.2	35.3	26.7	13.4	4.9	0.4
Carp–O[a]	0	19.8	40.4	22.3	11.6	4.8	1.0
Carp–C[b]	0	13.3	47.9	23.0	12.4	2.9	0.3
Eel	0	15.0	36.2	28.3	16.1	4.0	0.4
Herring	0.3	11.0	31.4	40.0	13.4	3.6	0.3
Menominee	0.1	5.9	27.0	40.3	19.8	6.3	0.5
Perch	0.2	13.1	38.7	29.1	13.8	4.5	0.5
Pickerel	0.3	11.0	39.9	31.6	13.9	2.9	0.3
Smelt	5.2	13.2	33.8	30.4	12.2	4.8	0.5
Trout	0.1	9.3	28.6	40.5	16.9	4.4	0.2
Whitefish	1.4	31.9	26.6	23.2	13.4	3.3	0.3

[a] Open fishery.
[b] Closed fishery.

Table 4. Percentage distribution of PCB congeners in various species of fish from the Great Lakes

Congener	Bass	Bullhead	Carp–O[a]	Carp–C[b]	Eel	Herring	Menominee	Perch	Pickerel	Smelt	Trout	Whitefish
138	18.3	8.9	10.1	11.1	14.0	14.0	18.5	13.5	13.6	11.7	19.9	10.9
153	12.7	3.5	6.6	7.1	8.9	19.0	12.1	8.4	8.7	9.2	12.9	7.0
90	11.3	5.8	14.6	17.2	8.8	11.2	9.3	13.0	16.1	15.2	7.1	9.7
110	6.7	4.5	7.2	7.4	7.5	6.8	5.0	11.0	9.8	7.3	5.7	5.9
180 + 193	8.2	4.0	4.9	4.7	6.5	4.8	10.4	5.8	4.8	5.4	5.5	6.1
118	3.3	5.2	6.8	8.5	8.2	5.7	5.3	5.3	4.4	3.8	7.2	4.5
99	3.5	3.6	6.0	7.2	6.9	4.0	4.1	5.2	4.9	4.1	4.1	3.5
187	4.6	2.3	3.0	2.4	3.2	2.9	3.5	3.4	3.8	2.8	3.3	3.1
170	4.1	1.6	2.6	2.1	3.1	2.6	4.4	2.8	3.1	2.6	3.6	2.8
52	1.5	1.4	3.5	2.4	4.0	2.4	1.2	2.8	2.9	3.3	1.6	6.9
41	0.7	2.0	3.2	2.1	3.2	2.1	1.3	1.9	1.4	2.5	1.5	11.8
66	1.1	3.1	4.7	3.1	3.1	1.4	0.9	3.0	1.7	1.8	1.9	2.4
105	1.1	1.8	2.7	3.4	3.3	1.9	2.1	2.1	1.8	1.5	2.7	1.9
201	2.5	1.5	2.4	1.4	1.8	1.9	3.0	2.5	2.1	2.4	2.5	1.6

[a] Open fishery.
[b] Closed fishery.

to those previously found for whole trout in Lake Superior (2), whereas the amounts of DDTs, mirex, HCB, octachlorostyrene, and chlordane in the present sampling of trout are one-fifth to one-tenth of those reported for Lake Ontario (3, 5). The concentrations of all pesticides determined, with the exception of mirex, were below the International Joint Commission objectives (10).

The percentage distribution of PCB homologues determined in the various species is given in Table 3. The predominant congeners are the hexa- and pentachlorinated PCBs, although whitefish contains a higher percentage of the tetrachlorinated congeners than other species. Bass and menominee contained relatively low proportions of the tetrachlorinated and higher proportions of heptachlorinated congeners. The literature contains little data comparing congener distribution among different species of fish, although Oliver and Niimi (11) showed that smelt and salmonids from Lake Ontario contained similar proportions of congeners, as in the present study.

The proportions of the most abundant individual congeners are shown in Table 4. Bass, menominee, and trout contained relatively large amounts of the hexachlorinated congener No. 138; the predominant congener in carp, pickerel, and smelt was the pentachlorinated biphenyl No. 90. In perch, No. 138 and 90 were present in similar amounts. The large percentage of tetrachlorinated biphenyl in whitefish is represented mostly by congeners No. 52, 41, and 66. In contrast to the present data, No. 153 was the most abundant congener in a study of Lake Ontario trout (5) and in various species of fish from Wisconsin (6). A larger sampling is required to determine whether these differences are due to lake-to-lake variation such as that observed for PCB congeners in various species from different lakes adjacent to Lake Ontario and Georgian Bay (12).

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DAIRY PRODUCTS

Liquid Chromatographic Method for Determination of Iodine in Milk: Collaborative Study

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Nine laboratories participated in an AOAC International/International Dairy Federation collaborative study on a liquid chromatographic (LC) method for determination of iodine in milk. Liquid milk is passed through a 25 000 MW membrane filter to remove protein and insoluble material. Iodine (in the form of iodide) in the clear filtrate is separated by reversed-phase ion-pair LC and is detected electrochemically. Participants analyzed 2 commercial pasteurized whole milks and 5 nonfat dry milk powders in blind duplicate. Each sample was tested in duplicate on 2 days. Repeatability and reproducibility standard deviations (s_r and s_R, respectively) and repeatability and reproducibility relative standard deviations (RSD_r and RSD_R, respectively) for determinations of iodine in whole milk (mean recovery, 86.7%) were as follows: s_r, 22 μg/L; s_R, 22 μg/L; RSD_r, 8.2%; and RSD_R, 8.3%. For powdered milk (mean recovery, 91%), the values were as follows: s_r, 0.14 μg/g; s_R, 0.22 μg/g; RSD_r, 9.0%; and RSD_R, 12.7%. The method was adopted first action by AOAC International.

Iodine is an essential micronutrient and is necessary for proper functioning of the human thyroid gland. Through secretion of its iodine-containing hormones (thyroxine and triiodothyronine), the thyroid gland maintains the level of metabolism in tissues that is optimal for their normal function. The lack of these hormones causes poor mental and physical development in children and enlargement of the thyroid (goiter) in adults. Excessive intake of iodine can cause toxic goiter (thyrotoxicosis).

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The recommendation was approved by the General Referee and the Committee on Foods I and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

Iodine is a normal trace constituent in milk in the range of 100 to 500 μg/L. It is generally present in milk as iodide (I^-). Iodophores are organic compounds containing covalently bound iodine, which release iodine gradually and serve as disinfectants in the dairy industry. Much higher iodine levels are found in milk when iodophor contamination occurs during processing.

The most commonly used methods for measuring iodine in milk are catalytic/colorimetric analysis and ion-selective electrode (ISE) analysis. The catalytic/colorimetric method (1) is lengthy and requires special care (2) to avoid low recovery of iodine. The ISE method (3) is the simplest and most rapid but is subject to interferences from sulfur-containing amino acids. Neutron activation analysis (NAA) (4, 5) is useful as a reference method but requires a nuclear reactor.

In 1979, Heckman conducted an interlaboratory study of the radiochemical NAA and catalytic/colorimetric methods for iodine in foods at the low part-per-million level (6). Not only did the results of the 2 methods differ, but each method showed poor reproducibility among the laboratories involved. Results of that study confirmed the lack of a definitive method for the measurement of iodine in foods (as of 1979 technology), and Heckman recommended further work to improve the methods or to develop a new one.

Because no AOAC method was available, in 1985 AOAC requested development of a liquid chromatographic (LC) method for the determination of iodine in milk. The present report details the development, collaborative study, and performance of the LC method.

Method Development

The LC method of Hurst et al. for determining iodide (7, 8), which uses ion-pair LC and electrochemical detection, was taken as a starting point for the method reported here. The mobile phase used by Hurst contained phosphate buffer and an ion-pair reagent with EDTA added to reduce detector noise. We removed the EDTA because the detector noise level was acceptable without it. To further simplify the mobile phase, the concentrations of phosphate buffer and ion-pair reagent were reduced to 10mM and 1.0mM, respectively, and the acetoni-

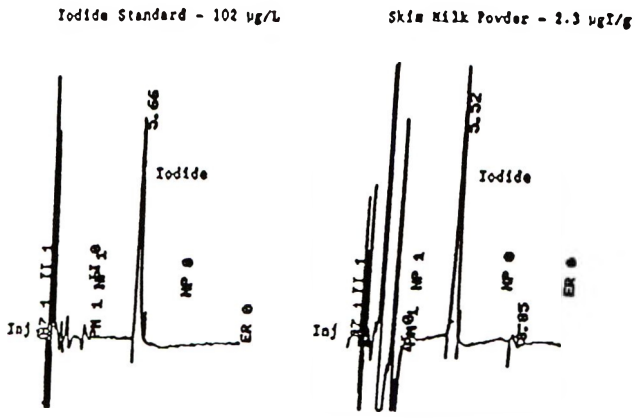

Figure 992.22. Typical liquid chromatograms of iodide in iodide standard solution and in powdered milk sample analyzed on 110 × 4.7 mm id PARTISPHERE C_{18} 5 µm, 2 mL/min flow rate, 60 µL injection, BAS LC-4B detector, 10 nA full scale, +50 mV.

trile content was increased to 32%. These conditions resulted in good chromatographic response, with adequate sensitivity and minimal noise (Figure **992.22**). A sample preparation procedure was developed for liquid and powdered milks by using exclusion membrane filtration (25 000 MW) to yield a clear filtrate for LC analysis.

Two methods for total iodine (epithermal NAA [ENAA] and Fischer catalytic/colorimetric [2]) were identified and evaluated outside Ross Laboratories as reference methods to establish the true levels of iodine in the milk samples for the study. ENAA (9) is an improved form of NAA that does not require radiochemical sample preparation before NAA analysis, uses epithermal neutrons to give more favorable activation of iodine, and uses an improved detection material with better wavelength resolution. These advantages give improved accuracy and sensitivity for iodine determination in the low part-per-million range. The Fischer catalytic method improves the accuracy of the catalytic method by taking extra precautions to minimize volatilization losses of iodine. Results for the 2 reference methods agreed well on the 7 milk samples in the collaborative study.

In 1987, after the proposed LC method was tested extensively at Ross Laboratories, it was evaluated at 3 outside laboratories on powdered and liquid milk samples. The method performed satisfactorily and, with minor modifications, was ready for collaborative study.

The proposed method quantitates iodine in the form of iodide (I^-) in the milk sample. After membrane filtration of the liquid samples, the clear filtrate is injected onto the LC system for separation of the iodide using reversed-phase ion-pair chromatography followed by detection by dc amperometry.

Collaborative Study

Design of the Study

In 1987, the International Dairy Federation (IDF) was contacted, and they agreed to cosponsor the study. A collaborative study design was submitted to AOAC in March 1988 and was approved. The study was designed to evaluate both the reproducibility and accuracy of the method on 2 liquid whole milk and 5 milk powder samples. Each collaborator received the 7 samples in blind duplicate and tested each sample in duplicate on 2 days. To establish total iodine levels, the 7 samples were also analyzed by ENAA at the University of Virginia and by the catalytic/colorimetric method (2) by Peter Fischer at Health and Welfare Canada.

Preparation of Study Samples

The 7 milk samples were selected to cover a wide range of iodine concentrations. The 2 whole milk samples (Liquids 1, 2) were commercial pasteurized whole milk (3.5% fat) from Columbus, OH, retail stores. The samples were transferred to 8 oz metal cans and were sterilized in the Ross pilot plant for storage and shipment to collaborators. The powdered milk samples

Table 1. Source of powdered milk samples for collaborative study on LC method for iodine in milk

Powder	Source	Certified iodine content (dry weight basis)
1	Skim milk control sample C. Olieman, Dutch Institute for Dairy Research, Ede, The Netherlands	—
2	Reference Standard CRM 150 H. Marchandise, Commission on European Communities, Brussels, Belgium Spiked skim milk powder	1.29 µg/g (Refs 10, 11) (dry 2 h at 0 in. Hg, 102°C
3	NBS Reference Standard 1549 Nonfat milk powder Robert Alvarez, NBS, Gaithersburg, MD	3.38 µg/g (Ref. 12) (dry 48 h at 30 in. Hg, 20–25°C)
4	Skim milk powder, Province of Quebec Carla Barry, Agriculture Canada, Ottawa, ON, Canada	—
5	Spiked skim milk powder Reference Standard CRM 151	5.35 µg/g (Refs 10, 11) (dry 2 h at 0 in. Hg 102°C)

were nonfat dry milk from North America and Europe (Table 1). Those samples were transferred on an as-is basis to tightly capped, 15 mL plastic containers for shipment.

Study Participants

The collaborative study was initiated with 12 laboratories from 4 countries. Participants analyzed a practice milk sample to become familiar with the method. Three of the collaborators had Dionex PAD II electrochemical detectors and experienced serious drift problems because of interactions of the mobile phase with the reference electrode, and they could not report results. Attempts to resolve the problem with input from Dionex were unsuccessful. (Dionex has since developed an improved reference electrode for the PAD II detector, which can be used with this mobile phase.)

The remaining 9 laboratories successfully analyzed the practice sample and were sent instructions, report forms, and the 7 collaborative study samples in blind duplicate. Two additional researchers from Ross Laboratories took part in the study as collaborators. Special care was taken so that these participants received no more instructions or assistance than did the other 7 laboratories.

992.22 Iodine (as Iodide) in Pasteurized Liquid Milk and Skim Milk Powder Liquid Chromatographic Method

First Action 1992

(Applicable to determination of iodine, as iodide, in liquid and powdered milk)

Method Performance:

Iodine, 303, 308 μg/L, in liquid milk

Mean recovery of total iodine = 87%

$s_r = 22$; $s_R = 22$; $RSD_r = 8.2\%$; $RSD_R = 8.3\%$

Iodine, 0.5–4.6 μg/g, in powdered skim milk

Mean recovery of total iodine = 91%

$s_r = 0.14$; $s_R = 0.22$; $RSD_r = 9.0\%$; $RSD_R = 12.7\%$

(*Caution*: Observe common safety procedures for operating centrifuge. Acetonitrile is extremely flammable and a skin irritant. If skin contact occurs, immediately flush affected area with large amounts of water.)

A. Principle

Iodine in milk samples is measured only in form of iodide (I^-). Protein and insoluble material in liquid milk samples are removed by passing through 25 000 MW cutoff membrane. Iodide in clear filtrate is separated by using reversed-phase ion-pair liquid chromatography (LC) and is selectively detected electrochemically with silver working electrode at 0 to +50 mV applied potential. Iodide is quantitated against external iodide standards.

B. Apparatus

(**a**) *Membrane cones.*—25 000–30 000 MW cutoff (Amicon CENTRIFLO, CF–25, Amicon Corp., Danvers, MA, and Millipore Ultrafree PF, Bedford, MA, are suitable).

(**b**) *Conical membrane support.*—To support membrane cone in centrifuge tube (Amicon No. CS1A is suitable).

(**c**) *Guard column (optional).*—C_{18} cartridge, 15 × 3.2 mm.

(**d**) *Analytical column.*—5 μm C_{18} to yield acceptable k′ and column efficiency (see **E**) (PARTISPHERE C_{18}, 110 × 4.7 mm, Whatman Inc., Clifton, NJ, is suitable).

(**e**) *Membrane filters.*—47 mm, NYLON-66 1.2 μm, 0.5 μm.

(**f**) *Centrifuge tubes.*—50 mL, 27 mm id, conical, disposable plastic, with screw cap.

(**g**) *Centrifuge.*—Capable of holding 50 mL centrifuge tubes and delivering 1000 rcf centrifugal force (rcf = 0.0000118 × radius (cm) × rpm × rpm). Lower rcf force can be used if centrifugation time is increased.

(**h**) *LC system.*—LC pump capable of 5000 psi and 5 mL/min flow rate; valve injector capable of 50–200 μL injections; electrochemical LC detector with silver working electrode at 0 to +50 mV potential (BAS LC-4B, Bioanalytical Systems, Inc., West Lafayette, IN, is suitable) or pulsed amperometric detector (PAD) (Dionex Corp., Sunnyvale, CA, is suitable) may be used with solvent compatible silver electrode kit; strip chart recorder or electronic integrator/plotter.

C. Reagents

(*Note*: Iodide is unstable in light; store all iodide solutions away from extended exposure to white light.)

(**a**) *Iodide reference standard solution.*—0.100 ± 0.001M sodium or potassium iodide solution (12.69 g iodide/L), recommend standardization traceable to certified reference material. Discard 12 months after standardization or 6 months after opening container. (Iodide reference standard traceable to National Institute of Standards and Technology [NIST] Standard Reference Material is available on special order from Ricca Chemical Co, Arlington, TX).

(**b**) *Reagent-grade chemicals.*—Dibasic sodium phosphate, 85% phosphoric acid, *n*-amyl alcohol (octanol may be substituted), and reagent alcohol (90% ethanol, 5% methanol, 5% propanol) (pure ethanol or 2-propanol may be substituted).

(**c**) *Acetonitrile.*—LC grade.

(**d**) *Hexadecyltrimethylammonium chloride solution.*—25% in H_2O (0.78M) hexadecyltrimethylammonium chloride (cetyltrimethylammonium chloride) (Aldrich Chemical, Milwaukee, WI, is suitable supplier).

(**e**) *LC mobile phase.*—10.0mM dibasic sodium phosphate, 1.0mM cetyltrimethylammonium chloride, 32% acetonitrile, pH = 6.8. Dissolve 1.42 ± 0.05 g dibasic sodium phosphate in ca 600 mL H_2O in 1 L beaker. Add 1.3 mL hexadecyltrimethylammonium chloride solution and mix. Add 320 mL acetonitrile and mix well. Adjust pH of mobile phase to 6.8 ± 0.1 with phosphoric acid solution. Dilute to 1 L with H_2O and mix well. Filter solution through 0.5 μm NYLON-66 membrane filter. (Mobile phase may be slightly cloudy before filtration. Prefiltering through 1.2 μm filter aids significantly in filtration through 0.5 μm filter. Dilute mobile phase slightly with H_2O or acetonitrile to adjust to desired iodide retention time.) Store in tightly capped container. Discard after 1 year.

(**f**) *50% acetonitrile solution.*—Add 500 mL acetonitrile to 1 L volumetric flask. Dilute to volume with H_2O and mix well. Store in stoppered glass container. Discard after 2 years.

(**g**) *20% alcohol solution.*—Add 200 mL reagent alcohol to 800 mL H_2O; mix well.

(**h**) *Stock iodide standard solution.*—101.5 mg/L. Pour ca 10 mL iodide reference standard solution, (**a**), into 50 mL beaker. Pipet 8.00 mL iodide standard into 1 L volumetric flask. Dilute to volume with H_2O; mix well. Store at room temperature and protect from white light. Discard after 30 days.

(**i**) *Intermediate iodide standard solution.*—1015 µg/L. Dilute 10.00 mL stock iodide standard solution, (**h**), to 1 L with H_2O. Mix well. Protect from white light. Discard after 7 days.

(**j**) *Working iodide standard solutions.*—254, 152, 102, and 20.3 µg/L. Dilute 25.00, 15.00, 10.00, and 2.00 mL intermediate standard solution, (**i**), to 100 mL with H_2O in separate volumetric flasks. Mix well. Protect from white light. Discard after 7 days.

D. Preparation of Sample

Soak new membrane cones in 20% alcohol solution ≥1 h. Remove cone from alcohol solution, drain, mount in support, and place in 50 mL centrifuge tube. Centrifuge 5–10 min at 900–1000 rcf to remove excess liquid from membranes. Invert and drain cone to confirm that no appreciable amount of liquid remains in cone before use. Place prepared cones into membrane supports mounted in clean, labeled centrifuge tubes for sample analysis.

For powdered milk samples.—Accurately weigh 4–4.5 g milk powder to nearest 0.01 g. Place in beaker containing 70–80 mL H_2O. Stir briskly 5–10 min to fully wet and mix milk powder. Visually verify complete wetting and mixing of powder. Transfer sample quantitatively to 100 mL volumetric flask, add 1 drop *n*-amyl alcohol to reduce foaming, and dilute to volume with H_2O. Mix well.

For liquid milk samples.—Dilute 10 mL sample with 10 mL H_2O and mix well.

Prepare 2 cones/sample.—Fill membrane cone with diluted powdered or liquid sample, above, to within 5 mm of cone top. Centrifuge samples at 900–1000 rcf 15–20 min (if centrifuge will not attain 900 rcf, use highest rcf available and increase time to attain enough filtrate for LC analysis). Inject filtrate from each cone separately on LC system. Soak used membrane cones and supports immediately in hot water. Flush cones well with stream of hot water to remove sample residue. Store in 20% alcohol solution. Membrane cones may be re-used several times.

E. LC Analysis

For new LC column, wash column with ca 50 mL 50% acetonitrile solution. Pump 30 mL mobile phase through column, discarding eluent. Turn on working electrode of detector and verify proper applied potential. Begin recycling mobile phase, and pump mobile phase through system at 2 mL/min until stable baseline is obtained (1 h minimum). Stable retention time and peak area or height reproducibility of 3% for consecutive injections of 254 µg/L standard iodide solution indicates equilibrated and properly operating system.

Iodide peak capacity factor (k′) should be 3–10, and column efficiency should allow quantitation of 20.3 µg/L working iodide standard solution. If using integrator, adjust parameters for proper integration of iodide peak, but do not integrate negative "tail" of iodide peak (see Figure **992.22**). If using manual peak heights, adjust injection volume or detector sensitivity for ≥70% full scale peak height for 254 µg/L working iodide standard solution. Adjust applied potential to EC detector working silver electrode within 0 to +50 mV to optimize peak shape and sensitivity.

When LC system is equilibrated, inject working iodide standard solutions and samples. Wait ≥5 min after iodide peak elutes before next injection. Distribute working iodide standard solution injections throughout run to assure adequate quantitation of samples and to account for any drift in system. (*Note*: We recommend injecting all 4 working iodide standard solutions every 6–8 sample injections.)

Mobile phase may be recycled between sample analyses or when standard solutions alone are being injected. However, when samples are being injected, do not recycle column eluent, because this can reduce column life and may lead to interfering peaks and gradually drifting baseline. During extended intervals between use, flush column and system with 50% acetonitrile solution, and reequilibrate column with mobile phase before next use. In routine use, recycle mobile phase at low flow rate (0.2 mL/min) to maintain system readiness.

F. Calculations

(*Note*: Silver electrode may yield negative tailing on iodide peak; see Figure **992.22**). Measure peak heights from baseline extended from baseline immediately before iodide peak. To accomplish this when using integrator equipped with negative peak function, activate negative peak function ca 1 min before iodide peak elutes and deactivate shortly before end of run.

Measure peak responses (areas or heights) of iodide peaks in chromatograms of working standards. (Peak area is preferred to peak height because peak shape can vary during run. Visually verify that each iodide peak is being detected reasonably well.) Perform linear least-squares analysis on relationship between standard concentration (µg/L) vs peak area (height) to obtain calibration line. (Use only the 4 working iodide standard solutions for calibration line; do not use zero [0, 0].) Correlation coefficient (r) of line should be ≥0.99.

For each sample injection, calculate iodide concentration, C, in µg/L, in sample filtrate from sample peak response and calibration line.

Calculate iodide level, I_L, in original liquid sample as follows:

$$I_L\ (\mu g/L) = C \times 2$$

where 2 = dilution factor of original sample.

Calculate iodide level, I_P, in original powdered milk sample as follows:

$$I_P\ (\mu g/g) = C \times 0.100L/W$$

Table 2. Collaborative results for LC determination of iodine in liquid and powdered milk[a]

Lab.	Day	Powder, µg iodide/g					Liquid, µg iodide/g	
		1	2	3	4	5	1	2
1	1	0.530	1.02	2.84	2.39	4.75	333	326
		0.507	1.02	2.75	2.40	4.74	327	335
	2	0.576	1.04	2.54	2.32	4.09	310	311
		0.532	0.971	2.47	2.57	3.78	323	305
2[b]	1	0.691	0.726	2.52	2.43	4.43	248	259
	2	0.480	1.20	2.49	2.44	3.78	308	296
3	1	0.624	1.20	2.17	2.24	3.78	248	258
		0.715	1.34	2.48	2.38	3.99	274	278
	2	0.544	1.16	2.43	2.13	3.84	254	267
		0.527	1.13	2.32	2.29	4.01	270	282
4	1	0.594	1.00	2.59	2.49	4.61	271	285
		0.531	0.938	2.46	2.49	4.52	280	275
	2	0.508	1.01	2.58	2.45	4.22	277	280
		0.608	0.986	2.72	2.60	4.25	282	269
5	1	0.613[b]	1.15[b]	2.66[b]	2.57[b]	4.69[b]	294[b]	286[b]
	2	0.547[b]	1.07[b]	2.91	2.75	4.86	197	278[b]
				2.79	2.83	4.93		
	3[c]	—	1.07	2.44[b]	2.47[b]	4.08[b]	—	—
			1.13					
6	1	0.657	1.17	2.10	2.33	4.18	267	256
		0.642	1.15	2.10	2.30	4.18	272	262
	2	0.543	1.10	1.98	2.20	4.47	276	272
		0.543	1.09	2.07	2.22	4.57	282	279
7	1	0.469	0.897	1.70	1.85	4.08	266	252
		0.403	0.935	1.77	1.88	4.17	270	252
	2	0.453	0.950	1.83	1.82	3.85	277	150[d]
		0.453	0.932	1.91	1.78	3.92	267	269
8[e]	1	0.719	0.727	2.02	2.41	4.27	273	260
	2	0.284	0.409	2.11	2.13	4.03	261	271
9	1	0.338	1.09	2.43	2.48	4.34	337	330
		0.360	1.03	2.40	2.49	4.28	338	336
	2	0.509	1.05	2.12	2.19	4.32	308	323
		0.613	1.01	2.20	2.16	4.29	329	306

[a] Samples were distributed as blind duplicates. Except where noted, each data point is the result from a single filtration and injection.
[b] Analyst pooled the 2 filtrates before injection.
[c] Data for Day 3 were not used in estimation of between-day and between-laboratory variances.
[d] Data point was not used in statistical analysis; it should not have been reported because of likely error in procedure.
[e] Analyst reported a single result for each day (single filtration and injection).

Table 3. Collaborative data used to estimate between-day and between-laboratory variance for LC method for iodine in liquid and powdered milk[a]

Lab.	Day	Powder, µg iodide/g					Liquid, µg iodide/g	
		1	2	3	4	5	1	2
1	1	0.530	1.02	2.84	2.39	4.75	333[b]	326[b]
	2	0.576	1.04	2.54	2.32	4.09	310[c]	311[c]
2	1	0.691	0.726[d]	2.52	2.43	4.43	248	259
	2	0.480	1.20[e]	2.49	2.44	3.78	308	296
3	1	0.624	1.20	2.17	2.24	3.78	248	258
	2	0.544	1.16	2.43	2.13	3.84	254	267
4	1	0.594	1.00	2.59	2.49	4.61	271	285
	2	0.508	1.01	2.58	2.45	4.22	277	280
5	1	0.613	1.15	2.66	2.57	4.69	294	286
	2	0.547	1.07	2.91	2.75	4.86	197	278
6	1	0.657	1.17	2.10	2.33	4.18	267	256
	2	0.543	1.10	1.98	2.20	4.47	276	272
7	1	0.469[b]	0.897	1.70	1.85[b]	4.08	266	252
	2	0.453[f]	0.950	1.83	1.82[g]	3.85	277	269
8	1	0.719	0.727[b]	2.02	2.41	4.27	273	260
	2	0.284	0.409[g]	2.11	2.13	4.03	261	271
9	1	0.338[b]	1.09	2.43	2.48	4.34	337[b]	330[b]
	2	0.509[f]	1.05	2.12	2.19	4.32	308[c]	323[c]

[a] Data identified as Cochran, Grubbs single, or Grubbs pair outliers were removed from the data set. See text for further information.
[b] First.
[c] Data identified as Grubbs pair outlier (high); removed from the data set.
[d] Second.
[e] Data identified as Cochran outlier; removed from the data set.
[f] Data identified as Grubbs pair outlier (low); removed from the data set.
[g] Data identified as Grubbs single outlier; removed from the data set.

where W = sample weight, g; and $0.100L$ = volume of diluted powdered milk.

Calculate mean of single determinations for filtrate from each membrane cone for each sample; round mean to 3 significant figures.

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

Results and Discussion

Data reported by the 9 collaborators are shown in Table 2. Each laboratory should have reported 4 results for each of the 7 samples: 2 within-day results (single filtration and injection) from each of 2 days of analyses. Laboratory 8 reported only single within-day results for each day. Laboratories 2 and 5 pooled their replicate filtrates before analysis and reported only a single result for each day. Laboratory 5 also reported data for

Table 4. Statistical analysis of collaborative results for LC determination of iodine in milk

	Powder, µg iodide/g					Liquid, µg iodide/g	
Statistic	1	2	3	4	5	1	2
			Source of variance				
Within-day	0.0017	0.0015	0.0078	0.0054	0.0081	77.6	62.4
Between-day	0.0174	—	0.0094	0.0090	0.0608	881.5	95.1
Between-lab.	—	0.0069	0.1060	0.0149	0.0468	—	14.0
Total variance	0.0191	0.0084	0.1232	0.0293	0.1157	959.1	171.5
Mean	0.565	1.07	2.33	2.37	4.25	266	271
			Standard deviations[a]				
Within-day	0.041	0.039	0.088	0.073	0.090	8.8	7.9
Between-day	0.132	—	0.097	0.095	0.257	29.7	9.8
Between-lab.	—	0.083	0.326	0.122	0.216	—	3.7
s_r	0.138	0.039	0.131	0.120	0.272	31.0	12.6
s_R	0.138	0.092	0.351	0.171	0.348	31.0	13.1
RSD_r, %	24.4	3.6	5.6	5.1	6.4	11.7	4.7
RSD_R, %	24.4	8.6	15.1	7.2	8.2	11.7	4.8
			Degrees of freedom				
Within-day	12	12	13	13	13	12	11
Between-day	7	9	9	8	9	7	7
Between-lab.	6	8	8	7	8	6	6

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

a third day, which was not used in the statistical analysis because it would imbalance the data unnecessarily.

Statistical Treatment

The SAS statistical computing package (13) was used to provide the estimates of the 3 variance components of the method: between-laboratory, between-day, and within-day variances. Cochran and Grubbs outliers were removed according to AOAC guidelines for collaborative study procedures (14).

Within-Day Precision

Data (Table 2) from only the 6 participants who completed the analyses according to the study design (Laboratories 1, 3, 4, 6, 7, and 9) were used to calculate within-day variance and within-day precision (s_{wd}). When the sequence of outlier tests was applied to these data, no Cochran outliers were found for pairs of within-day results. An estimate of within-day variance was then made on the data set from the 6 laboratories.

Between-Day and Between-Laboratory Precision

For the estimates of between-day and between-laboratory variance, the use of as many laboratories as possible is desirable. However, only a small lack of balance in the data set can perturb the variance estimates to a significant degree (15). The pooled values reported by Laboratories 2 and 5 were judged to

Table 5. Summary of statistical analysis of variance of collaborative data for LC determination of iodine in milk

Variance source	% of total variance (min.–max.)	Av. % of total variance
Powders		
Within-day	6–18	12
Between-day	0–91	36
Between-lab.	0–86	52
Liquids		
Within-day	8–36	22
Between-day	56–92	74
Between-lab.	0–8	4

Table 6. Summary of repeatability and reproducibility of LC method for iodine in milk

Statistic	Range over samples	Average
Powders		
s_r, µg/g (RSD_r)	0.04–0.27 (4–25%)	0.14 (9.0%)
s_R, µg/g (RSD_R)	0.09–0.35 (7–24%)	0.22 (12.7%)
Liquids		
s_r, µg/L (RSD_r)	13–31 (5–12)	22 (8.2%)
s_R, µg/L (RSD_R)	13–31 (5–12)	22 (8.3%)

Table 7. Accuracy of the LC method for determination of total iodine in milk

	Powder, μg iodine/g					Liquid, μg iodine/g	
Statistic	1	2	3	4	5	1	2
ENAA analysis, mean ± SD	0.527 ± 0.053	1.11 ± 0.08	3.21 ± 0.13	2.92 ± 0.09	4.63 ± 0.14	301 ± 16	312 ± 16
Fischer catalytic method, mean ± SD	0.535 ± 0.005	1.09 ± 0.02	2.96 ± 0.06	2.78 ± 0.03	4.65 ± 0.03	305 ± 2	303 ± 2
Av. of data for ENAA & catalytic method	0.531	1.10	3.09	2.85	4.64	303	308
LC method, av. (as-is basis)	0.565	1.07	2.33	2.37	4.26	266	271
Bias of LC method (LC – total)	+0.034	–0.03	–0.76	–0.48	–0.38	–37	–37
LC recovery of total iodine, % [(LC/total) × 100]	106.4	97.3	75.4	83.2	91.8	87.8	87.8

be equivalent to a single replicate result from the group of 6 laboratories from Laboratory 8. For this reason, only results from the first analysis on each day were used to provide data from all 9 laboratories (Table 3). This maximized the degrees of freedom for estimating between-laboratory variance and maintained a balanced data set. The sequence of outlier tests (Cochran, Grubbs single, and Grubbs pair) was applied to the data set, and the sequence was started over when an outlier was found.

The Cochran test was run to identify exceptionally large variation among laboratory day-to-day duplicate results. No outlier duplicates were found.

The single-value Grubbs test was run to identify a laboratory with an extreme average result. Low outliers were found for Laboratory 8/Powder 2 and Laboratory 7/Powder 4, and these results were removed from the data set.

The Cochran test was rerun, and the results for Laboratory 2/Powder 2 were found to be outliers and were removed from the data set. All remaining data passed the single-value Grubbs test.

The pair-value Grubbs test was run to identify pairs of laboratories with extreme average results. For Liquids 1 and 2, Laboratories 1 and 9 were high outliers, and for Powder 1, Laboratories 7 and 9 were low outliers. These outliers were removed from the data set.

The Cochran test found no outliers on the remaining data.

The single-value Grubbs test then identified Laboratory 8/Powder 1 as an outlier. The pair-value Grubbs test identified Laboratories 3 and 5/Liquid 1 as outlying pairs. None of these was removed from the data set, because 2 of the 9 laboratories for Powder 1 and Liquid 1 were already discarded as outliers, and removal of additional laboratories would have violated the "2/9" rule. No Grubbs outliers (single or pair) were found for the other 5 collaborative samples. Outlier testing was suspended at this point.

Table 8. Summary of accuracy data of LC method for iodine in milk

Statistic	Range over samples	Average
Powders		
Bias	+0.03 to –0.76 μg/g	–0.32 μg/g
Recovery	75–106%	90.8%
Liquids		
Bias	–37 μg/L	–0.037 μg/g
Recovery	87.8%	87.8%

The remaining data (Table 3) were used to calculate between-laboratory variance and (between-day + within-day) variance. The between-day variance was then calculated by subtracting the within-day variance from the (within-day + between-day) variance. The between-day precision (s_{bd}) and between-laboratory precision (s_L) were calculated. The results of this analysis are shown in Table 4.

Method Repeatability and Reproducibility

The method repeatability (s_r) reflects 2 sources of variability: within-day and between-day.

$$s_r^2 = s_{wd}^2 + s_{bd}^2$$

The method reproducibility (s_R) reflects all 3 sources of variability: within-day, between-day, and between-laboratory.

$$s_R^2 = s_{wd}^2 + s_{bd}^2 + s_L^2 = s_r^2 + s_L^2$$

Results of these calculations are shown in Table 4.

Summary of Method Precision

Table 4 shows the results of the statistical analysis of the collaborative study data. The total variance of the method is distributed among within-day, between-day, and between-laboratory sources in various proportions (Table 5). Table 6 summarizes method repeatability and reproducibility.

Summary of Method Accuracy

The certified iodine contents of the 3 reference standard milk powders were not used, because the moisture contents of these powders were not measured according to the National Bureau of Standards (now NIST) and Community Bureau of Reference procedures. The accuracy of the method is estimated by the bias (LC iodine – total iodine by reference methods) and by the percent recovery (LC results as percent of total iodine in

Table 9. Method performance criteria

Criteria	Powdered milk	Liquid milk
Applicable iodine level	0.4–10 µg/g	20–1000 µg/L
Minimum detectable level	0.2 µg/g	10 µg/L
Average iodine level tested	3.0 µg/g	300 µg/L
Average repeatability, s_r (RSD_r)	0.14 µg/g (9.0%)	22 µg/L (8.2%)
Average reproducibility, s_R (RSD_R)	0.22 µg/g (12.7%)	22 µg/L (8.3%)
Average method bias	–0.3 µg/g	–42 µg/L
Average recovery of total iodine	91%	87%

the samples) of the LC iodine measurements. These estimates are shown in Table 7 and summarized in Table 8.

The relatively high recoveries of total iodine by the LC method indicate that about 90% of the iodine in milk is in the free iodide (I^-) form.

Performance Criteria of Method

The performance criteria of the LC method are summarized in Table 9.

Collaborators' Comments and Poststudy Revisions

Comments were sent by 6 of the 9 collaborators reporting results. No major problems with the method were reported. The method performed well with the variety of LC columns and electrochemical detectors used (Table 10).

Three collaborators reported difficulties with the Amicon membrane cones used to clean up the milk samples before analysis. The cones leak occasionally and, after several uses, tend to plug up and must be cleaned or replaced. Instead of using the Amicon cones, one collaborator (Laboratory 8) successfully used Millipore Ultrafree PF filter units (30 000 NMWL) to achieve a clear filtrate. This alternative cleanup technique qualifies as "equivalent" to the Amicon cones. Another collaborator (Laboratory 7) obtained equivalent results on the study samples by using centrifugation at 20 000 × *g* to yield a clear supernatant for injection on the LC system. This alternative approach is promising and merits further investigation.

Laboratory 8 modified the detector by using a porous carbon working electrode at –200 mV in the electrochemical detector, along with postcolumn flow switching, and reported acceptable data. This modified detection could be equivalent to the one specified in the method if the iodide is detected selectively, which apparently it is on the basis of the data. This detection system needs further investigation before it can be included in the method.

Recommendation

We recommend that the LC method for iodide in milk be adopted first action.

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Kim Ponto and Terry Daniels, Ross Laboratories, Columbus, OH

David Sakai, Calreco Inc., Van Nuys, CA

Total Iodine Analyses

Paul Beneche, Reactor Facility, University of Virginia, Charlottesville, VA (ENAA analysis)

Table 10. Columns and detectors used in collaborative study on LC method for determination of iodine in milk

Lab.	LC column packing	Particle diam., µm	Electrochemical detector	Applied potential, mV
1	Whatman Partisphere ODS	5	Waters 460	0
2	Brownlee V18–103	3	BAS LC–4B	0
3	Supelcosil LC–18	5	BAS LC–4B	+50
4	Whatman Partisphere ODS	5	BAS LC–4B	0
5	Waters µBondapak C–18	10	BAS	0
6	Brownlee V18–103	3	BAS LC–4B	0
7	Waters Novapak C18	5	Metrohm 656	0
8	Whatman Partisphere C18	5	Coulochem 5100A (carbon electrode)	–200
9	Whatman Partisphere ODS	5	BAS LC–4B	0

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DRUG RESIDUES IN ANIMAL TISSUES

Simultaneous Determination of Xylazine and Its Major Metabolite, 2,6-Dimethylaniline, in Bovine and Swine Kidney by Liquid Chromatography

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A liquid chromatographic (LC) method is described for the simultaneous determination of xylazine (XY) and its major metabolite, 2,6-dimethylaniline (2,6-DMA), in bovine and swine kidney in the 25–100 ppb range. XY and 2,6-DMA are extracted from kidney with chloroform, followed by cleanup on an acidic Celite 545 column. A μBondapak phenyl column is used for LC separation with UV determination at 225 nm. The mobile phase is a mixture of acetonitrile, water, sodium acetate, and acetic acid. Mean recoveries from bovine kidney were 78.3% for XY, with a standard deviation (SD) of 7.45 and a coefficient of variation (CV) of 9.51%, and 87.2% for 2,6-DMA, with an SD of 8.38 and a CV of 9.61%. Mean recoveries from swine kidney were 80.8% for XY, with an SD of 5.92 and a CV of 7.33%, and 86.7% for 2,6-DMA, with an SD of 6.16 and a CV of 7.10%.

Xylazine (XY) is used widely in foreign countries as a tranquilizer for both domestic and wild animals. It is used not only therapeutically but also to reduce aggressiveness and activity associated with livestock breeding. A special application is its use to treat animals during transport to slaughterhouses to avoid death and loss of meat quality caused by stress reactions. Administration of this drug to major food-producing species has never been officially approved by the U.S. Food and Drug Administration, and recent concerns over the possible carcinogenicity of XY and its major metabolite, 2,6-dimethylaniline (2,6-DMA), have created a need to monitor the fate of XY in bovine and swine tissue. The chemical structures of XY and its metabolite, 2,6-DMA, are given in Figure 1.

A literature search revealed that no method is available that will simultaneously determine XY and its metabolite. Typical approaches for determining XY have involved the use of gas chromatographs equipped with specific detectors, column extraction, spectrophotometry, and liquid chromatography (LC) (1–6). In 1989, Van Ginkel et al. (7) reported an LC method that uses UV spectrophotometry to determine tranquilizers in pig kidneys and thin-layer chromatography to confirm their identities. In the same year, Keukens and Aerts (8) reported an LC procedure with UV and fluorescence detection for the determination of tranquilizers in swine kidneys; this analysis involves extraction with acetonitrile and cleanup on a Sep-Pak C_{18} cartridge.

The purpose of this study was to develop a rapid chemical method for the simultaneous determination of XY and 2,6-DMA in bovine and swine kidneys. A number of methods for determining XY in different products (2, 6, 7, 9–12) were investigated for their applicability to the problem. This paper describes the developed method, which uses solvent extraction and solid-phase extraction cleanup followed by LC.

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METHOD

Apparatus

(a) *Pasteur pipet.*—Disposable, 5.75 in. (14.6 cm).

(b) *Centrifuge bottle.*—250 mL with glass stopper, 29/26 (No. K-322000-0021, Kontes, Vineland, NJ 08360).

(c) *Rotary vacuum evaporator.*—Buchi/Brinkmann Rotavap-R (Brinkmann Instruments, Inc., Westbury, NY 11590) with water bath at 55°C, or equivalent.

(d) *Glass microfiber filters.*—Whatman GF/C, 15.0 cm (Cat. No. 28497–787, VWR Scientific, Denver, CO 80239).

(e) *Nylon filters.*—Phenomenex 25 mm, 0.45 μm (Part No. AFO–0414, Phenomenex, Torrence, CA 90501).

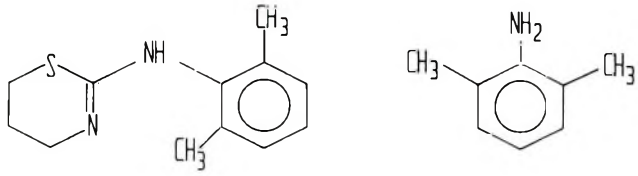
(f) *Chromatographic tube.*—22 × 300 mm (K–420550–233, Kontes).

(g) *Centrifuge.*—Model DPR–6000 (International Equipment Co., Needham Heights, MA 02914).

(h) *LC column.*—Waters μBondapak phenyl, 10 μm, 3.9 mm × 30 cm (Millipore–Waters, Milford, MA 01757).

(i) *Liquid chromatograph.*—Waters Model 510 LC pump and Rheodyne injector with 50 μL loop (Millipore–Waters).

(j) *UV/vis spectrophotometric detector.*—Shimadzu SPD–6AV module for LC; cell volume, 8 μL; light source, deuterium (D_2) lamp; absorbance range, 0.005 AUFS (Shimadzu Corp., Kyoto, Japan). Operating conditions: chart speed,



XYLAZINE 2,6-DIMETHYLANILINE

Figure 1. Chemical structures of XY and its major metabolite, 2,6-DMA.

0.25 cm/min; mobile phase flow, 1 mL/min; column temperature, ambient; injection volume, 50 µL.

(**k**) *Recorder.*—Dual channel SE–120 strip chart recorder set at 10 mV (BBC-Metrawatt/Goerz, Broomfield, CO 80020).

(**l**) *Boiling flasks.*—250 and 500 mL flat-bottom flasks, with T 24/40 joint (Cat. No. 29112–108 and 29112–120, VWR Scientific).

(**m**) *Tissuemizer.*—Model SDT–1810, equipped with Model TR–10 power control and SDT–182EN shaft and generator (Tekmar Co., Cincinnati, OH 45222–1856).

(**n**) *Blender.*—Waring, or equivalent.

Reagents

(**a**) *Xylazine standard.*—2-(2,6-Dimethylphenylamino)-4*H*-5,6-dihydrothiazine hydrochloride, 99% pure (Sigma Chemical Co., St. Louis, MO 63178). *Stock solution.*—100 µg/mL in methanol. Dilute aliquot of stock solution to desired working standard concentration with methanol. *Fortification standard solution.*—10 µg/mL in methanol. Keep all standard solutions in amber glassware and store under refrigeration.

(**b**) *2,6-Dimethylaniline standard.*—99% pure (Cat. No. D14,600-5, Aldrich Chemical Co., Inc., Milwaukee, WI 53201). *Stock solution.*—100 µg/mL in methanol. Dilute aliquot of stock solution to desired working standard concentration with methanol. *Fortification standard solution.*—10 µg/mL in methanol. Keep all standard solutions in amber glassware and store under refrigeration.

(**c**) *Solvents.*—ACS reagent grade HCl; distilled-in-glass methanol, *n*-hexane, chloroform, acetonitrile, and petroleum ether. Petroleum ether used in the extraction and column preparation steps should be prepared by washing with water (water saturated).

(**d**) *LC mobile phase.*—Acetonitrile–water–2M sodium acetate–1M acetic acid (32 + 64 + 2 + 2).

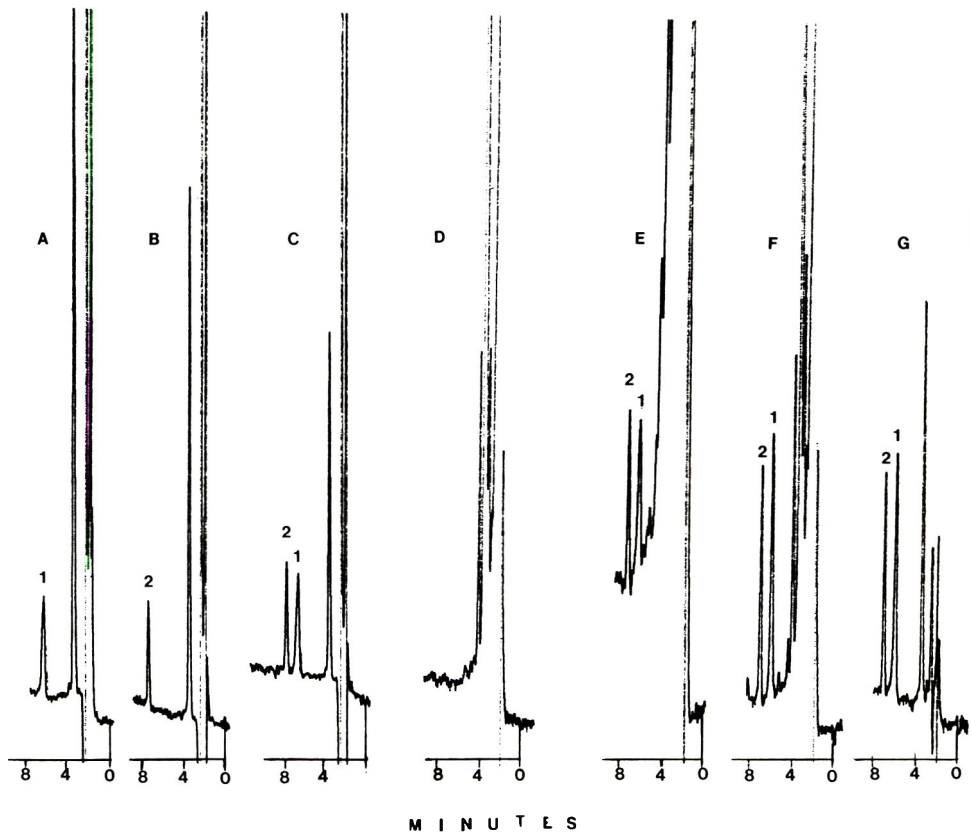


Figure 2. Liquid chromatograms from analysis of bovine kidney: (A) 4.97 ng XY standard injected (peak 1), (B) 4.94 ng 2,6-DMA standard injected (peak 2), (C) XY and 2,6-DMA mixed standard (peaks 1 and 2), (D) control kidney, (E) control kidney fortified at 25 ppb XY and 2,6-DMA (peaks 1 and 2), (F) control kidney fortified at 100 ppb XY and 2,6-DMA (peaks 1 and 2), and (G) mixed standard: 9.87 ng XY and 9.84 ng 2,6-DMA injected (peaks 1 and 2).

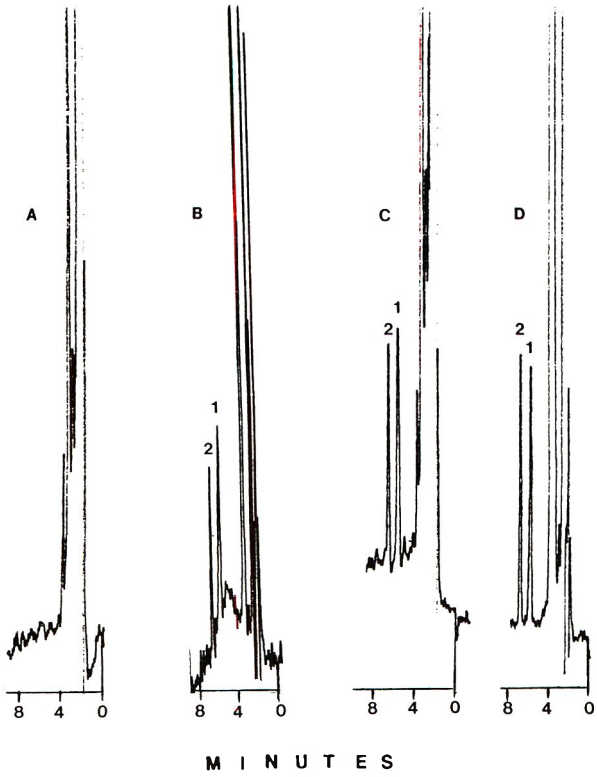


Figure 3. Liquid chromatograms from analysis of swine kidney: (A) control kidney, (B) control kidney fortified at 25 ppb XY and 2,6-DMA (peaks 1 and 2), (C) control kidney fortified at 100 ppb XY and 2,6-DMA (peaks 1 and 2), and (D) mixed standard: 9.87 ng XY and 9.84 ng 2,6-DMA injected (peaks 1 and 2).

(e) *Hydrochloric acid solution.*—1N. Add 83.4 mL ACS grade HCl to ca 500 mL water. Let solution cool, and dilute to 1 L with water.

(f) *Acid-washed Celite 545.*—Johns-Manville, Denver, CO 80217. Purify as follows: Slurry ca 250 g Celite with HCl–water (1 + 1) (ca 800 mL) and heat on steam bath several hours. Stir occasionally with glass rod. Let mixture settle and pour off acidic solution. Reslurry and heat again for several hours. Decant acidic solution. Wash Celite with water until pH of washings is neutral. Wash Celite with successive 250 mL portions of methanol, *n*-hexane, and petroleum ether. Place washed Celite in shallow dish, heat on steam bath to remove residual solvents, and dry at 105°C until material is powdery and solvent-free.

Sample Preparation

Bovine/swine kidney.—Cut away as much fat from kidney as possible and discard fat. Homogenize kidney in Waring blender. Weigh 50–75 g homogenate into Whirl-Pak bag. Seal bag and keep tissue frozen (–70°C) until analysis.

Recovery Studies

For recovery determinations of 100, 50, and 25 ppb standards, fortify three 20 g portions of homogenized control bovine/swine kidney tissue with 0.2, 0.1, and 0.05 mL of each fortification standard, respectively. Perform recovery determinations in conjunction with analyses of unfortified bovine/swine kidney tissue.

Table 1. Recovery of XY and 2,6-DMA from bovine kidney

	XY			2,6-DMA	
Added, ppb	Rec., ppb	Rec., %	Added, ppb	Rec., ppb	Rec., %
98.7	96.3	97.6	98.4	94.5	96.0
	80.8	81.9		100.2	101.8
	78.9	79.9		88.8	90.2
	79.0	80.0		100.5	102.1
	80.1	81.2		90.7	92.2
	84.3	85.4		96.7	98.3
Mean	83.2	84.3		95.2	96.8
SD		6.80			4.91
CV, %		8.07			5.08
49.4	34.6	70.0	49.2	38.2	77.6
	36.3	73.5		39.0	79.3
	34.4	69.6		43.1	87.6
	37.9	76.7		43.0	87.4
	40.1	81.2		44.3	90.0
	38.8	78.5		39.2	79.7
Mean	37.0	74.9		41.1	83.6
SD		4.69			5.31
CV, %		6.26			6.35
24.7	21.5	87.0	24.6	20.9	85.0
	18.5	74.9		19.7	80.1
	20.2	81.8		20.3	82.5
	17.6	71.3		21.2	86.2
	16.9	68.4		18.6	75.6
	17.3	70.0		19.1	77.6
Mean	18.7	75.6		20.0	81.2
SD		7.36			4.16
CV, %		9.74			5.13

Extraction

Weigh 20 g homogenized kidney tissue into 250 mL centrifuge bottle. Add 70 mL chloroform. Homogenize tissue for 1–1.5 min at medium to high speed. Rinse Tissuemizer blade with ca 5 mL chloroform, and collect washings in bottle. Centrifuge ca 10 min at 1800 rpm. Dislodge tissue cake by tilting bottle and tapping bottle with fingers or large glass stirring rod. Decant supernatant through folded glass fiber filter into 500 mL flask containing 1 mL 1N HCl. Repeat extraction by adding 70 mL chloroform to homogenate. Break up tissue cake with blade and homogenize 1–1.5 min. Rinse blade with ca 5 mL chloroform, and collect washings in bottle. Centrifuge and then decant supernatant through filter into 500 mL flask containing first supernatant. Rinse filter with ca 30 mL chloroform, and rotoevaporate contents of flask to dryness at 55°C. Add ca 25 mL methanol to flask and evaporate contents of flask to dry-

ness. Add 25 mL petroleum ether to warm flask and swirl contents of flask to dissolve fatty extract.

Column Chromatography

Column preparation.—Place small pledget of glass wool in constricted portion of stem of 22 × 300 mm chromatographic tube, and place glass wool pad ca 5 mm thick in bottom of large portion of tube. Blend 0.5 mL water uniformly with 2 g Celite. Transfer mixture to tube with aid of powder funnel and tamp mixture lightly with packing rod. Blend 1.0 mL 1N HCl uniformly with 3 g Celite. Transfer mixture to tube on top of original 2 g Celite and tamp packing lightly. Place small pledget of glass wool on top of column.

Transfer 25 mL petroleum ether extract to column. Complete transfer of extract to column with 3 additional 10 mL portions of petroleum ether; let each portion enter sorbent bed before adding the next. Wash flask with two 50 mL portions of methanol–petroleum ether (1.5 + 98.5) and add wash solution to column. Let first volume of wash solution enter sorbent bed before adding the second. Collect wash solution as it passes through column and discard. After all of wash solution has passed through column, rinse stem of chromatographic tube with wash solution.

Place 250 mL flat-bottom flask under column. Elute column with two 50 mL portions of methanol. Rinse tip of chromatographic tube with methanol, collect washings in flask, and rotoevaporate contents of flask to dryness. Add ca 25 mL methanol to flask and evaporate contents of flask to complete dryness. Quantitatively dissolve residue in 4–10 mL mobile phase by swirling flask. Filter concentrated eluate through nylon filter attached to 5 mL syringe; collect filtered eluate in small glass-stoppered Erlenmeyer flask.

Liquid Chromatography

Mixed working standard solution(s).—Pipet appropriate volume of each fortification standard solution into same 50 mL pear-shape boiling flask. Add 1 mL 1N HCl and 25 mL methanol, and evaporate to dryness. Add additional 25 mL methanol and again evaporate to dryness. Dissolve standard(s) in 4–10 mL mobile phase for LC.

Determination.—Inject 50 µL concentrated eluate into liquid chromatograph. Dilute concentrated eluate with mobile phase as necessary for quantitation. Bracket each set of eluate injections with injections (50 µL) of mixed working standard solutions. Determine concentrations of XY and 2,6-DMA in concentrated eluate by comparing responses obtained for eluate with those obtained for mixed working standard solution(s). If concentrated eluate contains late-eluting components, increase flow rate of mobile phase for several minutes or until components are eluted from LC column. Readjust flow rate and let system equilibrate before next injection.

Results and Discussion

The bovine/swine kidneys used for this study were purchased from a local meat market. The prepared tissue was stored in a freezer at −70°C approximately 6 months before use.

Table 2. Recovery of XY and 2,6-DMA from swine kidney

XY			2,6-DMA		
Added, ppb	Rec., ppb	Rec., %	Added, ppb	Rec., ppb	Rec., %
98.7	80.3	81.4	98.4	85.9	87.3
	78.9	79.9		87.2	88.6
	77.7	78.7		85.9	87.3
	80.8	81.9		91.6	93.1
	78.3	79.3		92.8	94.3
	89.0	90.2		97.2	98.8
Mean	80.8	81.9		90.1	91.6
SD		4.25			4.63
CV, %		5.18			5.06
49.4	37.6	76.1	49.2	40.0	81.3
	38.5	77.9		37.2	75.6
	38.9	78.7		39.1	79.5
	37.0	74.9		43.3	88.0
	36.9	74.7		44.1	89.6
	40.1	81.2		39.9	81.1
Mean	38.2	77.2		40.6	82.5
SD		2.51			5.30
CV, %		3.25			6.43
24.7	21.9	88.7	24.6	19.8	80.5
	21.2	85.8		21.9	89.0
	19.1	77.3		20.3	82.5
	18.6	75.3		22.1	89.8
	23.8	96.4		23.0	93.5
	18.9	76.5		19.9	80.9
Mean	20.6	83.3		21.2	86.0
SD		8.41			5.44
CV, %		10.09			6.33

Both compounds under investigation were determined by measurement of UV absorbance. The wavelength of maximum absorption for XY and 2,6-DMA was below 225 nm. Measurement of absorbance at 225 nm was selected to eliminate background interference.

In this study, determination of 2,6-DMA presented the greatest challenge. During the enrichment step, losses of 2,6-DMA occurred because of the compound's great volatility. To solve this problem, 2,6-DMA was stabilized by addition of 1N HCl to the flask containing the chloroform extract before rotoevaporation. The analytes were retained on an acidic Celite column that was washed with petroleum ether and methanol–petroleum ether (1.5 + 98.5) to remove residual fatty materials and other interferences. The analytes were stripped from the column with methanol, and the eluate was evaporated to dryness. The residue was dissolved in the mobile phase, and the solution was filtered before LC analysis. The analytes are sta-

ble in the mobile phase and can be stored under refrigeration overnight.

The LC mobile phase used in this procedure, a mixture of acetonitrile, water, 2M sodium acetate, and 1M acetic acid, was a modification of the mobile phase used by Etter et al. (6). With the LC column and mobile phase used in the procedure, we were able to obtain baseline separation of the analytes with little or no interference at the retention times of XY and 2,6-DMA shown in Figures 2 and 3. By using column chromatography for cleanup, we were able to process several samples at the same time without having to deal with the formation of emulsions.

Figure 2 shows typical UV chromatograms of XY, 2,6-DMA, mixed standards, control bovine kidney, and XY and 2,6-DMA recovered from control (fortified) bovine kidney.

Figure 3 shows typical UV chromatograms of control swine kidney, XY, and 2,6-DMA recovered from control (fortified) swine kidney, and XY and 2,6-DMA mixed standard.

Table 1 shows the recoveries of XY and 2,6-DMA from control bovine kidney fortified at 3 levels. Mean recoveries of 2,6-DMA were higher than those of XY at all 3 fortification levels. Mean recoveries of both compounds were greatest at the highest level of fortification.

Table 2 shows the recoveries of XY and 2,6-DMA from control swine kidney fortified at the same levels as the bovine kidney. The recovery pattern was the same as that shown in Table 1.

Mean recoveries of 2,6-DMA from swine and bovine kidney were 91.6 and 96.8%, respectively, for the highest fortification level, and 86.0 and 81.2%, respectively, for the lowest fortification level. Mean recoveries of XY from swine and bovine kidney were 81.9 and 84.3%, respectively, for the highest fortification level, and 83.3 and 75.6%, respectively, for the lowest fortification level. In all cases, recoveries of XY and 2,6-DMA were closer at the 2 lower fortification levels.

The results indicate that this LC procedure is adequate for determination of XY and 2,6-DMA in swine and bovine kidney. Although developed for kidney tissue, the method may be applicable to other matrixes, e.g., urine, blood, and other tissues, with or without slight modifications.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Method for Determination of Sulfamethazine Residues in Milk: Collaborative Study

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Seven laboratories participated in a collaborative study of a liquid chromatographic (LC) method for determination of sulfamethazine (SMZ) residues in raw milk that were previously frozen. The milk is extracted with chloroform, the chloroform is evaporated, and the residue is suspended in hexane and extracted with 0.1M KH_2PO_4 (PDP) solution. The PDP extract is analyzed by LC on a C_{18} column with methanol–0.1M PDP (30 + 70) as mobile phase. Individual laboratories were instructed to analyze 5 replicates each of control milk, fortified control milk at 2 levels, and 3 blind samples. Blind samples included raw milk fortified with SMZ at 10 and 20 ppb and 1 sample containing SMZ residue from a dosed cow. For blind fortified samples containing 10 ppb SMZ, average recovery and relative standard deviations for repeatability and reproducibility (RSD_r and RSR_R) based on the results from 6 of the 7 participating laboratories were 8.21 ppb, 7.16%, and 23.16%, respectively. Similar data, including results from a seventh participant who reported instrumental problems but was not eliminated by the Dixon outlier test, were 9.13 ppb, 8.38%, and 31.94%, respectively. These results demonstrate that the method is suitable for the determination of SMZ residues in milk at 10 ppb and above. The method was adopted first action by AOAC International.

A liquid chromatographic (LC) method for determination of sulfamethazine (SMZ) residues in milk was developed in this laboratory (1). The method was used in a limited nationwide survey of shelf milk for SMZ residues in 1988. Results of that 10-city survey corroborated the need for a validated method for the determination of SMZ residues in milk. A trial was conducted in 6 U.S. Food and Drug Administration (FDA) laboratories and a laboratory of Health and Welfare Canada. The results are reported here as a collaborative study. The method (1) was also used in a dairy cattle feeding study (2) to determine if feed contamination could be the source of the SMZ residues observed in the survey.

Collaborative Study

The 7 collaborators were supplied instructions and a copy of the method, which was essentially the same as that in Ref. 1. The frozen samples were subsequently packed in dry ice and shipped to the collaborators with instructions that they be kept frozen until analysis.

Control raw milk was collected and analyzed by the authors. A portion was subdivided and frozen. Other portions were fortified at 2 levels, 10 and 20 ppb, and each of these portions was subdivided and frozen. Studies in our laboratories indicate that freezing milk and holding it at −80°C for up to 1 year does not significantly influence the SMZ levels. The cow was dosed with sulfamethazine, and milk was collected every 12 h postdosage. Test portions of the milk were analyzed until the level of sulfamethazine in the test portion was about 14 ppb. That milk was subdivided, and the subdivided portions were used in the study as incurred milk samples. The portions of milk were labeled with code numbers and identified as control, fortified, or incurred milk. Portions of the prepared samples were thawed and reanalyzed. The frozen samples were sent to the collaborators.

Collaborators were requested to prepare standards, calibrate chromatographs, and check responses for linearity. To become familiar with the method, the collaborators were requested to analyze 2 control milk samples, to fortify control milk at 5 and at 10 ppb SMZ, and to analyze 2 samples of each fortified control milk. After this initial familiarization, they were instructed

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The recommendation was approved by the General Referee and the Committee on Drugs and Related Topics and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

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to evaluate the method by analyzing 5 replicates of control milk, 5 replicates each of the 2 blind samples, and 5 replicates of the incurred milk.

992.21 Sulfamethazine Residues in Raw Bovine Milk—Liquid Chromatographic Method

Final Action 1992

(Applicable to determination of 10–20 ppb sulfamethazine in raw bovine milk.)

Method performance:

10 ppb fortified, blind coded

$s_r = 0.59$; $s_R = 1.92$; $RSD_r = 7.16\%$; $RSD_R = 23.16\%$

20 ppb fortified, blind coded

$s_r = 1.18$; $s_R = 3.84$; $RSD_r = 6.76\%$; $RSD_R = 22.05\%$

14.8 ppb incurred, blind coded

$s_r = 0.83$; $s_R = 2.74$; $RSD_r = 5.62\%$; $RSD_R = 18.51\%$

A. Principle

Milk is extracted with chloroform. Chloroform is evaporated, and residue is suspended in hexane and extracted with KH_2PO_4 solution. Sulfamethazine is separated by liquid chromatography (LC), using deactivated-phase C_{18} column and detected by UV absorption at 265 nm.

B. Apparatus

(**a**) *LC system.*—With injector, column heater, UV/vis detector (set to 265 nm), and integrator. Operating conditions: mobile phase isocratic flow rate, 1.5 mL/min; injection volume, 100 µL; run time, 15 min with 1 min equilibration between runs; column temperature, 35.0 ± 0.2°.

(**b**) *LC columns.*—250 × 4.6 mm id column and 20 × 4.6 mm id guard column, both reversed-phase (deactivated) octadecyldimethylsilyl, 100 Å pore diam., 5 µm particle size; and 0.5 µm low-dispersion precolumn filter with 3 mm diam. (Supelcosil LC-18-DB, Supelco, Inc., Bellefonte, PA 16823, was found suitable).

(**c**) *Rotatory evaporator.*—Set at 32 ± 2° (Buchi, Laboratory Techniques Ltd, Flawil, Switzerland, was suitable supplier).

(**d**) *Vortex mixer.*

(**e**) *Polypropylene tubes.*—50 mL.

(**f**) *Pipettors.*—1 mL, 10 mL, and 100 µL.

(**g**) *Fluted filter paper.*—12.5 cm coarse particle retention with fast flow rate (Schleicher & Schuell Cat. No. 588 was found to be suitable).

(**h**) *Nylon filter.*—0.4 µm porosity Nylon 66 (N66) filter (Supelco, Inc., PA, is suitable).

(**i**) *Pear-shaped flasks.*—100 mL, glass with 24/40 standard taper neck, with stoppers (Kontes Glass Co, Vineland, NJ, is suitable supplier).

C. Reagents

(**a**) *Sulfamethazine (SMZ) standard.*—Analytical reference grade.

(**b**) *Potassium dihydrogen phosphate (PDP) solution.*—0.1M. Dissolve 27.2 g potassium dihydrogen phosphate (LC grade) in H_2O, dilute to 2 L, mix, and filter through N66 filter. Store at room temperature. Discard after 3 months.

(**c**) *Solvents.*—Methanol and hexane, LC grade; chloroform, distilled in glass grade. (*Caution*: Use fume hood with adequate ventilation when using chloroform. Avoid contact with eyes, skin, and clothing, and avoid inhalation. Chloroform is possible carcinogen.)

(**d**) *LC solutions.*—(*1*) *Mobile phase.*—Dilute 600 mL N66-filtered methanol to 2 L with PDP solution, and mix well. (*2*) *Flush solution.*—Dilute 1200 mL methanol to 2 L with H_2O, mix, and filter through N66 filter. Store mobile phase and flush solution at room temperature; discard after 3 months.

(**e**) *Sulfamethazine standard solutions.*—(*1*) *Master solution.*—Accurately weigh ca 100 mg (to nearest 0.1 mg) sulfamethazine standard at room temperature in glass weighing boat, transfer quanititatively to 100 mL volumetric flask, dissolve in methanol, dilute to volume with methanol, and mix well. (*2*) *Intermediate solution.*—10 000 ng/mL. Transfer 1.0 mL master solution by 1.0 mL volumetric pipet into 100 mL volumetric flask, dilute to volume with H_2O, and mix well. (*3*) *Fortification (100 ppb standard) solution.*—1000 ng/mL. Transfer 10 mL intermediate solution to 100 mL volumetric flask by 10 mL volumetric pipet, dilute to volume with H_2O, and mix well. (*4*) *20 ppb standard solution.*—200 ng/mL. Dilute 20 mL 100 ppb standard solution to 100 mL with H_2O, and mix well. (*5*) *10 ppb standard solution.*—100 ng/mL. Dilute 10 mL 100 ppb standard solution to 100 mL with H_2O, and mix well. (*6*) *5 ppb standard solution.*—50 ng/mL. Dilute 5 mL 100 ppb standard solution to 100 mL with H_2O, and mix well.

Refrigerate all standard solutions below 10°; discard after 3 months.

D. Samples

Refrigerate milk below 10°. If milk will not be analyzed within few days, store below −40° in ca 50 mL portions in polypropylene tubes. Mix raw milk gently before sampling.

E. Extraction

Place fluted filter in 75 mm funnel, wash with 5 mL chloroform, and discard chloroform. Place 100 mL pear-shaped flask under funnel as receiver.

Pipet 10 mL milk sample into 125 mL separatory funnel, and add 50 mL chloroform. Shake mixture vigorously for 1 min. Carefully vent through stopper. Shake for 1 min, vent, and let phases separate 1 min. Shake 1 min, vent, and shake for 1 min. Vent and let phases separate for ≥5 min. Drain chloroform layer, and filter into pear-shaped flask. Rinse filter 2× with 5 mL chloroform; collect washings in pear-shaped flask.

Evaporate chloroform solution in pear-shaped flask just to dryness on rotatory evaporator at 32 ± 2°. Add 5 mL hexane to flask, stopper, and dissolve residue by agitating vigorously on vortex mixer ca 1 min. Immediately add 1.0 mL PDP solution to flask, agitate vigorously on vortex mixer 3 or 4× at intervals of ca 1 min over minimum of 15 min. Use Pasteur pipet to remove aqueous layer. Store extract in glass tube or autoinjector vial for injection.

F. Quantitation

Use flush solution to rinse autoinjector and to clean LC system and column. Chromatograph at least 3 levels of standards (5, 10, and 20 ppb), and use linear-regression analysis of standard curve to calculate concentrations. Quantify levels of analyte by peak height measurement, P. Standard curve should have correlation coefficient of ≥0.98 (if less, repeat standards injections). Note 10-fold concentration of residues (10 mL milk extracted into final 1 mL PDP) when calculating SMZ concentration in original sample; i.e., standard or final extract with 100 ng/mL concentration is equivalent to 10 ppb SMZ in milk.

Sulfamethazine retention time varies with instrument, column, column temperature, and batch of mobile phase but should be 4.5–6 min. However, with variables held constant, retention time is very stable and should not vary more than ca 15 s during a day. Bracketing samples with standards serves both as source of standard curve and as instrumental stability check.

$$\text{SMZ, ppb} = (P - b)/m$$

where P is peak height of unknown, and b and m are y-intercept and slope, respectively, from corresponding linear regression equation.

(*Note*: Occasionally in some shelf milk samples, a broad late-eluting peak appears in subsequent run. Using autosampler with only 1 min equilibration, this peak does not coelute with SMZ peak. To avoid coeluting peaks if making randomly spaced manual injections, increase run time by few minutes so broad peak elutes in solvent front of subsequent run. This peak has not been identified.)

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

CAS-57-68-1 (sulfamethazine)

Results and Discussion

Results obtained by the collaborators are shown in Table 1. Contents of samples 1–3 were known to the analysts. In some instances, the analysts did not follow instructions on the number of samples to analyze; therefore, some of the preliminary study data are also included for information in these sections. One collaborator analyzed 6 replicates instead of 5, because that was standard operating procedure in his laboratory. For the blind samples (4–6), some of the collaborators reported values corrected for response determined in the control milk. Only uncorrected values are reported in Table 1, because known control milk would not be available for a commercial sample. The collaborators reported results as parts per billion for the control milk fortified by the Associate Referee.

On reanalysis in this laboratory, 1 of the samples produced an emulsion during extraction. The cause of the problem was not established, but collaborators were instructed to discard the sample. The sample was replaced, and all the collaborators discarded the problem sample, except one who had already analyzed the sample when we notified him. His results are reported in Table 1, but the values were not used in calculating average recoveries for the blind sample of milk fortified at 10 ppb.

Because of a conflict between the write-up of the method and the instructions to the collaborators, different laboratories performed the familiarization study using either 5 and 10 ppb or 10 and 20 ppb fortification levels. We do not believe that this is a problem because the actual collaborative samples were the same for each of the collaborators, and the same method was followed with the actual collaborative study.

The analytical method (1) required a special wash procedure for glassware because of the observation in our laboratory that cross contamination at the parts-per-billion level can occur with glassware from general laboratory stock. This is especially true if the laboratory also analyzes tissue for residues of SMZ, which are usually present at the parts-per-million level. To minimize this, we rinse all previously cleaned glassware with 1N HCl, then with water, and finally with methanol. Also, all stopcocks are removed from separatory funnels during these final rinsing steps.

We do not recommend the more drastic cleaning procedures recommended by 2 of the participants, but we do emphasize that special care must be taken to ensure the cleanliness of the glassware. Also, despite the background interference observed by the participating laboratories, we do not recommend correction of the results for background, because control milk would not be available for commercial samples as a true reference.

Several participants commented that the method was either "straightforward," "easy to follow," or "relatively easy," or that they "encountered no major problems with the procedure." One collaborator commented that the vortexing step in the final extraction was extremely tedious when processing a large number of milk samples; however, we feel this step is necessary and should not be changed at this time.

We think data from laboratory B (Table 1) should not be used in calculating results of the collaborative study, because the collaborator reported difficulties with his chromatograph and because of the poor appearance of the chromatograms. The relative standard deviations were good, but the recoveries were substantially higher than those of the other collaborators. One other laboratory reported values significantly higher than the average, but there were no specific observations to justify discarding the values.

Average percent recoveries for all of the laboratories, even when the questioned data were included, were judged satisfactory for residues at 10–20 ppb levels. The average within-laboratory recoveries for blind samples fortified at the 10 and 20 ppb levels were 86.4 and 84.8%, respectively. The average value determined for the blind milk samples containing incurred SMZ was 14.8 ppb. The within-laboratory relative standard deviations (RSD_r) for the 3 blind samples ranged from 3.1 to 11% at 10 ppb, 2.2 to 11.9% at 20 ppb, and 2.5 to 11.4% for the incurred milk.

For blind fortified samples containing 10 ppb SMZ, average recovery and relative standard deviation values for repeatability (RSD_r) and reproducibility (RSD_R) based on the results from 6 of the participating laboratories were 8.23 ppb, 7.16%, and 23.16% respectively. Similar results based on all 7 participating laboratories were 9.13 ppb, 8.38%, and 31.94% (Table 2). For the incurred samples, the results were 14.8 ppb,

Table 1. Collaborators' results for quantitation by LC method of sulfamethazine (ppb) fortified and incurred raw milk

Sample	Collaborator						
	A	B	C	D	E	F	G
1. Control milk	0.0	1.89	trace	2.0	1.6	0.0	1.1
		4.51	trace	1.8	0.0	0.0	1.1
		5.01	trace	1.7	0.0	2.3	1.0
		2.88	trace	1.8	0.0	0.0	1.0
		3.04	trace	1.5	2.7	0.0	0.9
			trace		0.0		0.8
			trace				1.8
			trace				1.9
			trace				0.07
							0.08
							0.3
							0.3
2. Control, fortified at 5 ppb by analyst	4.3	5.8	ND[a]	6.2	3.8	3.3	4.8
	3.9	5.2		6.1	3.8	3.4	4.7
	4.6	11.1		6.0	3.5	3.4	4.5
	4.6	3.6		5.7	2.8	3.6	4.8
	4.6	4.7		5.4	3.5	3.4	4.6
	4.4				3.5	3.9	4.6
	4.3				4.5	4.0	5.2
					4.6	3.7	5.4
						3.7	
3. Control, fortified at 10 ppb by analyst	8.5	ND[a]	8.1	9.6	ND[a]	7.2	8.8
	8.4		8.4	8.9		7.6	8.8
			8.8	9.5		6.8	8.7
			7.5	9.4		7.3	8.8
			8.9	9.8			9.9
				7.2			9.0
				7.6			8.8
							8.7
4. Blind, fortified at 10 ppb	12.0	13.0	6.7	9.6	5.8	9.0	8.5
	10.4	14.9	6.2	9.0	6.6	7.6	7.8
	11.6	13.4	6.5	9.2	5.3	8.4	8.7
	11.7	16.6	6.5	7.8	6.8	8.9	8.8
	11.3	14.0	6.7	7.4	6.3	8.2	8.8
							8.7
5. Blind, fortified at 20 ppb	22.8	13.8	15.0	15.2	16.3	17.0	14.8
	26.4	15.3	14.2	15.7	15.4	17.2	16.0
	26.0	15.1	13.9	16.1	15.6	16.8	16.3
	24.7	16.1	14.8	16.0	14.2	16.5	20.7
	24.2	14.9	15.6	15.8	14.7	16.2	17.7
							17.6
6. Blind, incurred SMZ	20.4	21.2	11.6	11.8	13.3	13.9	14.0
	18.8	22.5	12.0	14.8	14.1	13.3	14.3
	19.9	22.0	13.0	14.9	14.1	14.0	14.9
	20.5	21.8	11.7	14.9	14.2	14.8	14.2
	19.9	21.1	11.1	16.3	14.4	14.3	14.7
							14.8

[a] ND = No data submitted in this category.

Table 2. Statistical evaluation of collaborators' data for quantitation by LC method of sulfamethazine fortified and incurred in raw milk

	Control milks		Blind samples		
Statistic	5 ppb[a]	10 ppb[a]	10 ppb[a]	20 ppb[a]	Incurred
Mean rec., ppb	4.56	8.50	9.13	17.07	15.7
s_r	1.03	0.66	0.77	1.14	0.80
s_R	1.38	0.90	2.91	3.63	3.61
RSD_r, %	22.67	7.80	8.38	6.64	5.09
RSD_R, %	30.33	10.56	31.94	21.28	22.89

[a] Amount added.

5.62%, and 18.51% based on 6 laboratories and 15.7 ppb, 5.09%, and 22.89% based on 7 laboratories.

These results demonstrate that the method is suitable for determination of SMZ residues in raw milk at 10 ppb. These values for RSD_R fall on the curve that Hall and Selinger (3) call the Horwitz trumpet. Horwitz (4, 5) predicted a 32% between-laboratory coefficient of variation for 10 ppb concentration levels.

Recommendation

We recommend that the LC method be adopted first action for the determination of sulfamethazine residues in raw milk at the 10 ppb level.

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ENVIRONMENTAL ANALYSIS

Pararosaniline as a New Chromogen for the Extractive Spectrophotometric Determination of Trace Amounts of Hydrogen Sulfide in Air

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A sensitive spectrophotometric method for the determination of trace amounts of hydrogen sulfide (H_2S) in air, after fixing in a zinc acetate–disodium ethylenediaminetetraacetate–sodium hydroxide solution, is described. The reaction of iodate with the fixed H_2S in the presence of acid and an excess of chloride leads to the formation of ICl_2^- ions. The resulting ICl_2^- species forms an ion par with pararosaniline cation, and the product is extracted into isopentyl alcohol. The color system obeys Beer's law over the range of 0 to 5.0 μg H_2S. The molar absorption coefficient of the color system is 1.8×10^4 L mol^{-1} cm^{-1}. The coefficient of variation is 3.3% for 10 determinations at 3.0 μg H_2S. The effect of interfering gases on the determination is discussed. The method was applied to the determination of residual amounts of H_2S present in a laboratory fume cupboard, and results were compared to those obtained by the widely used methylene blue method. The current method can be used to determine residues as low as 0.2 μg H_2S.

Hydrogen sulfide, released by industrial processes and by the decay of organic matter on land and in water, is one of the principal compounds involved in the environmental sulfur cycle. Because of the high toxicity and foul odor associated with this gas, ambient air quality standards prescribe a limit of 4 μg/m^3 for hydrogen sulfide (1).

The methylene blue method is widely used for the determination of H_2S, but difficulties arise from the formation of small amounts of other similar dyes and also from the dimerization of methylene blue dye (2).

Many sensitive methods were reported for the determination of H_2S based on its reducing property (3–5). Recently, 2 methods were reported that exploit the interaction of fixed H_2S with iodate in a chloride-containing acidic medium. The method reported by Balasubramanian and Kumar (6) is based on the reaction of the fixed H_2S with iodate in an acidic medium containing excess chloride to form ICl, which is stabilized as ICl_2^- ion. The ICl formed iodinates 2′,7′-dichlorofluorescein, resulting in the formation of 2′,7′-dichloro-4′,5′-diiodofluorescein. The iodinated product is extracted into 15% isopentyl acetate–isopentyl alcohol and measured spectrophotometrically at 535 nm. The color system was stable up to 90 min after extraction.

Selvapathy et al. (7) reported a sensitive method based on ion-pair formation between the iodine–chloro complex and pyronine-G cation. The iodine–chloro complex was generated by the interaction of fixed H_2S with iodate in a chloride-containing acidic medium. The ion pair was extracted into benzene, and the absorbance was measured at 535 nm. Even though the method is sensitive, it has a disadvantage of high blank absorbance, and absorbance measurements must be completed within 10 min after extraction of the ion pair because of poor color stability.

We describe the study and evaluation of the variables that govern the formation of the ion pair between ICl_2^-, generated by the iodate and the fixed H_2S reaction, and the pararosaniline (PRA) cation. This evaluation provides the basis for a sensitive spectrophotometric method for the determination of H_2S.

Experimental

Apparatus

The absorbance was measured by a Carl Zeiss PMQ II spectrometer with 10 mm quartz cells. Frittered glass bubblers with suitable suction devices were used for trapping H_2S from air. The flow rate of the air was measured by using a rotameter.

Reagents

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

(**a**) *Standard H_2S solution, 1 μg/mL.*—Dissolve 0.127 g $Na_2S{\cdot}9H_2O$ in 100 mL water, and standardize the solution iodimetrically (8). The hydrogen sulfide content of this solution will be 200 μg/mL. Dilute a suitable volume of this solution with water to give a solution containing 1 μg/mL H_2S. This solution must be prepared fresh daily.

(**b**) *PRA hydrochloride solution, 0.001%.*—Dissolve 0.025 g of the dye (BDH, England) in 125 mL 5M sulfuric

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acid, and dilute to 250 mL with water in a calibrated flask. Dilute 25 mL of this solution to 250 mL with 2.5M sulfuric acid in a calibrated flask. The solution is stable for 1 month if stored in a brown bottle at room temperature.

(c) *Formaldehyde solution, 0.2% m/v.*—Dilute 0.5 mL 40% formaldehyde solution to 100 mL with water.

(d) *Sulfuric acid, 4.75M.*—Dilute 280 mL sulfuric acid (specific gravity, 1.84) to 1 L with water.

(e) *Zinc acetate–disodium ethylenediaminetetraacetate (Na_2EDTA)–sodium hydroxide trapping solution.*—Dissolve 20 g Na_2EDTA and 10 g zinc acetate in ca 800 mL water. Adjust pH of the solution to ca 7.2. Add 4 g sodium hydroxide, and dilute solution to 1 L with water in a calibrated flask. pH of the final solution is 12.7. This solution contains 1% zinc acetate, 2% EDTA, and 0.4% NaOH.

(f) *Acetic acid, 1M.*—Dilute 6 mL acetic acid (specific gravity, 1.05) to 100 mL with water.

(g) *Potassium iodate solution, 0.4% m/v.*

(h) *Sodium chloride solution, 6% m/v.*

(i) *Sulfamic acid solution, 5% m/v.*

(j) *Isopentyl alcohol (extraction solvent).*

Procedure

Sampling

Air samples were collected by drawing 10–100 L air through a fritted glass bubbler containing 10 mL zinc acetate–Na_2EDTA–sodium hydroxide solution for 20–200 min at a flow rate of 0.5 L/min. The solution was made up to 25 mL with the trapping solution before the determination of H_2S.

Determination

Five milliliters 0.4% potassium iodate, 2 mL 4.75M sulfuric acid, and 1 mL 6% sodium chloride were placed in a 50 mL calibrated flask. A 10 mL aliquot of the zinc acetate–Na_2EDTA–sodium hydroxide solution, containing not more than 5.0 μg H_2S, was placed in a test tube. This solution was then introduced into the 50 mL calibrated flask through a long-stemmed funnel, with the tip kept well immersed in the reagent solution to avoid the loss of hydrogen sulfide. The solution was mixed, 2.5 mL 0.001% PRA solution was added, and the final volume of the solution was made up to 50 mL with water and allowed to stand 5 min. It was then transferred into a 125 mL separatory funnel and extracted 1 min with 5 mL isopentyl alcohol. The organic layer was separated, transferred into a test tube, and treated with ca 1 g anhydrous sodium sulfate to remove trace amounts of water. The absorbance of the organic extract was measured at 560 nm in 10 mm cells against a reagent blank carried through the entire procedure. The concentration of H_2S was established by reference to a calibration graph prepared by treating 0–5 mL standard H_2S solutions (0–5 μg H_2S) with 10 mL zinc acetate–Na_2EDTA–sodium hydroxide trapping solution and following the above procedure.

Table 1. Extractability of the ion pair into various solvents (H_2S = 3 μg)

Set No.	Solvent	Absorbance	
		Blank vs solvent	Sample vs blank
1	Ethylacetate[a]	0.010	0.070
2	CCl_4	0.002	0.002
3	1-Butanol[a]	0.100	0.220
4	Isobutyl alcohol[a]	0.070	0.190
5	IBMK	0.020	0.115
6	Isopentyl alcohol	0.025	0.305

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

Results and Discussion

H_2S was fixed in the modified trapping solution developed by Balasubramanian and Kumar (6). The trapping solution consists of 1% zinc acetate–2% Na_2EDTA–0.4% NaOH and permits a sampling rate of 0.5 L/min for 6 h. Preliminary studies were performed using 5 mL 0.4% potassium iodate, 2 mL 4.75M sulfuric acid, 1 mL 6% sodium chloride, and 2.5 mL 0.001% PRA solution. The resulting solution was treated with 3.0 μg H_2S in 10 mL of the above mentioned trapping solution and diluted to 50 mL with water. The solution was transferred into a 125 mL separatory funnel and extracted for 1 min with isopentyl alcohol. Results indicated that the extraction of the ion pair into the solvent was selective because the absorbance of the blank at 560 nm was quite low and that of the sample was high.

The optimum acidity for the formation of ICl_2^- species was first established. A constant and maximum absorbance was obtained when the acidity was 0.255–0.355M in sulfuric acid. Above 0.355M, the absorbance of the sample decreased; below 0.255M, an increase in blank absorbance was observed. Therefore, a solution maintained at 0.305M in sulfuric acid was selected as the optimum acidity for the reaction.

Similar studies revealed that 3 mL 0.4% potassium iodate, 1.5 mL 0.001% PRA, and 0.5 mL 6% sodium chloride were sufficient to provide a constant and maximum absorbance. However, we recommend the use of 5 mL 0.4% potassium iodate, 2.5 mL 0.001% PRA, and 1 mL 6% sodium chloride.

The formation of the ion pair was almost instantaneous, and mixing of the 2 phases for about 30 s was sufficient for quantitative extraction of the ion pair into the isopentyl alcohol solvent. The color system was stable for 72 h after extraction.

The extraction behavior of 5.0 mL of various solvents was tested for the extraction of the ion pair. No extraction was observed in benzene and *n*-hexane. The extraction behaviors of other solvents are shown in Table 1. Only isopentyl alcohol extracted maximum quantities of the ion pair and kept the absorbance of the blank low. The absorption spectra of the ion pair formed from different concentrations of H_2S are shown in Figure 1. Curve A shows the absorption spectrum of the reagent blank (2.5 mL 0.001% PRA). Curve F shows the spectrum of an identical concentration of PRA prepared in isopentyl alcohol. Comparison of curves A and F indicate that free PRA does not get extracted into isopentyl alcohol under experimental conditions. Curves B, C, D, and E show the absorption spectra

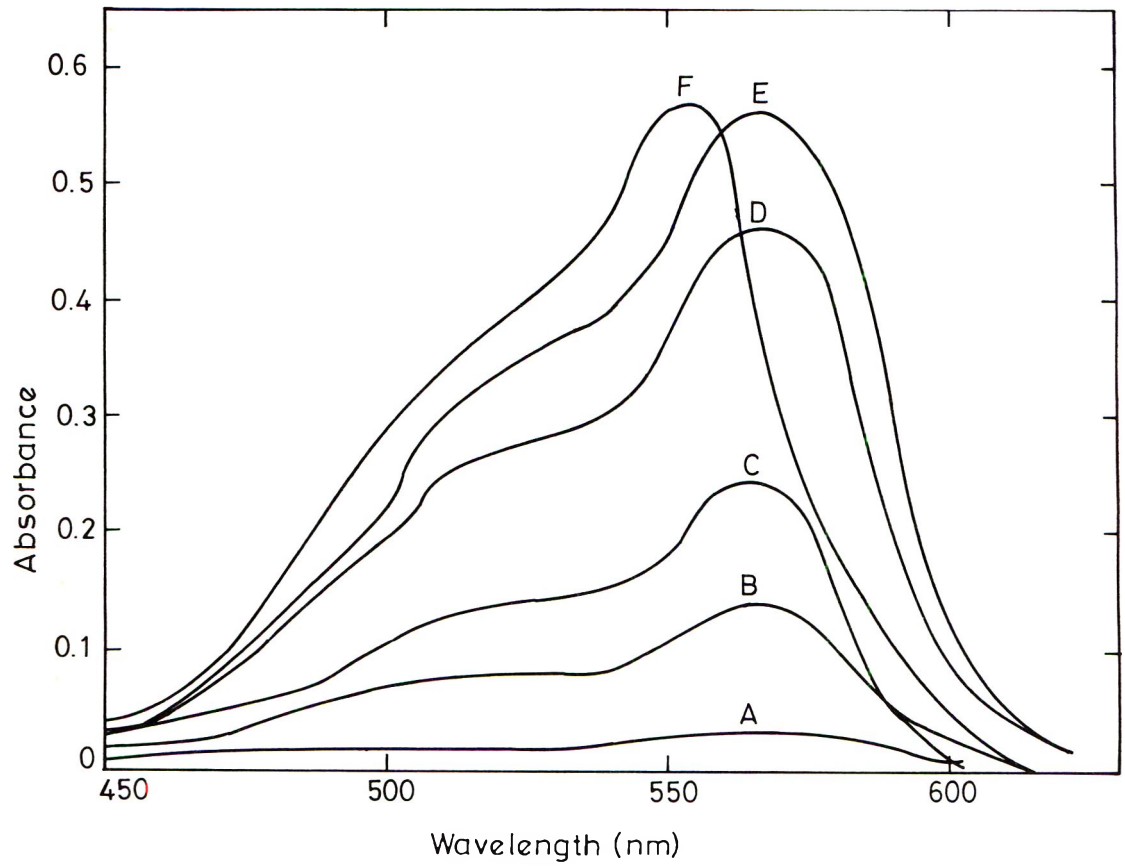

Figure 1. Absorption spectra measured against solvent blank: A, reagent blank; B, 1μg H_2S; C, 2μg H_2S; D, 4μg H_2S; E, 5 μ H_2S; F, 3.09×10^{-5}M pararosaniline in isopentyl alcohol.

resulting from the extraction of the ion pair formed between ICl_2^-, generated by various amounts of H_2S and PRA.

The calibration graph was rectilinear over the 0–5.0 μg H_2S range. The molar absorption coefficient was 1.8×10^4 L mol^{-1} cm^{-1}. The precision of the proposed method was evaluated by establishing the concentration of 10 standard samples containing 3.0 μg H_2S. The mean recovery was 2.99 μg, with a relative standard deviation of 3.3%.

Nature of the Extracted Species

In the present study, an attempt was made to form an ion pair between ICl_2^-, generated by the reaction of H_2S with iodate in an acidic medium containing chloride ions, and PRA instead of between ICl_2^- and cationic dyes. As already reported, the methods based on the formation of an ion pair between rhodamine 6G (4) or pyronine G (7) and an anionic chloro-complex of iodine suffer from high blank absorbance values (0.10 A for rhodamine 6G method and 0.16 A for pyronine G method) and poor color stability (absorbance measurements to be completed within 10 min after extraction by pyronine G).

Recently, we established the formation of ICl_2^- species (6) from the reaction between H_2S and iodate in an acidic medium containing an excess of chloride ions (in accordance with equation 1). We used the ICl_2^- species for the iodination of 2′,7′-dichlorofluorescein.

$$2IO_3^- + H_2S + 4H^+ + 4Cl^- \rightarrow 2ICl_2^- + H_2SO_4 + 2H_2O \qquad (1)$$

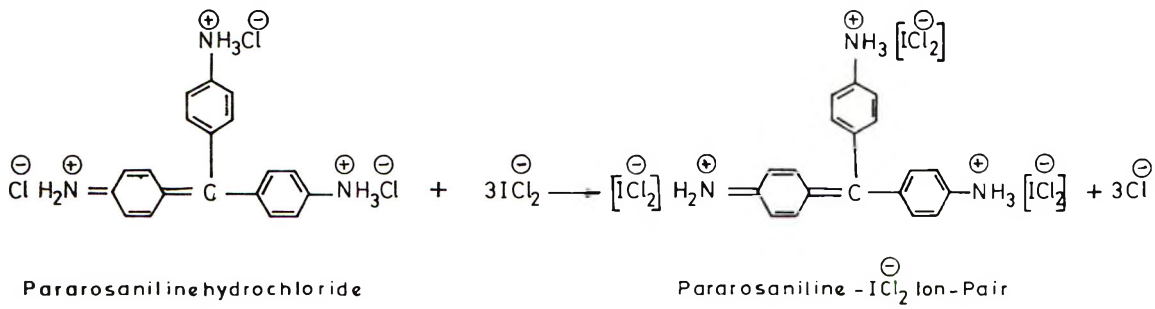

Figure 2. Species responsible for color.

In the present study, when 2′,7′-dichlorofluorescein was replaced by PRA, an absorbance value of 0.305 A for 3 μg H_2S was observed on extraction with isopentyl alcohol. This observed absorbance may result from the extraction of the iodinated PRA or the ion pair formed between ICl_2^- and the PRA cation. Experiments were conducted to establish the nature of the species responsible for the observed absorbance. Using potassium iodate, PRA, and 3 μg H_2S solution, the color was developed and extracted into isopentyl alcohol. The corresponding reagent blank was also prepared and extracted into isopentyl alcohol. The organic layers from samples and blank were equilibrated with 5 mL neutral 5% potassium iodide aqueous solution. This resulted in the formation of a yellow color in the aqueous layer of the sample, which on treatment with starch solution turned blue, indicating the presence of iodine. No such blue color was observed in the blank. The formation of iodine in the sample can be explained as a result of the interaction of iodide with ICl_2^- anion (in accordance with equation 2), which was present as an ion pair with the PRA cation in the organic layer.

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

If free iodate were extracted as an ion pair with PRA, then this ion pair can react with iodide to generate iodine and give a blue color with starch solution. This possibility of iodine generation was ruled out because no blue color was observed in the blank. When experiments were conducted using twice the optimum concentration of iodate, no blue color was observed in the blank, which indicated that there is no extraction of free iodate as an ion pair with PRA.

If the ion pair extracted into the organic layer was an iodinated PRA, it could not generate iodine on treatment with aqueous neutral potassium iodide. Hence, the observed color in the organic layer results from the ion pair between ICl_2^- and the PRA cation. In addition, the composition of the ion pair was established by the mole-ratio method. The result indicated that the mole ratio of PRA:ICl_2^- was 1:3 (Figure 2). The extracted ion pair of ICl_2^- and PRA was stable for 72 h from the time of extraction. The color system had a low blank absorbance (0.024 A), unlike the reported ion pair systems of the anionic-chloro complex of iodine with cationic dyes (4, 7).

Effect of Interfering Species

The effect of common air pollutants on the determination of 3 μg H_2S was studied by introducing the gas under examination into the trapping solution in the form of anions together with H_2S. A concentration of 5000 μg formaldehyde did not interfere with the determination of 3 μg H_2S when introduced as an aqueous solution. Nitrogen dioxide, when present at any level, caused a low recovery of H_2S. The negative interference seen with levels of nitrogen dioxide (0–1000 μg) was eliminated by the addition of 2.0 mL 5.0% sulfamic acid to the trapping solution upon completion of sampling. Sulfur dioxide interfered seriously at all levels and caused positive errors, because it is fixed as sodium sulfite in the trapping solution. This interference (0–100 μg) was eliminated by converting the fixed sulfite to a more stable hydrogen sulfite addition compound of formaldehyde. The conversion was achieved after sampling was completed by adding 1.0 mL 0.2% formaldehyde and 3.0 mL 1M acetic acid (which lowers the pH of the solution to 4.90) to the trapping solution.

Table 2. Determination of residual H_2S in a laboratory fume cupboard (sampling rate, 0.5 L/min)

Set No.	Volume of air sampled, L	H_2S found, μg: Methylene blue method		H_2S found, μg: Pararosaniline method	
1	100	9.50	(68.3)[a]	9.25	(66.5)
2	100	20.00	(143.8)	20.00	(143.8)
3	100	12.25	(88.1)	12.75	(91.7)
4	180	40.05	(160.0)	40.95	(163.6)
5	180	36.90	(147.4)	37.35	(149.2)

[a] Values in parenthesis are ppb H_2S.

Application of the Method

The proposed method was used to determine the residual concentration of H_2S in a laboratory fume cupboard after a 6 h qualitative analysis class in which H_2S was used as a precipitating reagent for metal ions. Air samples were collected in 10 mL zinc acetate–Na_2EDTA–NaOH trapping solution at a rate of 0.5 L/min. The final volume of the solution, after sampling, was made to 25 mL with the trapping solution. Suitable aliquots of this solution were then analyzed by the proposed procedure and the standard methylene blue method. The aliquots used for the proposed procedure ranged from 2.5 to 5 mL, and 10 mL aliquots were used for the methylene blue method. With the proposed method, 2 mL 5% sulfamic acid, 1 mL 0.2% formaldehyde solution, and 3 mL 1M acetic acid were added to the aliquots of the sampled solutions to overcome the interference from nitrogen dioxide and sulfur dioxide. Results are shown in Table 2; concentrations of H_2S obtained with both methods were comparable.

Conclusion

Levels of hydrogen sulfide as low as 0.2 μg can be determined by using the proposed procedure. The calibration graph is rectilinear from 0 to 5 μg H_2S. The coefficient of variation is 3.3% for 10 determinations of 3 μg H_2S. The color system is stable for 72 h after extraction into isopentyl alcohol. The application of the proposed method to the determination of H_2S present in a laboratory fume cupboard demonstrated the usefulness of the method.

Acknowledgment

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FEEDS

Rapid Determination of Supplemental Vitamin E Acetate in Feed Premixes by Capillary Gas Chromatography

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A rapid capillary gas chromatographic method was developed for determination of supplemental α-tocopherol acetate (containing and verified at 50–5000 mg/kg feed) in various kinds of feed premixes. The method is based on simple *n*-hexane extraction of feed samples pretreated with a solution of acetic acid in ether for matrix breakdown followed by column cleanup on deactivated alumina to eliminate background interference. Fused silica chromatography is completed in 3 min. The within-day precision expressed as the relative standard deviation for recovery examples was 3.4, 2.9, and 4.0% for high, medium, and low concentrated feed premixes, respectively; extraction recovery was 99–110%. The storage stabilities of standards and feed premix preparations were found to be excellent up to 3 months and up to 60°C.

Many kinds of feed premixes for treatment of animal diseases or promotion of animal performance are used throughout the world. These premixes are composed of a wide spectrum of substances, including vitamin E. In Europe, vitamin E is administered in the form of vitamin E acetate (DL-α-tocopherol acetate); the concentration typically ranges from 50 mg/kg of feed premix for animal health promotion to 5000 mg/kg for medicated feed supplements. Typical doses in the United States are 4 times as high. Because vitamin E acetate is sensitive to oxidation, quantitative analytical control of feed premixes is required (1–5) after manufacturing and during storage. The analysis of feed premixes, however, requires preliminary matrix breakdown and separation steps to free the analyte and eliminate interfering compounds found in the matrix. Formerly, the vitamin E acetate was saponified (3, 4, 6, 7), but this step produced the alcohol, eliminating the capability for simultaneous determination of the 2 moieties. Avoidance of the saponification step (1) is helpful because the identification of vitamin E alcohol (DL-α-tocopherol) formed from the decomposition of the acetate due to storage conditions (4) or found as a natural component is useful. The gas chromatographic (GC) method is preferred over the colorimetric method because of its higher precision and specificity (8, 9). Liquid chromatographic (LC) methods are now also available for oil-soluble vitamins in tablet form (6, 10) and for other analytical samples (11).

The method presented here is a simple, micro-sized sample preparation method for feed premixes. A variety of feed premixes can be analyzed equivalently, which we feel is an important consideration. Extraction and cleanup are performed in a small tube and plastic pipet tip. Simultaneous determinations can be made of vitamin E acetate and, if desired, the residual or breakdown product, vitamin E alcohol. Fused silica capillary analytical chromatography for the system presented here gives results in 3 min, which is a very short time compared to the 30 and 6 min required for packed and glass capillary column analyses, respectively, as previously reported (3–5, 7, 9).

The utility of the assay is demonstrated by the determination of the supplemental vitamin E acetate in 3 types of premixes. For this paper, all recoveries were done at the 500 mg/kg level. Typically, the premixes for poultry and sheep contain low concentrations of supplemental vitamin E acetate at the 300 mg/kg level. In this case, vitamin E acetate is mixed in a cereal matrix together with protein and feed supplements. These premixes also contain vitamins A and D and macro and micro mineral supplements. The premix for pigs, which contains about 90% cereal with peas, faba beans, yeast, and mineral supplements, typically contains vitamin E acetate at 500 mg/kg. The highest content of supplemental vitamin E acetate is in medicated feed supplements containing Therapeuthan E. This preparation has been developed for therapeutic purposes and contains such drugs as furazolidone, tetracycline hydrochloride, dichlorodihydroxychinolidine, and glucose with $CaCO_3$, in addition to vitamin E acetate at the 3000–5000 mg/kg level.

METHOD

Principle

Supplemental vitamin E acetate was extracted by *n*-hexane after short treatment of finely powdered premixes in 1% acetic

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acid in ether. The extract was cleaned up on deactivated alumina and quantitated by internal standard GC with flame ionization detection.

Apparatus

(**a**) *Gas chromatograph.*—Hewlett-Packard 5880A level IV-calculation capability with 22 Kbytes RAM capacity. Operating conditions: detector and injector temperatures, 300°C; initial column temperature, 200°C; hold time, 0.5 min; programmed at 25°C/min to 255°C, held 1 min, and then post-programmed (for column cleanup) at 25°C/min to 325°C, and held 1 min; inlet hydrogen carrier pressure, 25 kPa for 1:15 split; flow velocity, 80 cm/s at 100°C; FID detection nitrogen and air make-up, 30 and 430 mL/min, respectively; detector attenuation, adjusted for FSD from the injection of 1 µL of the internal standard solution. Under these conditions, squalane and vitamin E acetate had retention times of 1.5 and 2.7 min, respectively.

(**b**) *Chromatographic column.*—Fused silica column, 12.5 m × 0.32 mm id and 0.12 mm film thickness (CP Sil 5CB, Chrompack, The Netherlands), connected by 1 m retention gap column with butt connector (Supelco, Switzerland) to split/splitness injection port.

(**c**) *Horizontal laboratory shaker.*—(Labora, Czechoslovakia).

(**d**) *Evaporation module.*—Reacti-therm, heating (Pierce Chemical Co., Rockford, IL 61105).

(**e**) *Extraction tubes.*—15 × 120 mm (ca 15 mL) with Teflon-lined caps (Labora).

(**f**) *Centrifuge.*—IEC, or equivalent.

(**g**) *Vials.*—5 mL Reacti-vials (Supelco).

(**h**) *Tips.*—5 mL pipet tip (Eppendorf), or equivalent.

Reagents and Standards

(**a**) *Solvents.*—*n*-Hexane p.a., cyclohexane, isopropanol (Loba Feinchemie, Austria).

(**b**) *Acetic acid solution.*—1% (v/v) in redistilled ether (Chemapol, Czechoslovakia).

(**c**) *Internal standard solution.*—Squalane; 1 mg/mL in hexane (Fluka AG, Switzerland). *Column performance evaluation.*—DL-α-Tocopherol (vitamin E alcohol). *Standard.*—Vitamin E acetate (DL-α-tocopheryl acetate, minimum 97% purity by UV, Fluka AG, Switzerland); stock standard solutions prepared at 0.1, 0.5, 1.0, 2.5, 5.0, and 8.35 mg/mL.

(**d**) *Extractant solutions.*—Hexane–cyclohexane (1 + 1) and hexane–isopropanol (1 + 1).

(**e**) *Alumina.*—Chromatographic grade, 60–80 mesh (Reanal, Hungary) deactivated by 7% deionized water to Brockmann activity IV.

Caution: Ether peroxidizes very easily. Test all batches immediately before use by shaking a few milliliters with a solution of a few crystals of potassium iodide in glacial acetic acid (or water). If there is any evidence of brown color (from I_2 produced), reject that batch for use. Ether is easily separated from its peroxide by distillation, but be careful not to let the distillation pot go to dryness. Peroxides can also be destroyed by chemical means.

Column Performance Evaluation

Prepare 1 mg/mL solution of α-tocopherol and α-tocopheryl acetate in *n*-hexane. Inject 1 µL into GC. Calculate resolution R and accuracy of determination for 2 injection mode from chromatogram by using HP 5880 BASIC programming for 0.04 and 0.02 min peak width, respectively, or other integrator techniques set at maximum sensitivity. The resolution is:

$$R = 1.18(RT(2)–RT(1))/(WIDTH(1) + WIDTH(2))$$

where RT(2) and RT(1) are retention times of α-tocopherol acetate and α-tocopherol and WIDTH(1) and WIDTH(2) are peak widths at 1/2 height, respectively. Resolution must be >1.5 for complete resolution of 2 peaks.

This evaluation was performed to ensure separation of vitamin E acetate from its alcohol, a frequent contaminant due to instability, particularly from storage (Figure 1).

Determination of Relative Response Factor

Prepare 1 mg/mL response standard solution of α-tocopheryl acetate, using internal standard solution. Inject 1 µL into GC system. Calculate response factor (RF) as follows:

$$RF = (PA_{is} / PA_{rs}) \times (C_{rs} / C_{is}) \times P$$

where PA_{is} and PA_{rs} = peak area of internal standard and reference standard, respectively; C_{rs} and C_{is} = concentrations in mg/mL of reference standard and internal standard, respectively, in response standard solution; and P = % purity of reference standard.

Sample Preparation

Into 15 mL extraction tube, weigh 1 g finely powdered feed premix previously stored in plastic bag. Add 1 mL of a solution of 1% acetic acid in ether and 10 mL *n*-hexane. Heat 10 min at 70°C in dry box under sealed Teflon-lined caps. Place extraction mixture in horizontal shaker for 20 min at 60 strokes/min. Centrifuge 10 min at 200 × *g* and then pass hexane layer through 2 g deactivated alumina packed in 5 mL Eppendorf pipet tips for cleanup. Repeat extraction twice with 10 mL hexane each time and evaporate combined hexane fractions under gentle stream of nitrogen at 50°C just to dryness. Take up residues with 1 mL internal standard solution and inject 1 µL into GC. If desired, samples of higher concentrations can be analyzed by adjusting internal standard concentration or split ratio in GC.

Calibration Curve and Calculation

Prepare 6-point calibration curve with 6 entries on each level, starting from 0.1, proceeding through 0.5, 1.0, 2.5, 5.0 to 8.35 mg/mL. Evaporate 1 mL of each solution to dryness with gentle nitrogen stream; then dissolve in 1 mL internal standard solution. Inject 1 µL for analysis. Calculate concentrations in unknown samples from slope and intercepts determined from least squares regression graph of concentration vs area of vitamin E acetate to internal standard ratio.

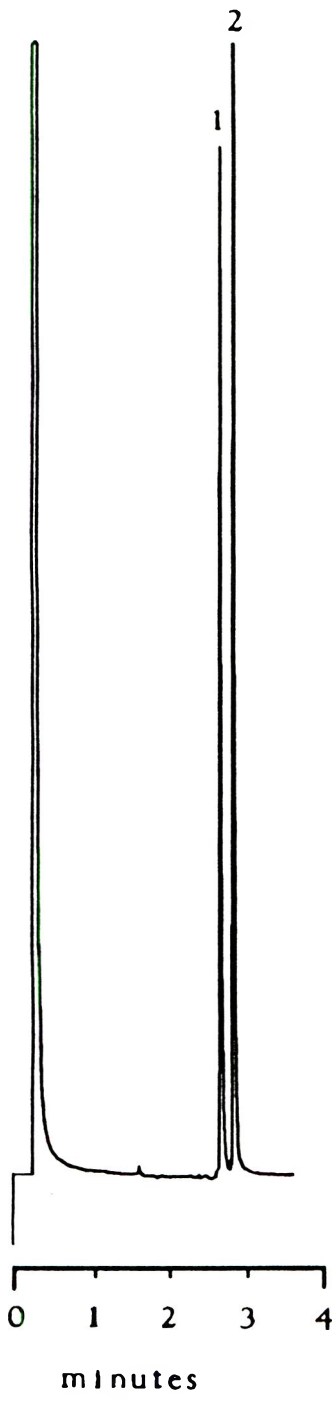

Figure 1. Chromatogram for resolution measurement of vitamin E and vitamin E acetate standards at 1 mg/mL. Peaks: 1 = vitamin E, 2 = vitamin E acetate. See Table 1 for resolution data.

Recovery Studies

The extraction efficiencies for vitamin E acetate were determined by spiking 50 g finely powdered premix samples with 10 mL of a 2.5 mg/mL solution of vitamin E acetate in hexane. This corresponds to a spiking level of 500 mg/kg feed premix. After thorough mixing in an air-pressured plastic bag and 24 h of storage, six 1 g sample aliquots were analyzed, containing the equivalent of 0.5 mg added vitamin E acetate per sample. During the extraction of premix for sheep, a coelution of sulfur, which had been added for better wool growth, was observed. Therefore, extraction of this premix was tested by a more polar solvent system for comparison: hexane–cyclohexane (1 + 1) and hexane–isopropanol (1 + 1).

Stability Studies

Stability studies were conducted of standard solutions, and feed premixes containing vitamin E acetate underwent short- and long-term storage. Standards prepared in hexane were stored at 4°C and run in the gas chromatograph on days 2, 4, and 25 to determine the combination of solution and instrumental response. Dosed feed premixes at the 3 levels studied (low, medium, and medicated concentrations) were prepared, stored at 4°C, and analyzed on days 1, 2, and 3 of storage. Because feed premixes may often be stored on the farm at less than ideal conditions, the manufactured medicated level batches were stored at 5, 25, 35, and 60°C for 3 months before analyses. In addition, a tropicalization test was run for 3 months at 95% humidity and 12 h cycling between 20 and 40°C.

Results

Column Performance

A 12.5 m fused silica column, as described, was used to separate vitamin E alcohol from the acetate in <3 min. Resolutions of ≥3.3 min with low variability were obtained (Table 1).

The variability of peak areas for a 1 µg autosampler injection was about 7% relative standard deviation (Table 1), which shows instrumental and not method performance. Only a slightly higher variability, 10.5–12.7%, was found for hand injection.

Recovery Curves, Precision, and Accuracy

The calibration graphs were developed for a wide concentration range (150–12 650 mg/kg). Table 2 gives information about calibration of standards by this method. Calibration of 151–12 650 mg vitamin E acetate/kg hexane gave somewhat linear results. Resultant data was positive (23%)

Table 1. Column performance evaluation and accuracy in the determination of vitamin E and vitamin E acetate[a]

	Vitamin E alcohol	Vitamin E acetate
Integrator setting	0.02	0.04
Resolution, min (%RSD)[b]	3.43 (2.62)	3.25 (2.69)
RSD, %, by injection method[c]		
Manual injection	10.45	12.72
Auto injection	7.52	6.90

[a] Results from mean of 6 determinations.
[b] RSD = SD × 100%/mean.
[c] Injection – 1 µg on column (0.02 min. integration setting).

Table 2. Summary of calibration data used in the analysis of supplemental vitamin E acetate[a]

Recovery analysis			
Recovery and precision for between estimations			
Concentration levels (mg/kg)			
Actual	Measured (SD)[b]	Av. dev. of mean from actual concn, %	Precision for within (RSD, %)
151	186 (22)	+23.2	7.2
758	709 (56)	−6.2	3.1
1520	1383 (67)	−8.7	1.9
3790	3390 (269)	−10.6	4.3
7580	7530 (265)	−0.6	3.3
12650	9900 (841)	−21.7	2.2
Linear regression analysis			
Day	Slope	Y-intercept	Corr. coef., %
1	1.824	−0.106	0.997
2	1.895	−0.028	0.999
3	2.185	−0.310	0.985

[a] Within-precision data were calculated from 6 entries of freshly prepared vitamin E standards without matrix for each level on the separate days.
[b] Mean ± SD.

for accuracy at the lowest concentration with respect to the actual prepared concentration, and all other values were negative (≤22% for the highest level). The precision variation of 6 calibration samples was very favorable, ranging from 1.9 to 7.2% relative standard deviation. Regression analyses of peak area response were fairly consistent over the 3 days studied.

Recovery Studies

The extraction recovery achieved for 1 g samples containing 0.5 mg vitamin E acetate (500 mg/kg level) is shown in Table 3. The recovery was 98.9% for high concentration-type premix, 102.8% for medium-type, and 110.1 and 100.2% for low-type. An inter-precision between 6 separate samples was 9.4% for high concentration, 7.3% for medium concentration, and 5.1 and 13.9% for low concentration, respectively. Precision of the premix for sheep was 13.9% because the mix contained elemental sulfur.

Sulfur is the most probable cause of the lower precision in analysis of the low-type premix because it is soluble in hexane and coelutes with the analyte during the cleanup step. Extracting solvent, when evaporated, gave well-defined dry residues except for the premix samples intended for sheep, which contained sulfur. The extracted residues for samples containing sulfur were yellow and sticky. Accordingly, this premix was extracted by 2 more polar solvent systems. However, in both cases, the extraction efficiencies were lower with mixed solvent systems (Table 3) than with the pure hexane extraction,

Table 3. Recoveries of supplemental vitamin E acetate from medicated feed supplement and feed premixes

Extraction	Sample	Rec., %[a]	Precision (RSD%)	
			Inter-	Intra-
n-Hexane	Medicated feed supplement-type premix[b]	98.9	9.4	3.4
n-Hexane	Medium concentration-type premix for pigs	102.8	7.3	2.9
n-Hexane	Low concentration-type premix for broilers	110.1	5.1	4.1
n-Hexane	Low concentration-type premix for sheep	100.2	13.9	10.0
n-Hexane–cyclohexane (1 + 1)	Low concentration-type premix for sheep	66.4	27.9	18.9
n-Hexane–isopropanol (1 + 1)	Low concentration-type premix for sheep	80.3	19.8	14.5

[a] Vitamin E acetate at 0.5 mg per sample (500 mg/kg level), $n = 6$.
[b] Therapeuthan E.

Table 4. Storage stability of extracted dosed samples and prepared standards at 4°C[a]

Days of storage	Low concn	RSD, %	Med. concn	RSD, %	Medicated concn	RSD, %
	Content of vitamin E acetate in premix and feed supplemental samples, mg/kg					
1	77.6	6.2	536	3.9	3390	2.3
2	55.9	13.6	499	4.5	3310	2.2
3	59.2	13.4	492	6.4	3610	4.8
Int. day[b] composite	64.3 ± 12	11.6	509 ± 24	5.0	3440 ± 152	3.3
	Content of vitamin E acetate in standards, mg/kg					
2	—	—	202	2.4	12850	4.1
4	—	—	190	5.0	11970	5.6
25	—	—	212	3.6	14470	4.5
Int. day[b] composite	—	—	202 ± 11	3.8	13100 ± 1268	4.8

[a] Results are calculated as the mean of 6 determinations.
[b] Overall RSD computed as $(1/3\ \Sigma RSD_i^2)^{1/2}$ (root mean square).

and no improvement was observed in either recovery or precision.

Storage Stability Studies

The data of Table 4 show stability of standard solutions over a period of ≤25 days.

With dosed premixes, Table 4 shows a substantial drop in the extracted concentration at the low concentration and a small drop at the medium concentration level after 1 day. The change in low and medium concentrations of feed premix after day 1 may be the result of a very small initial loss of vitamin E acetate. Table 5 also shows that at manufactured medicated levels, there are no losses at ≤60°C during ≤3 months storage, and under the 3-month tropicalization test.

Discussion

A simple and rapid hexane extraction of supplemental vitamin E acetate from various kinds of feed premixes gives excellent recoveries. Vitamin E acetate is a slightly polar compound, sensitive to light and oxidation. A quick saponification step with acetic acid was used to break down the premix matrix. The sample was heated only 10 min at 70°C. Other recently published methods use a 30 min heating at 70°C (11). This step could replace the AOAC Soxhlet extraction (7) with a boiling solvent and, thus, prevent thermal degradation of the analyte. The combined hexane fractions of the saponified product were cleaned up by sorption on deactivated alumina. Triterpene squalane was chosen as an internal GC standard because it has a short retention time well in front of the studied analyte (Figure 2). Vitamin E alcohol can be determined simultaneously, however, as the saponification referred to here affects only the matrix and not the acetate moiety. Fused silica capillary analysis with short column, high injection temperature, and high carrier flow allows rapid elution of the analyte compared to its performance on a packed or glass capillary column. The value of the average response factor of 1.407 is higher than that found in packed column analysis where hexadecyl palmitate was

Table 5. Stability of supplemental vitamin E acetate in manufactured medicated feed supplement therapeuthan E[a]

Batch	Content of Vitamin E acetate, mg/kg	Accuracy of determination (RSD%) Inter-	Intra-
	No. 1		
5°C	5560	10.3	5.1
25°C	4190	13.4	4.1
35°C	4450	9.8	5.2
60°C	5090	6.6	1.2
Tropic. test	5056	9.5	6.6
Overall RSD, %[b]		10.2	4.8
	No. 2		
5°C	4870	9.5	6.5
25°C	5090	16.2	3.9
35°C	5650	10.5	6.8
60°C	4270	17.8	2.3
Tropic. test	4750	11.6	6.0
Overall RSD, %[b]		13.5	5.4
	No. 3		
5°C	4630	8.5	3.0
25°C	4850	6.5	5.0
35°C	4760	3.1	1.9
60°C	5330	4.0	3.8
Tropic. test	4650	5.0	2.4
Overall RSD, %[b]		5.7	3.1

[a] Results are calculated from 6 determinations.
[b] Computed as $(1/5\ \Sigma RSD_i^2)^{1/2}$ (root mean square).

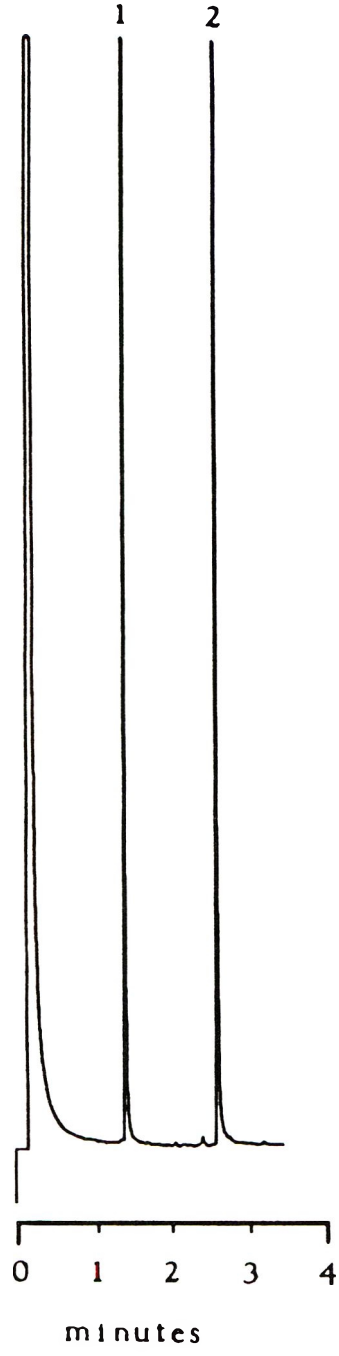

Figure 2. Determination of relative response factor. Peaks: 1 = squalane (basis), 2 = vitamin E acetate. RF = 1.407 + 0.008 (RSD = 0.54%) (n = 10).

used as internal standard (8). Under the conditions of analysis, no significant interfering peaks appeared in the chromatograms.

This capillary analysis method shows the clear, unambiguous separation of the vitamin E alcohol from the acetate, even at retention times <4 min. Small quantities of the alcohol form can be present in mixtures, resulting in quantitative errors, if the separation is not good. Conventional regression calibration was used in this work. More accurate and precise data could be obtained by attention to such details as constant variance across the graph and proper transformation details (12). This is being explored for a future publication.

The variability of the calibration data between the various levels (Table 2) can be explained as follows: Vitamin E acetate is a volatile component that could have undergone loss when taken to dryness. The relatively higher precision of recovery samples could be a result of the "keeper" action of matrix. The fall-off at the top, however, may have been due to column overload.

The extraction recovery was 98.9% for medicated feed supplement, 102.8% for medium, and 110.1 and 100.0% for the low class of premixes. The 110.1% recovery of analyte in premix for pigs in our laboratory is common for commercial vitamin tablets. A 138% recovery was claimed for an LC method for vitamin E acetate in multivitamin tablets (13). The within-day precision expressed as the relative standard deviation was 3.4, 2.9, and 4.0% for high, medium, and low concentration feed premixes, respectively.

The goal of this method was validation for a wide variety of matrixes rather than a longer calibration range. Premixes for poultry and sheep are different from those for pigs. Premixes for medicated purposes are different for still other reasons.

The drop in concentration exhibited at the lowest levels on the third day in Table 2 and, with fresh standards, the fourth day in Table 4, may have been a result of adsorption of analyte on vial walls or of auto-oxidation of the lowest concentrations. However, the drop at days 3 and 4 and the rise in content after 25 days of storage (from possible solvent evaporation) are not of great enough significance to show a statistical difference at the 95% level of confidence. Barring repetition of the tests, it is still recommended that fresh standards be prepared on every third day.

The homogeneity of sample sets was characterized by average "inter" relative standard deviation. For determination of representative analyte content at the 95% confidence level with a maximum error of 5%, 6 collected samples are sufficient (14). Only one batch (No. 2) of medicated feed supplement Therapeuthan E was more heterogeneous. Therefore, a higher number of samples may have to be taken for analysis in this case. No significant decrease of analyte concentration during stability tests was observed in all sample data sets. In stability tests, vitamin E alcohol was found only in the 60°C thermal stability test. By applying the van't Hoff rule for decay reaction, it would be possible to submit a 4-year expiration date for Therapeuthan E.

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FEEDS

Influence of Water on the Near Infrared Spectra of Model Compounds

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The application of near infrared spectroscopy (NIRS) to high-moisture samples (i.e., silages) has shown that accuracy does not match that of dried materials. Examination of spectra does not indicate absorbance levels to be the problem. The objective of this effort was to determine the effect of water on the spectra of model compounds. Near infrared spectra were taken using a Digi-Lab FTS-65 Fourier transform spectrometer. Liquids were examined by transmission and solids by reflectance. Examination of organic acids, alcohols, ketones, amines, and amides showed that the presence of water causes shifts in spectral wavelengths not related to OH or NH groups. The most significant shifts were for alcohols and ketones (up to 10+ nm at 90% H_2O) and least for acids. These peak shifts increased with increasing amounts of water and varied within individual spectra and among the compounds tested. As solids, sugars and amino acids had many sharp peaks in their spectra. As solutions, however, the sharp spectral features disappeared, resulting in large broad peaks. The spectra of polymers such as starch, cellulose, and casein did not appear to be significantly altered by the presence of water (0–50%, w/w), although they often appear to alter the water spectra. Variations in water content, physical state, and concentrations of components, when combined with these results, may help explain the decrease in accuracy of NIRS determinations that occurs with high-moisture samples.

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Although near infrared reflectance spectroscopy (NIRS) has been used extensively for the analysis of dried feedstuffs—literally hundreds of references are available—less research has been done on the analysis of moist agricultural materials such as silage (1–3). However, the application of NIRS to high-moisture samples (i.e., silages) has shown that accuracy does not match that of dried materials (4, 5). As shown in Table 1, the *r*-squares are consistently higher and the SE (SEC and SEP) consistently lower for dried materials than for wet. Examination of spectra does not indicate absorbance levels to be the problem. As shown in Figure 1, although the absorbances are much greater for wet silages than for dried, the maximum values are still well within the dynamic range of modern scanning spectrometers. By using dry ice to grind samples (3), it was shown that NIRS could be used with undried silages to determine many components of interest. Thus acceptable results were achieved for neutral and acid detergent fibers, crude protein, dry matter, in vitro digestible dry matter (DM), and several other components, although the results were not as good as results for dried samples.

A second study (4) examined the question of how sample presentation (area scanned), sample grind, spectral region scanned (680–1234, 1100–2498, and 680–2498 nm), and the particular chemistry of interest (DM, fiber, protein, etc.) interact to influence the quality of NIRS analysis. Although the results showed that the accuracy of NIRS determinations on wet silages could be affected by these parameters, again the best results were achieved with dried samples. Similar conclusions

Table 1. NIRS results for silages using various grinds and cells with samples scanned on a scanning monochromator from 1100 to 2498 nm with 10 nm between wavelengths[a]

	Calibration, $n = 98$		Validation, $n = 47$			
Sample	Grind[b]	Cell[c]	R[b]	SEC[d]	r[b]	SEP[e]
Acid detergent fiber	Dry	Round	0.96	1.49	0.96	1.48
	VIT	Long	0.96	1.61	0.95	1.83
	WIL	Long	0.95	1.73	0.92	2.13
Crude protein	Dry	Round	0.99	0.58	0.99	0.61
	VIT	Long	0.98	0.90	0.97	1.06
	WIL	Long	0.98	0.78	0.97	0.94

[a] Reeves III, J.B., and Blosser, T.H. (1991) *J. Dairy Sci.* **74**, 882–895.
[b] Grind: VIT = Ground with dry ice in a Vita-Mix food juicer and scanned in wet state, DRY = dried at 60°C, ground in a Wiley grinder to pass a 20 mesh screen, and scanned in a dry state, WIL = Ground wet in a Wiley grinder with a 3/8 in. screen and scanned in a wet state.
[c] CELL: Long = 6 in. by 2 ft vertical cell, Round = 2 in. diameter non-rotating cell.
[d] SEC = Standard error of calibration.
[e] SEP = Standard error of performance.

were drawn from a study using undried in situ-generated samples (5).

The question then remains as to why NIRS results for wet samples are of a lower accuracy than those for the same samples after drying. Because real samples, such as silages, are very complex mixtures of monomers (i.e., volatile fatty acids, ethanol, etc.) and polymers (i.e., cellulose, proteins, etc.) and dealing with such a complex mixture is very difficult, a study was designed in which model materials (i.e., mixtures of water and single compounds) were used to try to determine the basis for the performance of NIRS with wet materials. The study investigated the effect of water on the near infrared (NIR) spectra of 5 classes of materials including simple liquids, crystalline water-soluble solids, water-soluble and insoluble polymers, and the effect of solutes on the NIR spectra of water itself.

Experimental

Near infrared spectra were recorded with a Digi-Lab (BIO-RAD, Cambridge, MA) FTS-65 Fourier transform spectrometer. Two methods were used to obtain spectra: diffuse reflectance for solids and wet solids, using sulfur (sublimed, 100 mesh) as a standard and transmission (pathlength of ≤0.1 mm) for solutions of liquids and solids, with dry CO_2-free air as the standard. Liquid solutions were prepared at various volume/volume dilutions from 100% to 0% depending on the

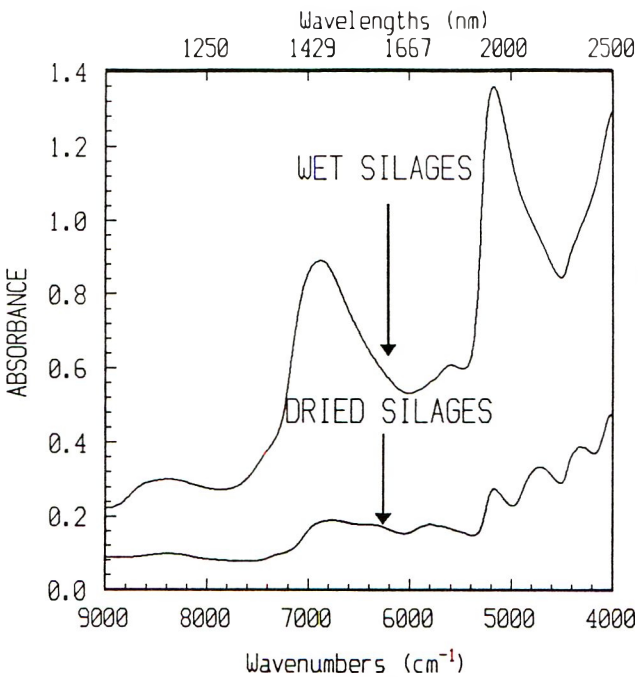

Figure 1. Averaged spectra of 145 wet and dried silages scanned on a scanning monochromator.

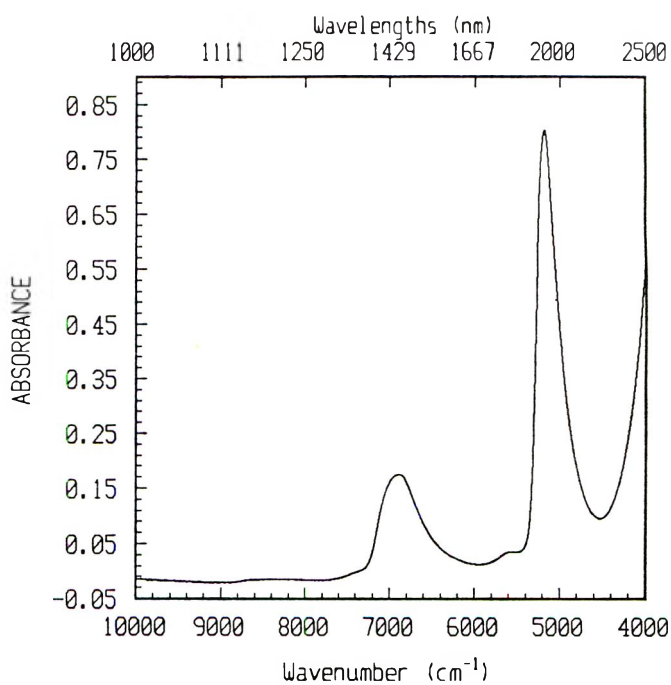

Figure 2. Near infrared spectrum of water.

solubility of the material in question. For solids, either percent water on a weight basis or saturated solutions (prepared by shaking excess material with water at room temperature) were investigated. Deionized water and reagent grade or better chemicals were used in all studies. Sugars and amino acids were the biologically active forms.

Although spectra were recorded from 10 000 cm^{-1} (1000 nm) to 4000 cm^{-1} (2500 nm) at a resolution of 4 cm^{-1} corresponding to 0.4 nm at 1000 nm, 1 nm at 1750 nm, and 2.5 nm at 2500 nm, only data from 5000 (2000 nm) to 4000 cm^{-1} (2500 nm) will be discussed. Instrumental noise levels were <0.1% or 100 μOD. This region was chosen for study because water does not absorb strongly there, but organic molecules do. Figure 2 shows the NIR spectrum of water. It can be seen that between 5000 and 4000 cm^{-1} there is a valley where water does not absorb. Spectral subtraction (unless otherwise noted) was used to remove the spectrum of water, and data were examined closely to be sure that effects noted were not due to spectral distortions caused by overlapping of the water and model compound spectra. Second derivative spectra and the effect of temperature on spectra were also examined.

Results and Discussion

Liquids

Several ketones, alcohols, acids, and amines were examined as solutions with water (0–95%). Several spectra of acetone/water mixtures, after subtraction of the water spectra, are presented in Figure 3.

The following conclusions can be drawn:

1. The wavelengths of some of the acetone peaks change or shift with water content.

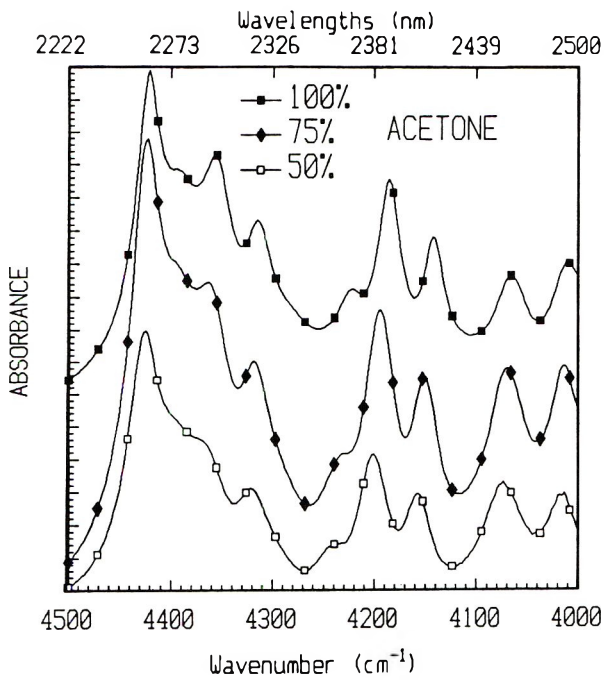

Figure 3. Spectra of acetone and 75% and 50% acetone in water (v/v) (after spectral subtraction of water).

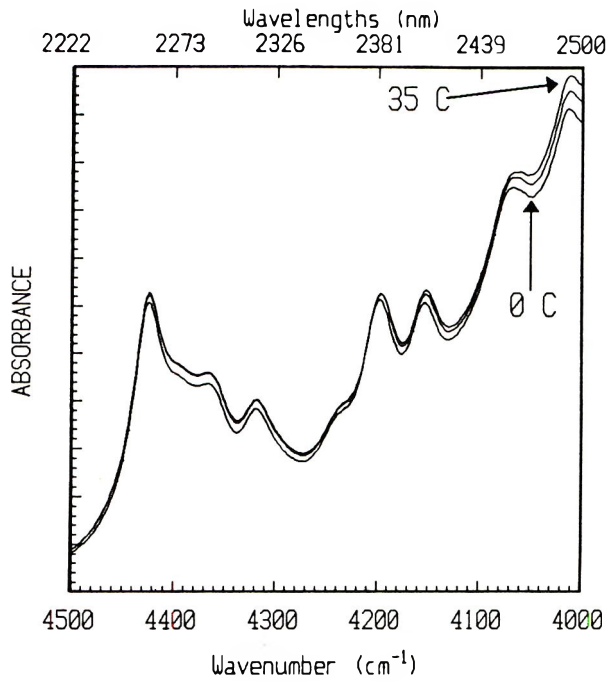

Figure 4. Spectra of 60% acetone in water (v/v) at 0, 19, and 35°C (without spectral subtraction of water).

2. The degree of shift depends on the peak in question and water concentration.

3. The shifts are in the opposite direction of what would be expected from the overlapping of water peaks with acetone.

At a water concentration of 50% the shifts for acetone peaks varied from ca 2 cm^{-1} (1 nm) at 4390 cm^{-1} (2278 nm) to ca 20 cm^{-1} (11 nm) at 4225 cm^{-1} (2367 nm). It should also be noted that although the amount of shifting increased in going from

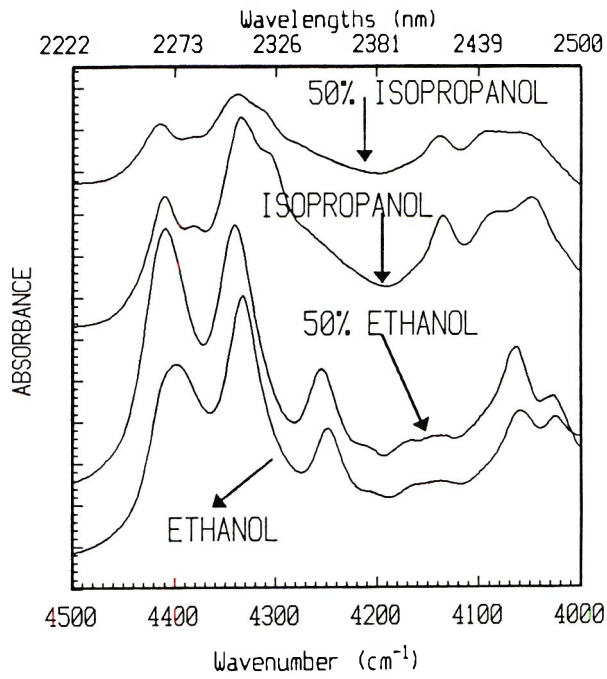

Figure 5. Spectra of ethanol and isopropanol and their 50% (v/v) solutions with water (after spectral subtraction of water).

4000 cm^{-1} (2500 nm) to 4225 cm^{-1} (2367 nm), it then became erratic, with both decreases and increases. Also (data not shown), no peak ever disappeared entirely (even at 95% water). The shifting in acetone came as a surprise; acetone, with only methyl groups and a carbonyl which is too weak to be seen, was thought to be an unlikely candidate for such shifts.

Comparisons of acetone/water spectra (water not subtracted) performed at 0, 19, and 35°C (Figure 4) showed that temperature changes could not account for the shifts found, as the spectra were virtually identical over a temperature range of 35°C and the spectra used in this study were all taken over a range of a few degrees at most. The same results were found for 100% acetone and for other acetone/water mixtures.

For ethanol and isopropanol (Figure 5), the shifts were generally half or less of those found with acetone, although at 4332 cm^{-1} (2308 nm), the shift was about the same.

For acetic and propionic acids (Figure 6), the shifts were only on the order of 2 or 3 cm^{-1} (1 or 2 nm) at the most, and a small peak at 4266 cm^{-1} (2344 nm) appeared to be lost at the 50% water level. Notice that for the 50% spectra in Figure 6, the spectra appear to become noisy from 4100 to 4000 cm^{-1}. This problem is caused by the subtraction of the water spectra and appears mostly between 4100 and 4000 cm^{-1} and again between 5000 and 4900 cm^{-1}, where absorptions due to water (Figure 2) become large. Changes in the water spectrum induced by the solute make it impossible to completely and accurately remove the effects of water. As will be seen later, the problem varies with the solute in question because of the relative concentrations of the solute and water; accordingly, saturated solutions were used for solids, thus maximizing contribution of the solute. Changes in the spectra of *n*-butylamine

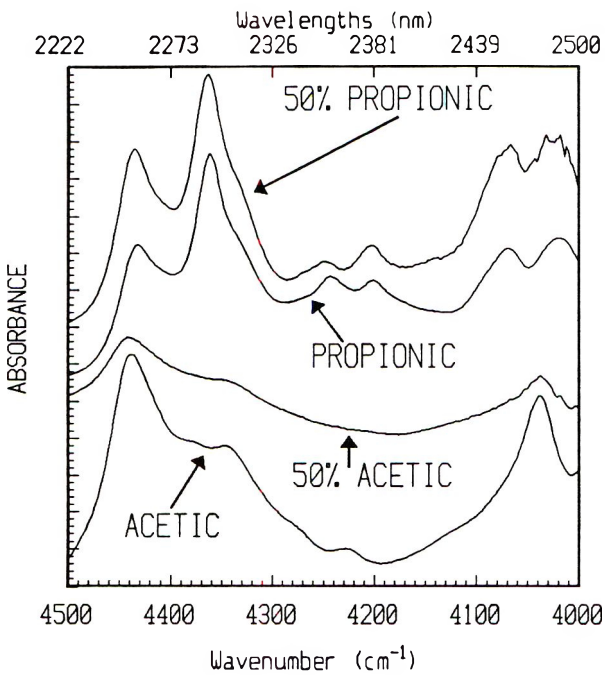

Figure 6. Spectra of acetic and propionic acids and their 50% (v/v) solutions with water (after spectral subtraction of water).

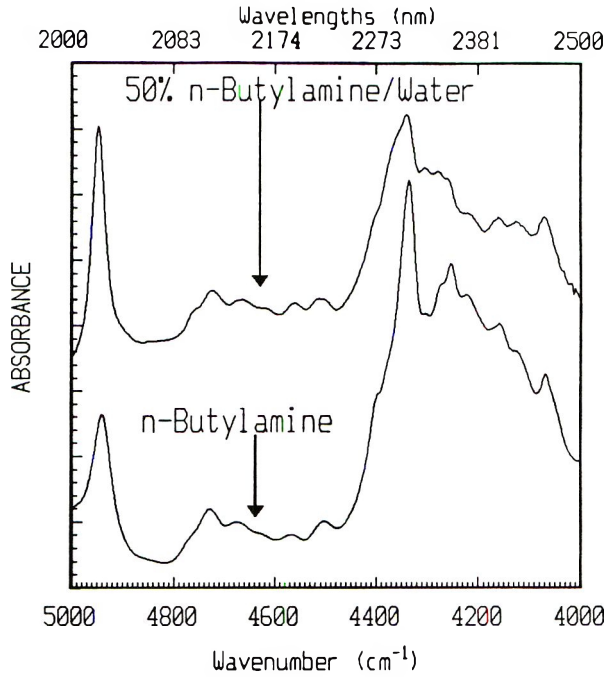

Figure 7. Spectra of *n*-butylamine and 50% (v/v) *n*-butylamine in water (after spectral subtraction of water).

(Figure 7) and formamide (Figure 8) were likewise small, although they were greater than for acetic acid. For *n*-butylamine, there was also a change in the spectra in the 6500 cm^{-1} region, where the water spectrum is relatively flat (Figure 9). In this case, a new peak appeared.

Finally, examination of similar compounds (i.e., ethanol and isopropanol, acetic and propionic acids, etc.), indicated that al-

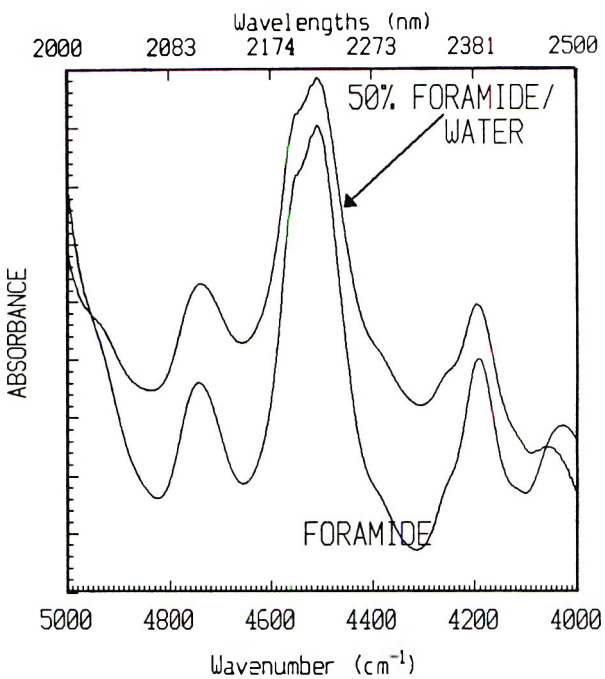

Figure 8. Spectra of formamide and 50% (v/v) formamide in water (after spectral subtraction of water).

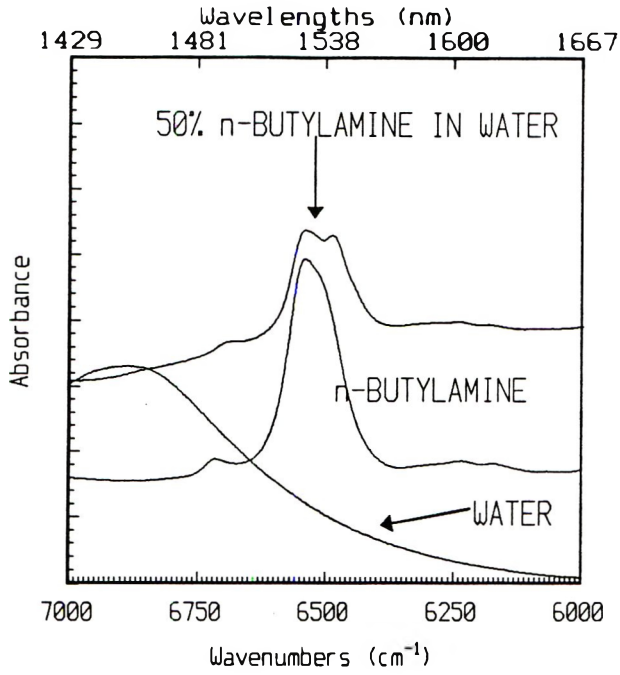

Figure 9. Spectra of water, *n*-butylamine, and 50% (v/v) *n*-butylamine in water (after spectral subtraction of water).

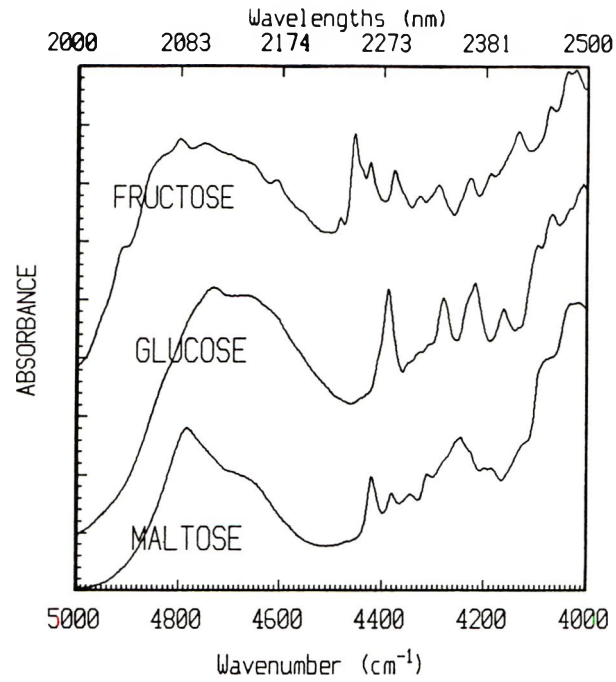

Figure 10. Spectra of crystalline fructose, glucose, and maltose.

though the degree of shifts was often similar (i.e., the spectra of organic acids did not seem to be altered greatly by the presence of water), the exact amounts of shifts could vary between similar compounds even when peaks occurred in virtually the same place. For example, ethanol and isopropanol both have a peak at 4411 cm^{-1} (2267 nm), but although the ethanol peak shifted only 0.6 cm^{-1}, the isopropanol peak shifted 4 cm^{-1} (0.5 and 2 nm, respectively) at the 50% water level. It should be noted that on a molar basis, the isopropanol was present only to the extent of 75% of the ethanol, and thus the higher water/solute ratio could account for some of these differences. However, the magnitude of the difference makes this seem unlikely, based on observations of shifts vs water concentration. Also on a molar basis, the ratio of water to methyl groups was less for isopropanol than for ethanol, and the ratio of solute to water for isopropanol was virtually the same as for acetone, where the shifting was much greater.

A common thread to these shifts would appear to be the polarity of the solute molecules. The more polar the solute, the less the effect of water because of a reduction in the influence of hydrogen bonding. Efforts to determine the exact pattern and degree of these shifts would be very difficult, because for most series of compounds, the materials rapidly become water-insoluble as the chain length increases. This, combined with the difficulty of spectral subtraction at the very high water levels (90–99%) required for insoluble materials, makes results difficult to substantiate. The next ketone after acetone (2-butanone), for example, could only be used at the extremes (10 and 75% water).

Solids

Monomers.—Except for alcohols and the volatile fatty acids, most of the monomers found in silages are solids. Figure 10 shows the spectra of glucose, fructose, and maltose from 5000 to 4200 cm^{-1} (2000 to 2381 nm). As can be seen, each

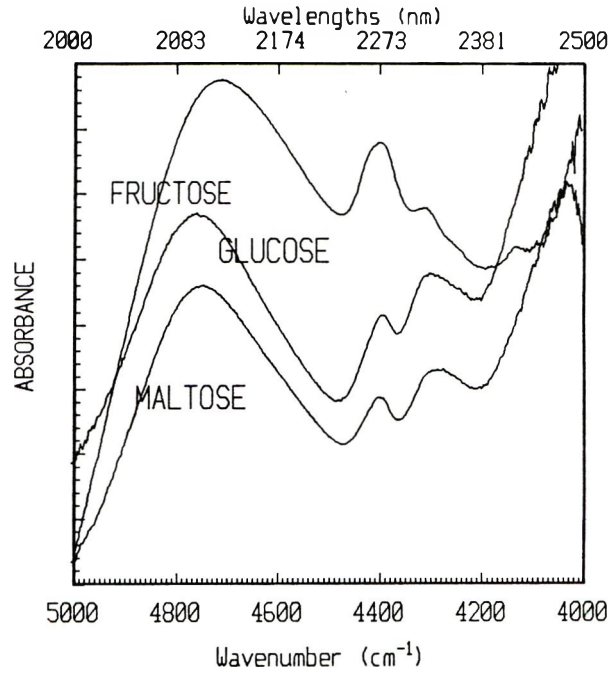

Figure 11. Spectra of saturated fructose, glucose, and maltose water solutions (after spectral subtraction of water).

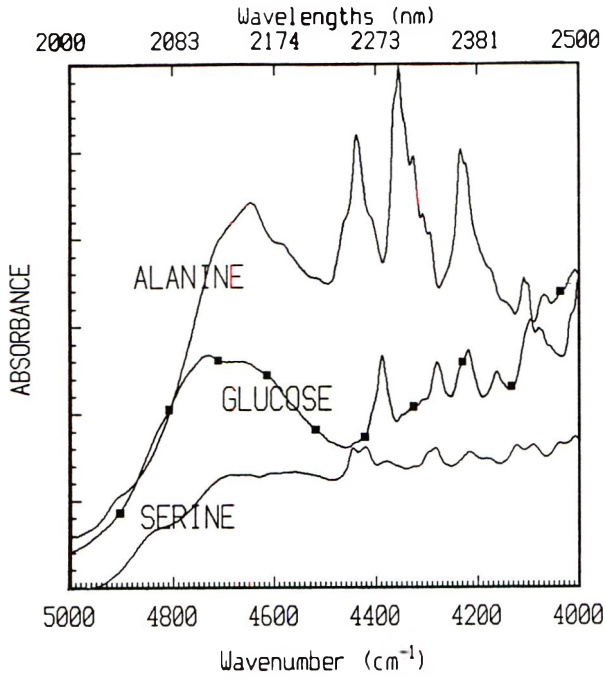

Figure 12. Spectra of crystalline alanine, serine, and glucose.

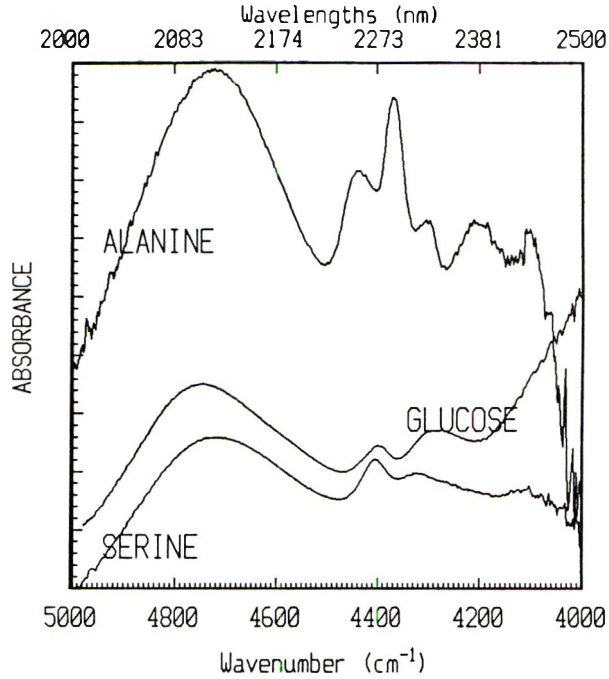

Figure 13. Spectra of saturated alanine, serine, and glucose water solutions (after spectral subtraction of water).

sugar (dry sample) has a distinctive spectrum with many distinct peaks.

Figure 11 shows the difference spectra of the same sugars as generated from spectra of saturated solutions. Two features stand out: first, most of the distinctive spectral features are gone, as though a smoothing operation was applied to the spectra, and second, the spectra now appear much more alike.

Examination of the amino acids alanine and serine showed similar effects (Figures 12 and 13). In addition, both serine and alanine, like glucose, showed a large shift (ca 44 cm^{-1} or 20 nm) in the position of the main spectral hump centered around 4700 cm^{-1} (2128 nm) when dissolved in water (Figure 13). The resulting difference spectra (for saturated solutions) for the 2 amino acids and the 3 sugars were very similar in this region as a result (Figure 13).

Finally, crystalline urea, although not showing the spectral details found for sugars and amino acids, did show significant spectral shifts when dissolved in water (Figure 14).

Considering all the materials in question, it would appear that much of the fine spectral features in these compounds (as solids) is due to crystal structure. When the compound is dissolved, this structure is lost. Hydrogen bonding with water can also result in a loss of distinctiveness for the hydroxyl groups on the sugars, thus producing molecules which spectrally look much more alike. Differences in the effects between molecules can be explained as differences in the relative importance of these 2 factors. For amino acids and sugars, dissolution results in a loss of crystallinity, but hydroxyl groups are a much more prominent feature of the sugar molecules. Urea behaves differently because it does not have any C-H or C-OH groups. Future studies will be needed to determine whether or not this is simply a water effect.

Polymers.—Many of the components of silages are polymers that make up most of the silage dry weight. These include proteins, cellulose, hemicelluloses, and sometimes starch. For this discussion, efforts were concentrated on soluble starch, cellulose, and casein as a model protein. The spectrum of cellulose (100–50% cellulose by weight) from 5000 to 4500 cm^{-1} (2000 to 2222 nm) resembled the spectra of dissolved

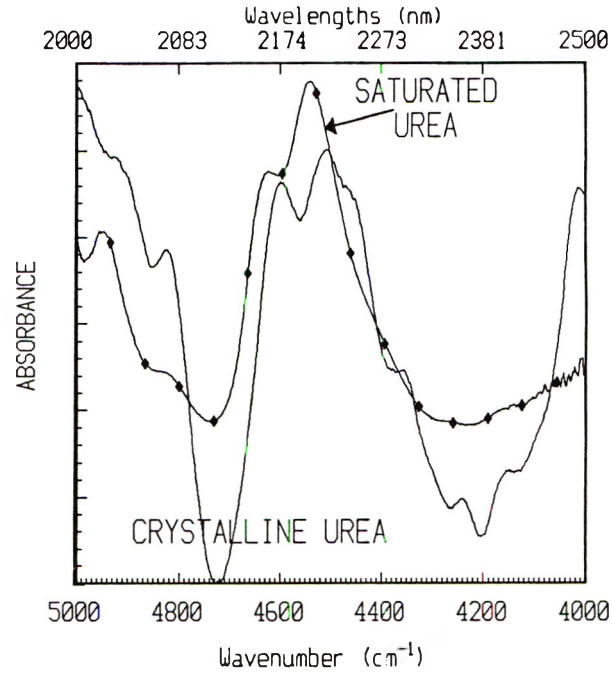

Figure 14. Spectra of crystalline urea and its saturated solution in water (after spectral subtraction of water).

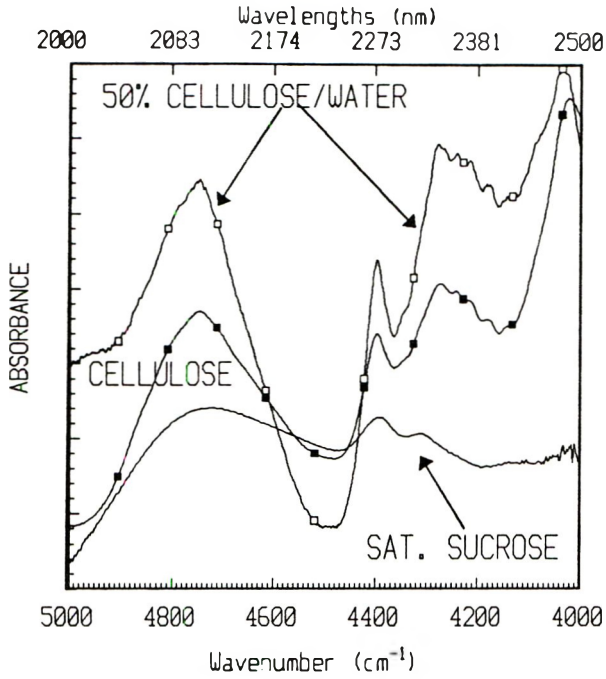

Figure 15. Spectra of cellulose, cellulose with 50% water (w/w), and saturated sucrose solution (after spectral subtraction of water).

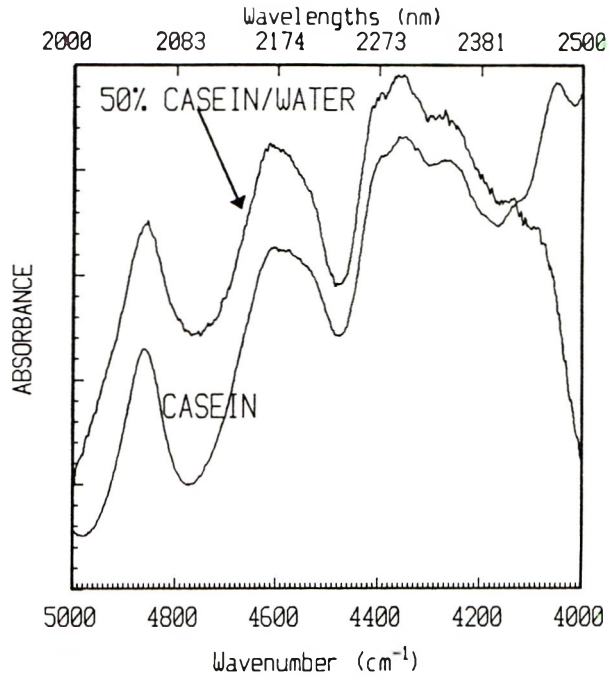

Figure 16. Spectra of casein and 50% casein (w/w) with water (after spectral subtraction of water).

sugars to a remarkable degree (Figure 15) and showed little or no change due to the presence of water. From 4500 to 4000 cm^{-1} (2222 to 2500 nm), the spectrum showed more detail than did the spectra of dissolved sugars but little or no change with increasing water content. The spectrum of casein showed very little fine detail (Figure 16), but did show some shifting with increasing water content and indications that the presence of water actually added information to the spectrum. As water was added (from 10 to 50% by weight), the spectrum appeared to become noisy with many small peaks. Close examination of this spectrum and of derivative spectra indicated that the spectral changes might be real and not just noise. However, studies using other proteins will be required to answer this question definitively. If real, the changes involved were over very small ranges (5–10 cm^{-1}, 2 to 5 nm) and only on the order of 0.001–0.002 absorbance units.

Even at levels of 95% water, it was possible to work with the spectra of acetone and see the trends in spectral changes. Wet polymers, however, were found to be difficult materials to work with, often producing noisy spectra and spectra for which it was very difficult to perform spectral subtraction. One problem with polymers is their state in agricultural samples. For example, soluble starch can be examined in at least 4 states: dry solid; an actual solution, made by dissolving a small amount of starch at room temperature; a wet powder, like cellulose mixtures, made by mixing starch and water at room temperature; and a gel made by boiling starch and water mixtures.

All 4 states of soluble starch were studied. At room temperature, so little starch dissolved that no indication of starch was seen in the spectrum, except for the fact that the curvature of the water spectrum between the water bands was so altered that spectral subtraction was nearly impossible. As shown in Figure 17, the curvature was altered, particularly between 5000 and 4000 cm^{-1} (2000 to 2500 nm).

Spectral subtraction then often resulted in negative curvatures in this region in the resulting difference spectra. This problem persisted in mixtures containing large amounts of starch (50–90%), indicating that the starch was significantly altering the water spectrum. Work with gels and mixtures did show shifts in the starch spectrum with increasing water con-

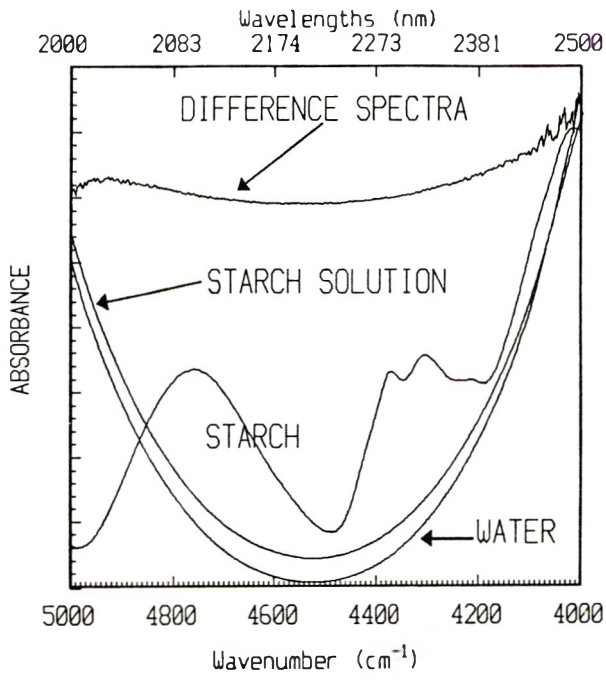

Figure 17. Spectra of starch (S), saturated starch solution, water (Y), and difference spectrum (S–Y).

tent which were more significant than those seen with cellulose. Also, examination of corn starch spectra showed small changes more like those on cellulose, indicating that solubility might be an important factor. Finally, the similarity of the spectra of carbohydrate polymers (wet or dry) to those of wet sugars supports the importance of crystallinity to the spectral results.

Conclusions

The work presented here indicates that several types of spectral alterations are caused by the presence of water in NIR spectra. Liquids showed shifts in the absorption bands, which depended on the class of compound in question (acid, alcohol, etc.) and the particular compound within that class. The same was true of solids; in addition, there was the added factor of solubility and loss of crystallinity for monomer-type materials. Finally, changes in the spectrum of the water were caused by the presence of these substances. Any or all of these factors may play a role in the degradation of the NIRS results found when working with high moisture materials, such as silages, which may vary greatly in their water content.

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FERTILIZERS

Comparison of Texas Liquid Sampler with Missouri Bottle for Sampling Liquid Fertilizers: Collaborative Study

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Six laboratories participated in a collaborative study designed to qualify the Texas liquid sampling tube as a device for sampling liquid fertilizers. The Texas sampler is a polyethylene tube with a stainless steel check valve on one end. The tube, which is threaded so that it can be expanded in length, enables acquisition of a uniform profile across a liquid sample. The 6 collaborators sampled 33 loads of fertilizer, 8 of which were slurries, using both the Texas tube and the Missouri bottle (AOAC 969.01). The resulting subsamples were analyzed for total nitrogen, available phosphorus, and soluble potash. Analysis of the data showed no significant ($p < 0.05$) difference in results. This sampling procedure, using the Texas liquid sampler, was adopted first action by AOAC International as a suitable means of sampling liquid fertilizers.

Method **969.01** (*Official Methods of Analysis* (1990) 15th Ed., AOAC International, Arlington, VA) prescribes 2 ways of sampling liquid fertilizers: (*a*) if the liquid is clear, the sample can be taken from the surface or through a direct tap and collected in a glass or polyethylene bottle, or (*b*) a sample container can be lowered into the well-mixed material and filled. No particular specifications are given for the sample container.

Fluid fertilizers with suspended materials can be sampled as described for clear liquids or they can be taken using a Missouri or Indiana sampling bottle (Figure **969.01A**). For some time, the Office of the Texas State Chemist has used a third method for sampling both clear liquid fertilizers and suspensions. The device consists of a polyethylene tube fitted with a stainless steel check valve on one end (Figure **969.01B**). The tube is threaded so it can be conveniently expanded in length. This so-called Texas liquid sampler, simple in construction and relatively inexpensive, provides acquisition of a uniform profile across a liquid sample. Because of the advantages offered by the Texas liquid sampler, the Office of the Texas State Chemist collaborated with others to determine whether this sampler provides samples that are equivalent to those taken by the Missouri bottle. Equivalency means that there are no statistically significant differences between analytical results on samples taken with this device and those taken with the Missouri bottle.

Submitted for publication June 2, 1992.

The recommendation was approved by the General Referee and the Committee on Feeds, Fertilizers, and Related Topics and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

Collaborative Study

Thirty-three loads (39 samples, 6 loads were sampled in duplicate) of liquid fertilizer were sampled by 6 different laboratories. One sample was taken by the method for use of the Missouri bottle (**969.01**); the other was taken according to the directions for using the Texas sampler, i.e., the sample was taken by inserting the Texas tube vertically downward and slowly withdrawing it while removing excess liquid from the exterior. Each sample was emptied into a plastic container of such size that the sample filled the container to no more than 75%. The object of the study was to determine the variability of the sampling techniques not confounded with interlaboratory variations in analytical methodology, so all samples were sent to the Office of the Texas State Chemist for analysis in that laboratory. Before sampling, the suspensions were shaken vigorously for 30 min and sampled immediately. Nitrogen was determined by the modified comprehensive method (**978.02**), available P_2O_5 by the direct gravimetric quinolinium molybdophosphate method (**960.03E**), and soluble K_2O by the volumetric sodium tetraphenylboron/oxalate extraction method (**958.02**). Of the 33 loads, 8 were suspensions or slurries and 1 contained sediment.

969.01 Sampling of Liquid Fertilizers—Direct, Missouri, or Indiana Sampling Bottle Methods

Final Action

(In absence of free ammonia)

(**a**) *Clear solutions.*—Mixed liquids and N solutions. Secure sample directly from mixing vat, storage tank, or delivery tank after thorough mixing. Take sample from surface or through direct tap. Flush direct tap, or delivery line and faucet, and collect sample in glass or polyethylene container. Alterna-

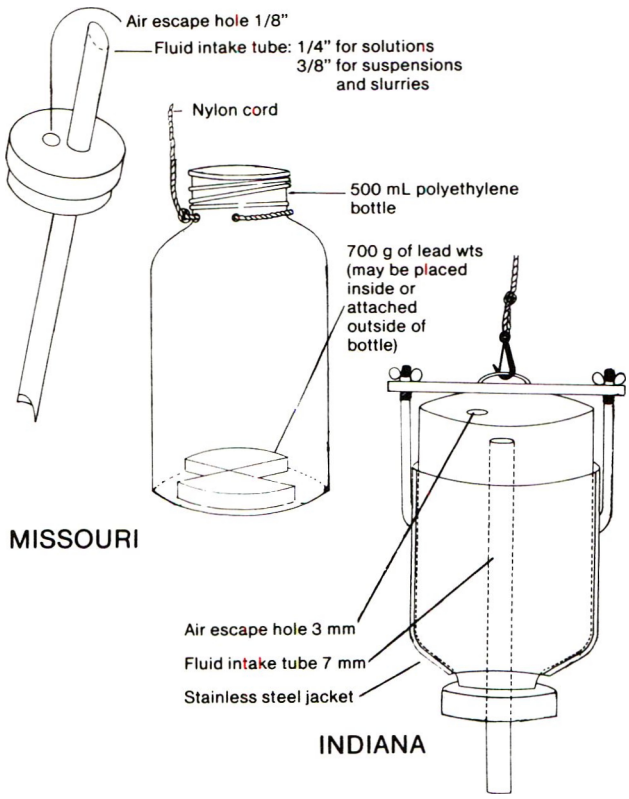

Figure 969.01A. Missouri and Indiana weighted restricted-fill fluid fertilizer sampling bottles designed to fill while being lowered (and raised) in storage tanks.

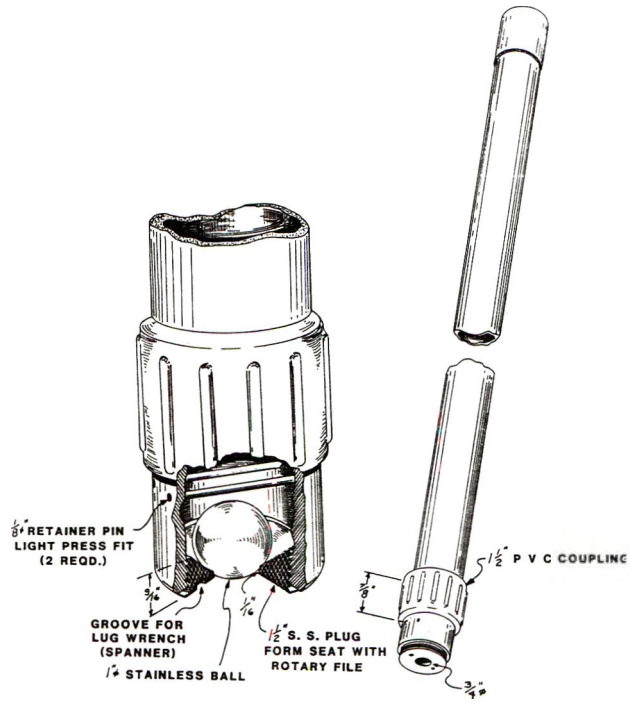

Figure 969.01B. Schematic of Texas liquid sampler.

tively, lower sample container into well-mixed material through port in top of tank and let fill. Seal container tightly.

(**b**) *Fluid fertilizers with suspended material.*—Salt suspensions and slurries. Agitate material in storage until thoroughly mixed (15 min usually adequate) before taking sample. Sample directly as in (**a**), or use 500 mL Missouri or Indiana sampling bottle, Figure **969.01A**. Lower sampling bottle from top opening to bottom of tank and raise slowly while filling. Transfer to sample bottle and seal tightly.

Alternatively, secure sample from tap on recirculation line after agitating *and* recirculating simultaneously until thoroughly mixed. Draw sample while recirculating. If recirculation line is attached to manifold delivery line, allowing cross-contamination, pump ca 30 cm (1′) or 2000 L (500 gal.) into temporary storage tank, and then sample from recirculation line as above or from delivery line. Transfer to sample bottle and seal tightly.

Ref.: JAOAC **52**, 592(1969).

Texas Liquid Sampler Method

First Action 1992

(Applicable to sampling of clear liquid fertilizers and fertilizer suspensions in absence of free ammonia)

A. Apparatus

Texas liquid sampler.—42 in. polyethylene tube, threaded on both ends, one to fit stainless steel check valve (see Figure **969.01B**) and one to accept additional lengths of tubing (Rick-Mar Metal Fabricators, Bryan, TX, is suitable source).

B. Sampling

Assemble sampler by screwing together enough lengths of tubing to reach bottom of fertilizer container.

Mix liquid fertilizers as required. Agitate fertilizer suspensions vigorously 30 min before sampling. Sample immediately. Insert sampler vertically downward into liquid fertilizer to depth required, and slowly withdraw sampler while stripping excess liquid from exterior with gloved hand or rag. Hold index finger over check valve and pour contents of sampler into plastic container of such size that sample fills container to only ca 75%. Clean after use by flushing with water.

Ref.: J. AOAC Int. (1993) **76**, July/August issue

Results and Discussion

Results of the analyses are shown in Table 1. All data were used in the paired *t*-test, even though 21 of the 196 duplicate analyses exceeded the 99% confidence limit for the average range of duplicates (ARD) that we expect within our laboratory. Data were retained because 17 of the 21 outlying duplicate results were for analyses of suspensions. Our ARD does not include many suspensions, and the wider ARD reflects problems in sampling from the laboratory subsample. However, 1 pair of nitrogen values was not used in calculating the statistic that estimates the ability of a given technique to secure a dupli-

Table 1. Analysis of liquid fertilizer samples taken by collaborators to compare Texas tube and Missouri bottle samplers[a]

		Nitrogen, %		Available P_2O_5, %		Soluble K_2O, %	
Coll.	Sample[b]	Tube	Bottle	Tube	Bottle	Tube	Bottle
1	1	9.80	9.76	33.07	33.03	—	—
		9.78	9.80	33.11	33.02	—	—
	2	9.57	9.59	32.69	32.68	—	—
		9.61	9.58	32.60	32.62	—	—
	3a[c]	10.55	10.61	29.52	29.64	—	—
		10.71	10.56	29.64	29.52	—	—
	3b	10.39	10.69	29.69	29.63	—	—
		10.49	10.49	29.63	29.74	—	—
	4a[c]	2.83	2.86	8.23	7.94	27.13[d]	25.89[d]
		2.83	2.84	8.11	7.74	26.94	25.57
	4b	2.92	2.82	8.18	7.83	26.17[d]	26.75[d]
		2.87	2.81	8.35	7.82	26.67	25.99
	5a[c]	4.74	4.74	14.17	14.07	16.06[d]	16.04[d]
		4.74	4.72	14.10	14.02	16.49	16.57
	5b	4.74	4.75	13.96	14.10	16.45	16.24[d]
		4.74	4.74	13.98	14.11	16.57	16.41
2	1	18.31	18.71	23.70	23.07	—	—
		18.40	18.77	23.73	23.06	—	—
	2	10.68	10.66	34.96	34.94	—	—
		10.68	10.67	35.04	35.12	—	—
	3	8.86	8.86	18.15	18.09	9.08	9.13
		8.76	8.88	18.13	18.09	9.04	9.07
	4	8.76	8.80	18.18	18.08	9.01	9.24
		8.68	8.73	18.15	18.12	8.98	9.25
	5	8.72	8.91	17.93	17.96	9.11	9.18
		8.87	8.90	17.98	18.05	9.18	9.26
	6	32.05	32.11	—	—	—	—
		32.10	32.14	—	—	—	—
3	1	11.90	12.06[d]	—	—	6.17	6.13
		11.82	11.56	—	—	6.12	6.01
	2[e]	11.72	11.82	—	—	—	—
		11.64	11.68	—	—	—	—
	3[c]	2.62	2.58	—	—	25.11	25.63
		2.63	2.60	—	—	25.28	25.68
	4	9.67	10.02	—	—	9.52	10.42
		9.64	9.82	—	—	9.44	10.22
	5	8.78	8.81	1.95	1.96	6.47	6.54
		8.71	8.71	1.95	1.95	6.47	6.45
	6[c]	14.66[d]	16.60[d]	3.95	4.05	19.20[d]	16.94[d]
		14.24	15.56	4.02	4.14	19.03	17.04
	7	9.18	9.37	3.11	3.13	6.49	6.47
		9.23	9.37	3.14	3.08	6.35	6.34

Table 1. ***(Continued)***

Coll.	Sample[b]	Nitrogen, %		Available P_2O_5, %		Soluble K_2O, %	
		Tube	Bottle	Tube	Bottle	Tube	Bottle
4	1	10.18	10.25	33.37	33.25[d]	—	—
		10.26	10.23	33.34	33.57	—	—
	2	10.13	10.17	29.63[d]	30.20[d]	—	—
		10.24	10.17	30.03	29.98	—	—
	3[f]	26.98	26.93	—	—	—	—
		26.93	26.97	—	—	—	—
	4	2.23	2.24	5.56	5.56	12.04	12.03
		2.23	2.24	5.54	5.53	12.03	12.05
	5	10.42	10.42	—	—	10.12	10.12
		10.45	10.47	—	—	10.00	10.18
5	1a[c]	23.73	20.36	6.87	6.38	—	—
		23.69	20.61	6.87	6.40	—	—
	1b	23.42	20.54	6.88	6.44	—	—
		23.78	20.54	6.88	6.43	—	—
	2	15.08	14.97	4.28	4.34	3.83	3.80
		15.08	14.94	4.23	4.19	3.82	3.77
	3	18.19	18.08	7.65	7.68	3.97	4.00
		18.12	18.09	7.68	7.78	4.05	3.92
	4	18.11	18.02	7.82	7.76	4.04	4.07
		18.01	17.99	7.84	7.81	4.12	4.14
	5	23.76	23.72	2.94	2.97	1.97	1.99
		23.72	23.63	2.98	3.05	1.99	2.04
6	1a[c]	15.71	14.80[d]	4.32	4.27	12.28	13.13[d]
		15.67	17.91	4.34	4.34	12.33	12.81
	1b	15.67	15.64	4.27	4.40	12.85[d]	12.32[d]
		15.66	15.64	4.34	4.39	12.29	12.93
	2a[c]	3.17	3.19	6.32	6.35	—	—
		3.20	3.17	6.30	6.24	—	—
	2b	3.20	2.66	6.31	6.27	—	—
		3.19	3.19	6.33	6.32	—	—
	3[c]	3.32	3.10	7.59	7.53	11.95	11.78
		3.33	3.31	7.54	7.61	11.90	11.74
	4[c]	2.00	2.00	5.73	5.69	15.17[d]	15.46[d]
		2.04	1.96	5.74	5.66	14.43	14.46
	5[c]	3.05	3.08	7.61	7.60	13.46	13.51
		3.06	3.06	7.66	7.52	13.45	13.81

[a] All samples were analyzed in author's laboratory.
[b] Thirty-three loads were sampled. Six loads were sampled in duplicate and those samples are designated, e.g., 3a and 3b.
[c] Sample (or duplicate sample) was a suspension.
[d] Duplicate analyses exceeded 99% confidence limit for average range of duplicates expected in author's laboratory. All data were used in statistical evaluation.
[e] Contained sediment.
[f] Also contained sulfur: sulfur, % = 4.03 and 3.95 for tube and 4.01 and 3.97 for bottle.

Table 2. Repeatability estimates

Analyte	Tube	Bottle
Nitrogen	0.07 (0.03)	0.08 (–)
Available P_2O_5	0.06 (0.05)	0.04 (–)
Soluble K_2O	0.29 (0.23)	0.30 (0.17)

cate sample from the bulk material. This was done because those data contributed about 25% of the total variance to the value.

Estimates of repeatability shown in Table 2 represent both variance contributions from sampling and from performing the determinations. The variances from sampling alone are in parentheses.

Negative results indicate that the component of variation from sampling is less than that allocated to the laboratory analysis. Overall, repeatability was equivalent for the bottle and the tube.

The statistical data from the standard 2-tail paired *t*-test, summarized in Table 3, show that there are no significant ($p < 0.05$) differences in results for analyses of samples taken with the Missouri bottle and those taken with the Texas tube. Note also that the 95% confidence level for the tests includes a necessary condition for deciding that there is no difference between methods of sampling.

Recommendation

I recommend that the sampling procedure, using the Texas liquid sampler, be adopted first action by AOAC International for sampling liquid fertilizers.

Acknowledgments

I thank the following collaborators:

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D. Terry, Division of Regulatory Services, University of Kentucky, Lexington, KY

Table 3. Statistical evaluation of analytical data for liquid fertilizer samples taken by Texas tube and Missouri bottle samplers

	Nitrogen		Available P_2O_5		Soluble K_2O	
Statistic	Tube	Bottle	Tube	Bottle	Tube	Bottle
No. laboratories	6	6	6	6	6	6
No. samples	39	39	32	32	24	24
Mean, %	11.28	11.18	13.72	13.65	11.97	11.93
Mean difference $\times 10^{-2}$	9.32		6.26		4.37	
95% confidence interval $\times 10^{-2}$	–15.9 to 34.5		–0.78 to 13.3		–20.1 to 28.9	
Two-tail test:						
P actual	0.459		0.0798		0.716	
P at <0.05	0.251		0.0704		0.245	

FOOD ADDITIVES

Simultaneous Determination of Flavor Enhancers Inosine 5′-Monophosphate and Guanosine 5′-Monophosphate in Food Preparations by Derivative Spectrophotometry

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A derivative spectrophotometric method was developed for the quantitative determination of 2 flavor enhancers, inosine 5′-monophosphate (IMP) and guanosine 5′-monophosphate (GMP), in the presence of monosodium glutamate (MSG). Procedures for determining IMP and GMP singly and in binary mixtures are described. Overlapping absorption spectra of both compounds were resolved by using first-derivative spectrophotometry. By measuring the first-derivative signals of IMP and GMP at 253 and 248 nm, respectively, simultaneous determination was possible for IMP and GMP at 5–40 μg/mL, in the presence of up to 5000 μg/mL MSG. The method was satisfactorily used to determine IMP and GMP in several food preparations.

The most frequently used flavor enhancers include L-aminoacids, such as glutamic acid (monosodium glutamate) (MSG) at 0.2–0.8%, and 5′-nucleotides, such as guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), and xanthine 5′-monophosphate (XMP) at 0.01% (1).

These compounds have pronounced flavors by themselves and can be detected clearly at high concentrations. Because high concentrations of MSG have been associated with a nervous system sickness known as "Chinese restaurant syndrome" (1), methods of diminishing the MSG concentration used in foods have been pursued.

Kodama (2), in 1913, described the synergistic flavor effect produced of MSG added to mixtures of 5′-nucleotides, and Kuminaka (3), in a review of a series of experiments with 5′-nucleotides, described its synergistic action in combination with MSG. MSG is currently used as a flavor enhancer in dehydrated soups and related products, together with small quantities of IMP or GMP (4). Because producers have been frequently using mixtures of IMP and GMP instead of IMP or GMP alone (4), the determination of IMP and GMP in mixtures and in the presence of MSG is of current interest in the area of food analysis.

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Numerous methods for the determination of these two 5′-nucleotides have been proposed, including photometry (5, 6), liquid chromatography (7–11), ion exchange chromatography (12), and thin-layer chromatography (13). All of these methods have been used to determine IMP, GMP, or mixtures of both compounds in food preparations.

Resolution of mixtures by using instrumental approaches avoids time-consuming cleanup procedures or separation steps. Derivative spectrophotometry offers greater selectivity than normal spectrophotometry (14), and because it can resolve spectral overlapping of highly overlapping species, simultaneous determination of binary mixtures can be done. We have used this approach to resolve mixtures of oxytetracycline and doxycycline (15), carbaryl and chlorpyrifos (16), furfural and hydroxymethylfurfural (17, 18), and butylated hydroxyanisole and butylated hydroxytoluene (19), among others.

Because of their similar chemical structures, IMP and GMP have spectra with large overlaps, which prevent their determination by conventional spectrophotometry. The present paper reports on the simultaneous determination of these compounds in food preparations by recording the first-derivative of the absorption spectra and by using the zero-crossing technique (15–18, 20, 21).

Experimental

Reagents and Chemicals

(a) *Standard solutions.*—Prepare standard solutions of guanosine 5′-monophosphate (Sigma), inosine 5′-monophosphate (Sigma), and L-glutamic acid (monosodium salt) (Sigma) in deionized water.

(b) *Buffer solution.*—Prepare buffer solution of pH 4.0 from acetic acid and 0.5M sodium acetate.

Apparatus

(a) *Spectrophotometer.*—Beckman DU-64 connected via RS-232 to Olivetti PCS-286 microcomputer equipped with Beckman Data Leader software (22).

Inosine 5′-Monophosphate Determination

Place aliquot of sample containing up to 125 μg inosine 5′-monophosphate in 25 mL volumetric flask, add 3 mL acetate buffer solution (pH 4.0), and dilute to mark with deionized

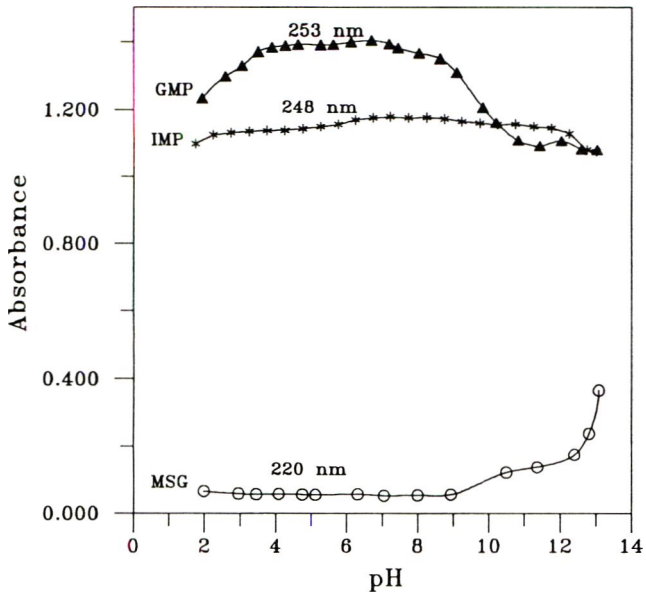

Figure 1. Influence of pH on absorbance of aqueous solutions of IMP (2.46×10^{-3}M), GMP (1.04×10^{-4}M), and MSG (4.24×10^{-2}M).

water. Record absorption spectra of samples between 200 and 350 nm against blank of water with 3 mL acetate buffer solution (pH 4.0). Smooth spectra through use of 7 experimental points and calculate the first-derivative spectra with $\Delta\lambda$ = 10 nm. Calculate first-derivative absorption spectra by the Savitzky and Golay simplified least squares method of spectral smoothing and differentiation (23, 24).

Determine IMP content from first-derivative signal at maximum wavelength of 256 nm and at minimum wavelength of 237.5 nm or by measuring the absolute peak-to-peak amplitudes, $^1D_{239.5} + {}^1D_{260.5}$.

Guanosine 5′-Monophosphate Determination

Place aliquot of sample containing up to 125 µg guanosine 5′-monophosphate in 25 mL volumetric flask, add 3 mL acetate buffer solution (pH 4.0), and dilute to mark with deionized water. Record absorption spectra of samples between 200 and 350 nm against blank of water with 3 mL acetate buffer solution (pH 4.0). Smooth spectra through use of 7 experimental points and calculate first-derivative spectra with $\Delta\lambda$ = 10 nm. Determine GMP content from first-derivative signal at maximum wavelength of 260.5 or 288 nm and at minimum wavelength of 239.5 nm or by measuring the absolute peak-to-peak amplitudes, $^1D_{237.5} + {}^1D_{256}$.

Analysis of IMP and GMP Binary Mixtures

Prepare samples in 25 mL volumetric flasks to contain 125–1000 µg IMP, 125–1000 µg GMP, and 3 mL acetate buffer solution (pH 4.0), and dilute to mark with deionized water. Record absorption spectra between 200 and 350 nm against blank prepared in absence of compounds, and obtain first-derivative spectra as described above. Determine IMP content from first-derivative spectrum by measuring first-derivative signal at zero-crossing point for GMP, at 253 nm, and comparing value with appropriate calibration graph. Determine GMP content by measuring first-derivative signal at zero-crossing point for IMP, at 248 nm, and comparing value with appropriate calibration graph.

Determination of IMP and GMP in Foods

For products in dry form, reduce 0.5 g to powder in mortar and mix with 25 mL deionized water in beaker. For products in liquid form, weigh 0.5 g into 50 mL beaker and mix with 25 mL deionized water.

Stir contents of beaker for 15 min and centrifuge. Transfer 15 mL to separatory funnel and add 5 mL water and 20 mL *n*-hexane. Shake vigorously for 5 min and let phases separate. Re-extract aqueous phase with 20 mL *n*-hexane and centrifuge aqueous phase. Transfer 15 mL into 25 mL volumetric flask, add 3 mL acetate buffer solution (pH 4.0), and dilute to mark with deionized water. Record absorption spectra and smooth them through use of 7 experimental points. Calculate first-derivative spectra with $\Delta\lambda$ = 10 nm, and determine IMP and GMP contents at $^1D_{253}$ and $^1D_{248}$, respectively.

Results and Discussion

Spectrophotometric analysis of IMP and GMP in different media and conditions have been proposed (5, 6). These compounds are usually determined by liquid chromatography with photometric detection (7–11).

The acid–base characteristics of these compounds were established with the object of developing a direct method for their determination in mixtures and in the presence of MSG. The influence of chemical and instrumental variables was also studied.

The influence of pH on the absorbance of IMP, GMP, and MSG in aqueous solution was investigated. Figure 1 shows that the determination of IMP and GMP can be carried out at pH 3.2–7. A pH of 4, obtained by adding 3 mL acetate buffer solution, was selected as optimum.

The absorption spectra of IMP, GMP, and MSG are shown in Figure 2. These spectra were recorded at between 200 and 350 nm at a scan rate of 500 nm min^{-1}. IMP and GMP have high absorbance in the UV region of the spectrum. IMP shows an absorption maximum at 248 nm, and GMP shows an absorption maximum at 253 nm and a shoulder at 275 nm. MSG has weak absorption at around 220 nm. The absorption spectra of IMP and GMP overlap, whereas MSG absorbs at lower wavelengths.

The possible interference of MSG on IMP and GMP analysis was studied. Up to concentrations of interference of 5000 µg/mL (125-fold excess), MSG did not interfere in the determination of 40 µg/mL of IMP or GMP by conventional spectrophotometry. For higher concentrations of MSG, a slight interference was observed. Because the usual concentration of IMP or GMP in foods is around 0.01% and the usual concentration of MSG in foods in the presence of IMP or GMP is less than 0.2% (20-fold excess), the interference from MSG in the quantification of IMP or GMP should be negligible.

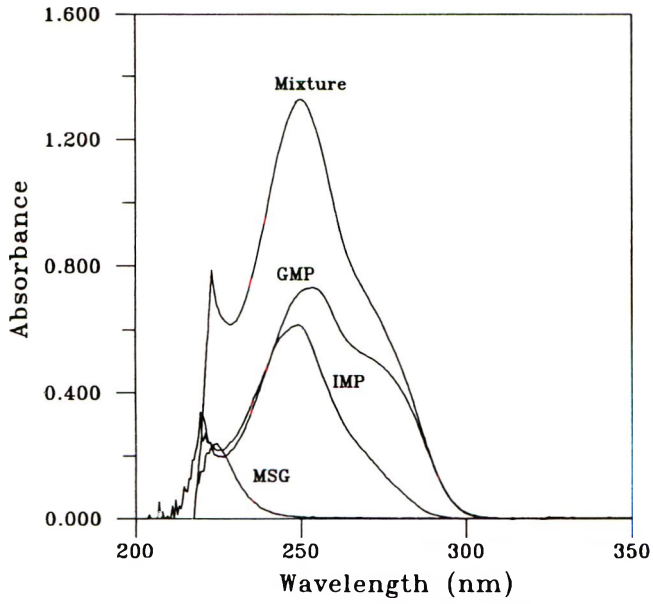

Figure 2. Absorption spectra of IMP (20 μg/mL), GMP (20 μg/mL), MSG (4000 μg/mL), and mixture of the 3 compounds at pH 4.0.

Figure 2 shows that the spectra of IMP and GMP have a large overlap, which prevents resolution of binary mixtures by conventional absorption spectroscopy. This overlap was resolved by using the first-derivative absorption spectra.

Instrumental Parameters

Instrumental parameters to register the absorption spectra and calculate the first-derivative spectra were selected. To record the spectra, a scan speed of 500 nm min gave the best signal-to-noise ratio.

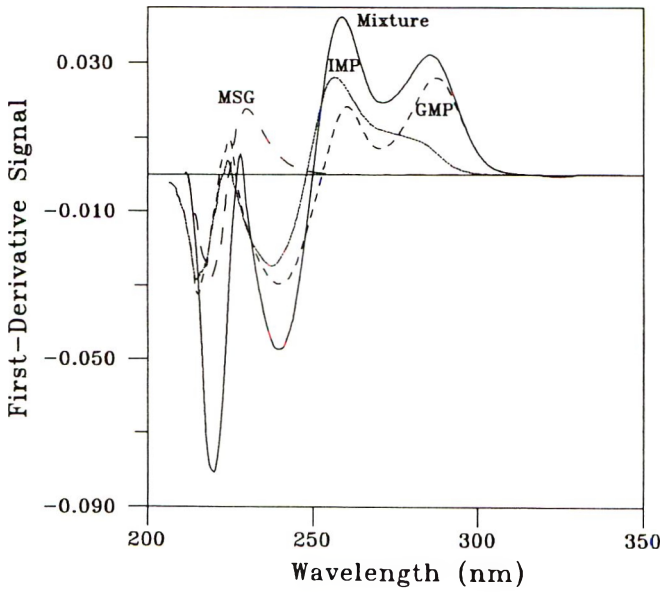

Figure 3. First-derivative spectra of IMP (20 μg/mL), GMP (20 μg/mL), MSG (4000 μg/mL), and mixture of the 3 compounds at pH 4.0.

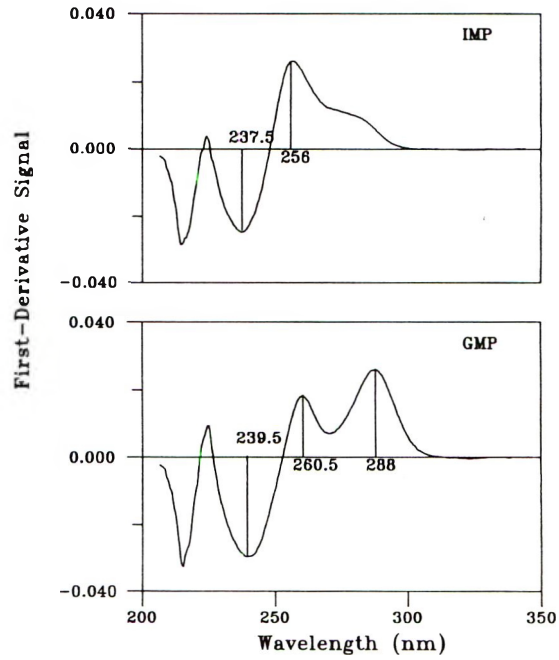

Figure 4. Wavelengths for different calibration graphs used for individual determination of IMP and GMP by first-derivative method.

When the derivative technique is used, other variables should be optimized, including the number of experimental points for smoothing the spectra, the optimal $\Delta\lambda$ for the derivation (23, 24), and the measurement wavelengths for the correct determination of both compounds. Our results show that, for IMP and GMP, and MSG, the signal-to-noise ratio is best when the spectra are smoothed by using 7 experimental points and when the first-derivative spectra is calculated with $\Delta\lambda$ = 10 nm.

First-Derivative Spectra

The first-derivative spectra of IMP, GMP, and MSG are shown in Figure 3. An interference study like that described earlier was carried out for the first-derivative spectra. MSG did not interfere in the determination of IMP but, at concentrations higher than 5000 μg/mL, interfered slightly in the determination of GMP. Different calibration graphs for the individual determination of IMP and GMP were obtained from the first-derivative spectra by using peak-to-peak amplitudes or peak-height-to-baseline measurements, as indicated in Figure 4.

If the derivative of a spectral band is equal to the sum of the derivatives of its individual bands, then the wavelengths chosen to measure the concentrations of IMP and GMP must have been suitable for the preparation of the calibration graphs for binary mixture analysis. These wavelengths were chosen by zero-crossing measurement, which involves measurement of

the absolute value of the total derivative spectrum of the mixture at an abcissa value (wavelength) corresponding to the zero-crossing of the spectrum of the interfering component, and vice versa. Figure 5 shows that the heights at 253 nm ($^1D_{253}$), the zero-crossing point of GMP, and at 248 nm ($^1D_{248}$), the zero-crossing point of IMP, are proportional to the concentrations of IMP and GMP, respectively.

Analytical Parameters

Methods are proposed for the individual determination of IMP and GMP by first-derivative spectrophotometry. Table 1 shows the equations of linear regression lines, correlation coefficients, detection limits, and determination limits (25, 26). These results indicate that all the selected derivative signals are suitable for the individual determination of IMP or GMP. The correlation coefficients and the intercepts on the axis (close to zero) indicate the good linearity of all the calibration graphs on the first-derivative measurements.

The analysis of IMP and GMP mixtures involves the construction of independent calibration graphs for each component. The signal for the first-derivative at 253 nm varies linearly with the IMP concentration in the range of 5–40 μg/mL, and the variation of the signal at 248 nm varies linearly with the GMP concentration in the range of 5–40 μg/mL.

For the determination of IMP at 253 nm, the relative standard deviation ($P = 0.05$, $n = 10$) and relative error were, respectively, 0.86 and 0.62% for IMP at 10 μg/mL and 0.31 and 0.28% for IMP at μg/mL. For determination of GMP at 248 nm, the relative standard deviation ($P = 0.05$, $n = 10$) and relative error were, respectively, 0.78 and 0.56% for GMP at 10 μg/mL and 0.27 and 0.19% for GMP at 28μg/mL.

The detection limits for IMP and GMP when determined simultaneously were 0.1μg/mL and 0.4 μg/mL, respectively. These values are the same as those reported for the chromatographic method with photometric detection (8).

The first-derivative method was used to analyze mixtures of IMP and GMP. Table 2 summarizes the results for several mixtures of IMP and GMP in different ratios.

Simultaneous Determination in Food Samples

The first-derivative method was used to analyze IMP and GMP in several food samples, as described in *Experimental*. To eliminate possible interferences, a previously treated sample was analyzed. Before proposing the procedure for the food samples, solvents that did not extract IMP and GMP were verified. Several solvents were assayed. Because IMP and GMP are not extracted in *n*-hexane, a solvent that can be used for the extraction of fats, it was selected for the procedure.

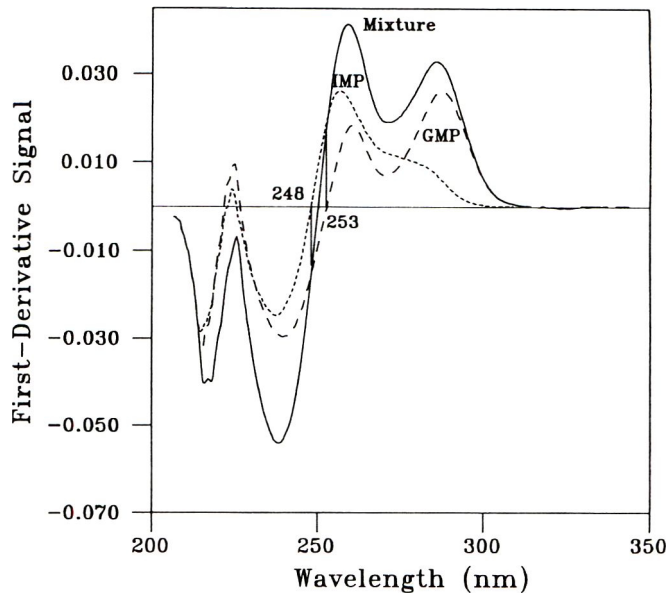

Figure 5. First-derivative spectra of IMP (20 μg/mL), GMP (20 μg/mL), and mixture of both compounds. The wavelengths for the simultaneous determination of both compounds, zero-crossing points, are marked.

Before adding IMP or GMP to the foods to be analyzed, a blank assay was performed; i.e., a portion of the food was taken and the previous treated sample was analyzed. When IMP or GMP was not present, no signal was obtained at the analytical wavelengths used, and no compounds were extracted. When IMP or GMP was present, a signal was obtained at the respective analytical wavelengths. This signal increased as the IMP or GMP concentration in the sample increased.

Table 3 summarizes the results of the recovery studies. The application of the procedure was tested by using the standard addition method. The calibration lines and the standard addition calibration lines gave the same slopes. In all cases, recovery values between 95 and 111% were obtained. The results can

Table 1. Individual determination of IMP and GMP by the first-derivative spectrophotometric method

Compound	Equation[a]	Correlation coefficient, r	Detection limit, μg/mL	Determination limit, μg/mL
IMP	$^1D_{256} = 0.0013C + 0.00002$	0.99999	0.124	0.415
	$^1D_{237.5} = 0.0013C + 0.0009$	0.99994	1.329	4.429
	$^1D_{237.5} + {}^1D_{256} = 0.0026C - 0.0009$	0.99999	0.653	2.178
GMP	$^1D_{288} = 0.0013C - 0.0002$	0.99999	0.103	0.344
	$^1D_{260.5} = 0.0009C + 0.00007$	0.99997	0.105	0.351
	$^1D_{239.5} = 0.0014C - 0.00009$	0.99997	0.971	3.237
	$^1D_{260.5} + {}^1D_{239.5} = 0.0024C - 0.00002$	0.99990	0.574	1.912

[a] ^1D = First derivate signal. C = IMP or GMP concentration, μg/mL.

Table 2. Determination of IMP (λ = 253 nm) and GMP (λ = 248 nm) in binary mixtures by the first-derivative spectrophotometric method

	IMP, μg/mL			GMP, μg/mL		
IMP:GMP ratio	Theoretical	Found	Rec., %	Theoretical	Found	Rec., %
1:2	5	5.5	110	10	10.8	108
1:1	10	10.8	108	10	10.6	106
2:1	20	21.5	107	10	10.4	104
1:4	5	5.5	110	20	22.4	111
1:2	10	10.8	108	20	22.4	111
1:1	20	21.3	106	20	22.4	111
1:2	10	10.9	109	20	19.3	97
2:1	10	10.4	104	5	4.2	84
4:1	20	20.7	104	5	4.5	90
2:1	20	20.9	104	10	9.6	96
1.5:1	15	16.1	107	10	10.6	103
2.5:1	25	26.9	107	10	10.5	106
1:1.5	10	10.9	109	15	14.4	96
1:2.5	20	21.2	106	25	24.6	98

be regarded as preliminary with respect to the application of the method to the analysis of foods. The degree of inaccuracy found is well tolerated for fast routine analysis. The amounts of IMP and GMP found are the same level as those usually found in such samples (8).

Conclusions

A first-derivative spectrophotometric method was developed for the determination of IMP and GMP in aqueous solutions. Derivative techniques can be used for the determination of individual compounds in turbid samples. In addition, the possible interference of MSG in the determination of these flavor enhancers was studied. At concentrations up to 5000 μg/mL, MSG interference was negligible.

The highly overlapping spectra of IMP and GMP were resolved by selecting the proper wavelengths for the measurement of each component without interference from the other. The first-derivative absorption spectra were used. The results of the analysis of IMP and GMP mixtures in several food

Table 3. Recovery of IMP and GMP in food preparations by the first-derivative spectrophotometric method

	IMP, μg			GMP, μg		
Food	Added	Found[a]	% Rec. ± SD[a]	Added	Found[a]	% Rec. ± SD[a]
Chicken vegetable soup	—	—	—	—	—	—
	200	215	108 ± 2	200	223	111 ± 1
	833	852	102 ± 3	980	975	99 ± 4
	1500	1558	104 ± 4	550	560	102 ± 2
Instant noodle soup	—	98	—	—	—	—
	882	1016	102 ± 2	882	967	110 ± 2
	441	591	110 ± 3	1588	1534	97 ± 3
	1765	1830	98 ± 3	245	232	95 ± 3
Fried tomato sauce	—	—	—	—	130	—
	200	192	96 ± 5	200	327	98 ± 4
	491	472	96 ± 7	491	630	102 ± 6
	1000	960	96 ± 3	1500	1638	101 ± 1
Consomme	—	90	—	—	36	—
	200	298	103 ± 4	441	488	102 ± 2
	882	970	99 ± 2	1000	1020	98 ± 3
	1500	1599	101 ± 2	245	287	102 ± 2

[a] Average of 3 independent experiments.

preparations and in the presence of high concentrations of MSG were satisfactory. The approach allows multicomponent determination without previous separation. Derivative techniques are simple and suitable for routine analysis of binary mixtures of compounds; complete resolution of species with highly overlapping absorption spectra is possible. They can be used for quality control of food preparations.

For simple binary mixtures, derivative spectrophotometric techniques allow direct determination of the individual compounds. However, for more complicated mixtures, multicomponent factor analysis methods are usually necessary. Different chemiometric procedures for the analysis of these commonly used food additives are currently under way in our laboratory.

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FOOD ADDITIVES

Gas Chromatographic/Mass Spectrometric Determination of Benzene in Nonstick Cookware and Microwave Susceptors and Its Migration into Foods on Cooking

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Benzene in poly(tetrafluoroethylene) (PTFE) coatings of nonstick cookware was determined by heating a small amount of coating in a sealed vial. Gas chromatography/mass spectrometry (GC/MS) was used to analyze the vial headspace for benzene. A small survey (26 samples) of retail nonstick cookware detected benzene ranging from 2 to 50 μg/dm^2 in 7 samples. Nonstick frying pans with various polymer coatings were obtained directly from 1 manufacturer. Benzene (6–30 μg/dm^2) was detected in a number of these samples and was attributed to the use of a phenylmethyl silicone ingredient that contained benzene at 360 mg/kg. To determine the possible transfer of benzene from these coatings during normal use, several foods (puddings, cakes, and roast potatoes) were prepared in previously unused cookware. The foods were analyzed by using static headspace GC/MS. Benzene was not detected in any of these foods at a limit of detection of 2 μg/kg. In related studies, the determination of benzene release from microwave susceptors was performed by heating the materials in a sealed system at 190°C for 4 min. Benzene release above 1 μg/dm^2 was not detected in 24 samples of susceptors. However, 1 specially supplied sample of nonmetallized susceptor released 10 μg/dm^2 benzene when heated above normal anticipated temperatures of usage (to 220°C). Foods such as french fries and pizza when cooked according to the manufacturer's instructions in susceptors contained no benzene with a limit of detection of 2 μg/kg. Even under abuse conditions of susceptors, the transfer of benzene to foods remained below this limit.

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The analysis of food contact materials previously led to the detection of benzene in polystyrene cups at up to 3.5 mg/kg (1). Benzene migration into water was not detectable when these cups were tested at 66°C (150°F). For a range of retail food packaging applications, benzene was detected in 11 different styrene-based polymers at levels from 0.11 to 1.7 mg/kg (2). However, the foods themselves were not examined. Other studies have been carried out on benzene in polypropylene food packaging materials, in paraffin waxes intended for food-contact use (3), and in commercial potato chip packages (4). Benzene has also been detected in certain food contact materials of thermoset polyester at levels from 0.3 to 85 mg/kg (2). The source of benzene was identified as the breakdown of an aromatic initiator used in producing the polymer. However, when foods were cooked in these thermoset food contact materials, the transfer of benzene and subsequent food contamination was very low (<0.01 to 0.09 mg/kg). Microwave susceptors have been an important topic for study. Although susceptors may release low levels of benzene when heated (5), a study in which susceptors were spiked with model compounds shows that the transfer of these model compounds to foods was negligible (6). Despite reports on the transfer of benzene from susceptors to foods such as popcorn (7, 8), there is little actual data on levels detected or the limits of detection at which measurements were carried out.

The present paper reports on the presence of benzene in PTFE-coatings of nonstick cookware and the liberation of benzene from microwave susceptors when heated beyond the expected real-use conditions (i.e., under abuse). Experiments have been carried out to detect benzene release from these food contact materials and to establish the extent of benzene transfer to foods prepared under normal and abuse conditions. Static headspace GC/MS has been used throughout with deuterated benzene as the internal standard, both for the analysis of the food contact materials and for the analysis of prepared foods.

METHOD

Reagents

(a) *Solvents.*—*N,N*-Dimethylacetamide (DMA) (99.5%) (BDH Ltd, Poole, UK).

(b) *Standards.*—Benzene (99.9+%) and deuterated benzene internal standard (d_6-benzene 99.5 atom %) (Aldrich Chemical Co., Poole, UK). **Note:** Benzene is a carcinogen and should be handled at all times with protective gloves in a fume hood.

(c) *Cookware.*—Purchased from retail outlets in Norwich, UK, area. Samples of known composition were supplied by commercial coating company.

(d) *Microwave susceptors, nonmetallized susceptor composites, and samples of adhesives used in their manufacture.*—Samples supplied with confidential formulation details by 3 manufacturers, as representative of susceptor materials for retail sale in Europe and the United States.

(e) *Microwave susceptor packaging and foods.*—Purchased from local grocer.

Apparatus

(a) *Engraving tool with carborundum tip.*—Burgess Powerline 72 (Argos Distributors, London, UK).

(b) *Domestic food processor.*—Braun multipractic Type 4233 (Argos Distributors, London, UK).

(c) *Glass headspace vials (20 mL).*—Chromacol 20-CV (Chromacol Ltd, London, UK).

(d) *PTFE-faced silicone rubber septa and aluminum crimp caps for headspace vials.*—(Chromacol Ltd).

(e) *Crimping device for sealing headspace vials.*—(Chromacol Ltd).

(f) *Laboratory fan-assisted oven thermostatted to 300°C.*—(Gallenkamp, Fisons Scientific Equipment, Loughborough, UK).

(g) *Oil bath.*—2 L Heat-resistant glass dish heated with immersion heater type IH/1 (Camlab. Ltd, Cambridge, UK) filled with Dow Corning silicone oil 210H/100CS (BDH Ltd).

(h) *Automated static headspace analyzer.*—Dani 3950 sampling unit (Dani SpA, Monza, Italy).

(i) *Combined GC/MS system.*—Carlo Erba Mega Series 5300 gas chromatograph coupled directly to VG 7070 EQ mass spectrometer (Fisons Instruments, Manchester, UK).

(j) *Capillary GC column.*—30 m × 0.33 mm id fused silica column with 1.8 μm phase of DB624 (J & W Scientific Inc., Fulsom, CA 95630).

Preparation of Calibration Solutions

Prepare solution of 20 μg/mL d_6-benzene in DMA as internal standard and solutions of 100, 20, 2, and 0.5 μg/mL d_0-benzene in DMA as working standards. Prevent loss of benzene by adding DMA to volumetric flasks and then injecting benzene under the surface, before making to mark with DMA. Store all standards at −20°C in screw capped vials.

Analysis of Nonstick Coatings

Determine volume of each cookware as mass of water required to fill to maximum level used in practice. Calculate food contact area by measuring various dimensions of cookware.

Wash cookware in soapy water, rinse with water, and dry as recommended in the manufacturer's instructions. Scrape known area (0.15–0.20 dm^2) of nonstick polymer coating from each cookware by using either scalpel or engraving tool. Transfer known weight of polymer sample to headspace vial. Add d_6-benzene internal standard solution by using syringe (10 μL). Seal vial with septum and aluminum ring and heat 30 min in oil bath at 250°C (for frying pan samples) or 230°C (for other cookware), with vial immersed to depth of 2–3 cm. Analyze headspace gas for benzene as described below.

Analysis of Nonstick Coatings for Benzene Release After Repeated Heatings

Analyze sample of nonstick coating for benzene release as described above and then cool to −20°C for 30 min, replace septum, and reheat sample for second period. Re-analyze headspace and then repeat procedure for third period. Because volume of headspace gas withdrawn for each analysis is small (0.2–0.3 mL) in relation to total volume of headspace vial, assume to first approximation that internal standard concentration remains unchanged throughout repeated analysis.

Analysis of Microwave Susceptors for Benzene Release

Cut 6 × 3.5 cm area of susceptor and place in 20 mL glass vial with longer side curled inside vial. Food contact surface of susceptor should touch side of glass vial to give good thermal contact and, thus, provide rapid heating to desired temperature. Add internal standard (10 μL of 20 μg/mL d_6-benzene in DMA), and seal vial with septum and aluminum ring. Immerse vial to depth of 4 cm in oil bath at 190°C for 4 min. Susceptor should be completely submerged. For testing under extreme conditions, heat samples for 10 min at 220°C.

Weigh 0.1 g sample of adhesive into vial and dry under gentle nitrogen stream for 15 min at 40°C. Add internal standard and heat at 190°C for 4 min.

Cool samples immediately to ambient temperature and analyze headspace by selected ion monitoring GC/MS as described below. If analysis is likely to be delayed by more than 3 h, store sample at −20°C.

Cooking Foods in Nonstick Cookware

Prepare foods such as "Yorkshire pudding," pate, quiche, cake, and roast potatoes, according to published recipes, and cook in nonstick cookware using times and temperatures recommended in recipe instructions. For each recipe, also cook in glass or metal cookware with no nonstick coating to act as appropriate control samples.

Heating Foods with Microwave Susceptors

Cook various microwave foods such as pizza, french fries, and pastry, whose packaging incorporates susceptors for heating, according to on-pack instructions. For each food, prepare blank by cooking/heating food on glass cookware, without susceptor present.

Sample Preparation

Weigh cooked food and then slurry with known mass of chilled distilled water by using food processor to achieve homogeneous mixture. Let foods such as cakes, which would nor-

mally be consumed at room temperature, cool before slurrying, and cover with aluminum foil during cooling. Weigh 2.0 g sample of food slurry into headspace vial, add internal standard solution (10 µL), and seal vial with septum and aluminum ring.

Prepare calibration standards in headspace vials from working standards to give 0.005–10.00 µg d_0-benzene per vial together with internal standard solution (10 µL). Inject primary standards close to bottom and down side of vials with vials cooled at −20°C to avoid loss of benzene. To each vial of blank food, add benzene for calibration purposes together with internal standard solution (10 µL). Seal with septum and aluminum crimp cap.

Instrumental Analysis

Let vials containing samples equilibrate in thermostatted bath of headspace sampler for at least 30 min at 100°C. Carry out automated headspace analysis by pressurizing vial with helium carrier gas, venting to sample loop, and then switching sample onto GC system. Operate with transfer manifold at 120°C, vial pressurization time of 10 s, auxiliary pressure of 0.2 bar, venting time of 5 s, and injection time of 2 s.

Carry out GC analysis isothermally at 50°C at helium carrier gas flowrate of 1 mL/min, and inject headspace with split ratio of 50:1 at injector temperature of 250°C. Operate mass spectrometer in electron impact mode at 70 eV with at source temperature of 250°C and trap current of 200 µA. Carry out selected ion monitoring at resolution of 500 (10% valley definition). Monitor ions at m/z 78 for benzene (d_0-benzene) and m/z 84 for deuterated benzene internal standard (d_6-benzene) with dwell times of 100 msec/mass and settling time of 20 msec. Under these conditions, benzene should be detected at 2 ng/vial with signal to noise ratio of 3:1. This performance is equivalent to around 0.01 µg/dm^2 for coatings and susceptors (0.2 dm^2/vial), and to 1–4 µg/kg food depending on food weight taken and proportion of water used to yield homogeneous slurry.

Retention time for putative benzene in samples should agree with time for standards, within variability of retention time established by repeat analysis of benzene standards. Measure peak areas by using data system but fit baselines manually.

Calculation of Results

Construct calibration graph from selected ion monitoring chromatograms of blank foods spiked with benzene and internal standard. Plot peak area ratios of d_0/d_6 for m/z 78 (d_0-benzene) and m/z 84 (d_6-benzene) against known weight ratio of added benzene to internal standard. Using calibration graph, interpolate benzene levels for samples.

Results and Discussion

Nonstick Cookware

The carborundum bit used to remove the polymer coating from nonstick cookware samples was also used to make "blank" scrapings from the outer surface of 1 pan. This outer surface was aluminum not coated with nonstick material. The GC/MS response for these blanks was equivalent to 1 µg/dm^2 benzene, which was attributed to the carborundum. These background values must be determined and allowed for in calculating migration levels. Table 1 shows the various retail cookware used to analyze benzene in nonstick coatings. Benzene release was below the limit of detection (1 µg/dm^2) for 19 of the 26 retail samples. However, benzene levels of 1.6 to 49.8 µg/dm^2 were determined for 7 samples sold under 3 different brand names. The specific weight of coatings for the cookware examined ranged from 50 to 200 mg/dm^2 although these figures are approximate only; some aluminum was unavoidably removed together with the coating. Benzene release was, therefore, in the range of 16–500 mg/kg coating.

Table 1. Benzene release from nonstick coatings of retail cookware articles

Manufacturer	Article type	No. samples	Benzene, µg/dm^2
A	Flan dish	1	19
A	Loaf pan	1	35
B	Sandwich cake tin	1	44
B	Cake pan	1	50
B	Cake tin	1	8
C	Cookware	5	<1
D	Cake tins	2	<1
E	Pie plate/cake tin	3	<1
F	Yorkshire pudding	1	<1
G	Oval dish	1	<1
H	Sauce pan	1	2
I	Pizza tray	1	2
J	Frying pan	1	<1
K	Cake and pie dishes	5	<1

To determine the likely source of benzene contamination, samples of nonstick cookware with polymer coatings of known composition were obtained directly from a coating company and analyzed. Benzene levels of 0.6, 2.6, and 30.2 µg/dm^2 were determined for polymer coatings of PTFE/polyethersulphone, PTFE/polyphenylene sulfide, and PTFE/phenylmethyl silicone, respectively. Furthermore, benzene levels of 11.9 and 6.4 µg/dm^2 were determined for samples containing a phenylmethyl silicone, whereas 2 samples with polymer coatings containing methyl silicones were found to have lower levels of benzene (0.8 and 0.6 µg/dm^2). This finding indicates that benzene release is associated principally with the phenylmethyl silicone component of the coatings. When this component was obtained from the coater and analyzed directly by using headspace GC/MS, benzene was detected at a concentration of 360 mg/kg. During the manufacture of nonstick cookware, a curing stage is included after application of the polymer coating, and the cookware is heated typically at 380°C for several minutes. This cure period is either insufficient to purge benzene from the coating, or benzene may arise as a later breakdown product of the phenylmethyl silicone although this breakdown must be limited because release ceases on repeated heating.

Table 2. Determination of benzene in foods prepared in nonstick cookware articles contaminated with benzene in the coatings

Article type	Benzene in coating,[a] μg/dm²	Food type	Cooking conditions	Benzene in food, μg/kg[b]
Six-cup tray	6–14	Pudding	45 min 180°C	<2
Loaf pan	10–22	Pate	60 min 160°C	<2
Cake tin	25–33	Quiche	40 min 180°C	<2
Loaf pan	9–10	Cake	60 min 160°C	<2
Cake tin	6–8	Cake	105 min 140°C	<2
Roasting	3–8	Potatoes	60 min 180°C	<2

[a] Heating conditions of 30 min at 230°C.
[b] Cooking experiments performed with 3 new items in each case.

Experiments were carried out to examine whether benzene contamination of nonstick cookware could be eliminated by pre-treating the cookware with oil. Previously unused cookware of a type that releases benzene when heated was filled with olive oil and heated 10 min in a laboratory oven at 250°C. The temperature of the oil reached 194°C. After cooling and removing the oil, the pan was washed in warm soapy water, rinsed with distilled water, dried, and examined. A benzene level of 8.4 μg/dm² was released by the nonstick coating compared with an initial release of 45 μg/dm². This finding indicates that pre-treating the cookware with oil could be a practical method of removing benzene from newly purchased cookware before food use although several treatments would be necessary for complete elimination.

Behavior of the nonstick coating under repeat-use conditions was also examined. For 1 frying pan coated with PTFE/phenylmethyl silicone, 14 μg/dm² benzene was released in the first heating, a further 11 μg/dm² in the second heating but only 2 μg/dm² in a third heating.

On the basis of benzene release from various polymers, the maximum possible benzene content in food was estimated if 100% transfer occurred. Depending on the anticipated use of the cookware, 1 to 560 μg/kg benzene could be released into food from the coatings of the retail cookware in Table 1. To examine actual transfer, a number of typical food items were prepared in nonstick cookware following normal recipes appropriate for the intended use of the cookware. The levels of benzene released from nonstick cookware coatings under simulated conditions are shown in Table 2 together with migration results for food prepared under real conditions of use. Benzene migration was not detectable (<2 μg/kg), and this finding indicates that transfer is a very inefficient process (<0.5% transfer). Volatile substances are released from the coating but not effectively trapped by the food, and portions that are trapped are likely to be lost subsequently by a process equivalent to steam distillation.

Benzene was detected in the blank foods (up to 5 μg/kg for fruit cake). These background values must be determined and allowed for in calculating migration levels. The analysis of foods spiked with known levels of benzene gave ion ratios for m/z 78/84 as predicted from the external calibration line, indicating no other significant matrix effects to account for.

Repeat-use conditions were investigated for 1 cookware. Three carrot cakes were cooked in succession in the cookware whose polymer coating had released 9.6 μg/dm² when heated. The level of benzene in the cake was < 2 μg/kg on each of the 3 uses. Thus, there was no obvious effect of repeat use.

Microwave Susceptors

For 24 samples of microwave susceptors and their component parts, including adhesives obtained directly from the manufacturers, benzene was not detectable (<0.01 μg/dm²) in 14 samples and was present only at low levels (0.01–0.06 μg/dm²) in 9 samples. The highest levels were found in an experimental nonmetallized composite susceptor that released 0.28 μg/dm² and in a solvent-based acrylic latex adhesive that released an equivalent of 0.1 μg/dm² on the basis of the recommended application rate of the adhesive to the susceptor. For 7 retail susceptor samples, one contained no detectable benzene and the remainder released 0.01–0.58 μg/dm². If 100% transfer occurred, even the highest level of benzene would only give rise to 4 μg/kg contamination. For reasons described above, actual transfer is expected to be very much lower than this worse-case calculation.

For the above experiments, a fiber-optic probe was used to measure the temperature at the susceptor surface, which rose to 190°C within 1 min of immersion of the vial containing the susceptor in the oil bath. A temperature of 190°C was selected as a typical operating temperature for a susceptor. However, the true temperatures reached at the surface of susceptors in actual use and the possibility of localized "hot-spots" are still uncertain. There is also the issue of whether or not conventional and microwave heating yield equivalent quantitative results because of microwave-induced crazing although this issue is likely to be significant for nonvolatile migration only. To take account of these uncertainties, an experiment was conducted by heating a susceptor sample to the higher temperature of 220°C. This sample released 10 μg/dm² benzene, or a level about 36 times higher than that observed at 190°C.

Results of the analysis of foods cooked or re-heated with microwave susceptors are shown in Table 3. In all cases, foods were cooked precisely according to the manufacturer's instructions and also under abuse conditions with either extended periods of heating or with heating at a higher microwave power than recommended. Although, in many cases, the foods ap-

Table 3. Determination of benzene in foods cooked with microwave susceptors

Food type	Microwave conditions		Benzene in food, μg/kg[a]
	Time, min	Power, W	
French fries	2.7	700	<2
	5.5[b]	700[b]	<2
	2.7[b]	1400[b]	<2
Pizza	6.5	700	<2
	9.7[b]	700[b]	<2
	4.0[b]	1400[b]	<2
Pie crust	8.5	700	<2
	11.0[b]	700[b]	<2
	6.0[b]	1400[b]	<2
French fries[c]	2.5	700	<2
	5.0[b]	700[b]	<2
	2.5[b]	1400[b]	<2
Pizza[d]	2.5	700	<2
	4.0[b]	700[b]	<2
	2.5[b]	1400[b]	<2

[a] Experiments performed in duplicate.
[b] Cooked under abuse conditions.
[c] Susceptor design with french fries individually browned.
[d] Pizza reheated on carbon-backed susceptor supplied directly by manufacturer.

peared unpalatable (dried out and slightly burnt) when cooked under abuse conditions, this did give the opportunity of examining whether even under extreme conditions the higher levels of benzene observed when the susceptors were heated alone would give rise to contamination of the foods. For all foods, benzene was not detectable above a limit of 2 μg/kg, indicating no significant initial uptake by the foods, or, alternatively, subsequent volatilization and loss to the atmosphere under steam distillation conditions (7).

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FOOD ADDITIVES

Liquid Chromatographic Method for the Determination of Nine Phenolic Antioxidants in Butter Oil: Collaborative Study

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Ten laboratories collaboratively studied a liquid chromatographic (LC) method for the determination of propyl, octyl, and dodecyl gallate (PG, OG, and DG, respectively), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) in butter oil. The 10 samples analyzed were spiked in matched pairs at about 100, 50, and 10 μg/g. In the method studied, antioxidants are extracted as in AOAC LC method 983.15, but different LC eluants are used to separate the 9 antioxidants. Results from 1 laboratory were rejected as not valid and were not included in any calculations. For the remaining 9 laboratories, the overall mean recoveries for PG, THBP, TBHQ, NDGA, BHA, OG, Ionox, BHT, and DG were 100.9, 97.8, 103.4, 95.4, 97.4, 93.6, 95.5, 79.0, and 96.2%, respectively. The overall reproducibility relative standard deviations were 8.55, 17.4, 25.6, 14.5, 6.60, 9.64, 10.8, 11.4, and 7.35%, respectively. The method was adopted first action by AOAC International as a modification of AOAC method 983.15.

In 1983, a collaborative study on a liquid chromatographic (LC) method for the determination of 7 antioxidants in oil and lard was reported (1), and the method was later adopted by AOAC (**983.15**) (2). The antioxidants studied were propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT). Where permitted, these antioxidants are generally regulated either singly or in combination at levels up to 200 μg/g in fats and oils. The AOAC collaborative study was conducted at about 200 μg/g to evaluate method performance at the regulatory level, at about 100 μg/g for combined antioxidant use, and at about 20 μg/g for lower level analyses (1). The method was based on a procedure first described in 1979 (3) in which octyl and dodecyl gallate (OG and DG) were studied in addition to the 7 above antioxidants. OG and DG were not included in the 1983 AOAC study because OG coeluted with Ionox-100 and BHT and DG were poorly separated. This was not considered a serious limitation in the method because Ionox-100 is permitted only in the United States, whereas OG is not permitted in the United States but is allowed in many European countries. Current increases in international trade, however, may require methodology to regulate all 9 antioxidants in various foods, including fats and oils.

Validated methodology for antioxidants is required to regulate their occurrence in butter oil. For example, the antioxidant TBHQ may be permitted in butter oil under certain circumstances in several countries but not in the various European countries. Furthermore, butter oil with added antioxidants may be restricted for use in the manufacture of bakery goods or confectionery products. Analysis of such butter oil must be conducted to ensure the absence of antioxidants if the oil is to be used in other applications.

Group E-43 of the International Dairy Federation (IDF) decided to study collaboratively AOAC method **983.15** to validate its applicability to butter oil. Several modifications in this method were to be introduced: evaluation of butter as a substrate; use of more efficient LC columns; use of a new nonaqueous eluant developed by the study coordinator, which effected the separation of all 9 antioxidants; and, with the newer LC columns, injection of only 10 μL.

A collaborative study of such a method, if successful, would provide the IDF with a validated method for the LC determination of 9 antioxidants in butter oil. Data would also be available for the possible extension of the AOAC LC method for antioxi-

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The recommendation was approved by the General Referee and the Committee on Residues and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

dants to include butter oil and, with modification of the chromatographic conditions, to include OG and DG.

Collaborative Study

The AOAC LC collaborative study reported in 1983 documented problems with the determination of TBHQ. TBHQ is the most readily oxidized of the 9 antioxidants. In some instances, recoveries were low because TBHQ can be lost during prolonged evaporation of the acetonitrile extract and also after the extract is transferred and adjusted to volume. The present study emphasized the need for rapid evaporation (<10 min) and injection of the extract as soon as possible after preparation.

The coordinator evaluated the separation of the 9 antioxidants on a variety of commercial columns packed with 3 or 5 μm spherical C_{18}-bonded silica. A list of columns that provided the desired baseline separation was sent to prospective collaborators. The method for collaboration, however, specified the columns in generic terms only as C_{18} reversed-phase columns with 5 μm (or less) spherical packing, but the method required baseline separation of the 9 antioxidants as shown in an accompanying chromatogram.

Butter oil for the collaborative study was prepared by heating butter at 65°C, decanting the oil, filtering (glass wool), and if required, centrifuging. The combined oil was then analyzed for interferences by using the method. Samples for the study were prepared by adding appropriate 1 or 2 mL aliquots of each antioxidant in 2-propanol to 95–120 g degassed butter oil in a 500 mL tared round-bottom flask. The 2-propanol was removed under vacuum with gentle heat and stirring. Individual 6 g aliquots, enough for 1 determination, were removed and placed in clean Teflon-lined screw-cap vials, sealed under nitrogen, and stored at −20°C. Each vial was labeled with a unique 3-digit random number. In total, 15 sets of collaborative samples were prepared. One set was reserved to monitor sample stability. Extra spiked material was analyzed to verify the spiking levels.

Ten samples were prepared, at 3 levels of about 100, 50, and 10 μg/g for each antioxidant. Using the split-level collaborative design originally described by Youden and Steiner (4), each of the 6 antioxidant spikes, as the 3 matched pairs, were distributed randomly among 6 of the 10 samples; the other 4 samples were blank for that particular antioxidant. Thus, the 9 antioxidants, as 6 spikes in 10 samples, gave either 5 or 6 antioxidants in each of the 10 samples.

Collaborators were supplied with the following: standards (150–200 mg each) of the 9 antioxidants; the 10 spiked samples with instructions for storage; about 35 g blank butter oil for practice pretrial recoveries; a copy of the method; forms for reporting results, raw data, and equipment used; and general instructions for a pretrial determination and other tests. Collaborators were instructed to run a blank gradient and to carry out a reagent blank determination, a blank oil determination, and finally, a practice recovery at 20 μg/g for each antioxidant. They were asked to proceed with the analysis of the collaborative samples only if recoveries were >90% (>80% for BHT).

Participants were instructed to extract and concentrate each sample, to make only 1 injection, to dilute the extract if required and reinject, and to alternate standard and sample injections. The calculated antioxidant level, average standard peak heights, sample peak heights, and dilution factors were to be recorded on the appropriate form. The collaborators were also requested to provide comments and copies of all chromatograms.

In total, 14 collaborators agreed to participate in the study. Samples were prepared in December 1989 and sent to participants in 1990 as follows: January (7 sets), February (1 set), March (4 sets), August (1 set), and September (1 set).

Analysis of the prepared samples by the study coordinator showed no loss of any antioxidant after storage at −20°C for 8 weeks. After 8 weeks at −20°C followed by 9 weeks at room temperature, only TBHQ spiked at 9.93 μg/g showed any loss (83.0% recovered) when the samples were analyzed. After 8 weeks at −20°C and a further 16 weeks at room temperature, the other antioxidants showed levels of 85–93% of the spiked concentrations. These results and the sample stability studies conducted previously (2) suggest that no appreciable loss of antioxidants in the samples should occur during storage before the analysis is conducted.

983.15 Phenolic Antioxidants in Oils, Fats, and Butter Oil—Liquid Chromatographic Method

First Action 1992

[Applicable to propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaretic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) at 20–200 μg/g in oils and fats and 10–100 μg/g in butter oil, and to octyl and dodecyl gallate (OG, and DG) at 10–100 μg/g in butter oil]

[*Caution*: See Safety Appendix, "Safe Handling of Organic Solvents," "Safe Handling of Special Chemical Hazards," and "Potential Hazards of Equipment, Vacuum." (2)]

Method performance:

See Tables **983.15A–C** for method performance data.

A. Principle

Antioxidants are extracted into acetonitrile. Extract is concentrated and diluted with 2-propanol. Antioxidants are separated by liquid chromatograph and measured by ultraviolet detection at 280 nm.

B. Apparatus

(**a**) *Gradient liquid chromatographic (LC) system.*—Equipped with 10 mV recorder or integrator to electronically measure peak heights, 10 μL sample loop injection valve, and detector to measure absorbance at 280 nm. Typical operating conditions: detector sensitivity, 0.05 AUFS; temperature, ambient; flow rate, 2.0 mL/min.

Table 983.15A. Method performance for phenolic antioxidants in oils, liquid chromatographic method

		Recovery		Repeatability		Reproducibility	
Antiox.	Mean added, μg/g[a]	Rec.	%	s_r	RSD_r, %	s_R	RSD_R, %
PG	193.7	184	95.2	16.0	8.66	16.0	8.66
PG	96.7	93.8	96.9	4.50	4.80	4.50	4.80
PG	19.4	17.6	90.9	2.01	11.5	2.52	14.3
THBP[b]	203.2	199	98.1	9.99	5.01	9.99	5.01
THBP	101.6	99.1	97.5	5.25	5.30	5.58	5.63
THBP	20.3	19.6	96.7	0.97	4.94	1.53	7.77
TBHQ	196.1	201	103	8.77	4.36	22.8	11.4
TBHQ	98.1	100	102	3.63	3.63	21.5	21.5
TBHQ	19.6	19.1	97.5	1.50	7.85	3.17	16.6
NDGA	93.8	91.7	97.8	5.06	5.51	6.39	6.97
NDGA	18.8	18.4	98.3	0.34	1.83	0.60	3.24
BHA	198.5	197	99.1	6.54	3.32	6.61	3.36
BHA	99.2	99.7	101	5.43	5.45	6.15	6.17
BHA	19.9	19.5	98.0	0.43	2.19	0.76	3.92
IONOX	208.2	198	95.3	17.7	8.92	19.4	9.76
IONOX	104.1	98.7	94.8	10.2	10.4	10.5	10.6
IONOX[c]	20.8	20.2	97.0	0.71	3.54	1.14	5.63
BHT[c]	202.9	170	83.8	3.50	2.06	4.54	2.67
BHT[c]	101.5	84.3	83.1	2.36	2.80	2.36	2.80
BHT	20.3	17.2	85.1	0.90	5.22	1.06	6.18

[a] "Added" antioxidants values measured to 4 significant figures, but rounded to nearest 0.1 μg/g.
[b] 2 of 7 laboratories rejected as outliers by the pair Grubbs test.
[c] 1 of 7 laboratories rejected as outlier by the single Grubbs test.

Table 983.15B. Method performance for phenolic antioxidants in lard, liquid chromatographic method

		Recovery		Repeatability		Reproducibility	
Antiox.	Mean added, μg/g[a]	Rec.	%	s_r	RSD_r, %	s_R	RSD_R, %
PG	96.9	90.1	93.0	3.18	3.53	3.18	3.53
PG	38.7	34.6	89.4	1.55	4.48	1.55	4.48
THBP	101.6	97.9	96.4	6.75	6.89	14.3	14.6
THBP[b]	40.7	36.4	89.5	1.35	3.71	1.47	4.06
TBHQ	98.1	95.6	97.5	7.71	8.07	22.1	23.2
TBHQ	39.3	35.0	89.0	6.04	17.3	11.6	33.2
NDGA	93.8	87.8	93.6	2.65	3.02	4.74	5.40
NDGA	37.5	35.3	94.2	1.06	3.01	1.79	5.06
BHA	99.2	97.4	98.2	2.49	2.56	3.72	3.82
BHA	39.7	38.3	96.6	1.90	4.97	1.90	4.97
IONOX	104.1	99.3	95.4	4.83	4.87	5.45	5.49
IONOX	41.7	40.7	97.7	3.49	8.56	4.90	12.0
BHT	101.5	87.9	86.6	4.63	5.27	4.90	5.58
BHT	40.6	34.6	85.2	1.11	3.22	1.17	3.39

[a] "Added" antioxidants values measured to 4 significant figures, but rounded to nearest 0.1 μg/g.
[b] 1 of 7 laboratories rejected as outlier by the single Grubbs test.

Table 983.15C. Method performance for phenolic antioxidants in butter oil, liquid chromatographic method

		Recovery		Repeatability		Reproducibility	
Antiox.	Mean added, µg/g[a]	Rec.	%	s_r	RSD_r, %	s_R	RSD_R, %
PG	92.1	89.3	97.0	4.76	5.33	6.08	6.81
PG	46.0	46.9	102	3.86	8.23	4.54	9.67
PG	9.20	9.53	104	0.450	4.72	0.875	9.17
THBP	87.0	82.3	94.6	5.34	6.48	11.2	13.6
THBP	43.6	42.8	98.1	3.77	8.81	6.68	15.6
THBP	8.69	8.7	101	1.39	15.9	2.00	22.9
TBHQ	105.9	111	105	10.9	9.78	24.3	21.8
TBHQ	52.8	51.8	98.2	2.01	3.87	11.0	21.2
TBHQ	10.5	11.3	107	1.55	13.8	3.82	34.0
NDGA[b]	96.5	93.0	96.4	6.27	6.74	6.27	6.74
NDGA	48.3	47.0	97.3	3.22	6.86	4.66	9.91
NDGA	9.63	8.9	92.5	2.11	23.7	2.39	26.8
BHA	101.3	96.3	95.2	8.49	8.81	8.49	8.81
BHA	50.6	48.8	96.5	2.29	4.70	2.50	5.12
BHA	10.1	10.2	101	0.515	5.06	0.597	5.87
IONOX	105.7	103	97.4	4.79	4.66	7.52	7.31
IONOX	52.9	50.2	94.9	3.77	7.51	4.13	8.24
IONOX	10.6	9.36	88.6	1.22	13.0	1.25	13.4
OG	89.2	86.3	96.8	3.80	4.40	4.37	5.06
OG	43.7	42.0	96.2	2.89	6.87	2.89	6.87
OG	8.76	8.19	93.5	1.69	20.6	1.69	20.6
BHT	96.5	76.7	79.4	9.16	12.0	9.51	12.4
BHT[b]	48.4	38.8	80.2	2.58	6.64	3.16	8.12
BHT[c]	9.65	7.47	77.4	0.443	5.94	1.03	13.8
DG	101.1	96.7	95.7	4.02	4.16	7.94	8.21
DG	50.6	48.8	96.5	2.98	6.12	3.05	6.24
DG	10.1	9.76	96.4	0.468	4.80	0.742	7.61

[a] "Added" antioxidants values measured to 4 significant figures, but rounded to nearest 0.1 µg/g.
[b] 2 of 9 laboratories rejected as outliers by the pair Grubbs test.
[c] 2 of 9 laboratories rejected as outliers; by the Cochran test, then by single Grubbs test.

(**b**) *LC column.*—Packed with C_{18}-bonded spherical (preferred) silica, or equivalent. Use guard column if desired. Must be capable of baseline resolution of all 9 antioxidants as shown in Figure **983.15**. Verify peak identities, if necessary, by injection of individual standards.

(**c**) *Glassware.*—Rinse all glassware with $CHCl_3$, acetone, and MeOH, successively, and blow dry with N_2.

C. Reagents

(**a**) *Solvents.*—Acetonitrile, 2-propanol, and hexane. Distilled-in-glass grade.

(**b**) *Mobile phases.*—(*A*) 5% acetic acid in H_2O (LC grade). (*B*) acetonitrile–methanol (1 + 1) (LC grade).

Run linear gradient, from 30% B in A to 100% B, over 10 min with hold until last antioxidant (DG) is eluted. For test solution only, increase flow rate to 4 mL/min at 100% B over 6 min or until nonpolar lipids are eluted. For test solutions and standards, return to 30% B in A over 1 min at 2 mL/min, and let baseline and pressure stabilize (ca 6 min). (*Note*: Reduce flow rate and proportionately increase rinsing and equilibration times if excessive back pressure results.) Run blank solvent gradient (no injection) to ensure no peaks interfering with any antioxidant are present. To remove or reduce peaks arising from elution solvent, A, replace inlet filter with prerinsed C_{18} solid-phase extraction cartridge and use in-line filter. If small interfering peaks are not eliminated, subtract peak height of gradient interference from that of relevant standard or test solution.

(**c**) *Antioxidants.*—BHA (2- and 3-BHA mixture), BHT, TBHQ, Ionox-100, THBP, and PG (PolyScience, Niles, IL 60648, USA, is suitable source); NDGA, OG, and DG (Aldrich Chemical Co., Inc., Milwaukee, WI 53201, USA, is suitable source); purity ≥97%.

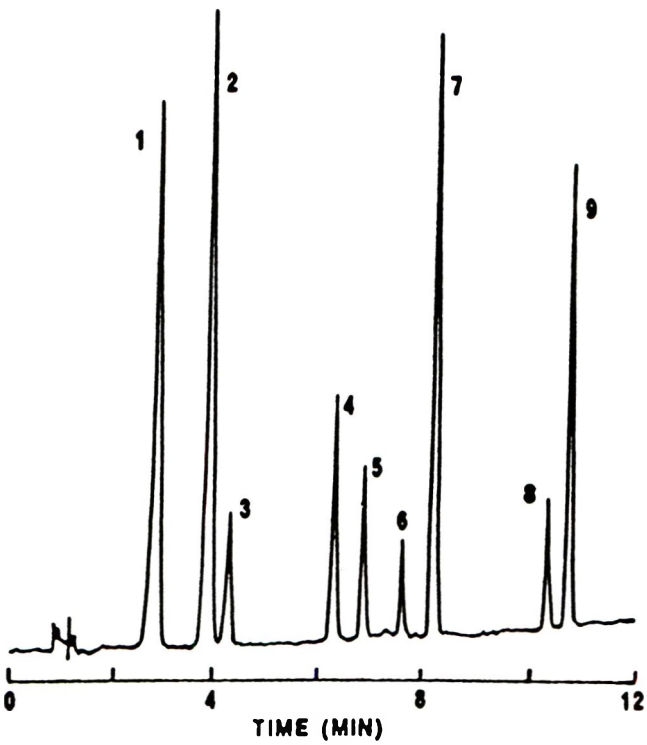

Figure 983.15. Chromatographic separation of antioxidant standards, ca 0.1 μg each antioxidant: 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, OG; 8, BHT; 9, DG.

(**d**) *Standard solutions.*—Prepare in 2-propanol–acetonitrile (1 + 1). (*Caution*: TBHQ is readily oxidized, especially in light. Refrigerate all antioxidant solutions and store out of direct light. Monitor TBHQ response relative to PG or THBP, and prepare fresh standards if response decreases more than 5%. (*1*) *Stock standard solution.*—1 mg/mL. Accurately weigh ca 50 mg to nearest 0.1 mg of each antioxidant, and transfer into single 50 mL volumetric flask. Dissolve, dilute to volume, and mix. (*2*) *Working standard solution.*—0.01 mg/mL (10 μg/mL). Pipet 1 mL stock standard solution into 100 mL volumetric flask, dilute to volume, and mix.

(**e**) *Extraction solvents.*—(*1*) *Saturated hexane.*—Saturate ca 300 mL hexane in separatory funnel by adding acetonitrile until 2 layers remain after shaking 2 min. Discard acetonitrile lower layer. (*2*) *Saturated acetonitrile.*—Saturate ca 300 mL acetonitrile in separatory funnel by adding hexane until 2 layers remain after shaking 2 min. Remove and discard hexane upper layer.

D. Determination

(**a**) *Extraction.*—Accurately weigh to nearest 0.01 g 50 mL beaker containing ca 5.5 g liquid or butter oil or ca 3.0 g lard or shortening (liquefied in bulk using 60° water bath or oven, and swirled or shaken to ensure homogeneity). Decant as much test portion as possible into 125 mL separatory funnel containing 20 mL (22.5 mL for lard or shortening) saturated hexane. Reweigh beaker to determine test portion weight. Swirl to mix test portion with hexane, and extract with three 50 mL portions of saturated acetonitrile. If emulsions form, hold separatory funnel under hot tap water 5–10 s. Collect extracts in 250 mL separatory funnel, and let combined extracts slowly drain into 250 or 500 mL round-bottom flask to aid removal of hexane–oil droplets. (*Note:* At this point, 150 mL acetonitrile extract may be stored overnight, refrigerated.)

Evaporate to 3–4 mL, using flash evaporator with ≤40° water bath, within 10 min. [*Notes*: (*1*) Prolonged evaporation time may cause TBHQ losses. To decrease evaporation time, use efficient vacuum source and water–ice condenser cooling. (*2*) Use 500 mL flask to reduce "bumping" losses. Take care to ensure quantitative transfer of extract after evaporation.] Using disposable pipet, transfer acetonitrile–oil droplet mixture to 10 mL glass-stoppered graduated cylinder. Rinse flask with small portions of nonsaturated acetonitrile. As rinse pools in flask bottom, pipet rinse to cylinder until 5 mL is collected. Rinse pipet through top, and continue to rinse flask with small portions of 2-propanol, transferring rinses to cylinder until 10 mL is collected. Mix cylinder contents. (*Note*: Delay in analyzing extracted sample may cause TBHQ loss.)

(**b**) *Chromatography.*—Using sample loop injection valve, inject 10 μL sample extract and elute with solvent gradient program for samples, **C(b)**. Before and after every 3–4 test injections, or more frequently if differences between standard peak heights are found to be >5%, inject 10 μL antioxidant working standard solution (10 μL/mL) and elute with solvent gradient program for standards, **C(b)**. For analyte peaks off-scale or >3× standard, quantitatively dilute test extract with 2-propanol–acetonitrile (1 + 1) and reinject. Identify peaks by comparison with retention times of standard.

For reagent blank determination, take 25 mL saturated hexane and follow extraction, (**a**), from "...extract with three 50 mL portions of saturated acetonitrile." Inject 10 μL reagent blank extract, and elute with solvent gradient program for samples. The reagent blank should have no peaks interfering with antioxidant determination.

Use electronically determined peak height, or measure peak height to 0.1 mm, by using blank gradient chromatogram as guide to follow baseline. Determine antioxidant peak heights and average antioxidant standard peak heights (from duplicate injections before and after test injection, corrected for gradient blank).

E. Calculation

Calculate concentration of antioxidant as follows:

$$\text{Antioxidant, ppm} = (R_x/R_s) \times (C_s/W_x) \times D$$

where R_x and R_s are peak heights from test portion and standard, respectively; C_s is concentration standard, μg/mL; W_x is test portion weight, g/mL, in undiluted 10 mL test extract; and D is dilution factor, if solution injected is diluted.

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

CAS-25013-16-5 (butylated hydroxyanisole)
CAS-128-37-0 (butylated hydroxytoluene)
CAS-500-38-9 (nordihydroguaiaretic acid)
CAS-121-79-9 (propyl gallate)
CAS-1034-01-1 (octyl gallate)
CAS-1166-52-5 (dodecyl gallate)

CAS-1421-63-2 (2,4,5-trihydroxybutyrophenone)
CAS-1948-33-0 (*tert*-butylhydroquinone)
CAS-88-26-6 (Ionox-100)

Results and Discussion

Results were reported by 10 of 14 collaborators. Those who reported results submitted the requested forms and copies of all chromatograms. After a review of the data, a short questionnaire was sent to the 10 participants requesting certain information relating to TBHQ stability and "system" peaks and asking them to review specific individual calculations and peak identifications. Results from this questionnaire were received from 9 of the participants.

Statistical Evaluation

When the results submitted by the 10 collaborators were visually compared, it was apparent that the results of Collaborator 2 were aberrant. The chromatograms indicated poor separation of BHT and DG. In 3 of the 10 samples, although the expected number of peaks were present, antioxidants were not correctly identified or not reported at all because the retention times had significantly decreased from previous samples. Standards were not run as required, and the changes in retention times went unnoticed. This collaborator reported, in response to the questionnaire, that problems observed with the LC equipment resulted in a decrease of retention times and in difficulty in identifying peaks. Statistical identification of outliers by the Cochran and Grubbs tests, as described below, showed results for Collaborator 2 to be outliers in 14 of the 27 pairs of results evaluated for outliers. Regretfully, because of these chromatographic problems described, all results from Collaborator 2 were judged to be "not valid" and were excluded from further statistical evaluation.

The reported data, excluding those of Collaborator 2, were then subjected to 2-sample plots of the matched pairs and to the statistical analysis as recommended by the International Union of Pure and Applied Chemistry Workshop on Harmonization of Collaborative Analytical Studies (2). This statistical procedure involves outlier removal by the Cochran and Grubbs (single and pair) tests with recycle; calculation of the mean and percent recoveries; and calculation of the repeatability and reproducibility, their standard deviations, and the corresponding International Organization for Standardization (ISO) repeatability and reproducibility values. A computer program (Lotus 1-2-3) developed and provided by J.G. Phillips (U.S. Dept of Agriculture, Philadelphia, PA; Chairman, AOAC Statistics Committee) was used for the outlier and statistical calculations. The collaborative results with the calculated statistical data for each of the 9 antioxidants studied are presented in Tables 1–9. The statistical results summarized in Table **985.15C** include standard deviations and relative standard deviations for repeatability and reproducibility. ISO repeatability (r) and reproducibility (R) values are shown in Tables 1–9 for each antioxidant.

The outliers identified by the Cochran and Grubbs tests and the s_r, s_R, RSD_r, and RSD_R values before and after outlier re-

Table 1. Collaborative results for the determination of PG (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	9	2	Total	Diff.	1	4	Total	Diff.	3	6	Total	Diff.
2	101.9	74.2	—	—	44.2	41.5	—	—	10.4	7.55	—	—
4	97.1	82.7	179.8	14.4	48.1	41.3	89.4	6.8	9.6	8.8	18.4	0.8
5	99.6	91.8	191.4	7.8	50.6	43.4	94.0	7.2	9.8	8.7	18.5	1.1
6	84.1	77.1	161.2	7.0	41.8	42.2	84.0	−0.4	10.4	8.7	19.1	1.7
7	102.2	83.2	185.4	19.0	52.8	88.8	103.3	−2.3	12.1	10.3	22.4	1.8
8	79.8	82.8	162.6	−3.0	43.3	58.3	106.6	−10.0	8.6	8.4	17.0	0.2
9	94.4	87.6	182.0	6.8	47.3	44.5	91.8	2.8	9.5	8.9	18.4	0.6
10	94.1	86.7	180.8	7.4	45.3	43.5	88.8	1.8	10.0	8.5	18.5	1.5
14	94.4	85.6	180	8.8	48.3	41.7	90.0	6.6	9.7	8.6	18.3	1.1
15	91.7	92.6	184.3	−0.9	49.2	47.3	96.5	1.9	10.5	10.5	21.0	0.0
No. labs.[b]	9[a]				9[a]				9[a]			
Mean lab. av.	89.3				46.9				9.53			
Added	97.2, 86.9				48.6, 43.3				9.67, 8.72			
Rec., %	97.0				102.0				103.6			
s_r	4.76				3.86				0.45			
s_R	6.08				4.54				0.87			
RSD_r, %	5.33				8.23				4.72			
RSD_R, %	6.81				9.67				9.17			
r	13.5				10.9				1.27			
R	17.2				12.8				2.48			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Number of laboratories retained after outliers removed.

Table 2. Collaborative results for the determination of THBP (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	3	6	Total	Diff.	1	8	Total	Diff.	7	4	Total	Diff.
2	80.0	ND[b]	—	—	41.8	32.8	—	—	8.0	6.4	—	—
4	90.6	81.8	172.4	8.8	44.0	39.1	83.1	4.9	8.7	7.8	16.5	0.9
5	91.8	82.0	173.8	9.8	46.4	43.1	89.5	3.3	8.77	8.00	16.8	0.8
6	62.1	61.4	123.5	0.7	22.0	33.2	55.2	−11.2	6.3	3.3	9.6	3.0
7	108.4	84.0	192.4	24.4	48.6	49.2	97.8	−0.6	8.9	9.9	18.8	−1.0
8	71.2	65.5	136.7	5.7	44.0	48.5	92.5	−4.5	9.1	13.4	22.5	−4.3
9	90.5	82.5	173.0	8.0	44.4	41.0	85.4	3.4	9.1	8.9	18.0	0.2
10	88.4	85.4	173.8	3.0	46.1	46.1	92.2	0.0	10.1	9.7	19.8	0.4
14	84.8	80.7	165.5	4.1	45.1	40.4	85.5	4.7	8.9	8.0	16.9	0.9
15	94.3	76.5	170.8	17.8	42.7	46.5	89.2	−3.8	9.08	9.41	18.49	−0.33
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	82.3				42.8				8.74			
Added	91.6, 82.4				46.0, 41.3				9.19, 8.19			
Rec., %	94.6				98.1				100.6			
s_r	5.34				3.77				1.39			
s_R	11.2				6.68				2.00			
RSD_r, %	6.48				8.81				15.9			
RSD_R, %	13.6				15.6				22.9			
r	15.1				10.7				3.94			
R	31.8				18.9				5.67			

a Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

Table 3. Collaborative results for the determination of TBHQ (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	5	8	Total	Diff.	7	2	Total	Diff.	1	4	Total	Diff.
2	ND[b]	ND	—	—	45.4	ND	—	—	ND	9.76	—	—
4	109.4	95.9	205.3	13.5	52.7	48.0	100.7	4.7	10.5	9.5	20.0	1.0
5	127.3	107.2	234.5	20.1	55.6	54.0	109.6	1.6	10.6	10.3	20.9	0.3
6	88.7	74.8	163.5	13.9	29.2	25.0	54.2	4.2	1.9	7.2	9.1	−5.3
7	139.0	161.5	300.5	−22.5	62.9	54.2	117.1	8.7	18.7	17.3	36.0	1.4
8	106.7	96.5	203.2	10.2	50.5	46.6	97.1	3.9	11.3	9.6	20.9	1.7
9	111.5	101.0	212.5	10.5	55.8	49.7	105.5	6.1	10.6	11.0	21.6	−0.4
10	162.7	129.4	292.1	33.3	67.3	67.9	135.2	−0.6	13.4	15.5	28.9	−2.1
14	110.1	96.0	206.1	14.1	53.5	48.4	101.9	5.1	10.4	9.6	20.0	0.8
15	105.2	79.8	185.0	25.4	59.6	52.1	111.7	7.5	12.78	12.6	25.3	0.1
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	111.3				51.8				11.3			
Added	111.7, 100.1				55.7, 49.8				11.1, 9.93			
Rec., %	105.1				98.2				106.8			
s_r	10.9				2.01				1.55			
s_R	24.3				11.0				3.82			
RSD_r, %	9.78				3.87				13.8			
RSD_R, %	21.8				21.2				34.0			
r	30.8				5.68				4.39			
R	68.8				31.02				10.82			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

Table 4. Collaborative results for the determination of NDGA (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	9	6	Total	Diff.	1	8	Total	Diff.	3	10	Total	Diff.
2	96.7	ND[b]	—	—	42.7	10.0	—	—	10.0	7.33	—	—
4	98.9	91.3	190.2	7.6	48.0	42.8	90.8	5.2	9.8	8.6	18.4	1.2
5	100.7	88.8	189.5	11.9	49.8	46.4	96.2	3.4	9.53	8.17	17.7	1.36
6	75.0	80.2	—[c]	—[c]	36.8	41.8	78.6	−5.0	9.2	ND	9.2	9.2
7	101.0	81.9	182.9	19.1	55.3	52.4	107.7	2.9	12.4	9.4	21.8	3.0
8	79.5	67.2	—[c]	—[c]	45.4	51.0	96.4	−5.6	9.6	8.3	17.9	1.3
9	99.8	86.5	186.3	13.3	45.9	45.4	91.3	0.5	9.9	9.0	18.9	0.9
10	93.5	86.1	179.6	7.4	49.0	42.3	91.3	6.7	9.7	6.6	16.3	3.1
14	97.8	85.6	183.4	12.2	48.8	43.0	91.8	5.8	9.6	8.7	18.3	0.9
15	90.4	99.5	189.9	−9.1	50.3	51.6	101.9	−1.3	10.18	11.8	21.9	−1.7
No. labs.[d]	7[a,c]				9[a]				9[a]			
Mean lab. av.	93.0				47.0				8.91			
Added	101.8, 91.2				50.9, 45.7				10.1, 9.13			
Rec., %	96.4				97.3				92.5			
s_r	6.27				3.22				2.11			
s_R	6.27				4.66				2.39			
RSD_r, %	6.74				6.86				23.7			
RSD_R, %	6.74				9.91				26.8			
r	6.27				9.12				5.98			
R	17.7				13.2				6.76			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Results of Collaborators 6 and 8 rejected as outliers by the pair Grubbs test and not included in statistical analysis.
[d] Number of laboratories retained after outliers removed.

Table 5. Collaborative results for the determination of BHA (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	9	6	Total	Diff.	7	2	Total	Diff.	1	10	Total	Diff.
2	95.8	ND[b]	—	—	45.0	ND	—	—	9.17	7.92	—	—
4	105.5	96.0	201.5	9.5	51.6	46.0	97.6	5.6	10.4	9.4	19.8	1.0
5	108.1	97.2	205.3	10.9	50.9	48.1	99.0	2.8	10.53	9.12	19.62	1.38
6	89.5	92.1	181.6	−2.6	48.0	41.9	89.9	6.1	9.9	9.4	19.3	0.5
7	117.2	81.3	198.5	35.9	55.2	41.4	96.6	13.8	11.2	10.5	21.7	0.7
8	87.1	85.3	166.4	−4.2	50.8	48.1	98.9	2.7	10.7	9.8	20.5	0.9
9	105.7	92.2	197.9	13.5	54.1	49.1	103.2	5.0	10.6	9.7	20.3	0.9
10	106.3	89.8	196.1	16.5	53.8	46.8	100.6	7.0	10.4	9.4	19.8	1.0
14	104.4	91.5	195.9	12.9	50.2	44.1	94.3	6.1	10.7	9.5	20.2	1.2
15	96.6	94.3	190.9	2.9	52.1	45.6	97.7	6.5	10.5	11.6	22.1	−1.1
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	96.3				48.8				10.2			
Added	106.8, 95.7				53.4, 47.7				10.7, 9.59			
Rec., %	95.2				96.5				100.5			
s_r	8.49				2.29				0.52			
s_R	8.49				2.50				0.60			
RSD_r, %	8.81				4.70				5.06			
RSD_R, %	8.81				5.12				5.87			
r	24.0				6.49				1.46			
R	24.0				7.06				1.69			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

Table 6. Collaborative results for the determination of Ionox-100 (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	5	2	Total	Diff.	7	8	Total	Diff.	3	10	Total	Diff.
2	ND[b]	ND	—	—	44.2	ND	—	—	15.8	7.32	—	—
4	104.1	92.0	196.1	12.1	50.2	46.4	96.6	3.8	10.5	9.2	19.7	1.3
5	112.4	104.9	217.3	7.5	53.8	56.2	110.0	−2.4	11.02	8.45	19.45	2.55
6	99.5	80.6	180.1	18.9	48.4	42.0	90.4	6.4	9.9	9.7	19.6	0.2
7	113.1	111.5	224.6	1.6	55.1	52.8	107.9	2.3	10.9	10.5	21.4	0.4
8	103.5	103.0	206.5	0.5	43.0	52.5	95.5	−9.5	8.8	8.3	17.1	0.5
9	106.4	94.7	201.1	11.7	50.4	46.4	96.8	4.0	9.2	10.0	19.2	−0.8
10	108.5	106.2	214.7	2.3	56.2	47.3	103.5	8.9	9.4	9.2	18.6	0.2
14	106.8	92.1	198.9	14.7	50.9	48.4	99.3	2.5	9.7	8.8	18.9	0.9
15	114.5	98.6	213.1	15.9	52.1	51.3	103.4	0.8	5.63	9.34	14.97	−3.71
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	102.9				50.2				9.36			
Added	111.7, 99.7				55.8, 50.1				11.1, 10.0			
Rec., %	97.4				94.9				88.6			
s_r	4.79				3.77				1.22			
s_R	7.52				4.13				1.25			
RSD_r, %	4.66				7.51				13.0			
RSD_R, %	7.31				8.24				13.4			
r	13.6				10.7				3.44			
R	21.3				11.7				3.54			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

Table 7. Collaborative results for the determination of OG (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	5	8	Total	Diff.	1	4	Total	Diff.	9	10	Total	Diff.
2	ND[b]	ND	—	—	37.1	33.0	—	—	7.34	5.69	—	—
4	90.4	80.4	170.8	10.0	42.7	39.1	81.8	3.6	8.7	7.7	16.4	1.0
5	91.7	83.2	174.9	8.5	49.1	40.9	90.0	8.2	9.46	7.76	17.22	1.7
6	92.6	88.1	180.7	4.5	38.6	42.8	81.4	−4.2	9.9	2.3	12.2	7.6
7	99.0	83.5	182.5	15.5	44.1	41.9	86.0	2.2	9.7	8.3	18.0	1.4
8	79.9	82.2	162.1	−2.3	46.1	35.8	81.9	10.3	7.8	9.0	16.8	−1.2
9	93.2	83.1	176.3	10.1	44.0	41.4	85.4	2.6	9.10	7.8	16.9	1.3
10	89.4	74.8	164.2	14.6	42.9	37.6	80.5	5.3	9.1	6.2	15.3	2.9
14	90.1	83.3	173.4	6.8	43.4	39.0	82.4	4.4	9.2	7.6	16.8	1.6
15	87.6	81.3	168.9	6.3	44.9	42.2	87.1	2.7	9.37	8.46	17.85	0.93
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	86.3				42.0				8.19			
Added	95.5, 82.9				46.2, 41.1				9.23, 8.29			
Rec., %	96.8				96.2				93.5			
s_r	3.80				2.89				1.69			
s_R	4.37				2.89				1.69			
RSD_r, %	4.40				6.87				20.6			
RSD_R, %	5.06				6.87				20.6			
r	10.8				8.17				4.78			
R	12.4				8.17				4.78			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

Table 8. Collaborative results for the determination of BHT (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	3	6	Total	Diff.	5	10	Total	Diff.	9	4	Total	Diff.
2	68.6	ND[b]	—	—	ND	22.3	—	—	8.57	5.71	—	—
4	86.1	77.8	163.9	8.3	41.5	38.8	80.3	2.7	8.1	7.6	15.7	0.5
5	91.1	74.0	165.1	17.1	42.5	35.3	77.8	7.2	8.43	8.13	16.56	0.3
6	78.9	72.9	151.8	6.0	39.3	39.1	78.4	0.2	7.1	5.9	13.0	1.2
7	50.9	77.8	128.7	-26.9	44.7	39.8	84.5	4.9	6.1	5.9	12.0	0.2
8	69.5	68.9	138.4	0.6	45.6	35.0	80.6	10.6	7.8	7.8	15.6	0.0
9	90.8	83.3	174.1	7.5	52.4	39.2	—[c]	—[c]	9.5	8.0	17.5	1.5
10	80.9	74.9	155.8	6.0	35.0	33.7	68.7	1.3	7.0	7.2	14.2	-0.2
14	80.0	67.9	147.9	12.1	112.8	47.5	—[c]	—[c]	20.2	20.6	—[d]	—[d]
15	84.6	70.1	154.7	14.5	37.9	35.8	73.7	2.1	10.37	16.8	—[d]	—[d]
No. labs.[e]	9[a]				7[a,c]				7[a,d]			
Mean lab. av.	76.7				38.9				7.47			
Added	101.6, 91.4				51.5, 45.8				10.2, 9.09			
Rec., %	79.4				82.2				77.4			
s_r	9.16				2.56				0.44			
s_R	9.51				3.16				1.03			
RSD_r, %	12.0				6.64				5.94			
RSD_R, %	12.4				8.12				13.8			
r	25.9				7.30				1.26			
R	26.9				8.94				2.92			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Results of Collaborators 9 and 14 rejected as outliers by the pair Grubbs test and not included in statistical analysis.
[d] Results of Collaborator 15 rejected as outlier by the Cochran test and then that of collaborator 14 rejected as outlier by the pair Grubbs test; results not included in statistical analyses.
[e] Number of laboratories retained after outliers removed.

moval are given in Table 10. Outliers were found in 3 situations: with NDGA at the highest level (2 laboratories), BHT at the medium level (2 laboratories), and BHT at the lowest level (2 laboratories). Results of laboratory 14 were determined to be outliers in 2 situations, both with BHT; results of laboratories 6, 8, 9, and 15 were each outliers in a single instance. Excluding all results of laboratory 2, these outlying data represent 2.5% of the total 243 pairs of results from the other 9 laboratories, the 9 antioxidants, and the 3 pairs of results (one pair at each level).

The method collaboratively studied in 1983 used the same extraction and concentration steps as the present method; the only significant differences are in the chromatography. These include the use of a more efficient analytical column, a 10 μL injection, and a different elution solvent B. The substrate analyzed was also different: butter oil vs vegetable oil or lard at levels one-half the earlier study. A comparison of the results of these 2 studies is given in Table 11. Only data at the same spiking level, i.e., 100 μg/g, are included. Results from the 1983 study were recalculated from the original raw data by using the AOAC statistical program to identify outliers and to calculate the performance parameters. Results show the recoveries of the present method to be similar to those of the earlier study, except for TBHQ. The repeatability and reproducibility relative standard deviations on the average, however, are slightly higher in the present study.

Four (2.7%) outliers were identified when the raw data from the earlier study (vegetable oil, 7 valid laboratories, 7 antioxidants, and 3 pairs of results) were recalculated by using the AOAC statistical program. This compares favorably to the 5 (2.5%) outliers of the present study. The statistical data for OG and DG, which were not included in the 1983 study, are included at the bottom of the table and compare favorably with those of the other gallate ester, PG.

In some cases, the method exceeds the expected reproducibility relative standard deviations for collaborative study given by Horwitz et al. (5), who suggested that at 10 μg/g, 11% is to be expected, and at 100 μg/g, 8% is to be expected. Samples spiked at 50 μg/kg would be expected to lie between the 11 and 8% values. The values determined for the present method are summarized in Table **983.15C**. At 100 μg/g, 3 of 9 results exceed the 8% criterion: THBP, TBHQ, and BHT (13.6, 21.8, and 12.4%, respectively). Four of 9 significantly exceed the 11% criterion for 10 μg/g: THBP, TBHQ, NDGA, and OG (22.9, 34.0, 26.8, and 20.6%, respectively), with Ionox-100 at 13.4% and BHT at 13.8% slightly exceeding the 11% figure. At 50 μg/kg, 2 of 9 (THBP and TBHQ at 15.6 and 21.2%, respectively) exceed the expected relative standard deviation. In total, 9 of 27 (33%) significantly exceeded the expected value.

Table 9. Collaborative results for the determination of DG (μg/g) in butter oil[a]

	Sample				Sample				Sample			
Coll.	3	8	Total	Diff.	5	2	Total	Diff.	7	6	Total	Diff.
2	84.2	ND[b]	—	—	ND	ND	—	—	8.63	ND	—	—
4	103.3	91.6	194.9	11.7	54.1	46.3	97.7	5.1	10.0	9.0	19.0	1.0
5	94.6	90.1	184.7	4.5	56.4	48.2	104.6	8.2	10.9	9.44	20.3	1.46
6	113.9	95.7	209.6	18.2	51.4	41.6	93.0	9.8	9.1	10.2	19.9	−0.5
7	119.0	104.0	223.0	15.0	58.4	44.8	103.2	13.6	11.0	10.2	21.2	0.8
8	89.1	83.6	172.7	5.5	49.3	48.4	97.7	0.9	9.7	8.9	18.6	0.8
9	99.0	93.4	192.4	5.6	51.5	46.5	98.2	5.2	10.2	10.0	20.2	0.2
10	97.4	86.5	183.9	10.9	48.6	43.5	92.1	5.1	8.7	7.9	16.6	0.8
14	98.6	91.7	190.3	6.9	53.3	45.3	98.6	8.0	10.3	9.8	20.1	0.5
15	94.8	94.7	189.5	0.1	46.6	46.4	93.0	0.2	9.61	10.1	19.7	−0.49
No. labs.[c]	9[a]				9[a]				9[a]			
Mean lab. av.	96.7				48.8				9.76			
Added	106.3, 95.8				53.5, 47.7				10.7, 9.56			
Rec., %	95.7				96.5				96.4			
s_r	4.02				2.98				0.47			
s_R	7.94				3.05				0.74			
RSD_r, %	4.16				6.12				4.80			
RSD_R, %	8.21				6.24				7.61			
r	11.4				8.44				1.33			
R	22.5				8.62				2.10			

[a] Results of Collaborator 2 excluded from all statistical analyses.
[b] Not detected.
[c] Number of laboratories retained after outliers removed.

In the previous study at 100 μg/kg, 2 of 7 (TBHQ and Ionox-100, at 21.5 and 10.6%, respectively) exceeded the expected 8%.

This comparison of the 1983 and the present collaborative studies, and the data in Table 11, suggest that at comparable spiking levels, method performance was slightly better in the earlier study. At equal sample spikes, apart from the reduced injection volume, the only difference lies in the substrate and the chromatographic conditions. It is not apparent how these factors could affect the method performance. The antioxidant TBHQ gave the highest relative reproducibility standard deviations.

Only in 1 instance was an added antioxidant not detected or reported by a collaborator: NDGA added at 9.63 μg/g. In several instances, antioxidants were reported (>1 μg/g) that were not added. These false positives were Ionox-100 at 3.0, 5.2, and 4.7 μg/g; BHT at 6.1 and 1.7 μg/g; OG at 1.1 and 2.1 μg/g; DG at 1.1 and 1.5 μg/g; and NDGA at 2.1 μg/g. These incidences and low levels of false positives reported are considered acceptable.

Collaborators' Comments

Comments, suggestions, and criticisms were received from most of the participating laboratories. The reported instrumen-

Table 10. Statistical data before and after outlier removal

			Repeatability		Reproducibility	
Antioxidant (spike)			s_r	RSD_r, %	s_R	RSD_R, %
NDGA (96.49μg/g)	Data processed	All data	6.47	7.26	9.21	10.3
	Data rejected	Labs. 8 and 6[a]	6.27	6.74	6.27	6.74
BHT (48.44μg/g)	Data processed	All data	14.5	32.7	17.2	38.9
	Data rejected	Labs. 9 and 14[b]	2.58	6.64	3.16	8.12
BHT (9.65μg/g)	Data processed	All data	1.68	17.6	4.76	49.7
	Data rejected	Lab 15 and 14[c]	0.44	5.94	1.03	13.8

[a] Results of Laboratories 6 and 8 rejected as outliers by the pair Grubbs test.
[b] Results of Laboratories 9 and 14 rejected as outliers by the pair Grubbs test.
[c] Results of laboratory 15 rejected as outlier by the Cochran test and then those of laboratory 14 by the single Grubbs test.

Table 11. Comparison of current and previous collaborative study results at 100 μg/g: butter oil (1991) vs vegetable oil (1983)

	Rec., %		RSD_r, %		RSD_R, %	
Antiox.	1991	1983	1991	1983	1991	1983
PG	97.0	96.9	5.33	4.80	6.81	4.80
THBP	94.6	97.5	6.48	5.30	13.6	5.63
TBHQ	105.1	94.8	9.78	3.63	21.8	21.5
NDGA	96.4	96.5	6.74	5.51	6.74	6.97
BHA	95.2	99.2	8.81	5.45	8.81	6.17
Ionox	97.4	96.0	4.66	10.4	7.31	10.6
BHT	79.4	83.1	12.0	2.80	12.4	2.80
OG	96.8	—	4.40	—	5.06	—
DG	95.7	—	4.16	—	8.21	—

tation, columns, and conditions are given in Table 12. Although the method sent to the collaborators specified a C_{18} column packed with a 5 μm spherical packing capable of baseline separation of the 9 antioxidants, several of the collaborators reported the use of columns packed with nonspherical (irregularly shaped) material. Except for Collaborator 2, whose chromatograms showed partial overlap of BHT and DG, baseline separations of all 9 antioxidants were obtained with all columns used. An example of an acceptable separation using a column packed with a nonspherical packing is given in Figure 1A. Also shown in Figure 1 are chromatograms of the antioxidant separation obtained by using columns with spherical packing material. Although the greater efficiency of the columns packed with the spherical material compared with the nonspherical material is evident, these results show that separation using the latter material can be acceptable. Therefore, the primary consideration in selecting a column for the antioxidant method should be that of acceptable separation, rather than column packing material.

Collaborator 2 reported difficulty in quantitatively transferring the sample extract from the 250 mL round-bottom flask to the 10 mL cylinder and suggested this step could be a source of errors. Collaborator 2 also reported that separation of BHT and DG was difficult. Samples were weighed directly into the separatory funnel.

Collaborator 4 noted an interfering peak in the blank gradient at the retention time of BHT that was attributed to the acetic acid in the aqueous mobile phase. This collaborator suggested the sample weighing procedure could be simplified by accurately weighing a 50 mL beaker containing about 5.5 g liquid butter oil and decanting as much oil as possible into a 125 mL separatory funnel containing 20 mL hexane. The weight of oil sampled is determined by reweighing the beaker.

Collaborator 5 reported that, although the retention times and peak heights did not vary appreciably during the course of the analysis, time was not lost by alternating standard and sample injections. The extractions and evaporations were carried out while the chromatograms were being run.

Collaborator 6 was not able to separate DG and BHT by using 1 of the columns recommended by the coordinator of the study. However, a satisfactory column was found. In the pretrial recoveries, low recoveries for TBHQ and THBP (80%) were noted.

Collaborator 7 also had difficulty in finding a satisfactory column to resolve BHT and DG. This collaborator started the gradient at 50%, rather than the 30% recommended by the

Table 12. Reported instrumentation, LC columns, and conditions used in study

Coll.	Instrument	Ht. meas.[a]	Column packing	Dia., μm	Dimensions, mm	Flow, mL/min
2	Hewlett Packard 1084B	M	LiChrosorb RP-18 (I)[b]	5	125 × 4.6	1.9
4	Waters 720/6000A	E	LiChrospher 100 RP-18	5	250 × 4.0	2
5	LKB 2152/2150	M	Supelcosil LC-18	5	150 × 4.6	2
6	Perkin Elmer Series 4	M	μBondapak C_{18} (I)	10	150 × 3.9	2
7	Tri-Rotar/GP-A 40	E	TSKgel ODS-120T	5	250 × 4.6	1
8	Waters 720	M	Spherisorb ODS	5	250 × 4.6	2
9	Perkin Elmer Series 3	E	LiChrosorb RP-18 (I)	10	250 × 4.0	2
10	Varian 5000	E	Supelcosil LC-18	5	150 × 4.6	2
14	Varian 5000	E	LiChrosorb RP-18 (I)	10	250 × 4.6	2
15	Spectra Physics 8100	M	Hypersil 5 ODS	5	150 × 4.6	2

[a] M, peak height measured manually; E, peak height measured electronically by integrator.
[b] I, irregularly-shaped packing material; all others are spherical.

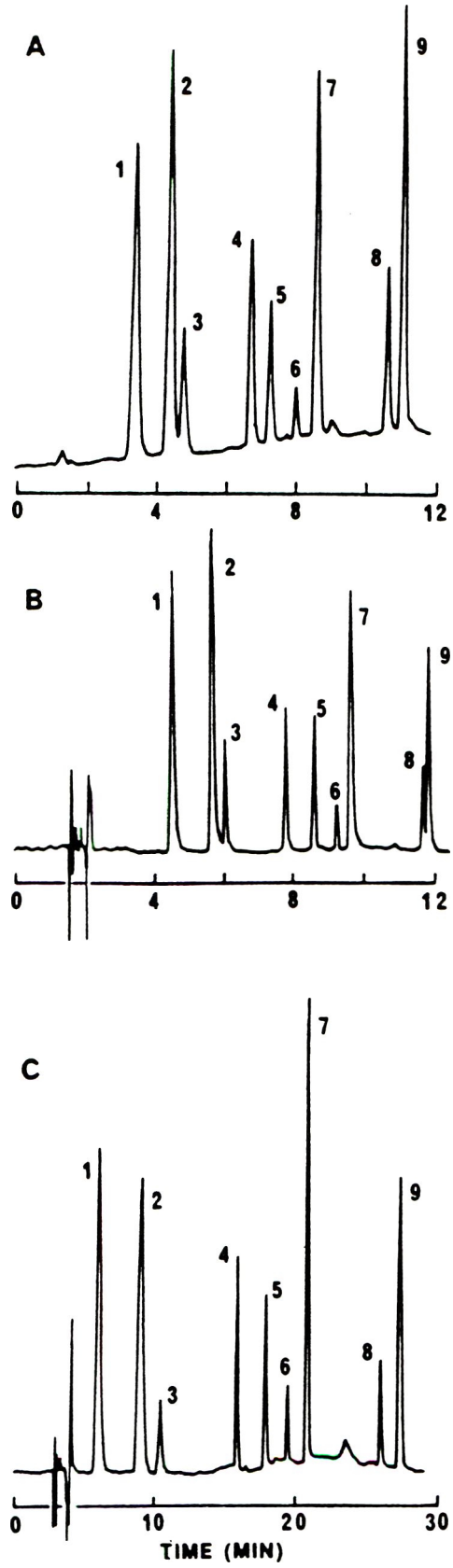

Figure 1. Typical collaborators' chromatograms of butter oil extracts illustrating separation of the following: 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHT; 6, Ionox-100; 7, OG; 8, BHT; and 9, DG. Chromatography was performed on different 250 × 4.6 mm id columns as follows: A, LiChrosorb RP-18 (10 μm irregular particle); B, Spherisorb ODS (5 μm spherical particle); and C, TSK ODS-120T (5 μm spherical particle).

method, and used a 1 mL/min flow rate because of excessive back pressure.

Collaborator 8 suggested changing the format of the method to a step-by-step presentation, as in the accepted IDF format. Other changes in terminology and the use of numbered sections were suggested for easier reference. The alternating injections of standards and samples were regarded as excessive because few variations in the peak heights and retention times of the standards were noted.

Collaborator 10 reported a "system" peak coeluting with BHT, most probably arising from the aqueous eluant. This interference was removed in an ingenious manner: a 6 mL BondElut C_{18} solid-phase extraction column was installed in place of the inlet filter on the aqueous mobile phase inlet line to retain the interference. This participant also observed "bumping" of the oil extract during the concentration step, which was reduced when using a 500 mL flask.

Collaborators 14 (BHT) and 15 (Ionox) both reported small interfering peaks arising from the gradient.

Studies in the Coordinator's Laboratory

The coordinator conducted several studies directed toward determining the reasons for the high reproducibility and repeatability standard deviations obtained for some analytes and by some laboratories.

Results of the questionnaire indicated that the method did not specifically alert the analysts to the possible degradation of antioxidants in the working standard.

TBHQ, compared with the other antioxidants in this study, is rapidly oxidized. The previous study (1) recognized this instability in sample extracts and alerted the analyst to the need for rapid evaporation of the sample extract. In the present collaborative study, however, it became evident that TBHQ in the 10 μg/mL standard solution was also subject to oxidation. The oxidized product, with a lower response, elutes between TBHQ and NDGA and increases as the parent TBHQ disappears. The apparent high recoveries obtained by laboratories 7 and 10 for this antioxidant were determined to arise from a loss of TBHQ in the working standard solution. A plot of the ratio of the TBHQ peak height to that of PG in the standard calculated from the data provided by 1 collaborator showed an approximate linear decrease in this ratio over 11 days. With this decrease in peak height of the TBHQ standard, there was a corresponding increase in recovery from 120% (day 3) to 146% and 156% (day 9) as the samples were analyzed over this period. The results for TBHQ from the other laboratory were also high, again probably as a result of oxidative loss of TBHQ in the standard. In this instance, the recoveries increased from 124 to 161% with a corresponding decrease in the TBHQ peak height in the standard. In the chromatograms supplied by the collaborator, the oxidation product increased as the parent TBHQ decreased.

The stability of TBHQ in the 10 μg/mL standard solution was studied in the coordinator's laboratory. After 2 days on the laboratory bench with normal fluorescent lighting during working hours, 9% decreases were noted in the peak height of TBHQ relative to THBP and PG; after 8 days, a 25% loss was observed. When exposed for 2 days at a distance of 10 cm from

a fluorescent light, a 32% loss was observed. TBHQ was lost from standards in sunlight at an undetermined but greater rate. Standards stored on the laboratory bench but wrapped in light-excluding aluminum foil showed no losses after 8 days. Statistical performance in the collaborative study would be improved if this decrease of TBHQ response in the standard was noticed and fresh standards were prepared.

With TBHQ in butter oil extracts on the laboratory bench with normal fluorescent lighting, more rapid losses occurred: 60% loss in 2 days at 28 μg/mL and 50% loss at 5 μg/mL. Complete loss occurred after 4–5 days in both extracts. In contrast, similar extracts containing TBHQ at 10 μg/mL showed only minor losses after 6–7 days when wrapped in light-excluding aluminum foil at room temperature (12% loss) or stored under refrigeration in the dark (8% loss). In an unprotected control extract, 50% loss of TBHQ was noted after 3 days, and complete loss occurred after 5–6 days.

The magnitude of a system peak, which may arise from nonpolar contaminants in the aqueous phase, depends on the volume of aqueous phase passing through the column during equilibration. If this volume varies, then any correction applied to an actual peak may be in error. This may have caused the outliers in the BHT results. As noted, Collaborator 10 reported the use of a C_{18} solid-phase extraction cartridge to trap contaminants in the aqueous eluant. In the coordinator's laboratory, the aqueous mobile phase was spiked with 3 antioxidants (Ionox-100, BHT, and DG) and with 3 phthalate esters [di-(2-ethylhexyl), di-*n*-butyl, and butyl benzyl phthalate]. The spiking level was such that 20 mL mobile phase passing through the analytical column during equilibration would give peaks equivalent to 0.1 μg of each antioxidant and 0.4 μg of each phthalate. Using another brand of C_{18} solid-phase extraction cartridge installed as described, no system peaks were observed with equilibration volumes of 60 mL, as evidenced by the breakthrough of any antioxidant or phthalate. The capacity of the cartridge was not determined.

Results of the coordinator's studies, combined with the information gained from the questionnaire, data sheets, chromatograms, and comments provided by each collaborator, indicated that the high recoveries for TBHQ can be avoided if analysts monitor the TBHQ peak height relative to those of other antioxidants in the working standard solution and prepare fresh standards as required. Similarly, system peak interferences arising from the aqueous mobile phase can be reduced or eliminated by installing a prerinsed C_{18} solid-phase extraction cartridge in place of the inlet filter.

Conclusions

Considering the complexity of the analysis, which involves extraction, concentration, transfer, and LC determination, the statistical results for the LC method for the 9 antioxidants in butter oil must be considered satisfactory. Overall mean recoveries for PG, THBP, TBHQ, NDGA, BHA, OG, Ionox, BHT, and DG were 100.9, 97.8, 103.4, 95.4, 97.4, 93.6, 95.5, 79.0, and 96.2%, respectively: overall RSD_R values were 8.55, 17.4, 25.6, 14.5, 6.60, 9.64, 10.8, 11.4, and 7.35%, respectively. The recoveries were generally over 95%, except for BHT, which, because of the partitioning, was about 80%. The RSD_R values were greater than the expected values of Horwitz et al. (5) in about 33% of the cases. These values were, on the average, also slightly greater than those of the 1983 collaborative study compared with samples spiked at the same level. The RSD_R and recovery values for OG and DG, 2 antioxidants not previously studied, were good. Using improved LC elution solvents and the currently available columns packed with spherical C_{18}-bonded silica, good separations of all 9 antioxidants were readily obtained. The comments provided by the collaborators, their response to a postcollaborative questionnaire, and several studies in the coordinator's laboratory suggested the method as provided to the participants for collaborative study could be amended and significantly improved. These additions include a reduction in the injection frequency of standards, a modification to the solvent inlet to reduce system peak interferences, a simplified sample weighing procedure, a warning to monitor the stability of TBHQ in standard solutions and to prepare fresh as required, and several other minor changes. Resulting improvements in the statistical performance of the method would be anticipated.

Recommendation

I recommend that AOAC method **983.15** be modified to include determination of the 9 antioxidants studied in butter oil and to use improved chromatographic conditions for all analyses included in the present method.

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FOOD COMPOSITION

Comparison of Kjeldahl Method for Determination of Crude Protein in Cereal Grains and Oilseeds with Generic Combustion Method: Collaborative Study

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Seven laboratories participated in a collaborative study to extend the applicability of the AOAC generic combustion method for determination of crude protein in animal feed (990.03) to include determination in cereal grains and oilseeds. In the study, method 990.03 was compared with the AOAC mercury catalyst Kjeldahl method for determination of protein in grains (979.09) and crude protein in animal feed (954.01). The study also evaluated the effect on the results of fineness of grind. For determination of crude protein in grains and oilseeds by the combustion method, standard deviations for repeatability and reproducibility ranged from 0.10 to 0.37 and from 0.25 to 0.54, respectively, and relative standard deviations for repeatability and reproducibility ranged from 0.77 to 2.57% and from 1.24 to 3.15%, respectively. The combustion method was adopted first action by AOAC International for determination of crude protein in cereal grains and oilseeds containing 0.2–20% nitrogen.

Kjeldahl nitrogen determination has been the standard method for over 100 years for determination of crude protein in a wide variety of products. During this time, the analytical chemist has endured its long analysis times and use of hazardous chemicals. The Dumas method, a combustion procedure, is another 100-year-old method for determining crude protein. The method does not use hazardous chemicals or require long analysis times, but it has not been as widely accepted as the Kjeldahl method. Modern advances in electronic instrumentation and computers have improved the capabilities of the Dumas method, making it faster, safer, and more reliable than the Kjeldahl method. In the improved Dumas method, nitrogen freed by pyrolysis at high temperature in pure oxygen is quantified by a thermal conductivity detector. Equivalent protein is calculated from the nitrogen value by a microprocessor. Analysis time varies from 4 to 11 min depending on the sample size and the instrument model.

A generic combustion method for determination of crude protein in animal feeds was collaboratively studied by Sweeney (1) and adopted by AOAC as method **990.03** (2). The present collaborative study was conducted to compare **990.03** with the AOAC mercury catalyst Kjeldahl methods (3) for determination of protein in cereal grains (**979.09**) and determination of crude protein in animal feed (**954.01**). The purpose of the study was to extend the applicability of method **990.03** to additional products.

The generic description in **990.03** allowed use of 3 different brands of equipment. All equipment had to meet the performance criteria in that method. Three manufacturers were represented in this study: LECO Corp., Perkin-Elmer Corp., and UIC, Inc.

The study also included samples to evaluate the effect of fineness of grind on results.

Collaborative Study

The experimental design addressed systematic error (interlaboratory bias), precision (within-laboratory repeatability using blind duplicates), and accuracy (recovery of known standards). Three protein concentration levels (8–13%, 17–23%, and 35–40%) were selected to represent the total range of protein found in the products being considered. Two different products were then selected from each concentration level. From these products, the blind duplicates and the samples for

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The recommendation was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

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

Table 1. Samples used in collaborative study of combustion method for determining crude protein in cereal grains and oilseeds

Concn level	Batch (type)	Sample	Estd protein, %	Screen size, mm	Description
1	1	1	35	1.0	Soybean 1
		6	35		
		16	35	2.0	
		25	35		
		12	40	1.0	Soybean 2
		22	40		
	2	4	20	1.0	Canola 1
		11	20		
		27	23	1.0	Canola 2
		29	23		
2	1	28	18	1.0	Sunflower
		30	18		
		5	13	1.0	Wheat 1
		17	13		
		13	13	2.0	
		15	13		
		21	17	1.0	Wheat 2
		24	17		
	2	7	12	1.0	Barley
		20	12		
3	1	2	8	1.0	Corn
		10	8		
		3	8	2.0	
		18	8		
	2	9	9	1.0	Sorghum
		23	9		
Reference standard		19	71[a]	—	Nicotinic acid
		26	71[a]	—	
Reference standard		8	96[a]	—	Lysine-HCl
		14	96[a]	—	

[a] Calculated equivalent protein.

the alternative grind comparison were chosen. Two chemical reference materials were also selected for analysis. The total sample set was designed to provide 15 closely matched pairs (Table 1).

992.23 Crude Protein in Cereal Grains and Oilseeds Generic Combustion Method

First Action 1992

(Applicable to cereal grains and oilseeds containing 0.2–20% N, % crude protein, for wheat and its products = $N \times 5.70$, % crude protein, for other cereal grains and oilseeds = $N \times 6.25$)

Method Performance (estimated % crude protein):
Soybean, 35 and 40%
$s_r = 0.29$; $s_R = 0.47$; $RSD_r = 0.77\%$; $RSD_R = 1.24\%$
Canola, 20 and 23%
$s_r = 0.19$; $s_R = 0.39$; $RSD_r = 0.87\%$; $RSD_R = 1.79\%$
Sunflower, 18%
$s_r = 0.37$; $s_R = 0.54$; $RSD_r = 2.00\%$; $RSD_R = 2.94\%$
Wheat, 13 and 17%
$s_r = 0.15$; $s_R = 0.27$; $RSD_r = 0.99\%$; $RSD_R = 1.74\%$

Table 2. Effect of fineness of grind (1 vs 2 mm) on collaborative results for determination of protein (%) in soybean, wheat, and corn by combustion method

		Soybean		Wheat		Corn	
Lab.[a]	N	Mean	SD	Mean	SD	Mean	SD
1	4	34.76	0.32	13.10	0.16	8.74	0.18
2	4	35.01	0.48	13.38	0.50	8.96	0.48
3	4	35.63	0.43	13.50	0.22	9.10	0.16
4	4	34.94	0.19	13.43	0.31	9.07	0.21
5	4	34.84	0.40	13.13	0.16	8.72	0.08
6	4	34.55	0.34	13.37	0.13	9.14	0.15
7.1	4	35.53	0.39	13.51	0.15	9.03	0.25
7.2	4	35.52	0.40	13.69	0.15	9.48	0.14
7.3	4	34.94	0.78	13.41	0.30	9.05	0.27
Screen size							
1 mm	18	35.27	0.43	13.51	0.22	8.95	0.25
2 mm	18	34.88	0.56	13.27	0.30	9.11	0.33

[a] Laboratory 7 reported results for 3 different analyzer brands.

Barley, 12%
$s_r = 0.27$; $s_R = 0.40$; $RSD_r = 2.13\%$; $RSD_R = 3.15\%$
Corn, 8%
$s_r = 0.10$; $s_R = 0.26$; $RSD_r = 1.15\%$; $RSD_R = 2.88\%$
Sorghum, 9%
$s_r = 0.23$; $s_R = 0.25$; $RSD_r = 2.57\%$; $RSD_R = 2.84\%$
Lysine-HCl, 96%*
$s_r = 0.36$; $s_R = 0.72$; $RSD_r = 0.38\%$; $RSD_R = 0.75\%$
Nicotinic acid, 71%*
$s_r = 0.32$; $s_R = 0.83$; $RSD_r = 0.45\%$; $RSD_R = 1.18\%$
*Calculated equivalent protein.

A. Principle

Nitrogen freed by pyrolysis and subsequent combustions at high temperature in pure oxygen is quantified by thermal conductivity detection. Equivalent protein is calculated.

B. Apparatus

Any instrument or device designed to measure nitrogen by combustion provided that it meets system suitability requirements, **E**.

(**a**) *Furnace.*—Capable of maintaining minimum operating temperature of 950° for pyrolysis of sample in pure (99.9%) oxygen. Some systems may require higher temperatures.

(**b**) *Isolation system.*—Capable of isolating liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector. Device for converting NO_x products to N_2 or measuring N as NO_2 may be required and included in instrument.

(**c**) *Detection system.*—Capable of interpreting detector response as % nitrogen w/w. Features such as calibration of standard material, blank determination, and barometric pressure compensation may be included. Any required calibration must be based on theoretical % nitrogen in pure standard organic material such as EDTA.

(**d**) *Grinder.*—Capable of grinding samples to pass No. 20 sieve.

(**e**) *Analytical balance.*—Accurate to 0.01 mg.

(**f**) *Barometer.*—Hg type, readable to 0.1 mm.

C. Reagents

(**a**) *Accuracy standards.*—(*1*) *Nicotinic acid.*—99.9% minimum purity. (*2*) *Lysine-HCl.*—99.9% minimum purity (tryptophan, 99.9% minimum purity, may be substituted).

(**b**) *Calibration standards.*—EDTA, 99.9% minimum purity, or other suitable standard of equal purity.

D. Samples

Grind samples to suitable fineness (determined for each different material analyzed) to attain ≤2.0% relative standard deviation (RSD) for 10 successive nitrogen determinations for that material type.

$$RSD, \% = (s/N) \times 100$$

Table 3. Analysis of variances for soybean, wheat, and corn (with protein as dependent variable) due to model, laboratory, and fineness of grind effects

Statistic	Soybean	Wheat	Corn
Av. protein, %	35.08	13.39	9.03
R-square	0.61	0.55	0.58
Root MSE	0.387	0.222	0.226
P-value			
Model	0.0012	0.0054	0.0024
Laboratory	0.0036	0.0267	0.0033
Grind	0.0057	0.0033	0.0423

Table 4. Collaborative results for determination of crude protein (%) in cereal grains and oilseeds by Kjeldahl method 955.04 and generic combustion method

	Laboratory											
	1		3		5		2	4	6	7	8	9
Sample[a]	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Comb.	Comb.	Comb.	Comb.	Comb.	Comb.
1	34.7	34.7	36.0	35.7	34.9	34.9	34.8	34.9	34.8	35.7	35.8	35.1
2	8.8	8.7	9.0	9.1	8.7	8.8	8.6	9.1	9.2	9.0	9.3	8.8
3	8.6	8.6	9.0	8.9	8.7	8.8	9.7	8.9	9.1	8.8	9.5	9.0
4	20.6	20.4	21.4	21.2	20.7	20.7	21.1	20.8	20.7	21.0	20.9	20.8
5	13.3	13.2	13.6	13.5	13.2	13.2	13.6	13.6	13.3	13.4	13.9	13.2
6	35.0	35.0	35.8	35.8	35.1	35.1	35.3	35.2	34.7	35.6	35.9	35.9
7	12.4	12.5	13.0	13.0	12.6	12.6	12.9	13.0	12.8	13.0	13.6	12.5
8	95.5	95.4	96.6	96.1	96.1	96.2	96.3	95.5	94.3	96.0	95.4	93.6
9	8.5	8.6	9.0	9.0	8.7	8.4	9.1	8.6	8.9	8.9	9.4	9.1
10	8.7	8.7	9.1	9.1	8.7	8.6	8.7	8.9	9.0	9.0	9.5	9.0
11	20.6	20.6	21.3	21.4	20.4	20.4	20.7	20.5	21.0	20.9	20.9	21.3
12	40.0	40.9	41.8	41.7	41.3	40.9	41.7	41.1	40.4	41.6	41.8	41.0
13	12.8	12.9	13.6	13.4	13.1	13.0	13.6	13.5	13.3	13.6	13.6	13.7
14	95.7	95.5	96.4	96.0	95.9	95.8	95.7	95.3	94.9	95.9	95.5	94.8
15	13.0	13.0	13.3	13.3	13.1	13.0	12.6	13.0	13.3	13.4	13.6	13.1
16	34.0	34.3	34.8	35.0	34.4	34.3	35.5	34.7	34.1	35.0	35.0	34.5
17	13.5	13.3	13.9	13.8	13.3	13.3	13.7	13.6	13.6	13.7	13.7	13.6
18	9.0	9.0	9.3	9.3	9.1	8.7	8.9	9.4	9.3	9.4	9.6	9.4
19	62.5	71.0	65.7	70.8	71.3	71.3	69.8	69.5	70.4	71.6	71.6	71.2
20	12.6	12.6	13.0	13.1	12.6	12.6	12.5	12.3	12.9	13.5	13.2	12.2
21	17.1	17.0	17.5	17.4	17.0	16.9	17.3	17.3	17.3	17.9	17.4	17.7
22	40.4	40.5	41.1	41.1	40.4	40.6	41.0	40.8	40.2	41.8	41.0	41.1
23	8.8	8.7	9.0	9.0	8.8	8.6	8.9	9.2	9.0	9.1	8.8	8.9
24	16.8	17.1	17.6	17.6	17.0	17.0	17.2	17.2	17.3	18.1	17.6	17.2
25	34.9	34.9	36.0	36.0	35.0	35.1	34.4	34.9	34.7	35.9	35.5	34.2
26	69.0	70.9	66.0	71.1	70.9	71.4	69.0	69.3	70.7	71.4	70.7	71.4
27	23.0	23.0	23.9	23.7	23.1	22.9	23.3	22.3	23.0	24.0	23.8	23.0
28	18.5	18.8	19.7	19.6	18.0	18.3	18.6	20.1	19.4	18.5	18.2	17.9
29	22.6	23.0	23.6	23.6	23.1	22.9	23.2	22.8	23.4	24.0	23.8	23.4
30	18.1	18.2	18.8	18.6	18.1	17.7	18.1	17.4	18.8	18.5	18.0	17.9

[a] See Table 1 for sample description.

where s = standard deviation, N = mean % nitrogen.

Some materials may require analysis of larger sample sizes to achieve this precision, depending on fineness of grind attained.

E. System Suitability

System equipped as in **B(a)–(c)** must meet or exceed minimum performance specifications as follows:

(*1*) Capable of measuring nitrogen in materials containing 0.2–20% nitrogen.

(*2*) Demonstrate system accuracy based on 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations of nitrogen in lysine-HCl or tryptophan. Means of determinations must be ±0.15 of respective theoretical values, with standard deviations ≤0.15. System accuracy must not be tested with same material used for calibration.

F. Calculations

For wheat and its products:

$$\text{Crude protein, \%} = N \times 5.70$$

For other cereal grains and oilseeds:

$$\text{Crude protein, \%} = N \times 6.25$$

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

Results and Discussion

Seven laboratories participated in the study. One laboratory used 3 different brands of combustion nitrogen analyzers; 6 laboratories used the LECO® FP-428 model nitrogen analyzer (Leco Corp.). Although 8 laboratories were originally contacted to analyze the samples by the Kjeldahl method, only 3 were able to complete the Kjeldahl determinations for the study. Laboratories that used the mercury catalyst Kjeldahl

Table 5. Comparison of laboratory averages for determination of protein (%) by combustion method

Sample[a]	Av., Kjeldahl	Av., combustion	Kjel. – Comb.
1	35.19	35.16	0.03
2	8.83	8.95	–0.12
3	8.75	9.00	–0.25
4	20.89	20.84	0.05
5	13.36	13.44	–0.08
6	35.30	35.39	–0.09
7	12.67	12.88	–0.21
8	96.06	95.41	0.65
9	8.73	8.89	–0.16
10	8.82	8.95	–0.13
11	20.77	20.85	–0.08
12	41.03	41.24	–0.21
13	13.17	13.41	–0.24
14	96.01	95.47	0.54
15	13.12	13.13	–0.01
16	34.38	34.70	–0.32
17	13.54	13.58	–0.04
18	9.10	9.22	–0.12
19[b]	66.49	70.78	–4.29
20	12.75	12.78	–0.03
21	17.18	17.36	–0.18
22	40.66	40.90	–0.24
23	8.87	8.90	–0.03
24	17.14	17.36	–0.22
25	35.31	35.06	0.25
26[b]	68.62	70.65	–2.03
27	23.33	23.22	0.11
28	18.75	18.83	–0.08
29	23.09	23.35	–0.26
30	18.34	18.15	0.19
Average	28.01	28.26	–0.25
Average[b]	25.18	25.23	–0.05

[a] See Table 1 for sample description.
[b] Kjeldahl analysis of nicotinic acid (samples 19 and 26) showed significant difference from theoretical; results for those samples were eliminated from calculation of the second set of averages.

method and also had a nitrogen analyzer and were willing to participate in the collaborative study were extremely difficult to locate. The collaborative study by Sweeney (1) established a statistically sound correlation between the Kjeldahl methods and the combustion method. Because the purpose of the present study was to extend the applicability of the combustion method, the Kjeldahl data were not essential for validation.

The sample set (Table 1) consisted of 15 matched pairs of blind duplicates to establish the within-laboratory repeatability of the method. The samples were ground with a 1 mm screen, and 3 of the samples (soybeans, corn, and wheat) were also ground with a 2 mm screen to establish whether any significant difference existed due to fineness of grind or particle size.

The moisture content and oil content of cereal grains and oilseeds contribute to the difficulty in grinding these types of samples. Using the 0.5 mm screen specified in the combustion method for feeds (**990.03**) would necessitate predrying of the cereal grain and oilseed samples, thereby adding excessive sample preparation time. Sample size also becomes a critical consideration as the nonhomogeneity of the sample increases because of the nature of the material and/or the fineness of grind. The size of the ground sample analyzed must represent the sample as a whole. For these reasons, 1.0 and 2.0 mm screens were chosen to prepare the samples on an "as-is" basis.

Using the calculated *P*-values for each type of grain tested, there appears to be a statistically significant difference due to grind effects (Tables 2 and 3). The types of samples used in this study are very homogeneous when ground with a 1 mm screen, with the exception of sunflower seeds, which required additional care in blending (after grinding). Different products grind differently with the same size screen. Therefore, guidelines must be set for grinding each type of product. The traditional 1 g sample was chosen for the Kjeldahl method to decrease the variance of analysis due to sample variation and grinder effects. All but one of the combustion models tested accept sample sizes of at least 200 mg for the types of samples studied. Therefore, the recommended requirements for determining an instrument's precision were based on the following criteria: type of sample, fineness of grind, and sample size for the individual laboratory and the particular brand of analyzer used.

Three different brands of combustion analyzers and 2 different models of 1 brand of instrument were used in this collaborative study. The manufacturers' recommended sample sizes and analysis times varied considerably from instrument to instrument. In general, the sample size is proportional to the analysis time. The manufacturers' recommended sample sizes for the products tested were 20 mg for the Perkin-Elmer® (Perkin-Elmer Corp.), 150 mg for the LECO, and 500 mg for the Heraeus® (UIC, Inc.). A comparison to determine whether any significant differences exist between models due to sample size was not included in this collaborative study.

Results are shown in Table 4. A general observation is that the combustion method gives slightly higher protein results than does the Kjeldahl method. An average difference of –0.05% protein was obtained by comparing the Kjeldahl values with the combustion values (Table 5) after discarding the nicotinic acid data (poor recovery of nicotinic acid by several laboratories skewed the data). The combustion method data from the performance criteria procedure using standard reference materials (Table 6) showed a standard deviation for 10 analyses by each of the 9 laboratories of 0.03 for nicotinic acid, 0.02 for lysine-HCl, and 0.02 for tryptophan (2 laboratories). Data in Table 7 demonstrate the accuracy and precision of the combustion method in determining the nitrogen content of a sample.

Recommendations

I recommend that the scope of the generic combustion method for crude protein in feeds (**990.03**) be extended to include cereal grains and oilseeds. I also recommend that the combustion method be referenced as an alternative to the

Table 6. Performance of combustion method for determination of nitrogen in standard reference materials

Analysis No.	Nicotinic acid, av. % N	Lysine-HCl, av. % N	Tryptophan, av. % N
1	11.49	15.28	13.77
2	11.47	15.29	13.74
3	11.44	15.30	13.78
4	11.48	15.32	13.74
5	11.42	15.32	13.72
6	11.43	15.28	13.76
7	11.42	15.28	13.73
8	11.37	15.27	13.76
9	11.43	15.31	13.79
10	11.40	15.31	13.74
No. labs	9	9	2
Av., %	11.44	15.30	13.75
SD	0.03	0.02	0.02
Theoretical, %	11.38	15.34	13.71

Kjeldahl method for determining protein in cereal grains (**979.09**).

I further recommend that the following be substituted for C(*3*) when method **990.03** is applied to cereal grains and oilseeds:

A suitable fineness of grind must be determined (for each different material analyzed) to achieve a precision which gives a relative standard deviation (RSD) of ≤2.0% for 10 successive determinations of nitrogen. RSD, % = (SD/mean % N) × 100. Some materials may require analysis of larger quantities of the material to achieve this precision, depending on the attainable fineness of grind.

Acknowledgments

I thank the following collaborators for their contribution:

Table 7. Statistical summary of collaborative results for study of combustion method for determining crude protein in cereal grains and oilseeds

Sample	Description	s_r	s_R	RSD_r, %	RSD_R, %
1 & 6	Soybean	0.26	0.44	0.75	1.24
16 & 25[a]	Soybean	0.52	0.57	1.49	1.62
12 & 22	Soybean	0.32	0.49	0.78	1.20
Average		0.29	0.47	0.77	1.24
4 & 11	Canola	0.20	0.29	0.95	1.39
27 & 29	Canola	0.19	0.48	0.80	2.06
Average		0.19	0.39	0.87	1.79
28 & 30	Sunflower	0.37	0.54	2.00	2.94
5 & 7	Wheat	0.17	0.22	1.23	1.63
13 & 15[a]	Wheat	0.21	0.27	1.55	2.02
21 & 24	Wheat	0.14	0.31	0.82	1.78
Average		0.15	0.27	0.99	1.74
7 & 20	Barley	0.27	0.40	2.13	3.15
2 & 10	Corn	0.10	0.26	1.15	2.88
3 & 18[a]	Corn	0.32	0.33	3.50	3.66
9 & 23	Sorghum	0.23	0.25	2.57	2.84
8 & 14	Lysine-HCl	0.36	0.72	0.38	0.75
19 & 26	Nicotinic acid	0.32	0.83	0.45	1.18

[a] 2 mm grind. Results not used to calculate average for this sample type. All other grain and oilseed samples ground to 1 mm.

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FOOD COMPOSITION

Combustion Method for Determination of Crude Protein in Meat and Meat Products: Collaborative Study

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Twelve laboratories participated in a collaborative study to compare a combustion method with the AOAC mercury catalyst Kjeldahl method (928.08) for the determination of crude protein in meat and meat products. Three different combustion instruments were used; consequently, the combustion method for this study is written in generic terms describing the principle, the apparatus specifications, and the performance requirements needed. Fifteen sample pairs were used for the study; each pair consisted of the same commercial meat product from each of 2 different manufacturers. Protein content of all samples ranged from about 10 to 20%. In addition, nicotinic acid and lysine monohydrochloride were used as standards to assess combustion equipment performance. All laboratories and all instruments performed the combustion method satisfactorily on the basis of results for the standards. For the meat samples, repeatability standard deviations (s_r) ranged from 0.11 to 0.40 for the Kjeldahl method and from 0.12 to 0.41 for the combustion method; the repeatability relative standard deviations (RSD_r) ranged from 0.82 to 2.41% and from 0.60 to 2.23% for the Kjeldahl and combustion methods, respectively. Reproducibility standard deviations (s_R) ranged from 0.20 to 0.49 for the Kjeldahl method and from 0.18 to 0.46 for the combustion method, whereas the reproducibility relative standard deviations (RSD_R) ranged from 1.59 to 2.84% for the Kjeldahl method and from 1.32 to 3.35% for the combustion method. Overall grand means were 15.59% protein for the Kjeldahl method and 15.75% protein for the combustion method. The combustion method was adopted first action by AOAC International.

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This report was presented at the 106th AOAC Annual International Meeting, August 31–September 2, 1992, at Cincinnati, OH.

The recommendation was approved by the General Referee and the Committee on Foods I and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

Combustion methods for protein analysis that release nitrogen at high temperatures and quantitate the nitrogen by thermal conductivity were shown to be a practical alternative to the classical Kjeldahl method (1, 2). Several different manufacturers currently provide instruments that measure nitrogen in meat and meat products. The combustion method has inherent advantages over the Kjeldahl method in terms of speed (about 3 min per sample) and freedom from concentrated acid and base and the mercury catalyst.

Although the combustion method was studied and adopted for protein analysis of materials such as animal feeds (2, 3), it was not approved for meat and meat products. Because an alternative to the Kjeldahl method is of great interest to the meat industry, a collaborative study of the combustion method for meat and meat products was initiated.

Collaborative Study

Twelve laboratories, using 3 different commercially available combustion instruments, participated in the study. Nine laboratories used the LECO FP-428, 2 used the Foss Heraeus Macro-N Analyzer, and 1 used the Perkin-Elmer PE2410. To avoid requiring a particular manufacturer's instrument for this method, the combustion method was generally described with performance guidelines to be met for analysis of standard nicotinic acid and lysine hydrochloride. The standards and guidelines for accuracy were used to ensure that each instrument was capable of sufficient accuracy. The amino acid standards (Sigma Chemical Co., St. Louis, MO) and EDTA for instrument calibration were provided to each collaborator. Collaborators were asked to report the results from 10 successive analyses of each of the 2 amino acid standards, using the combustion and the mercury catalyst Kjeldahl methods.

Meat samples provided to collaborators consisted of 15 closely matched pairs, 30 samples in total. Samples were selected by choosing 15 typical commercial meat products and then choosing 2 commercial manufacturers of each product.

Samples were prepared by grinding in a tabletop meat grinder (Biro Model 8-22, Biro Co., Marblehead, OH), following U.S. Department of Agriculture (USDA) guidelines (4) to ensure uniform fineness of samples. The preparation consisted of passing emulsified meat products through the grinder (1/8 in. plate) twice and passing nonemulsified (coarse) products through 3 times. After grinding, ca 250 g units were double-bagged in polyethylene and frozen at −30°C for 72 h before shipment to collaborators. Each sample was identified with a random 3-digit number obtained from a random number table. No indication was made to collaborators of which samples were paired. Collaborators were instructed to keep the samples refrigerated (5°C or less) and to knead or massage each sample bag for 20 s before opening. Collaborators were encouraged to run all analyses within 1 week of receiving the samples.

Each collaborator was also provided a copy of the combustion method requirements for accuracy, a general description of the combustion method, and a set of report sheets to use for data recording. Sample size used by the various collaborators ranged from ca 200 mg to ca 900 mg.

992.15 Crude Protein in Meat and Meat Products—Combustion Method

First Action 1992

(Applicable to meat and meat products with 10–20% crude protein)

Method Performance:

s_r = 0.12–0.41; s_R = 0.18–0.46; RSD_r = 0.60–2.23%; RSD_R = 1.32–3.35%

A. Principle

Combustion method determines nitrogen released at high temperature into pure oxygen and measured by thermal conductivity. Nitrogen is converted to protein equivalent by using the appropriate factor, 6.25 for meat and meat products.

B. Apparatus

Note: Manufacturer's recommendations must be followed for safe and accurate operation of instruments. For proper laboratory precautions in handling compressed gases required for instruments, see "Compressed Gas Cylinders" in "Appendix: Laboratory Safety", *Official Methods of Analysis* (1990) 15th Ed.

(**a**) *Combustion instrument.*—Suitable for detecting 1–5% nitrogen (ca 5–30% protein) in meat and meat products to within ±0.15% of theoretical nitrogen content of standard, with standard deviation of ≤0.15 for 10 successive determinations on same standard. Instrument capable of analyzing single sample of at least 200 mg (to reduce impact of nonhomogeneity of meat and meat products). Instrument with oven capable of operating at ≥850° in pure oxygen (for complete release of nitrogen from samples), capable of isolating nitrogen from other combustion products (i.e., CO_2, H_2O) for subsequent quantitation, and capable of thermal conductivity measurement of nitrogen (Leco FP428, Leco Corp., St. Joseph, MI 49085, USA; Macro-N Analyzer, Foss Heraeus Analysensysteme GmbH, Hanau 1, Germany; and PE2410, Perkin-Elmer Corp., Norwalk, CT 06859, USA, are suitable). Calibrations, required by most instruments, must be conducted by using theoretical percent nitrogen in pure primary standard organic compounds, such as EDTA.

(**b**) *Food chopper.*—With 1/8 in. (or less) plate, capable of grinding meat samples.

C. Reagents

The following reagents are typical but may vary depending on instrument. Consult manufacturer's instructions for specific instruments.

(**a**) *Compressed oxygen gas.*—99.99%.

(**b**) *Compressed helium gas.*—99.99%.

(**c**) *Compressed inert gas.*—Nitrogen (or equivalent), oil and water free.

(**d**) *Nitrogen standard.*—Ethylenediaminetetraacetic acid (EDTA), 9.59% nitrogen, or other suitable organic material of high purity and known nitrogen content (e.g., nicotinic acid or lysine hydrochloride).

(**e**) *Quartz wool.*

(**f**) *Glass wool.*

(**g**) *Alumina oxide pellets.*

(**h**) *Anhydrous magnesium perchlorate ($MgClO_4$).*

(**i**) *Sodium hydroxide on silicate carrier.*

(**j**) *Cu sticks.*

(**k**) *Cu metal turnings.*

(**l**) *Al foil combustion cups.*

Reagents available from several commercial manufacturers of combustion analyzer instruments [See **B(a)**].

D. Preparation of Sample

Pass samples through grinder 2× in succession for emulsified meat products; mix thoroughly after each grinding. Pass 3× in succession for nonemulsified (coarse or whole muscle) products.

E. Determination

Set instrument operating parameters (oven temperature, oxygen flow, calibration values, etc.) according to manufacturer's instructions. Let furnace and instrument reach operating temperature and stabilize. Warm-up time may be ca 6 h from cold start. Establish system blanks as appropriate for analysis and calibrate to blanks if necessary. At least 5 blank analyses are recommended. Calibrate instrument by using 3–5 analyses of nitrogen standard as follows:

(*1*) Accurately weigh 110–150 mg EDTA to the nearest 0.1 mg, or equivalent amount of nitrogen if using other nitrogen standard, into tared combustion cup or foil and transfer cup or foil to open loading port on instrument. Enter or record protein conversion factor required, if appropriate for instrument used.

(*2*) Close port, move sample into furnace, and begin analysis.

(*3*) When analysis is complete (3–5 min), repeat sequence for next sample.

Table 1. Samples used for collaborative study of combustion method for determination of crude protein in meat and meat products

Sample pair	Sample No.	Estd protein, %[a]	Description	Supplier
1	377	17	Ground beef	Fareway
	741	17		HyVee
2	414	20	Lean ground beef	ISU Meat Lab
	573	20		Cub Foods
3	165	19	Ground pork	Cub Foods
	822	19		ISU Meat Lab
4	439	18	Ground turkey	Louis Rich
	946	18		Longacre
5	471	11	Frankfurters	Dubuque Beef
	759	11		Nissen Beef
6	298	12	"Light" frankfurters	Oscar Mayer
	582	12		Hormel
7	163	13	Turkey frankfurters	Jennie-O
	935	13		Schweigert
8	434	10	Bologna	HyVee
	723	10		Oscar Mayer
9	637	12	"Light" bologna	Oscar Mayer
	866	12		Kahns Bulk at Cub
10	198	14	Turkey bologna	HyVee
	598	14		Wilson
11	118	17	Canned ham	Dubuque
	777	17		Farmland
12	364	17	Ham, water added	Wilson
	629	17		Farmstead
13	255	20	Dried beef	Carl Buddig
	299	20		Wilson
14	513	13	Pork sausage	ISU Meat Lab
	856	13		Purnells Old Folks
15	647	14	Summer sausage	Huisken
	941	14		ISU Meat Lab

[a] Protein estimated from previous work with commercial meat products by assuming typical values would be found.

(*4*) Adjust instrument as necessary on the basis of results from nitrogen standard.

(*5*) Analyze samples by repeating steps (1) to (3).

(*6*) Read nitrogen results directly from instrument.

F. Calculation

$$\text{Crude protein, \%} = \text{\% nitrogen} \times 6.25$$

(*Note*: Results with this method average 1.01 × results with **928.08**.)

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

Results and Discussion

Sample pairs selected for the study are presented in Table 1. We anticipated that by choosing different manufacturers of the same product (for example, frankfurters) the protein content of each pair would be very similar but not identical, an objective of the Youden pair approach (5). Collaborators analyzed each sample once by the AOAC mercury catalyst Kjeldahl method (**928.08**) (1) and once by the combustion method. The data reported by each of the 12 laboratories are presented in Table 2. In general, sample pairs were closely matched, with about 5%

Table 2. Data for collaborative study on crude protein (%) determination in meat and meat products by AOAC Kjeldahl (K) method and a combustion (C) method

Sample			Laboratory											
Pair	No.	Method	1	2	3	4	5	6	7	8	9	10	11	12
1	377	K	16.58	16.19	16.06	17.08	16.17	16.64	16.54	16.80	16.50	16.53	16.33	16.32
		C	16.48	16.72	17.10	16.74	16.62	16.30	16.62	16.91	15.88	16.15	17.56	16.56
	741	K	17.25	16.81	16.63	18.00	16.87	17.05	17.23	16.88	17.13	16.83	16.60	16.64
		C	17.36	17.41	17.61	16.83	16.74	16.92	17.18	17.50	17.38	16.55	17.71	16.81
2	414	K	20.46	20.38	20.02	21.02	19.49	20.39	20.68	20.15	19.31	20.21	20.09	19.99
		C	20.25	20.59	20.31	20.40	20.77	19.92	20.59	20.30	18.56	20.65	20.26	20.03
	573	K	18.99	17.62	18.38	19.56	18.94	18.87	19.20	18.40	18.25	18.55	18.40	18.39
		C	18.71	18.96	18.80	18.41	18.88	18.76	19.29	18.84	18.44	18.99	19.52	18.81
3	165	K	19.73	19.12	19.58	20.33	18.95	19.63	20.57	19.53	19.38	19.71	19.38	19.45
		C	19.35	19.90	20.00	19.44	19.53	19.59	20.08	19.95	19.62	19.82	21.12	19.88
	822	K	19.77	19.56	19.23	20.30	19.10	19.73	19.74	19.51	19.50	19.72	19.36	19.45
		C	19.44	19.84	19.68	19.62	19.51	19.58	19.97	19.84	19.94	20.03	20.31	19.70
4	439	K	18.61	18.00	18.49	19.18	18.76	18.84	18.71	18.76	17.81	18.44	18.46	19.02
		C	18.54	19.06	18.87	18.63	18.61	18.73	18.68	19.02	17.63	17.98	19.34	18.72
	946	K	18.25	18.38	17.63	18.52	18.36	18.46	18.44	18.27	18.31	18.58	17.79	18.05
		C	18.36	18.21	17.30	18.16	18.47	18.14	18.68	18.35	18.25	18.37	19.25	18.68
5	471	K	11.65	11.44	11.92	11.82	11.35	11.49	11.71	11.32	10.75	11.55	11.46	11.65
		C	11.61	11.78	11.66	11.68	11.73	11.33	11.79	11.69	11.19	11.58	12.03	12.15
	759	K	11.65	11.50	11.08	11.63	11.56	11.32	12.04	11.76	10.94	11.33	10.98	11.03
		C	11.53	11.74	11.50	11.49	11.51	11.22	11.68	11.74	10.88	11.00	11.45	11.99
6	298	K	11.63	11.19	11.76	11.86	11.70	11.26	11.55	11.72	11.69	11.66	11.64	11.27
		C	11.57	11.70	11.89	11.54	12.16	11.78	11.90	11.74	11.81	11.74	11.88	11.92
	582	K	11.95	12.12	11.53	11.58	11.97	12.03	11.87	11.54	12.19	12.03	11.76	11.76
		C	11.78	12.09	11.97	11.78	11.78	12.10	11.94	11.71	12.19	12.25	12.18	11.76
7	163	K	13.44	13.19	13.13	13.40	13.47	13.33	13.30	13.51	12.88	13.40	13.42	13.33
		C	13.25	13.58	13.61	13.31	13.38	13.18	13.85	14.19	13.06	13.93	14.26	13.86
	935	K	13.60	13.38	13.44	13.37	13.82	13.24	13.46	13.68	12.75	13.36	13.48	13.31
		C	13.44	13.68	13.54	13.68	13.55	13.86	13.92	13.46	12.94	13.56	13.71	13.37
8	434	K	10.90	10.69	10.76	10.67	10.96	10.53	10.51	10.89	10.56	10.79	10.82	10.80
		C	10.69	10.99	10.03	10.63	10.86	10.83	10.98	11.55	10.69	10.85	11.86	11.18
	723	K	11.68	11.44	11.06	11.64	11.49	11.02	11.87	11.63	11.00	11.53	11.54	11.40
		C	11.45	11.67	11.50	11.34	11.46	11.43	11.58	11.55	11.25	11.68	12.14	12.04
9	637	K	11.68	11.50	11.42	11.42	11.41	11.11	11.82	11.88	11.69	11.54	11.28	11.25
		C	11.40	11.67	11.42	11.23	11.54	11.38	11.53	12.10	11.63	11.45	12.17	11.89
	866	K	13.94	13.56	13.53	13.55	13.68	13.32	13.63	13.41	13.69	13.45	13.56	13.57
		C	13.27	13.68	13.74	13.42	13.76	13.35	13.70	14.14	13.88	13.58	13.85	13.52
10	198	K	14.65	14.62	15.22	15.16	14.34	14.38	14.77	14.16	14.88	14.60	14.50	14.53
		C	14.55	14.78	14.66	14.79	14.47	14.42	14.81	15.02	14.56	14.52	14.95	14.59
	598	K	14.67	14.75	14.29	15.16	14.87	14.45	14.75	14.99	15.35	14.70	14.70	14.71
		C	14.66	14.98	14.78	14.92	14.73	14.48	15.01	15.50	15.00	14.83	14.98	14.50
11	118	K	17.04	16.81	16.98	17.45	16.67	16.51	17.27	17.13	18.06	16.98	16.84	16.93
		C	16.91	17.27	17.24	17.13	17.44	16.88	17.14	17.45	16.88	16.87	17.41	17.09
	777	K	16.50	16.69	16.23	16.92	16.50	16.54	16.22	17.04	16.00	16.46	16.44	16.30
		C	16.52	16.82	16.82	17.18	16.73	17.56	16.66	17.31	16.19	16.33	16.91	16.45

Table 2. *(Continued)*

Sample			Laboratory											
Pair	No.	Method	1	2	3	4	5	6	7	8	9	10	11	12
12	364	K	17.36	17.12	16.92	17.81	17.54	17.06	16.94	17.80	17.06	17.44	17.26	17.01
		C	17.08	17.64	17.68	18.00	18.03	17.11	17.42	18.05	17.01	17.44	17.90	17.53
	629	K	17.05	16.94	16.48	17.72	16.62	16.48	17.18	17.23	16.50	17.60	16.91	16.99
		C	17.04	17.30	16.84	18.46	17.00	17.71	16.95	17.27	16.56	17.12	17.41	17.12
13	255	K	19.83	19.50	19.56	20.29	19.09	19.61	20.49	19.42	19.81	19.46	18.98	19.07
		C	19.61	19.59	19.78	19.51	19.70	19.35	19.62	19.79	19.56	19.14	19.95	19.47
	299	K	20.49	20.44	20.08	21.37	20.83	20.39	20.41	20.64	20.25	20.16	19.95	20.27
		C	20.63	20.92	20.74	21.88	20.48	20.94	20.63	21.10	20.06	20.33	20.99	20.57
14	513	K	15.43	15.06	15.25	16.12	15.34	15.14	15.21	15.53	15.81	15.41	15.17	15.63
		C	15.13	15.82	15.65	15.42	15.36	15.18	15.82	16.16	15.88	15.53	15.86	15.84
	856	K	13.10	13.00	12.31	13.29	12.73	12.49	12.59	12.45	12.31	13.09	12.57	12.98
		C	12.81	13.23	12.99	12.93	12.77	11.84	13.37	13.06	12.44	12.85	13.24	13.09
15	647	K	14.44	14.50	14.00	14.55	14.34	14.20	14.00	14.24	13.81	13.97	14.52	14.17
		C	14.39	14.45	13.84	14.18	14.19	13.99	14.40	14.64	13.44	14.33	15.08	14.79
	941	K	19.69	19.19	19.14	20.34	19.43	18.75	19.36	19.63	19.50	19.31	18.99	19.18
		C	19.24	18.87	19.51	19.26	19.21	18.93	19.72	19.47	19.56	19.49	19.50	19.41

or less difference between the 2 samples of the pair. Three of the pairs (sample pairs 9, 14, and 15), however, showed more than 10% difference between the 2 samples of the pair. These samples are products (light bologna, pork sausage, and summer sausage, respectively) that are not uniform across the industry, and consequently, the greater differences were not surprising.

The performance testing of the combustion method by the collaborators showed that all laboratories performed well in combustion analysis of standard nicotinic acid and lysine hydrochloride (Table 3). Only 1 (laboratory 10) of the 24 means shown in Table 3 was outside the recommended ±0.15, and only 2 (laboratories 2 and 6) of 24 exceeded the recommended standard deviation of 0.15 (by 0.01 and 0.02, respectively).

Table 3. Collaborators' performance for combustion analysis of standards

	Nicotinic acid		Lysine·HCl	
Laboratory	Mean, % N[a]	SD[b]	Mean, % N[a]	SD[b]
1	11.32	0.13	15.34	0.04
2	11.31	0.09	15.22	0.17[c]
3	11.39	0.05	15.40	0.02
4	11.42	0.02	15.33	0.01
5	11.43	0.05	15.29	0.03
6	11.36	0.16[c]	15.32	0.03
7	11.49	0.03	15.36	0.02
8	11.46	0.06	15.32	0.02
9	11.39	0.02	15.34	0.02
10	11.26	0.03	15.08[c]	0.12
11	11.49	0.01	15.37	0.05
12	11.41	0.10	15.26	0.05
Theoretical	11.38		15.34	

[a] Mean of 10 determinations.
[b] SD = standard deviation.
[c] Value outside the performance requirements of ±0.15 respective theoretical %N values or ±0.15 standard deviation.

Examination of the data from both methods for gross outliers was done by preparing 2-sample X-Y plots of the 15 sample pairs (5).

No gross outliers were observed for the Kjeldahl values from any of the 12 laboratories. In addition, use of the Cochran test, the single Grubbs test, and the double Grubbs test showed that none of the Kjeldahl data exceeded critical values for these tests for outliers. For the combustion method, one sample pair (414, 573) from laboratory 9 was in the low quadrant of the X-Y plot and exceeded the critical value of the single Grubbs test. Consequently, for the combustion method, the data for this pair (414, 573) from laboratory 9 were excluded from the rest of the comparisons.

The calculated estimates of precision are shown in Table 4, with the sample pairs arranged in order of increasing protein content. Repeatability standard deviations (s_r) were very similar for the 2 methods, ranging from 0.11 to 0.40 for the Kjeldahl method and from 0.12 to 0.41 for the combustion method (Table 5). Reproducibility standard deviations (s_R), likewise, were similar, ranging from 0.20 to 0.49 and from 0.18 to 0.46 for the Kjeldahl and combustion methods, respectively. The repeatability relative standard deviations (RSD_r) ranged from 0.82 to 2.41% for the Kjeldahl method and from 0.60 to 2.23% for the combustion method. The ranges for the reproducibility relative standard deviations (RSD_R) were 1.59 to 2.84% for the Kjeldahl method and 1.32 to 3.35% for the combustion method.

Comparison of the sample means (Table 4) shows the means from the combustion method to be slightly higher for all

Table 4. Comparison of precision parameters for Kjeldahl and combustion methods for closely matched pairs in collaborative study on crude protein in meat and meat products

	Kjeldahl					Combustion				
Sample pair	Mean % protein	s_r	s_R	RSD_r, %	RSD_R, %	Mean % protein	s_r	s_R	RSD_r, %	RSD_R, %
8	11.09	0.19	0.22	1.75	2.02	11.26	0.25	0.38	2.19	3.35
5	11.46	0.28	0.32	2.41	2.84	11.58	0.14	0.29	1.20	2.50
6	11.72	0.27	0.27	2.28	2.28	11.88	0.18	0.18	1.53	1.53
9	12.54	0.16	0.20	1.31	1.59	12.64	0.16	0.27	1.23	2.14
7	13.36	0.11	0.22	0.82	1.68	13.59	0.29	0.33	2.14	2.46
14	14.08	0.27	0.32	1.90	2.29	14.26	0.25	0.37	1.75	2.58
10	14.72	0.30	0.30	2.04	2.07	14.22	0.12	0.24	0.79	1.60
15	16.80	0.29	0.33	1.75	1.99	16.83	0.35	0.36	2.08	2.12
1	16.76	0.22	0.37	1.29	2.22	16.90	0.28	0.41	1.65	2.43
11	16.77	0.40	0.40	2.37	2.37	16.99	0.28	0.36	1.64	2.14
12	17.13	0.24	0.37	1.40	2.16	17.40	0.34	0.43	1.97	2.49
4	18.42	0.33	0.34	1.77	1.86	18.50	0.41	0.46	2.23	2.47
3	19.60	0.22	0.39	1.11	1.99	19.76	0.12	0.26	0.60	1.32
2	19.41	0.36	0.49	1.89	2.53	19.64[a]	0.25	0.28	1.27	1.42
13	20.02	0.33	0.42	1.63	2.11	20.18	0.33	0.36	1.63	1.77

[a] Results by combustion method for sample pair 2 from laboratory 9 were excluded on basis of single Grubbs test.

pairs. When means are compared for each sample within pairs (Table 6), only one sample (941) resulted in a greater value by the Kjeldahl method. The average difference was 0.16% protein (0.025% N) between the 2 methods for all samples. Paired *t*-tests showed no significant difference between the methods. The grand means of all samples were 15.59% for the Kjeldahl method and 15.75% for the combustion method.

Comparing the 2 methods for analysis of the standards, nicotinic acid and lysine hydrochloride (Table 7), shows the greater difficulty experienced by several laboratories with the Kjeldahl method for the standards. Six of the 12 laboratories reported low values for nicotinic acid, and 5 had difficulties with the determinations on lysine hydrochloride. This difficulty is not unusual and has been observed previously (2). The combustion method, when applied to the standards, was more consistent than the Kjeldahl method when all 12 laboratories are considered.

Conclusions

The combustion method for crude protein performed very satisfactorily when applied to a variety of meat and meat products by 12 different laboratories. Repeatability and reproducibility standard deviations were equivalent for the 2 methods. Further, data for only one sample pair for the combustion method in one laboratory were excluded as outliers. This means that only 2 of the 360 data points collected for the combustion method were outliers, an outcome indicating that both the method and the laboratories involved performed very well.

In addition, 3 different combustion instruments were used in this study with no obvious effects on the results; therefore, the combustion method does not require a particular instrument. Minimum sample size and performance requirements for accuracy should be included when the method is applied to meat and meat products.

Table 5. Range of precision estimates for Kjeldahl and combustion methods in collaborative study on crude protein in meat and meat products

Measure	Kjeldahl	Combustion
s_r	0.11–0.40	0.12–0.41
s_R	0.29–0.49	0.18–0.46
RSD_r	0.82–2.41	0.60–2.23
RSD_R	1.59–2.84	1.32–3.35

Recommendation

We recommend that the combustion method be adopted first action for determination of crude protein in meat and meat products. To achieve homogeneity before sampling, a minimum sample size of 200 mg and careful sample preparation following USDA laboratory guidelines are recommended. Performance guidelines for combustion instruments should be ±0.15 of theoretical N content of the mean of 10 determinations of nicotinic acid and of lysine hydrochloride, with a standard deviation of ≤0.15. Safety precautions should be followed according to the respective manufacturer of the instrument used.

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Table 6. Comparison of sample means by combustion and Kjeldahl methods (all laboratories) for collaborative study on crude protein in meat and meat products

Sample pair	Sample No.	Mean protein, % Kjeldahl	Combustion	Difference (comb. – Kjeldahl)
1	377	16.54	16.64	0.10
	741	17.07	17.17	0.10
2[a]	414	20.18	20.37	0.19
	573	18.63	18.91	0.28
3	165	19.61	19.77	0.16
	822	19.58	19.75	0.17
4	439	18.59	18.67	0.08
	946	18.25	18.35	0.10
5	471	11.51	11.69	0.18
	759	11.40	11.48	0.08
6	298	11.58	11.80	0.22
	582	11.86	11.96	0.10
7	163	13.32	13.66	0.34
	935	13.41	13.56	0.15
8	434	10.74	10.93	0.19
	723	11.44	11.59	0.15
9	637	11.50	11.62	0.12
	866	13.57	13.66	0.09
10	198	14.65	14.68	0.03
	598	14.79	14.86	0.07
11	118	17.06	17.18	0.12
	777	16.49	16.79	0.30
12	364	17.28	17.57	0.29
	629	16.98	17.23	0.25
13	255	19.59	19.59	0.00
	299	20.44	20.77	0.33
14	513	15.43	15.64	0.21
	856	12.74	12.89	0.15
15	647	14.23	14.31	0.08
	941	19.38	19.35	–0.03
Overall		15.59	15.75	0.16

[a] Results of combustion method from laboratory 9 excluded.

Table 7. Collaborators' results of 10 determinations by combustion and Kjeldahl methods for standards (% protein)

Laboratory	Nicotinic acid Kjeldahl	Combustion	Lysine·HCl Kjeldahl	Combustion
1	71.45	70.75	95.91	95.89
2	70.21	70.72	94.02	95.10
3	69.99	71.18	94.63	96.27
4	59.54[a]	71.35	94.96	95.80
5	39.28[a]	71.43	93.33[a]	95.57
6	64.83[a]	70.98	93.52[a]	95.74
7	71.90	71.81	96.08	96.01
8	70.19	71.65	94.65	95.72
9	70.73	71.16	95.80	95.84
10	27.94[a]	70.37	93.17[a]	94.23
11	14.49[a]	71.79	77.33[a]	96.07
12	35.04[a]	71.28	99.00[a]	95.39
Mean	70.75	71.21	95.15	95.64
Theoretical	71.12		95.88	

[a] Excluded from mean.

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METALS AND OTHER ELEMENTS

Determination of Free and Bound Cd, Zn, Cu, and Ag Ions in Lobster (*Homarus americanus*) Digestive Gland Extracts by Gel Chromatography Followed by Atomic Absorption Spectrophotometry and Polarography

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Gel-permeation chromatography followed by atomic absorption spectrophotometric and polarographic analyses were used to measure free and bound divalent metal ions in lobster digestive gland extracts prepared with or without α-toluenesulfonyl fluoride, a protease inhibitor. Chromatography on Sephadex G-50 or G-100 yielded 3 UV-absorbing peaks, which corresponded, respectively, to the void volume, a medium molecular weight fraction, and a low molecular weight fraction that contained free divalent metal ions. In protease-inhibited extracts, only Zn^{+2} was found, whereas Cd, Cu, and Ag were bound in high and medium molecular weight fractions. Cd^{+2} and Zn^{+2} were rapidly released from their bound forms in the absence of a protease inhibitor, and their presence was confirmed by polarography with EDTA. Gel-permeation chromatography coupled with atomic absorption spectrophotometry offers a rapid method for following changes in the concentrations of bound and free metal ions during processing of shellfish-based foodstuffs.

Shellfish are highly desirable foodstuffs, and consumption of visceral tissues of most shellfish is common practice. However, shellfish accumulate toxic metal ions, such as Cd, Cu, Zn, and Ag in certain visceral tissues (1). For example, the digestive gland of American lobster (*Homarus americanus*), which is approximately 20% of the edible tissue, normally contains high Cd concentrations (1–80 μg/g wet weight) (2). Lobsters from contaminated areas may contain surprisingly high Cd concentrations, frequently exceeding 100 μg Cd/g wet weight in digestive glands of lobsters captured near a lead smelter (3). Cd^{+2} is normally protein-bound in biota, and various metal-binding proteins were isolated and characterized (4–6). A protease inhibitor must be used during their isolation (7), because Cd^{+2} is rapidly released by postmortem activity. Chou et al. (8) reported that approximately 50% of the total Cd in fresh frozen oyster tissue was present as Cd^{+2}. Cd was rapidly released from the digestive gland of giant scallop (*Placopecten magellanicus*) upon death (9).

A study of Cd uptake by rats fed a diet based on canned lobster digestive gland showed that uptake from this source was much less than that observed with a casein-based diet spiked with an equivalent concentration of an inorganic Cd salt (10). Polarographic assay of the canned diet showed that free Cd^{+2} was formed during processing. The concentration of Cd^{+2} was high enough to account for the observed uptake.

These observations led us to develop a method for measuring concentrations of free and bound forms of various metal ions in lobster digestive gland extracts by using gel-permeation chromatography followed by atomic absorption spectrophotometry (AAS) and polarography. The method can be used to monitor changes in the nature of various toxic metal ions during processing of certain foodstuffs.

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METHOD

Apparatus

(**a**) *Flame AAS system.*—Perkin-Elmer Model 403 equipped with single slot burner head and deuterium arc background correction.

(**b**) *Flameless AAS system.*—Perkin-Elmer Model 403 equipped with Model HGA 2100 graphite furnace and deuterium arc background correction.

(**c**) *Differential pulse polarographic analyzer.*—Princeton Applied Research Corp., Model 174.

(**d**) *Liquid chromatographic system.*—Pharmacia Type C column (100 cm × 26 mm id), LKB-Produckter Model II Uvcord flow-through detector, and Ultrorac Model 7000 fraction collector, with Marriotte bottle reservoir or PREX peristaltic pump to maintain pressure.

Reagents

All reagents were analytical grade. Quartz-distilled water was used throughout. α-Toluenesulfonyl fluoride (TSF) was obtained from Sigma Chemical Co. (St. Louis, MO). Metal ion standard solutions were obtained from Fisher Scientific (Canada).

Preparation of Extracts

Cd-contaminated lobsters were obtained from Belledune Harbour, New Brunswick, Canada. Clean lobsters were obtained from Heron Island, New Brunswick.

Gently homogenize 3 g digestive gland (from freshly killed animal) in 10 mL 1.0M ammonium acetate (pH 7.2, with or without 0.1mM TSF) in Potter-Elvehjem glass homogenizer with PTFE pestle for 1 min. Centrifuge homogenate 3 times at 30 000 × *g* for 30 min at 4°C, decanting after each sedimentation; filter through fritted disc (Millipore 4 xx-10-025-14) overlaid with 1 layer of flow adaptor nylon screen (Pharmacia)

Gel-Permeation Liquid Chromatography

Pack chromatographic column with either Sephadex G-100 or G-50 prepared according to manufacturer's instructions. Elute with 1M ammonium acetate, pH 7.2, at ca 15 mL/h. Monitor eluant at 250 nm, and collect 5 mL fractions.

Blue dextran, cytochrome C, and K^+, were used to calibrate column (Figure 1). Metal ions (Cd^{+2}, Zn^{+2}, Cu^{+2}, and Ag^+) were shown to elute quantitatively under these conditions (11).

Atomic Absorption Spectrophotometry

Metal ions in extracts and fractions were determined without digestion. Cd, Zn, and Cu were determined by flame AAS at 228.8, 213.8, and 324.8 nm for Cd, Zn, and Cu, respectively, using Cd electrodeless discharge lamp or hollow cathode lamps for Zn and Cu. Ag was determined by graphite furnace AAS using hollow cathode lamp at 328.1 nm. Method of standard additions was used throughout. Accuracy of these methods was confirmed through intercomparison studies (12, 13).

Polarography

Dilute 0.5–1.0 mL extract or 1 mL chromatographic fraction to 10 mL with 1M ammonium acetate, transfer to polarographic cell, and determine ions by using differential pulse operating mode at 2 mV/s potential scan rate, 2 s drop time, and 50 mV modulation amplitude.

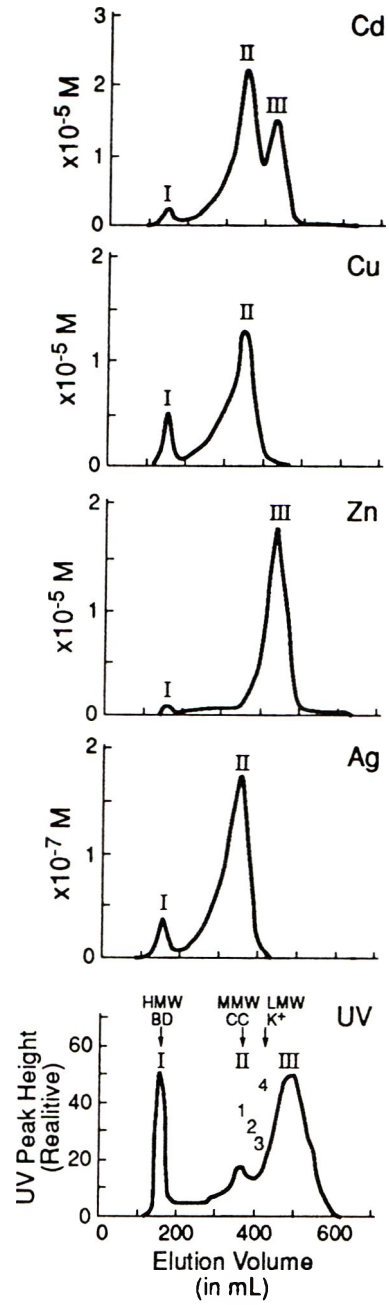

Figure 1. Sephadex G-100 (2.5 × 80 cm column; eluant, 1M ammonium acetate) elution profiles for contaminated lobster digestive gland extract (no TSF). Fractions 1–4 correspond to the region UV-II and UV-III (HMW, high molecular weight; MMW, medium molecular weight; LMW, low molecular weight).

Results and Discussion

Gel-Permeation Chromatography

Without protease inhibitor (TSF).—Figure 1 shows the 250 nm absorption chromatograms for Cd, Zn, Cu, and Ag of a Cd-contaminated lobster digestive gland extracted by using Sephadex G-100. Three major UV-absorbing peaks (UV-I, UV-II, and UV-III, in order of elution) were found. UV-I corresponded to high molecular weight material that eluted at the void volume; UV-II corresponded to medium molecular weight material within the molecular weight range of metallothionein or metal-binding proteins; and UV-III corresponded to low molecular weight material. AAS yielded 3 Cd-containing peaks (Cd-I, Cd-II, and Cd-III), 2 Cu-containing peaks (Cu-I and Cu-II), 2 Zn-containing peaks (Zn-I and Zn-III), and 2 Ag-containing peaks (Ag-I and Ag-II). Again, the designations I, II, and III refer to relative molecular weights of the metal-containing fractions. The absence of Zn in peak II and the presence of Cd in Peak III suggested that some degradation occurred during processing (4, 11). Without protease inhibition,

Table 1. Recovery and peak distribution of Cd^{+2}, Zn^{+2}, and Ag^{+} in lobster digestive gland extract (containing TSF) by gel-permeation chromatography/atomic absorption spectophotometry

Column packing	Metal ion	Metal ion in digestive gland, µg/g wet wt	Metal ion recovered, off column, %	Metal ion, %		
				UV-I	UV-II	UV-III
Sephadex G-50	Cd	87.3	98.1	1.5	98.5	—
	Zn	23.5	103.0	19.8	54.6	25.4
	Cu	6.34	110.0	67.3	32.7	—
	Ag	1.14	94.6	54.8	45.2	—
Sephadex G-100	Cd	87.3	96.8	2.0	98.0	—
	Zn	23.5	108.0	21.8	58.1	19.1
	Cu	6.34	101.0	71.2	28.8	—
	Ag	1.14	96.3	52.6	47.4	—

the distribution of Cd in extracts changed over time; rechromatography of Cd-II yielded only Cd-III (Cd^{+2}), probably because of the presence of autolytic and digestive enzymes. Bacterial degradation also may play a role, but this is unlikely because of the speed at which these changes occurred.

Obviously, the distribution of certain metal ions in lobster digestive gland extracts changes over time. To prevent proteolytic changes, extracts were prepared in the presence of 10^{-4}M TSF (7), a known protease inhibitor. Polarographic studies showed that TSF did not interfere with the determination of Cd^{+2}.

With protease inhibitor.—In the presence of TSF, no Cd+2 was found in the extracts (Table 1). Only 55% of the Zn was present in the medium molecular weight fraction (UV-II). These results confirm the suspicion that the presence of Cd^{+2} in extracts prepared without TSF resulted from postmortem enzymatic activity. No Cu^{+2} or Ag^{+} was present in the extracts. Upon chromatography on Sephadex G-100, 71.2% of the Cu was found in the high molecular weight fraction (UV-I) and 28.8% in the medium molecular weight fraction (UV-II), whereas 52.6% of the Ag was found in the high molecular weight fraction and 47.4% in the medium molecular weight fraction. Cu and Ag were present in the high molecular weight fraction in greater proportions than Cd and Zn. This probably reflects the higher affinities of Cu^{+2} and Ag^{+} for proteins compared with those of Cd^{+2} and Zn^{+2} (14, 15). Sephadex G-50 gave essentially the same results as G-100; both gave recoveries of the 4 metals ranging from 94.6 to 100%. However, Sephadex G-100 gave slightly better chromatographic resolution than did G-50.

Polarographic Studies on Gel-Permeation Chromatographic Fractions (No TSF)

Differential pulse polarograms (Figure 2) were recorded for fractions from the UV-II and UV-III peaks of Figure 1. Fraction 1, corresponding to the metal-binding protein peak (Cd-II), yielded polarograms with 3 undefined peaks. Fractions 2–4, corresponding to low-molecular weight species, showed increasing amounts of unbound Cd^{+2} and Zn^{+2}. The polarograms for fractions 2, 3, and 4 were increasingly more complex because of interference by other low-molecular weight substances. The polarogram for fraction 4 (UV-III) contained a Cd^{+2} reduction peak at –0.63 V, a Zn^{+2} reduction peak at –1.05 V, and an unidentified peak at –0.50 V. Figure 3 presents the results of titrating fraction 4 with EDTA. EDTA-bound Zn^{+2} and Cd^{+2} reduction waves are not seen in differential pulse polarography; therefore, the background contribution can be estimated. The addition of 0.3 µmoles EDTA to fraction 4 reduced the Cd^{+2} (0.068 µmoles) and Zn^{+2} (0.153 µmoles) peaks. The peak at –0.5 V remains and a new peak at –0.30 V

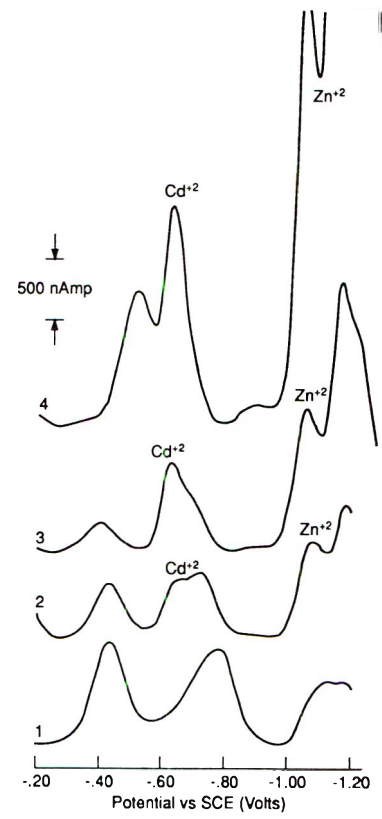

Figure 2. Polarograms corresponding to fractions shown in Figure 1. (All potentials were referenced to the saturated calomel electrode [SCE].)

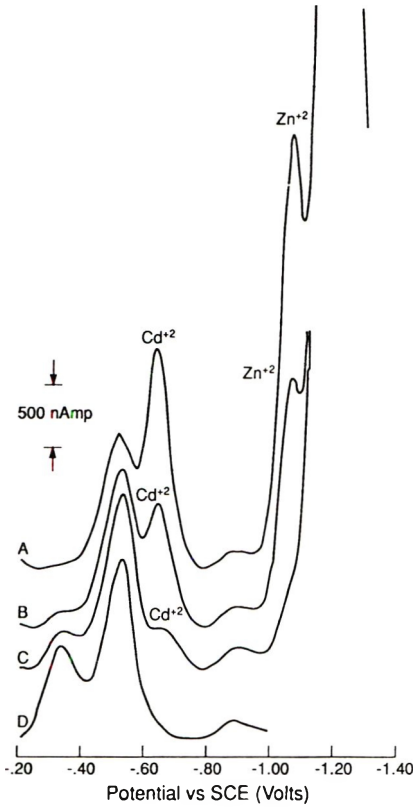

Figure 3. EDTA complexing studies of the polarograms of the UV-III peak of Sephadex G-100 (fraction 4 of Figure 1). Polarogram A, 1 mL fraction 4; polarogram B, 1 mL fraction 4 + 0.1 μmole EDTA; polarogram C, 1 mL fraction 4 + 0.2 μmole EDTA; polarogram D, 1 mL fraction 4 + 0.3 μmole EDTA. All run in 10 mL 1M ammonium acetate.

appears. The results confirm the presence of Cd^{+2} and Zn^{+2} in fraction 4.

Comparison of Chromatographic and Polarographic Results for Cd^{+2} in Digestive Gland Extracts

Cd^{+2} was determined in extracts prepared without TSF by polarography directly or by gel-permeation chromatography/AAS (Table 2). An extract prepared from a contaminated lobster (583 μg Cd/g wet weight) yielded 105 and 110 μg free Cd^{+2}/g by Sephadex G-50 and Sephadex G-100, respectively (20–21% free Cd^{+2}). Polarographic analysis yielded 102 μg free Cd^{+2}/g. In an extract from an uncontaminated lobster (8.54 μg Cd/g wet weight), chromatography yielded 7.27 μg free Cd^{+2}/g with Sephadex G-50 or G-100, and 6.96 μg free Cd^{+2}/g polarographically. Thus, the 2 methods showed good agreement. The uncontaminated lobster extract contained 81.5% free Cd^{+2}, compared to 17.5% in the contaminated lobster extract. Routine polarographic analysis of the extracts directly is not recommended because of the presence of substances that have a reduction potential differing by <0.2 V from that of Cd^{+2}, and the overlap of the Zn^{+2} peak by a large, uncharacterized peak at −1.15 V.

Our results show that Cd^{+2} and Zn^{+2}, bound to medium molecular weight proteins, are rapidly released post-mortem because of protease activity. Cd-binding protein isolated from lobster digestive gland has been shown to be highly reactive with a variety of ligands to release Cd^{+2} (16). A protease inhibitor is essential for recovering intact metal-binding proteins. Partially degraded extracts were useful for developing and studying chromatographic and polarographic methods for distinguishing free and bound metal ions such as Cd^{+2} and Zn^{+2}.

These results complement our earlier studies that showed rapid release of Cd^{+2} from scallop digestive gland (9) and offer methodology for determining levels of metal ions released during processing of raw foodstuffs into consumer products. Although detailed studies have not been made of the release of bound metal ions under various treatments, e.g., cooking, the results reported in this paper give ample evidence that toxicological evaluation of foodstuffs prepared from shellfish tissues containing levels of toxic elements of concern will require assessment of the forms of such metals and their bioavailability.

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Table 2. Determination of free Cd^{+2} in lobster digestive gland extracts (no TSF) by either polarography or gel-permeation chromatography followed by atomic absorption spectrophotometry (concentrations in μg/g wet wt)

Sample	[Cd] in intact tissue	Cd extracted from tissue, %	[Cd^{+2}] in tissue extract by polarography	[Cd^{+2}] in tissue extract by GPC[a]
A	583	90.3	102 (17.5%)	105 (G-50) 110 (G-100)
B	8.54	92.5	6.96 (81.5%)	7.27 (G-50) 7.27 (G-100)

[a] Gel-permeation chromatography.

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METALS AND OTHER ELEMENTS

Determination of Metals in Foodstuffs by Atomic Absorption Spectrophotometry after Dry Ashing: NMKL[1] Interlaboratory Study of Lead, Cadmium, Zinc, Copper, Iron, Chromium, and Nickel

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An interlaboratory study of a method for determination of lead, cadmium, zinc, copper, iron, chromium, and nickel in foodstuffs by atomic absorption spectrophotometry (AAS) after dry ashing at 450°C was conducted in 16 laboratories. The study was preceded by a practice round of familiarization samples and another round in which solutions were distributed and the metals were determined directly by AAS. The study included 5 different foods (liver paste, apple sauce, minced fish, wheat bran, and milk powder) and 2 composite diets. A single analysis was carried out on each sample. Suitable sample combinations were used as split level combinations for determination of the repeatability standard deviation. The relative reproducibility standard deviation for each of the elements ranged as follows: lead, 74–18% at 0.025–0.28 mg/kg; cadmium, 80–11% (0.002–0.51 mg/kg); zinc, 12–7% (44–72 mg/kg); copper, 47–10% (0.48–41 mg/kg); iron, 35–9% (2–228 mg/kg); chromium, 48–21% (0.008–0.22 mg/kg); nickel, 64–39% (0.025–0.39 mg/kg).

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[1] Nordic Committee on Food Analysis (Secretariat General, c/o Technical Research Centre of Finland, Food Research Laboratory, PO Box 203, SF-02151 Espoo, Finland).

This method was accepted as an official NMKL method at the 44th Annual Meeting of the Nordic Committee on Food Analysis, August 29–31, 1990, Gentotle, Denmark.

Most of the collaboratively tried and approved methods available today for trace element determinations are very specific and apply only to 1 or 2 elements, usually in a very specific matrix. Only a few methods exist that are approved for simultaneous determination of more than 1 element in more general types of food matrixes (1). For many elements commonly determined, there are no approved methods at all.

Most types of samples require a procedure to get the sample into solution before analysis by atomic absorption spectrophotometry (AAS). The 2 most commonly used techniques to accomplish this are dry ashing at a defined temperature, and wet digestion with mineral acid. Over the years, several investigators have pointed out the possible loss of analyte during dry ashing. Gorsuch (2, 3) showed that certain metals could be lost through volatilization or retention on silica crucible walls when

metallic standard solutions were added to the samples, or when metallic standard solutions were ashed with certain chlorides. Losses of Cd in specific sample tissues have been reported by Feinberg and Ducauze (4) and by Slabyj et al. (5). In the first case, however, the samples were ashed at 750°C, with H_2SO_4 as ashing aid. In the second case, the indications that dry ashing contributed to the poor results were not substantial. Koirtyohann and Hopkins (6) showed that no losses of Cd, Zn, Fe, or Cr through volatilization occurred when tissues were ashed at temperatures below 600°C. Loss by retention on crucible walls at an ashing temperature of 500°C was observed for Zn in porcelain crucibles. In platinum or silica crucibles, only insignificant retention was observed at an ashing temperature of 500°C. Using radioactive isotopes in biological materials, van Raaphorst et al. (7) demonstrated that no losses of Cd occurred by volatilization or retention at an ashing temperature of 450°C.

The papers cited above present strong indications that the method for dry ashing at a maximum temperature of 450°C presented here yields results free from losses by volatilization or retention. This method has been used for many years; moreover, numerous recovery studies and frequent use of certified reference materials have given no indication of systematic losses. Ashing aids and modification of the sample matrix during ashing have often been used to eliminate potential losses and/or to speed up the ashing procedure. This always increases the risk of contamination, however, and results in poorer detection limits.

Dry ashing is generally rather time-consuming; it usually takes a day or more before a result can be obtained, although very little attention from the analyst is necessary. Contamination can sometimes be a problem, however, because the samples are exposed to ambient air for long periods of time. One advantage of dry ashing is that the resulting ash can be dissolved in a small amount of diluent. This provides much better detection limits than wet digestion, especially when dry ashing is used with flame AAS.

Wet digestion methods are generally rapid; analysis may be completed within several hours, and the methods are not as sensitive to contamination, especially when closed decomposition vessels are used. The disadvantages of wet digestion are that only fairly small samples can be used and also that the solutions normally have to be strongly diluted before the analysis. This results in rather poor detection limits, especially when flame AAS is used.

The 2 decomposition techniques should, therefore, be considered complementary because both have advantages as well as drawbacks. The selection of the technique should be based on individual laboratory requirements.

AAS is now probably the most widely used technique for determination of metals in biological materials. AAS determinations are usually made by flame-AAS when the concentrations are high enough, or by graphite furnace (GFAAS) when the concentrations are low. It is probably not meaningful to try to define exactly when to use flame AAS or GFAAS. Both techniques should basically give the same result as long as the flame results are above the detection limit. In practice, flame AAS should be selected instead of GFAAS whenever possible because it is less time-consuming and also less sensitive to interference (e.g., background absorption).

The metals that were considered to be of greatest interest for this interlaboratory trial were the toxic metals lead and cadmium, for which many countries have established legal limits and for which low detection limits are of interest. Also of interest were the essential metals zinc, copper, iron, and chromium, for which there are recommendations regarding a safe and adequate daily intake. Nickel, which is potentially essential and also is the metal that causes most allergic eczemas, was also of great interest. Chromium and nickel also require good detection limits because of their low levels in most foods.

In 1986, the following 3 methods suggested by a working group within the Nordic Committee on Food Analysis (NMKL) were subjected to a pre-trial in which the participants were free to choose which method to use: (*1*) wet digestion with HNO_3 in an open vessel; (2) wet digestion with H_2SO_4/HNO_3, followed by extraction with APDC/MIBK; and (*3*) dry ashing at 450°C, according to the method described here. Of the responding laboratories, 15 used method 3, 5 used method 2, and 2 used method 1. The results by all 3 methods were similar, and the reproducibility between the laboratories was encouraging for further work. But because the interest was focused on the dry ashing method, that method was selected for the interlaboratory trial, and work on the 2 wet digestion methods was terminated.

In an attempt to elucidate the contribution by the determination step to the total variance in the results, a number of prepared solutions were sent to the participants and analyzed directly by AAS before the start of the interlaboratory trial. The results of this pre-trial will be covered under *Discussion*. The dry ashing method described here was based on the method by Dalton and Malanoski (8), using a modified pre-ashing apparatus from the method of Thiers (9). This trial was finished in 1989, and the method was approved by NMKL in 1990.

METHOD

For quantitative determination of metals (lead, cadmium, chromium, copper, iron, zinc, and nickel) in food products by dry ashing and atomic absorption spectrophotometry (AAS).

Principle

Samples are dried and then ashed at 450°C with gradual increase in temperature. Hydrochloric acid is added, and the solution obtained is evaporated to dryness. The residue is dissolved in 0.1M HNO_3, and the metal contents are determined by flame and graphite procedures.

Apparatus

(**a**) *Atomic absorption spectrophotometer.*—Equipped with background correction and graphite furnace; alternatively air-acetylene burner or nitrous oxide-acetylene burner.

(**b**) *Hollow cathode lamps.*—For all elements to be determined. EDL lamps are recommended for Pb and Cd because of improved light gain.

(**c**) *Furnace.*—Programmable or muffle furnace with thermostat. Separate pre-ashing device is required for muffle furnace. See (**d**–**h**).

(**d**) *Hot plate.*—With stepwise heating control, up to ca 300°C.

(**e**) *Lamp.*—IR 250 W, fixed to retort stand so as to allow adjustment of distance to plate.

(**f**) *Ceramic plate.*—Such as desiccator plate on low stand, with diameter suitable for hot plate.

(**g**) *Glass cover.*—Such as crystallizing dish, 185 mm diameter, 100 mm high.

(**h**) *Wash-bottle.*—Containing H_2SO_4 for purification of air.

(**i**) *Crucibles.*—Quartz or platinum, 50–75 mL.

(**j**) *Plastic bottles.*—With leak-proof closures, 100 mL.

All glassware and plasticware should be carefully cleaned and rinsed, e.g., with HNO_3 or HCl, to prevent metal contamination.

Reagents

Reagents should be of at least analytical reagent grade (p.a.), preferably Suprapur or similar.

(**a**) *Water.*—Redistilled or deionized (Millipore or similar quality).

(**b**) *Hydrochloric acid, 6M.*—Dilute 500 mL concentrated HCl (37%, w/w) with H_2O to 1000 mL.

(**c**) *Nitric acid, 0.1M.*—Dilute 7 mL concentrated HNO_3 (68%, w/w) with H_2O to 1000 mL.

(**d**) *Lead standard solution.*—1 mg/mL. Dissolve 1.000 g Pb in 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**e**) *Cadmium standard solution.*—1 mg/mL. Dissolve 1.000 g Cd in 14 mL H_2O + 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**f**) *Chromium standard solution.*—1 mg/mL. Dissolve 3.735 g K_2CrO_4 in 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**g**) *Nickel standard solution.*—1 mg/mL. Dissolve 1.000 g Ni in 14 mL H_2O + 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**h**) *Zinc standard solution.*—1 mg/mL. Dissolve 1.000 g Zn in 14 mL H_2O + 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**i**) *Copper standard solution.*—1 mg/mL. Dissolve 1.000 g Cu in 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**j**) *Iron standard solution.*—1 mg/mL. Dissolve 1.000 g Fe in 14 mL H_2O + 7 mL HNO_3 in 1 L volumetric flask. Dilute to volume with H_2O.

(**k**) *Working standard solutions.*—For graphite furnace analysis: Dilute standard solutions (**d**)–(**j**) with 0.1M HNO_3 to range of standards that covers linear range of element to be determined. Working standard solutions for flame analysis: Dilute standard (**d**)–(**j**) to range of standards that covers concentration of element to be determined. *Note*: Instead of preparing standards from metals or metal salts, commercially available standard solutions for AAS may be used.

Recommended Cleaning Procedure for Glass and Plasticware

Solutions.—NaOH solution: 130 g NaOH + 130 mL H_2O + 880 mL EtOH. Nitric acid: 500 mL concentrated HNO_3 + 4500 mL H_2O. Water should be redistilled or deionized (Millipore quality).

Wash first with H_2O and detergent; rinse with tap water. Wash with NaOH solution; rinse with deionized H_2O and then with HNO_3. Finally rinse 4-5 times with deionized H_2O.

Comments

The risk of contamination of the samples, especially with Pb, Cr, and Ni, must always be considered. Disposable plastic articles (like pipet tips) that are not normally washed should, therefore, be rinsed with acid before use.

Special attention should be paid to the crucibles. Quartz crucibles can be stored in HNO_3 and then rinsed with deionized H_2O before use. When necessary, these crucibles may also have to be boiled with acid before use. Platinum crucibles should preferably be heated until red hot and thereafter boiled with acid before use. Samples with a very high fat content should be ashed with great care to avoid self-ignition. If a programmable furnace is used, the samples should be pre-ashed according to dry ashing procedure B.

Procedures

Pre-treatment.—Homogenize sample with suitable apparatus. Check for leaching metals if apparatus consists of metal parts.

Dry ashing.—In crucible, weigh convenient amount of sample (10–20 g), to nearest 0.01 g, depending on kind of sample. Dry in drying oven, on water-bath, or on hot plate at 100°C,

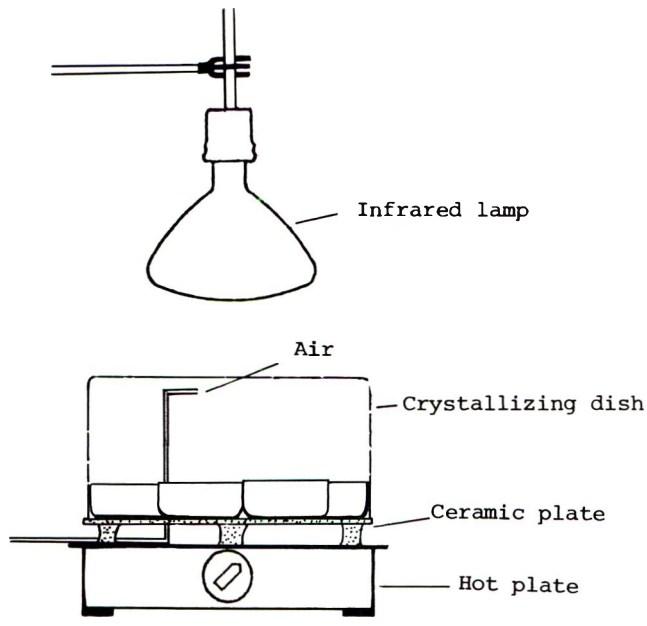

Figure 1. Apparatus for pre-ashing of samples.

Table 1. Instrumental parameters for flame determination

Element	Flame	Wavelength, nm
Fe	Nitrous oxide/acetylene, oxidizing	248.3
Cu	Air/acetylene, oxidizing	324.7
Zn	Air/acetylene, oxidizing	213.9

if there is risk of heavy boiling in ashing step. Proceed according to type of furnace.

A. Ashing in programmable furnace: Place sample in furnace at initial temperature ≤100°C. Increase temperature to 450°C at ≤50°C/h. Let sample stand overnight. Continue according to C.

B. Ashing in muffle furnace with thermostat (hot plate with ceramic plate and glass cover + IR lamp + washing bottle with H_2SO_4 + furnace). See Figure 1.

Cover sample with glass cover, place crucible on ceramic plate, and let purified air sweep over sample through glass tube. Place IR lamp 35 cm from sample and set hot plate at ca 100°C. Reduce distance as drying proceeds until sample can be assumed to be dry. Lamp should then be right down at cover.

Pre-ash sample by increasing temperature slowly and stepwise with IR lamp and hot plate. Final temperature on ceramic plate should then be ca 300°C. Time required for pre-ashing varies greatly, depending on type of sample. Put crucible in muffle furnace at 200-250°C and slowly raise temperature to 450°C at rate of ≤50°C/h. Let sample stand overnight.

C. Remove crucible from furnace and let it cool. Wet ash with 1–3 mL H_2O, and evaporate on water-bath or hot plate. Return crucible to furnace at ≤200°C and raise temperature stepwise to 450°C. Proceed with ashing at 450°C for ≥1–2 h. Repeat this procedure until sample is completely combusted, i.e., ash should be white/grey or slightly colored. Number of necessary repetitions varies greatly, depending on type of sample. Add 5 mL 6M HCl to crucible, ensuring that all ash comes into contact with acid. Evaporate acid on water-bath or hot plate. Dissolve residue in exact volume (10.0–30.0 mL) of 0.1M HNO_3. Swirl crucible with care so that all ash comes into contact with acid. Cover with watch glass and let sample stand for 1–2 h. Then stir solution in crucible thoroughly with stirring rod and transfer contents to plastic bottle.

Treat blanks similarly. Include 2 blanks per batch of samples.

Atomic Absorption Spectrophotometry

Select method to be used (flame or flameless) according to concentration of metal to be determined. Use flame whenever possible because this technique is less sensitive to interference than flameless AAS. Pb, Cd, Cr, and Ni in foodstuffs generally require graphite furnace AAS for determination. Zn, Cu, and Fe in most foodstuffs can be determined by flame AAS. Consult manual provided with instrument for wavelength, gas mixture/temperature program, and other instrumental parameters that are most appropriate for each metal. Always use background correction, and also when Cr is determined, unless proved to be unnecessary. When necessary, dilute sample solutions with 0.1M HNO_3.

Flame technique.—Derive metal contents of samples from calibration curves prepared from ≥3 standards. Table 1 exemplifies instrumental parameters for flame AAS.

Graphite furnace (flameless) technique.—Always use method of addition unless proved to be unnecessary. It is of utmost importance to make measurements in linear range when method of addition is used. Make measurements on peak area rather than peak height if possible. Table 2 shows examples of instrumental parameters applicable to Perkin-Elmer/HGA 500 instruments.

Table 2. Instrumental parameters for graphite furnace AAS

		Temperature program					
Element	Wavelength	Step 1	Step 2	Step 3	Step 4	Sample volume, µL	Type of graphite tube
Pb	283.3	Temperature, 130°	650	1900	2500	20	L'vov
		Ramp, 10 s	5	0	2		
		Hold, 30 s	10	2	2		
Cd	228.8	Temperature, 130°C	350	1200	2500	10	Uncoated
		Ramp, 1 s	5	0	2		
		Hold, 19 s	10	2	2		
Cr	357.9	Temperature, 130°C	1200	2300	2700	20	Pyrolytic-coated
		Ramp, 1 s	10	0	2		
		Hold, 19 s	10	2	3		
Ni	232.0	Temperature, 130°C	1200	2500	2700	20	Pyrolytic-coated
		Ramp, 1 s	5	0	2		
		Hold, 19 s	20	2	2		

Table 3. Reference analyses of samples 6 and 7, simulated diets

Metal	No. of labs	Methods of analysis[a]
Pb	12	AAS, ASV, ICP-MS
Cd	12	AAS, ASV, ICP-MS
Zn	8	AAS, ICP-MS, ICP, DCP, NAA
Cu	9	AAS, ICP-MS, ICP, DCP, NAA
Fe	7	AAS, ICP, DCP, NAA, UV-VIS
Cr	5	AAS, DCP
Ni	5	AAS, DCP

[a] AAS = Atomic Absorption Spectroscopy. ASV = Anodic Stripping Voltammetry. ICP = Inductively Coupled Plasma emission spectroscopy. ICP-MS = Inductively Coupled Plasma-Mass Spectrometry. DCP = Direct Current Plasma emission spectroscopy. NAA = Neutron Activation Analysis. UV-VIS= UV-VIS-Spectrophotometry.

Calculations and Evaluation of Results

Detection limit.—Calculate detection limit, D1, for each metal as follows:

$$D1 = 3 \times \text{standard deviations of mean of blank determinations. } (n = \geq 20)$$

Calculate concentration, K, of metal in sample according to formula:

$$K = \frac{(a - b) \times V}{m}$$

where K = concentration in sample (mg/kg); a = concentration in sample solutions (mg/L); b = mean concentration in blank solutions (mg/L); V = volume of sample solution (mL); m = weight of sample (g). If (a − b) is lower than detection limit, D1, substitute D1 for (a − b) to calculate limit of detection in sample.

If sample solution has been diluted, dilution factor must be taken into account. When running replicates, give average of results to 2 significant figures.

Interlaboratory Study

Test Materials

Test materials 1–5 were produced in Denmark under the guidance of the official adviser who was previously responsible.

Element added, in mg/kg

1. Liver paste, packed in 100 mL Al cans
2. Apple sauce, packed in 100 mL Al cans: Pb, 0.2; Ni, 0.2
3. Minced fish, packed in 100 mL Al cans: Pb, 0.5; Cd, 0.2; Cr, 0.2
4. Wheat bran, packed in 250 mL plastic bottles
5. Milk powder, packed in 100 mL plastic bottles

6 and 7. Composite diets D and E, packed in 50 mL plastic bottles.

The levels of Pb and Ni in apple sauce and of Pb, Cd, and Cr in minced fish were fortified as shown above to extend the range of these elements. The concentrations of the different elements ranged between 0.025 and 0.5 mg/kg for Pb, 0.001 and 0.6 mg/kg for Cd, 0.7 and 55 mg/kg for Zn, 0.2 and 45 mg/kg for Cu, 2 and 235 mg/kg for Fe, 0.01 and 0.2 mg/kg for Cr, and 0.01 and 0.3 mg/kg for Ni. These ranges cover the natural levels found in most foods.

Test materials 6 and 7, composite diets, consisted of different proportions of a number of foodstuffs, e.g., meat, liver, potatoes, milk, and flour. These 2 diets were originally produced as reference samples for another project (10, 11) and have previously been analyzed by a number of laboratories to establish reference values. Each element had been determined by at least 2 different techniques (see Table 3). To deduce the contribution of the AAS determination to the total analytical error, before the study, the participating laboratories were given 4 samples of aqueous solutions to determine the metals directly by AAS: 2 mixed standard solutions containing Pb, Cd, Cu, Fe, and Cr at 2 different levels and 2 solutions of dry-ashed pork and pig liver. The concentrations of the standards and the results of the determination are shown in Table 20 and Figures 2 and 3.

Homogeneity of the Test Materials

The within- and between-container variation was determined by 2-way analysis of variance (ANOVA) of duplicate determinations of 10 randomly selected containers from each type of sample. The results are presented in Table 4. The statistical test of homogeneity was based on a comparison between (i) the variation between determinations made within the containers pooled over all containers analyzed (error of method), and (ii) the variation between containers (error of method + inhomogeneity). These 2 variations will be equal if no inhomogeneity is present. Random variations, however, are generated that will sometimes cause the ratio (ii) divided by (i) to deviate from 1, even if no inhomogeneity is present. Hence, only large values for this ratio can indicate inhomogeneity. The F-distribution is used to compute p-values.

Normally p-values >0.05 are interpreted as if no inhomogeneity is indicated, whereas p-values <0.05 are normally interpreted as if inhomogeneity is present. However, in this latter case, there is a risk equal to the p-value of drawing the wrong conclusion because the p-value only gives the probability that random effects alone are the cause of the results. This means that the risk for a randomly caused statistical significance increases if many tests are performed at p-level 0.05. Forty-nine tests were performed at this level (Table 4), and, consequently, 2–3 random significant inhomogeneities could be expected. Inhomogeneity can still be present if it is evenly distributed between as well as within containers, which would result in a p-value >0.05. To some extent, this can be identified by high RSDs. "Normal" or low RSDs for which the p-value is <0.05 indicate that the inhomogeneity is probably insignificant, although the contrary is indicated by the p-value. The iron concentration in sample 2 was judged as too inhomogeneous to be analyzed in the collaborative trial.

From Table 4, it can be seen that in general the higher the CV%, the lower the concentration of the metal. Consequently, the highest concentrations generally had the lowest CV%, which is in accordance with expectations when the levels are close to the detection limits.

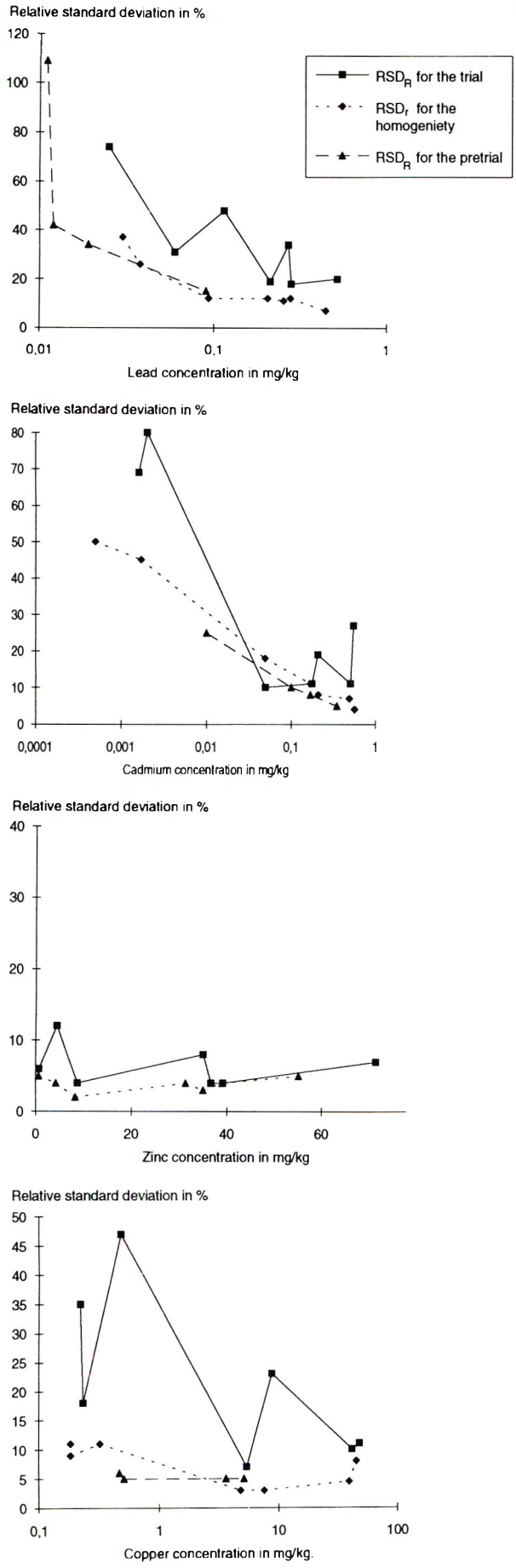

Figure 2. The relative standard deviations at different concentration levels of Pb, Cd, Zn, and Cu.

Comments

Very few comments on, or deviations from, the method were noted on the reply forms:

Laboratory 5 had taken 5–10 g sample and, after ashing, diluted it to 100 mL. The large volume results in a strongly reduced detection limit.

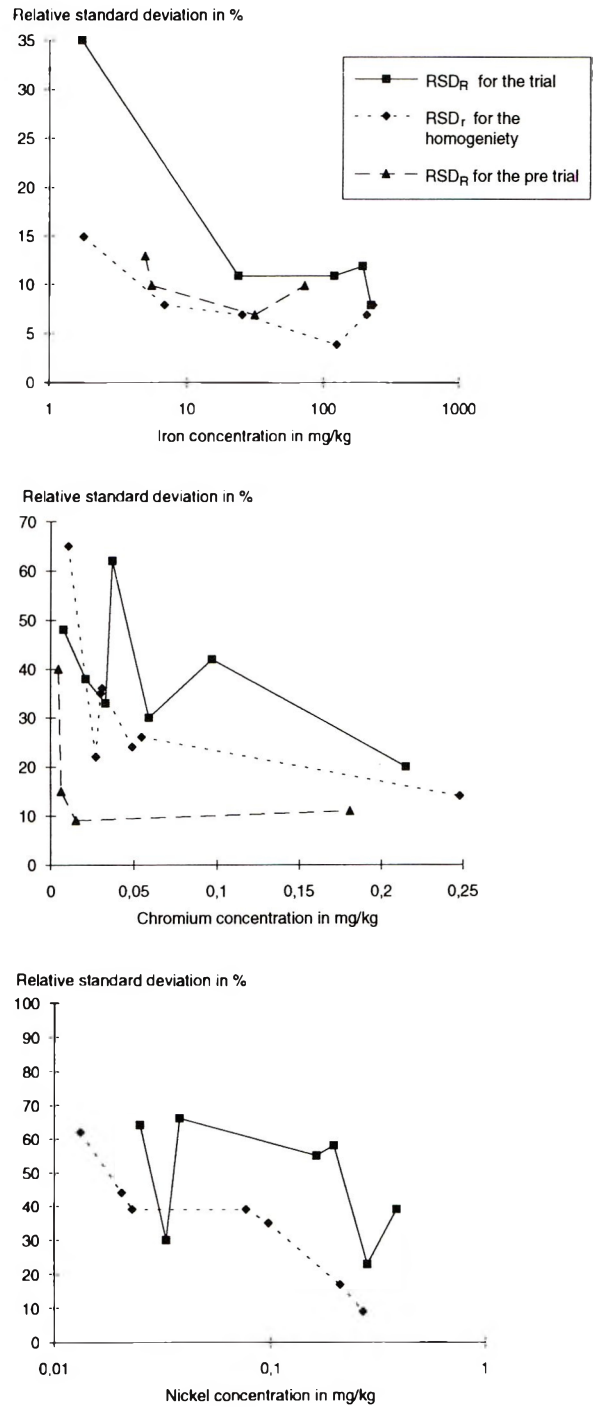

Figure 3. The relative standard deviations at different concentration levels of Fe, Cr, and Ni.

Table 4. Results (mg/kg) of the homogeneity study; means, *p*-value, and the relative standard deviation (RSD)

		Sample No.						
		1	2	3	4	5	6	7
Metal	Results	Liver paste	Apple sauce	Minced fish	Wheat bran	Milk powder	Diet 1	Diet 2
Pb	Mean	0.0375	0.278	0.444	0.0930	0.0298	0.204	0.253
	p	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
	RSD	26	12	6.8	12	37	12	11
Cd	Mean	0.0494	0.0017	0.210	0.170	0.0005	0.492	0.566
	p	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05
	RSD	18	45	8.1	11	50	6.6	4.0
Zn	Mean	8.29	0.639	4.22	55.1	31.3	35	39
	p	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
	RSD	2.3	5.3	3.7	5.3	3.8	2.9	4.1
Cu	Mean	4.78	0.184	0.184	7.53	0.320	39	45
	p	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01
	RSD	3.0	9.2	11	2.9	11	4.5	8.0
Fe	Mean	25.7	23.2	6.96	127	1.80	212	235
	p	<0.05	<0.01	>0.05	>0.05	>0.05	<0.05	<0.01
	RSD	6.6	13	7.7	4.4	15	7.1	8.5
Cr	Mean	0.0271	0.0546	0.248	0.0309	0.0106	0.030	0.049
	p	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	RSD	22	26	14	36	65	35	24
Ni	Mean	0.0206	0.211	0.0230	0.269	0.0132	0.098	0.077
	p	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	RSD	44	17	39	9.3	62	35	39

Laboratory 17 added 0.3 mL concentrated HNO_3 to the samples before the last round of ashing. According to the author's experience, this has no effect on the results.

Laboratory 20 cleaned the utensils with 3*N* HCl-EDTA.

Laboratory 25 claimed that sample 7 was not in the package.

The participants were instructed to report the results to 3 significant digits (i.e., 0.0111, 1.11, or 111). There were, however, a considerable number of deviations from this instruction in the replies. The results are presented in the tables as received.

Elimination of Outlying Laboratories/Results

The Kruskal-Wallis k-sample test was used to eliminate laboratories with extreme deviation. Outlying results were eliminated according to the 2-tailed method of Filliben (12), rather than with the use of Grubb's test, which is only 1-tailed. Laboratory 15 was eliminated by the Kruskal-Wallis test for all metals. Its results are, therefore, not included in the tables.

Results

Results were received from 16 of the original 17 participating laboratories. Not all of the laboratories determined all of the metals. All determinations were made as single determinations. Some results for Pb and Cd at very low concentrations were negative. This is usually a result of overcompensated background correction, but it can also be a result of random error at levels around or below the detection limit. All negative results were, however, eliminated as outliers according to the above mentioned tests.

Samples with nearby or split levels were used to calculate the repeatability (within-laboratory variation) and reproducibility (within- and between-laboratory variation) with the aid of variance component analysis. Samples 6 and 7, composite diets, had similar but not identical compositions and metal levels, and were, therefore, ideally suited for this purpose. In those instances in which samples had a unique level, only reproducibility was calculated.

The contamination level was controlled by blank determinations. The participants were instructed to carry out at least 5 blanks/metal. The mean value of the blanks was deducted from the readings before the results were calculated.

The grand mean of the blank determinations was decided for each metal on the basis of results only from laboratories that reported results acceptable according to the statistical evaluation. Limits of detection were calculated as 3 times the standard deviation of the mean of these blanks.

Table 5. Collaborative results (mg/kg) for lead in foods

	Sample No.						
Coll.	1	2	3	4	5	6	7
1	0.030	0.431	0.375	0.0403	—	0.153	0.248
2	0.10	0.41	0.68	0.045	0.006	0.25	0.063[a]
3	0.0698	0.113	0.503	0.0903	0.0163	0.236	0.270
4	0.0566	0.300	0.551	0.154	0.0254	0.261	0.289
5	0.0502	0.308	0.570	0.245	0.030	0.275	0.300
6	0.060	0.187	0.435	0.0740	0.038	0.150	0.267
7	0.078	0.257	0.414	0.150	0.243[a]	0.223	—
8	0.0453	0.296	0.570	0.102	0.0091	0.195	0.247
9	0.0707	0.158	0.539	0.0947	0.0579	0.241	0.388
10	0.0431	0.262	0.564	0.155	<0.0001[a]	0.214	0.277
12	0.046	0.24	0.70	0.104	<0.003	0.21	0.34
14	0.070	0.262	0.459	0.096	–0.007[a]	0.223	0.248
16	0.051	—	0.381	0.132	0.040	0.171	0.203
Mean	0.0593	0.269	0.518	0.114	0.0249	0.212	0.280
s_R	0.0183	0.092	0.104	0.054	0.0185	0.039	0.050
RSD_R	30.9	34.4	20.0	47.6	74.2	18.6	17.9
No. labs. after elimination of outliers	13	12	13	13	9	13	11
Ref. V						0.225	0.272
SD for Ref. V						0.023	0.043

[a] Outlying results according to Filliben.

The method used to determine the significance of the difference between the trial results and the reference values for samples 6 and 7 was based on a 2-sample *t*-test.

Lead

Fourteen of the participating laboratories performed Pb determinations. All of the determinations were made with background correction. Most of the analyses were carried out by GFAAS. Flame AAS was used by laboratory 6 for sample 3, by laboratory 7 for all samples, by laboratory 10 for samples 2, 3, 6, and 7, and by laboratory 11 for sample 2. Matrix modification was used by laboratories 2, 3, 6, 14, and 16. Laboratory 1 reported the level in sample 5 to be not detectable. Laboratory 11 reported problems with the background correction during the measurements. Laboratory 16 claimed that it had probably missed a dilution factor when calculating the result of sample 2. These results were, therefore, not included in the evaluation.

Results reported as below a laboratorys detection limit (<-values) are assumed in the calculations to be half of that detection limit.

The mean of the blank determinations was 0.0019 mg/L, range 0.0004–0.0055 mg/L. The limit of detection was calculated to be 0.0021 mg/L of sample solution, with a range of 0.0003–0.0047 mg/L. This corresponds to an average limit of detection in the actual sample of 0.0063 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Lead Results.—The reproducibility relative standard deviation (RSD_R) values for the various concentration levels (see Table 5 and Figure 2) show that the relative variation is level-dependent and decreases from 74 to 20% with increasing Pb levels. The RSD_R of the results from the pre-trial with ready-made solutions and the repeatability relative standard deviation (RSD_r) for the homogeneity test were approximately half of the RSD_R of the actual trial results (Figure 2). The homogeneity test resulted in an RSD_r similar to the results of the pre-trial.

For the samples with split levels, the repeatability (s_r) and the reproducibility (s_R) were the same (Table 6), which indicates that the variation within the concentration interval is caused mainly within the laboratory.

The results of samples 6 and 7 were not significantly different ($p>0.05$) from the reference values.

The results obtained by flame AAS were not significantly different from those obtained by GFAAS, which indicates that at the concentrations at which both techniques were used they yield basically the same result.

Table 6. Statistical results for lead (mg/kg) in samples with nearby or split levels

	Sample combinations	
Variable	1;5	2;6;7
Mean	0.0452	0.253
s_r	0.0183	0.0649
s_R	0.0183	0.0649
RSD_r	40.5	25.7
RSD_R	40.5	25.7

Table 7. Collaborative results (mg/kg) for cadmium in foods

	Sample No.						
Coll.	1	2	3	4	5	6	7
1[a]	0.038	0.000	0.130	0.106	–0.006	0.364	0.397
2	0.058	0.002	0.23	0.16	0.033[b]	0.50	0.22
3	0.0429	0.00287	0.213	0.184	–0.0017[b]	0.547	0.642
4	0.0451	0.0130[b]	0.213	0.170	0.0003	0.473	0.602
5	0.054	0.0007	0.212	0.173	0.0007	0.557	0.583
6	0.050	0.002	0.211	0.204	0.002	0.573	0.708
7	0.045	<0.003	0.159	0.135	0.032[b]	0.439	—
8	0.0417	0.0012	0.273	0.174	0.0000[b]	0.461	0.571
9	0.0507	0.00010	0.282	0.0744[b]	0.00142	0.614	0.771
10[a]	0.0631	0.002	0.245	0.394	0.231	0.515	0.610
11	0.0509	0.00300	0.160	0.184	0.00319	0.450	0.473
12	0.054	0.003	0.214	0.201	0.003	0.506	0.582
14	0.053	0.0010	0.182	0.168	0.0007	0.462	0.464
16	0.049	0.000	0.162	0.195	0.005	0.493	0.437
Mean	0.0495	0.0016	0.209	0.177	0.0020	0.506	0.550
s_R	0.0050	0.0011	0.040	0.020	0.0016	0.055	0.149
RSD_R	10.1	68.8	19.2	11.1	80.0	10.9	27.1
No. labs. after elimination of outliers	12	11	12	11	3	12	11
Ref. V						0.467	0.537
SD for Ref. V						0.052	0.059

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.
[b] Outlying results according to Filliben.

Cadmium

Fifteen of the participating laboratories performed Cd determinations, all with background correction. The major part of the determinations were made by GFAAS. Flame AAS was used by laboratory 6 for samples 3, 6, and 7, by laboratory 8 for all samples, by laboratory 10 for all samples, by laboratory 11 for samples 1, 3, 4, 6, and 7, and by laboratory 12 for samples 3, 4, 6, and 7. Matrix modification was used by laboratories 3, 6 (sample 5), 14, and 16. Laboratories 1 and 10 were eliminated as outliers.

The mean of the blank determinations was 0.0004 mg/L, range 0.00001–0.0022 mg/L. The limit of detection was calculated to be 0.0013 mg/L of sample solution with a range of 0.00003–0.0088 mg/L. This corresponds to an average limit of detection in the actual sample of 0.0039 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Cadmium Results.—The RSD_R values for the different concentration levels are level-dependent (see Table 7 and Figure 2) and decrease from approximately 70 to 11% with increasing concentration.

The RSD_R for the pre-trial solutions and the RSD_r for the homogeneity gave similar results except at the lowest concentrations.

In Table 8, for the samples with split levels, it can be seen that the RSD_r decreases more than the RSD_R as the concentration increases.

The results for samples 6 and 7 are not significantly different ($p>0.05$) from the reference values.

The results obtained by flame AAS were not significantly different from those obtained by GFAAS, which indicates that at the concentrations at which both techniques were used, they yield basically the same result.

Zinc

Fifteen of the participating laboratories performed Zn determinations. Laboratories 1, 13, and 14 did not use background correction. All determinations were made by flame AAS. Laboratories 9 and 10 were eliminated as outliers.

Table 8. Statistical results for cadmium (mg/kg) in samples with nearby or split levels

	Sample combinations		
Variable	2;5	3;4	6;7
Mean	0.0013	0.193	0.528
s_r	0.0013	0.0268	0.0876
s_R	0.0013	0.0337	0.111
RSD_r	72.0	13.9	16.6
RSD_R	72.0	17.5	21.0

Table 9. Collaborative results (mg/kg) for zinc in foods

	Sample No.						
Coll.	1	2	3	4	5	6	7
1	8.96	1.92[b]	4.25	69.5	32.1	37.3	39.0
2	12.2[b]	1.04[b]	5.60	69.3	35.8	36.0	38.0
3	8.31	0.679	3.29	75.6	28.0	36.1	39.9
4	9.08	1.15[b]	4.95	66.9	35.0	37.1	38.8
5	8.85	0.73	4.65	69.6	35.4	37.1	39.6
6	8.71	0.719	4.47	82.1	38.3	35.5	37.4
7	9.06	0.726	4.06	72.0	36.9	36.2	—
8	7.98	0.647	4.23	70.4	33.6	35.6	38.1
9[a]	10.4	0.773	4.79	35.2	4.43	41.7	24.1
10[a]	8.70	0.651	4.36	5.75	2.93	24.2	37.3
11	8.82	0.752	4.60	70.2	36.8	36.4	38.1
13	8.47	0.635	4.23	63.6	34.3	34.6	38.1
14	9.09	0.72	4.74	77.0	37.9	37.4	41.9
16	9.05	0.655	4.27	72.0	35.3	39.0	41.1
Mean	8.76	0.696	4.45	71.5	35.0	36.6	39.1
s_R	0.37	0.042	0.55	4.9	2.8	1.3	1.4
RSD_R	4.22	6.0	12.4	6.9	8.0	3.6	3.6
No. labs. after elimination of outliers	11	9	12	12	12	12	11
Ref. V						37.2	39.8
SD for Ref. V						4.0	4.2

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.
[b] Outlying results according to Filliben.

The mean of the blank determinations was 0.017 mg/L, range 0.0032–0.030 mg/L. The limit of detection was calculated to be 0.019 mg/L of sample solution, with a range of 0.000–0.039 mg/L. This corresponds to an average limit of detection in the actual sample of 0.057 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Zinc Results.—The RSD_R values for the different concentrations show no level dependence within the interval. The RSD_R for the trial results are generally slightly higher than the RSD_r for the homogeneity of the samples. See Table 9 and Figure 2.

For samples with split levels (Table 10), the s_r and the s_R were almost identical, indicating that the variation is caused mainly within the laboratories. The results of samples 6 and 7 are not significantly different ($p>0.05$) from the reference values.

The results obtained without background correction are not systematically higher or significantly different from those obtained with background correction.

Copper

Fifteen of the participating laboratories performed Cu determinations. Laboratories 6, 11, 13, and 14 did not use background correction. Laboratory 1 did not submit the information. All of the laboratories used flame AAS. Laboratory 1 was eliminated as outlier. The mean of the blank determinations was 0.015 mg/L, range 0.000–0.060 mg/L. The limit of detection was calculated to be 0.036 mg/L, range 0.000–0.072 mg/L of sample solution. This corresponds to an average detection limit in the actual sample of 0.108 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Copper Results.—The RSD_R values for the different concentrations (see Table 11 and Figure 2) show a level dependence. The variations for at least 2 of the samples are larger than was expected within the interval. The main reason for this is that laboratories 2, 3, and 9 produced some results that could be suspected of being outliers but that happened to fit the normal distribution. The RSD_R for the pre-trial solutions is on the same level as the RSD_r for the homogeneity.

For samples with split levels (Table 12), the difference between s_r and s_R increases with increased concentrations in the samples. This shows that the between-laboratory variation becomes more important as the concentration increases within

Table 10. Statistical results for zinc (mg/kg) in samples with nearby or split levels

	Sample combinations	
Variable	1;3	5;6;7
Mean	6.60	36.9
s_r	0.283	1.88
s_R	0.283	1.96
RSD_r	4.3	5.1
RSD_R	4.3	5.3

Table 11. Collaborative results (mg/kg) for copper in foods

	Sample No.						
Coll.	1	2	3	4	5	6	7
1[a]	5.12	0.0958	0.400	10.1	0.803	40.8	44.0
2	5.25	0.27	0.25	5.53	0.36	30.5	34.5
3	3.47[b]	0.245	0.0313	10.2	0.0857	40.8	48.6
4	4.97	0.193	0.179	8.26	0.542	38.9	45.8
5	5.85	0.27	0.24	10.2	0.39	46.7	52.8
6	5.40	0.248	0.291	10.7	0.620	40.6	46.3
7	4.93	0.257	0.218	7.65	0.710	41.3	—
8	4.80	0.255	0.182	8.50	0.316	42.1	47.7
9	5.63	0.01[b]	0.159	4.06	0.130	45.8	56.0
10	6.09	0.285	0.316	9.98	0.564	41.0	49.2
11	5.27	0.241	0.192	9.38	0.705	46.9	48.4
13	5.21	0.231	0.247	9.13	0.801	39.0	46.1
14	5.52	0.17	0.30	9.41	0.69	40.6	46.7
16	5.43	0.143	0.286	10.7	0.446	43.8	51.8
Mean	5.36	0.234	0.222	8.75	0.482	41.4	47.8
s_R	0.38	0.043	0.077	2.00	0.225	4.2	5.2
RSD_R	7.1	18.4	34.6	22.9	46.7	10.1	10.9
No. labs. after elimination of outliers	12	12	13	13	13	13	12
Ref. V						40.1	45.6
SD for Ref. V						1.5	2.5

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.
[b] Outlying results according to Filliben.

the interval measured. The results of samples 6 and 7 are not significantly different ($p>0.05$) from the reference values.

The results obtained without background correction are not systematically higher or significantly different from those obtained with background correction.

Iron

Fourteen of the participating laboratories performed Fe determinations. Background correction was not used by laboratories 13 and 14. All laboratories used flame AAS. Laboratory 11 was eliminated as an outlier.

The mean blank level was 0.108 mg/L, range 0.010–0.590 mg/L. The limit of detection was calculated to be 0.267 mg/L, range 0.000–2.00 mg/L of sample solution. This corresponds to an average detection limit in the actual sample of 0.800 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Iron Results.—The RSD_R for the different concentrations (Table 13 and Figure 3) show a level dependence at the lowest concentrations. The RSD_R for the pre-trial solutions and the RSD_r for the homogeneity of the samples are on the same level as the trial results.

For samples with split levels (Table 14), the s_r and s_R were identical for sample combination 3:5, indicating that the variation at this concentration is mainly caused within the laboratories. In combination 6:7, s_r is lower than s_R, which indicates that the within-laboratory variation becomes less significant at higher concentrations.

The results of samples 6 and 7 are not significantly different ($p>0.05$) from the reference values.

The results obtained without background correction are not systematically higher or significantly different from those obtained with background correction.

Chromium

Ten of the participating laboratories performed Cr determinations. The participants were instructed to use background correction (BC) if possible. Laboratories 6 and 11 analyzed sample 3 by flame AAS. All other determinations were made by GFAAS. Laboratory 14 used matrix modification.

Four laboratories did not use BC. As can be seen in Table 15, the results for samples 1–3 are all of similar magnitude. For samples 4–7, however, the importance of BC at low con-

Table 12. Statistical results for copper (mg/kg) in samples with nearby or split levels

	Sample combinations		
Variable	2;3;5	1;4	6;7
Mean	0.315	7.05	44.6
s_r	0.126	1.42	1.57
s_R	0.137	1.54	4.73
RSD_r	40.0	20.1	3.5
RSD_R	43.5	21.8	10.6

Table 13. Collaborative results (mg/kg) for iron in foods

Coll.	Sample No. 1	3	4	5	6	7
1	23.7	6.60	140	2.61	204	219
2	25.8	6.3	129	1.9	211	202
3	18.9	7.12	133	1.19	146	223
4	25.1	6.59	111	2.25	192	208
5	27.3	5.99	118	1.57	240	261
6	24.6	6.48	127	1.98	205	250
7	28.2	4.15[b]	98.5	2.42	176	—
8	23.3	5.52	136	0.877	207	253
9	20.1	6.17	56.6[b]	0.516	201	215
10	22.2	5.81	105	1.64	202	216
11[a]	27.6	12.6	144	2.40	242	242
13	24.3	6.47	114	2.25	195	214
14	26.6	10.3[b]	123	1.57	220	246
16	22.3	6.08	136	1.85	166	232
Mean	24.0	6.28	122	1.74	197	228
s_R	2.7	0.44	13	0.61	24	20
RSD_R	11.2	7.0	10.7	35.1	12.2	8.8
No. labs. after elimination of outliers	13	11	12	13	13	12
Ref. V					193	212
SD for Ref. V					16	19

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.
[b] Outlying results according to Filliben.

centrations can be seen. The results produced with BC are compiled in Table 16. Laboratory 10 was eliminated as an outlier.

The mean of the blank determinations was 0.0034 mg/L, range 0.0004–0.012 mg/L. The limit of detection was calculated to be 0.012 mg/L, range 0.0004–0.052 mg/L of sample solution. This corresponds to an average detection limit in the actual sample of 0.035 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL. One of the participants had a very large variation in the blanks, which resulted in the rather high detection limit. Omitting these blanks, the detection limit comes to 0.009 mg/kg.

Comments on the Chromium Results.—The RSD_R values for the different concentrations (see Table 16 and Figure 3) show a certain level dependence. The RSD_r for the homogeneity is of the same magnitude, whereas the RSD_R for the pre-trial solutions is on a lower level. It can be assumed that the variation is caused by contamination in the ashing procedure.

Table 14. Statistical results for iron (mg/kg) in samples with nearby or split levels

Variable	Sample combinations 3;5	6;7
Mean	3.82	212.2
s_r	0.440	17.4
s_R	0.440	22.6
RSD_r	11.5	8.2
RSD_R	11.5	10.7

For samples with split levels (Table 17), the s_r and s_R were almost identical, indicating that the variation is caused within the laboratory.

The results of samples 6 and 7 are not significantly different ($p>0.05$) from the reference values. The reference value, however, is based on relatively few results (see Table 3).

Nickel

Nine of the participating laboratories performed Ni determinations. All laboratories used BC. Laboratory 6 analyzed sample 2 by flame AAS. All other analyses were made by GFAAS.

The mean of the blank determinations was 0.0079 mg/L, range 0.0001–0.030 mg/L. The limit of detection was calculated to be 0.0088 mg/L, range 0.0006–0.041 mg/L. This corresponds to an average detection limit in the actual sample of 0.026 mg/kg, assuming a sample weight of 10 g and dilution to 30 mL.

Comments on the Nickel Results.—The RSD_R values for the different concentrations (see Table 18 and Figure 3) show a level dependence. As was the case for Cr, it can be assumed that contamination during the ashing procedure is the reason for this variation.

Table 15. Results for Cr in foods from all collaborators, with and without background correction

Coll.	Sample No. 1	2	3	4	5	6	7	BC
1	0.0221	0.0852	0.236	0.0534	0.0683	0.114	0.0500	N
3	0.0677	0.0820	0.263	0.0299	0.0127	0.0486	0.0716	Y
4	0.0192	0.0800	0.225	0.0284	0.0038	0.0251	0.0442	Y
6	0.061	0.086	0.227	0.148	0.091	0.123	0.125	N
8	0.0214	0.0527	0.186	0.0204	0.0056	0.0357	0.0529	Y
9	0.0562	0.109	0.156	0.0115	0.0106	0.0188	0.0827	Y
10[a]	0.147	0.187	0.990	0.105	0.0491	0.167	0.795	Y
11	0.0470	0.0540	0.244	0.900	0.206	0.274	0.364	N
14	0.022	0.162	0.246	0.016	0.006	0.035	0.043	Y
16	0.055	0.138	0.196	0.116	0.042	0.122	0.211	N
Mean	0.041	0.094	0.220	0.147	0.050	0.088	0.116	
s_R	0.020	0.036	0.034	0.286	0.067	0.082	0.108	
RSD_R	48	38	16	195	134	92	93	

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.

For the samples with split levels (Table 19), the s_r and s_R are similar for the 2 lowest concentrations. At the higher concentration, the s_r is lower than the s_R.

The results for sample 6 are significantly higher ($p>0.05$) than the reference value. The reference value, however, is based on relatively few results.The single result obtained by flame analysis for sample 2 was lower than the results obtained by graphite furnace analysis, but not so low as to be classified as an outlier.

Discussion

The results of the pre-trial with ready-made solutions (Table 20) gave several indications:

(1) At similar concentration levels in the standard solutions and the sample solutions, there was no significant difference in the variance for Cd, Cu, Fe, and Cr. At the lowest concentration (0.0106 ± 0.0012 mg/L of standard solution and 0.008 ± 0.008 mg/L of sample solution), the variance for Pb was significantly higher (variance ratio 48) in the sample solution, which indicates that the higher mineral content in the sample solution causes greater variance in the result. This is due to several factors, among which is the correction process for the background absorption.

(2) When the concentrations of the sample solutions of pork and liver were multiplied by a factor, 2.5, to give the approximate concentration in the actual samples and then compared with similar concentrations in samples from the

Table 16. Collaborative results (mg/kg) for chromium in foods after elimination of laboratories not using background correction

Coll.	Sample No. 1	2	3	4	5	6	7
3	0.0677	0.0820	0.263	0.0299	0.0127	0.0486	0.0716
4	0.0192	0.0800	0.225	0.0284	0.0038	0.0251	0.0442
8	0.0214	0.0527	0.186	0.0204	0.0056	0.0357	0.0529
9	0.0562	0.109	0.156	0.0115	0.0106	0.0188	0.0827
10[a]	0.147	0.187	0.990	0.105	0.0491	0.167	0.795
14	0.022	0.162	0.246	0.016	0.006	0.035	0.043
Mean	0.037	0.097	0.215	0.021	0.0077	0.033	0.059
s_R	0.023	0.041	0.044	0.008	0.0037	0.011	0.018
RSD_R	62.2	42.3	20.5	38.1	48.1	33.3	30.5
No. labs. after elimination of outliers	5	5	5	5	5	5	5
Ref. V						0.049	0.063
SD for Ref. V						0.027	0.023

[a] Laboratory eliminated by the Kruskal-Wallis k-sample test.

Table 17. Statistical results for chromium (mg/kg) in samples with nearby or split levels

Variable	Sample combinations		
	4;5	1;6	2;7
Mean	0.0145	0.0350	0.0780
s_r	0.0062	0.0160	0.0333
s_R	0.0062	0.0181	0.0333
RSD_r	43	46	43
RSD_R	43	52	43

Table 19. Statistical results for nickel (mg/kg) in samples with nearby or split levels

Variable	Sample combinations		
	1;3;5	6;7	2;4
Mean	0.0313	0.180	0.33
s_r	0.0173	0.0944	0.0849
s_R	0.0173	0.1033	0.116
RSD_r	55.3	52.5	25.3
RSD_R	55.3	57.5	34.6

trial, the variance of Cu, and to a varying degree Cd, Fe, and Cr, was significantly higher for the trial samples than the pre-trial samples, as shown in Table 21. It may then be assumed that the ashing procedure contributes to the total variance for these metals. Lead, being notoriously difficult to determine, apparently receives the major part of the variance, at least at very low concentrations, from the AAS-determination. It must, however, be emphasized that this pre-trial was of a limited nature and that the conclusions, therefore, are only indicative.

If the instrumental parameters are well optimized, there is no reason why flame and graphite furnace analyses should not give similar results if the concentrations are at a sufficient level. This is shown to be the case by the results for Pb and Cd, for which both techniques were used with no significant increase in s_R. The importance of background correction at low concentrations is demonstrated by the chromium results. At higher concentrations, as for Zn, Cu, and Fe, results that are not systematically too high can be obtained without background correction. This must, of course, be verified in each individual case.

With the exception of Pb and Cr, the difference between the s_r and the s_R for the split-level sample combinations increased with increasing concentrations. For Pb, Cd, Zn, Cu, Fe, and Cr, the results for samples 6 and 7 showed very good agreement with the reference values. It can, therefore, be assumed that no loss of these metals occurred during the dry ashing. For Ni, the s_R was very large (presumably due to contamination) but overlapping the range for the reference value. It would have been desirable to have an extended range of Ni in the trial; however, the results show conclusively that Ni determinations at low concentrations are difficult.

The RSD_Rs for all the metals determined agreed reasonably well with what Horwitz et al. (13) showed to be generally expected at different concentrations. When the RSD_R ratios (HORRAT) of the RSD_R found/RSD_R predicted (14) are calculated, ratios between 0.5 and 2 indicate acceptable precision of the method according to the International Union of Pure and Applied Chemistry (15). The mean ratios for the different metals were: Pb 1.5 (range 0.9–2.7), Cd 1.1 (0.4–2.0), Zn 0.6 (0.4–

Table 18. Collaborative results (mg/kg) for nickel in foods

Coll.	Sample No.						
	1	2	3	4	5	6	7
1	0.0295	0.239	0.0149	0.323	0.0803	0.0919	0.0900
3	0.0422	0.352	0.0291	0.590	0.0339	0.139	0.220
4	0.021	0.271	0.0158	0.276	0.0295	0.0622	0.123
6	0.026	0.170	0.007	0.267	0.044	0.418	0.082
8	0.0263	0.212	0.0209	0.330	0.0374	0.136	0.0614
9	−0.009[a]	0.306	0.0344	0.153	0.724[a]	0.313	0.222
10	0.0367	0.301	0.0319	0.501	0.0027	0.266	0.306
14	0.049	0.377	0.016	0.528	−0.005[a]	0.171	0.096
16	0.115[a]	0.316	0.059	0.517	0.232[a]	0.173	0.266
Mean	0.033	0.283	0.025	0.387	0.038	0.197	0.163
s_R	0.010	0.066	0.016	0.150	0.025	0.114	0.090
RSD_R	30.3	23.3	64.0	38.8	66.2	57.9	55.2
No. labs. after elimination of outliers	7	9	9	9	6	9	9
Ref. V						0.095	0.106
SD for Ref. V						0.011	0.026

[a] Outlying results according to Filliben.

Table 20. Results of the analysis of aqueous solutions analyzed directly by AAS, prior to the collaborative trial (mg/L)

Sample	Metal	Expected level	Measured level	s_R	n
Std soln 1	Pb	0.010	0.011	0.001	11
	Cd	0.100	0.102	0.013	11
	Cu	0.50	0.51	0.03	13
	Fe	5.0	4.9	0.4	12
	Cr	0.010	0.015	0.001	4
Std soln 2	Pb	0.090	0.094	0.010	10
	Cd	0.005	0.005	0.001	10
	Cu	5.0	5.1	0.3	13
	Fe	75	75	6.4	12
	Cr	0.15	0.18	0.02	3
Soln of ashed pork	Pb	—	0.008	0.008	11
	Cd	—	0.008	0.001	10
	Cu	—	0.47	0.03	13
	Fe	—	5.7	0.46	12
	Cr	—	0.006	0.001	4
Soln of ashed pig liver	Pb	—	0.019	0.006	11
	Cd	—	0.080	0.005	10
	Cu	—	3.6	0.19	13
	Fe	—	32	2.1	13
	Cr	—	0.005	0.002	4

1.0), Cu 1.4 (0.6–2.6), Fe 1.4 (0.6–2.4), Cr 1.5 (1.0–2.4), and Ni 2.1 (1.1–2.8).

Conclusions

The results of the interlaboratory trial, at the concentrations tested, correspond to the requirements for reproducibility that, according to Horwitz et al., can be put on a method. The comparison of the trial results for reference samples 6 and 7 with their reference values was generally very good. The method results in low detection limits, making the method suitable for quantitative analysis at low concentrations. The HORRATs show that the precision of the method is acceptable for Pb, Cd, Zn, Cu, Fe, and Cr. The HORRAT for Ni is not as good as for the other metals; there is, however, no collaboratively tried method available today that provides as good or better results for these metals. On the basis of these conclusions, the method must be considered to give acceptable results for the elements determined.

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Table 21. Comparison of results (mg/kg) for samples from the pretrial by AAS and the collaborative trial

Metal	Pretrial samples		Trial samples		Variance ratio
Pb	0.048 ± 0.015	(12)	0.059 ± 0.018	(13)	1.4
	0.020 ± 0.021	(12)	0.025 ± 0.018	(9)	<1
Cd	0.0020 ± 0.0028	(11)	0.0020 ± 0.0016	(8)	<1
	0.200 ± 0.012	(11)	0.177 ± 0.020	(11)	2.8
			0.209 ± 0.040	(12)	11
Cu	9.0 ± 0.48	(13)	8.8 ± 2.0	(13)	17
Fe	14.3 ± 1.2	(13)	6.3 ± 0.4	(11)	<1
			24 ± 3	(13)	5
	80 ± 5	(13)	122 ± 13	(12)	2.6
Cr	0.012 ± 0.005	(4)	0.008 ± 0.004	(5)	<1
	0.015 ± 0.002	(4)	0.021 ± 0.008	(5)	16

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MICROBIOLOGICAL METHODS

Detection of *Salmonella* in Dry Foods Using Refrigerated Pre-Enrichment and Enrichment Broth Cultures: Interlaboratory Study

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An interlaboratory study was performed in 11 laboratories to validate the use of pre-enrichment and tetrathionate brilliant green (TBG_{35}) and selenite cystine (SC_{35}) enrichment cultures refrigerated 72 h at 2–5°C for greater analytical flexibility in the detection of *Salmonella* in dry foods. Productivities of refrigerated pre-enrichment and enrichment cultures were compared with that of the AOAC/*Bacteriological Analytical Manual* (BAM) procedure using 4 food types: whole egg powder, milk chocolate, animal feed, and instantized skim milk powder. Uninoculated and inoculated samples were included in each food group. There was complete agreement between the results obtained by the standard AOAC/BAM procedure and the 2 refrigeration procedures. Of 660 samples tested, the AOAC/BAM procedure identified 393 contaminated samples that were readily detected from the corresponding refrigerated pre-enrichment cultures and from the combined productivity of homologous refrigerated TBG_{35} and SC_{35} cultures. Refrigeration (72 h) of pre-enrichment or enrichment cultures for greater analytical flexibility and laboratory productivity in the examination of dry foods is under review for adoption by AOAC International.

The AOAC/*Bacteriological Analytical Manual* (BAM) procedures that use a pre-enrichment step require 4 days to yield presumptive evidence of foodborne *Salmonella* on plating media. Such procedures include overnight enrichment of food samples in a nonselective broth medium (pre-enrichment), enrichment (24–48 h) in one or more selective broth media, and plating of enrichment cultures onto 2 or more plating media (1–4). Although direct enrichment (no pre-enrichment) of materials known or suspected to contain large populations of endogenous flora can provide presumptive evidence of product contamination within 3 days (2), numerous reports underlined the low productivity of this analytical approach (5, 6). The need for more rapid, less laborious methods of detection led to significant advances in developmental research and to the marketing of diagnostic kits based on enzyme-linked immunosorbence, nucleic acid hybridization, immunoimmobilization, and genus-specific biochemical reactions (5, 7). Although the performance characteristics of many of these novel technologies are impressive, these systems require 3 or more days to presumptively identify a *Salmonella*-contaminated food (8).

In standard AOAC/BAM procedures involving a pre-enrichment step, initiation of sample analyses is restricted to Mondays and Tuesdays if weekend work is to be avoided and if analyses are to be interrupted only by refrigeration of incubated plating media. The ability to reliably recover *Salmonella* from pre-enrichment and enrichment broth cultures refrigerated 72 h would not only double the number of weekdays on which analyses could be initiated (i.e., Monday to Thursday inclusively) but also provide greater analytical flexibility. The present study was undertaken to extend the existing data base on this novel approach (9, 10) and to further validate refrigeration of pre-enrichment and enrichment broth cultures as a means of increasing laboratory productivity and timeliness of results. The study was designed in accordance with AOAC requirements for collaborative studies (11).

Interlaboratory Study

Whole egg powder, milk chocolate, animal feed, and instantized skim milk powder were selected, because they can be naturally contaminated with *Salmonella* spp., and the novel refrigeration approach needed to be evaluated under different pre-enrichment conditions. Animal feed was included in the present study to challenge the sensitivity of the refrigeration procedure through large incident populations of competitive microflora. Each laboratory received 60 test samples consist-

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Table 1. Characteristics of inoculated test samples

Sample	*Salmonella* spp.	Inoculation level	Most probable number/25 g[a]
Whole egg powder	*S. senftenberg* (suc$^+$)	High	1.8
	S. enteritidis	Low	0.9
Milk chocolate	*S. enteritidis*	High	10.8
	S. hadar	Low	10.8[b]
Animal feed	*S. typhimurium*	High	10.8
	S. senftenberg (suc$^+$)	Low	3.8
Instantized skim milk powder	*S. hadar*	High	23.2[b]
	S. typhimurium	Low	10.8

[a] Determined by coordinating laboratory at the time of collaborators' sample analysis.
[b] Higher than targeted levels of contamination result from greater than anticipated strain stability.

ing of 15 × 25 g samples of each of the 4 test materials. Each subset included 5 replicate samples inoculated with high-level contamination (10–25 salmonellae/25 g), 5 samples with low-level contamination (1–5 salmonellae/25 g), and 5 uninoculated controls (Table 1). The *Salmonella* strains were obtained from a collection of foodborne isolates maintained by the coordinating laboratory. Test samples previously prepared in the coordinating Health Protection Branch laboratory in Ottawa, Canada, were shipped by overnight courier service to each laboratory as a single consignment 2 weeks before commencement of the study. Upon receipt, samples were stored at room temperature pending analysis. Bacteriological culture media (Difco Laboratories Inc., Detroit, MI) were shipped separately from a Difco warehouse in Wisconsin concurrently with the shipment of test samples.

Laboratories initiated the analysis of all egg powder and milk chocolate samples (total of 30) on a predetermined date according to a rigid experimental protocol. Concurrently, 3-tube most probable number (MPN) determinations of *Salmonella* in homologous test materials were conducted by the coordinating laboratory. Analysis of the remaining milk powder and animal feed samples was undertaken by participating laboratories 2 weeks following initiation of the study; ancillary MPN determinations by the coordinating laboratory were conducted at this time. Collaborators submitted test results to the coordinating laboratory at the conclusion of the study.

Preparation of Bacterial Inocula

Individual *Salmonella* strains (Table 1) stored at room temperature in a semi-solid agar medium (12) were each inoculated into 9.0 mL nutrient broth and incubated 16–18 h at 35°C. A loopful of each broth culture was then subcultured into fresh nutrient broth and incubated as described above. Each broth culture was diluted in peptone water to yield a viable cell concentration of 10^4–10^5 salmonellae/mL. One milliliter portions from each of these diluted cultures were then added to separate 500 mL freeze-drier vessels containing 100 mL of sweetened condensed milk–distilled water (1 + 2, v/v). Contents were shell-frozen (–60°C) in ethanol for ca 15 min and then freeze-dried up to 5 days in a VirTis Consol 12 unit (VirTis Co., Inc., Gardiner, NY 12525). The lyophilized material was removed from the freeze-drying vessel and briefly blended to a fine powder. This inoculum material was then stored at room temperature and periodically assessed by the 3-tube (1.0, 0.1, and 0.01 g) MPN procedure to establish inoculum stability as measured by the temporal variations in levels of viable salmonellae.

Inoculation and Shipment of Samples

Several weeks before commencement of the study, replicate 25 g samples of each of the 4 test materials were distributed in individual 4 oz (125 mL), plastic, screw cap containers (Can-Lab, Pointe Claire, Québec, H9R 5L4, Canada). Appropriate portions of seed inoculum (0.5–2.0 g) were added to each sample container (Table 1). Contents of each container were mixed by shaking and then stored at room temperature pending shipment to collaborators.

For shipment, sample containers were packaged in reinforced cardboard boxes approved by the Canadian government for the transportation of infectious materials. Five boxes, each containing 12 sample containers, were delivered to each participating laboratory by overnight courier service.

Sample Analysis

Each test sample was analyzed by the standard AOAC/BAM methods (1, 2). Pre-enrichment and selective enrichment broth cultures arising from these analyses were immediately refrigerated 72 h at 2–5°C. Refrigerated cultures were then vortexed and directly tested for the presence of salmonellae according to the standard AOAC/BAM method (1, 2).

For pre-enrichment, the content (25 g) of each sample container was added to a separate 500 mL bottle or flask containing 225 mL of one of the following pre-enrichment broths; lactose broth (whole egg powder and animal feed), brilliant green water (instantized skim milk powder), and skim milk broth with added brilliant green (milk chocolate). Whole egg powder was dissolved gradually by successive additions of 4 portions of lactose broth (15, 10, 10, and 190 mL), whereas instantized milk powder was gently layered onto the surface of brilliant green water (no mixing). Samples of milk chocolate and animal feed were thoroughly mixed into their respective broth me-

dia and allowed to stand at room temperature for 60 min before adjusting the pH to 6.8 ± 0.2 with 1N NaOH or HCl. The pH of the layered instantized milk powder samples was not adjusted. All pre-enrichment mixtures were then incubated 24 ± 2 h at 35°C. Replicate portions (1.0 mL) from each pre-enrichment culture were inoculated into 10 mL tetrathionate brilliant green (TBG_{35}) and into 10 mL selenite cystine (SC_{35}); both enrichment broths were incubated 24 ± 2 h at 35°C. The pre-enrichment culture used to inoculate the TBG_{35} and SC_{35} media was immediately refrigerated 72 h at 2–5°C. Replicate loopsful of the TBG_{35} and SC_{35} enrichment cultures were then streaked onto Hektoen enteric (HE), bismuth sulfite (BS), and xylose lysine desoxycholate (XLD) agar media; inoculated plates were incubated 24 ± 2 h at 35°C. TBG_{35} and SC_{35} enrichment cultures used to inoculate the 3 plating media were immediately refrigerated 72 h at 2–5°C. Three or fewer suspect colonies from each plating medium were screened biochemically on triple sugar iron (TSI) and lysine iron (LI) agars and in urea broth. Presumptively positive isolates were further screened serologically using polyvalent flagellar (H) and somatic (O) agglutination reactions. Additional biochemical tests were used if deemed necessary by analysts.

Contents of pre-enrichment cultures previously refrigerated 72 h at 2–5°C were suspended by vortex, and replicate portions (1.0 mL) were inoculated into 10 mL of fresh TBG_{35} and SC_{35}. Subsequent selective enrichment, isolation on plating media, and biochemical/serological screening of presumptive *Salmonella* isolates followed the standard AOAC/BAM procedure (1, 2). Similarly, TBG_{35} and SC_{35} cultures previously refrigerated 72 h were thoroughly mixed to suspend the enriched material and were plated directly on a separate set of HE, BS, and XLD agar media. Suspect colonies were screened biochemically and serologically as previously described.

Analysis of Data

Upon completion of the analytical work, collaborators submitted results to the Associate Referee for collation and statistical analysis. Data were examined by the logistic regression approach using the CATMOD procedure (13, 14) for interactions between the following experimental variables: 3 analytical methods (standard AOAC/BAM, refrigerated pre-enrichment, and refrigerated enrichment), 3 levels of inoculation (high, low, and negative controls), 11 participating laboratories, 4 food types (egg powder, milk chocolate, animal feed, and milk powder), and 2 selective enrichment (TBG_{35} and SC_{35}) conditions. All statistical testing was conducted at the 5% level of significance.

Salmonella in Dry Foods, Recovery from Refrigerated Broth Cultures

The novel refrigeration approach is an alternative analytical procedure for the detection of *Salmonella* in dry foods. Following refrigeration (2–5°C) of pre-enrichment or selective enrichment cultures for 72 h, the refrigerated broths are further tested as described in **967.26B**, and presumptive *Salmonella* isolates are identified as in **967.26C** and **967.27** (1).

Principle

The ability of *Salmonella* spp. to survive refrigerated storage (72 h) of pre-enrichment and selective enrichment cultures could increase the number of weekdays on which AOAC/BAM analyses could be initiated without engendering weekend work. Currently, sample analyses need to be undertaken on Mondays or Tuesdays if interruption of the analytical work is to be restricted to the refrigeration of incubated plating media. The demonstrated reliability of the proposed refrigeration approach would not only provide greater analytical flexibility but also increase laboratory productivity by extending the days on which analysts could initiate sample testing to Wednesdays and Thursdays. For example, a food sample pre-enriched on Wednesday morning would yield TBG_{35} and SC_{35} cultures on Friday morning; such enrichment cultures could then be refrigerated over the weekend (72 h), and directly plated onto agar media the following Monday. Similarly, the pre-enrichment culture arising from the analysis of a food on Thursday would be refrigerated over the weekend, and replicate portions of the refrigerated culture inoculated into TBG_{35} and SC_{35} the following Monday. The novel refrigeration approach would, therefore, facilitate a more even distribution of analytical work and greater laboratory productivity and result in a more rapid release of food products.

Method Performance

See Table 2.

Apparatus

(**a**) *Refrigerated incubator.*—Capable of maintaining 4 ± 1°C. A laboratory refrigerator with adequate temperature control could also be used.

(**b**) *Glassware and equipment.*—As required for preparation, dispensing, and inoculation of bacteriological media and preparation of reagents as described in **967.25–967.27**.

Table 2. Method performance

Result		Percent
Agreement	(RP)[a]	100
	(RE)[b]	100
False neg.	(AOAC/BAM)[c]	0
False neg.	(RP)[d]	0
False neg.	(RE)[d]	0

[a] Relates to the number of samples found to be positive by the standard AOAC/BAM procedure and by the refrigerated pre-enrichment (RP) approach.

[b] Similar to footnote a above, where RE relates to the combined productivities of refrigerated TBG_{35} and SC_{35} for individual samples.

[c] Relates to the number of samples found to be positive by the refrigerated (RP or RE) approach and negative by the standard AOAC/BAM procedure divided by the total number of positive samples.

[d] Relates to the number of samples found to be positive by the standard AOAC/BAM procedure and negative by the RP or RE approach.

Media and Reagents

(a) *Bacteriological media.*—Liquid and agar media for the isolation and identification of *Salmonella* as described in **967.25A**.

(b) *Reagents.*—Diagnostic reagents required for determinant biochemical reactions as described in **967.25B**.

Analysis of Sample

(a) *Pre-enrichment.*—Test products are pre-enriched in a noninhibitory broth medium to provide for the repair and growth of stressed or injured *Salmonella* (5, 6). The following protocol is intended for dry products whose analysis is to be initiated on Wednesdays or Thursdays.

Dry products.—Pre-enrich 25 g sample of product in 225 mL appropriate pre-enrichment broth medium as described in **967.26A** or in the BAM, Chapter 7 (2). If sample pre-enrichment is initiated on Thursday, refrigerate the incubated pre-enrichment culture on Friday for the weekend (72 h); resume analysis the following Monday with selective enrichment (**b**), isolation (**d**), and identification (**f**). If sample pre-enrichment is initiated on Wednesday, proceed with selective enrichment on Thursday (**b**) and refrigerate enrichment cultures on Friday (**c**).

(b) *Selective enrichment.*—Transfer 1 mL of incubated pre-enrichment culture into tube containing 10 mL TBG_{35} broth, and transfer 1 mL into tube containing 10 mL SC_{35}, as in **967.26B(a)**. Incubate both enrichment broths 24 ± 2 h at 35°C.

(c) *Refrigeration of enrichment cultures.*—Following incubation, refrigerate the TBG_{35} and SC_{35} cultures on Friday for the weekend (72 h); resume analysis the following Monday with isolation and identification.

(d) *Isolation.*—Streak a loopful of TBG_{35} culture onto BS, HE, and XLD plating media. Repeat this procedure with the homologous SC_{35} culture. Incubate inoculated plates 24 ± 2 h at 35°C. BS should also be examined after 48 h of incubation.

(e) *Treatment of suspicious colonies.*—Pick with sterile needle 2 or more typical or suspicious colonies as described in **967.26B(b)** from BS, HE, and XLD plating medium. Inoculate each isolate into TSI and LI agar slants, and screen for presumptive *Salmonella* reactions as indicated in **967.26C**.

(f) *Identification of isolates.*—Presumptive *Salmonella* isolates exhibiting typical or suspect TSI and LI reactions are confirmed biochemically and serologically as described in **967.27**.

Results

Whole Egg Powder

MPN determinations of *Salmonella* in whole egg powder yielded 1.8/25 g and 0.9/25 g for the high and low levels of inoculation, respectively (Table 1). The lower than targeted level of *S. senftenberg* (sucrose-positive) in highly contaminated samples likely arose from the antibacterial action of egg powder components during the storage of inoculated samples. Populations of background flora enumerated on plate count agar (24 h at 35°C) by the coordinating laboratory at the time of sample analyses occurred at levels of <100 CFU/g. In all laboratories, samples found to be contaminated by the standard AOAC/BAM procedure were also detected from the refrigerated pre-enrichment and enrichment cultures (Table 3). False-negative reactions were not encountered with this food product. Results also underlined the equivalence of refrigerated TBG_{35} and SC_{35} cultures for the detection of positive samples. Except for a single instance of recovery of *S. enteritidis* from refrigerated TBG_{35} but not SC_{35} (Collaborator 4, sample 7), both refrigerated enrichments consistently yielded parallel results. Uninoculated control samples were negative by all methods.

Milk Chocolate

MPN determinations of *Salmonella* in milk chocolate yielded 10.8/25 g for both the high and low levels of inoculation (Table 1). The instability of the *S. hadar* seed inoculum at the time of sample inoculation required an estimation of the inoculum to milk chocolate samples to obtain the desired level of contamination at the time of analysis. A lower than anticipated death rate of *S. hadar* in milk chocolate (and skim milk powder) resulted in higher than targeted levels of contamination. Enumeration of background flora in uninoculated milk chocolate by the coordinating laboratory yielded <100 CFU/g. Refrigerated pre-enrichment and enrichment cultures detected all contaminated samples identified by the standard AOAC/BAM procedure (Table 4). False-negative reactions were not encountered with this food product, and homologous refrigerated TBG_{35} and SC_{35} cultures consistently showed equal productivity (data not shown). A single uninoculated control sample (Collaborator 1, sample 14) yielded *Salmonella* by the AOAC/BAM and paired refrigerated broth cultures. Although the milk chocolate product was tested extensively and found to be negative by the coordinating laboratory, the possibility that few nonhomogeneously distributed salmonellae in the bulk material may have escaped detection cannot be dismissed. However, the report by Collaborator 1 of an isolate belonging to somatic group D_1 and exhibiting a flagellar G complex in sample 14 (corresponding to *S. hadar*) more strongly suggests that the sample was erroneously labeled or subjected to cross-contamination during preparation or sample analysis.

Animal Feed

MPN determinations of *Salmonella* in animal feed yielded 10.8/25 g and 3.8/25 g for the high and low levels of inoculation, respectively (Table 1). Concurrent enumeration of background flora on standard plate count agar (24 h at 35°) showed levels of 2.9×10^4 CFU/g. All positive samples detected by the standard AOAC/BAM procedure were identified from the refrigerated pre-enrichment and enrichment cultures (Table 5). False-negative results were not associated with this product. Homologous refrigerated TBG_{35} and SC_{35} cultures consistently showed equal productivity, except for one sample inoculated with a high level of *S. typhimurium* (Collaborator 9, sample 5), where the target organism was only recovered from the

Table 3. Detection of *Salmonella* in whole egg powder

Coll.	Positive samples by analytical method[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Standard culture (AOAC/BAM)															
1	+[b]	+	+	+	+	–[c]	–	+	+	+	–	–	–	–	–
2	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–
4	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–
6	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–
7	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–
8	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–
9	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–
10	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–
11	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
Refrigerated pre-enrichment															
1	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–
2	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–
4	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–
6	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–
7	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–
8	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–
9	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–
10	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–
11	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
Refrigerated enrichment[d]															
1	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–
2	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–
4	–	+	+	+	+	+	+[e]	+	+	+	–	–	–	–	–
5	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–
6	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–
7	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–
8	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–
9	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–
10	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–
11	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–

[a] Samples 1–5 were inoculated at a high level; samples 6–10 were inoculated at a low level; samples 11–15 were uninoculated controls.
[b] Detection of *Salmonella* by one or more enrichment-plating conditions.
[c] *Salmonella* not detected in sample.
[d] Results based on combined productivity of refrigerated TBG_{35} and SC_{35} cultures.
[e] Recovery of *Salmonella* from refrigerated TBG_{35} culture only.

Table 4. Detection of *Salmonella* in milk chocolate

Coll.	Positive samples by analytical method[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Standard culture (AOAC/BAM)															
1	+[b]	+	+	+	+	+	+	+	+	+	–[c]	–	–	+	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated pre-enrichment															
1	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated enrichment[d]															
1	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–

[a–d] See footnotes in Table 3.

refrigerated TBG_{35} culture. Uninoculated control samples were negative by all methods.

Instantized Skim Milk Powder

MPN determinations of *Salmonella* in milk powder yielded 23.2/25 g and 10.8/25 g for the high and low levels of inoculation, respectively (Table 1). Uninoculated product showed background flora at 2.3×10^3 CFU/g. All positive samples detected by the standard AOAC/BAM procedure were also identified from the corresponding refrigerated pre-enrichment and

Table 5. Detection in *Salmonella* in animal feed

Coll.	Positive samples by analytical method[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Standard culture (AOAC/BAM)															
1	+[b]	+	+	+	+	+	+	–[c]	+	+	–	–	–	–	–
2	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
4	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–
6	+	+	–	+	+	–	–	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
9	+	+	+	+	+	–	+	–	–	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated pre-enrichment															
1	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
2	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
4	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–
6	+	+	–	+	+	–	–	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
9	+	+	+	+	+	–	+	–	–	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated enrichment[d]															
1	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
2	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
4	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–
6	+	+	–	+	+	–	–	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
9	+	+	+	+	+[e]	–	+	–	–	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–

[a–d] See footnotes in Table 3.
[e] Recovery of *Salmonella* from refrigerated TBG_{35} culture only.

Table 6. Detection of *Salmonella* in instantized skim milk powder

Coll.	Positive samples by analytical method[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Standard culture (AOAC/BAM)															
1	+[b]	+	+	+	+	+	+	+	+	+	–[c]	–	–	–	–
2	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated pre-enrichment															
1	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
2	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Refrigerated enrichment[d]															
1	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
2	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+[e]	+	+	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–

[a–d] See footnotes in Table 3.
[e] Recovery of *Salmonella* from refrigerated SC_{35} culture only.

enrichment cultures (Table 6). False-negative results were not encountered with this product, and uninoculated control samples were negative by all methods. Homologous refrigerated TBG_{35} and SC_{35} cultures yielded parallel results with all but one positive sample, where *S. typhimurium* was recovered only from the refrigerated SC_{35} culture of a low-level-inoculated sample (Collaborator 3, sample 8).

Productivity of Plating Media

Of the 3 plating media used in the present study, BS consistently identified more positive samples than HE and XLD (Table 7). The productivities of HE and XLD were comparable under homologous test conditions, and none of the plating media was unduly affected by the use of refrigerated pre-enrich-

Table 7. Detection of *Salmonella* under different enrichment-plating conditions

Sample	Total samples positive[a]	Selective enrichment	Salmonella positive samples[b]								
			AOAC/BAM			RP			RE		
			BS	HE	XLD	BS	HE	XLD	BS	HE	XLD
Egg powder	81	TBG_{35}	79	60	61	81	64	63	81	61	62
		SC_{35}	80	61	60	79	62	61	79	60	60
Milk chocolate	110	TBG_{35}	109	110	110	110	110	108	110	110	110
		SC_{35}	109	110	109	108	108	104	108	110	109
Animal feed	93	TBG_{35}	92	69	76	92	70	75	82	70	76
		SC_{35}	92	73	74	91	67	76	92	66	74
Instantized milk powder	109	TBG_{35}	109	108	107	109	108	108	107	102	107
		SC_{35}	109	105	108	108	107	108	108	100	105
Total	393	TBG_{35}	389	347	354	392	352	354	380	343	355
		SC_{35}	390	349	351	386	344	349	387	336	348

[a] *Salmonella* detected by one or more analytical condition.
[b] RP = refrigerated pre-enrichment culture; RE = refrigerated enrichment culture; BS = bismuth sulfite agar; HE = Hektoen enteric agar; XLD = xylose lysine desoxycholate.

ment and enrichment broth cultures. However, no single enrichment-plating condition identified all 393 positive samples.

Discussion

Summary data from the 11 participating laboratories underlined the excellent sensitivity of the refrigeration method for detection of foodborne *Salmonella* (Table 8). Of 660 samples tested, 393 were found to be contaminated by the standard AOAC/BAM procedure. These positive samples were all identified from the corresponding refrigerated pre-enrichment cultures and from the combined productivities of homologous TBG_{35} and SC_{35} cultures. Moreover, homologous TBG_{35} and SC_{35} cultures yielded parallel results in all but 3 cases, where *Salmonella* could only be isolated from 2 TBG_{35} and 1 SC_{35} cultures. The latter difference was not found to be statistically significant (data not shown). The potential impact of the 2 levels of contamination on the sensitivity of the refrigeration approach also was not significant (data not shown).

Reliable recoveries of *Salmonella* from the 3 types of refrigerated pre-enrichment media used in the present study, and from refrigerated nutrient (9) and buffered peptone water (10, 15) broths in earlier studies, predicate a general application of the 72 h refrigeration approach to all nonselective broth media for the detection of *Salmonella* in dry foods. However, varying selectivity levels encountered in different enrichment media preempt a similar generic statement on the reliability of refrigerated enrichment cultures. Although data on the combined productivity of refrigerated TBG_{35} and SC_{35} (Table 8) and of TBG_{43} and SC_{35} (9, 10) clearly support the use of these paired enrichment conditions, endorsement of other refrigerated enrichment media must await validation. Interestingly, preliminary results on the recovery of salmonellae from refrigerated Rappaport-Vassiliadis (3) enrichment cultures of low- and high-moisture foods are encouraging (16).

The ability of individual plating media to recover *Salmonella* from refrigerated broth cultures with a sensitivity similar to that obtained in the standard AOAC/BAM procedure indicates that the refrigeration approach does not unduly challenge the performance of plating media (Table 7). The greater productivity of BS was not totally unexpected given its higher level of selectivity, its sensitive system for the detection of

Table 8. Recovery of *Salmonella* from refrigerated broth cultures

Product	No. samples tested	No. samples positive				
		AOAC/BAM	Refrigerated pre-enrichment	Refrigerated enrichment[a]	X^2	p-value
Whole egg powder	165	81	81	81	0.00	1
Milk chocolate	165	111[b]	111[b]	111[b]	0.00	1
Animal feed	165	93	93	93	0.00	1
Instanizedt skim milk powder	165	109	109	109	0.00	1

[a] Based on combined results from refrigerated TBG_{35} and SC_{35} cultures.
[b] Includes a single uninoculated control sample that yielded *Salmonella* by the AOAC/BAM and homologous refrigerated pre-enrichment and enrichment cultures (see Table 4).

gaseous hydrogen sulfide production, and its saccharide-independent identification of salmonellae (5). The latter characteristic likely played a determinantal role in the ability of many collaborators to identify, on HE and XLD, the sucrose-positive strain of *S. senftenberg* inoculated into egg powder and animal feed samples (Table 1).

The present study established the sensitivity of the novel refrigeration approach for the detection of *Salmonella* in dry foods. The procedure allows sample analysis on 4 consecutive weekdays (Monday to Thursday) rather than the current 2-weekday (Monday and Tuesday) limitation imposed by AOAC/BAM methods. The refrigeration approach not only provides for greater analytical flexibility and productivity through the analysis of more samples and more uniform distribution of the analytical workload, but it also facilitates the more timely release of *Salmonella*-negative products. A timer-controlled incubator/refrigerator could also be used to further enhance the analytical flexibility provided by the refrigeration approach.

Recommendation

On the strength of the results of this study and earlier comparative data (9, 10), the use of refrigerated pre-enrichment and paired refrigerated TBG_{35} and SC_{35} or TBG_{43} and SC_{35} enrichment cultures for the detection of *Salmonella* in dry foods is recommended for first action adoption.

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MICROBIOLOGICAL METHODS

AutoMicrobic System for Biochemical Identification of *Listeria* Species Isolated From Foods: Collaborative Study

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A collaborative study was conducted to evaluate the performance of the AutoMicrobic System Gram-Positive Identification (GPI) and Gram-Negative Identification (GNI) test kits to biochemically characterize *Listeria* spp. Thirteen laboratories each tested 97 food and environmental isolates, representing the 7 species of *Listeria*, as well as 11 additional genera of Gram-positive rods. Each collaborator inoculated both a GPI and a GNI card with a pure culture of each organism. The AutoMicrobic System identified the isolates and printed out the biochemical results. The GPI card is used to obtain a species identification and a mannitol reaction result, and the GNI card is used to obtain rhamnose and xylose reaction results. Organisms are classified into species groups and can be further distinguished on the basis of hemolysis or nitrate reduction tests. The AutoMicrobic System method correctly classified 90.8% of the *Listeria* spp. isolates and 100% of the non-*Listeria* isolates. The AutoMicrobic System method was adopted first action by AOAC International for the biochemical characterization of *Listeria* spp. isolated from food and environmental sources.

The *Bacteriological Analytical Manual* (BAM) (1) method for identification of *Listeria* spp. from primary isolation media involves a number of conventional biochemical tests, in addition to hemolytic, serological, and mouse pathogenicity tests. Some of the conventional biochemical tests require an incubation period of up to 7 days. The AutoMicrobic System (bioMerieux Vitek, Inc., Hazelwood, MO) is an automated microbial identification system that uses miniaturized test kits containing up to 30 different biochemical tests each. Test kits are incubated in the system and scanned hourly for turbidity and/or color changes. Positive test results are analyzed by the system computer, which then provides an organism identification. Several workers (2–5) investigated the use of the AutoMicrobic System for identifying members of the family *Enterobacteriaceae*. The AutoMicrobic System also has identification kits for Gram-positive organisms (6–9), *Bacillus* spp. (10), yeasts (11, 12), and anaerobes. With the use of 2 different AutoMicrobic System identification kits, the Gram-Positive Identification (GPI) card, and the Gram-Negative Identification (GNI) card, *Listeria* spp. can be identified biochemically in 24 h. Further tests, such as β-hemolysis and Christie-Atkins-Munch-Peterson (CAMP) reaction on sheep blood agar, or nitrate reduction, as specified in BAM, are necessary to differentiate some of the species of *Listeria*.

This study was conducted to collaboratively evaluate the performance of the GPI and GNI cards in the AutoMicrobic System method for the biochemical identification of *Listeria* spp. isolates from foods.

Collaborative Study

A total of 13 laboratories participated in this collaborative study, consisting of industry, government, and private testing laboratories involved in the testing of food products. Ninety-seven cultures (Table 1), along with 6 American Type Culture Collection (ATCC) GNI card control and 6 ATCC GPI card control isolates, were shipped to the collaborators either on brain heart infusion (BHI) agar slants or on trypticase soy agar with 5% sheep blood plates. All of the isolates were tested by both the conventional biochemical method and the AutoMicrobic System method at the Kraft General Foods Technology Center, Glenview, IL; the collaborating laboratories tested the isolates by the AutoMicrobic System method only.

The collaborators were provided with enough materials and test kits to identify the isolates. The collaborators were instructed to streak each culture onto a plate of trypticase soy agar with 5% sheep blood, incubate the plates for 24 h at 35 ± 1°C,

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The recommendation was approved by the General Referee and the Committee on Microbiology and Extraneous Materials and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

Table 1. Isolates used in collaborative study of automated systems for biochemical identification of *Listeria* spp. isolates

Organism	No. isolates	Source[a]	Serotype[b]	Culture No.[c]
		Listeria		
L. monocytogenes	30	sc[d] from FDA (14), ATCC strain 15313 (1), Brie cheese (2), Mexican cheese (1), ice cream (2), sc from FRI (5), shrimp (1), environmental isolate (4)	—[e]	1, 2, 3, 9, 12–14, 27, 33, 34, 36–46, 88–97
L. ivanovii	10	sc from FDA (2), sc from USDA (6), environmental isolate (2)	unknown (10)	4, 18–24, 30, 35, 47–49
L. innocua	10	sc from FDA (1), sc from USDA (3), environmental isolate (6)	1, 4(4), 4(6)	5, 15–17, 25, 26, 28, 29, 31, 32
L. seeligeri	5	sc from FDA (2), ATCC strain 35967 (1), Brie cheese (2)	1(5)	6–8, 10, 11
L. welshimeri	6	sc from USDA (4), radish (1), potato (1)	unknown (6)	50–53, 58, 59, 84–87
L. grayi	4	ATCC strain 19120 (1), ATCC strain 25400 (1), ice cream (2)	NA[f]	54, 55, 60, 61
L. murrayi	3	ATCC strain 25401 (1), ATCC strain 25402 (1), ATCC strain 25403 (1)	NA	56, 62, 63
		Non-*Listeria*		
Jonesia denitrificans	1	ATCC strain 14870 (1)	NA	57
Kurthia zopfii	2	ATCC strain 33403 (1), ATCC strain 6900 (1)	NA	64, 65
Bacillus cereus	2	ATCC strain 14579 (1), KGF sc (1)	NA	66, 67
Bacillus subtilis	2	ATCC strain 6633 (1), KGF sc (1)	NA	68, 69
Erysipelothrix rhusiopathiae	2	sc from Vitek (2)	NA	73, 74
Brevibacterium linens	2	KGF sc (2)	NA	78, 79
Brochothrix thermosphacta	1	ATCC strain 11509 (1)	NA	70
Corynebacterium bovis	1	ATCC strain 7715 (1)	NA	71
Corynebacterium flavescens	1	ATCC strain 10348 (1)	NA	72
Gemella hemolysans	1	ATCC strain 10379 (1)	NA	75
Cellulomonas cartae	1	ATCC strain 21681 (1)	NA	76
Cellulomonas flavigena	1	ATCC strain 482 (1)	NA	77
Propionibacterium shermanii	1	KGF sc (1)	NA	82
Propionibacterium freudenreichii	1	KGF sc (1)	NA	83
Lactobacillus coryneformis	1	ATCC strain 25602 (1)	NA	80
Lactobacillus helviticus	1	ATCC strain 15009 (1)	NA	81

[a] Number in parentheses indicates the number of different organisms from a single source. FDA = U.S. Food and Drug Administration; ATCC = American Type Culture Collection; FRI = Food Research Institute; USDA = U.S. Dept of Agriculture; KGF = Kraft General Foods.
[b] Number in parentheses indicates the number of strains of a single serotype.
[c] Additional culture members represent replicates of certain strains, included because of previously demonstrated variability (unpublished data).
[d] sc = stock culture.
[e] Serotypes: 1(7), 1A(1), ½A(1), 1B(2), ½B(1), 3A(1), 3B(2), 4(10), 4B(2), Flag. A(1), Flag. D(1), Flag. C(1).
[f] NA = not applicable.

and check for purity. An isolated colony from the plate was picked and streaked onto a BHI agar slant, and the slants were incubated for 24 h at 35 ± 1°C. Biochemical identification was performed on the incubated slant as described in the method. The result for each isolate was reported to Kraft General Foods by each collaborator. Species group identifications were given codes as follows: LM = *L. monocytogenes/L. innocua* group, LI = *L. ivanovii/L. seeligeri* group, LW = *L. welshimeri*, LG = *L. grayi/L. murrayi* group, O = unidentified/non-*Listeria* group. Each result was treated as a Bernoulli trial, with the result obtained by the AutoMicrobic System method classified as either "agrees with conventional" or "disagrees with conventional." The methods used for constructing the confidence intervals for the binomial parameter were those of Johnson and Kotz (13) and Brownlee (14).

992.19 *Listeria* sp. Biochemical Identification Method (Vitek GPI and GNI)

First Action 1992

This method is for presumptive generic identification of foodborne *Listeria* species and screening and elimination of non-*Listeria* isolates. For speciation of some *Listeria*, analyst must perform CAMP, β-hemolysis, and/or nitrate reduction tests as described in current edition of *Bacteriological Analytical Manual* (BAM).

Caution: Potential biohazard. Bacteria used in method are potentially pathogenic microorganisms. Extreme care should be taken in handling and pipetting bacterial solutions. Reaction products, bacterial cultures, tubes, pipets, and test trays should be placed in biohazard bags and sterilized in autoclave before disposal according to laboratory guidelines. All surfaces should be decontaminated with appropriate disinfectant; hands should be washed with bactericidal soap.

Method performance:

See Table **992.19A** for method performance data.

A. Principle

Method is automated in-vitro testing system that uses incubator chamber with optical reader, filler/sealer unit for test kit inoculation, and computer. Gram-positive identification cards (GPI) and Gram-negative identification cards (GNI) each contain 30 biochemical tests, as listed in Tables **992.19B** and **992.19C**. Positive results of preliminary off-line tests (e.g., catalase and oxidase) are entered on card. Optical reader, within temperature-controlled incubator, scans each card hourly and measures turbidity and/or color changes in biochemical tests and transmits readings to computer. Computer analyzes and interprets changes, reporting results of biochemical tests in cards as positive or negative, and classifies organism to genus and/or species. Method uses GPI and GNI cards, each targeted to identify single group of microorganisms, to identify single organism (GPI card for preliminary identification of *Listeria* spp. and 2 reactions on GNI card for final determination to species group). CAMP reaction and β-hemolysis on sheep blood agar and/or nitrate reduction tests are necessary to differentiate species within 3 of groups (Table **992.19D**).

B. Apparatus

(a) *Cotton swabs, sterile.*—Place nonsterile cotton-tipped swabs in autoclavable container and sterilize 30 min at 121°.

Table 992.19A. Method performance for *Listeria* in foods, biochemical identification method (Vitek GNI and GPI)

	Method error rate		
Organism	Correct	Incorrect	Total
Listeria spp.[a]	835	77	912
Non-*Listeria* spp.[b]	252	0	252

[a] Sensitivity rate: 835/912 = 91.55%.
[b] Specificity rate: 252/252 = 100%.

Table 992.19B. Biochemical tests in Gram-positive identification card

Microaerophilic		
Growth control	Urea	Trehalose
Bacitracin	Tetrazolium red	Arabinose
Optochin	Novobiocin	Pullulan
Hemicellulase	Dextrose	Pyruvate
6% NaCl	Lactose	Inulin
10% Bile	Mannitol	Melibiose
40% Bile	Raffinose	Melezitose
Esculin	Salicin	Cellobiose
Arginine negative control	Sorbitol	Ribose
Arginine	Sucrose	Xylose

Table 992.19C. Biochemical tests in Gram-negative identification card

Aerobic	Microaerophilic
DP-300(2-4-4′-Trichloro-2′-hydroxydiphenyl ether)	Raffinose
Glucose (oxidative)	Sorbitol
Growth control	Sucrose
Acetamide	Inositol
Esculin	Adonitol
Plant indican	*p*-Coumaric acid
Urea	H_2S
Citrate	ONPG
Malonate	Rhamnose
Tryptophan	Arabinose
Polymixin B	Glucose (fermentative)
Lactose/10% lactose	Arginine
Maltose	Lysine
Mannitol	Base control
Xylose	Ornithine

Table 992.19D. Species group determination from GPI and GNI cards

Organism designation		GPI card		GNI card	
		Identification	Mannitol result	Rhamnose result	Xylose result
LM	*L. mono*	*Listeria* spp.	–	+	–
	L. innocua	*Listeria* spp.	–	–	–
LI	*L. ivanovii*	*Listeria* spp.	–	–	+
	L. seeligeri				
LW	*L. welshimeri*	*Listeria* spp.	–	+	+
LG	*L. grayi*	*Listeria* spp.	+	+	–
	L. murrayi	*Listeria* spp.	+	–	–
O	Non-*Listeria*	Unidentified	+ or –	+ or –	+ or –
		Other species	+ or –	+ or –	+ or –

(**b**) *Glass slides.*—25 × 75 × 1 mm.

(**c**) *Incubator.*—Capable of maintaining 35 ± 2°.

(**d**) *Inoculating loops.*—3 mm, for use in **E**(*1*).

(**e**) *Inoculating needles.*

(**f**) *Pipets.*—10 mL serological, sterile, with cotton plug.

(**g**) *Tubes.*—12 × 75 mm, sterile, and 16 × 125 mm.

(**h**) *Automated system.*—Filling/sealing unit, incubator chamber with optical reader, and computer (AutoMicrobic System or AutoMicrobic Junior System, bioMerieux Vitex, Inc., 595 Anglum Dr. Hazelwood, MO 63042).

(**i**) *Filling stand.*—Plastic stand to hold test kit and transfer tube assembly.

(**j**) *Filling module rack.*—Plastic rack to hold 10 filling stands.

(**k**) *Permanent marker.*—Blue or black, fine tip.

(**l**) *GPI card.*—90 × 57 × 4 mm plastic card with 30 chambers containing dehydrated growth or biochemical test media for Gram-positive tests. Monitor GPI card performance by using the following: *Streptococcus pyogenes*, ATCC 19615; *Enterococcus faecalis*, ATCC 29212; *Streptococcus pneumoniae*, ATCC 27336; *Enterococcus durans*, ATCC 6056; *Streptococcus bovis*, ATCC 9808; *Staphylococcus xylosus*, ATCC 29971; and *Streptococcus equi*, ATCC 9528. Use fresh stock for each performance test and subculture 2× on consecutive days on trypticase soy agar-5% sheep blood plates at 35° prior to card inoculation.

(**m**) *GNI card.*—90 × 57 × 4 mm plastic card with 30 chambers containing dehydrated growth or biochemical test media for Gram-negative tests. Monitor GNI card performance by using the following: *Proteus mirabilis*, ATCC 7002; *Pseudomonas aeruginosa*, ATCC 27853; *Serratia odorifera*, ATCC 33077; *Klebsiella pneumoniae*, ATCC 13883; *Acinetobacter* cal. bio. *anitratus*, ATCC 19606; *Shigella sonnei*, ATCC 25931; and *Bordetella bronchiseptica*, ATCC 10580. Use fresh stock for each performance test and subculture 2' on consecutive days on trypticase soy agar-5% sheep blood plates at 35° before card inoculation.

(**n**) *Transfer tubes.*—L-shaped plastic tubes, 80 mm long, to transfer culture suspension from tube into card chambers. Included with GPI and GNI test cards.

(**o**) *Sealer plugs.*—4 × 4 × 26 mm plastic plugs to seal card chambers, for systems without sealer unit (for use with AutoMicrobic Junior Systems only).

(**p**) *Applicator sticks, sterile.*—Place nonsterile wooden applicator sticks in autoclavable container and sterilize 30 min at 121°.

C. Culture Media and Reagents

Use distilled or deionized water throughout.

Culture media are available from variety of commercial sources.

(**a**) *BHI agar slants.*—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4 \cdot 12H_2O$, 2.0 g glucose, and 15.0 g agar in 1 L H_2O. Heat to boiling to dissolve medium completely. Dispense 8–10 mL portions into 16 × 125 mm tubes. Autoclave 15 min at 121°. Final pH must be 7.4 ± 0.2. Before medium solidifies, incline tubes to obtain 5–6 cm slant.

(**b**) *Turbidity standards.*—1% $BaCl_2$–1% H_2SO_4 (1 + 99) (McFarland nephelometer scale, No. 1.0) and (0.5 + 99.5) (McFarland nephelometer scale, No. 0.5).

(**c**) *Hydrogen peroxide solution, 30%.*

(**d**) *Saline, sterile.*—0.45%. Dissolve 4.5 g NaCl in 1 L H_2O and autoclave 15 min at 121°. Final pH must be 5.5–8.0. Aseptically dispense 1.8 mL portions to 12 × 75 mm sterile disposable tubes. Cap tubes and store at 4°.

(**e**) *Trypticase soy agar–5% sheep blood plates.*—Dissolve 14.5 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal, 5.0 g NaCl, 14.0 g agar, and 1.5 g growth factors in 1 L H_2O. Autoclave 15 min at 121° and cool to 55°. Add 5% defibrinated sheep blood and pour into plates.

D. General Instructions

Store GNI and GPI cards unopened in protective liners at 2–8°. (Note: Do not freeze.) Do not use cards after expiration

date. Before removing cards from liners, visually check liner for holes or cracks in foil material. Discard card if liner is damaged.

Materials and methods outlined are intended for use only with Vitek AutoMicrobic System. Unsatisfactory results or misidentifications may result from use of equipment or reagents from other sources.

E. Preparation of Inocula

(*1*) Streak culture for isolation onto trypticase soy agar–5% sheep blood plate. Incubate 24 h at 35 ± 2°. Transfer well-isolated colony to BHI agar slant, and incubate 24 h at 35 ± 2°.

(*2*) Perform catalase test from BHI slant. Remove small portion of growth from BHI agar slant with applicator stick and place on slide. (*Note*: Wire inoculating loops may give false reaction; do not use.) Add 1 drop 30% hydrogen peroxide to growth on slide. Immediate bubbling indicates positive test for catalase. No bubbling is negative reaction. Discard any catalase-negative cultures. (*Caution*: Avoid skin contact with hydrogen peroxide. Should contact occur, flush affected area with water.)

(*3*) Let GPI and GNI cards come to room temperature. Remove GPI card from liner and mark sample number with marker. Use marker to fill in upper left circular depression in card to indicate culture is catalase positive (no mark in depression would indicate catalase-negative reaction). Disregard printed red circle on GPI card. (*Note*: Improper marking of circular depression corresponding to catalase test result will give incorrect isolate identification.) Remove GNI card from liner and mark same sample number. Disregard upper left circular depression on GNI card.

F. Inoculation and Reading of Results

(*1*) Label two 12 × 75 mm tubes containing 1.8 mL 0.45% saline with sample number, and place into 2 consecutive filling stands.

(*2*) Using sterile swab, inoculate first tube with growth from BHI agar slant to achieve at least No. 0.5, but no greater than No. 1.0 McFarland standard. Use vortex mixer to uniformly suspend cells.

(*3*) Using fresh swab, inoculate second tube with growth from same BHI agar slant to achieve No. 1.0 McFarland standard. Use vortex mixer to uniformly suspend cells.

(*4*) Using aseptic technique, firmly insert short end of transfer tube into side port of GPI card with long part of tube directed toward bottom section of card.

(*5*) Place mated GPI card/transfer tube unit in filling stand with long part of transfer tube inserted into first saline tube. Properly inserted transfer tube extends well into sample.

(*6*) Using aseptic technique, repeat (*4*) and (*5*) with GNI card, placing mated GNI card/transfer tube unit in filling stand with long part of transfer tube inserted into second saline tube.

(*7*) Position filling stands in filling module rack, and place rack into filling module of automated system. Subject card/transfer tube assembly to filling cycle. Without removing cards from filling stands, inspect both cards to verify proper fill has been achieved. If either card has not filled properly, discard it and repeat (*1*)–(*7*) using fresh inoculum, transfer tube, and card.

(*8*) Seal cards. If automated system has sealer modules, insert card/transfer tube unit on filling stand into sealer module; insertion simultaneously severs transfer tube from card and seals card port. For automated system without sealer modules, remove transfer tube manually and use blue sealer plug to seal card.

(*9*) Load both sealed cards into reader/incubator tray and proceed with tests. (*Note*: Interval between filling cards and placement in reader/incubator should not exceed 15 min.)

(*10*) Sterilize, by autoclaving, used transfer tube remnants, tubes, and cards before disposal.

(*11*) After incubation cycle, data terminal prints status report for each card in reader/incubator tray, with normalized percent probability for first and second choice identifications.

G. Interpretation of Results

Consult GPI and GNI card reports for species group. Consult Table **992.19D** for species group determination. The 11 tests (on GPI card) whose positive results are considered definitive for generic identification of *Listeria* species are as follows: bacitracin, opticin, 6% NaCl, 10% bile, 40% bile, esculin, tetrazolium red, glucose, salicin, trehalose, and cellobiose.

H. Report Messages

Several types of messages may appear on final GPI or GNI printed identification report to explain why identification cannot be made or to qualify identification statements.

Card inoculation error.—GPI card analysis was terminated after initial reading because of unacceptably low levels of light transmission. Inoculate new card after determining reason(s) for unacceptable reading from following list.

(*1*) Inoculum turbidity exceeded No. 1.0 McFarland standard.

(*2*) Card not placed in reader/incubator within 15 min recommended time interval.

(*3*) Card not checked to confirm proper fill was achieved.

Insufficient growth or nonviable organism.—All biochemical tests in GPI or GNI card were negative. Reasons for insufficient growth include the following:

(*1*) Organism is nonviable. Confirm by aseptically sampling peptone base well (No. 1) on GPI card and/or growth control well (No. 3) on GNI card and subculturing onto blood agar.

(*2*) Insufficient number of organisms in inoculum. Repeat test with inoculum equivalent to No. 1.0 McFarland standard for GPI card, or No. 2.0 McFarland standard for GNI card.

(*3*) Isolate is strict aerobe and will not grow in microaerophilic environment of GPI card.

Questionable inoculum.—Fermentative glucose well in GNI card did not become positive until 13 h or later in incubation cycle, usually indicating low-level contamination. Check purity of isolate by aseptically sampling growth control well (No. 3) of GNI card and subculturing to blood agar.

Unidentified organism.—GPI or GNI results do not sufficiently resemble any stored in respective data bases. Possible causes include the following:

Table 2. Collaborators' results for biochemical identification of *Listeria* spp. isolates by automated system

Culture No.[a]	Correct ID[b]	1	2	3	4	5	6	7	8	9	10	11	12
1	LM												
2(A)	LM												
3	LM												
4(A)	LI		O[c]			O	O			O			
5	LM												
6	LI												
7	LI												
8	LI												
9	LM												
10	LI												
11	LI												
12	LM												
13	LM												
14	LM												
15	LM												
16	LM												
17	LM												
18	LI					O	O			O			
19	LI												
20	LI												
21(A)	LI	O	O			O	O			O	O		
22	LI												
23	LI		O			O	O			O	O		
24(A)	LI		O				O			O			
25	LM												
26	LM												
27	LM												
28	LM												
29	LM												
30	LI								LM				
31	LM												
32	LM	LG											
33	LM												
34	LM												
35	LI		O				O		O	O	O		
36	LM												
37	LM												
38	LM												
39	LM												
40	LM	LI											
41	LM	LI											
42	LM								O				
43	LM												
44	LM												
45	LM						O						
46(2B)	LM												
47(4B)	LI		O			O	O			O			
48(21B)	LI		O			O	O	O		O	O		O
49(24B)	LI		O				O			O	O		
50(A)	LW												
51(A)	LW												
52(A)	LW												
53(A)	LW												
54	LG			O	O				O		O		O
55	LG			O	O								

Table 2. ***(Continued)***

Culture No.[a]	Correct ID[b]	1	2	3	4	5	6	7	8	9	10	11	12
56	LG		O		LM			O					O
57	O												
58	LW											O	
59	LW									LM			
60	LG	O		O	O			O	O	O	O	O	
61	LG	O		O	O			O			O	O	O
62	LG	O	O			O	O					O	
63	LG	O	LM			O	O				O		
64	O												
65	O												
66	O												
67	O												
68	O												
69	O												
70	O												
71	O												
72	O												
73	O												
74	O												
75	O												
76	O												
77	O												
78	O												
79	O												
80	O												
81	O												
82	O												
83	O												
84(50B)	LW												
85(51B)	LW												
86(52B)	LW												
87(53B)	LW												
88	LM												
89	LM												
90	LM												
91	LM												
92	LM												
93	LM												
94	LM												
95	LM												
96	LM		O										
97	LM												

[a] (A) = first replicate; (B) = second replicate of culture number indicated, e.g., (2B).
[b] LM = *L. monocytogenes/L. innocua* group; LI = *L. ivanovii/L. seeligeri* group; LW = *L. welshimeri*; LG = *L. grayi/L. murrayi* group; O = unidentified/other organism group.
[c] Presence of a code indicates a misidentification.

(*1*) Inoculating card contains mixed culture. Confirm by subculturing growth control well in either card to blood agar.

(*2*) Inoculating card contains isolate belonging to species or group not included in data base.

(*3*) Isolate is rare or unusual biotype.

(*4*) Inoculating card contains isolate that cannot be uniformly suspended in 0.45% saline.

(*5*) Inoculating card contains suspension less turbid than No. 0.5 McFarland standard for GPI card or No. 1.0 for GNI card.

When "Unidentified organism" message appears for GPI card, repeat test with same culture isolate stored in refrigerator. (*Note*: Because Gram-positive organisms are used in GNI card, receiving "Unidentified organism" message for GNI card is highly likely. Disregard such messages on GNI card.)

Good confidence, marginal separation.—Biochemical results for pair of organisms listed in either GPI or GNI report have good likelihood for identification but closely resemble both species patterns in data base.

Questionable biopattern.—Biochemical results of GPI or GNI card only marginally resemble 2 species or species groups in data base; isolate identification may be third taxon not listed on GPI or GNI report.

See notes 1–3 etc.—Messages are explained in appropriate Technical Bulletin accompanying either GPI or GNI card.

I. Confirmation

Organisms placed in LM category are identified as *Listeria monocytogenes* or *Listeria innocua*; in LI category, as *Listeria ivanovii* or *Listeria seeligeri*; in LW category, as *Listeria welshimeri*; and in LG category, as *Listeria grayi* or *Listeria murrayi*. Organisms in O category are classified as non-*Listeria* species. Further tests should be conducted to distinguish species within each category (refer to current edition of BAM).

Ref.: J. AOAC Int. (1993) **75**, July August issue.

Results

The total number of *Listeria* spp. isolates tested was 76 (Table 1), including 8 duplicates of strains that were shown to give variable results with the AutoMicrobic System (unpublished data). The strains chosen were isolated from food and environmental sources and represented all of the *Listeria* spp. serogroups available. The number of non-*Listeria* isolates totalled 21 (Table 1). The latter isolates were chosen on their ability to interfere with *Listeria* spp. during culture and isolation. The 97 isolates were tested by 13 collaborators on a total of 1261 GPI and GNI cards. All of the collaborators completed all of the isolates submitted. A single collaborator had contamination in all of the test kits, and these data were omitted. As a result, a total of 912 GPI/GNI test kits were tested with the *Listeria* spp. isolates, and 252 kits were tested with the non-*Listeria* isolates.

Collaborators' results for the cultures (Table 2) are presented as identified or misidentified. Table 3 summarizes the collaborators' results. Of the 912 *Listeria* spp., 828 were correctly identified; 84 replicates were misidentified. The sensitivity rate (number of *Listeria* found positive by the method divided by the number of *Listeria* positive) was 91.55% (Table 3). The numbers used to calculate this rate (835/912 vs 828/912) differed from the number shown correct for *Listeria* spp., because 7 replicates were incorrect but still classified as *Listeria* spp. (Table 2). The specificity rate (number of *Listeria* found negative by the method divided by the number of *Listeria* negative) was 100.0%.

The false-positive rate, which is the number of misclassified non-*Listeria* spp. divided by the total number of positives (misclassified non-*Listeria* spp. plus correctly identified *Listeria* spp.), was 0.0%. The false-negative rate, which is the number of misclassified *Listeria* spp. divided by the total number of negatives (misclassified *Listeria* spp. plus correctly classified non-*Listeria* spp.), was 25.0%. The majority of the misidentifications were either *L. ivanovii* (40), *L. grayi* (21), or *L. murrayi* (15) replicates. None of the 252 non-*Listeria* replicates tested was misidentified as *Listeria* spp. Misidentifications for *L. ivanovii* resulted from salicin-negative reactions, and *Staphylococcus auricularis* was identified on the GPI card. The misidentifications for *L. grayi* and *L. murrayi* were probably the result of too few positive reactions. In either case, better results can be obtained by repeating the test with the original, older culture. Because of the psychrotrophic nature of *Listeria* spp., we recommend that unidentified organisms be retested by using refrigerated cultures rather than fresh cultures. The system software can be modified to indicate this requirement.

Only 4 of the 72 LG replicates (0.05%) gave mannitol-negative results, and this test should be considered definitive for separating *L. grayi* and *L. murrayi* from the remaining *Listeria* species. Stuart and Welshimer (15) proposed a new classification scheme, which would place the organisms *L. grayi* and *L. murayi* in a new genus, *Murraya*, based on their ability to ferment mannitol.

For 7 of the 912 replicates (0.007%), the misidentifications resulted from an erroneous reaction in the GNI card for xylose or rhamnose, and the organism was misclassified to the wrong species group. This was not restricted to any 1 particular species but occurred within all of the species groups. Eleven of the tests in the GPI card are considered definitive for the generic identification of *Listeria* species and gave a positive result: bacitracin, opticin, 6% NaCl, 10% bile, 40% bile, esculin, tetrazolium red, glucose, salicin, trehalose, and cellobiose.

The results obtained with the conventional method (1) were as follows: sensitivity rate, 84.2%; specificity rate, 100%; false-negative rate, 36.3%; and false-positive rate, 0%. Of the 76 *Listeria* species replicates tested with the conventional method, 6 LM replicates, 2 LI replicates, 3 LW replicates, and 1 LG replicate were misidentified. None of the 21 non-*Listeria* replicates was misidentified by the conventional method.

Table 3. Summary of collaborators' results for biochemical identification of *Listeria* spp. isolates by automated system

Species group/organism	No. tested	No. correct	No. incorrect	% Correct
LM	492	486	6	98.8
L. monocytogenes	372	367	5	98.6
L. innocua	120	119	1	99.2
LI	216	175	41	81.0
L. ivanovii	156	116	40	74.3
L. seeligeri	60	59	1	98.3
LG	84	48	36	57.1
L. grayi	48	27	21	56.2
L. murrayi	36	21	15	58.3
LW	120	119	1	99.2
L. welshimeri	120	119	1	99.2
O	252	252	0	100
Non-*Listeria* spp.	252	252	0	100
Total isolates	1164	1080	84	93

Conclusion

The AutoMicrobic System method provides an objective, rapid alternative to conventional biochemical tests. The accuracy of the GPI and GNI 2-card method compares favorably with the conventional method (1) for biochemical characterization of *Listeria* species. Reports of unidentified organisms on the GPI card should be investigated with either a retest of the original culture or by conventional biochemical tests (1). As is the case with the conventional method, CAMP, β-hemolysis, and/or nitrate reduction tests (1) are required for final confirmation of some of the *Listeria* species.

Recommendation

The results of this collaborative study indicate that the AutoMicrobic System method, using both the GPI and GNI test kits, is a reliable alternative to the conventional method (1) for the biochemical characterization of foodborne *Listeria* species and for the screening and elimination of non-*Listeria* isolates. For speciation of some *Listeria*, the analyst must perform CAMP, β-hemolysis, and/or nitrate reduction tests as outlined in BAM (1). This automated system provides the following data:

(*1*) Cultures classified as *Listeria monocytogenes/Listeria innocua* are confirmed as *Listeria monocytogenes*, if the culture demonstrates β-hemolysis and enhanced hemolysis with the *Staphylococcus aureus* streak of the CAMP test, or *Listeria innocua*, if the culture is negative for β-hemolysis and enhanced hemolysis with the *S. aureus* streak of the CAMP test.

(*2*) Cultures classified as *Listeria ivanovii/Listeria seeligeri* are confirmed as *Listeria ivanovii*, if the culture demonstrates β-hemolysis and enhanced hemolysis with the *Rhodococcus equi* streak of the CAMP test, or *Listeria seeligeri*, if the culture is positive for β-hemolysis and enhanced hemolysis with the *S. aureus* streak of the CAMP test.

(*3*) Cultures classified as *Listeria welshimeri* are identified as *Listeria welshimeri*.

(*4*) Cultures classified as *Listeria grayi/Listeria murrayi* are confirmed as *Listeria grayi*, if the culture is unable to reduce nitrate, or *Listeria murrayi*, if the culture is able to reduce nitrate.

(*5*) Cultures classified as non-*Listeria* are disposed of as non-*Listeria* spp.

Cultures that do not conform to any of the above criteria should be characterized by additional tests specified in *Bergey's Manual of Systematic Bacteriology* (16) or sent to a reference laboratory for definitive identification.

We recommend that the AutoMicrobic System method be adopted first action for the purposes described.

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MICROBIOLOGICAL METHODS

Comparison of MICRO-ID *Listeria* Method with Conventional Biochemical Methods for Identification of *Listeria* Isolated From Food and Environmental Samples: Collaborative Study

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Fourteen laboratories participated in a collaborative study to evaluate the ability of the MICRO-ID *Listeria* identification method to correctly identify *Listeria* isolated from food and environmental sources. Each collaborator received 60 isolates consisting of 51 *Listeria* and 9 non-*Listeria* cultures. All isolates were identified by conventional biochemical analyses in the principal laboratory. Cultures were checked for purity by Gram staining and examined for oxidase and catalase activities. Only Gram positive, oxidase negative, catalase positive cultures were tested with the method. Colonies from trypticase soy agar with 0.6% yeast extract were suspended in 4.6 mL physiological saline to a MacFarland No. 1 turbidity standard and used to inoculate the test strip. In addition, the hemolytic reaction of each isolate was determined by using the Christie-Atkins-Munch-Peterson (CAMP) test and by stabbing sheep blood agar. Identification of *Listeria* is based on the octal code obtained from the strip and the hemolytic reaction of the isolate. The MICRO-ID *Listeria* method agreed with conventional biochemical identification for 98.0% of *L. monocytogenes*, 77.1% of *L. seeligeri*, 90.0% of *L. ivanovii*, 96.4% of *L. grayi*/*L. murrayi*, 73.9% of *L. welshimeri*, and 100% of *L. innocua* isolates. A large percentage of errors in identification of the *L. seeligeri* and *L. ivanovii* cultures was caused by inaccurate reading of the CAMP and hemolysis tests rather than errors in the test strip. The method was adopted first action by AOAC International.

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The recommendation was approved by the General Referee and the Committee on Microbiology and Extraneous Materials and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, Jan/Feb issue.

Screening of food for *Listeria* sp. has increased in magnitude over the last several years because of the recognition of *L. monocytogenes* as the causative agent of a number of foodborne disease outbreaks (1). The conventional U.S. Food and Drug Administration (FDA) method found in the *Bacteriological Analytical Manual* (2) for the identification of *Listeria* sp. requires the use of standard biochemical and serological tests, which can take up to 7 days to complete. Therefore, acceptance of rapid and reliable methods for the species identification of *Listeria* isolates would be of benefit. Several diagnostic kits for the rapid identification of *Enterobacteriaceae* from foods and clinical samples have been evaluated (3–5). A collaborative study by Poelma et al. (3) led to the adoption by AOAC of 3 of the diagnostic kits as alternatives to conventional biochemical methods for presumptive generic identification of foodborne *Salmonella*. Later, a collaborative study by Keelan et al. (4) evaluated the MICRO-ID system (Organon Teknika Corp., Durham, NC) for identification of foodborne and clinical enterics, and the method was adopted by AOAC (**989.12**) (6). Knight et al. (5) collaboratively evaluated use of the Gram Negative Identification Card, which was also adopted by AOAC.

More recently, Organon Teknika Corp. developed a version of MICRO-ID, MICRO-ID *Listeria*, which allows the analyst to identify members of the genus *Listeria*. The MICRO-ID *Listeria* method, which includes several conventional tests, identifies the various species of the genus *Listeria* in 24–48 h. The API 20 Strep system (Analytab Products) identifies gram-positive isolates to the genus level (7). The objective of the present study was to collaboratively evaluate the MICRO-ID *Listeria* method for species identification of *Listeria* isolated from food, environmental, and other sources.

Collaborative Study

Bacterial Isolates

Sixty bacterial isolates (Table 1) and 5 control isolates were shipped overnight to each collaborator on slants of trypticase soy agar with 0.6% yeast extract (TSA-YE). Each isolate was identified by a 1 or 2 digit code.

Table 1. Bacterial isolates used for collaborative evaluation of MICRO-ID *Listeria* method

Organism	Culture No.	No. isolates	Source[a]
	Listeria		
L. monocytogenes	1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 23, 26, 28, 30, 32, 33, 37, 39, 42, 44, 47, 50, 52, 56, 59	25[b]	ATCC, USDA, FDA, food
L. seeligeri	2, 18, 22, 38, 46	5	ATCC, USDA, FDA, food
L. ivanovii	4, 10, 35, 41, 54	5	ATCC, USDA, environment
L. murrayi	40, 53, 57	3	ATCC
L. grayi	25, 49, 60	3	ATCC
L. welshimeri	20, 24, 27, 48, 55	5	ATCC, USDA
L. innocua	12, 14, 21, 36, 45	5	ATCC, FDA, USDA, food, environment
	Non-*Listeria*		
Bacillus sp.	6, 8, 16, 29	4	ATCC
Brevibacterium	51	1	food
Brocothryx	31	1	ATCC
Cellulomonas	34, 43	2	ATCC
Jonesia	58	1	ATCC

[a] ATCC = American Type Culture Collection, USDA = U.S. Dept of Agriculture, FDA = U.S. Food and Drug Administration.
[b] Represents serotypes 1, 1a, 1b, ½a, ½b, ½c, 2, 3, 3a, 4a, 4b, 4c, 4d, 4e, flagellar A, flagellar D, and flagellar C.

Collaborators were provided with a sufficient number of test strips to identify all isolates. Concurrently, each isolate was identified by conventional biochemical methods (2) at Organon Teknika Corp., Durham, NC.

Isolates were shipped to collaborators in 3 sets of 20 cultures, ca 2 weeks apart. Collaborators streaked each culture onto a TSA-YE plate and incubated the plates for 24 h at 30°C. Cultures were Gram stained and examined for oxidase and catalase activities. Only pure, Gram positive, oxidase negative, catalase positive cultures were tested with the method. Colonies from each TSA-YE plate were suspended in 4.6 mL of sterile, physiological saline so that the turbidity was equivalent to at least a MacFarland No. 1 standard. These suspensions were used to inoculate test strips, which were incubated for 24 h at 37°C.

Identification of *Listeria* sp. by using the method requires that the hemolytic reaction of the isolate be known. Therefore, collaborators tested for hemolysis by using the Christie-Atkins-Munch-Peterson (CAMP) and the stab hemolysis tests. Identification of *Listeria* was based on the octal code obtained from the strip and the hemolytic reaction of the isolate. On completion of analyses, collaborators reported the octal code and culture identification as described in the method. In addition, each isolate was identified by conventional biochemical methods (2) at the principal laboratory and compared with the results from collaborators using the test strip. Results for the comparison of *Listeria* sp. were tabulated as correctly identified (I); U, unidentified; M, misidentified; and N, not determined.

992.18 *Listeria* species Biochemical Identification Method (MICRO-ID *Listeria*)

First Action 1992

This method is for the presumptive generic identification of *Listeria* species isolated from foods and environmental samples. Use of commercial biochemical kit as alternative to conventional biochemical testing is based on demonstration in analyst's laboratory of adequate correlation between biochemical kit and conventional biochemical tests as described in current edition of *Bacteriological Analytical Manual* (2).

Caution: Potential biohazard. Bacteria used in method are potentially pathogenic microorganisms. Extreme care should be taken in handling and pipetting bacterial solutions. Reaction products, bacterial cultures, tubes, pipets, and test trays should be placed in biohazard bags and sterilized in autoclave before disposal according to laboratory guidelines. All surfaces should be decontaminated with an appropriate disinfectant; hands should be washed with bactericidal soap.

Method Performance

See Table **992.18A** for method performance data.

A. Principle

Method depends on principle that test inoculum contains preformed enzymes that can be detected after 24 h at 37°. Test strip contains filter paper discs impregnated with chemical substrate/reagents specific for enzymes endogenous to *Listeria* sp. Substrates are converted to metabolic end products that react with detection system to produce visible color changes. Tests

Table 992.18A. Method performance for identification of *Listeria* and selected non-*Listeria* isolates, MICRO-ID *Listeria* test

Organism	Correct	% Correct	Misidentified	Unidentified	Total
			Listeria		
L. monocytogenes	339	98.0	3	4	346
L. seeligeri	54	77.1	15	1	70
L. ivanovii	63	90.0	6	1	70
L. murrayi	39	92.9	1	2	42
L. grayi	42	100.0	0	0	42
L. welshimeri	51	73.9	15	0	69
L. innocua	70	100.0	0	0	70
			Non-*Listeria*		
Bacillus sp.	56	100.0	0	0	56
Brevibacterium	14	100.0	0	0	14
Brocothryx	10	71.4	4	0	14
Cellulomonas	26	92.8	2	0	28
Jonesia	13	92.8	1	0	14

Overall sensitivity and specificity rates for MICRO-ID test *Listeria* identification by organism type

Type	Total	Correct	% Correct	
Listeria	709	658	92.8	Sensitivity rate
Non-*Listeria*	126	119	94.4	Specificity rate

included in strip are listed in Table **992.18B**. Each test is reported as either positive or negative. Typical results for each *Listeria* sp. are given in Table **992.18C**.

B. Apparatus

(**a**) *MICRO-ID Listeria Identification System.*—(*1*) Plastic trays containing 15 biochemical tests (*See* Table **992.18B**). (*2*) Coding forms. (*3*) Color guide. Available from Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704, USA. Components of kit are intended for use only with MICRO-ID *Listeria* identification system.

(**b**) *Test tubes.*—Sterile test tubes, 16 × 100 mm or larger, one per isolate to be identified.

(**c**) *Inoculating loops.*—3 mm loops with handle. Sterile.

(**d**) *Culture dishes.*—100 × 15 mm polystyrene Petri plates (disposable). Sterile.

(**e**) *Incubator.*—Non-CO_2. Capable of 30 and 37°, maintained to ±0.5°.

(**f**) *Support racks.*—To hold test kit units (Organon Teknika Corp. is suitable source.)

(**g**) *MacFarland No. 1 turbidity standard.*—Prepare 1% H_2SO_4 solution (dilute 1.04 mL conc. H_2SO_4 to 100 mL with H_2O). (*Caution*: H_2SO_4 is very corrosive; avoid contact with body and laboratory materials.) Prepare 1% $BaCl_2$ solution (dilute 1.2 g $BaCl_2 \cdot 2H_2O$ to 100 mL with H_2O). Add 1.0 mL 1% $BaCl_2$ solution to 99.0 mL 1% H_2SO_4 solution with stirring. Dispense 8.0 mL in 16 × 100 mm tubes; cap and mix well.

C. Culture Media and Reagents

Use distilled or deionized H_2O throughout.

(**a**) *Trypticase soy agar with 0.6% yeast extract (TSA-YE) plates.*—15 g trypticase or tryptone (pancreatic digest of casein), 5 g soytone (papaic digest of soybean meal), 5 g NaCl, 6 g yeast extract, and 15 g agar per 1 L H_2O. (Or use commercially available materials.) Heat to boiling to dissolve ingredients completely. Sterilize by autoclaving 15 min at 121°. Cool medium to 50° and aseptically pour ca 20 mL/Petri plate or 6–8 mL/slant.

(**b**) *Physiological saline.*—Dissolve 8.5 g NaCl in 1 L H_2O. Sterilize by autoclaving 15 min at 121°. Final pH must be 6.0 ± 0.5. Do not use saline containing preservatives such as Na azide or other bacterial growth inhibitors.

(**c**) *20% KOH solution.*—Slowly add 20 g KOH pellets to 60 mL H_2O. Stir to dissolve. Dilute with H_2O to 100 mL. Store KOH solution in tightly closed container when not in use. (Caution: Caustic reagent. Avoid contact with body and laboratory materials.)

(**d**) *5% TSA sheep blood agar plates.*—Combine 15.0 g trypticase, 5.0 g phytone peptone (papaic digest of soybean meal), 5.0 g NaCl, and 15.0 g agar; dilute to 1 L with H_2O. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve. Autoclave 15 min at 121° and cool to 48° in water bath. Add 50 mL sterile defibrinated sheep blood per L medium. Mix well and dispense

Table 992.18B. Biochemical assays contained in MICRO-ID *Listeria* test

Test	Positive reaction	Negative reaction
Voges-Prokauer, VP	Pink to red	Light yellow
Nitrate reductase, N	Red	Colorless to light pink
Phenylalanine deaminase, PD	Green	Light yellow
Hydrogen sulfide, H_2S	Light brown	White
Indole, I	Pink to red	Light yellow to orange
Ornithine decarboxylase, OD	Purple to red-purple	Amber to yellow
Lysine decarboxylase, LD	Purple to red-purple	Amber to yellow
Malonate utilization, M	Green to blue	Yellow
Urease, U	Orange to red-purple	Yellow
Esculin hydrolysis, E	Brown to black	Colorless or beige
β-Galactosidase, ONPG	Light yellow to yellow	Colorless
Xylose fermentation, XYL	Yellow to amber	Red-purple to purple
Rhamnose fermentation, RHAM	Yellow to amber	Red-purple to purple
Mannitol fermentation, MANN	Yellow to amber	Red-purple to purple
Sorbitol fermentation, SORB	Yellow to amber	Red-purple to purple

18–20 mL into Petri dishes or 6–8 mL/slant. Commercially prepared sheep blood agar plates are available.

(**e**) *3% Hydrogen peroxide solution.*—Dilute 1 mL 30% hydrogen peroxide solution with 9 mL H_2O. (Caution: 30% hydrogen peroxide is a strong oxidizer. Avoid all contact with body. Contact with other materials may cause fire.)

(**f**) *Cytochrome oxidase test reagent.*—Tetramethyl-*p*-phenylenediamine dihydrochloride (Becton Dickinson, Microbiology Systems, Cockeysville, MD 21030, USA, is suitable source) or dissolve 1.0 g *p*-amino dimethylphenylenediamine monohydrochloride/100 mL H_2O.

(**g**) *CAMP test organisms.*—(*1*) β-Hemolytic *Staphylococcus aureus* (NCTC 1748). (2) *Rhodococcus equi* (NCTC 1621). Streak each culture from frozen stock or fresh slants on separate 5% TSA sheep blood agar plates, and incubate 24 h at 35–37°.

D. System Suitability

The following organisms are used as quality control indicators: *Listeria monocytogenes*-ATCC 19111, *Listeria seeligeri*-ATCC 35967, *Listeria grayi*-ATCC 25400, *Streptococcus cremoris*-ATCC 19257, and *Streptococcus mitis*-ATCC 6249. The biochemical reactions for each *Listeria* organism are given in Table **992.18C**. No positive reactions should be observed with the *Streptococcus* species.

E. Preparation of Inocula

Select single isolated colony from primary isolation plate and streak on TSA-YE plate or slant. Incubate 24–26 h at 30°. Perform Gram stain, catalase, and oxidase tests below on isolated colonies from TSA-YE.

(**a**) Perform Gram stain on 24 h TSA-YE agar cultures as **966.24(e)**.

(**b**) For catalase test, transfer loopful of isolated colony to glass slide, and add 1–2 drops 3% hydrogen peroxide solution. Evolution of gas bubbles (effervescence) indicates catalase positive culture.

(**c**) For oxidase test, transfer loopful of colony to filter paper. Add few drops of oxidase test reagent to smear. If cells turn light to dark blue, culture is oxidase positive. Alternatively, add

Table 992.18C. Reactions of *Listeria* sp. using MICRO-ID *Listeria* test[a]

	MICRO-ID *Listeria* reactions																	
	CAMP																	
Strain	Sa[b]	Re[c]	Hemolysis	VP	N	PD	H_2S	I	OD	LD	M	U	E	ONPG	XYL	RHAM	MANN	SORB
L. monocytogenes	+	−	+	+	−	−	+	−	−	−	−	−	+	±	−	+	−	±
L. seeligeri	+	−	+	+	−	−	+	−	−	−	−	−	+	−	+	−	−	±
L. ivanovii	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	−	−	±
L. grayi	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	+	±
L. murrayi	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	±	+	±
L. welshimeri	−	−	−	+	−	−	+	−	−	−	−	−	+	−	+	±	−	±
L. innocua	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	±	−	±

[a] See Table **992.18B** for abbreviations.
[b] Hemolysis enhancement in the vicinity of *S. aureus*.
[c] Hemolysis enhancement in the vicinity of *R. equi*.

several drops of *p*-amino dimethylphenylenediamine monohydrochloride reagent to colony. A positive reaction is a color change of pink to red to black within 20 min.

Only Gram positive, oxidase negative, catalase positive cultures should be tested with this method. *Note*: Sterile tubes and sterile saline are required. Saline should be freshly prepared and must have pH of 6.0 ± 0.5.

Pipet 4.6 mL physiological saline into sterile tube. Aseptically transfer growth from TSA-YE plate into tube and mix using vortex mixer. Inoculum preparation is complete when turbidity of suspension is equivalent to at least MacFarland No. 1 turbidity standard.

F. Inoculation

(**a**) Place strip on flat surface. Do not remove clear plastic tape covering test wells. Open cover and pipet ca 0.3 mL cell suspension, *Preparation of Inocula*, into each inoculation well at top of unit. Close cover and stand strip upright in support rack. Check that cell suspension is in contact with all substrate discs, but do not moisten detection discs.

Incubate all strips at 35–37°. After 4 h, examine esculin (E) and rhamnose (R) reactions, and record preliminary readings on coding forms. Reincubate strips an additional 20 h at 35–37°.

(**b**) For CAMP test (on each isolate), streak β-hemolytic *S. aureus* and *R. equi* [*Culture Media and Reagents*, (**g**)*1* and *2*, respectively] vertically on sheep blood agar plate. Separate vertical streaks so test strains may be streaked horizontally between them without touching them. Streak test strains parallel to each other and perpendicularly between streaks of *S. aureus* and *R. equi*. Streaks of test strains should be no closer than ½ in. apart and ¼ in. from *S. aureus* and *R. equi* streaks. See Figure **992.18**. Incubate plate 24 h at 35°. After incubation, examine plates for hemolysis in zone of influence of vertical streaks. Hemolysis exhibited by *L. monocytogenes* and *L. seeligeri* is enhanced in vicinity of *S. aureus* streak, whereas *L. ivanovii* hemolysis is enhanced near *R. equi* streak. *L. seeligeri* hemolysis may be weak and difficult to read; see *Reading of Results*, (**b**). All other *Listeria* sp. remain nonhemolytic in this test.

(**c**) For stab hemolysis test, stab each test isolate into sheep blood agar by using sterile wire or small loop. Incubate plate 24 h at 35–37°. Hemolysis by *L. seeligeri* strains is more easily detected by this test.

G. Reading of Results

(**a**) *Test strip.*—After 24 h incubation, place strip flat, open lid, and add 2 drops (ca 0.1 mL) 20% KOH solution to inoculation well of Voges-Proskauer test only.

Close lid and hold strip upright. Ensure that KOH solution flows into VP test solution. Rotate strip clockwise 90° so upper detection discs in first 5 wells are moistened. Hold strip upright and tap gently on lab surface to dislodge any suspension trapped under upper discs. Read all reactions immediately, except VP test, as positive or negative according to color changes listed in Table **992.18B**. Let color develop in VP well for ca 10 min. Read color of upper disc for first 5 tests and color of organism suspension for remaining 10 tests.

(**b**) *CAMP and β-hemolysis tests.*—Hemolysis reactions are more easily detected by holding sheep blood plates up to light for examination. Record results of enhanced hemolysis reactions in region of *S. aureus* and *R. equi* streaks. Record hemolysis reactions around each stab on sheep blood agar plate.

H. Interpretation of Results

Differentiation of *Listeria* species is based on octal code, CAMP test, and hemolysis reaction results. Enter test results in appropriate spaces on coding form as either positive or negative. To determine octal code for each isolate, add numerical values for each group of 3 tests and enter sum in appropriate space on "Sum of Positive Values" form. Enter zero if all tests in group of 3 are negative. Locate octal code in table provided in package insert with strip. Identify organisms based on octal code, CAMP, and hemolysis results. Alternatively, use Table **992.18C**.

Ref.: J. AOAC Int. (1993) **76**, July/August issue.

Results

All test isolates were identified by the FDA method for the identification of *Listeria* sp. (2) at Organon Teknika. All *Listeria* isolates were small Gram-positive rods and exhibited motility in wet mount and sulfide indole motility agar. They were cytochrome oxidase negative, catalase positive, and did not hydrolyze urea. *Listeria* isolates produced an acid slant and butt in triple sugar iron agar without production of H_2S and used dextrose, esculin, maltose, and were MR and VP positive. *Lis-*

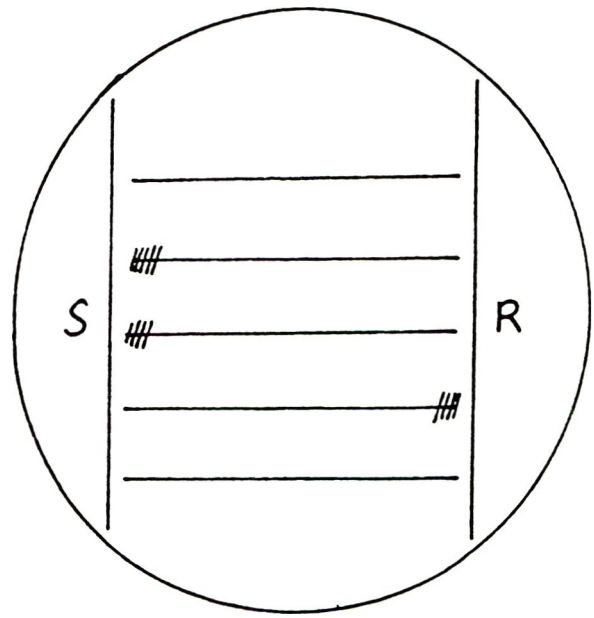

Figure 992.18. CAMP test for *Listeria monocytogenes* (2): Inoculation pattern of sheep blood agar plate. Horizontal lines represent streak inoculation of 5 test strains. Vertical lines represent streak inoculation of *Staphylococcus aureus* (S) and *Rhodococcus equi* (R). Hatched lines indicate (diagrammatically only) locations of hemolysis enhancement regions.

Table 2. Collaborators' results for identification of bacterial isolates by MICRO-ID *Listeria* method[a]

	Collaborator													
Culture No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2	M	M	I	I	I	I	I	I	M	I	I	U	I	I
3	I	I	I	I	I	I	I	I	I	I	I	U	I	I
10	I	I	I	I	I	I	I	I	I	I	I	U	I	I
13	N	I	I	I	I	I	I	I	I	I	I	I	I	I
15	I	I	I	I	I	I	I	I	I	I	I	I	U	I
17	N	I	I	I	I	I	I	I	I	I	I	I	I	I
18	M	M	I	I	I	M	I	I	I	I	I	I	I	I
20	I	I	I	I	I	M	I	I	M	M	I	U	I	I
22	M	M	I	M	I	I	I	I	I	I	I	M	I	I
23	N	I	I	I	I	I	I	I	I	I	I	U	I	I
24	M	I	I	M	M	I	I	I	M	I	I	I	I	I
27	M	I	I	M	M	I	I	I	M	I	I	I	I	I
30	I	I	I	I	I	I	I	I	I	I	I	I	I	M
31	M	I	M	I	I	I	I	I	I	M	I	I	I	M
34	I	I	M	I	I	I	I	I	I	I	I	I	I	I
35	I	I	I	I	I	I	I	I	I	I	I	I	I	M
38	I	M	I	M	I	I	I	I	I	I	I	I	I	I
39	I	I	I	I	I	I	I	I	I	I	I	U	I	I
40	I	I	I	I	I	U	I	I	I	I	I	I	I	I
41	M	I	I	I	I	I	I	I	I	I	I	I	I	M
43	I	I	I	M	I	I	I	I	I	I	I	I	I	I
46	I	M	M	I	I	I	I	I	I	I	I	U	I	I
48	M	I	I	I	I	I	I	I	I	I	I	M	I	I
50	I	I	I	N	I	I	I	I	I	I	I	I	I	I
53	I	U	I	I	I	I	I	I	I	I	I	I	I	I
54	M	I	I	I	I	I	I	I	I	I	M	I	I	M
55	M	I	M	I	M	I	I	I	M	I	I	M	I	I
57	I	U	I	I	I	M	I	I	I	I	I	I	I	I
58	I	I	M	I	I	I	I	I	I	I	I	I	I	I

[a] A total of 60 cultures were analyzed; those not listed were identified by each collaborator: I = identified; M = misidentified; N = not determined; U = unidentified.

teria were negative for nitrate reduction except for the 3 *L. murrayi* isolates. *L. welshimeri*, *L. monocytogenes*, 2 *L. innocua* isolates used rhamnose, and 3 *L. innocua* isolates used mannitol. *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* isolates used xylose. *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* isolates produced hemolysis in sheep blood stabs and were positive to the CAMP test. *L. monocytogenes* and *L. seeligeri* exhibited enhanced hemolysis at the *S. aureus* stab, and *L. ivanovii* showed enhanced hemolysis at the *R. equi* streak. None of the non-*Listeria* isolates produced biochemical results identical to any *Listeria* sp.

Data from Table 2 classify cultures as identified (I), misidentified (M), unidentified (U), and not determined (N) by each collaborator. These data are summarized by organism in Tables 3 and 4, which include both uncorrected and corrected values for the percentage of agreement with conventional culture technique. The values were corrected when the strip gave the correct octal code, yet the organism was misidentified because of a mistake in a conventional technique (CAMP, stab hemolysis, Gram stain, catalase, and/or oxidase tests).

Initial analysis of raw data indicated that the MICRO-ID *Listeria* method agreed with conventional identifications on 100% of the *L. innocua* and *L. grayi* isolates, 98.0% of the *L. monocytogenes* strains, 92.9% of the *L. murrayi* cultures, 90.0% of the *L. ivanovii* isolates, 77.1% of the *L. seeligeri* organisms, and 73.9% of the *L. welshimeri* strains (Table 3). When errors in conventional microbiological tests were identified, the percentage of agreement between the method and cultural identification improved to 98.6% for *L. monocytogenes* and *L. ivanovii*, 91.4% for *L. seeligeri*, and 81.2% for *L. welshimeri* (Table 4). In total, *Listeria* sp. were identified correctly 92.7% of the time (95.9% when corrected for errors in conventional techniques). In addition, 5.6% of the non-*Listeria* isolates were misidentified as either *L. murrayi* or *L. grayi* (Table 5). Four of 14 *Brocothryx* isolates, 2 of 28 *Cellulomonas*, and 1 of 14 *Jonesia* isolates were misidentified. The number of misidentifications for all isolates tested ranged from 0 to 13 per collaborator, with an average number of 4.6 per collaborator and 7.7 per 100 isolates examined.

Table 3. Identification of *Listeria* sp. by collaborators using MICRO-ID *Listeria* method

Organism	No. isolates examined	No. isolates identified	Correct ID No.	Correct ID %[a]
L. monocytogenes	346	342	339	98.0
L. seeligeri	70	68	54	77.1
L. ivanovii	70	69	63	90.0
L. murrayi	42	40	39	92.9
L. grayi	42	42	42	100
L. welshimeri	69	69	51	73.9
L. innocua	70	70	70	100
Total	709	700	658	92.8

[a] % correct identification = (No. correct/no isolates examined) × 100.

Table 5. Misidentification of non-*Listeria* sp. by collaborators using MICRO-ID *Listeria* method[a]

Genus	No. isolates examined	No. isolates misidentified	% Mis-ID[b]
Bacillus sp.	56	0	0.0
Brevibacterium	14	0	0.0
Brocothryx	14	4	28.6
Cellulomonas	28	2	7.1
Jonesia	14	1	7.1
Total	126	7	5.6

[a] Non-*Listeria* sp. were misidentified as either *L. murrayi* or *L. grayi*.
[b] % Mis-ID = (No. isolates misidentified/No. isolates examined) × 100.

Discussion

The MICRO-ID *Listeria* method compared favorably with conventional biochemical methods for identification of *Listeria* to the genus and species level. The use of the method also reduced the time required to identify *Listeria* to the species level from 7 days to 24–48 h. As mentioned, misidentifications could be separated into 2 groups: those resulting from the test strip and those resulting from conventional techniques (CAMP, stab hemolysis, etc.). More errors were associated with the CAMP and stab hemolysis tests than with other conventional tests. These tests accounted for 2 of 3 misidentifications of *L. monocytogenes*, 10 of 14 misidentifications of *L. seeligeri*, and 5 of 6 misidentifications of *L. ivanovii*. The analyst must be familiar with the hemolytic reactions of *Listeria* sp. and competent with the CAMP and stab hemolysis tests before using the method. The quality of the blood used in the medium for these tests is also very important, because partially hemolyzed blood plates can adversely affect test results.

Moreover, *L. welshimeri* isolates were correctly identified at a lower percentage, 81.2%, than were other *Listeria* sp. *L. welshimeri* was misidentified almost exclusively as *L. innocua* because of a false-negative xylose fermentation reaction. The xylose reaction is the slowest of all the biochemical tests. If an inoculum is low in cell density, the xylose reaction can be delayed, resulting in a false-negative result in the 24 h incubation period. Analysts should be certain that the turbidity of the suspension is at least a MacFarland No. 1 turbidity standard, and turbidity can be as high as a MacFarland No. 2 to avoid this problem. *L. ivanovii* and *L. seeligeri*, also xylose fermentors, did not exhibit false-negative xylose reactions.

Of the 346 *L. monocytogenes* isolates analyzed in the study, 333 (96.2%) were rhamnose- and esculin-positive after only 4 h of incubation, indicating presumptive *L. monocytogenes* cultures. Therefore, approximately 3.8% of the *L. monocytogenes* isolates would have been missed if the strips had not been incubated for the full 24 h period.

One other observation is worth noting. Five misidentifications were caused by a false-positive nitrate test: 3 for *L. murrayi*, 1 for *L. monocytogenes*, and 1 for *L. seeligeri*. *L. murrayi* is the only *Listeria* sp. that can reduce nitrate (2). However, *L. murrayi* does not give a positive reaction for nitrate reduction by the test strip. The reason for these false-positive nitrate tests is unclear.

Table 4. Corrected values for identification of *Listeria* sp. by collaborators using MICRO-ID *Listeria* method

Organism	No. errors	No. CE[a]	% ID	% corrected ID[b]
L. monocytogenes	3	2	98.0	98.6
L. seeligeri	14	10	77.1	91.4
L. ivanovii	6	5	90.0	98.6
L. murrayi	1	0	92.9	92.9
L. grayi	0	0	100	100
L. welshimeri	18	5	73.9	81.2
L. innocua	0	0	100	100
Total	42	22	92.7	95.9

[a] Number of errors due to conventional microbiology techniques.
[b] % corrected ID = [(No. correct ID + No. CE)/No. isolates examined] × 100. See Table 3.

Recommendation

On the basis of the results of this collaborative study, the MICRO-ID *Listeria* method, which includes several conventional tests, is a suitable alternative to FDA-approved conventional biochemical methods (2) for the species identification of foodborne and environmental *Listeria* and for screening and elimination of non-*L. monocytogenes* isolates. We recommend that this method be adopted first action for the purposes described.

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MICROBIOLOGICAL METHODS

Total Bacterial Count in Raw Milk Using the BactoScan 8000

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The BactoScan 8000 (Foss Electric) automatically performs a total count of bacteria in raw milk. It was designed for fast, repeatable counting. The apparatus counts the individual bacteria (IBC) instead of colony forming units (CFU). IBC provides more information on the state of milk hygiene than the CFU count. The repeatability, s_r, was 0.033 log (IBC/μL), which is equivalent to a coefficient of variation of 7.9% (IBC/μL). The carryover, which is the contamination one sample imposes on the next, was 0.19%. The BactoScan 8000 was calibrated against the reference method of counting colonies on a Petri dish. The correlation coefficient for 429 milk samples was $r = 0.88$, and the standard deviation of the residuals, s_{yx}, was 0.259. The detection limit was 15 CFU/μL. We recommend establishing a system of payment to farmers based on the bacteriological quality of their milk as determined by the BactoScan 8000. Because results are obtained in IBC/μL, analytical differences resulting from conversion to CFU/μL are eliminated.

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Because many methods are available for evaluating the bacteriological quality of raw milk, the choice of method depends on the aim of the evaluation. When analyzing a small number of milk samples, the cost of the process and its labor intensiveness are of secondary importance; when a very large number of samples are involved, as in the case of the payment for quality made to farmers, a high level of automation and reduction of laboratory costs are very important.

The BactoScan 8000 (Foss Electric) automatically assesses the bacteriological quality of ≤80 samples of raw milk per hour.

Because of the treatment undergone by the samples, the instrument counts individual bacteria (IBC/μL) rather than colony forming units (CFU/μL).

Because use of this instrument is becoming more widespread, comparisons with the reference method, which is based on counting colonies in a Petri dish, should be carried out in different milk-producing areas. Dasen et al. (1) and O'Connor and O'Riordan (2) evaluated the BactoScan 8000 in France and Ireland, respectively, where cattle raising is highly developed and most farmers produce milk of very high hygienic quality with a very low microbial count. In this study, the BactoScan 8000 was evaluated in Spain, one of the countries where the bacteriological quality of milk varies widely.

Experimental

BactoScan 8000

After the milk samples are heated to 40°C in a water bath, they are placed in the apparatus, which automatically takes 2.5 mL milk, places it in the mixing chamber, and adds 7.5 mL of a lysing solution. The lysing solution is an alkaline solution of EDTA disodium salt, which binds the calcium to dissolve the casein micelles, and a detergent, which dissolves the fat and breaks the membranes of the somatic cells without altering bacteria. The liquid then passes to a centrifuge (42 600 rpm) where the bacteria are separated with the aid of 2.2 mL of a saccharose–glycerol solution, which creates a density gradient. The suspension of separated bacteria is then incubated 3 min at 40°C with 3 mL of a proteolytic enzyme solution to eliminate any remaining proteins. Finally, the bacteria are stained with 1 mL of an acridine orange solution and deposited as a film on a disc, which is rotated under the objective of an epifluorescence microscope equipped with a xenon lamp. The light pulses produced by each stained bacterium are counted and displayed on the screen as IBC/μL, which can be converted into CFU/μL once the apparatus has been calibrated.

After analysis of one sample, all parts of the apparatus contaminated with milk are automatically cleaned.

The necessary chemicals are supplied by the manufacturer as a kit.

Colony Counting on a Petri Dish

International Dairy Federation (IDF) Standard 100-A (3) was followed. All determinations were carried out in duplicate.

Inhibitor Detection

The brilliant black reduction test was used according to IDF Bulletin No. 220 (4). Samples in which inhibitors were detected were not used to calibrate the instrument.

Samples

Bacteria were counted by using the BactoScan 8000 and the reference method on 429 milk samples, covering a range of 25 to 49 547 IBC/μL. Samples were obtained from dairy farmers with refrigeration tanks in Galician, Spain, and were collected according to Spanish regulations (5).

Table 1. Repeatability standard deviation and coefficient of variation for different bacterial contents

IBC/μL	s_r, log (IBC/μg)	CV, %
<200	0.054	13.2
300	0.040	9.6
1000	0.030	7.2
10000	0.013	3.0
35000	0.009	2.1
Total	0.033	7.9

Results and Discussion

Repeatability

Repeatability was assessed according to IDF Standard No. 128 (6) with samples of differing bacterial content. Table 1 lists the values for standard deviation (s_r) in log (IBC/μL). The repeatability was better for samples with a high bacterial content, and the value obtained for a large group of samples with a wide range of bacterial contents was $s_r = 0.033$, which is equivalent to a coefficient of variation of 7.9% (IBC/μL). The repeatability is much better with the BactoScan 8000 than with the reference method.

Carryover

Carryover is the error a sample causes in the bacterium count of the sample immediately following. Carryover was quantitated by counting bacteria in a sample of milk with a high bacterial content and then counting bacteria in a sterile blank. Table 2 shows examples of the results obtained. The percent carryover is calculated from the following equation:

$$\% \text{ carryover} = (\Sigma B_i/\Sigma M_i) \times 100$$

where ΣB_i is the sum of the values in the blanks, and ΣM_i is the sum of the values in the samples.

In tests performed with a range of bacterial contents, the carryover was ≤0.19%; this is very close to the value obtained by Dasen et al. (1) for the same apparatus. Our carryover value improves considerably upon the values obtained by Grappin et al. (7) and Nieuwenhof and Hoolwerf (8) (1.2 and 1%, respectively) from earlier models of the BactoScan.

Once the value of the carryover is obtained, it can be introduced into the software of the instrument; correction of results is automatic from then on.

Calibration

A logarithmic correlation was found between the values obtained with the BactoScan 8000 (IBC/μL) and with the reference method (CFU/μL). Linear and polynomic fits were then compared. No significant differences were found between the 2 types of calibration; therefore, the linear calibration, which is the simpler of the 2 types, was chosen. The resulting calibration equation was as follows:

Table 2. Carryover at 2 levels of bacterial content

Sample	IBC/μL	IBC/μL
Milk	18927	52387
Blank	29	101
Milk	17783	49505
Blank	24	89
Milk	18381	47930
Blank	20	81
Milk	16003	47458
Blank	20	103
Milk	16793	47841
Blank	36	66
Milk	18098	46498
Blank	21	88
Milk	17433	43526
Blank	14	75
Milk	18830	47915
Blank	27	83
Milk	17355	47605
Blank	15	80
Milk	17675	48725
Blank	27	62
Carryover, %	0.13	0.19

$$\log(\text{CFU}/\mu\text{L}) = 0.40042 + 0.70440 \times \log(\text{IBC}/\mu\text{L})$$

where correlation coefficient, r, = 0.88 and residual standard deviation, s_{yx}, = 0.259 (Figure 1); these values are better than those found by Grappin et al. (7) for the earlier version of this instrument (r = 0.84 and s_{yx} = 0.29). Our values are also better than those found by Dasen et al. (1) (s_{yx} = 0.2765) and O'Connor et al. (2) (r = 0.71 and s_{yx} = 0.27) from the BactoScan 8000.

Our study was compared with other work by classifying the samples studied into 1 of 3 categories of bacterial content (0–100, 101–500, and >500 CFU/μL), transforming the results obtained with the BactoScan 8000 into CFU/μL by using eq. 1, and comparing them with the value obtained with the reference method. It was found that 319 samples (74.4%) stayed in the same category, 109 samples (25.4%) changed by 1 category, and 1 sample (0.2%) changed by 2 categories.

The percentage of samples classified in the same category by both methods is less than the 79.9% obtained by Grappin et al. (7) in France. The reason for this might be the large number of samples in France that have a very low bacterial content and that fall comfortably within the 0–100 CFU/μL category. Spanish milk has a much wider spread of bacteriological quality, and many samples lie close to the borderlines between categories.

The differences between the results obtained with the BactoScan 8000 and the reference method can be linked to the following processes, which will affect one method or the other:

(1) The existence of different levels of bacterial grouping signifies that CFU, which is counted by the reference method, varies for the same total IBC.

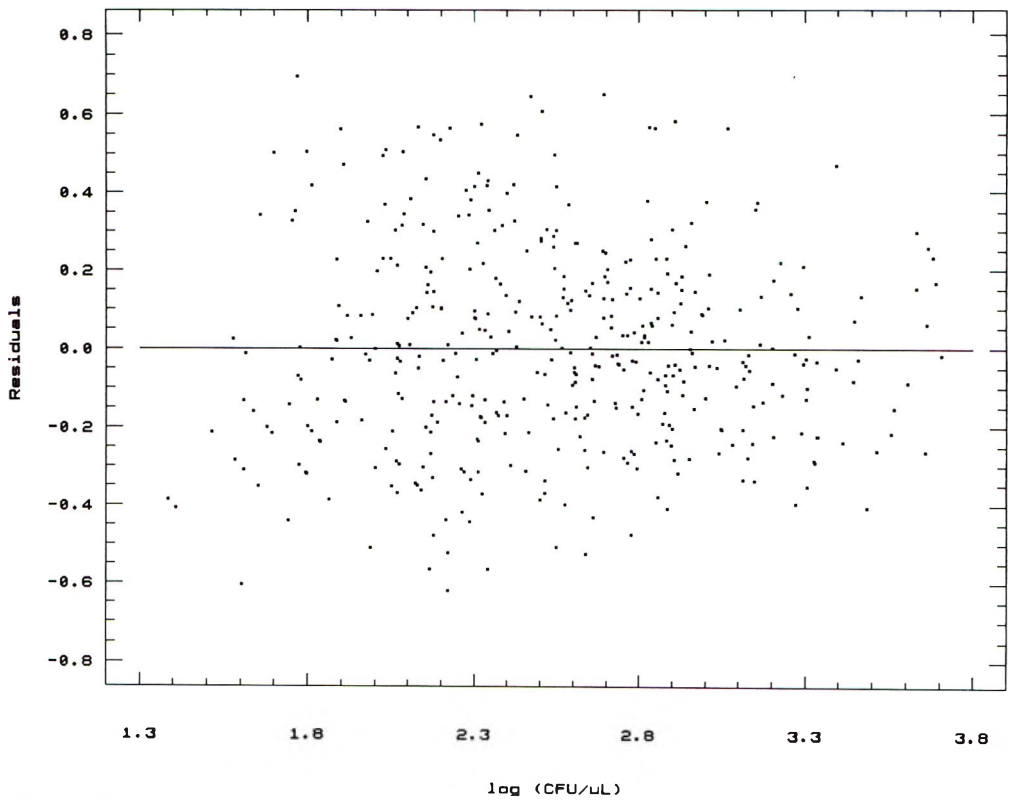

Figure 1. Distribution of residuals.

(2) Not all bacteria flourish in the conditions of the reference method: incubation temperature, pH, redox potential, and chemical composition of medium.

(3) Some bacteria do not stain with acridine orange and, therefore, cannot be counted with the BactoScan 8000.

Limit of Detection

In general, the limit of detection is defined as the concentration (in this case, CFU/μL) that gives a signal in the instrument that is significantly different from that given by a blank. The limit of detection was calculated by using the criterion of Miller and Miller (9): If the calibration line is of the form $y = a + bx$, the limit of detection equals the ordinate at the origin plus 3 times the residual standard deviation.

In our case:

$$\text{Limit of detection} = 0.40042 + 3 \times 0.259 = 1.1774$$

Converting from logarithmic units gives 15 CFU/μL, which is better than that found by Grappin et al. (7) for the earlier version of this apparatus.

Conclusions

Both repeatability and carryover are better than the manufacturer's specifications for the BactoScan 8000.

Correlation between the BactoScan 8000 and the reference method is high, especially in view of the poor repeatability of the latter, because it often places duplicate samples in different categories.

By using the value of the residual standard deviation and the logarithmic relationship between the 2 methods, we found that, for $p = 0.95$, the confidence interval ranges from the value obtained with the BactoScan 8000 divided and multiplied by 3.22, giving, for the example of 500 CFU/μL, a confidence interval ranging from 155 to 1610 CFU/μL.

The BactoScan 8000 is, in our opinion, highly suitable for quantitation of the bacteriological quality of milk, because it improves markedly upon the repeatability of the reference method. The BactoScan 8000 also counts the total number of bacteria; this feature provides more information on the state of milk hygiene than does counting the number of colonies that the bacteria are capable of forming. Therefore, we recommend setting up a system of paying for milk on the basis of IBC/μL values given directly by this apparatus. If the values do not have to be converted into CFU/μL, the analytical errors due to the conversion are avoided.

The mean number of bacteria per colony for all the samples was 5.6 IBC/CFU; for a milk sample with little bacterial content (IBC/μL = 100), the number was 1.6 IBC/CFU; for a sample with a high bacterial content (IBC/μL = 20 000), the number was 7.4 IBC/CFU.

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MYCOTOXINS

Ochratoxin A in Cow's Milk and in Human Milk with Corresponding Human Blood Samples

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A method for determining ochratoxin A in milk has been elaborated in which the sample was subjected to a liquid–liquid extraction step and then purified on a silica gel column packed in a Pasteur pipet. The purified samples were analyzed by ion-pair liquid chromatography with fluorescence detection. The detection and quantitation limits for determination of ochratoxin A in cow's milk were 10 and 40 ng ochratoxin A/L milk, respectively. The same limits were valid for the analysis of human milk. A total of 36 cow's milk and 40 human milk samples were analyzed. All samples were collected in Sweden. Ochratoxin A was found in 5 (14%) of the cow's milk samples (range 10–40 ng/mL) and in 23 (58%) of the human milk samples (range 10–40 ng/L). Blood samples were collected from the mothers who gave milk samples. A total of 39 samples were analyzed. All blood samples contained ochratoxin A in concentrations exceeding the quantitation limit (60 ng/L blood). The mean concentration of ochratoxin A in the samples was 167 ng/L blood (range 90–940 ng/L). The concentration of ochratoxin A in human milk was ≤0.1 of that in the human blood.

Ochratoxin A is a secondary metabolite produced by *Penicillium* and *Aspergillus* species. The mycotoxin has been encountered as a contaminant in cereals, different kinds of beans (coffee, soya, cocoa), and maize. Ochratoxin A has carcinogenic, nephrotoxic, teratogenic, and immunotoxic properties. In the kidneys, its major target is the proximal convoluted tubules, and the toxin has been connected with endemic nephropathies in livestock as well as in humans (for reviews see 1–2).

After consumption of ochratoxin A-contaminated food and feed, the toxin is distributed into different tissues such as blood, kidney, liver, and muscle (for review see 1). In cows the bioavailability of ochratoxin A is low, as the microflora in the rumen of the cow hydrolyzes ochratoxin A to the less toxic metabolite ochratoxin α (3–6). Therefore, the ochratoxin A concentration in cow's milk is expected to be very low. Because ochratoxin A is now considered to be a potent carcinogen in rats (7), concern has increased about the daily intake of ochratoxin A by humans. Risk assessments of the intake of ochratoxin A have been performed, and different tolerable daily intakes (TDIs) have been suggested (1, 8–9). With regard to these suggested values, small amounts of ochratoxin A in milk can be of importance; for consumers of large quantities of cow's milk, even low concentrations of ochratoxin A in the milk can contribute to a significant portion of the total intake of the toxin.

Investigations in Germany (10) and Italy (11) have shown that ochratoxin A can be found in human milk. The concentrations of ochratoxin A found in the positive samples were low in the German study (range 17–30 ng/L) but very high in the Italian investigation (range 1700–6600 ng/L). Infants consuming the ochratoxin A-contaminated milk found in Italy clearly exceed the suggested TDIs. Furthermore, these proposed TDIs have been calculated for adults, and it is not known whether infants are more susceptible than adults to the toxicity of ochratoxin A.

In the present study, a novel method for purifying milk samples was developed. Cow's milk samples were collected from different lines of tank trucks from dairies in different regions of Sweden, and were analyzed for the occurrence of ochratoxin A. Human milk samples were collected, together with blood samples from the very same mothers, with the aim of examining the correlation between the concentration of ochratoxin A in milk and blood.

Experimental

Apparatus

(a) *Liquid chromatography system.*—Two pumps (LC-6A), auto injector (SIL-9A), column oven (CTO-6A), and fluorescence detector (RF 551) (Shimadzu Corp., Kyoto, Japan).

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Chromatography data system and controller (Model 727, Axxiom Chromatography, Inc., Calabasas, CA).

(b) *Milk pump.*—For collection of human milk samples (Egnell & Co. kB, Trollhättan, Sweden).

Reagents

(a) *Chemicals.*—Ochratoxin A was produced according to Fuchs et al. (12). Ochratoxin A methyl ester was prepared by esterification in methanol with 10% boron trifluoride (13). Silica gel, Si-60, 40–63 μm, was purchased from Merck-Schuchardt & Co., Germany. All water used was distilled and run through a $MilliQ_{PLUS}$ (Millipore Intertech, MA) system. All other chemicals used were of pro analysis grade and purchased from Merck-Schuchardt & Co.

(b) *Potassium phosphate buffer.*—7.5 mL 1M KH_2PO_4 and 61.5 mL 0.5M K_2HPO_4 were mixed and diluted to 1000 mL with water to provide buffer at pH 7.5 and ionic strength 0.1.

(c) *Standards.*—Ochratoxin A standards in milk were prepared by adding known amounts of stock solution of ochratoxin A to portions of pooled milk. The stock solution was 10^{-4}M ochratoxin A in 40 mM Tris–H_2SO_4 buffer at pH 7.5 (Tris = tris(hydroxymethyl)-aminomethane); it was calibrated spectrophotometrically at 380 nm, using the extinction coefficient 5680 $M^{-1}cm^{-1}$ (14). For the analysis of blood samples, standards were prepared in 40 mM Tris–H_2SO_4 buffer at pH 7.5 because no ochratoxin A-free blood samples were found.

Milk and Blood Samples

(a) *Cow's milk.*—Cow's milk samples were collected from different lines of tank trucks from 5 different dairies located in different parts of Sweden: Lycksele in the north, Östersund in the middle, Uppsala in the middle south, Visby on the island of Gotland, and Malmö in the south. Six 1 L samples from 6 different tank truck lines were collected from each dairy. The milk samples were collected before the milk from different tank trucks were mixed and before the milk was pasteurized; thus, the samples represent the milk from a limited number of producers. The fat content of the milk was ca 4% but could differ between different samples, as the fat content of the milk was not standardized before the samples were collected. The samples were collected in June 1991. Six additional samples were collected from the dairy in Lycksele in July 1991. The samples were kept at –20°C before analysis.

(b) *Human milk.*—Human milk samples were collected in late 1990 and early 1991 from 40 lactating women living in the county of Västerbotten, in the middle north of Sweden. The milk was collected by a milk pump 6 weeks after delivery and 2–3 h after the previous feed. The hands and breasts were thoroughly washed with water before milking. Approximately 100 mL milk was collected from one breast into polypropylene test tubes, and the samples were stored at –20°C until analysis.

(c) *Human blood.*—Human blood samples were collected from the women giving milk at the same time the milk samples were collected. The samples (5 mL) were collected in evacuated blood collection tubes (Venoject, Terumo Corp., Tokyo, Japan) containing heparin. The tubes were turned 20 times after collection to prevent coagulation and were thereafter kept at –20°C until analysis.

Purification of Cow's Milk Samples

Extraction.—To a 50 mL nylon centrifuge tube, 2 mL milk, 5 mL ethanol, and 0.5 mL 1M HCl were added. Chloroform (5 mL) was added to the tube and ochratoxin A was extracted into the organic phase by inverting the tube continuously for 10 min (20 rpm). The mixture was centrifuged 10 min at 10 000 × *g*. A 4 mL portion of the chloroform phase was transferred from the bottom of the tube to a 15 mL glass centrifuge tube. The chloroform phase was washed once with 2 mL water to remove the methanol distributed into the chloroform. A white emulsion formed during the washing was broken by centrifuging the tube. The chloroform phase turned slightly yellow.

Purification on silica gel.— A 200 mg portion of silica gel was packed in a Pasteur pipet. Glass wool was placed in the lower end of the Pasteur pipet to support the silica gel and 300 mg Na_2SO_4 was added to the top of the column. The column was washed with 1.5 mL chloroform. The chloroform phase from the sample extraction (see above) was transferred to the column, and the solvent was drained to the top of the column. The column was washed with 3 mL chloroform. Ochratoxin A was eluted with 3 mL chloroform–formic acid (95 + 5, v/v). The eluate was washed with 2 mL water. The solvent was removed by evaporation under a gentle stream of nitrogen in a water bath at 40°C.

Purification of Human Milk

Extraction.—A 2 mL portion of human milk was treated with methanol, 1M HCl, and chloroform as described above for the extraction of cow's milk. Ochratoxin A was extracted into the organic phase, the mixture was centrifuged, and 4 mL of the chloroform phase was withdrawn from the bottom of the tube. The chloroform phase was extracted once with 1.5 mL freshly prepared 1% aqueous $NaHCO_3$ (w/v) and once with 1.5 mL 1% aqueous $NaHCO_3$–MeOH (1 + 1, v/v). The aqueous phases were pooled and acidified with 0.5 mL 1M HCl. The white precipitate formed was removed by extraction with 2 mL hexane as described by Hult and Gatenbeck (15). Ochratoxin A was extracted from the aqueous phase with 3 mL chloroform.

Purification on silica gel.—The extract was purified on a silica gel column as described for the purification of cow's milk.

Purification of Human Blood Samples

The blood samples were purified as described by Breitholtz et al. (13): 1 mL blood, 10 mL 0.1M MgCl–0.05M HCl solution, and 5 mL chloroform were mixed in a nylon centrifuge tube. Ochratoxin A was extracted into the chloroform phase by continuously turning the tube end over end for 10 min. The tube was centrifuged 10 min at 10 000 × *g*, and the chloroform phase was transferred to another tube and extracted twice with 40 mM Tris–H_2SO_4 buffer at pH 7.5. The Tris phases were pooled and acidified with formic acid. Chloroform was added and the ochratoxin A was extracted into the organic phase. The

Table 1. Occurrence of ochratoxin A in cow's milk collected from different lines of tank trucks from 5 different dairies in Sweden[a]

Dairy		Ochratoxin A, No. samples		
		<10 ng/L	10–40 ng/L	Total
Lycksele	June	4	2	6
	July	6	0	6
Östersund		5	1	6
Uppsala		5	1	6
Visby		6	0	6
Malmö		5	1	6
Total		31	5	36

[a] Six 1 L samples from different production lines were collected from each dairy in June 1991. An additional set of samples was collected in July 1991 from the dairy in Lycksele.

chloroform phase was drawn off and evaporated to dryness under a gentle stream of nitrogen in a water bath at 40°C.

Derivatization of Ochratoxin A for Confirmation

The samples were purified and the solvent was evaporated as described above for the different types of samples. The methyl ester of ochratoxin A was prepared as described by Breitholtz et al. (13). Methanol containing 20% boron trifluoride was added to the samples, which were kept at 65°C for 60 min. The samples were cooled, and cold water was added. The methyl ester was extracted twice with 1.5 mL chloroform, and the chloroform phase was washed with water and evaporated under nitrogen.

Liquid Chromatography (LC)

The LC analyses were performed at an alkaline pH by the same ion-pair technique used by Breitholtz et al. (13). The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol–potassium phosphate buffer mixture. The ratios of methanol to potassium phosphate buffer were 51:49 and 54:46 (v/v) for the determination of ochratoxin A and ochratoxin A methyl ester, respectively. The samples were dissolved in 300 µL mobile phase and 100 µL was injected on the column.

The determinations were performed at 380 nm excitation wavelength, 450 nm emission wavelength, and 30°C. The column was Spherisorb S3ODS2 (C_{18}), 4.6 × 150 mm (Hichrom Ltd, Reading, Berkshire, UK), with 3 µm particles. The total flow rate was 0.8 mL/min.

Standard curves were based on the results from the analyses of spiked samples. The curves were calculated by the least-squares method.

Results

Liquid Chromatography Method

Standard curves of ochratoxin A in the different matrixes (milk or blood) were used for calculating the limits of detection and quantitation, with the whole sample preparation and procedure of analysis taken into account. The analytical signal was plotted on the y-axis and the concentration on the x-axis. Using the commonly used definition of the detection limit and the blank signal plus 3 standard deviations of the blank, and adjusting for the standard deviation of the standard curve in the y-direction ($\alpha = 0.001$), the detection limits were calculated to be: (a) 10 ng ochratoxin A/L in cow's milk and human milk, and (b) 10 ng ochratoxin A/L in blood. Limits of quantitation were determined by adding 10 standard deviations of the blank to the blank signal and adjusting for the standard deviation of the standard curve in the y-direction. The limit of quantitation in cow's and human milk was 30 ng ochratoxin A/L. In blood, the limit of quantitation was 40 ng ochratoxin A/L, when calculated by this method.

Slightly higher detection and quantitation limits were calculated by using weighted regression lines as bases for the calculations of the limits (16). The standard deviation of the blank was estimated as the standard deviation of the appropriate standard curve in the y-direction, and the blank signal was estimated as the value of the intercept. The calculations were based on data collected during the days the samples were analyzed. Standard curves of ochratoxin A in the different matrixes (cow's milk, human milk, and blood, respectively) were performed on 3 occasions each. Each standard curve on each occasion consisted of 5 points. The detection limit of ochratoxin A in cow's and human milk by this second method of calculation was 10 ng ochratoxin A/L. The quantitation limit was 40 ng ochratoxin A/L milk. In blood, the detection and quantitation limits were 20 and 60 ng ochratoxin A/L, respectively.

The recoveries for the overall procedures were 85 and 75% for cow's and human milk, respectively, in the concentration range tested (10–500 ng ochratoxin A/L). For blood samples, the recovery was 90% in the range 500–1000 ng ochratoxin A/L. This recovery was in accordance with the recovery determined in spiked Tris–H_2SO_4 buffer samples. In the range 50–500 ng/L, the recovery of ochratoxin A in Tris buffer increased from 80 to 90%.

Occurrence of Ochratoxin A

Cow's milk.—A total of 36 milk samples were analyzed. Five of these samples (14%) contained ochratoxin A in the concentration range 10–40 ng ochratoxin A/L (Table 1). No samples contained >40 ng ochratoxin A/L. No difference was seen in the contamination level of ochratoxin A in the milk from the different dairies.

Human milk.—Ochratoxin A was found in 23 (58%) of the 40 analyzed samples. No samples were found above the quantitation limit, i.e., 40 ng ochratoxin A/L (Table 2).

Human blood.—A total of 39 samples were analyzed. All of these samples had ochratoxin A contents over the quantitation limit of 60 ng ochratoxin A/L (Table 2). The mean concentration was 167 ng ochratoxin A/L blood (range 90–940 ng/L).

Confirmation of Ochratoxin A-Positive and -Negative Samples

The presence of ochratoxin A in the samples was confirmed by the formation of the ochratoxin A methyl ester. Seven of the

Table 2. Occurrence of ochratoxin A in human blood and breast milk collected from the same mothers in late 1990 and early 1991[a]

	Ochratoxin A, mg/L			
	Ochratoxin A		Ochratoxin A methyl ester	
Sample	Blood	Milk	Blood	Milk
1	90	<10	110	—[b]
2	100	10–40	—	10–40
3	100	10–40	—	—
4	100	10–40	—	—
5	110	<10	—	—
6	110	<10	120	—
7	110	<10	—	—
8	110	<10	—	—
9	110	10–40	—	—
10	110	10–40	—	—
11	120	<10	—	—
12	120	<10	—	—
13	120	10–40	—	—
14	120	10–40	—	—
15	130	<10	—	—
16	130	<10	—	—
17	130	10–40	—	—
18	130	10–40	—	—
19	140	<10	—	—
20	140	<10	—	—
21	140	10–40	—	—
22	150	<10	—	—
23	150	<10	—	—
24	150	10–40	—	—
25	160	<10	—	<10
26	160	<10	—	10–40
27	160	10–40	—	—
28	170	10–40	—	—
29	180	<10	—	—
30	180	10–40	—	—
31	180	10–40	—	<10
32	180	10–40	—	<10
33	190	10–40	—	—
34	190	10–40	200	—
35	200	10–40	—	—
36	200	10–40	310	10–40
37	240	<10	270	—
38	270	10–40	320	—
39	940	10–40	1040	10–40
40	—[c]	10–40	—	—

[a] The presence of ochratoxin A was confirmed by formation and chromatography of its methyl ester.
[b] Not determined.
[c] Missing sample.

blood samples were selected for derivatization. All these samples were confirmed to contain ochratoxin A (Table 2). Because of the very small amounts of ochratoxin A in the milk samples (Tables 1 and 2), the identity of ochratoxin A in these samples was difficult to confirm. Of 4 ochratoxin A-positive cow's milk samples, 3 were confirmed to contain ochratoxin A in the range 10–40 ng/L. No ochratoxin A methyl ester was detected in the fourth sample. One ochratoxin A-negative cow's milk sample was derivatized; no ochratoxin A methyl ester was detected in this sample. Five ochratoxin A-positive human milk samples were included in the derivatization procedure; in 3 of these 5 samples, the ochratoxin A methyl ester was detected in the concentration range 10–40 ng/L (Table 2). Of 2 derivatized ochratoxin A-negative human milk samples, one showed an ochratoxin A methyl ester content in the range 10–40 ng/L; no methyl ester was detected in the other sample. None of 3 negative standards included in the derivatization procedure showed a methyl ester content above the detection limit.

Discussion

The method for purification of cow's milk samples is short and easily performed. But when the same procedure was used for the purification of human milk, the samples started to foam after they were dissolved in the mobile phase. Therefore, some extra steps were incorporated in the extraction procedure, although no disturbances were seen in the chromatograms during analysis of the foaming samples.

The transfer of ochratoxin A into mammalian milk has been studied in only a few cases. Galtier et al. (17) have shown that the ochratoxin A concentration in rabbits is 7–8 times as high in the plasma as in the milk. Mortensen et al. (18) did not detect any transfer of ochratoxin A into the milk of sows that had been fed ochratoxin A-contaminated barley. Galtier and Alvinerie (5), Hult et al. (3), and Kiessling et al. (6) have shown that ochratoxin A is degraded to ochratoxin α by the microbial flora in the rumen of cows. In the study by Hult et al., only a minor amount of ochratoxin A remained unaffected after 4 h. When Ribelin et al. (4) treated cows with ochratoxin A, traces of ochratoxin α were found in the milk of all cows, but ochratoxin A was detected in the milk only when the daily dose of ochratoxin A exceeded 1.66 mg/kg body weight. The samples were analyzed by thin-layer chromatography. In the present investigation, ochratoxin α was not determined. In view of the degradation process of ochratoxin A in the rumen, the low concentrations of ochratoxin A still found in cow's milk in this study show that cattle feed is frequently contaminated with ochratoxin A.

Ochratoxin A contamination of feed grain in Sweden varies with the season (19). After long storage, the ochratoxin A contamination of the grain frequently increases. In the present investigation, the milk samples were collected in June and July. During both of these periods, the cows were fed grain from the autumn 1990 harvest. Thus, the feed had been stored for a long period, and a higher ochratoxin A contamination level was expected than if the samples had been collected in the autumn.

Occurrence of ochratoxin A in human milk has been reported from Germany and Italy. In Germany, Gareis et al. (10) analyzed 36 milk samples and found 4 of them (11%) to be ochratoxin A-positive (range 17–30 ng/L). In our study, the concentration range of the ochratoxin A-positive human milk samples was 10–40 ng/L milk. This is similar to the levels

found by Gareis et al. (10) in Germany, but the frequency of ochratoxin A-positive samples was higher in Sweden, i.e., 23 of 40 samples (58%). In Italy, Micco et al. (11) found that 9 of 50 milk samples (18%) contained ochratoxin A. The concentration range in this case was very high, i.e., 1700–6600 ng ochratoxin A/L milk.

In the present investigation, all the ochratoxin A-positive samples of human milk and blood fall into narrow concentration ranges. Therefore, no fair estimation about the concentration dependence between blood and milk could be reached. We concluded that the concentration level of ochratoxin A in human milk is roughly ≤0.1 of that in human blood (Table 2).

During the last few years, the concern about human exposure to ochratoxin A and its toxicity to human beings has increased. Several health risk assessments have been performed, and different TDIs have been suggested. The present investigation indicates that the very low TDI (0.2 ng/kg body weight) suggested by Kuiper-Goodman and Scott (1) can be exceeded by consumers of large quantities of milk, e.g., teenagers consuming cow's milk and infants consuming human milk. The high TDI (4.2 ng/kg body weight) suggested by Kuiper-Goodman and Scott (1), the TDI (5 ng/kg body weight) suggested by the Nordic group (9), and the provisional tolerable weekly intake (112 ng/kg body weight; 16 ng/kg body weight per day) proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (8) are not exceeded by the intake of milk at the average consumption rate (0.42 L/day) (20) and at the ochratoxin A concentration levels found in milk in this study, although the cow's milk samples were collected in a season when ochratoxin A contamination was expected to be high. As the cow's milk samples were collected before the milk from different production lines were mixed, the amount of ochratoxin A found in cow's milk in this investigation may exceed the concentration level of ochratoxin A in the milk that reaches the consumers.

On the other hand, consumption of cow's milk has not previously been considered to be a source of ochratoxin A intake. The average ochratoxin A concentration of 10 ng/L found in this investigation corresponds, at an average milk consumption, to an intake of ochratoxin A on the same level as from pork meat. Intake calculations for ochratoxin A based on >1000 analyses of Swedish food items shows that cereal-based food is the major dietary source of ochratoxin A (21). Considering that children are big consumers of milk, however, our results indicate that cow's milk can contribute to about one-fifth of their total ochratoxin A intake. The high levels of ochratoxin A found in human milk in Italy by Micco et al. (11) indicate that even the high TDIs could be exceeded by breast-fed infants.

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PESTICIDE FORMULATIONS

Rapid and Accurate Method for Determination of Niclosamide Released from Molluscicidal Formulations

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A rapid, simple, and reproducible method was developed for quantitative determination of niclosamide ethanolamine salt (NES) — the most widely used molluscicide against snail vectors of schistosomiasis — in both distilled and natural canal waters. The biocide is extracted completely from water samples by passing through Sep-Pak C_{18} cartridge and eluting the cartridge with methanol. The eluate is then analyzed directly for NES by UV/vis spectrophotometry at 330 nm. This method was found sensitive enough to detect 0.1–5 ppm, and it is highly reproducible (standard deviation, 0.006–0.012). The standard curve obeyed Beer's law. The method also successfully measured the release rate of NES from controlled-release elastomeric formulations. The release study suggests that NES loading and certain rubber additives are not the key factors that govern the release of the biocide.

Niclosamide (NES), the 2-aminoethanol salt of 2',5-dichloro-4-nitrosalicylanilide, is widely used as a wettable powder to control aquatic snails. These snails are the intermediate hosts of organisms that cause schistosomiasis (1). NES is also used in the Canadian Great Lakes region to control larvae of the parasitic sea lamprey (2).

Several colorimetric (3–6) and gas chromatographic (7, 8) methods were reported for the analysis of NES in water following treatments for snail or lamprey control. However, these methods are limited by problems with sensitivity and reproducibility (9). They also require the time-consuming hydrolysis and/or derivatization of NES before determination. In addition, many of the methods (3–7) cannot analyze NES in natural water without interferences from its hydrolysis products (8).

A lengthy liquid chromatographic (LC) method was described (8), where the biocide was extracted from large water samples (500–1000 mL) by using 3 successive portions of ethyl acetate (200, 100, and 100 mL) and then analyzed over an NH_2-Bondapak column with absorbance detection at 313 nm. Another LC method (9) used a lengthy procedure to detect one concentration of NES (0.1 ppm) in combination with another biocide (λ_{max}, 330 nm). The method did not give data for a standard curve needed for detection of unknown concentrations of NES. However, on testing the technique (9) in this laboratory, a correlation between peak height and NES concentration could not be shown (Emara, L.H., unpublished results).

During a search for a simple and accurate method for NES, the spectrophotometric method of Farrington (3) was examined. Our study (10) indicated that the data (3) do not obey Beer's law, the maximum absorption used (λ_{max}, 385 nm, $CHCl_3$) was erroneous and proved to be 335 nm (broad), the extraction of niclosamide with $CHCl_3$ is not quantitative, and the transformation of the compound to its amine salt is not likely to occur within the time stated. Therefore, the results were not reproducible (standard deviation ±0.062–0.204 for the 0.2–5 ppm range). To remedy this, I developed a UV/spectrophotometric method (10) that determined 0.3–6 ppm NES in water. *n*-Amyl alcohol provided a direct and quantitative extraction of NES from water samples, and direct measurement was performed (λ_{max}, 335 nm). The method is reproducible and was used to measure the release of NES from controlled-release systems (11). However, mud, masses of microorganisms, and other contaminants interfered with the method when applied to field waters. When samples of such waters are shaken with amyl alcohol, the formation of heavy emulsions cannot be avoided, and the time taken for separation of phases becomes appreciably longer. Determination of NES in natural waters is still difficult, and a practical and accurate method is needed to monitor concentrations in snail and lamprey control programs.

In this study, a fast, simpler, and sensitive method was developed. A reversed-phase column is used to extract, concentrate, purify, and measure (UV) NES in one step. The method is applicable to 0.1–5 ppm NES in distilled and natural canal waters.

Because controlled-release formulation is currently considered the most safe and effective pesticide application, the method was designed to be applicable to the research in this area. Several novel NES elastomeric compositions were prepared and subjected to determination of NES release pattern.

Experimental

Apparatus

(a) *UV-vis spectrophotometer.*—Ultrospec II 4050 (LKB).

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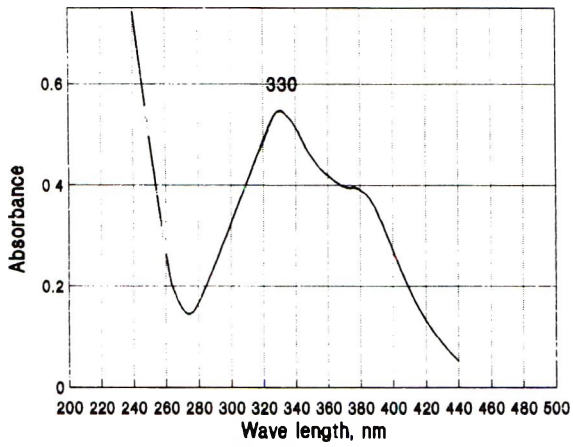

Figure 1. UV spectrum of NES, (4.1 μg/mL in methanol).

(**b**) *Reversed-phase column.*—Sep-Pak C_{18}, Disposable Cartridges (Waters Assoc., Inc.).

(**c**) *Syringe.*—Hamilton Gastight, 10 mL, gas and liquid tight syringe (Waters).

Reagents

(**a**) *NES.*—Technical grade (Bayer, A.G.)

(**b**) *Solvent.*—Methanol, gradient grade (Merck).

(**c**) *Standard NES solution.*—(*1*) *Stock solution.*—Dissolve 100 mg NES in sufficient methanol, and dilute to 100 mL with methanol. Prepare fresh weekly. (*2*) *Intermediate solution.*—10 μg/mL (ppm). Transfer 10 mL stock solution to 1 L volumetric flask, and dilute to volume with water. (*3*) *Working solution.*—0.1–5 μg/mL. Dilute aliquots of the intermediate solution with distilled water to prepare a series of standard concentrations covering 0.1–5 μg/mL. (*4*) *Field working solutions.*—0.1–5 μg/mL. Dilute aliquots of the intermediate solution as above using natural canal water instead of distilled water.

Extraction and Determination

Condition a Sep-Pak RP-C_{18} cartridge with 2 mL methanol and 5 mL water. Force 10 mL aliquots slowly into the cartridge, each representing different concentration of working solution, using a gas tight syringe. Pass 5 mL water for washing. Elute the pure NES by injecting 3 mL methanol. Measure the eluant absorbance at 330 nm (Figure 1) against a blank of methanol. Construct the standard curve.

Repeat the above procedure using the field working solution. A blank for this is prepared as follows: Force 10 mL aliquot of natural canal water free of NES into the cartridge. Elute with methanol, and use the eluant as blank solution.

Preparation of NES Controlled-Release Formulations

NES was uniformly incorporated in elastomeric natural rubber (NR) matrix as follows: NR, 100 g; chalk, 50 g; dicumyl peroxide, 4 g; and NES, 8 and 15 g, were accurately weighed. Ingredients were mixed on a two-roller mill (diameter, 170 mm; working distance, 300 mm; speed of slow roll, 24 rpm; and gear ratio, 1:1.4) (12). The rheometric characteristics were determined by using an oscillating disc rheometer (13). The vulcanizates of the mixture were prepared by subjecting them to their optimum time for cross-linking, 13 and 12 min for 8 and 15 NES parts per hundred (phr) of NR, respectively.

This method produced rubber sheets with a content of 22 and 40 mg NES per a 4 × 1 × 0.1 cm piece of 8 and 15 phr NES formulation, respectively.

Table 1. NES absorbance at 330 nm using reversed-phase chromatography

Standard solution, ppm	1st trial	2nd trial	3rd trial	Mean	SD
0.1	0.036	0.042	0.030	0.036	0.006
0.3	0.055	0.052	0.060	0.056	0.004
0.5	0.087	0.080	0.075	0.081	0.006
0.8	0.105	0.111	0.125	0.114	0.010
1.0	0.128	0.130	0.134	0.130	0.003
2.0	0.248	0.245	0.251	0.248	0.003
2.5	0.333	0.310	0.322	0.322	0.012
3.0	0.401	0.392	0.397	0.397	0.005
5.0	0.660	0.657	0.643	0.653	0.009

Determination of NES Release Rate

A flow-through method was adopted (14). A sample (4 cm length, 1 cm width, and ca 1 mm thickness) of NES vulcanized rubber sheet was placed in a flow cell connected with miniflow peristaltic pump. Distilled water (100 mL) was circulated inside the cell at constant flow rate (20 mL/min) in a closed circuit. Both the flow cell and the circulating solution were maintained at 23 ± 0.1°C by means of refrigerated circulator. Samples (10 mL) were taken from the release medium at appropriate time intervals and rapidly replaced with equal volumes of water. The concentration of NES in the release medium was estimated by using the proposed method. The same procedure was performed using a rubber vulcanizate sample containing no NES to serve as a blank control.

Results and Discussion

Results are shown in Table 1. Beer's law is obeyed in the concentration range used, as shown by the computed constants in Table 2. Although a high correlation coefficient was determined for the 0.1–5 ppm range, a slope for the lower range (0.1–0.8 ppm) and another for the higher range (1–5 ppm) were found. These slopes possessed higher correlation coefficients than that obtained for the whole range (0.1–5 ppm). Us-

Table 2. Constants of the standard curve of NES

Concn range, ppm	Correlation coefficient	Slope	Intercept
0.1–5.0	0.9989	0.126	0.0129
0.1–0.8	0.9994	0.1126	0.0239
1.0–5.0	0.9995	0.132	−0.0065

Table 3. Absorbance of the first and second eluate at 330 nm (NES recovery in parenthesis)

NES, ppm	First eluate (rec., %)	Second eluate
0.1	0.036 (107)	0
0.3	0.055 (92)	—
	0.059 (103)	—
0.8	0.125 (112)	0
	0.112 (98)	—
	0.117 (103)	—
2.0	0.253 (98)	—
	0.245 (95)	—
4.1	0.547 (102)	—
5.0	0.657 (100)	0

ing the relevant constants from the 2 slopes for each concentration range will result in more precise recoveries.

The unknown concentrations were calculated from the following linear equation:

$$Y = Ax + B$$

where Y is the absorbance, A = slope, B = intercept, and x = unknown concentration.

The reproducibility of the method was checked at selected concentrations covering the 0.1–5 ppm range. The absorbance data of replicates for different concentrations (Table 1) show that the low standard deviation values indicated the high validity and reproducibility of the method at low concentrations of NES.

The extracted standard solution was re-extracted on another Sep-Pak C_{18} column and eluted with a second portion of the eluting solvent, methanol. The data reveal that a single extraction step by this technique safeguards 100% elution of the biocide (Table 3). The recovery over the 2 concentration ranges (Table 3) was high; therefore, quantitation of NES is highly accurate and precise.

Natural canal waters were collected during December 1991 from 2 locations in Egypt: the Nile branch at El-Quanater (pH, 8; turbidity, 8 NTU) and the El-Zomor canal, a sub-branch of the Nile (pH, 7.4; turbidity, 5 NTU). Blanks of field working

Table 4. NES absorbance at 330 nm using reversed-phase chromatography with natural waters

Standard solution, ppm	El-Quanater	El-Zomor
0.1	0.039	0.042
0.5	0.089	0.079
0.8	0.123	0.131
2.0	0.307	0.311
3.0	0.423	0.435
5.0	0.66	0.643

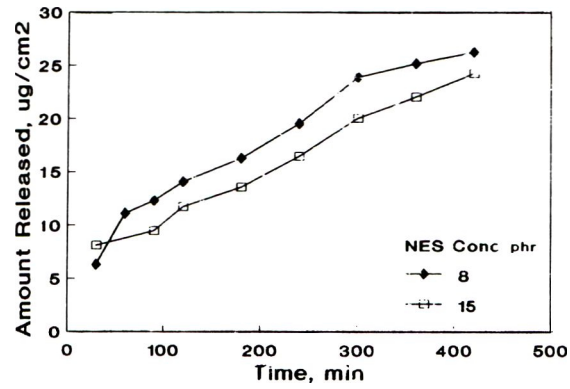

Figure 2. Effect of NES concentration on the release rate from natural rubber matrixes (Each point represents a mean of 3 replicates).

solutions of these canals gave absorbance of 0.037 and 0.024 (against methanol), respectively. The results of the constructed standard curve for these field waters are presented in Table 4. The data reveal high correlation between absorbance and NES concentration. The relatively low absorbance of these natural water blanks allows for reproducible determination of NES in the field.

I found that a water sample volume of 10 mL is sufficient to monitor NES concentrations of 0.1–5 ppm in the field during molluscicidal operation. The concentration gradient of NES over a distance of a canal will show where the NES controlled-release preparation should be located to achieve the minimum effective molluscicidal concentrations.

Because the effective concentration of NES is about 0.3–1 ppm, correlation of absorbance to concentration was made in the concentration range of 0.1–5 ppm. This was considered a useful range for the evaluation of different sustained-release formulations and the assessment of their performance.

Reversed-phase column chromatography efficiently extracted NES from water samples, separated contaminants present in water canals, and saved time (each sample needs 3–5 min for analysis).

NES Controlled-Release Formulations

The method also determined (14) microamounts of NES released from controlled-release formulations. The release pattern of NES was evaluated from natural rubber vulcanizates, with 8 and 15 phr NES loadings. The rubber matrixes also contained chalk as filler. Instead of the conventional rubber additives (11), peroxide was added as a cross-linking agent. Blank values for rubber vulcanizates containing no NES were found to be zero. No significant difference was found in the NES re-

leased from 3 separate samples representing each vulcanizate sheet. This indicates that NES was evenly distributed in the sheets. The release rate of NES from these matrixes was quantified and is presented in Figure 2. Loading of 8 phr NES was found to produce a release slightly greater than the 15 phr NES. Data also show that the presence of the peroxide as a cross-linking agent does not alter the release behavior. This finding is consistent with matrixes prepared with sulfur as a cross-linking agent (L.H. Emara, unpublished data). The release study suggests that certain unknown factors (other than NES loading and rubber additives) might control the release of the biocide from the rubber matrixes. The possibility of these factors should be investigated.

The diffusion-controlled mechanism of NES release was found to obey the Higuchi square-root-of-time equation (15).

A rapid, inexpensive, and accurate method was developed for NES in prepared samples and in the field, with determination as low as 1/3 to 1/10 the minimum effective molluscicidal concentration. The method also evaluated novel controlled-release elastomeric formulations.

The method is safer than previous methods, because the volume of possibly infected field samples is reduced to 10 mL. Also, the liquid–liquid extraction steps are omitted and substituted by a single injection into the cartridge.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Methyl 2-Benzimidazolecarbamate in Wine by Competitive Inhibition Enzyme Immunoassay

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A benomyl polyclonal enzyme immunoassay (EIA) commercial kit was used to quantitate methyl 2-benzimidazolecarbamate (MBC), a degradation product of benomyl in wine. Total analysis time, including sample preparation, was 30 min. As many as 8 samples can be analyzed simultaneously with a limit of quantitation of 5 ppb. The assay logarithmic response was linear from 0.4 to 26 ppb MBC. Intra-assay percent coefficients of variation (%CVs) ranged from 2.4 to 13 for standards and from 7.4 to 21 for actual wine samples. Interassay %CVs varied from 2.6 to 15 for the standards and from 6.9 to 23 for the samples. Average recovery from samples spiked at 10–10 000 ppb was 93% for evaporated red and white wines. MBC was determined in 134 different wines by immunoassay and liquid chromatography (LC). Of these samples, 98 were positive for MBC by both methods with a correlation coefficient (r) of 0.986. The other 36 samples had MBC levels that either were not detectable by either procedure or were below the 10 ppb limit of quantitation for LC. Concentrations of MBC in wine ranged from 5 to 1329 ppb, with the majority ranging from 10 to 300 ppb. Also, a mini-study was conducted using the plate EIA format.

Ever since the procymidone problem, concern about the presence of pesticides in wine has increased worldwide. One such pesticide, methyl 2-benzimidazolecarbamate (MBC; carbendazim), is a fungicide used in Europe and is also a major breakdown product of benomyl, a fungicide used widely in the United States. These systemic benzimidazole fungicides may be used for either pre- or postharvest treatment of fruits and vegetables to prevent Botrytis disease. Recently, questions have arisen about the safety of benomyl and MBC (1–3). Therefore, it is important to thoroughly monitor MBC residues in foods for proper dietary risk assessment.

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Previous methods for detecting MBC in wine have been reviewed (4, 5); most are spectrophotometric and chromatographic procedures. However, enzyme immunoassay technology is emerging as an important tool in pesticide residue determination (6). In fact, many scientists now believe that adequate monitoring of pesticides in our food supply will require immunoassay technology to play an increasingly key role (6). Previously, 3 EIA methods had been developed for MBC in foods, but none were developed for wine (7–9).

This paper describes a rapid, inexpensive, and sensitive EIA method for the quantitation of MBC in wine. Such a method should be very useful for monitoring MBC levels in all types of wine.

METHOD

Reagents and Standards

(**a**) *Reagents.*—All reagents pertaining to preparation of immunogens for raising antisera to MBC were previously described (8). All solvents were LC grade (VWR, Boston, MA 02101).

(**b**) *Analytical standard.*—MBC pesticide standard was obtained from U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

(**c**) *Pesticide standard stock solution.*—Weigh 10.0 mg MBC into 50 mL volumetric flask, dissolve, and dilute to volume with methanol.

(**d**) *Diluting solvent for tube immunoassay.*—Place 25 mL mobile phase (**h**) into 250 mL volumetric flask and dilute to volume with skim or nonfat dry milk (prepare according to label directions).

(**e**) *Pesticide standards for tube immunoassay.*—(*1*) *Intermediate standard.*—Remove 0.1 mL aliquot from corresponding stock solution (**c**), place into 50 mL volumetric flask, and dilute to volume with 50 mL diluting solvent (**d**). (*2*) *Working standard.*—Remove 5, 10, 20, 40, 80, 160, and 320 μL aliquots

for standard curve determination from intermediate standard (*1*) and place each into a separate 20 mL scintillation vial containing 0.5 mL organic wine (made from grapes grown without pesticides) that was previously evaporated to dryness with nitrogen at room temperature. Dilute each vial to 5 mL total volume with diluting solvent (**d**); then sonicate to dissolve residue.

(**f**) *Pesticide standards for plate immunoassay.*—(*1*) *Intermediate standard.*—Remove 0.1 mL aliquot from corresponding stock solution (**c**) and place into 50 mL volumetric flask. Dilute to volume with reverse osmosis (RO) water. (*2*) *Working standard.*—Remove 2.5, 5, 10, 20, 40, and 80 µL aliquots for standard curve determination from intermediate standard (*1*) and place into separate 20 mL scintillation vials. Dilute each vial to 5 mL with RO water.

(**g**) *MBC standards for LC.*—(*1*) *Intermediate standard.*—Take 1.0 mL aliquot from MBC stock and place into 50 mL volumetric flask. Dilute to volume with LC mobile phase. (*2*) *Working standards.*—Remove 1.0, 2.0, 4.0, and 8.0 mL aliquots for standard curve determination from MBC intermediate standard solution and place into separate 50 mL volumetric flasks. Dilute to volume with LC mobile phase.

(**h**) *Mobile phase.*—Acetonitrile–methanol–water–monoethanolamine (260 + 70 + 500 + 0.1).

(**i**) *Sample extraction solvent.*—Use 5 mL ethanol with 15 mL 2M ammonium chloride adjusted to pH 10 with 14.5N ammonium hydroxide. Partition into methylene chloride.

(**j**) *Conjugate synthesis and antisera.*—Follow methods described previously (8).

Apparatus

(**a**) *Liquid chromatograph.*—510 pump (Waters Associates, Milford, MA 01757); Valco pneumatic injector (VICI Instruments, Houston, TX 77255); Waters 470 fluorescence detector and HP 1040A photodiode array detector-integrator system (Hewlett-Packard, Avondale, PA 19311). Operating conditions: injection volume, 5 µL; flow rate, 1.0 mL/min; fluorescence, excitation at 286 nm and emission at 305 nm; attenuation, 8; gain, 100; filter, 1.5 s.

(**b**) *Chromatographic column.*—Ultracarb 30 ODS, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Torrance, CA 90501).

(**c**) *EnviroGard™ EIA Benomyl Tube Kits.*— Polystyrene test tubes coated with MBC antibodies and enzyme conjugate (horseradish peroxidase bound to MBC) together with hydrogen peroxide as substrate and tetramethylbenzidine as chromogen (Millipore Corp., Bedford, MA 01730).

(**d**) *EnviroGard™ Benomyl Plate Kits.*—96-well microtiter plate coated with MBC antibodies and enzyme conjugate (horseradish peroxidase bound to MBC) together with hydrogen peroxide as substrate and tetramethylbenzidine as chromogen (Millipore).

Tube Immunoassay of MBC

Prepare MBC standards as described in (**e**)(2). For samples, remove 0.1 mL wine and evaporate to dryness under nitrogen. Add 1 mL diluting solvent described in (**d**) and sonicate.

Analyze standards and samples by adding 160 µL to coated tubes of tube kit, followed by 160 µL enzyme conjugate (up to 8 samples with 2 controls can be prepared simultaneously). Incubate tubes for 10 min at room temperature. Rinse tubes with water to remove unreacted sample and enzyme conjugate. Add 320 µL substrate-chromogen mixture (1 + 1). Incubate tubes for 10 min; then add 1 drop 2.5N sulfuric acid (stop solution) to stop reaction; color changes from blue to yellow.

Measure intensity of yellow solutions by reading tubes at 450 nm. Alternatively, use conventional spectrophotometer set at 450 nm. Run 2 control tubes (one at beginning and other at end) with each set of 8 samples to calculate $\%B_O$ values of standards and samples (100 × absorbance at 450 nm of standard or sample/average absorbance at 450 nm of negative control comprised of 0.1 mL evaporated organic wine/mL diluting solvent, [**d**]). Use average value for negative control. Run standard curve before samples and again after last samples. (Time difference from beginning samples to end may range from 10 to 1 h. Although this procedure is not standard practice, we have shown that it is effective and saves time and money.) Use average of both standard runs to quantitate MBC in samples. Prepare standard curve and use it to calculate unknowns. Use semilog (3-cycle) to plot curve, where y-axis is $\%B_O$ and x-axis is MBC concentration on log scale.

Plate Immunoassay of MBC

Prepare MBC standards as described in (**f**)(2). For samples, add 9.9 mL RO water to 0.1 mL wine. A further 10-fold dilution in RO water may be needed.

Analyze standards and sample by adding 80 µL to each well of plate kit (**d**), followed by 80 µL enzyme conjugate (a total of ≤96 samples, standards, and controls can be run simultaneously). Incubate plate for 60 min at room temperature. Rinse plate 4 times with water to remove unreacted sample and enzyme conjugate. Add 160 µL substrate-chromogen mixture (1 + 1). Incubate plate for 30 min; then add 1 drop 2.5N sulfuric acid to each well to stop reaction (color changes from blue to yellow).

Measure intensity of yellow solutions by reading plate at 450 nm in Millipore Microwell Strip Reader or full plate reader. Calculate $\%B_O$ values of standards and samples (100 × absorbance at 450 nm of standard or sample/average absorbance at 450 nm of negative control comprised of RO water). Use average values of standards to prepare standard curve for calculating samples. Prepare standard curve and calculate MBC concentration in samples as described for tube assay.

LC Determination of MBC

Add 5 mL wine to 50 mL conical polypropylene centrifuge tube followed by 20 mL extraction solvent (**i**) and 20 mL methylene chloride. Shake tube vigorously for 5 min in wrist shaker. Centrifuge for 3 min at 5000 × g. Remove bottom layer (methylene chloride) into 20 mL scintillation vial containing 0.5 g sodium sulfate. Dry 10 mL aliquot of methylene chloride under nitrogen. Dissolve residue in 1 mL mobile phase (**h**) and transfer to 1.5 mL polypropylene centrifuge tube. Centrifuge 5 min at 10 000 × *g*. Inject 5 µL sample, using LC conditions

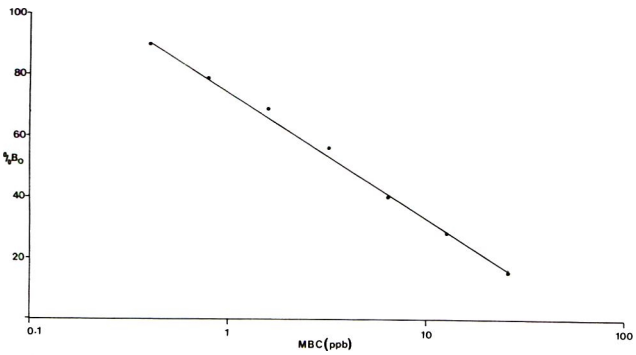

Figure 1. Typical standard curve for MBC tube EIA in organic wine.

described in *Apparatus* (**a**) and (**b**) and *Reagents and Standards* (**h**).

Results and Discussion

The typical standard curve for MBC (Figure 1) shows a linear relationship between the logarithm of the MBC concentration (0.4–26 ppb MBC) and the corresponding $\%B_0$ values at 450 nm. Samples with a concentration >26 ng/mL (indicated by $\%B_0$ <13) are simply diluted.

Food matrix effects are quite common in immunoassay (9), and wine is no exception. To help prevent such effects, 0.1 mL wine samples were evaporated under nitrogen and diluted to volume with 1 mL diluting solvent (**d**). In addition to reducing the matrix effect, the milk solution helped to maintain the pH at neutrality (wine is acidic) and also increased the solubility of the MBC. Control samples should contain 0.1 mL evaporated organic wine/mL milk and mobile phase solution. It is easy to find organic wine, but it should be tested by LC before use.

The limit of quantitation for evaporated wine samples by the tube assay was 5 ppb, determined by using the procedure developed in 1980 by a committee of environmental chemists (10). The limit of quantitation for the plate assay was 50 ppb.

Further studies with the tube immunoassay showed that if a lesser degree of accuracy is acceptable, the wine can be added directly to skim milk (0.1 mL wine/0.9 mL milk) and analyzed directly. Failure to evaporate the wine gives less accurate results (determined from a spiking study using nonevaporated and evaporated wine), yielding a high bias of approximately 6–37% in the MBC concentration of wines depending upon the composition of the wine. The major differences in MBC concentrations between the nonevaporated and evaporated wines were encountered at the first (1 + 10) dilution, because the matrix effect will be greatest at this level. Ethanol contributes to the matrix effect; consequently, the evaporation step is beneficial. However, other unidentified components add to the matrix effect. Preliminary results indicate that anthocyanins may be some of the unidentified components.

As with any analytical technique, precision within and between days is crucial. Reproducibility results for the MBC tube immunoassay for standards and wine are presented in Tables 1–3. Table 1 shows the consistency data obtained from analyzing MBC standards fortified with 0.1 mL evaporated red and white organic wine. Intra- and interassay %CVs ranged from 2.4 to 15, with most CVs <10. In fact, the interassay study was conducted over an entire year with several different lots of tube immunoassay kits.

Table 1. Reproduciblity of the MBC tube immunoassay for standards prepared in skim milk containing 10% LC mobile phase

MBC standard, ppb	CV, % (intra-assay)[a]	CV, % (interassay)[b]
0.4	2.4	2.6
0.8	5.7	4.8
1.6	7.1	7.6
3.2	7.6	8.1
6.4	11	8.3
13	13	12
26	12	15

[a] Percent coefficients of variation based on 6 determinations in 1 day.
[b] Percent coefficients of variation based on 21 determinations performed on 21 different days.

MBC was determined by tube EIA in a wide variety of 13 commercial wines, with MBC concentrations varying from 6.3 to 930 ppb. The results of intra- and interassay study are given in Table 2 for the evaporation process. Most %CVs were ≤15 (range 7.4–23). The intra- and interassay %CVs for this method are very comparable.

Reproducibility at the interassay level of the nonevaporation process was also studied for 6 commercial wines. The %CVs ranged from 6.9 to 23 (Table 3). These 6 wines correspond to wines from Table 2. By comparing the interassay re-

Table 2. Reproducibility of the MBC tube immunoassay for evaporated wine samples

Wine	MBC, ppb	CV, % (intra-assay)[a]	CV, % (interassay)[b]
Blueberry No. 1*	14	16	20
White No. 1*	32	9.4	15
Apple	59	11	11
Apple-blueberry	217	7.4	12
White No. 2	155	15	16
Red No. 1	219	12	13
Red No. 2	927	13	8.3
White No. 3	102	21	20
Red No. 4	172	13	9.3
Red No. 5	15	10	7.8
Red No. 6	8.4	10	14
Red No. 7	5.0	21	23
Blueberry No. 2*	930	15	8.0

[a] Percent coefficients of variation based on 6 determinations in 1 day except for results marked with asterisk, which are based on 5 determinations.
[b] Percent coefficients of variation based on 6 determinations performed on 6 different days.

Table 3. Reproducibility of the MBC tube immunoassay for wine samples not evaporated[a]

Wine	MBC, ppb	CV, % (interassay)
Red No. 4	190	6.9
White No. 3	129	11
Red No. 2	983	18
Red No. 5	24	22
Red No. 7	7.3	23
Red No. 6	11	14

[a] Mean and percent coefficients of variation are based on 6 determinations performed on 6 different days.

sults, the precision seems to be similar regardless of whether samples are evaporated before analysis. However, as mentioned earlier, the wine samples in Table 3 have higher MBC concentrations than those in Table 2. As discussed earlier, there is a matrix effect with nonevaporation, especially at the first (1 + 10) dilution.

Recovery studies were also performed on evaporated spiked organic wines. Results are given in Table 4. Spiking levels varied from 10 to 10 000 ppb, and recoveries ranged from 81 to 105% (mean recoveries for red and white wines were the same, 93%). Thus, the accuracy was acceptable. Reproducibility was also adequate in this study. Percent CVs ranged from 4 to 16, with all but one ≤13.

Cross-reactivity of the benomyl antibody (9) varies with other benzimidazole and thiophanate-type fungicides; MBC has the same reactivity as benomyl. Thiabendazole is sufficiently reactive to be detected at residue levels, but we have never seen it in wine, although our LC method will quantitate thiabendazole at the low ppb level.

Table 4. Accuracy of MBC tube immunoassay for red and white wine samples evaporated

MBC added, ppb	MBC found, ppb	Mean rec., %	CV, %
10	10	100[a]	13[a]
20	18	90	13
100	86	86	4.0
500	527	105	7.0
2000	1864	93	7.0
10000	8080	81	8.0
10	8.7	87[b]	16[b]
20	19	95*	13*
100	97	97	10
500	420	84*	5.5*
2000	1860	93	11
10000	10400	104	8.6

[a] Red wine series of means and percent coefficients of variation are based on 5 determinations.

[b] White wine series of means and percent coefficients of variation are based on 5 determinations except samples marked with asterisk are based on 4 determinations.

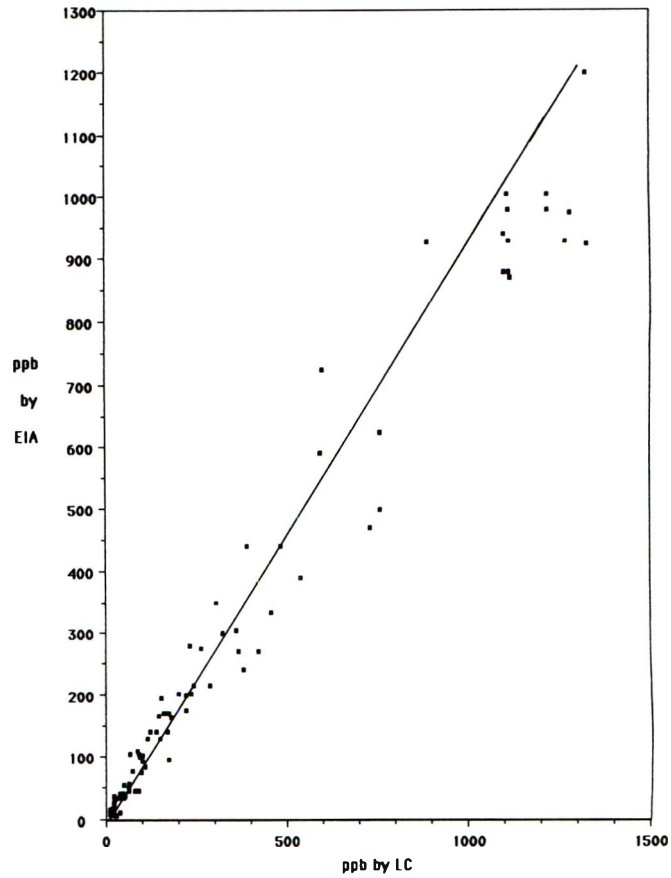

Figure 2. Correlation between MBC concentrations as determined by tube EIA and LC in wine.

A correlation study between the tube EIA and LC was performed on 134 wine samples. Ninety-eight samples were positive for MBC by both methods, with a correlation coefficient of 0.986 (Figure 2) ($y = 1.23 \times -9.58$). This agreement is acceptable, especially considering the number and variety of wines analyzed and the wide range of MBC levels found (10–1329 ppb). Furthermore, the data for this study were collected over a year. The MBC concentrations of the other 36 samples either were not detectable by either method or were below the detection limit of 10 ppb for the LC.

Results for all 134 wine samples are given in Table 5. Wine was analyzed from 8 countries; the largest numbers of samples were from Switzerland and the United States. Seventy-four were red wines, 41 were white, and 19 were comprised of apple, blueberry, apple-blueberry, and strawberry. Of the 74 red wines, 73% contained detectable MBC concentrations by both procedures; 68% of the white wines were positive by both EIA and LC.

A mini-study between the plate assay and the tube assay was conducted with 11 wines. The results are shown in Table 6. Of these 11 samples, 9 were positive for MBC by the tube assay and 8 by the plate EIA. The plate test does not detect MBC below 50 ppb because of matrix effects; therefore, 1 sample was below its detection limit. However, the correlation was 0.938 ($y = 0.943 \times 127$) for the 8 samples that were demon-

Table 5. Comparison of tube immunoassay and LC for the determination of MBC in wine[a]

Type of wine	Country	MBC		Type of wine	Country	MBC	
		Immunoassay	LC			Immunoassay	LC
1R	Switzerland	ND	ND	56R	Switzerland	5	ND
2R	Switzerland	6.4	ND	57R	Switzerland	ND	ND
3W	Switzerland	ND	ND	58R	Switzerland	167	144
4R	Switzerland	5.0	ND	59R	Switzerland	94	101
5R	Switzerland	12	13	60W	Switzerland	275	262
6R	Switzerland	18	16	61W	Germany	ND	ND
7R	Switzerland	9.0	ND	62W	USA	ND	ND
8R	Switzerland	ND	ND	63W	USA	ND	ND
9R	Switzerland	163	180	64R	France	ND	ND
10R	Switzerland	140	170	65R	France	ND	ND
11R	France	ND	ND	66W	USA	ND	ND
12W	Chile	45	89	67BA	USA	15	17
13R	France	45	81	68W	USA	33	36
14R	France	36	53	69A	USA	55	47
15W	USA	5.4	ND	70AB	USA	140	137
16W	Switzerland	55	62	71R	Switzerland	240	381
17R	Switzerland	203	201	72R	Switzerland	270	367
18R	Switzerland	12	12	73R	Switzerland	170	171
19R	Switzerland	17	13	74R	Switzerland	6.8	ND
20W	Switzerland	440	483	75R	Switzerland	7.8	24
21R	Switzerland	57	62	76W	Switzerland	175	220
22R	Switzerland	203	235	77W	Switzerland	130	149
23W	Switzerland	300	320	78R	Switzerland	390	539
24W	Switzerland	130	115	79R	Switzerland	15	ND
25W	Switzerland	215	285	80R	Switzerland	12	37
26W	USA	12	10	81R	France	110	85
27W	Germany	6.4	24	82R	France	140	122
28W	USA	15	22	83W	Australia	8.0	ND
29R	USA	ND	ND	84W	Italy	ND	ND
30W	Germany	ND	ND	85W	Germany	ND	ND
31W	Switzerland	105	67	86W	France	14	IF
32R	Switzerland	85	108	87W	France	42	38
33R	Switzerland	ND	ND	88W	USA	42	47
34R	Switzerland	195	512	89W	USA	34	50
35R	Switzerland	ND	ND	90W	USA	10	11
36R	Switzerland	215	241	91R	Switzerland	75	96
37R	Switzerland	270	421	92R	Switzerland	14	10
38R	Switzerland	ND	ND	93R	Switzerland	15	10
39R	Switzerland	927	888	94R	Switzerland	95	172
40R	Switzerland	590	594	95W	Switzerland	170	170
41W	USA	ND	ND	96R	Switzerland	ND	ND
42S	USA	ND	ND	97R	Switzerland	925	1329
43W	Spain	170	157	98R	Switzerland	100	93
44W	USA	6.0	ND	99W	Switzerland	105	90
45B	USA	ND	ND	100R	Switzerland	35	39
46R	Italy	50	63	101W	USA	15	22
47R	France	21	20	102A	USA	880	1100
48R	France	15	20	103A	USA	940	1100
49W	USA	36	19	104A	USA	980	1114
50W	USA	10	11	105A	USA	930	1114
51R	Switzerland	975	1284	106A	USA	980	1114
52W	Switzerland	1200	1329	107A	USA	980	1222
53R	Switzerland	1005	1222	108B	USA	880	1114
54R	Switzerland	625	758	109B	USA	870	1118
55R	Switzerland	305	357	110B	USA	930	1270

Table 5. *(Continued)*

Type of wine	Country	MBC	
		Immunoassay	LC
111R	Switzerland	350	303
112R	Switzerland	725	600
113R	Switzerland	6.8	ND
114R	Switzerland	280	232
115R	Switzerland	6.3	28
116R	Switzerland	5.0	ND
117R	Switzerland	102	101
118R	Switzerland	470	732
119W	Switzerland	440	390
120W	Switzerland	78	74
121B	USA	930	1270
122B	USA	930	1270
123B	USA	930	1270
124B	USA	45	61
125R	USA	19	21
126B	USA	28	22
127W	USA	42	47
128R	Switzerland	31	25
129R	Switzerland	201	222
130R	Switzerland	500	757
131R	Switzerland	335	454
132R	Switzerland	1005	1110
133R	Switzerland	9.4	ND
134R	Switzerland	ND	ND

[a] ND = none detected at a detection limit of 5 ppb for immunoassay and 10 ppb for LC; IF = none detected because of interference; R = red wine; W = white wine; A = apple wine; BA = apple-blueberry wine; B = blueberry wine; and S = strawberry wine.

strated to contain MBC by both EIAs. The plate immunoassay allows for a greater number of samples to be analyzed.

For determining MBC in wine, the immunoassay method is adequate either as a screening procedure or for quantitation. The nonevaporation method should be used for screening, because it is fast but less accurate. However, for quantitation by EIA, the evaporation step must be used to obtain the accuracy needed. Therefore, immunoassay, a very reproducible, rapid, and inexpensive technique, could be used as a confirmation technique for MBC or for a more precise method of collecting dietary MBC data for risk assessment studies.

Table 6. Comparison of the plate and tube immunoassay for MBC determination in wine[a]

Wine sample	MBC, ppb	
	Tube immunoassay	Plate immunoassay
1R	ND	ND
2R	540	595
3R	975	1056
4R	1200	1042
5R	1005	1227
6W	275	311
7W	390	489
8R	12	ND
9R	ND	ND
10R	925	1131
11R	500	648

[a] ND = none detected at a detection limit of 5 ppb for the tube assay and 50 ppb for the plate assay; R = red wine; W = white wine.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Optimization of Experimental Conditions for the Supercritical Carbon Dioxide Extraction of Pesticide Residues from Grains

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The efficiency of a supercritical carbon dioxide extraction system was investigated for the extraction at different pressures and temperatures of fortified grain samples containing organochlorine, organophosphorus, and organonitrogen pesticides from grain matrixes. The extractor unit was constructed using a gas booster pump and a Florisil sorbent trap for extracting and isolating the residues of interest. Using 20 g samples, extractions were performed between 40° and 80°C with pressures from 2000 to 10 000 psig. In most cases, pesticide recoveries exceeding 80% were recorded over the above range of temperature and pressure. Excellent recoveries and precision were recorded for an incurred methyl chlorpyrifos residue at the 0.043 ppm level. An improved liquid chromatographic assay was also developed for the analysis of carbofuran in grain samples.

The analysis of pesticide residues in grains intended for human consumption (1) or in the production of fermentation-derived chemicals (2) is of increasing importance because of tolerance and revised-action levels developed by regulatory agencies. In addition, regulatory agencies (3) are increasing scrutiny of traditional pesticide sample workup and analysis methodologies because of the disposal problems and environmental impact of the organic solvents used in such methods. Supercritical fluid extraction (SFE) is a viable alternative to traditional organic solvent-based methods used in the analysis of pesticide residues (4–6).

Several researchers (7–9) demonstrated the efficacy of SFE of selected pesticides from grain and crop matrixes. However, optimal extraction conditions applicable to many types of pesticides need to be identified so that the extraction procedure can be used as a multiresidue analysis screening method.

Theoretical schemes were applied by several investigators (10, 11) in an attempt to optimize extraction conditions for SFE. Currently, the most effective way to optimize the extraction step for multiresidue analysis is to run a series of experiments that carefully delineate factors contributing to optimum pesticide recoveries. The present study was undertaken to define optimum conditions for the extraction of several classes of pesticides commonly found in wheat, including methoxychlor, chlorpyrifos, dieldrin, malathion, pirimiphos-methyl, dimethoate, parathion-methyl, and carbofuran.

Samples obtained from the Federal Grain Inspection Service (FGIS) were fortified at 2 spiking levels (5.0 and 0.1 ppm) and extracted by using supercritical carbon dioxide (SC-CO_2) at combinations of 3 different pressures and temperatures. The extracted analytes were collected after decompression of SC-CO_2 on a sorbent-filled tube containing Florisil. Conventional elution solvents (12) were applied to desorb the pesticides from the sorbent cartridge before additional sample cleanup and/or direct analysis by gas chromatography (GC) or liquid chromatography (LC).

Experimental

Apparatus and Reagents

The extraction apparatus was similar in construction and operating principle to a previously described device (13). Modifications consisted of the insertion of an alumina cleanup column to purify the extraction fluid before the compressor, the placement of the extractor vessel in a Bendix 2600 GC oven, and the installation of Florisil-filled trap after the micrometering valve (Figure 1). One of 2 gas booster compressors were used depending on the desired extraction pressure.

(a) *Compressors.*—Models AGT-62/152 or AGC-30 (Haskel Engineering Corp., Burbank, CA) were used to generate the CO_2 pressure required for the extractions. The Model AGC-30 was used for the extractions at 2000 psi, because it provided better pressure control in this region.

(b) *Alumina cleanup column.*—A column for cleaning up the extraction fluid was constructed from 316 SS tubing (Part No. 15-009, Autoclave Engineers, Erie, PA), pressure rated to

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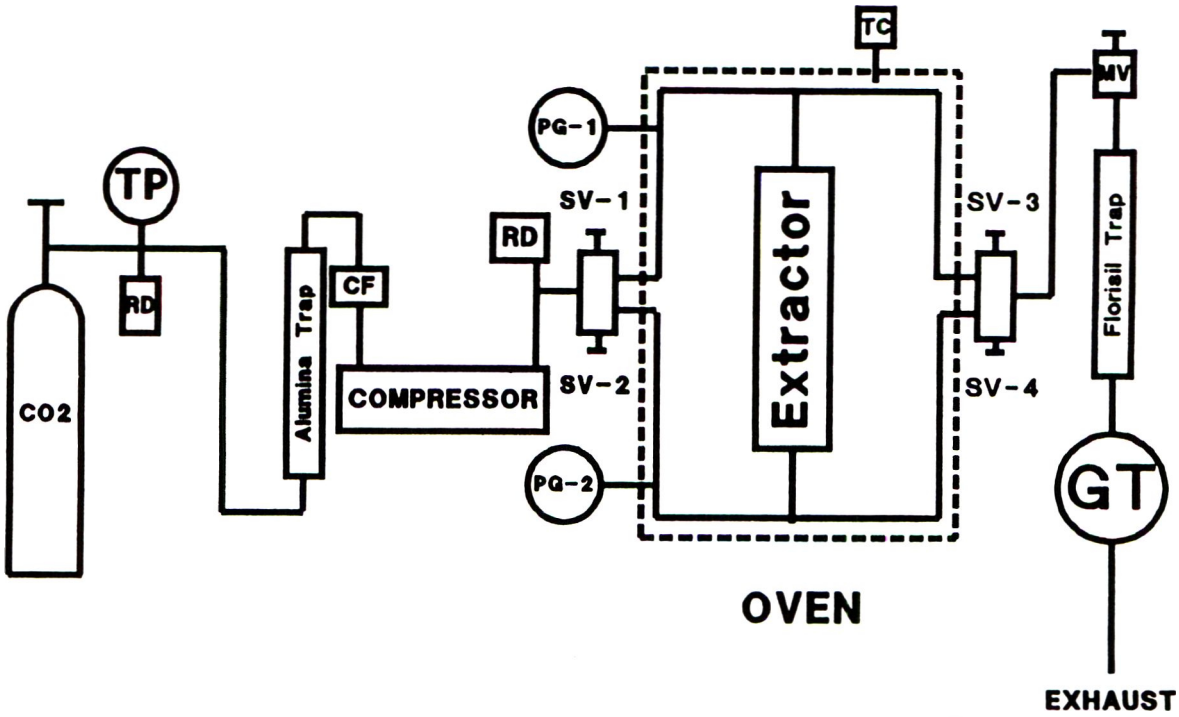

Figure 1. Supercritical fluid extraction apparatus with sorbent trap option: TP = tank pressure, RD = rupture disk, CF = check valve and filter, PG = pressure gauge, SV = switching valves, TC = thermocouple, MV = micrometering valve, and GT = gas totalizer.

76 MPa (11 000 psi) at room temperature, with dimensions of 56 × 1.75 cm id, filled with Alumina C held in place by glass wool plugs.

(c) *Florisil trap.*—A 316 SS column, 30.4 × 0.95 cm id, containing activated Florisil held in place by glass wool plugs was used as the pesticide trap.

(d) *Concentrator system.*—A miniature concentrator system consisting of a distilling trap adapter (No. 5226, Ace Glass, Inc., Vineland, NJ) and a micro evaporative concentrator (No. 6709, Ace Glass, Inc.) was constructed.

(e) *Gel permeation chromatograph.*—A gel permeation chromatographic (GPC) system, Auto-Prep 1002B (ABC Laboratories, Inc., Columbia, MO), equipped with a 30 × 2.5 cm id column (ABC Laboratories) slurry packed with 33 g Bio-Beads SX-3 resin (200–400 mesh, Bio-Rad Laboratories, Richmond, CA) and compressed to a bed length of ca 20 cm, was used for further cleanup of the extracted fractions. The elution solvent was methylene chloride–hexane (50 + 50, v/v) pumped at a flow rate of 5.0 mL/min at an operating pressure range of 8 to 11 psig. The GPC system was set up to execute a 12 min dump, 16 min collect, and 0 min wash cycle. The sample loading filter consisted of a 5.0 µm prepdisc membrane filter (Bio-Rad).

(f) *Gas chromatograph 1.*—A Varian Model 3600 GC system, equipped with an Ni-63 electron capture detection (ECD) system, a flame photometric detection (FPD) system using a phosphorus-specific filter (526 nm), a septum-equipped programmable injector (SPI), a packed-column injector, and an 8100 autosampler, was used for the analysis of specific pesticides. The packed-column injector was modified for the direct flash vaporization injection onto a wide-bore fused silica column as previously described (14). A 2 m × 0.53 mm id, presilanized, uncoated fused silica retention gap (No. 1602535, J&W Scientific, Inc., Folsom, CA) was connected to the modified inlet through a universal glass connector (No. 2-0479, Supelco, Inc., Bellefonte, PA) to a 15 m × 0.53 mm id, 1.0 µm/min film thickness, DB-17 fused silica column (No. 125-1712, J&W Scientific, Inc). A 10 m × 0.53 mm id, 1.0 µm film thickness, DB-1301 fused silica column (No. 125-1312, J&W Scientific, Inc.) was connected to a DB-17 column through a universal glass connector and then to a makeup gas fitting (No. 103462, Scientific Glass Engineering, Austin, TX) for connection to the FPD system. A 2 m retention gap was then connected to the SPI inlet and connected to a universal glass connector that was fastened to a 15 m × 0.53 mm id, 1.5 µm film thickness, DB-5 fused silica column (No. 125-5012, J&W Scientific, Inc.). The DB-5 column was then connected to the makeup gas fitting for the ECD system. Flow rates of ca 10 and 15 mL/min of helium, respectively, were used for the DB-5 and DB-17+DB-1301 columns. A makeup gas flow rate of 15 mL/min nitrogen was used for each detector. The following temperature parameters were used for ECD in Method 1: SPI inlet, initial 60°C with 0 min hold, programmed at 275°C/min to 240°C final temperature with 15 min hold; ECD system, 300°C; column, 100°C with 1 min hold, programmed at 10°C/min to 250°C final temperature with 6 min hold. The following temperature parameters were used for FPD in Method 2: inlet, 220°C; FPD system, 300°C; column, 150°C with 1 min hold, programmed at 5°C/min to 200°C final temperature with 5 min hold.

(g) *Gas chromatograph 2.*—A Tracor Model 540 GLC equipped with a Hall electrolytic conductivity detector

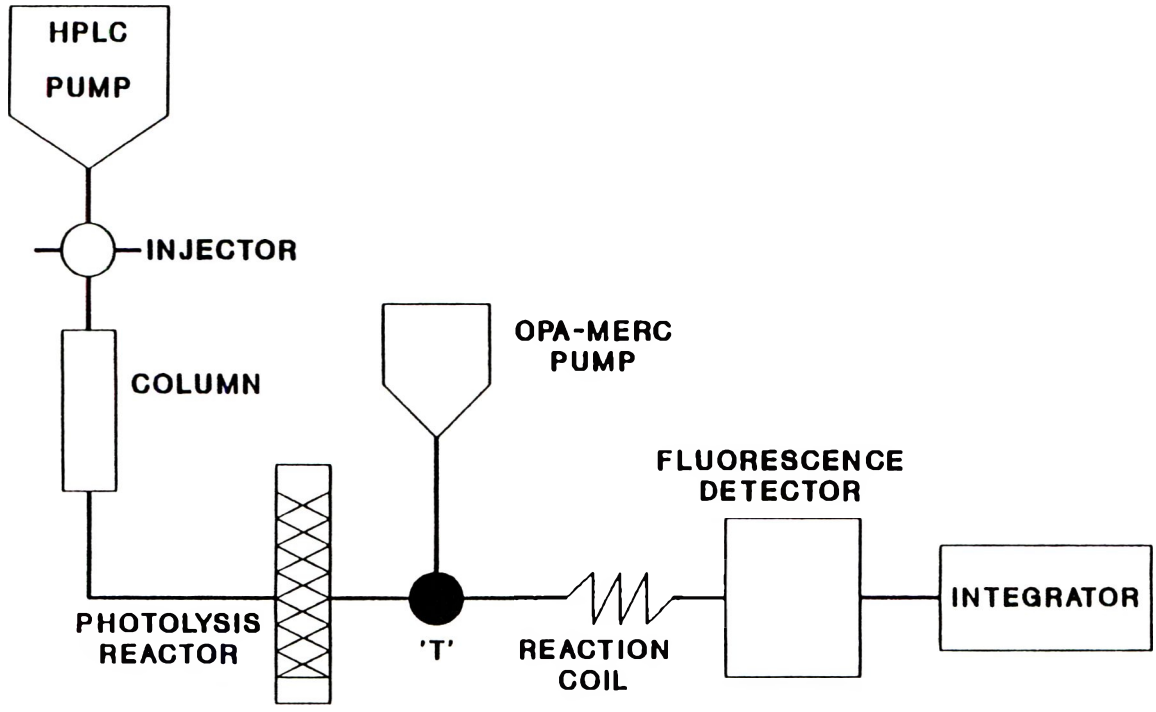

Figure 2. LC apparatus for carbofuran determination.

(HECD) was also used for pesticide analysis after SFE. A 2 m × 0.53 mm retention gap was connected to the modified packed column inlet as noted above and then through a universal glass connector to a 30 m × 0.53 mm id, 1.5 μm film thickness, DB-1 fused silica column (No. 125-1012, J&W Scientific, Inc.). The DB-1 column was connected to the HECD without makeup gas. Hydrogen was used as the carrier gas at a flow rate of ca 30 mL/min. Reaction gas flow rate for the furnace was 50 mL/min hydrogen. Solvent flow rate for the cell was 0.4 mL/min *n*-propanol. The following temperature programming parameters were used: inlet, 220°C; detector base, 260°C; column, 200°C; furnace, 900°C.

(**h**) *Liquid chromatograph.*—The basic schematic for the LC system used for the analysis of carbofuran in the extracts is shown in Figure 2. This is a modification of a previously described system (15) consisting of an injector, guard column, column, and column oven. Specific chromatographic parameters used are as follows: an Adsorbosphere C-8 column packed with 5 μm packing in a 150 × 4.6 mm cartridge, along with a Adsorbosphere C-8 guard column. Injections were 15 μL into an acetonitrile–water (40 + 60) mobile phase pumped through the column at a flow rate of 1.0 mL/min. The column temperature was 40°C.

The postcolumn photolysis unit consisted of a Model 80-1178-01 BHK Lamp, 17 cm × 9 mm (BHK, Inc., Pomona, CA), with a Model 90-001-01 power supply (BHK Inc.). The Teflon sleeve surrounding the photodegradation lamp was prepared by weaving 3.7 m × 0.5 mm id × 1/16 in. od Teflon tubing into a 3 dimensional coil. Alternatively, one may purchase a 10 ft × 0.5 mm id commercial delay coil (Part No. 5-9206, Supelco, Inc., Bellfonte, PA). The coiled Teflon tubing was placed over the lamp and connected at one end to the column outlet and at the other end to the mixing tee, which was a 10.25 mm id × 1/16 in. od fitting (No. ZT1C, Valco Instruments, Inc., Houston, TX). A low flow rate pump (Model 396-31, LDC/Milton Roy, Riviera Beach, FL) was used for the addition of *o*-phthalaldehyde–mercaptoethanol (OPA–MERC). The reaction coil consisted of 3.0 m × 0.3 mm id. Teflon tubing and was made into 3 dimensional coil or purchased as a delay coil from Supelco, as noted above. A Shimadzu RF 503 fluorescence detector (Shimadzu, Inc., Columbia, MD), or equivalent, was used to detect the carbofuran.

(**i**) *Carbon dioxide.*—Welding grade CO_2 (National Welding Supply Co., Bloomington, IL) was used for all of the extractions. Impurities in the CO_2 were removed by the use of an in-line trap filled with Alumina C sorbent.

(**j**) *Alumina C.*—1 kg (Part No. 02103-99, Universal Scientific Inc., Atlanta, GA) was heated to 230°C for 1 h to achieve maximum activity.

(**k**) *Florisil.*—A 60/80 mesh fraction of PR grade Florisil (Floridin Co., Berkely Springs, WV) was used for trapping the extracted analytes. The sorbent activity was determined as described in PAM I, Section 121.3.

(**l**) *Solvents.*—Pesticide or LC grades of the following solvents were used in this study: ethyl acetate, acetone, hexane, methylene chloride, and acetonitrile. Milli-Q reagent grade water (Millipore Corp., Bedford, MA) was used throughout the experiments.

(**m**) *LC reagents.*—Derivatization and detection agents: OPA and MERC, reagent grade (Sigma Chemical Co., St. Louis, MO). OPA–MERC reagent was prepared by dissolving 75–80 mg OPA in 2 mL methanol and then adding this to 500 mL 0.01M pH 10.5 borate buffer. The solution is then

mixed, 0.5 mL MERC is added, and the solution is mixed again.

(n) *Pesticide standards.*—Pesticide standards used in spiking the wheat samples were obtained from the U.S. Environmental Protection Agency (EPA), Analytical Chemistry Section, Beltsville, MD, or the U.S. Deptartment of Interior, Fish Wildlife Research Center, Chemistry Section, Laurel, MD. Analytical standards were prepared using pesticides obtained from EPA, Pesticide and Industrial Chemicals Repository, Research Triangle Park, NC. All samples and standards were filtered with 0.22 µL/min nylon polypropylene-encased 13 mm id filters (Alltech Associates, Inc., Deerfield, IL) before LC analysis.

Preparation and Spiking of Grain Samples

Approximately 2500 g of a 5000 g wheat sample was ground on a FN 3100 mill equipped with a 1 mm screen. Forty 50 g samples were then weighed into individual containers for pesticide fortification. Spiking solutions for each pesticide were prepared by weighing 100 mg of each pesticide in a beaker, transferring the pesticide into a 100 mL volumetric flask using ethyl acetate, and diluting to the mark to yield a stock solution of 1 µg/µL.

The above solution was used to prepare spiked samples at the 5 and 0.1 ppm level. For the 5 ppm spikes, a 50 g wheat sample was spiked with 250 µL stock solution using a Hamilton No. 1725 gas-tight 250 µL syringe (Hamilton Co., Reno, NV). The open glass bottle was then placed in a hood for 5–6 min until the ethyl acetate was evaporated. The bottle was then capped with an aluminum foil-lined cover. For preparing the 0.1 ppm spikes, the stock solution was diluted 1:10. A 50 µL aliquot of this solution was sampled using a Hamilton No. 805, gas-tight 50 µL syringe and added to a 50 g wheat sample in a glass bottle. The 0.1 ppm spiked wheat samples were then placed in a hood for 2–3 min to let the ethyl acetate evaporate. The ground wheat samples were then thoroughly mixed after fortification and again when sampling for SFE.

Extraction Procedure

A wheat sample (20 g) was weighed into a 250 mL beaker and then poured through a small funnel into the extraction vessel that contained a glass wool plug at the bottom. After filling the extraction vessel with the wheat sample, another glass wool plug was placed on top of the sample after the plug was used to remove any additional sample adhering to the walls of the beaker or funnel. The extraction vessel was then assembled and placed in the oven (Figure 1). Extraction pressure and temperature were set at the desired values and the CO_2 flow commenced. The CO_2 flow rate was set to ca 5 L/min (decompressed flow) as measured under ambient conditions on a dry test meter. Approximately 150 L CO_2 was used to complete the extractions at 2000 psi, whereas 100 L CO_2 was used for the 5000 and 10 000 psi extractions. The extracted analytes were collected on the described Florisil trap after the SC-CO_2 was decompressed. Acetone (50 mL) was used to elute the adsorbed pesticides from the Florisil bed at ambient temperature, and this extract was pooled with a 10 mL acetone rinse of the micrometering valve (MV in Figure 1). Typical extraction times were 20–30 min.

Sample Cleanup and Analysis

The 20 g wheat samples fortified at 5 ppm were analyzed by diluting the SFE extract to 100 mL in acetone. A 4 mL portion of this solution was diluted to 10 mL with acetone. The organophosphorus pesticides in this solution were quantitated with external standards using FPD. The organochlorine pesticides were quantitated with external standards using ECD and/or the HECD. Chlorinated and organophosphate pesticide standards diluted from 1.0 mg/mL stock solution in acetone to a final concentration of 0.4 µg/mL were used for this purpose.

The 20 g wheat samples fortified at 0.1 ppm were analyzed by diluting the SFE extract to 100 mL in acetone. A 50 mL portion of this solution was pipetted into a Kuderna-Danish evaporator (250 mL) with a 10 mL graduated concentrator tip. The sample was then evaporated to <5 mL using a Snyder column and steam bath. *n*-Hexane (50 mL) was then added through the Snyder column and the eluate was reconcentrated. This sample eluate was then concentrated to a small volume (<3 mL) with a distilling trap adapter and micro-evaporative concentrator. The volume was adjusted to 5 mL with *n*-hexane, diluted to 10 mL with methylene chloride, and thoroughly mixed. Then, 5 mL of each sample was cleaned-up by the GPC method described by Hopper (16). The sample eluate was transferred to a Kudema-Danish (250 mL) with a 4 mL graduated concentrator tube and concentrated to a small volume (<3 mL). The eluate was further concentrated (<1 mL) as previously described and diluted to a final volume of 2 mL with acetone. The organophosphorus and organochlorine pesticides were quantitated by GC as described in *Apparatus and Reagents*.

Also, duplicate 20 g wheat samples fortified at 5 and 0.1 ppm were extracted in duplicate by the conventional 35% water–acetonitrile procedure (17). Sample extracts were partitioned with 100 mL petroleum ether and the recovered volumes were recorded. All sample extracts were then diluted to 100 mL with petroleum ether. The organophosphorus and organochlorine pesticides contained in the 5 ppm fortifications were quantitated, without further dilution, by GC as described in *Apparatus and Reagents*. The sample extracts from the 0.1 ppm fortifications were analyzed by the procedure as described in *Apparatus and Reagents*, except that sample eluates were diluted to a final volume of 1 mL in acetone. Wheat sample blanks were also analyzed during each procedure to check for interfering responses. Methyl chlorpyrifos was found as an incurred residue in the wheat at a sub-part-per-million level.

For the determination of carbofuran by LC, the extract from the 5.0 ppm spiked wheat sample was diluted to 100 mL with acetone. Two milliliters of this solution was then pipetted into a 5 mL graduated cylinder and evaporated to dryness with nitrogen at 40°C. The resulting residue was dissolved in acetonitrile–water (1 + 1) and filtered for LC before analysis.

For the 0.1 ppm spiked wheat samples, the extract representing 20 g of SFE-extracted wheat was diluted to 100 mL with acetone. A pipet was then used to transfer 25 mL of the above solution to a 250 mL Kudema-Danish evaporator. Hex-

Table 1. Pesticide recoveries (%) at 2000 psi extraction pressure as a function of temperature (150 L CO_2)

		40°C		60°C		80°C	
	Spike level, ppm	A	B	A	B	A	B
Dimethoate	5.0	75	76	74	75	32	26
	0.1	91	92	82	86	53	63
Methyl parathion	5.0	76	71	78	71	64	65
	0.1	103	100	87	89	84	87
Pirimiphos-methyl	5.0	93	82	102	93	90	89
	0.1	106	102	99	99	93	94
Chlorpyrifos	5.0	95	84	105	97	93	89
	0.1	104	97	97	96	95	97
Malathion	5.0	81	78	84	78	74	72
	0.1	111	108	103	99	95	94
Dieldrin	5.0	102	89	97	96	93	92
	0.1	98	87	84	85	90	90
Methoxychlor	5.0	104	88	99	94	74	68
	0.1	110	90	78	81	75	75
Carbofuran	5.0	82	74	80	81	77	73
	0.1	95	90	87	90	85	81

ane (30 mL) was added, and the solution was evaporated on a steam bath to 3 mL; an additional 30 mL portion of hexane was added during the evaporation. The cooled liquid was transferred to a 15 mL centrifuge tube and evaporated to dryness using a stream of nitrogen at 40°C. Two milliliters acetonitrile–water (1 + 1) and 0.5 mL hexane previously saturated with acetonitrile–water (1 + 1) were added. The mixture was shaken 30 s and centrifuged at 1000 rpm for several minutes to clarify the layers. The lower aqueous layer was withdrawn and filtered before LC analysis.

The apparatus shown in Figure 2 was used to assay the wheat sample extracts. Sample chromatograms were compared with a chromatogram obtained from the analysis of a standard solution of carbofuran. A 1.0 mg/mL carbofuran stock solution prepared in methanol was used to prepare 2 working standards: 1 µg/mL, used to assay the 5 ppm spiked samples, and 0.25 µg/mL, used to assay the 0.1 ppm fortified wheats.

Results and Discussion

Extractions were performed at 3 different temperatures and 2 fortification levels in the wheat. Experiments were run using all possible combinations of the above variables. Duplicate runs were made under each experimental condition, for a total of 36 extractions.

Some experimental optimization was done before the initiation of the above extractions. These experiments were run to determine a suitable extraction flow rate, minimize the time of extraction, ascertain the effect of the total volume of extraction fluid on pesticide recovery, and to determine the required amount of trapping sorbent to avoid breakthrough of the analyte off the sorbent bed. Extraction times of 20–30 min and a CO_2 flow rate of 5 L/min at all extraction pressures and temperatures yielded high analyte recoveries. Experiments using dual Florisil traps (5 and 10 g each) aligned in series indicated that a single trap filled with 5 g Florisil was adequate to trap the pesticides under the above conditions.

Results for SFE of wheat samples at 3 extraction pressures are summarized in Tables 1–3. Listed within each table are the pesticide recoveries at the 3 designated temperatures: 40, 60, and 80°C. Recoveries at both fortification levels are listed side by side for comparison. The letters A and B designate percent recovery value for each of 2 separate extractions under identical conditions of the same spiked wheat sample.

The overall extraction results tabulated in Tables 1–3 are very encouraging. With the exception of a few extractions, pesticide recoveries in most cases were >80%. Tables 1–3 also reveal that somewhat higher recoveries were achieved when extracting at 5000 and 10 000 psi as opposed to 2000 psi, regardless of the extraction temperature. At the 2000 psi extraction pressure, recoveries from the extraction of the 0.1 ppm spiked wheats are higher than those obtained from extracting the 5.0 ppm fortifications. This trend is not evident at the 5000 and 10 000 psi extraction conditions.

The agreement between duplicate extractions at the same fortification levels under identical extraction conditions was good (Tables 1–3). Although not shown with the data in Tables 1–3, the appearance of an incurred residue of methyl chlorpyri-

Table 2. Pesticide recoveries (%) at 5000 psi extraction pressure as a function of temperature (100 L CO_2)

		40°C		60°C		80°C	
	Spike level, ppm	A	B	A	B	A	B
Dimethoate	5.0	86	96	84	95	78	94
	0.1	88	87	82	101	77	84
Methyl parathion	5.0	97	100	95	104	88	98
	0.1	89	89	92	103	91	93
Pirimiphos-methyl	5.0	95	100	98	104	92	100
	0.1	96	95	101	108	99	100
Chlorpyrifos	5.0	92	102	98	107	98	102
	0.1	97	97	105	113	99	101
Malathion	5.0	102	113	104	116	111	109
	0.1	93	95	102	109	96	97
Dieldrin	5.0	90	91	91	91	92	98
	0.1	95	91	104	104	93	91
Methoxychlor	5.0	96	99	91	98	96	101
	0.1	94	94	85	107	97	103
Carbofuran	5.0	93	99	86	86	93	102
	0.1	89	97	97	98	92	95

fos in the wheat allowed an estimate to be made of the reproducibility of the above SFE technique. The level of this incurred residue was determined 18 times when the 0.1 ppm spiked wheat samples were extracted. The average value for the incurred residue was 0.040 ± 0.003 ppm, with a relative standard deviation of 7.92%. These values are more representative of the true precision afforded by the SFE technique and are excellent considering the sub-parts-per-million level of the incurred residue.

SFE results for wheat samples and traditional water–acetonitrile extraction method (17) results for wheat samples are compared by the data in Table 4 and results tabulated in Tables 1–3. The data show that SC-CO_2 extraction is equivalent to the liquid solvent extraction procedure for the listed pesticides. Dimethoate and carbofuran were not recovered using the PAM procedure; however, extraction with SC-CO_2 yields excellent recoveries for both pesticides at 5000 and 10 000 psi and the chosen extraction temperatures. Note that the incurred methyl chlorpyrifos residue, determined upon analysis of the 0.1 ppm spiked wheats, is in excellent agreement with the results obtained by SFE.

The extractions performed at 2000 psi on 5 ppm spiked wheat samples (Table 1) yielded lower recoveries (70–80%) for dimethoate, carbofuran, malathion, and methyl parathion with respect to the other 4 pesticides. At 40 and 80°C, pesticide recoveries dropped relative to recoveries obtained at 5000 psi (Table 2). Dimethoate, in particular, could only be recovered at a 29% level at 2000 psi and 80°C. The above trends are not as prevalent for the 0.1 ppm spiked wheat samples extracted at 2000 psi; recoveries were above 80% for all of the pesticides, except dimethoate and methoxychlor at 80°C. The reason for this subtle difference in pesticide recoveries at the 0.1 and 5.0 ppm levels is not apparent; however, the difference may be related to the need for additional CO_2 or higher pressure to enhance the recovery of the larger amount of pesticide at the 5.0 ppm level.

Recovery results in Tables 2 and 3 at the 5000 and 10 000 psi pressure levels are excellent, averaging between 80 and 110% for all of the pesticides studied over the entire temperature range. The lowest extraction temperature possible should be used to avoid thermal degradation of the analytes. In addition, using the lowest pressure possible reduces the costs associated with high fluid compression and pressure ratings on the equipment.

Lower extraction pressures also minimize the amount of coextracted lipid matter carried along with the target analytes. Wheat contains about 10% oil, which can potentially be extracted under the conditions used in this study. Less than 20% of this oil is extracted at 10 000 psi and 80°C (18). Extractions performed on the 5.0 ppm fortified samples also had appreciable amounts of coextracted lipid material; however, these extracts required no further sample cleanup, because the extracts could be directly diluted and injected onto the described chromatographic instrumentation at levels that were not deleterious to chromatographic efficiency or detection.

In summary, the above study shows that SFE coupled with sorbent trapping of the target analytes is a viable technique for the extraction of pesticides from grain matrixes. Some caution

Table 3. Pesticide recoveries (%) at 10 000 psi extraction pressure as a function of temperature (100 L CO_2)

		40°C		60°C		80°C	
	Spike level, ppm	A	B	A	B	A	B
Dimethoate	5.0	96	82	84	80	94	101
	0.1	79	79	75	101	87	89
Methyl parathion	5.0	107	94	97	99	104	111
	0.1	81	80	89	106	90	94
Pirimiphos-methyl	5.0	104	93	100	108	115	113
	0.1	89	82	95	111	101	101
Chlorpyrifos	5.0	110	96	93	97	111	108
	0.1	87	88	95	111	101	104
Malathion	5.0	99	96	95	101	113	113
	0.1	85	81	96	115	97	96
Dieldrin	5.0	90	91	92	88	90	94
	0.1	77	84	101	107	103	88
Methoxychlor	5.0	92	96	97	92	89	99
	0.1	76	81	90	107	90	105
Carbofuran	5.0	94	99	93	86	95	100
	0.1	84	86	81	99	88	89

should be exercised, however, because, with the exception of the methyl chlorpyrifos, all of the reported recovery data are on fortified samples. SFE of incurred residues is preferred to SFE of spiked samples whenever possible. The technique can be translated onto commercial instrumentation, and many of the steps can be automated. In addition, desorption of the analytes from the Florisil trap may be possible by using SC-CO_2, thereby further reducing the use of organic solvents even further in the described procedure. An integrated extraction/cleanup method for pesticide residue analysis was recently described by France et al. (19). Additional selectivity for multiresidue analysis can be achieved by varying the collection sorbent, thereby fractionating individual pesticide classes and coextracted moieties.

Table 4. Pesticide recoveries (%) from the PAM, Vol. 1, sec. 212.13C, extraction procedure

	5.0 ppm		0.1 ppm	
	A	B	A	B
Dimethoate	NF[a]	NF	NF	NF
Parathion-methyl	98	99	101	97
Pirimiphos-methyl	103	104	100	91
Chlorpyrifos	100	101	94	93
Malathion	100	99	105	116
Dieldrin	84	89	106	108
Methoxychlor	98	101	90	89
Carbofuran	NF	NF	NF	NF
Chlorpyrifos-methyl[b]	—	—	0.040	0.039

[a] NF = Not found.
[b] Incurred residue in parts per million.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Evaluation of Soxtec Extraction Procedure for Extracting Organic Compounds from Soils and Sediments

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A study was carried out to evaluate the Soxtec extraction of 29 target compounds (7 nitroaromatic compounds, 3 haloethers, 7 chlorinated hydrocarbons, and 12 organochlorine pesticides) from spiked sandy clay loam and clay loam. The study also compared 2 solvent mixtures (hexane–acetone (1 + 1) and methylene chloride–acetone (1 + 1)) and investigated the effect on method recovery of 5 factors (matrix type, spike level, anhydrous sodium sulfate addition, total extraction time, and immersion/extraction time ratio), their 2-way interactions, and a 3-way interaction (matrix × spike × time). Of the 5 factors investigated, matrix type, spike level, and total extraction time had a significant effect on method performance at the 5% significance level for 16 of the 29 target compounds (4 compounds were not recovered at all, and analysis of variance was not significant at the 5% significance level for 9 compounds). Anhydrous sodium sulfate addition and immersion/extraction time ratio had insignificant effects for all but one of the target compounds. This effect may have been a random occurrence. The data indicate that the 2 solvents performed equally well and 4 compounds were not recovered at all. These 4 compounds were apparently lost from the spiked matrix. Experiments were performed to determine compound recovery at each step in the extraction procedure. Limited experimental work was performed with 64 basic/neutral/acidic compounds spiked onto clay loam and with 3 standard reference materials certified for polynuclear aromatic hydrocarbons. From the 64 compounds spiked onto clay loam at 6 mg/kg, 20 had recoveries of greater than 75%, 22 had recoveries from 50 to 74%, 12 had recoveries from 25 to 49%, and 10 had recoveries of less than 25%.

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U.S. Environmental Protection Agency (EPA) Method 3540 (Soxhlet extraction) and EPA Method 3550 (sonication extraction) are approved methods for the extraction of semivolatile organics from solids, such as soils, sediments, sludges, and hazardous wastes (1). Soxhlet extraction, which has been used for many years, usually requires extraction times between 8 and 24 h and fairly large amounts of solvents (300 mL for a 10 g soil sample). Nonetheless, many samples can be extracted side-by-side, and the equipment is not overly expensive. Sonication extraction requires much less time (approximately 10 min per sample) but still requires large amounts of solvents (300 mL for a 10 g soil sample); the technique is quite labor intensive. In both cases, the sample extracts usually require cleanup and concentration. An alternative method for the extraction of solids was recently proposed (2). This 3-stage method uses a commercially available extraction system (Soxtec). In the initial extraction stage, a thimble containing the sample is immersed into the boiling solvent and kept there for approximately 60 min. In the second stage, the thimble is elevated above the boiling solvent, and the sample is extracted as if it were in a Soxhlet apparatus, usually for 60 min or less. In the third stage, the solvent is evaporated directly in the Soxtec apparatus (this step requires only 10–15 min). The Soxtec technique requires only 20% of the volumes of solvents used in the Soxhlet or sonication methods, is faster than the Soxhlet method (requires approximately 2 h per sample), and is less labor-intensive than the sonication method. The extracts obtained by Soxtec extraction do not require filtration and can be concentrated directly in the Soxtec apparatus.

This procedure was evaluated using sandy clay loam and clay loam matrixes spiked with a 29-compound mixture consisting of 7 nitroaromatic compounds, 3 haloethers, 7 chlorinated hydrocarbons, and 12 organochlorine pesticides, and a 64-compound mixture consisting of the EPA basic/neutral/acidic compounds currently on the Hazardous Substances List (1). Two extraction solvent mixtures, hexane–acetone (1 + 1) and methylene chloride–acetone (1 + 1), were compared, and the

Table 1. Average retention times of 29 target compounds used in this study[a]

Compound No.	Compound name	Concn, ng/mL	Av. retention time, min	
			DB-5	DB-1701
1	1,3-Dichlorobenzene	1000	3.75	3.66
2	1,2-Dichlorobenzene	1000	4.19	4.29
3	Nitrobenzene	400	5.00	6.38
4	Benzal chloride	100	5.96	6.69
5	Benzotrichloride	100	7.82	7.96
6	4-Chloro-2-nitrotoluene	100	9.67	11.20
7	Hexachlorocyclopentadiene	100	10.40	9.70
8	2,4-Dichloronitrobenzene	100	11.19	13.08
9	3,4-Dichloronitrobenzene	100	11.55	13.25
10	Pentachlorobenzene	100	14.73	14.60
11	2,3,4,5-Tetrachloronitrobenzene	100	17.85	19.28
12	Benefin	100	18.24	19.67
13	alpha-BHC	100	18.58	20.41
14	Hexachlorobenzene	100	18.91	18.76
15	delta-BHC	100	20.83	25.76
16	Heptachlor	100	22.77	22.91
17	Aldrin	100	24.09	23.95
18	Isopropalin	100	25.33	26.72
19	Heptachlor epoxide	100	25.60	26.54
20	*trans*-Chlordane	100	26.48	27.70
21	Endosulfan I	100	26.94	27.53
22	Dieldrin	100	27.90	28.84
23	2,5-Dichlorophenyl-4′-nitrophenyl ether	300	28.41	30.43
24	Endrin	100	28.67	29.59
25	Endosulfan II	100	29.02	31.25
26	*p,p′*-DDT	100	30.72	31.68
27	2,3,6-Trichlorophenyl-4′-nitrophenyl ether	300	31.09	33.08
28	2,3,4-Trichlorophenyl-4′-nitrophenyl ether	300	32.61	34.89
29	Mirex	100	34.12	33.43
IS	Pentachloronitrobenzene	500	20.15	21.05

[a] The number of determinations was 8. GC operating conditions were as follows: 30 m × 0.53 mm id DB-5 (0.83 μm film thickness) and 30 m × 0.53 mm id DB-1701 (1.0 μm film thickness) connected to a press-fit Y-shaped inlet glass splitter. Temperature program: 100°C (2 min hold) to 275°C (6 min hold) at 5°C/min; injector temperature, 250°C; detector temperature, 320°C; helium carrier gas, 6 mL/min; nitrogen makeup gas, 20 mL/min. The compounds are listed in the order of elution from the DB-5 column.

Table 2. Average percent recoveries and percent RSDs for 64 basic/neutral/acidic compounds extracted from spiked clay loam by Soxtec extraction with hexane–acetone (1 + 1)[a]

Compound No.	Compound name	Scan No.	Quantitation ion, *m/z*	IS used in quantitation, *m/z*	Av. % rec.	%RSD
1	Phenol	530	94	IS-1	47.8	5.6
2	Bis(2-chloroethyl) ether	536	93	IS-1	25.4	13
3	2-Chlorophenol	544	128	IS-1	42.7	4.3
4	Benzyl alcohol	575	108	IS-1	55.9	7.2
5	2-Methylphenol	586	108	IS-1	17.6	6.6
6	Bis(2-chloroisopropyl) ether	589	45	IS-1	15.0	15
7	4-Methylphenol	599	108	IS-1	23.4	6.7
8	*N*-Nitroso-di-*n*-propylamine	601	70	IS-1	41.4	6.2
9	Nitrobenzene	616	77	IS-2	28.2	7.7
10	Isophorone	638	82	IS-2	56.1	4.2
11	2-Nitrophenol	647	139	IS-2	36.0	6.5
12	2,4-Dimethylphenol	649	107	IS-2	50.1	5.7
13	Benzoic acid	656	122	IS-2	40.6	7.7
14	Bis(2-chloroethoxy)methane	657	93	IS-2	44.1	3.0
15	2,4-Dichlorophenol	670	162	IS-2	55.6	4.6
16	1,2,4-Trichlorobenzene	678	180	IS-2	18.1	31
17	Naphthalene	684	128	IS-2	26.2	15
18	4-Chloroaniline	689	127	IS-2	55.7	12
19	4-Chloro-3-methylphenol	733	107	IS-2	65.1	5.1
20	2-Methylnaphthalene	749	142	IS-2	47.0	8.6
21	Hexachlorocylopentadiene	769	237	IS-3	19.3	19
22	2,4,6-Trichlorophenol	776	196	IS-3	70.2	6.3
23	2,4,5-Trichlorophenol	781	196	IS-3	26.8	2.9
24	2-Chloronaphthalene	794	162	IS-3	61.2	6.0
25	2-Nitroaniline	804	65	IS-3	73.8	6.0
26	Dimethyl phthalate	821	163	IS-3	74.6	5.2
27	Acenaphthylene	835	152	IS-3	71.6	5.7
28	3-Nitroaniline	843	138	IS-3	77.6	5.3
29	Acenaphthene	852	153	IS-3	79.2	4.0
30	2,4-Dinitrophenol	852	184	IS-3	91.9	8.9
31	4-Nitrophenol	857	109	IS-3	62.9	16
32	Dibenzofuran	866	168	IS-3	82.1	5.9
33	2,4-Dinitrotoluene	866	165	IS-3	84.2	5.4
34	2,6-Dinitrotoluene	828	165	IS-3	68.3	5.8
35	Diethyl phthalate	886	149	IS-3	74.9	5.4
36	4-Chlorophenyl-phenyl ether	896	204	IS-3	67.2	3.2
37	Fluorene	899	166	IS-3	82.1	3.4
38	4-Nitroaniline	900	138	IS-3	79.0	7.9
39	4,6-Dinitro-2-methylphenol	904	198	IS-4	63.4	6.8
40	*N*-Nitrosodiphenylamine	907	169	IS-4	77.0	3.4
41	4-Bromophenyl-phenyl ether	942	248	IS-4	62.4	3.0
42	Hexachlorobenzene	959	284	IS-4	72.6	3.7
43	Pentachlorophenol	974	266	IS-4	62.7	6.1
44	Phenanthrene	990	178	IS-4	83.9	5.4
45	Anthracene	994	178	IS-4	96.3	3.9
46	Di-*n*-butyl phthalate	1037	149	IS-4	78.3	40
46	Fluoranthene	1104	202	IS-4	87.7	6.9
47	Pyrene	1126	202	IS-5	102	0.8
48	Butylbenzyl phthalate	1186	149	IS-5	66.3	5.2
49	3,3′-Dichlorobenzidine	1264	252	IS-5	25.2	11
50	Benzo(a)anthracene	1273	228	IS-5	73.4	3.8
51	Bis(2-ethylhexyl) phthalate	1264	149	IS-5	77.2	4.8
52	Chrysene	1280	228	IS-5	76.2	4.4
53	Di-*n*-octyl phthalate	1378	149	IS-6	83.1	4.8

Table 2. *(Continued)*

Compound No.	Compound name	Scan No.	Quantitation ion, *m/z*	IS used in quantitation, *m/z*	Av. % rec.	%RSD
54	Benzo(*b*)fluoranthene	1495	252	IS-6	82.7	5.0
55	Benzo(*k*)fluoranthene	1501	252	IS-6	71.7	4.1
56	Benzo(a)pyrene	1586	252	IS-6	71.7	4.1
57	Indeno(1,2,3-*cd*)pyrene	2003	276	IS-6	72.2	4.3
58	Dibenzo(*a,h*)anthracene	2010	278	IS-6	66.7	6.3
59	Benzo(*g,h,i*)perylene	2128	276	IS-6	63.9	8.0
60	1,2-Dichlorobenzene[b]	582	146	IS-1	0	—
61	1,3-Dichlorobenzene[b]	559	146	IS-1	0	—
62	1,4-Dichlorobenzene[b]	564	146	IS-1	0	—
63	Hexachloroethane[b]	612	119	IS-1	0	—
64	Hexachlorobutadiene[b]	772	225	IS-2	0	—
IS-1	1,4-Dichlorobenzene-d_4	561	152		—	—
IS-2	Naphthalene-d_8	682	136		—	—
IS-3	Acenaphthene-d_{10}	849	164		—	—
IS-4	Phenanthrene-d_{10}	988	188		—	—
IS-5	Chrysene-d_{12}	1276	240		—	—
IS-6	Perylene-d_{12}	1602	264		—	—

[a] The number of determinations was 3. Soxtec operating conditions were as follows: immersion time, 45 min; extraction time, 45 min; sample size, 10 g; spike level, 6 mg/kg per compound.
[b] Possibly lost from the spiked soil prior to extraction.

effect on method recovery of 5 factors (matrix type, spike level, anhydrous sodium sulfate addition, total extraction time, and immersion/extraction time ratio), their 2-way interactions, and a 3-way interaction (matrix × spike × time) were investigated. Finally, a spiked clay loam and several standard reference materials were extracted by this Soxtec procedure to provide data on method performance for other compounds of interest.

Experimental

Apparatus

(a) *Extraction system.*—Soxtec HT-6 extraction system with temperature-controlled oil bath (Tecator, Inc., Silver Spring, MD 20904).

(b) *Evaporative concentrator.*—Kuderna-Danish apparatus with 10 mL concentrator tube, 500 mL evaporation flask, and 3-ball macro Snyder column.

(c) *Water bath.*—With concentric-ring cover; heated to 98 ± 2°C.

(d) *Gas chromatograph (GC).*—Varian 6000 (Varian Associates, Sunnyvale, CA 94089), equipped with 2 constant-current, pulsed-frequency electron capture detectors and 2 megabore fused-silica, open-tubular columns (30 m × 0.53 mm id × 0.83 μm film thickness DB-5 column and 30 m × 0.53 mm id × 1.0 μm film thickness DB-1701 column), connected to press-fit, Y-shaped inlet glass splitter (J & W Scientific Inc., Folsom, CA 95630), was used to determine the 29 target compounds. Columns were temperature programmed from 100°C (2 min hold) to 275°C (6 min hold) at 5°C/min; injector temperature, 250°C; detector temperature 320°C; helium carrier gas, 6 mL/min; nitrogen makeup gas, 20 mL/min.

(e) *Gas chromatograph/mass spectrometer (GC/MS).*—Finnigan 4510B (Finnigan MAT, San Jose, CA 95134), interfaced with data system for data acquisition and processing and equipped with 30 m × 0.32 mm id DB-5 fused-silica, open-tubular column (1 μm film thickness) was used to analyze the extracts obtained from the 3 standard reference materials and from the clay loam spiked with the 64 basic/neutral/acidic compounds. Column was temperature programmed from 40°C (4 min hold) to 300°C (6 min hold) at 8°C/min; injector temperature, 270°C; interface temperature, 270°C.

Materials

(a) *Standards.*—Analytical reference standards of the 29 target compounds (Table 1) and the 64 basic/neutral/acidic compounds (Table 2) were obtained from the EPA Pesticides and Industrial Chemicals Repository (Research Triangle Park, NC 27709), or purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201); Ultra Scientific, Inc. (Hope, RI 02831); and Chem Service, Inc. (West Chester, PA 19381). Pentachloronitrobenzene, 2-bromobiphenyl, and decafluorobiphenyl were purchased from Aldrich Chemical Co., Inc. The 29 target compounds were obtained as neat materials; the 64 basic/neutral/acidic compounds were obtained as 4 composite solutions in methylene chloride and methylene chloride–toluene (95 + 5) at concentration of 2 mg/mL. The purities of all compounds were greater than 98%. Stock solutions of each of the 29 target compounds were prepared in pesticide-grade hexane at 1 mg/mL; working calibration standards were prepared by using serial dilution of composite stock solution prepared from the individual stock solutions. Working calibration standards of the basic/neutral/acidic compounds were also prepared

Table 3. Physicochemical parameters of soils used in this study

Parameter	Sandy clay loam	Clay loam
Nitrate, ppm	14	42
Ammonia, ppm	5	8
Phosphate, ppm	20	42
Potassium, ppm	100	230
Calcium, ppm	2380	2430
Magnesium, ppm	604	828
pH	7.5	7.4
Cation exchange capacity, meq/100 g	14.6	21.3
Organic carbon, %	0.1	1.8
Sand, %	57.6	33.6
Silt, %	21.8	35.4
Clay, %	20.6	31.0
Moisture content, %	2.6	10.6

by using serial dilution (with methylene chloride) of the composite stock solution made from the 4 individual composite stock solutions.

(b) *Hexane, acetone, methylene chloride.*—Nanograde, or pesticide grade.

(c) *Sodium sulfate.*—Anhydrous and reagent grade, heated 4 h at 400°C, cooled in desiccator, and stored in glass bottle.

(d) *Sample matrixes.*—Sandy clay loam and clay loam samples were obtained from the Sandoz Crop Protection Division (Gilroy, CA 95021), standard reference marine sediments HS-3 and HS-4 were from the National Research Council of Canada (Halifax, Nova Scotia, Canada), and PAH-contaminated soil SRS103-100 were from Fisher Scientific (Pittsburgh, PA 15219). Standard reference materials were dry. Clay loam and sandy clay loam matrixes contained 10.6 and 2.6% water, respectively. Physicochemical parameters are presented in Table 3 for the sandy clay loam and clay loam but were not available for the standard reference materials.

Procedures

Spiked samples (10 g each) of clay loam and sandy clay loam were extracted in a Soxtec extractor Model HT-6 with 50 mL hexane–acetone (1 + 1) or 50 mL methylene chloride–acetone (1 + 1). The final volume of extract was 1 mL. Details of the Soxtec procedure are presented elsewhere (3).

Spiking of the samples with the 29 target compounds or 64 basic/neutral/acidic compounds was performed as follows: The sample was weighed into an aluminum cup, and a concentrated stock solution (100–1000 µL) in hexane (for the 29 target compounds) or methylene chloride (for the 64 basic/neutral/acidic compounds) was added to the sample with a syringe while making sure that the solution did not contact the aluminum cup. The 2 surrogate compounds (2-bromobiphenyl and decafluorobiphenyl) were spiked into all samples (prior to extraction) at

Table 4. Experimental design for optimization of Soxtec extraction

Experiment No.	Matrix	Spike level, mg/kg[a]	Total time, min[b]	Ratio of immersion time to extraction time
1, 2	Clay loam	0.1	60	0.5
3, 4	Clay loam	0.1	60	2.0
5, 6	Clay loam	1.0	60	0.5
7, 8	Clay loam	1.0	60	2.0
9, 10	Clay loam	0.1	120	0.5
11, 12	Clay loam	0.1	120	2.0
13, 14	Clay loam	1.0	120	0.5
15, 16	Clay loam	1.0	120	2.0
17, 18	Sandy clay loam	0.1	60	0.5
19, 20	Sandy clay loam	0.1	60	2.0
21, 22	Sandy clay loam	1.0	60	0.5
23, 24	Sandy clay loam	1.0	60	2.0
25, 26	Sandy clay loam	0.1	120	0.5
27, 28	Sandy clay loam	0.1	120	2.0
29, 30	Sandy clay loam	1.0	120	0.5
31, 32	Sandy clay loam	1.0	120	2.0
33, 34, 35, 36	Clay loam	0.5	90	1.0
37, 38, 39, 40	Clay loam	0.5	90	1.0
41, 42, 43, 44	Sandy clay loam	0.5	90	1.0
45, 46, 47, 48	Sandy clay loam	0.5	90	1.0

[a] Spiking was performed by adding 1 mL acetone solution containing the target compounds (except as noted below) at either 1 µg/mL or 10 µg/mL to a 10 g sample. Compounds 23, 27, and 28 were spiked at 0.3 mg/kg (low level) or 3.0 mg/kg (high level), compound 3 at 0.4 mg/kg (low level) or 4.0 mg/kg (high level), compounds 1 and 2 at 1.0 mg/kg (low level) or 10 mg/kg (high level), and the surrogate compounds at 1.0 mg/kg. Anhydrous sodium sulfate (10 g) was added to all odd-numbered samples. The moisture content was adjusted to 20% for all samples.

[b] Total time is the sum of immersion and extraction time.

Table 5. Average percent recoveries and percent RSDs for 29 target compounds extracted from spiked clay loam by Soxtec extraction with methylene chloride–acetone (1 + 1) and hexane–acetone (1 + 1)[a]

		Methylene chloride–acetone (1 + 1)				Hexane–acetone (1 + 1)			
		DB-5		DB-1701		DB-5		DB-1701	
Compound No.	Compound name	Av. % rec.	% RSD	Av. % rec.	% RSD	Av. % rec.	% RSD	Av. % rec.	% RSD
	Nitroaromatics								
3	Nitrobenzene	25.2	21.5	45.7	11	45.7	11	77.1	18
6	4-Chloro-2-nitrotoluene	28.3	16	41.6	27	74.7	11	92.8	17
8	2,4-Dichloronitrobenzene	37.1	18	39.9	18	68.0	7.7	115	8.0
9	3,4-Dichloronitrobenzene	35.9	26	54.3	16	65.4	7.8	78.3	8.3
11	2,3,4,5-Tetrachloronitrobenzene	87.2	3.4	89.8	2.3	90.5	6.9	122	4.6
12	Benefin	100	4.4	84.8	3.4	52.7	6.1	82.0	3.7
18	Isopropalin	95.6	5.1	95.2	8.8	69.7	24	97.5	6.9
	Haloethers								
23	2,5-Dichlorophenyl-4′-nitrophenyl ether	93.3	4.4	106	5.3	99.7	2.0	61.4	6.5
27	2,3,6-Trichlorophenyl-4′-nitrophenyl ether	83.1	11	96.9	5.3	104	5.6	97.2	2.0
28	2,3,4-Trichlorophenyl-4′-nitrophenyl ether	90.1	6.0	94.3	5.3	109	6.1	91.6	1.3
	Chlorinated hydrocarbons								
7	Hexachlorocyclopentadiene	0[b]	—	0	—	27.8	22	46.0	21
10	Pentachlorobenzene	51.8	7.2	58.7	8.9	65.2	11	48.6	12
14	Hexachlorobenzene	81.7	2.1	85.6	1.8	52.5	15	81.7	7.1
	Organochlorine pesticides								
13	alpha-BHC	94.1	4.1	91.8	6.3	94.9	5.5	94.9	5.5
15	delta-BHC	103	5.7	BI[c]	—	104	9.7	BI[c]	—
16	Heptachlor	92.7	8.2	89.4	3.0	73.6	6.5	87.1	5.4
17	Aldrin	70.7	3.3	70.7	3.3	78.2	5.7	78.2	5.7
19	Heptachlor epoxide	96.7	6.0	91.0	4.2	92.9	14	92.4	0.6
20	*trans*-Chlordane	98.7	3.8	95.8	4.2	82.4	10	85.8	2.2
21	Endosulfan I	87.5	14.6	92.8	4.3	91.4	13	90.5	2.0
22	Dieldrin	73.4	8.1	73.4	8.1	68.8	2.6	90.3	10
24	Endrin	119	4.6	119	4.6	67.1	9.3	97.2	2.0
25	Endosulfan II	89.5	6.1	89.5	6.1	90.3	10	90.3	10
26	*p,p′*-DDT	41.1	16	BI[c]	—	78.2	20	61.4	6.5
29	Mirex	99.5	4.4	106	7.4	97.7	6.2	84.0	5.1

[a] Soxtec operating conditions were as follows: immersion time 60 min; extraction time 60 min; the sample size was 10 g clay loam; the spike level was 0.05 mg/kg per compound, except for compounds 23, 27, and 28 at 0.15 mg/kg, compound 3 at 0.2 mg/kg, and compounds 1, 2, and the surrogate compounds at 0.5 mg/kg. The number of determinations was 4, and the moisture content of the samples was 10.6%. Compounds 1, 2, 4, and 5 were not recovered and are not included in the table.

[b] Possibly lost from the spiked soil prior to extraction.

[c] Not able to determine because of background interference.

1 mg/kg. Mixing was performed with the tip of disposable glass pipette. After the solvent had completely evaporated (approximately 15 min), the spiked sample was transferred to a precleaned thimble for Soxtec extraction. The extractions were started approximately 1 h after the addition of the spikes to the samples.

For all samples identified in Table 4, the sample water content was adjusted to 20% by weight. The water was added to these samples before spiking with the target compounds. The 20% water content was chosen arbitrarily and was intended to represent typical wet soil. For samples that required the addition of anhydrous sodium sulfate to the spiked matrix, equal

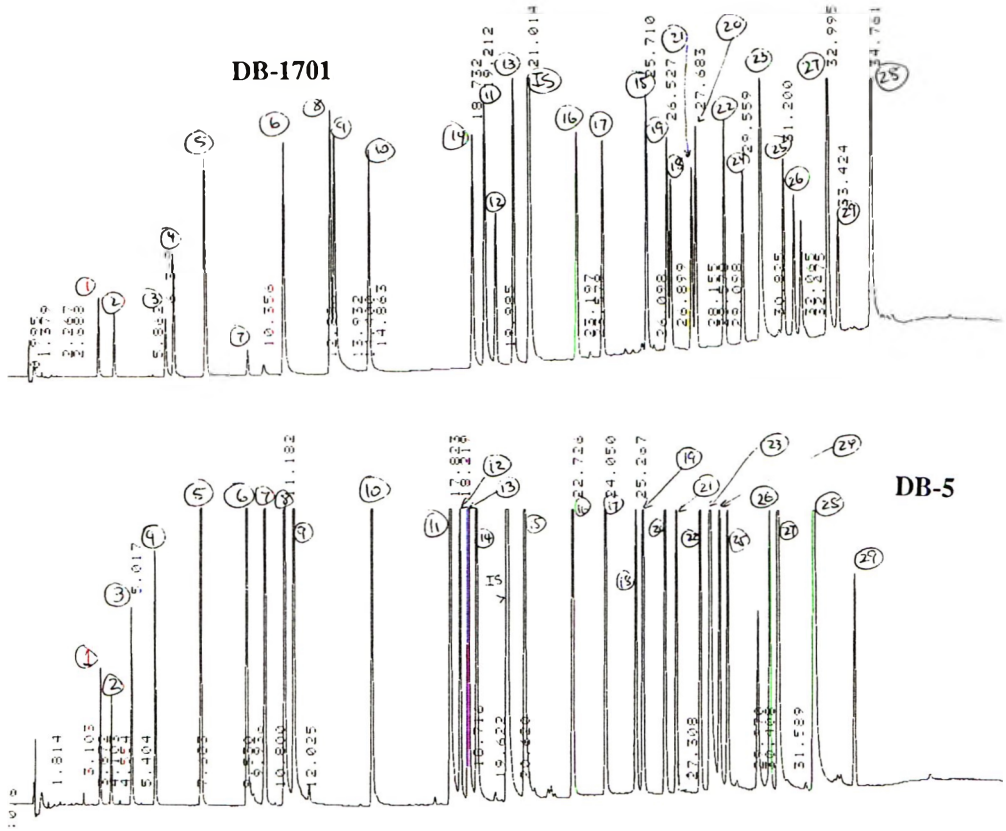

Figure 1. GC/ECD chromatograms of composite standard (concentration, 100 ng/mL) containing the 29 target compounds.

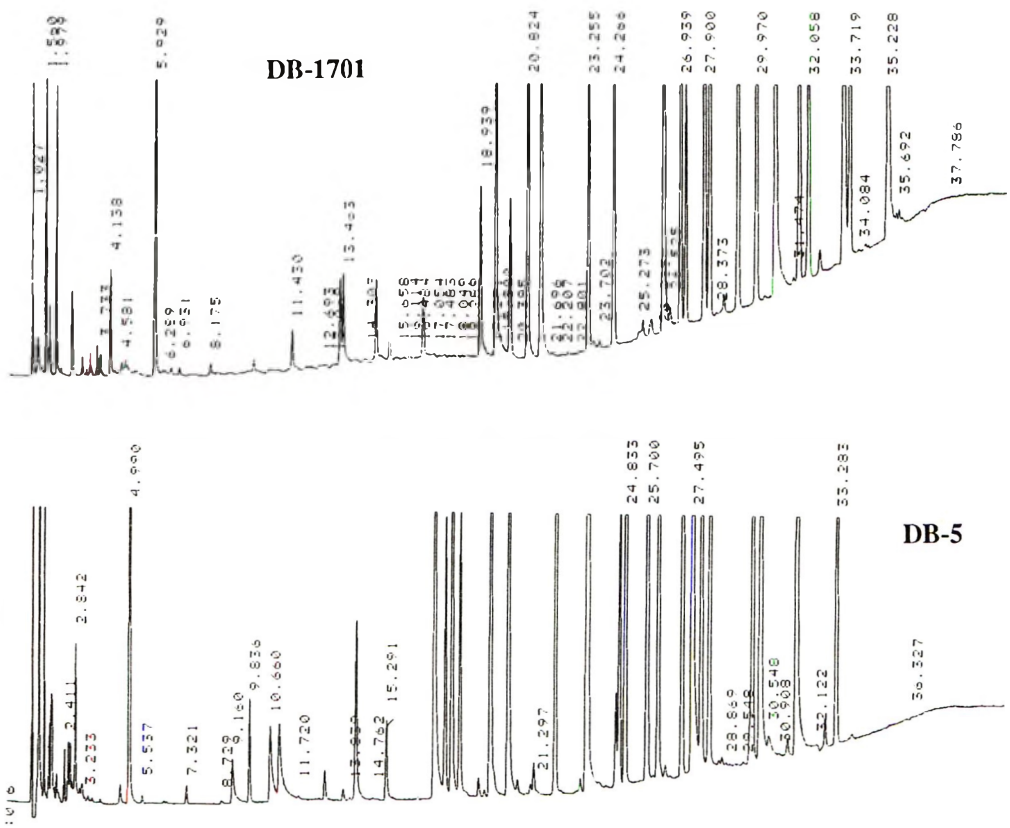

Figure 2. GC/ECD chromatograms of extract from sandy clay loam spiked with the 29 target compounds at 1 mg/kg and extracted in a Soxtec apparatus with hexane–acetone (1 + 1).

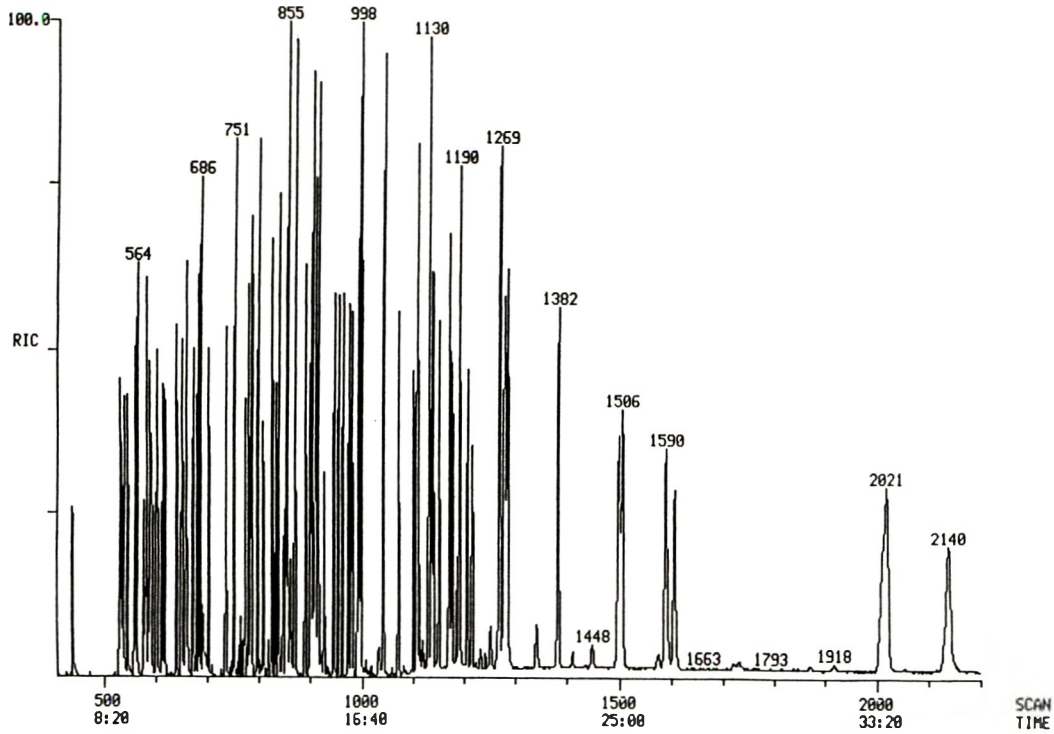

Figure 3. GC/MS chromatogram of composite standard containing the 64 basic/neutral/acidic compounds (concentration, 50 μg/mL) and the 6 internal standards listed in Table 2.

amounts of sample and anhydrous sodium sulfate (10 g each) were thoroughly mixed.

Experimental Design

The experimental design consisted of 48 experimental runs. In the first 32 experiments identified in Table 4, 2 matrix types were used (clay loam and sandy clay loam) and extractions were performed at 2 spike levels designated as low and high (0.1 and 1 mg/kg, respectively). The experiments were performed with and without the addition of anhydrous sodium sulfate to the matrix (the anhydrous sodium sulfate was added to bind water present in the soil matrix) at 2 conditions for total extraction time (60 and 120 min) and 2 conditions for immersion/extraction time ratio (0.5 and 2.0). Four replicate extractions were conducted with spiked clay loam (experiments 33–40 in Table 4) and spiked sandy clay loam (experiments 41 through 48 in Table 4) (spike level 0.5 mg/kg), with and without the addition of anhydrous sodium sulfate, using hexane–acetone (1 + 1), an immersion time of 45 min, and an extraction time of 45 min (Table 4).

Statistical Analysis

All statistical analyses were performed by using the general linear model procedure (PROC GLM) of the SAS statistical software package (4). To investigate the effect on method performance of the 5 variables (matrix type, spike level, anhydrous sodium sulfate addition, total extraction time, and immersion/extraction time ratio), the recovery data were analyzed by analysis of variance (ANOVA). A 5% significance level was used to determine whether a factor or an interaction was statistically significant.

The initial ANOVA model included the 5 factors mentioned earlier, their 2-way interactions (matrix × spike, matrix × Na_2SO_4, matrix × time, matrix × ratio, spike × Na_2SO_4, spike × time, spike × ratio, Na_2SO_4 × time, Na_2SO_4 × ratio, and time × ratio), and a 3-way interaction (matrix × spike × time). This model was evaluated separately for each compound. The main factors and their interactions that were not significant at the 5% significance level were eliminated, and the ANOVAs were rerun. The 2-way interactions that remained significant in the model are identified in the *Results and Discussion*.

GC and GC/MS Methods

The GC system was assembled as previously described. The sample extracts containing the 29 target compounds were analyzed by using gas chromatography with electron capture detection (ECD). The GC system was calibrated by using the internal standard technique with pentachloronitrobenzene as internal standard. All GC analyses were performed by using the dual-column, dual-detector arrangement. The data presented in this manuscript, with the exception of those given in Table 5, are the DB-5 column data with a few exceptions (e.g., outliers and matrix interferences), for which we used the DB-1701 column data. The average retention times of the 29 compounds on the DB-5 and DB-1701 columns are presented in Table 1. GC/ECD chromatograms of a composite standard of the 29 target compounds are shown in Figure 1. As shown in Figure 1, the best separation of the 29 compounds was achieved on the

Table 6. Average percent recoveries and percent RSDs for 29 target compounds extracted from spiked soil samples by Soxtec extraction with hexane–acetone (1 + 1)[a]

		With anhydrous sodium sulfate, 10 g				Without anhydrous sodium sulfate			
		Clay loam		Sandy clay loam		Clay loam		Sandy clay loam	
Compound No.	Compound name	Av. % rec.	% RSD	Av. % rec.	% RSD	Av. % rec.	% RSD	Av. % rec.	% RSD
	Nitroaromatics								
3	Nitrobenzene	18.2	25.4	6.9	12.6	17.3	4.2	8.0	23.6
6	4-Chloro-2-nitrotoluene	22.7	10	6.7	14.5	20.4	5.1	6.2	19.0
8	2,4-Dichloronitrobenzene	42.2	11	23.5	13	35.2	7.6	21.2	15
9	3,4-Dichloronitrobenzene	43.5	13	23.4	14	34.9	15	20.4	11
11	2,3,4,5-Tetrachloronitrobenzene	66.6	6.2	58.3	6.0	55.9	6.7	50.4	6.0
12	Benefin	75.9	4.9	71.9	6.9	62.6	4.8	62.7	2.9
18	Isopropalin	113	2.3	103	4.6	102	4.3	105	2.3
	Haloethers								
23	2,5-Dichlorophenyl-4′-nitrophenyl ether	126	2.8	112	5.1	110	4.1	112	2.1
27	2,3,6-Trichlorophenyl-4′-nitrophenyl ether	123	2.3	106	4.9	110	4.8	110	2.8
	Chlorinated hydrocarbons								
7	Hexachlorocyclopentadiene	5.3	31	8.4	18	4.1	15	7.8	23
10	Pentachlorobenzene	18.6	20	17.3	11	13.7	7.3	14.8	13
14	Hexachlorobenzene	37.3	11	35.1	13	26.9	13	25.1	5.7
	Organochlorine pesticides								
13	alpha-BHC	73.6	4.6	64.6	4.7	58.2	7.3	54.8	4.8
15	delta-BHC	109	3.4	97.9	4.1	95.8	4.6	99.2	1.3
16	Heptachlor	63.2	6.4	61.5	10	46.9	9.2	49.1	6.3
17	Aldrin	121	7.6	122	5.2	97.7	12	102	7.4
19	Heptachlor epoxide	104	2.6	95.3	4.2	90.4	4.4	93.6	2.4
20	*trans*-Chlordane	103	2.0	96.6	4.0	90.1	4.5	95.0	2.3
21	Endosulfan I	107	2.6	100	4.9	96.3	4.4	101	2.2
22	Dieldrin	152	1.6	104	4.5	129	4.7	104	1.9
24	Endrin	114	1.9	102	4.8	102	4.5	106	3.7
25	Endosulfan II	114	3.4	102	4.6	104	4.1	105	0.4
26	*p,p′*-DDT	134	2.1	109	4.5	134	2.1	111	2.0
29	Mirex	115	2.4	105	4.7	104	5.3	108	2.2

[a] Soxtec operating conditions were as follows: immersion time, 45 min; extraction time, 45 min; sample size, 10 g clay loam; spike level, 0.5 mg/kg per compound, except for the surrogate compounds at 1.0 mg/kg; compounds 23, 27, and 28 at 1.5 mg/kg; compound 3 at 2.0 mg/kg; and compounds 1 and 2 at 5.0 mg/kg. The number of determinations was 4, and the moisture content of the samples was 20%. Compounds 1, 2, 4, and 5 were not recovered and are not included in the table.

DB-5 column; on the DB-1701 column, 3 compound pairs (8 and 9, 18 and 19, and 20 and 21) were only partially resolved. To estimate the agreement between the DB-5 and the DB-1701 measurements, the ratios (values not shown) were calculated of the average percent recoveries reported in Table 5 for the DB-5 and DB-1701 data.

For the column labeled "methylene chloride–acetone (1 + 1)" in Table 5, the ratios of DB-5 data to DB-1701 data ranged from 0.55 to 1.18, with 86% of the values ranging from 0.8 to 1.18. Ratios in the 0.8–1.2 range indicated good agreement between the 2 data sets. A linear regression analysis was performed with these data (the DB-5 data were plotted on the x-axis and the DB-1701 data were plotted on the y-axis) and a correlation coefficient of 0.965 was obtained for 23 data points. For the column labeled "hexane–acetone (1 + 1)" in Table 5, the ratios of DB-5 data to DB-1701 data ranged from 0.59 to 1.62, with only 50% of the values ranging from 0.8 to 1.2. The ratios of DB-5 data to DB-1701 data from the 48 experiments identified in Table 4 were usually in the 0.7–1.3 range. In those experiments, the compounds were spiked at levels 10 and 20 times higher than those in Table 5, and, thus, the extracts could be diluted 10-fold for GC/ECD analysis. This 10-fold dilution

Table 7. Verification of compound recoveries (%) at various steps in the Soxtec procedure

Compound No.	Compound name	Evaporation step[a]	Soxtec method (no matrix)[a]	Soxtec method (with matrix)[a,b]
		Nitroaromatics		
3	Nitrobenzene	89.4	108	80.5
6	4-Chloro-2-nitrotoluene	88.8	101	79.1
8	2,4-Dichloronitrobenzene	87.3	100	80.4
9	3,4-Dichloronitrobenzene	82.1	92.8	72.0
11	2,3,4,5-Tetrachloronitrobenzene	91.4	109	87.6
12	Benefin	88.9	101	82.6
18	Isopropalin	86.6	109	96.6
		Haloethers		
23	2,5-Dichlorophenyl-4′-nitrophenyl ether	89.5	111	103
27	2,3,6-Trichlorophenyl-4′-nitrophenyl ether	96.3	119	105
28	2,3,4-Trichlorophenyl-4′-nitrophenyl ether	87.7	109	101
		Chlorinated hydrocarbons		
1	1,3-Dichlorobenzene	92.9	103	38.6
2	1,2-Dichlorobenzene	106	134	93.5
4	Benzal chloride	110	108	60.6
5	Benzotrichloride	90.3	112	47.6
7	Hexachlorocyclopentadiene	62.6	69.9	30.1
10	Pentachlorobenzene	95.9	109	76.5
14	Hexachlorobenzene	104	121	83.6
		Organochlorine pesticides		
13	alpha-BHC	96.0	114	88.6
15	delta-BHC	88.5	117	86.3
16	Heptachlor	97.5	120	94.3
17	Aldrin	93.3	110	91.8
19	Heptachlor epoxide	98.6	124	96.7
20	*trans*-Chlordane	96.0	116	94.5
21	Endosulfan I	94.8	123	92.3
22	Dieldrin	98.6	118	96.2
24	Endrin	104	124	111
25	Endosulfan II	103	124	104
26	*p,p′*-DDT	99.7	115	BI[c]
29	Mirex	93.3	118	108

[a] Single determinations.
[b] The spiked clay loam was extracted immediately following addition of the spike.
[c] Not able to determine because of background interference.

resulted in almost no background interferences (Figure 2), and, thus, the agreement between the DB-5 and the DB-1701 data was better.

The sample extracts containing the 64 basic/neutral/acidic compounds and the extracts from the 3 standard reference materials were analyzed by gas chromatography/mass spectrometry. The GC/MS system was calibrated by using the internal standard technique with 6 internal standards: 1,4-dichlorobenzene-d_4 (IS-1), naphthalene-d_8 (IS-2), acenaphthene-d_{10} (IS-3), phenanthrene-d_{10} (IS-4), chrysene-d_{12} (IS-5), and perylene-d_{12} (IS-6). In the case of GC/MS analysis, separation of the 64 compounds was not as critical in the GC/ECD analysis because compounds can still be identified and quantitated from the mass spectral information acquired during the analysis. Table 2 presents the retention times (given as scan numbers), the quantitation ions, and the various internal standards used for quan-

Table 8. Percent recovery statistics for the Soxtec extraction procedure

Compound No.	No. of runs	Av. rec.	Median rec.	SD	% RSD	Minimum rec.	Maximum rec.
			Nitroaromatics				
3	32	23.7	16.9	19.6	82.8	0.0	81.5
6	32	26.2	25.2	12.8	48.9	4.0	58.7
8	32	35.3	34.5	14.5	41.0	8.2	73.1
9	32	34.2	33.1	13.5	39.5	7.9	66.3
11	32	61.8	63.2	10.2	16.5	36.8	79.3
12	32	64.3	66.6	15.5	24.2	37.8	88.2
18	32	89.5	90.7	9.3	10.3	63.2	107
			Haloethers				
23	32	101	103	9.9	9.8	70.3	118
27	32	99.7	100	8.7	8.7	76.3	116
28	32	89.1	91.5	18.0	20.2	63.5	118
			Chlorinated hydrocarbons[a]				
7	32	6.2	6.0	4.0	64.1	0.0	15.1
10	32	25.7	25.2	9.7	37.9	7.8	44.9
14	32	44.5	44.6	8.6	19.3	23.3	59.0
			Organochlorine pesticides				
13	32	73.6	72.3	13.9	18.9	46.6	106
15	32	99.0	102	10.1	10.2	64.9	117
16	32	58.8	57.6	12.1	20.6	42.4	80.8
17	32	75.0	65.9	25.2	33.6	43.6	115
19	32	88.2	88.6	12.4	14.1	53.7	123
20	32	84.8	84.9	10.6	12.5	59.9	106
21	32	94.0	93.6	11.7	12.4	60.5	120
22	32	99.8	100	9.8	9.8	82.2	122
24	32	101	102	9.8	9.7	76.0	120
25	32	104	106	10.6	10.3	72.2	122
26	32	117	114	18.6	15.9	89.1	158
29	32	99.3	99.7	10.3	10.3	69.9	115

[a] Compounds 1, 2, 4, and 5 were not recovered and are not included in the table.

titating the 64 compounds. A GC/MS chromatogram of a composite standard containing the 64 basic/neutral/acidic compounds (concentration, 50 µg/mL) is shown in Figure 3.

Results and Discussion

Method Performance for 29 Target Compounds

The method performance data for the 29 target compounds extracted from spiked clay loam and sandy clay loam are presented in Tables 5 and 6. The 2 solvent mixtures used for extraction performed equally well. The individual recoveries for the DB-5 column ranged from 0 to 119% (with 16 values above 80%) for methylene chloride–acetone and from 27.8 to 109% (with 11 values above 80%) for hexane–acetone, respectively. The individual recoveries for the DB-1701 column ranged from 0 to 119% (with 15 values above 80%) for methylene chloride–acetone and from 46.0 to 122% (with 17 values above 80%) for hexane–acetone, respectively. For further investigation, hexane–acetone was chosen over methylene chloride–acetone. Because GC/ECD was used for the determinations, the hexane–acetone extracts could be analyzed directly without performing a solvent exchange.

Anhydrous sodium sulfate was added to the matrix (clay loam and sandy clay loam) to bind the water, and the effect on method performance was evaluated. Average recoveries increased approximately 9% for the clay loam (Table 6) but were only 2% for the sandy clay loam. These values were obtained by plotting recoveries for the experiment on clay loam mixed with sodium sulfate on the x-axis and recoveries for the experiment without sodium sulfate on the y-axis. The regression equation has a slope of 0.914 and a correlation coefficient of 0.9925. In the case of the sandy clay loam, the slope of the

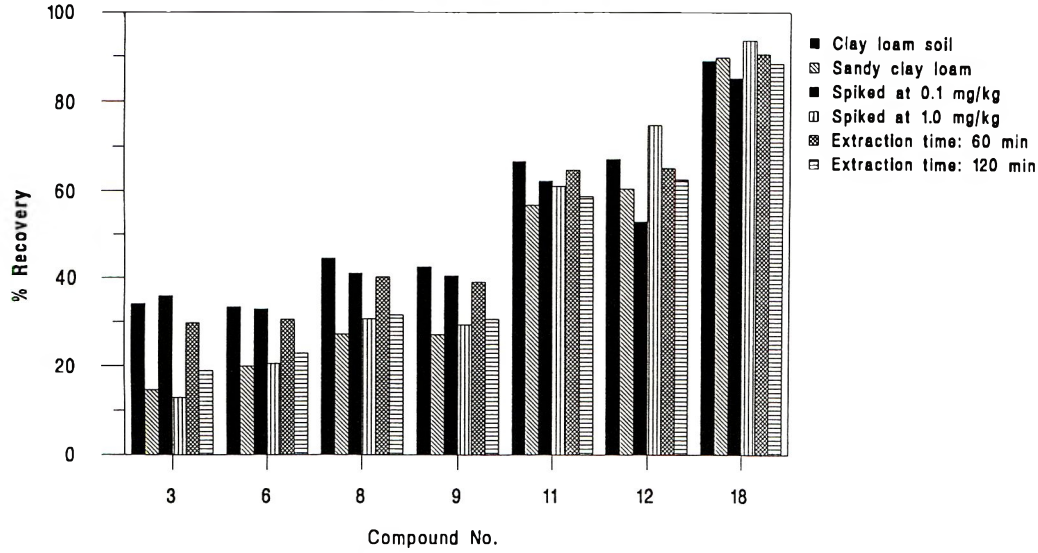

Figure 4. Average recoveries of nitroaromatics (compounds 3, 6, 8, 9, 11, 12, and 18) as a function of matrix, spike level. and extraction times (data from experiments 1 through 32 in Table 4).

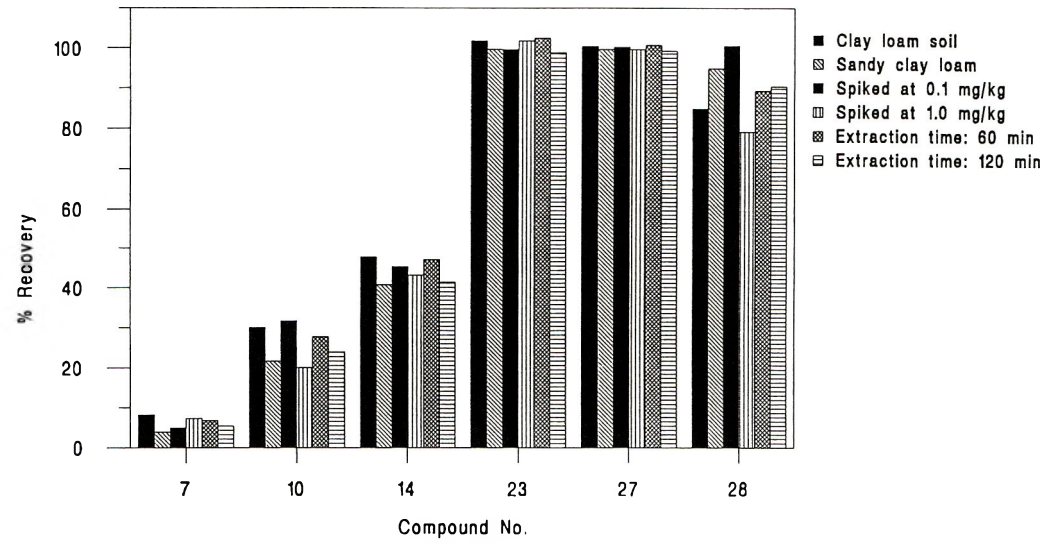

Figure 5. Average recoveries of chlorinated hydrocarbons (compounds 7, 10, and 14) and haloethers (compounds 23, 27, and 28) as a function of matrix, spike level, and extraction times (data from experiments 1 through 32 in Table 4).

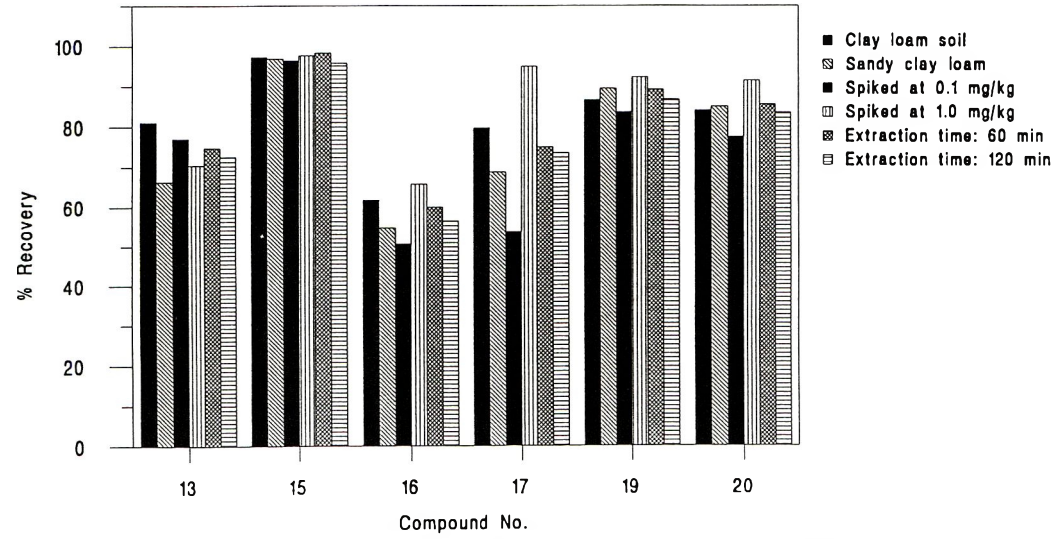

Figure 6. Average recoveries of organochlorine pesticides (compounds 13, 15, 16, 17, 19, and 20) as a function of matrix, spike level, and extraction times (data from experiments 1 through 32 in Table 4).

Table 9. Significant terms (at 5% level) from SAS PROC GLM for percent recovery model containing all primary effects and 2-way and 3-way interaction effects

Compound No.	No. of runs	R^2, %[a]	RMSE[b]	Main effects					2-way interactions						3-way interactions, matrix × spike × time
				Matrix	Spike	Na_2SO_4	Time	Ratio	Matrix × spike	Matrix × Na_2SO_4	Matrix × time	Matrix × ratio	Spike × time	Na_2SO_4 × time	
Nitroaromatics															
3	32	89.4	6.97	X	X	—	X	—	X	—	—	—	X	—	—
6	32	81.6	5.78	X	X	—	X	—	—	—	—	—	—	—	—
8	32	75.9	7.47	X	X	—	X	—	—	—	—	—	—	—	—
9	32	80.2	6.32	X	X	—	X	—	—	—	—	—	—	—	—
11[c]	32	63.6	6.99	X	—	—	X	—	—	—	—	—	X	—	X
12[c]	32	83.6	6.75	X	X	—	X	—	X	—	—	—	—	—	—
18[a]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
Haloethers															
23[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
27[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
28[c]	32	75.3	9.57	X	X	—	—	—	—	—	X	—	—	—	—
Chlorinated hydrocarbons[d]															
7	32	65.2	2.56	X	X	X	X	—	—	—	—	—	—	X	—
10	32	89.8	3.69	X	X	—	X	—	—	—	—	X	—	—	X
14	32	48.6	6.58	X	—	—	X	—	—	—	—	—	X	—	—
Organochlorine pesticides															
13[c]	32	39.6	11.20	X	X	—	—	—	—	—	—	—	—	—	—
15[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
16[c]	32	75.3	6.58	X	X	—	X	—	—	—	X	—	X	—	—
17[c]	32	87.2	9.34	X	X	—	—	—	—	—	—	—	—	—	—
19[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
20[c]	32	54.3	7.40	—	X	—	—	X	—	—	—	—	—	—	—
21[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
22[c]	32	55.7	6.86	X	—	—	—	—	—	X	—	—	—	—	—
24[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
25[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—
26[c]	32	65.2	11.32	X	X	—	—	—	—	—	—	—	—	—	—
29[c]	32	Model not sig.	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] R^2 = percent of the variance in the data explained by the model.
[b] RMSE = root mean square error is the estimate of the standard deviation of the error term in the model.
[c] Overall percent recovery exceeds 50% for these compounds.
[d] Compounds 1, 2, 4, and 5 were not recovered and are not included in the table.

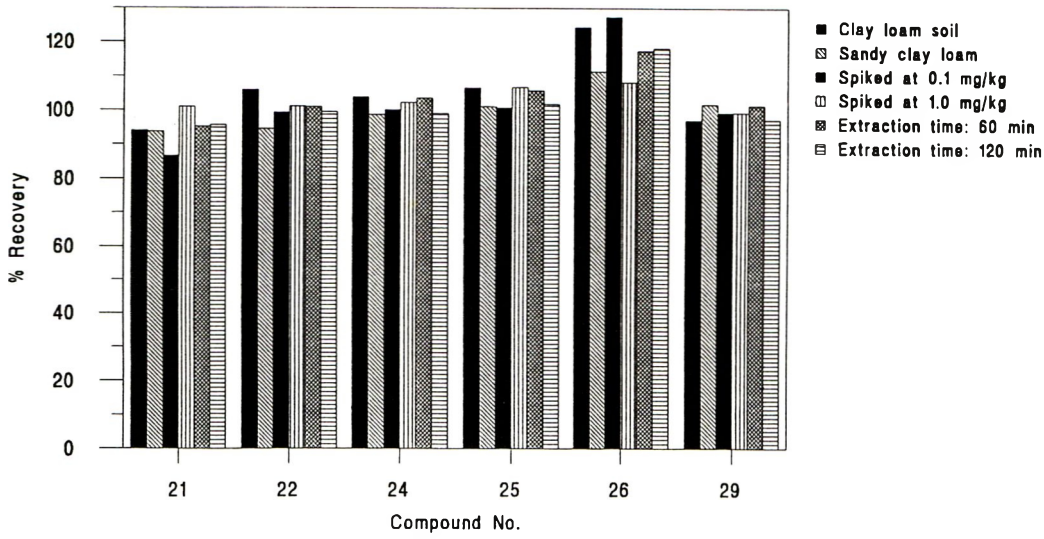

Figure 7. Average recoveries of organochlorine pesticides (compounds 21, 22, 24, 25, 26, and 29) as a function of matrix, spike level, and extraction times (data from experiments 1 through 32 in Table 4).

linear regression equation is 1.018 and the correlation coefficient is 0.9892. However, the 9% increase in recoveries when anhydrous sodium sulfate was used with the clay loam matrix is within the error of the GC measurement. Thus, we cannot claim that the addition of anhydrous sodium sulfate had any effect on recovery.

Table 6 shows that Compounds 1, 2, 4, and 5 could not be recovered even though they were recovered at each step in the Soxtec procedure (Table 7). Table 7 indicates that, when the compounds are spiked directly into the extraction solvent and then the solvent in the Soxtec apparatus is evaporated, compound recoveries were greater than 85%, except for 3,4-dichloronitrobenzene at 82.1% and hexachlorocyclopentadiene at 62.6%. Furthermore, the compounds were spiked into the extraction solvent, and then the solution was subjected to a typical Soxtec extraction but with an empty thimble (immersion and extraction steps were performed 45 min each); thus, we attempted to mimic an extraction from which we eliminated the matrix. Compound recoveries, except for hexachlorocyclopentadiene, were greater than 92.8%. The low recovery of hexachloropentadiene (69.9%) may be due to possible decomposition during the experiment (the Soxtec apparatus was covered with aluminum foil during the experiment to minimize possible photolysis of the target compounds). Experiments were also performed in which the 29 compounds were spiked directly into the clay loam matrix and then immediately extracted. Be-

Table 10. Percent recoveries of compounds extracted from SRS103–100 standard reference material by Soxtec extraction with hexane–acetone (1 + 1)[a]

Compound name	Certified value, mg/kg[b]	Concn measured in laboratory, mg/kg	Rec., %
Naphthalene	32.4 ± 8.2	41	127
2-Methylnaphthalene	62.1 ± 11.5	79	127
Acenaphthylene	19.1 ± 4.4	21	110
Acenaphthene	632 ± 105	683	108
Dibenzofuran	307 ± 49	378	123
Fluorene	492 ± 78	456	92.7
Phenanthrene	1618 ± 348	1315	81.3
Anthracene	422 ± 49	551	131
Fluoranthene	1280 ± 220	1040	81.3
Pyrene	1033 ± 289	714	69.1
Benzo(*a*)anthracene	252 ± 38	240	95.2
Chrysene	297 ± 26	272	91.6
Benzo(*k* + *b*)fluoranthene	152 ± 22	180	118
Benzo(*a*)pyrene	97.2 ± 17.1	78	80.2
Pentachlorophenol	965 ± 374	1072	111

[a] Single determinations. Soxtec operating conditions were as follows: immersion time, 45 min; extraction time, 45 min; sample size, 10 g.
[b] Value reported by the manufacturer.

Table 11. Percent recoveries of compounds extracted from HS-3 and HS-4 marine sediments by Soxtec extraction with hexane–acetone (1 + 1)[a]

Compound name	HS-3			HS-4		
	Certified value, mg/kg[b]	Concn measured in laboratory, mg/kg	Rec., %	Certified value, mg/kg[b]	Concn measured in laboratory, mg/kg	Rec., %
Naphthalene	9.0 ± 0.7	4.3	47.8	0.15	—[c]	—
Acenaphthylene	0.3 ± 0.1	0.5	167	0.15	—[c]	—
Acenaphthene	4.5 ± 1.5	5.8	129	0.15	—[c]	—
Fluorene	13.6 ± 3.1	7.3	53.7	0.15	—[c]	—
Phenanthrene	85 ± 20	38.2	44.9	0.68 ± 0.08	0.58	85.3
Anthracene	13.4 ± 0.5	10.1	75.4	0.14 ± 0.07	0.18	129
Fluoranthene	60 ± 9	30.8	51.3	1.25 ± 0.10	1.1	88.0
Pyrene	39 ± 9	16.8	43.1	0.94 ± 0.12	0.9	95.7
Benzo(*a*)anthracene	14.6 ± 2.0	8.2	56.2	0.53 ± 0.05	0.38	71.7
Chrysene	14.1 ± 2.0	8.1	57.4	0.65 ± 0.08	0.50	76.9
Benzo(*a*)pyrene	7.4 ± 3.6	3.6	48.6	0.65 ± 0.08	0.38	58.5
Benzo(*b*)fluoranthene	7.7 ± 1.2	5.5	71.4	0.70 ± 0.15	0.50	71.4
Benzo(*k*)fluoranthene	2.8 ± 2.0	4.9	175	0.36 ± 0.05	0.48	133
Benzo(*g,h,i*)perylene	5.0 ± 2.0	2.8	56.0	0.58 ± 0.22	—[c]	—
Dibenzo(*a,h*)anthracene	1.3 ± 0.5	1.2	92.3	0.12 ± 0.05	—[c]	—
Indeno(1,2,3-*cd*)pyrene	5.4 ± 1.3	2.8	51.9	0.51 ± 0.15	—[c]	—

[a] Single determinations. Soxtec operating conditions were as follows: immersion time, 45 min; extraction time, 45 min; sample size, 10 g.
[b] Value reported by the manufacturer.
[c] Below quantification limit.

cause all compounds (including compounds 1, 2, 4, and 5) were recovered in this experiment (Table 7), the low recoveries of compounds 1, 2, 4, 5, and 7 were attributed to possible evaporation from the aluminum cup or from the thimble during the 1 h period when the spike was allowed to equilibrate with the matrix. Therefore, we concluded that these compounds can be extracted from the matrix by the Soxtec procedure described in the present paper.

Method precision (percent RSDs) was determined from the 4 replicate determinations (Tables 5 and 6) at 2 spike levels, 0.05 and 0.5 mg/kg, except as listed in footnote a of Tables 5 and 6. With samples spiked at 0.05 mg/kg and with hexane–acetone as extraction solvent, the percent RSDs ranged from 2.0 to 24%, with 23 of the 26 values under 15%. At higher spike levels, the percent RSDs are usually lower, as shown in Table 6.

The summary statistics for the 32 experiments performed to estimate the effect on method performance of matrix type, spike level, anhydrous sodium sulfate addition, total extraction time, and immersion/extraction time ratio are presented in Table 8. The individual recovery data are presented elsewhere (3). These results indicate clearly that the method performs better for the organochlorine pesticides (average recoveries ranged from 58.8 to 117%) and haloethers (average recoveries ranged from 89.1 to 101%) than for the nitroaromatic compounds (average recoveries ranged from 23.7 to 89.5%) and the chlorinated hydrocarbons (average recoveries ranged from 0 to 44.5%). In the case of the 12 organochlorine pesticides, 9 compounds had average recoveries (based on 4 determinations) above 84.8% and the other 3 compounds had average recoveries of 58.8, 73.6, and 75.0% (Table 8). Two of the nitroaromatic compounds (compounds 3 and 6) had low recoveries averaging about 25%, 2 had average recoveries of 34.2 and 35.3%, and 3 compounds had average recoveries of 61.8, 64.3, and 89.5%. Four of the chlorinated hydrocarbons were not recovered, because as mentioned earlier, they were probably lost from the soil by evaporation before Soxtec extraction.

ANOVA was used to evaluate the effects on method performance of matrix type, spike level, anhydrous sodium sulfate addition, total extraction time, and immersion/extraction time ratio. Table 9 summarizes the ANOVA for each of the 4 groups of compounds. The compounds with a statistically significant effect on method recovery were marked with "X". From this information, the following observations can be made: the order of importance of the variables (based on the number of compounds affected) is matrix > spike > time; anhydrous sodium sulfate addition and immersion/extraction time ratio are insignificant, except for one compound in each case, where the effect may have been a random occurrence; the six 2-way interactions and the one 3-way interaction affected the recoveries of only a few compounds, and no conclusions could be drawn about their overall effect. ANOVA was not significant at the 5% significance level for 9 compounds (compounds 15, 18, 19, 21, 23, 24, 25, 27, and 29). Either the recovery of these 9 compounds was not affected by any of the factors or interactions, or the recovery was independent of the level of the factors considered.

Figures 4 through 7 show the average recoveries of 25 of the 29 compounds as a function of matrix type, spike level, and total extraction time. The compounds were grouped as ni-

troaromatics (Figure 4), chlorinated hydrocarbons and haloethers (Figure 5), and organochlorine pesticides (Figures 6 and 7).

For 6 of the 7 nitroaromatic compounds (Figure 4), significant variations were found in recovery depending on matrix type, spike level, and total extraction time. Except for compound 18 (which did not show any significant variations in recovery), the recoveries were significantly higher for the clay loam soil than for the sandy clay loam soil. Likewise, recoveries were significantly higher for the 0.1 mg/kg spike level (except for compound 12 for which the opposite is true) and the 60 min extraction time.

Recoveries of the chlorinated hydrocarbons (compounds 7, 10, and 14 in Figure 5) were significantly higher for the clay loam soil than for the sandy clay loam soil; shorter extraction times seemed to give significantly higher recoveries, and the effect of the spike level was inconclusive (we were not able to find a trend in recovery as a function of spike level).

In the case of the haloethers (compounds 23, 27, and 28 in Figure 5), we found no significant effect of matrix type, spike level, and total extraction time on recovery for compounds 23 and 27; however, for compound 28, significantly higher recoveries were found for the sandy clay loam and the 0.1 mg/kg spike level than for the clay loam and the 1.0 mg/kg spike level.

In the case of the organochlorine pesticides (Figures 6 and 7), 6 compounds (compounds 15, 19, 21, 24, 25, and 29) did not show any significant variation in recovery as a function of matrix type, spike level, or total extraction time. For the other 6 compounds (compounds 13, 16, 17, 20, 22, and 26) in Figures 6 and 7, recoveries were significantly higher for the clay loam soil and the 1 mg/kg spike level (except for compound 13), and recoveries did not appear to vary with the total extraction time.

Method Performance for the Basic/Neutral/Acidic Compounds

Method performance data for the 64 basic/neutral/acidic compounds extracted from spiked clay loam soil with hexane–acetone (1 + 1) and analyzed by GC/MS are presented in Table 2. From the 64 compounds spiked onto clay loam at 6 mg/kg, 19 had recoveries of greater than 75%, 24 had recoveries ranging from 50 to 74%, 11 had recoveries ranging from 25 to 49%, and 10 had recoveries of less than 25%. From these latter 10 compounds, 5 compounds were not recovered at all; they were probably lost, because of their volatilities, from the spiked clay loam before the samples were transferred to the Soxtec extractor. The method precision in this case was comparable to the precision reported for the 29 target compounds. Percent RSDs ranged from 0.4 to 40%, with 57 of the 59 values under 19%.

Method Performance for the Standard Reference Materials

The recoveries of compounds extracted from 3 standard reference materials by Soxtec extraction with hexane–acetone (1 + 1) and analyzed by GC/MS are presented in Tables 10 and 11. GC/MS chromatograms of each extract are included in Figure 8. The recoveries ranged from 69 to 131% for the SRS103-

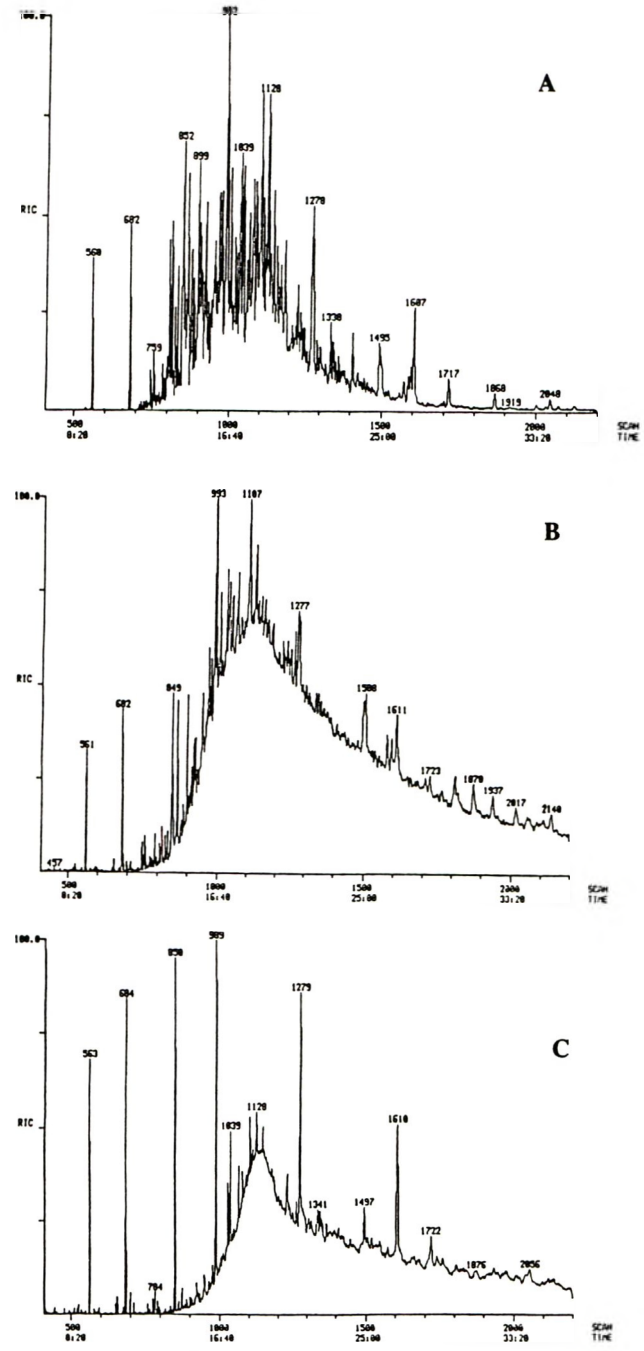

Figure 8. GC/MS chromatograms of SRS103-100 standard reference soil (A), HS-3 marine sediment (B), and HS-4 marine sediment (C) extracted by Soxtec extraction with hexane–acetone (1+1).

100 standard reference soil, from 43 to 175% for the HS-3 standard reference marine sediment, and from approximately 59 to 133% for the HS-4 standard reference marine sediment. The recoveries in excess of 100% can be explained by the wide concentration ranges reported for these standard reference materials by the manufacturer (e.g., we reported 175% recovery for benzo(*k*)fluoranthene for which the certified concentration is 2.8 ± 2 mg/kg). In addition, as shown in Figure 8, these matrixes are very complex, and recoveries in excess of 100% could also be due to hydrocarbon interferences. Unfortunately, no secondary ions could be used to quantitate PAHs by GC/MS

because they give strong molecular ions and only very few fragment ions that have relative intensities of only 10% of those of the molecular ions, or less.

Conclusions

A Soxtec extraction procedure for extracting organic compounds from soils and sediments was evaluated with 2 soil matrixes spiked with 29 compounds of environmental significance. The data indicate that the average recovery of 28 compounds (one compound could not be quantitated because of matrix interference) was 85.1% when extracted from freshly spiked soil with hexane–acetone (1 + 1). Four compounds were not recovered at all when the spiked soil samples were allowed to equilibrate with the spike for 1 h.

Limited experimental work performed with 64 basic/neutral/acidic compounds spiked on clay loam samples and with 3 standard reference materials certified for polynuclear aromatic hydrocarbons indicates that the recoveries of the basic/neutral/acidic compounds were good (except for the more volatile compounds) and that the repeatabilities were comparable to those obtained for the 29 target compounds previously described.

This technique requires only approximately 20% of the volumes of solvents used in the Soxhlet or the sonication methods, and it is faster than the Soxhlet method and less labor-intensive than the sonication method; therefore, it is proposed as an alternative extraction technique to the Soxhlet or the sonication procedures currently recommended by EPA.

Acknowledgment

We thank Philip Calvi of Tecator, Inc., for supplying a Soxtec apparatus for use in this study.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Liquid Chromatographic Method for Determination of Diquat and Paraquat Herbicides in Potatoes: Collaborative Study

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A liquid chromatographic (LC) method for the determination of diquat and paraquat herbicides/desiccants in potatoes was collaboratively studied in 6 laboratories. Analytes are extracted from 5 g sample with dilute acid by using a microreflux procedure; the hydrolysate is adjusted to pH 9–10 and passed through a disposable silica cartridge for rapid cleanup and preconcentration. Analytes are separated on a reversed-phase LC column and are measured as their heptanesulfonate ion pairs by UV detection. Each collaborator determined diquat and paraquat at 4 levels (0.05, 0.1, 0.5, and 1.0 ppm) in blind duplicate samples plus 2 blind negative control samples. Potatoes, obtained from each participant's region, were spiked by the collaborators with unknown aqueous solutions containing no analyte or a mixture of diquat and paraquat standards. Repeatability and reproducibility relative standard deviations (RSD_r and RSD_R) averaged 17.1 and 29.0%, respectively, for determination of diquat and 10.8 and 29.5%, respectively, for paraquat. For analysis of standard solutions, RSD_r and RSD_R values were 6.3 and 12.0%, respectively, for diquat and 7.3 and 13.9%, respectively, for paraquat. Accuracy, measured by comparison with true spiking values (absolute recovery) averaged 77.6 and 76.2% for diquat and paraquat, respectively, and ranged from 71.8 to 88.0% for both compounds. The method was adopted first action by AOAC International.

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The recommendation was approved by the General Referee and the Committee on Residues and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, Jan/Feb issue.

The herbicides diquat (1,1′-ethylene-2,2′-bipyridinium) and paraquat (1,1′-dimethyl-4,4′-bipyridinium) are used in their salt forms as preharvest crop desiccants on potato plants. Residues of diquat and paraquat were reported in potato tubers at levels that vary with the variety of potato, the agronomic conditions under which the potatoes are grown, and the time of treatment (1).

A thorough investigation by the Associate Referee of methods reported up to 1987 for determination of diquat and paraquat in various matrixes resulted in the development of novel methods (2, 3). After extensive, satisfactory in-house use and some interlaboratory application success, one of the methods (2) was deemed most suitable. No collaborative study had been reported for diquat or paraquat in plant matrixes, and so a study was initiated on the liquid chromatographic (LC) method (2) using an interlaboratory collaborative evaluation outlined by AOAC (4). The results of the study are the subject of the present report.

Collaborative Study

Design of Study

The study design and details of the method were approved in 1985 by an AOAC statistician before a study protocol and test solutions were distributed. Six collaborators were sent 18 glass ampoules, labeled A–R, that contained diquat/paraquat aqueous solutions or solvent blanks. Silanized ampoules were used to reduce adsorption by the glass container. Ampoules K–R were to be analyzed first (against the collaborator's own standards) as neat solutions to determine if any losses (sorption or degradation) of standard occurred before analysis. Collaborators were to use ampoules A–J for spiking potatoes according to instructions.

Collaborators were required to establish competence with the method by analysis of their own spiked potatoes (triplicate analysis at the 0.1 ppm level) before initiating the collaborative study. Background interferences or apparent bioincurred residues of diquat and paraquat were determined with the same method by analyzing in triplicate the batch of potatoes used for the collaborative study. Collaborators were also requested to

Table 992.17. Method performance for diquat and paraquat residues in potatoes, liquid chromatographic method

				Repeatability		Reproducibility	
Herbicide	Added, ppm	Rec., ppm	Rec., %	s_r	RSD_r, %	s_R	RSD_R, %
Diquat	0.05	0.044	88	0.013	30.4	0.013	30.4
	0.10	0.075	75	0.013	17.2	0.026	33.9
	0.5	0.359	71.8	0.048	13.3	0.103	28.8
	1.0	0.754	75.4	0.056	7.5	0.171	22.7
Paraquat	0.05	0.040	80	0.004	10.8	0.016	40.1
	0.10	0.072	72	0.015	20.5	0.027	38.0
	0.5	0.361	72.2	0.014	3.8	0.077	21.2
	1.0	0.805	80.5	0.067	8.3	0.152	18.9

prepare calibration curves for both compounds during periods of analysis (if analyses were not completed in 1 time period).

Collaborators were requested to purchase locally available potatoes at the time of analysis. They were also to supply their own primary standards and prepare them for use as described. Standard solutions and spiking solutions were to be stored in a refrigerator until required for analysis. Collaborators were sent study instructions and report forms. Collaborators were to perform the analyses using the method precisely as instructed, and to return all chromatograms, raw data, and a completed report of concentrations determined *without* correction for recoveries determined from their standards (K–R).

Standard solutions A–R were prepared from an aqueous stock solution containing desiccated diquat dibromide and paraquat dichloride (>99% stated purity obtained from Agriculture Canada) as discussed in the collaborative protocol and reference 2. Aqueous serial dilutions were prepared for spiking solutions, and 1 mL of each was pipetted into 2 mL glass ampoules. All glassware was silanized before use to prevent adsorption of analyte by the glass (2, 3).

Preliminary Practice Analyses

Before analysis of the collaborative samples, the participants were asked to use the method to determine diquat and paraquat in spiked (0.1 ppm level) and unspiked potatoes, to do triplicate analyses (against their own standards), and to check that the retention times of standards agree with those from the spiked samples or with controls containing endogenous residues. To ensure accurate quantitation, the peak sizes for sample residue and reference external standard should match within ±25%. The standard curve should be linear between 0.1 and 1.0 μg/mL, as described in the method.

Collaborators were instructed not to proceed further unless the recovery at 0.1 ppm was ≥75% for both diquat and paraquat spiked potatoes. If this was not possible or if problems arose, collaborators were requested to contact the Associate Referee for assistance. To determine if sorptive or degradation losses occurred in the spiking solutions before their use on potatoes, collaborators were requested to first quantitate vials K–R against the standards prepared for the practice runs. Solutions K–R were transferred to separate plastic vials; each ampoule and ampoule tip was washed with two 1 mL portions of water; the volume was reduced to dryness under nitrogen and made to 1 mL with LC mobile phase for quantitation. Dilutions of these samples were prepared as required using LC mobile phase as diluent.

992.17 Diquat and Paraquat Residues in Potatoes—Liquid Chromatographic Method

First Action 1992

Method Performance

See Table **992.17** for method performance data.

A. Principle

Samples are extracted with acid. Extract is applied to silica and eluted with acidic methanol. Diquat and paraquat are separated from coextractives by reversed-phase liquid chromatography (LC) and detected as heptanesulfonate ion pairs by dual channel UV detection.

B. Apparatus

(**a**) *Blender.*—With glass jar and explosion proof motor.

(**b**) *Heater.*—Dry bath capable of maintaining 100°, containing 2 aluminum heating blocks (each 75 × 102 × 42 mm, with 17 mm diameter tube openings in 3 × 4 matrix), stacked (each containing 12 openings with the top block drilled out) (Fisher Isotemp Model 147, Fisher Scientific, Pittsburgh, PA 15219, USA, is suitable).

(**c**) *Centrifuge.*—Capable of operating at centrifugal force of 402 × *g* and maintaining 5 ± 1°.

(**d**) *Evaporator.*—Operating at room temperature, with stainless steel Luer-Lok needles or single line using Pasteur pipet connected to source of nitrogen (N-Evap Analytical Evaporator, Organomation Assoc., Inc., Berlin, MA 01503, USA, is suitable).

(**e**) *Polyethylene vials.*—15 mm id × 55 mm.

(**f**) *Disposable silica cartridges.*—Containing 0.65 g silica, 1 cm id × 2 cm cartridges (Waters Chromatography Div., Millipore Corp., Milford, MA 01757, USA, is suitable source). Check elution profile by using standards and spiked potato extract; ≥85% recovery of analytes is acceptable.

(**g**) *LC system.—(1) Mobile phase delivery system.*—LC pump, capable of 3 mL/min delivery, 6000 psi upper pressure limit. (*2*) *Injector.*—With 100 µL injection loop (Rheodyne, Inc., Cotati, CA 94931, USA, is suitable source). (*3*) *Guard column.*—C_{18} cartridge. Condition before use by soaking in methanol 10 min. (*4*) *Analytical column.*—7 mm id × 305 mm column, containing poly(styrene-divinylbenzene) packing, 10 µm particle size (PRP-1, Hamilton Co., Reno, NV 89502, USA, is suitable). Column efficiencies: N (number of theoretical plates), 684 for diquat and 758 for paraquat.

(**h**) *UV detector.*—Capable of measuring absorbance at 254 and 313 nm filters. Adjust for steady baseline, ≤12% drift/h, and noise level, ≤5% at 0.01 AUFS, at range setting of 0.1 or 0.05 AUFS (e.g., for Waters detector).

(**i**) *Recorder.*—1 mV strip chart recorder with dual pens.

(**j**) *Connecting tubing.*—1.6 mm od × 0.18 mm id stainless steel tubing to connect injector, precolumn, analytical column, and detector.

(**k**) *Injection syringe.*—250 µL with Luer-Lok tip and blunt needle, 0.5 mm id × 5 cm.

C. Reagents

(**a**) *Diquat and paraquat standards.*—(*Caution*: These compounds have high oral toxicity.) Obtain diquat as dibromide salt [dication weight = (184.0/344.0) × sample weight]) and paraquat as dichloride salt [dication weight = (186.2/257.2) × sample weight], both >99% purity. Heat diquat and paraquat salts 3 h at 110°; then, cool in desiccator containing desiccant; repeat heating/cooling to constant weight on analytical balance. Store dried salts in sealed glass vials inside desiccator containing desiccant. Use anhydrous standards to prepare standard solutions.

(**b**) *Diethylamine.*—98% purity.

(**c**) *Orthophosphoric acid.*

(**d**) *Silanizing solution.*—10% (v/v) silanizing reagent in hexane, for deactivating glassware (Surfasil, Pierce, Rockford, IL 61105, USA, is suitable).

(**e**) *Organic solvents.*—Methanol, hexane. Use "distilled-in-glass" grade.

(**f**) *Sodium heptanesulfonate.*—98% purity (Eastman Kodak Co., Rochester, NY 14652, USA, is suitable source).

(**g**) *Sodium hydroxide solutions.*—1M and 6M aqueous NaOH solutions.

(**h**) *Hydrochloric acid solutions.*—1M, 2M, and 5M aqueous HCl solutions.

(**i**) *Methanol–HCl solution.*—8% Methanol. Add 8 mL methanol to 100 mL volumetric flask and dilute to volume with 5M HCl. Prepare fresh daily.

(**j**) *Potato control samples.*—Commercially obtain potatoes. Store at 5° before use.

D. LC Operating Parameters

(**a**) *LC mobile phase.*—Mix 13.5 mL orthophosphoric acid, 10.3 mL diethylamine, and 3.0 g sodium heptanesulfonate per liter in H_2O; filter through 0.45 µm (nylon) filter. Prepare mobile phase in 3–5 L quantities. Place 5 µm stainless steel filter, attached to polyethylene tubing, directly into mobile phase and pump at 3.0 mL/min; allow up to 1 h for system to equilibrate or until baseline drift is ≤12%/h.

Make 1 or 2 injections of mobile phase to elute accumulated coextractives before injecting standards and samples each day and after every 5–10 repetitive injections. This procedure should permit complete resolution between paraquat and a potential coextractive peak before it. Resolution should be optimized if retention time for coextractive peak increases by ≥2 min. Resolution may be improved by adding 1.5–7% methanol to mobile phase or by washing column with H_2O and then methanol, 5 min each, at 5 mL/min, followed by 10 min re-equilibration with mobile phase at 3 mL/min. After analysis, flush column with methanol and then water, as above, flush injector and loop with 0.5–1.0 mL methanol, and recycle mobile phase overnight.

(**b**) *Injection.*—Draw up 150–200 µL standard or sample extract and slowly fill loop, leaving 25–50 µL in syringe. Leave syringe in injector and inject; depress event marker for recorder injection mark. Remove syringe and clean with 2–3 rinses of fresh mobile phase.

E. Glassware Treatment

Treat all glassware with silanizing solution by rinsing surfaces in contact with analytes and pouring off excess. Dry silanized glassware under nitrogen and rinse thoroughly with hexane and water. After treated glassware is used in this procedure, soak it in 5M HCl at room temperature overnight, or at 60° for 30 min, and wash in detergent to remove residues of diquat and paraquat adsorbed onto glass surfaces.

F. Preparation of Standards and Standard Curve

(**a**) *Standards.*—Prepare standard solutions of diquat and paraquat for use as external standards for LC quantitation at concentrations of 0.62, 1.25, 6.25, and 12.5 µg cation/mL in silanized flasks by diluting with LC mobile phase. (*Note*: Condition pipet tips or volumetric pipets used for solution transfer by drawing up and expelling solution 20× before taking aliquot for dilution.) Store in silanized volumetric flasks at 5° until use.

(**b**) *Standard curve.*—Prepare standard curve by using diquat and paraquat standards mixed together at equal concentrations ranging between 10 and 100 ng per injection (i.e., 0.1, 0.3, 0.5, and 1.0 µg cation/mL mobile phase solution). At range setting of 0.01 AUFS, 2.5 ng of diquat and paraquat should be detectable (S/N = 3/1) at 313 and 254 nm, respectively. Standard curve should be linear between 0.1 and 1.0 µg/mL.

G. Sample Preparation

Wash and scrub whole potatoes to remove adhering soil particles; quarter and macerate potatoes in blender 60 s at high speed. Continue analysis on test samples as described in **H**.

H. Extraction and Cleanup

Weigh 5 g duplicate samples into glass tubes (16 mm × 125 mm); add 5 mL 2M HCl. Mix with vortex mixer 60 s, and place unstoppered tubes in heating block to reflux 1 h at 100°. Remove tubes and add 6 mL H_2O. Centrifuge 10 min at 1600 rpm, and use Pasteur pipet to transfer supernate into

Table 1. Collaborative study data, samples A–J, for LC determination of paraquat and diquat (ppm) in potatoes[a]

	1.0 ppm[b]		0.50 ppm		0.10 ppm		0.05 ppm		Neg. control	
Lab.	A	F	D	J	B	I	E	G	C	H
					Paraquat					
1	0.938	0.862	0.352	0.378	0.085	0.099	0.042	0.0435	ND[c]	ND
4[d]	0.895	0.991	0.495	0.463	0.101	0.10	0.063	0.069	ND	ND
8	0.61[d]	0.50	0.28	0.27	NR[e]	NR	NR	NR	ND	ND
9[d]	0.77	0.69	0.30	0.29	0.07	0.045	0.04	0.035	ND	ND
10	0.875	0.905[f]	0.350	0.33	0.055	0.022	0.022	0.027[g]	ND	ND
11	0.748	0.880	0.415	0.412	0.063	0.079	0.026	0.036	ND	ND
					Diquat					
1	0.9005	0.825	0.363	0.401	0.102	0.115	0.047	0.046	0.000	0.000
4[d]	0.791	0.884	0.469	0.454	0.075	0.082	0.039	0.072	0.109[h]	0.007[h]
8	0.45[d,i]	0.45[d,i]	0.30	0.15	NR	NR	NR	NR	0.047[h]	0.030[h]
9[d]	0.70	0.84	0.29	0.28	0.071	0.04	0.04	0.035	ND	ND
10	0.872	0.790[f]	0.350	0.31	0.056	0.042	0.019	0.044[g]	ND	ND
11	0.815	0.930	0.489	0.452	0.076	0.093	0.049	0.045	ND	ND

[a] Paired samples are blind duplicates. For each sample, results are means of duplicates, each of which is based on 2 injections unless otherwise indicated.
[b] "True" value.
[c] Not detected.
[d] Results of single, not duplicate, analysis.
[e] Not reported.
[f] Two results were given; the first was indicated in analyses.
[g] Result is based on 1 injection.
[h] Reported background level of apparent diquat (0.013 and 0.038 ppm); data for diquat-spiked samples were corrected for these 2 laboratories.
[i] Single Grubbs outlier.

25 mL beaker. Add 5 mL 2M HCl to residue (pellet); mix with vortex mixer 60 s. Reextract 30 min under reflux at 100°. Dilute with 6 mL H_2O, centrifuge as above, and combine supernates.

Adjust combined supernates to pH 9–10 by pH meter (microelectrode, ±0.1 pH units) by using 6M and 1M NaOH and 1M HCl. Because diquat degrades at this pH, immediately apply extract to dry disposable silica cartridge using 25 mL Luer-Lok glass syringe at ca 1 drop/s. Use same syringe to elute extract by adding 5 mL H_2O (discard eluate), 5 mL methanol (discard eluate), and 5 mL 8% methanol in 5M HCl. Collect final eluate in 15 mL graduated glass-stoppered centrifuge tube and, if necessary, adjust volume to 5.0 mL by adding methanol and mix. Transfer 1.0 mL final eluate to polyethylene vial (15 mm id × 55 mm). Place vial in 40–50° H_2O bath and evaporate to dryness under nitrogen. Add 1.0 mL mobile phase to dissolve residue.

I. LC Determination

Make duplicate 100 µL injections. Peak areas should agree within 10% for duplicate injections. Make duplicate injections of standard solution (containing both diquat and paraquat) at levels similar to anticipated level in sample solution (or controls, if contaminated) after every 2nd sample injection.

J. Calculations

Diquat and paraquat are weighed as anhydrous salts and analyzed as di-cations. Use molecular weights, 184 and 186.2, for diquat and paraquat, respectively, in calculations of µg analyte/g potato (ppm).

Using external standard(s):

$$\text{Analyte, ppm} = [(R_x \times C_s \times D)/(R_s \times W_x)]$$

where C_s = diquat or paraquat, as dication, µg/mL; R_x and R_s = average peak areas/100 µL injected (diquat at 313 nm, paraquat at 254 nm) for sample and standard, respectively; W_x = weight per volume of injected sample, g/mL; D = dilution factor, if 1 mL solution for injection is diluted.

Ref.: J. AOAC Int. (1993) **76**, July/August issue
CAS-2764-72-9 (diquat)
CAS-4685-14-7 (paraquat)

Results and Discussion

Collaborators returned reports within 24 months (1, 4, 11, 13, 13, and 24 months). Raw data and results of tests for outliers are shown in Table 1 for analyses of paraquat and diquat in samples A–J. Summary statistics are given in Tables 2 and 3 for samples A–J and K–R, respectively.

Statistical Treatment of Data

Levels of diquat and paraquat reported by collaborators (Table 1) are shown as ppm found compared with ppm added. Two statistical analyses were done: one for test samples A–J (Table 2), which is of primary interest in method evaluation, and

Table 2. Summary statistics for collaborative data on LC determination of diquat and paraquat (ppm) in potatoes, samples A–J

	Paraquat					Diquat				
Statistic	A, F	D, J	B, I	E, G	Mean	A, F	D, J	B, I	E, G	Mean
Sample mean	0.805	0.361	0.072	0.040		0.754	0.359	0.075	0.044	
"True" value	1.0	0.5	0.10	0.05		1.0	0.5	0.10	0.05	
No. labs	6	6	5	5		5	6	5	5	
s_r	0.067	0.014	0.015	0.040		0.056	0.048	0.013	0.013	
RSD_r, %	8.3	3.8	20.5	10.8	10.8	7.5	13.3	17.2	30.4	17.1
r	0.189	0.039	0.042	0.012		0.159	0.135	0.037	0.037	
s_R	0.152	0.077	0.027	0.016		0.171	0.103	0.026	0.013	
RSD_R, %	18.9	21.2	38.0	40.1	29.5	22.7	28.8	28.8	30.4	27.7
R	0.430	0.217	0.077	0.046		0.484	0.292	0.292	0.037	

one for solutions K–R (Table 3) in preliminary practice analyses. The raw data for solutions K–R are not shown but were analyzed for completeness and to provide summary information, which may be useful in the final evaluation of the method. Because these were practice test solutions, the usual summary statistics are not relevant in describing method performance. Test samples A–J were a series of blind duplicates with their "true" values (after spiking) as shown in Table 1.

The format of the data, as provided in the data reporting forms, for samples A–J required 2 replicates; each replicate comprised results of 2 injections. Deviations are as follows: data for 1 rather than 2 replicates were provided by Collaborators 4 and 9 for all samples; data for only 1 replicate for sample A and no detected residues for samples B, I, E, and G were provided by Collaborator 8; results of 2 complete analyses for sample F were provided by Collaborator 10; and results for sample G by Collaborator 10 were based on only 1 injection.

For each sample, calculations were done by the Collaborator and checked by the Associate Referee; mean values were entered. These were analyzed statistically as provided without regard to the numbers of replicates or injections done by the Collaborators.

Statistical analyses were based on AOAC guidelines (4). The Cochran test was used first to test for homogeneity of within-laboratory variances, and any outliers were indicated and deleted. The Grubbs test was applied to laboratory means to determine any outliers, i.e., abnormally high or low values. The test for single outliers was done first, and if no outliers were found then the paired test was done. Summary statistics were calculated with and without outliers; a single Grubbs outlier was found for diquat at the 1.0 ppm (A, F) level (Lab. 8, Table 1).

Samples A–J

For paraquat, the repeatability relative standard deviation (RSD_r) ranged from 3.8% (samples D, J) to 20.5% (samples B, I) (Table 2). The reproducibility relative standard deviation (RSD_R) ranged from 18.9% (samples A, F) to 40.1% (samples B, G).

For diquat, the RSD_r values ranged from 7.5% (samples A, F) to 30.4% (samples E, G). The latter is much higher than the rest because of the relatively large variation between blind duplicates for Collaborators 4 and 10. The RSD_R values ranged from 22.7% (samples A, F) to 30.4% (samples B, I). For sam-

Table 3. Summary statistics for collaborative data on LC determination of diquat and paraquat (ppm) in standards, samples K–R

	Paraquat					Diquat					
Statistic	K, L	M, N	O, P[a]	Q, R	Mean	K, L	K, L	M, N	O, P	Q, R	Mean
Sample mean	0.986	0.485	0.102	0.050		0.946	0.916[b]	0.4520	0.094	0.049	
"True" value	1.0	0.50	0.10	0.05		1.0	1.0	0.5	0.1	0.05	
No. labs	6	6	5	5		6	5	6	5	6	
s_r	0.097	0.027	0.006	0.0047		0.093	0.020	0.026	0.0086	0.0039	
RSD_r, %	10.1	5.5	6.1	4	7.3	9.8	2.29	5.7	9.2	8.0	6.3
r	0.276	0.076	0.018	0.010		0.262	0.057	0.073	0.024	0.011	
s_R	0.153	0.076	0.014	0.005		0.128	0.092	0.055	0.012	0.006	
RSD_R, %	15.8	15.6	13.6	10.8	13.9	13.6	10.1	12.3	13.3	12.2	12.0
R	0.433	0.214	0.039	0.015		0.363	0.261	0.157	0.035	0.017	

[a] Results for laboratory 1.
[b] Values in this column were calculated after results for laboratory 10 were deleted on the basis of the Cochran test.

Table 4. Absolute recoveries (%) calculated from collaborators' data on test samples A–J in study on LC determination of diquat and paraquat in potatoes

Analyte	Spiking level ("true value"), ppm				Mean
	1.0	0.5	0.1	0.05	
Paraquat	80.5	72.2	72.0[a]	80.0[a]	76.2
Diquat	75.4[a]	71.8	75.0[a]	88.0[a]	77.6

[a] Recovery based on data from 5 laboratories; all other data based on data from 6 laboratories. Absolute recovery = $(C_{spm}/C_{sdp}) \times 10^2$, where C_{spm} = concentration determined for spiked potatoes by method and C_{sdp} = concentration for samples and standards, true values.

ples E and G, the reproducibility and repeatability statistics for diquat are the same (Table 2), because the among-collaborator variation was less than the average within-collaborator variation. Collaborators would have a negative contribution to reproducibility, which is not possible theoretically, and therefore is set to zero.

Samples K–R

The standard solutions (K–R) were to be analyzed before use of the spiking solutions (A–J). Although these results may provide some useful information on the method, the summary statistics (e.g., repeatability and reproducibility) should not be used in summarizing method performance.

Recoveries

Table 4 shows the absolute recovery data for samples A–J for all laboratories at 4 spiking levels. Recoveries were 71.8–88.0% and 72.0–80.5% for diquat and paraquat, respectively. RSD_R means were 12.0 and 13.9%, respectively (Table 3). Analysis of samples K–R showed little or no loss of analyte in the spiking solutions, because these values were very close to the "true" values (Table 4).

Deviations from Protocol

Table 5 lists deviations to the LC protocol and the resultant retention times of diquat and paraquat. Slight deviations were reported for UV wavelengths, 257 and 310 nm vs 254 and 313 nm (recommended); this should have no significant effect on the accuracy of the method. Laboratory 8 used a different LC column: C_{18} Lichrosorb (250 × 4 mm, 10 µm particle size) instead of the PRP-1 column. This change was made because of baseline stability problems with the PRP-1 column. Retention times on this column were 7.18 and 8.00 min for diquat and paraquat, respectively.

Other retention times varied from the expected (24.9 and 29.6 min for diquat and paraquat, respectively) and must have been caused by different column characteristics (variability for the same column), because flow rates were never greater than 3 mL/min (recommended) and yet retention times were usually less than those expected (Table 5).

All laboratories stored their samples at 0–4°C except Laboratory 11 (room temperature). Laboratory 8 performed the practice spiking procedure at 10.0 ppm instead of the suggested 0.1 ppm. Laboratory 4 found it necessary to add 5% methanol to the mobile phase to effect separation of diquat and paraquat from interfering coextractives.

All diquat and paraquat standards used by collaborators were ≥98.5% purity and usually ≥99.6%; final data were not corrected for purity of collaborator external standards. Three laboratories used peak height (Laboratories 1, 8, and 10) and 3 used peak area (Laboratories 4, 9, and 11) for calculating sample concentrations; all standard curves were linear, and regression analysis gave correlation coefficients of ≥0.99 for detector response (absorbance) vs concentration of analyte. Only 2 laboratories (Laboratories 1 and 9) were capable of analyzing samples for diquat and paraquat simultaneously. Other laboratories required twice as many injections for analysis of both compounds in each sample due to the lack of a dual UV detector.

Table 5. Deviations from method protocol and other variations in collaborative study on LC determination of diquat and paraquat in potatoes

Lab.	Analysis[a]	Column	UV[b]	Flow rate, mL/min	RT[c]	Response measured	Temp, °C[d]	Time, days[e]
1	Simult.	PRP–1[f]	313/254	2.0	26.1/28.5	Peak ht	3	102
4	Sep.	PRP–1[f]	310/257	3.0	16.6/17.0	Peak area	3	189[g]
8	Sep.	C18[h]	313/254	3.0	7.18/8.00	Peak ht	3	398
9	Simult.	PRP–1[f]	310/257	2.5	16.3/16.4	Peak area	0	30
10	Sep.	PRP–1[f]	313/254	3.0	16.5/17.0	Peak ht	3	673
11	Sep.	PRP–1[i]	310/257	0.7	13.9/15.0	Peak area	22	334

[a] Simult. = simultaneous analysis of diquat and paraquat; Sep. = separate analysis of diquat and paraqaut (i.e., separate injections).
[b] UV = UV detector wavelengths, µm, for diquat/paraquat.
[c] RT = Retention times, min, for diquat/paraquat.
[d] Storage temperature for solutions.
[e] Time required for completion of analysis (from date samples were sent, samples received within 12 days).
[f] PRP-1, 10 µm, 300 × 7 mm, presumably as specified in the protocol.
[g] 189 days to extraction of samples plus 144 days for completion of analysis of residues in plastic vials.
[h] C18 Lichrosorb, 10µm, 250 × 4 mm.
[i] PRP-1, 5 µm, 150 × 4 mm.

Laboratory 4 reported extracting all samples, subsampling the extract, etc., and storing residues in the plastic vials at 4°C until required for analysis (144 days postextraction). This appeared to have no effect on accuracy or precision of their data. This laboratory and Laboratory 11 did not use a refrigerated centrifuge, but no significant effect on data was noted. Other laboratories filtered the mobile phase through a 0.45 μm nylon filter before use; this step should be done routinely and has been included explicitly in this report. Laboratory 4 also filtered samples through 0.45 μm disposable filters before LC/UV analysis; this was not found to be crucial by others (collaborators and Ref. 3). The different storage temperatures and times used for spiking solutions (Table 4) did not appear to result in degradation or sorptive losses, because results of analyses for standard solutions (K–R, Table 3) were similar to "true" values.

Collaborator's Comments

Collaborator 1 recommended leaving the mobile phase running overnight (0.2 mL/min) to reduce stabilization of the LC/UV detector the next day; this was intended as such in the protocol, either by keeping the system running as suggested or by recycling at 3.0 mL/min. This detail has been explicitly indicated in the present report.

Collaborator 4 found fewer baseline drifting problems when standard working solutions were made up in fresh mobile phase just before use during analysis; this may be related to an LC/UV problem, because no other collaborator reported such problems. This laboratory also suggested cautionary notes regarding the oral toxicity of diquat and paraquat, as well as the instability of diquat at high pH; this information has been included in the method.

Collaborator 8 reported problems with the silica cartridge plugging after the first 10 mL of supernatant extract and inserted a pledget of silanized glass wool in the syringe; they showed that recoveries were unaffected by this change.

Collaborator 11 recommended explicit description of adjusting the final silica cartridge eluate volume to 5 mL with methanol before removal of an aliquot; this detail has been included in the present method.

Recommendations

Generally, peak height or peak area can be used for quantitation depending on the quality of the chromatographic peaks. Several method variables may be modified: (1) resolution of interferences from analytes may be improved by decreasing the LC flow rate or by adding an organic modifier (e.g., 5% methanol) to the mobile phase; (2) limits of detection may be lowered by using larger subsamples of the final silica cartridge eluate; and (3) filtering extracts after centrifugation and/or before LC injection may improve method performance.

Although the method is somewhat time consuming and great care must be observed at each step because of the inherent profound adsorption of the analytes to glass surfaces, the statistical analyses of this study support its acceptability. I recommend that the LC method for the determination of diquat and paraquat in potatoes be adopted first action.

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PESTICIDE RESIDUES IN ANIMAL TISSUES

Supercritical Fluid Extraction of Poultry Tissues Containing Incurred Pesticide Residues

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A method using supercritical carbon dioxide to extract fat from poultry tissue was developed. Tissues used in this study were peritoneal fat, breast tissue, leg and thigh tissue, and liver from chickens fed rations containing heptachlor, dieldrin, and endrin. The fat was isolated from the peritoneal tissue by supercritical fluid extraction (SFE) and by thermal rendering. The fat was removed from the breast tissue, leg and thigh tissue, and liver by SFE and solvent extraction. The results indicate that recoveries of organochlorine pesticide from the peritoneal, breast, leg, and thigh tissues by SFE extraction are equivalent to those obtained by conventional extraction methods. The pesticide recoveries by SFE extraction of the liver were higher than those obtained by solvent extraction.

Analytical methods used to determine chlorinated pesticides in animal tissues have been applied to the isolated fatty material containing the incurred pesticide residues (1–6). Regulatory agencies and analytical laboratories currently use organic solvents to extract the fat from the animal tissue (6–7). Recently, there has been considerable concern about the health hazards associated with the use of organic solvents as well as the impact of their subsequent disposal on the environment (8). Supercritical fluid extraction (SFE) has been used to separate the lipid material containing pesticide residue from animal tissue (9) and fish tissue (10) as an alternative to solvent extraction. The present paper describes a method in which the fat from poultry (chickens) that have been fed 3 chlorinated pesticides has been extracted both by supercritical CO_2 and by conventional extraction methods. Quantitative recovery of the pesticides by the SFE extraction method is compared to the recovery by conventional solvent extraction techniques and by thermal rendering of fat.

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The reported method, which can be generically classified as "off-line" SFE, is preferred to an "on-line" SFE method (11), because the latter is difficult to accomplish in the presence of lipid coextractives. The described SFE technique has been applied successfully to relatively large fat and dehydrated tissue samples. The study is also unique in that SFE was performed on biological tissues that contained actual incurred residues, rather than fortified tissue.

Experimental

Apparatus

Gas chromatograph. — Hewlett-Packard GC 5890 equipped with ^{63}Ni electron capture detector (ECD) (Hewlett-Packard, Avondale, PA); 2 m × 4 mm id glass column (GP 1.5% SP-2250/1.95% SP-2401) (Supelco, Bellefonte, PA). Temperatures: oven 200°C, injector 220°C, and detector 350°C. Helium flow rate, 40 mL/min.

Pressure apparatus.—Haskel air-driven compressor (Burbank, CA); Autoclave micro-metering valve (Erie, PA).

Chemicals and Reagents

Petroleum ether and hexane were obtained from J.T. Baker, Inc. (Phillipsburg, NJ). Neutral alumina, Brockman Activity 1 (Fisher Scientific, Pittsburgh, PA) was heated to 800°C for 4 h, cooled, and activated by adding 5% distilled water by weight. Pesticide standards were purchased from Supelco. Carbon dioxide with a purity of 99.95% was obtained from National Welding Supply, Bloomington, IL.

Production of Incurred Residues

Chicken samples containing incurred chlorinated pesticide residues were generated by L. Rowe (College Station, TX). Nine chickens (22-month-old White Leghorn breeder hens) were fed diets containing 0.45 ppm each of heptachlor,

dieldrin, and endrin for 55 days to provide a concentration of 0.51 ppm of each pesticide (12); 5 control chickens were fed a pesticide-free diet. The chickens were sacrificed 2 days after withdrawal from the pesticide-treated feed. Previous feeding studies of chlorinated hydrocarbon pesticides had indicated that concentration of the residues reached a plateau after 6–7 weeks of feeding (12). The chicken tissue was removed from the bones and portioned into breast, leg/thigh, peritoneal fat, and liver tissue. The tissue samples were individually packaged, frozen, and sent to the National Center for Agricultural Utilization Research (NCAUR) in Peoria, IL. These samples were used in this study.

Extraction

Samples were prepared identically for both conventional fat separation techniques (13) and SFE. The peritoneal fatty tissue was ground with the aid of a Kitchen Aid food grinder and divided into 2 portions. One part was SFE-extracted to separate the fat, and the other part was thermally rendered at 80°C for 3 h to separate the fat.

The breast and leg/thigh muscle were ground and divided into 3 portions. Two parts were oven-dried at 50°C until the moisture content of the muscle was <5% by weight. One of the dried portions was extracted by SFE. The other portion was solvent-extracted by mixing it with 100 mL petroleum ether (PE) in a blender for 5 min; the solution was filtered, and the solvent was removed by evaporation on a steam bath. When ca 10 mL solvent was left, the remainder of the PE was removed by a nitrogen stream. The third portion of ground tissue was dried and extracted in 1 step by mixing PE and Na_2SO_4 in a blender; the solvent was decanted from the tissue, and the fat was recovered after solvent removal by using a steam bath and nitrogen flow as described previously.

The liver was ground, oven-dried at 50°C, and divided into 2 portions; 1 portion was extracted by SFE, and the other was extracted with PE in a blender. The PE extract was decanted from the liver tissue, and the PE was removed by evaporation on a steam bath followed by nitrogen drying.

The SFE extractions were performed in an extraction apparatus previously described (9). Two stainless steel extraction tubes (1.75 × 30.5 cm and 1.75 × 56 cm) were used, depending on the size of the sample. The peritoneal fat samples (25–30 g) were spread on glass wool supports, which were placed on plastic sheets (4 × 28 cm); the sheets were then rolled to fit into the extraction tubes (1.75 cm id). The extraction tubes (pressure rated at 10 000 psi) were then put into a converted GC oven, and the temperature was gradually raised to 80°C as the pressure was raised to 10 000 psi and maintained by the micrometering valve located before the collection flask. The flow rate of the CO_2 gas was measured at the exit port of the collection flask by an American dry test meter (Philadelphia, PA).

Extraction fluid flow rates for the peritonal fat ranged from 10 to 20 L/min, as measured under ambient conditions, and the extraction times varied from 30 to 40 min. The extracted fat, containing the incurred residues, was collected in glass round-bottom flasks. Ground meat tissue (ca 20–30 g) and liver samples (7–10 g) were placed directly in the extraction tubes. The same temperature and pressure were used to extract the ground meat and liver samples as were used for the peritoneal fat. The CO_2 flow rates were kept at 2–4 L/min for ca 2 h for the ground meat samples and 2 L/min for 45 min for the liver samples. Crude fat was determined on all tissues (7), and analysis indicated that >96% of the theoretical fat was removed by SFE extractions (9). SFE extractions of fat from control chickens were made to determine the optimum conditions for each tissue type.

Table 1. ECD/GC results for poultry tissues extracted with supercritical carbon dioxide[a]

		Pesticide, ppm in lipid extract		
Chicken No.	Tissue type	Heptachlor epoxide	Dieldrin	Endrin
1	Fat	0.88	2.93	2.32
1	Leg/thigh	0.91	2.82	2.24
1	Breast	1.04	3.06	2.34
2	Fat	0.80	2.57	2.41
2	Leg/thigh	0.91	2.53	2.15
2	Breast	1.09	2.21	1.75
3	Fat	0.96	2.73	2.08
3	Leg/thigh	0.82	2.30	1.75
3	Breast	1.42	2.68	1.98
4	Fat	1.12	3.00	2.32
4	Leg/thigh	1.08	2.86	2.22
4	Breast	1.56	3.08	2.24
CV, %[b]	Fat	6.1	4.1	3.7
CV, %	Leg/thigh	3.7	5.3	5.8
CV, %	Breast	3.9	3.8	3.5

[a] Differences determined by an analysis of variance.
[b] CV, coefficient of variation (av).

Cleanup and Analysis

The pesticide residues were separated from the fat and determined by Food Safety and Inspection Service method 5.002, a micro alumina column method for the separation of chlorinated hydrocarbons (13). For GC analysis, aldrin was added as an internal standard to the injection solution in isooctane, and a 2.0 µL aliquot was injected. Analyses were made in duplicate to determine repeatability.

Results and Discussion

Pesticide residue concentration was determined in lipid material extracted from the individual chicken tissues. From the 14 chickens sampled, the average weight % fat content of the peritoneal fatty tissue (F) was 88%; 24% fat was extracted from the leg/thigh (L/T) tissue, 5.8% from the breast (B), and 11% from the liver.

Pesticide residues were not found in any of the tissues of the 5 control chickens fed the pesticide-free diet. Pesticide concentrations in the 3 tissue types are shown in Table 1. Comparisons

Table 2. Analytical results for peritoneal fat processed by SFE or thermal rendering[a]

	Pesticide, ppm in lipid extract					
	Heptachlor epoxide		Dieldrin		Endrin	
Chicken No.	SFE[b]	REN[c]	SFE[b]	REN[c]	SFE[b]	REN[c]
1	0.88	0.97	2.93	2.91	2.39	2.39
2	0.80	0.79	2.57	2.43	2.41	2.26
3	0.96	1.08	2.73	3.15	2.08	2.57
4	1.12	0.83	3.00	2.73	2.32	2.40
5	0.86	0.96	2.92	3.09	2.32	2.40
6	0.70	0.74	2.38	2.46	2.06	2.23
7	0.56	0.62	2.33	2.30	2.00	2.11
8	0.59	0.57	1.97	1.97	1.95	1.93
9	0.66	0.60	2.34	2.19	2.38	2.17
CV, %[d]	4.2	3.7	5.0	2.0	3.3	2.3

[a] Comparison by paired *t*-tests found no differences between extraction methods.
[b] SFE, supercritical fluid extraction.
[c] REN, thermal rendering.
[d] CV, coefficient of variation (av).

were tested within an analysis of variance at $P < 0.05$. Heptachlor epoxide formed from metabolized heptachlor was higher in the breast muscle than in the peritoneal fat and leg/thigh tissue. There were also significant differences ($P < 0.05$) in the heptachlor epoxide residue between chickens. Chickens 1 and 4 had higher levels of dieldrin than chickens 2 and 3. There were no significant differences ($P < 0.05$) in the amounts of dieldrin or endrin residues present in the fat extract of the 3 tissue types.

The concentrations of the pesticides from the peritoneal fat extracted by SFE and from thermal rendering of the fat are compared in Table 2. A paired *t*-test comparing the 2 extraction techniques found no significant difference ($P < 0.05$) between the 2 methods for any of the 3 pesticides studied. Each value in Table 2 is the average of 2 determinations. At the bottom of the table is the average coefficient of variation (CV), showing the repeatability of the GC determinations.

Table 3 compares the data from the 3 extraction methods (PE, dehydration achieved by addition of Na_2SO_4 to PE and extracted; DPE, sample air-dried and extracted with petroleum ether; and SFE, supercritical fluid-extracted) for the breast and the leg/thigh tissues.

No significant differences ($P < 0.05$) were found by an analysis of variance for the PE vs DPE, PE vs SFE, or DPE vs SFE for any of the 3 pesticides. Chicken 7 generally tended to have lower levels of pesticide residues than the other 2 chick-

Table 3. Results from poultry tissue extracted by supercritical fluid vs solvent extraction methods[a]

		Pesticide, ppm in lipid extract								
		Heptachlor epoxide			Dieldrin			Endrin		
Chicken No.	Tissue type	PE[b]	DPE[c]	SFE[d]	PE	DPE	SFE	PE	DPE	SFE
6	Breast	0.80	0.80	0.86	2.79	2.77	2.79	2.42	2.34	2.39
6	Leg/thigh	0.71	0.76	0.80	2.61	2.80	2.71	2.30	2.41	2.36
7	Breast	0.52	0.48	0.46	2.09	2.02	2.08	1.70	1.58	1.70
7	Leg/thigh	0.52	0.52	0.49	2.00	1.96	1.80	1.72	1.80	1.65
9	Breast	0.67	0.70	1.01	2.40	2.35	2.04	2.32	2.29	2.13
9	Leg/thigh	0.58	0.61	0.74	2.20	2.26	2.22	2.13	2.15	2.10
CV, %[e]	Breast	3.7	3.0	1.4	4.3	7.3	3.7	4.0	7.5	3.8
CV, %	Leg/thigh	3.1	3.0	3.1	6.8	5.2	5.5	5.4	6.5	5.6

[a] Differences determined by an analysis of variance.
[b] PE, Na_2SO_4 added, extracted with petroleum ether.
[c] DHE, dehydrated, extracted with petroleum ether.
[d] SFE, supercritical fluid extraction.
[e] CV, coefficient of variation (av).

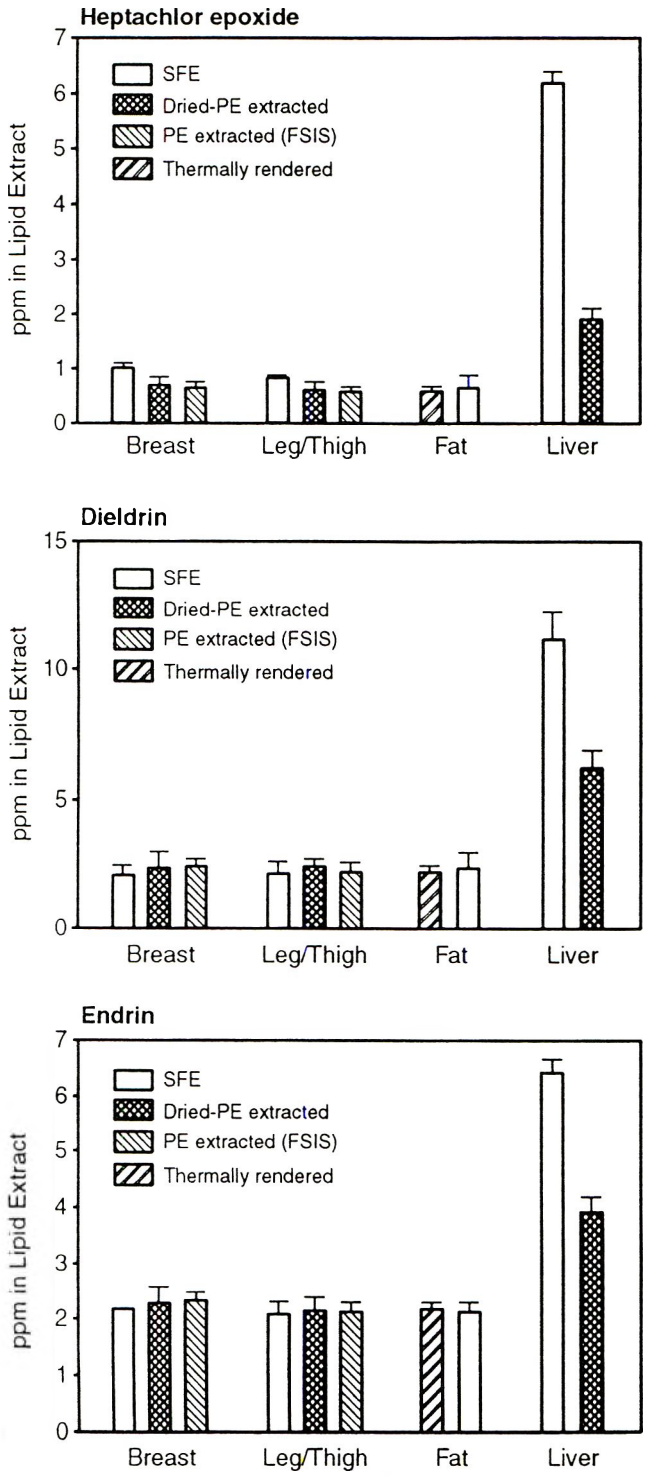

Figure 1. Comparison of extraction methods on poultry tissues from chicken No. 9. Standard deviation is indicated by the marks at the top of each bar.

ens studied. For these data, where a difference was found between tissues, higher levels of dieldrin were found by PE extraction and higher levels of endrin by SFE extraction for breast tissues than for leg/thigh tissues. The average CV of each extraction method for all analyses of the breast and leg/thigh samples was 4.6% for PE extraction, 5.4% for DPE extraction, and 3.8% for SFE extraction.

Pesticide residue levels from the fat extract of chicken 9 were plotted, and Figure 1 shows the concentrations determined by 4 methods of extraction of all the tissue types. The standard deviation for the duplicate GC determinations is shown at the top of each individual bar. The data trend for this chicken was representative of all of the chickens studied. Peritoneal fat extracted by the 2 methods of thermal rendering and SFE showed no difference in the pesticide concentrations. Also, there was no significant difference among the 3 extraction methods in the amount of pesticide found in the breast and leg/thigh tissues, as was also reported in the previous tables. However, the data in Figure 1 indicated that the amount of each pesticide residue found in the liver was greater by SFE than by solvent extraction. This result is possibly due to the superior mass transport properties exhibited by supercritical fluids vs conventional organic solvents (14), i.e., SC-CO_2 is able to penetrate the liver tissue more effectively and to extract the pesticides, and, thus, yielded higher analyte recoveries from all the livers that were extracted.

The incurred residues found in the poultry tissues were 2-6 times their concentration in the residue-inducing feed. This is in agreement with the results of Cummings et al. (12); however, the lower values found for heptachlor epoxide relative to dieldrin (15, 16) and endrin may be due to a difference in the rate of metabolic conversion of heptachlor to its epoxide within the chicken's body.

Overall, with the exception of the liver, the analytical data suggest that the distribution of the individual chlorinated pesticides in the fat from different types of hen tissue is relatively constant. This is consistent with the detoxifying role of the liver in the mammalian body, as reported by Groves et al. (17).

The results of this study indicate that supercritical fluid extraction is as effective as conventional thermal rendering or solvent extraction techniques. This research has the potential for establishing a "solventless" extraction method, eliminating the cost associated with hazardous solvent disposal, and avoiding the exposure of laboratory personnel to such chemical agents.

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TOXICITY TESTING

Evaluation and Application of a Bioluminescent Bacterial Genotoxicity Test

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The Mutatox® test (commercial name for the bioluminescent bacterial genotoxicity test) has been shown to be a good alternative to the Ames test. The test uses dark mutants of luminous bacteria (*Vibrio fischeri*) and determines the ability of various genotoxic agents to restore the luminescence by inducing mutation. It provides a rapid screening test which can be used to assay the genotoxicity of large numbers of pure and complex compounds. The test is completed in 1 day, and by serially diluting the compound, dose response data plus toxicity data can be generated for a number of samples simultaneously. For the direct assay (without exogenous metabolic activation), the positive controls selected were 3,6-diaminoacridine (proflavine) and *N*-methyl-*N'*-nitro-nitrosoguanidine. For the S-9 assay, which incorporated the microsome fraction (S-9) from rat liver as an exogenous metabolic activation system, the positive controls selected were aflatoxin B_1 and benzo(a)pyrene. This study also indicated that methyl-imidazo-quinoline and tryptophan pyrolysates were genotoxic in the presence of S-9 activation, aflatoxin B_1 epoxide and fumonisin B_1 showed direct genotoxic activity, and aflatoxin B_2 and ochratoxin A were not genotoxic.

The human population is exposed to an ever-increasing number of food additives, drugs, cosmetics, and chemical substances which are of great social and economic importance. In conjunction with the introduction of each new chemical, an equally great probability exists for the incidence of adverse effects upon the population. Substances that are not natural to a food may also become a part of the food by several routes, and may be consumed by humans and thus raise questions of safety. Toxicity testing has become a major concern nationally, because of the extensive legislation that now mandates such testing, as well as internationally because of the need for mutually acceptable products as a prerequisite to economic cooperation in the marketing of foods and chemicals (1, 2).

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Genotoxicity testing is one of the important branches of a toxicity testing system. In recent years, thousands of chemicals have been tested for genotoxic activity as a result of the development of rapid, relatively inexpensive genotoxicity screening tests. Many genotoxicity tests are now available that use diverse organisms from bacteria to mammalian cells in culture (3). The Ames test, also called *Salmonella*/mammalian microsomal test, has been widely used as a screening test by food scientists and environmentalists (4). Most recently, Ulitzur and his colleagues have developed a new genotoxicity test that uses a bacterial system and bioluminescent technique. The bioluminescence test (BLT) for genotoxic agents uses dark mutants of luminous bacteria to determine the ability of the tested agent to restore luminescence by inducing mutation. The restored luminescence can be measured by using photometric instrumentation (5).

The current study was conducted to evaluate the Mutatox® test (the commercial version of the BLT for genotoxic agents) in our laboratory, to use the test to screen some chemical compounds that may be found in foods or feeds, to confirm its reliability, and to contribute to a data base for the test.

METHOD

Reagents

(a) *Solvents.*—Acetone, acetonitrile, dimethyl sulfoxide (DMSO), ethanol, and methanol were all LC grade from Fisher Scientific Co., Fairlawn, NJ.

(b) *Water.*—Millipore double deionized water (Millipore Co., Bedford, MA) was used for water reagent in all tests.

(c) *Chemicals.*—Benzo(*a*)pyrene (B(a)P), aflatoxin B_1, aflatoxin B_2, fumonisin B_1, ochratoxin A, 3,6-diaminoacridine (proflavine), *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG), glucose-6-phosphate (G-6-P), and β-nicotinamide adenine dinucleotide phosphate disodium salt (NADP) were obtained from Sigma Chemical Co., St. Louis, MO. The heterocyclic amines, methyl-imidazo-quinoline (MeIQ) and tryptophan pyrolysates (Trp-P-1 and Trp-P-2), were supplied by W. Fiddler (USDA, Philadelphia, PA).

Mutatox® Test

The Mutatox test, which was developed by Microbics Co. (Carlsbad, CA), is the commercial version of the BLT for genotoxic agents described by Ulitzur (5). The Microbics Co. has

lyophilized the bacteria culture, modified the assay medium, and optimized the assay protocol to provide a simple and reliable system for the detection of genotoxic agents. Aseptic technique is not necessary for the test. The Microbics Co. provided all the reagents that were needed for the test, including the bacterial culture and assay medium.

(**a**) Bacterial culture.—A dark mutant, M 169, of *Vibrio fischeri* was prepared by Ulitzur. The Microbics Co. has lyophilized the bacteria culture, which is stable at –20°C. This culture has been developed for different purposes: *(1)* for direct assay (the test includes the tested chemical without exogenous metabolic activation), and *(2)* for S-9 assay (the test includes the tested chemical with the microsome fraction (S-9) from rat liver as an exogenous metabolic activation system). Before each test the lyophilized culture is rehydrated by adding 1 mL prechilled deionized water into the culture vial and mixing gently to obtain a homogenous suspension. The rehydrated culture was always used immediately after it was prepared.

(**b**) *Assay medium.*—The Mutatox test was performed in the growth medium designated as M 169 growth medium, which is also available in the lyophilized form prepared by Microbics Co. The powdered medium is stable at –20°C. For each test, the proper amount of lyophilized assay medium was weighed and dissolved in a premeasured amount of prechilled deionized water. The concentration of the assay medium in the deionized water was 3% (w/v). The assay medium has also been developed for the direct assay and the S-9 assay. The assay medium was prepared fresh for each test.

(**c**) *S-9 preparation.*—The S-9 preparation contained rat liver microsomes induced by Aroclor 1254 and was purchased from Molecular Toxicology Inc. (Annapolis, MD). The S-9 preparation is stable if kept in an ultralow freezer (–70°C). For the S-9 assay, the S-9 preparation was thawed and used immediately. The concentration of S-9 preparation in the assay medium was 0.5% (v/v).

(**d**) *S-9 cofactor.*—In addition to S-9 preparation, the S-9 assay also required the S-9 cofactor, which included 0.25 mmol NADP and G-6-P in the assay medium.

(**e**) *Sample preparation.*—Different concentration ranges of tested chemicals were prepared to determine in which range the genotoxicity occurs with the Mutatox test. For water-insoluble chemicals, the solvent systems that are compatible with the test are acetone, acetonitrile, DMSO, ethanol, and methanol.

(**f**) *Control preparation.*—For each test, concurrent positive and negative controls should be used in the same system as that used for the tested chemical. Both a solvent and an untreated (blank) control should be used as negative controls. Proflavine or MNNG was used as a positive control for the direct assay, and aflatoxin B_1 or B(a)P was used as a positive control for the S-9 assay.

(**g**) *Luminescence determination.*—The photometer used for the bioluminescence determination in the Mutatox test is the Tri-Carb® Model 1600 TR liquid scintillation analyzer (Packard Instrument Co., Meriden, CT). The analyzer was operated at the single photon count setting. Each sample preparation should be read at least once each hour up to 24 h.

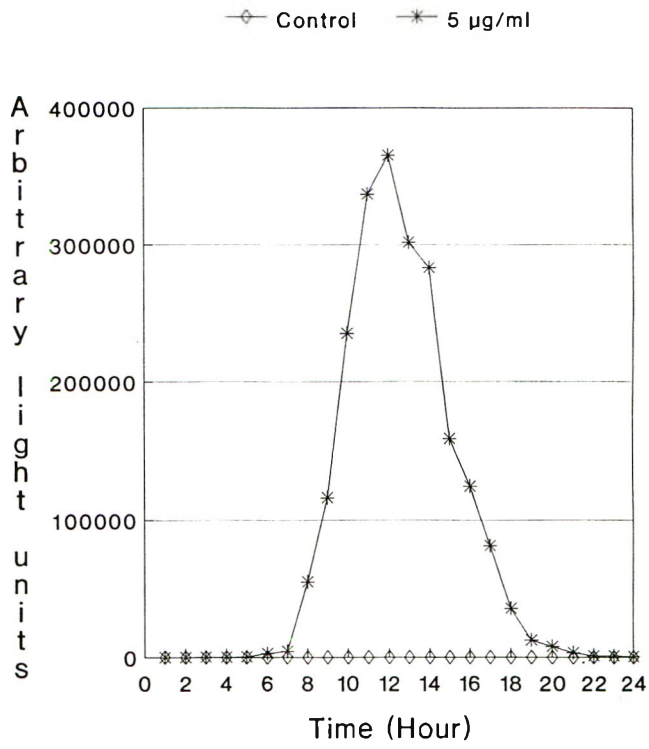

Figure 1. Typical result of Mutatox test for proflavine.

Assay Protocol

The test protocol steps, which are all performed at room temperature, are as follows:

(**a**) *(1) For the direct assay.*—Add 2 mL assay medium to scintillation vials. *(2) For the S-9 assay.*—Prepare S-9 preparation and S-9 cofactor mixture in the assay medium. Then add 2 mL of the assay medium mixture to scintillation vials.

(**b**) Add desired concentrations of the tested chemical(s) to scintillation vials in triplicate and shake gently.

(**c**) Add 20 µL of the rehydrated bacterial culture to each scintillation vial.

(**d**) Cap the scintillation vials and shake them for 20–30 min.

(**e**) For each test, prepare a blank control, concurrent solvent control, and positive control.

(**f**) Measure light emission automatically by using liquid scintillation analyzer hourly up to 24 h.

Analysis of Results

To evaluate the activity of the chemical in question is to determine the maximal luminescence value that has been reached at any time during the test. A significant genotoxic activity can be defined when the maximal luminescence developed due to the chemical in question is ≥3 times the maximal luminescence of the corresponding control (5).

Experimental Design

In this study, each tested chemical was prepared in triplicate in each Mutatox test and was examined for genotoxicity 3

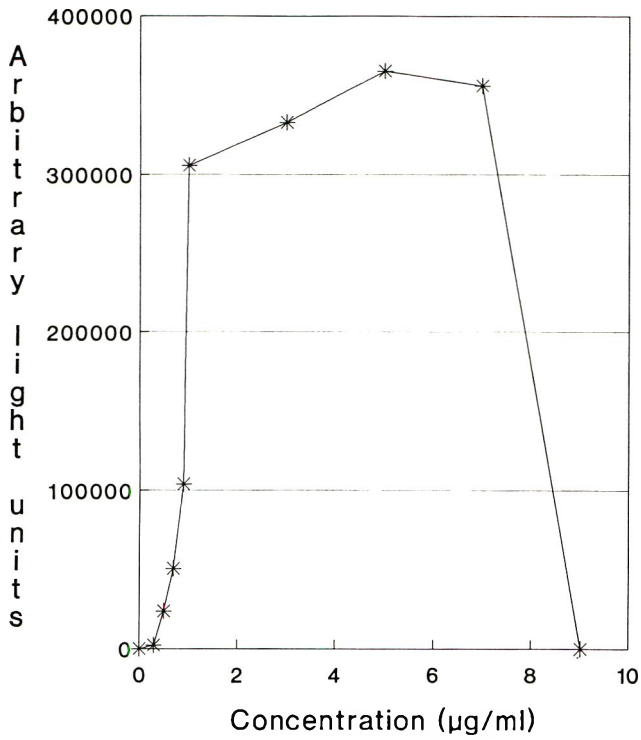

Figure 2. Typical dose-response result of proflavine by using Mutatox test.

times by the test. Results reported represent 1 typical result from 3 tests for each tested chemical.

Results

The efficiency of the chosen positive controls was examined. Figure 1 represents a typical result of the Mutatox test when comparing the effects of proflavine and a blank control. In the presence of 5 µg/mL proflavine, the restored luminescence reached its maximum around 12 h. In the blank control without proflavine, the spontaneous luminescence did not change significantly up to 24 h. The luminescence increase for the proflavine treatment is several orders of magnitude higher than that of the blank control. The genotoxic activity of proflavine has thus been confirmed.

For the Mutatox test, it is possible to obtain not only can a positive or negative result for genotoxicity, but also the dose-response within a certain concentration range of the tested chemical. Figure 2 shows the dose-response of proflavine from the test. From 0.3 to 5 µg proflavine/mL, the luminescence increased as the concentration increased. We also observed that when the concentrations of proflavine were further increased up to 9 µg/mL, the presence of proflavine eliminated the bacterial growth. The concentration of a certain tested chemical at which it will inhibit the bacterial growth is defined as the toxic concentration.

As shown in Table 1, the response of MNNG in the Mutatox test was also examined. Again, a positive response of genotoxic activity has been confirmed for MNNG at concentrations between 0.03 and 0.1 µg/mL. The toxic concentration of MNNG was 0.3 µg/mL.

The S-9 assay was conducted by using aflatoxin B_1 and B(a)P. Figure 3 shows the result of aflatoxin B_1 at 3 µg/mL. In the presence of S-9 activation, the luminescence of aflatoxin B_1 treatment increased dramatically around 10 h; without S-9 activation the maximal luminescence of aflatoxin B_1 treatment was similar to that of the blank control. These results demonstrate that S-9 activation is necessary for the aflatoxin B_1 to exhibit genotoxic activity. As shown in Table 1, the S-9 activation is also necessary for the B(a)P to show a positive response in the Mutatox test. Like the direct assay, the S-9 assay can also provide different kinds of information about certain chemicals, such as genotoxicity response, dose-response, and toxic concentration.

From the experiments with proflavine, MNNG, aflatoxin B_1, and B(a)P, the positive controls were established for further study with other chemicals to be tested. Table 1 summarizes the results of all the positive controls. The study indicated that: *(1)* the onset times for the maximal luminescence were different for different chemicals, *(2)* the genotoxic or toxic concentration was different for different chemicals, and *(3)* for all chemicals tested, a confirmed result can be obtained within 24 h.

Table 2 presents the results of tests with some of the mycotoxins selected for the Mutatox test. As indicated in the positive control study (see Figure 3), aflatoxin B_1 responded positively for genotoxic activity in the presence of S-9 activation. Within the concentration range 0.01–50 µg/mL, aflatoxin B_2 did not show genotoxic activity for both direct and S-9 assays. The toxic concentration for aflatoxin B_2 was 50 µg/mL. Epoxide aflatoxin B_1, a derivative of aflatoxin B_1, was synthesized according to the method described by Baertschi et al. (6) and evaluated for genotoxic activity. For the direct assay, epoxide aflatoxin B_1 exhibited genotoxicity between 2 and 10 µg/mL;

Table 1. Mutagenicity of proflavine, MNNG, aflatoxin B_1 and B(a)P

Chemical	Direct assay	S-9 assay	Concn range for positive response, µg/mL media	Toxic concn, µg/mL media
Proflavine	+	NT[a]	0.3–7.0	9.0
MNNG	+	NT	0.03–0.10	0.3
Aflatoxin B_1	–	+	0.10–5.00	8.0
B(a)P	–	+	0.001–0.700	1.0

[a] NT = not tested.

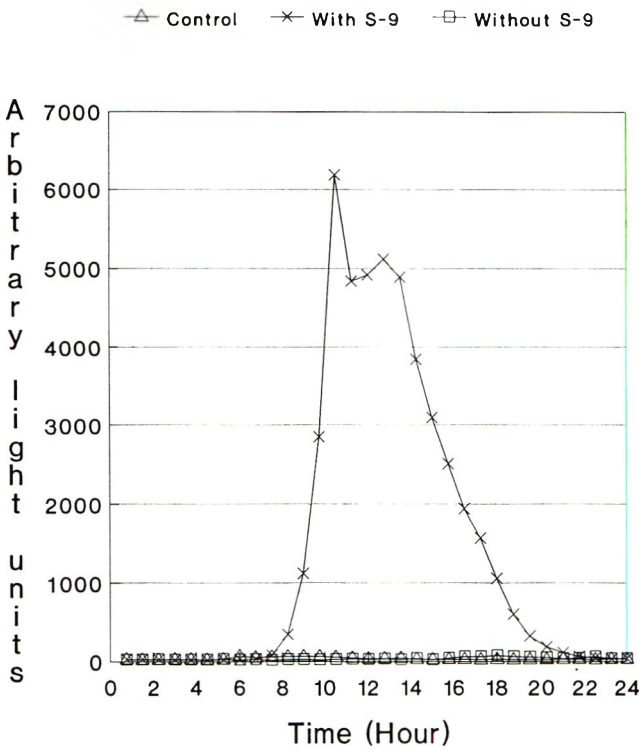

Figure 3. Typical result of Mutatox test for aflatoxin B_1 at 3 μg/mL.

at 20 μg/mL, the epoxide aflatoxin B_1 played an inhibitory role for bacterial growth. In the Mutatox test, ochratoxin A was not genotoxic with or without activation by S-9 fraction. The toxic concentration for ochratoxin A was 50 μg/mL. Fumonisin B_1 showed genotoxic activity without the activation of S-9 fraction. The concentration range which responds positively was between 5 and 20 μg/mL; the toxic concentration was 40 μg/mL.

Heterocyclic amines such as MeIQ, Trp-P-1, and Trp-P-2 were tested for genotoxicity in the Mutatox test. All 3 compounds responded negatively for the direct assay and positively for the S-9 assay (see Table 3). The concentration ranges that gave a positive response were 0.5–10 μg/mL, 0.5–7.5 μg/mL, and 0.5–7.5 μg/mL for MeIQ, Trp-P-1, and Trp-P-2, respectively. The toxic concentration was 30 μg/mL for MeIQ and 40 μg/mL for both Trp-P-1 and Trp-P-2.

Discussion

The success of a genotoxicity assessment program depends strongly on the efficiency and reliability of the screening system, for most of the decisions regarding individual chemicals must be made on the basis of short-term screening tests. The BLT for genotoxic agents has been developed by Ulitzur (5) and further commercialized and designated as the Mutatox® test by Microbics Co., CA. Based on current understanding of the regulatory control of *V. fischeri lux* system, 3 related mechanisms may explain the ability of the mutant strain to produce light after exposure to genotoxic agents. The transduction of the luminescence luciferase operon is under continuous repression, probably by an intercistronic repressor. Restoring the luminescence of the repressed dark mutant can theoretically be achieved by 3 independent events: *(1)* blocking the formation of the repressor, *(2)* inactivating the repressor of the luminescence system, and *(3)* changing the physical configuration of the DNA, thus allowing unrepressed transcription of luciferase operon. Direct mutagen agents (either base substitution or frame shift), DNA damaging agents, DNA intercalating agents, and DNA synthesis inhibitors will restore the light emitting stage of the strain and be measured by a luminator (5, 7, 8). Ben-Issak et al. (9) suggested that the BLT, unlike the Ames test, is not affected by the presence of amino acids or other nutrients; thus it is possible to assay complex organic matter such as foodstuffs and biological fluids. Ulitzur (5) found that the BLT detects all the 46 tested chemicals that are known to be active in the Ames test. Moreover, many carcinogenic agents that are not active in the Ames test are detected by the test.

Mycotoxins are intimately associated with almost every food or feed and can be produced by contaminating fungi at every stage of harvesting, production, and storage of food. The widespread presence of mycotoxins creates serious environ-

Table 2. Mutagenicity of some mycotoxins

Mycotoxin	Direct assay	S-9 assay	Concn range used, μg/mL media	Toxic concn, μg/mL media
Aflatoxin B_1	–	+	0.01–50 (0.1–5.0)[a]	8
Aflatoxin B_2	–	–	0.01–50	50
Epoxide aflatoxin B_1	+	ND[b]	0.10–50 (2–10)[a]	20
Ochratoxin A	–	–	0.01–50	50
Fumonisin B_1	+	ND	0.01–50 (5–20)[a]	40

[a] The concentration range responded positive.
[b] ND = not determined within the concentration range used.

Table 3. Mutagenicity of some heterocyclic amines

Chemical	Direct assay	S-9 assay	Concn range for positive response, μg/mL media	Toxic concn, μg/mL media
MeIQ	–	+	0.5–10.0	30
Trp-P-1	–	+	0.5–7.5	40
Trp-P-2	–	+	0.5–7.5	40

mental and economic problems. Aflatoxin B_1 is one of the most potent naturally occurring mutagens. Most laboratory animals respond to the toxic and carcinogenic effects of aflatoxin B_1. The Fisher strain of rat is probably the most sensitive mammal; 1 ppb in the diet can elicit a carcinogenic response (10). The rainbow trout is the most sensitive fish known; <4 ppb in the diet will produce a significant incidence of liver cancer (11). Aflatoxin B_1 can also induce mutations in bacterial, algal, fungal, and insect species and in mammalian cell culture (12). Metabolism of aflatoxins is considered a prerequisite for carcinogenicity and mutagenicity. It is well established that the chemical site implicated as responsible for the biological activity of aflatoxins is the C_2-C_3 double bond in the dihydrofurofuran moiety of these molecules (13). Reduction of this bond, yielding 2,3-dihydroaflatoxin B_1 (aflatoxin B_2), results in a 500-fold decrease in mutagenicity and a 150-fold decrease in carcinogenicity (14, 15). The ultimate carcinogen and mutagen in the metabolic activation of aflatoxin has been postulated to be the aflatoxin B_1-2,3-epoxide. This compound has been proposed to be a highly active metabolite that reacts with nucleophilic sites of macromolecules (16). Metabolic activation by the S-9 fraction was necessary for the genotoxic activity of aflatoxin B_1 in the Mutatox test, whereas aflatoxin B_1-epoxide exerted genotoxicity in the direct Mutatox assay. Aflatoxin B_2 did not show genotoxic activity in either the direct or the S-9 assay of the test. The results with ochratoxin A were similar to those of Kuczuk et al. (17) and Wehner et al. (18), who showed that ochratoxin A was not mutagenic with or without S-9 activation by the Ames test. Gelderblom et al. (19) isolated and purified 2 new mycotoxins, fumonisins B_1 and B_2, from culture material of *Fusarium moniliforme* MRC 826. Fumonisin B_1 has been shown to exhibit cancer-promoting activity in a short-term cancer initiation-promotion bioassay with diethylnitrosamine-initiated rats and the induction of gamma-glutamyltranspeptidase-positive (GGT^+) foci as an endpoint after 4 weeks of promotion. The genotoxicity of fumonisin B_1 has been demonstrated in the study. Results indicated that fumonisin B_1 is a direct genotoxic agent for which the metabolic activation system is not necessary.

Study by Ohgaki et al. (20) indicated that hepatocellular carcinomas and adenocarcinomas were found in the intestines of mice given 0.04% MeIQ. Matsukura et al. (21) found that a high incidence of liver tumors was induced between experimental days 402 and 621 in mice given Trp-P-1 and Trp-P-2 at a concentration of 0.02%. Trp-P-1 and Trp-P-2 were also found to induce liver tumors in rats (22). Heterocyclic amines are not themselves mutagenic to *Salmonella typhimurium* strains, but they exert mutagenic activity in the presence of S-9 fraction (23). Kato and Yamazoe (24) found that synthetic hydroxyamine derivatives of heterocyclic amines were mutagenic in the absence of the S-9 activation system and suggested that cytochrome P450 in the S-9 fraction may convert the heterocyclic amines to their hydroxyamine derivatives and thus exert mutagenicity. Our study further confirmed the necessity of the S-9 fraction activation system for heterocyclic amines, such as MeIQ, Trp-P-1, and Trp-P-2, to exhibit genotoxicity in the Mutatox test.

Our experiments suggest that the Mutatox test correlates well with the results of the *Salmonella*/mammalian microsomal test and other genotoxicity tests. The Mutatox test is much easier to conduct. Minimum personnel training and laboratory facility requirements are other advantages. In the Ames test, results are obtained by counting the colonies of bacteria. Because any unexpected contamination by other bacteria may affect the outcome significantly, aseptic technique is necessary. The dark mutant of luminous bacteria used in the Mutatox test, however, only responds to genotoxic agent and then luminescence is restored. Unless the contamination contains genotoxic agent(s), the luminescence will not be altered, thus affecting the outcome of the test. The Mutatox test provides a rapid general screening test which can be used to assay large numbers of pure chemicals and complex samples. The test can be performed in 1 day, and by serially diluting the compound, dose response data plus toxicity data can be generated for a number of samples simultaneously.

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VETERINARY ANALYTICAL TOXICOLOGY

pH Method for Determination of Cholinesterase in Whole Blood: Collaborative Study

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A collaborative study of an indirect method for determination of cholinesterase activity was conducted with cattle blood. Ten collaborators analyzed 8 samples of varying degrees of cholinesterase inhibition. The sample is diluted with a buffer, and the pH change that results from the hydrolysis of acetylcholine is monitored. Repeatability relative standard deviations (RSD_r) ranged from 6.6 to 8.6%, and reproducibility relative standard deviations (RSD_R) ranged from 10.7 to 17.3% for mean activities of 0.60–0.40 ΔpH/h. All samples that were completely inhibited were detected. The method was adopted first action by AOAC International for determination of cholinesterase activity in whole bovine blood.

Cholinesterase inhibition in blood is a clinical sign used to determine whether animals have had exposure to anticholinesterase agents such as organophosphate or carbamate pesticides. Inhibition of the cholinesterase enzymes (AChE) and pseudocholinesterase enzymes (PChE) (Enzyme Commission names: EC 3.1.1.7, acetylcholine acetylhydrolase; EC 3.1.1.8, acylcholine acetylhydrolase) interferes with normal neural transmission at the synapse, causing repetitive firing of the neuron because of lack of degradation of the transmitter acetylcholine. Animals exhibiting clinical signs show profuse salivation, abdominal cramps, diarrhea, excessive lacrimation, sweating, dyspnea, miosis, pallor, cyanosis, and incontinence of urine and feces. Also seen is excessive stimulation of skeletal muscles, manifested by twitching of the muscles in the face, eyelids, and tongue; general musculature exhibiting a seizure activity may be seen. The hyperactivity is often followed by weakness and paralysis and may result in depression. Excessive secretions in the respiratory tract will cause coughing in animals. Death usually results from hypoxia and erratic heartbeat (1).

Measurement of AChE and PChE is widely used to detect exposure to anticholinesterase agents (1). In blood, AChE and PChE are found in varying amounts for domestic animals (2, 3). Use of certain substrates will distinguish these forms, but in diagnostic testing, a rapid, reproducible, indirect method is needed. Although a quantitative method for determining activity exists (**991.10**) (4), many laboratories routinely use the indirect, simpler technique described here. Determining the activities of AChE and PChE in a single measurement is desired and will support diagnoses of organophosphate and/or carbamate pesticide poisonings.

A preliminary interlaboratory study of cholinesterase methods (5) provided data that supported the decision to conduct collaborative studies of 2 different methods (direct and indirect techniques). Diagnostic toxicology laboratories routinely use one or the other, but rarely both. A study of the direct method was completed (6). Described here is a collaborative study of the indirect method, anecdotally known as the Modified Michel method (7, 8), which uses incubation of whole blood at room temperature with the substrate acetylcholine bromide. The substrate is hydrolyzed by AChE and PChE to produce acetic acid and choline bromide. The acetic acid causes a change in pH that is measured over 1 h. When an inhibitor is present, less acetic acid is produced and little or no change in pH is observed (9). Organophosphates are irreversible inhibitors of cholinesterase and usually cause total inhibition of the enzyme; carbamates are reversible inhibitors and may cause only partial inhibition of the enzyme. Inhibition is dependent on dose (10).

Collaborative Study

Samples for the study were prepared from a pool of heparinized cattle whole blood (300 mL) obtained from a clinically normal animal that had no known recent exposure to inhibitors. The pool was divided into three 100 mL lots. Two of the lots were treated with a dilute solution of paraoxon solution to obtain the desired degree of inhibition. The third lot was not

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The recommendation was approved by the General Referee and the Committee on Feeds, Fertilizers, and Related Topics and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, Jan/Feb issue.

treated. Each lot was divided into 2 mL aliquots and frozen (−10°C) until use.

The proposed method was submitted to 11 participating laboratories, along with 2 blind duplicates of uninhibited blood, 3 replicates of partially inhibited blood, and 3 replicates of totally inhibited samples. Samples were shipped frozen on dry ice, and collaborators were instructed to keep them frozen until the time of analysis. Collaborators were also provided with certified buffers, saponin solution, enzyme buffer, and substrate solution.

992.20 Cholinesterase in Blood pH Method

First Action 1992

(Applicable for determining cholinesterase activity in whole bovine blood, <0.1–0.60 ΔpH/h)

Method Performance:

s_r = 0.035–0.04; s_R = 0.064–0.069; RSD_r = 6.6–8.6%; RSD_R = 10.7–17.3%

A. Principle

Whole blood samples are lysed with saponin to release cholinesterase and pseudocholinesterase and diluted in barbital buffer. Lysed samples are incubated with acetylcholine bromide, which is hydrolyzed by cholinesterase and pseudocholinesterase to yield acetic acid and choline bromide. Acetic acid produced is measured by monitoring change in pH over 1 h. Cholinesterase activity is reported as ΔpH/h.

B. Apparatus and Reagents

(**a**) *pH meter.*—Capable of reading 0.01 pH unit, with single junction pH combination electrode.

(**b**) *Certified pH buffers.*—Commercial certified calibration buffers, pH 4.0 and pH 7.0.

(**c**) *Acetylcholine substrate.*—0.11M. Dissolve 2.00 g acetylcholine bromide in 100 mL H_2O. Store, in 4.0 mL portions, at 4° (stable 1 week) or at −10° (stable 1 year).

(**d**) *Saponin solution.*—0.01%. Dissolve 0.1 mL saponin in 1.00 L H_2O.

(**e**) *Enzyme buffer.*—0.02M sodium barbital, 0.004M KH_2PO_4, 0.6M KCl. Dissolve 4.12 g sodium barbital (controlled substance in United States, see *Code of Federal Regulations* 160.107), 0.55 g KH_2PO_4, 44.73 g KCl, and 28 mL 0.1N HCl in H_2O and dilute to 1 L.

(**f**) *External control sample.*—Obtain several hundred milliliters of cattle whole blood in heparin from animal(s) known not to be exposed to inhibitors. Store at −10° in 2 mL aliquots. Ensure blood is not inhibited by method **991.10**. Establish mean cholinesterase activity by using following method on 20 replicates. Acceptable external control sample has results within 2 standard deviations of mean.

C. Calibration of pH Meter

Calibrate pH meter, according to manufacturer's instructions, by using certified pH 4.0 and 7.0 buffers before pH measurements. Buffers must be at room temperature for calibration.

D. Procedure

Dilute 1.0 mL heparinized blood to 10 mL with saponin solution in 17 × 100 mm tube, and mix 10 s on vortex mixer. Transfer 2.0 mL diluted sample to 17 × 100 mm tube containing 2.0 mL enzyme buffer and vortex mix 10 s.

Prepare blanks by adding 2.0 mL saponin solution to 2.0 mL enzyme buffer. Analyze 1 blank and 1 external control sample for every 10 samples.

Calibrate pH meter and measure pH of blanks, external controls, and samples. Record as pH1 for each, respectively. Rinse electrode with water and pat dry between samples. Add 0.4 mL acetylcholine substrate to blanks, external controls, and samples. Immediately record time and vortex mix each tube 10 s. Leave at room temperature. After ca 50 min, recalibrate pH meter. At 1 h, measure pH of blanks, external controls, and samples. Record as pH2 for each, respectively.

E. Calculations

$$\text{Cholinesterase, } \Delta pH/h = pH1 - pH2$$

If ΔpH/h for blank is not zero, subtract average blank value from external controls and samples. For samples <0.1 ΔpH/h, report as <0.1.

Ref.: J. AOAC Int. (1993) **76**, July/August issue

Results and Discussion

Eleven laboratories experienced with the method participated in the study. The collaborators received 8 frozen whole blood samples along with 2 pH calibration buffers, saponin solution, enzyme buffer, and enzyme substrate. The samples were arranged as 2 blood blind replicates (not inhibited), 3 partially inhibited blood blind replicates, and 3 totally inhibited blood blind replicates. This type of sampling was chosen to reflect a range of actual diagnostic samples.

Results were received from 10 laboratories (Table 1); results from 2 laboratories were not included because of their failure to correctly follow the procedure by using different chemical reagents and by altering the sample dilution ratio. The statistical analysis (Table 1) was done according to the recommendations of the AOAC statistical manual (11). Data from all laboratories were in good agreement. There were no rank-sum outlier laboratories. Sample 3 for laboratory G was determined to be an outlier by the Dixon test and was not included in the statistical calculation. The repeatability (RSD_r) and reproducibility (RSD_R) relative standard deviations were 6.6 and 10.7%, respectively, for the uninhibited samples and 8.6 and 17.3%, respectively, for the partially inhibited group. Collaborators were instructed to report <0.1 for samples with readings less than 0.1. All completely inhibited samples were reported correctly.

All collaborators were experienced with the method. No comments were offered.

Recommendation

The Associate Referee recommends that the pH method for determination of cholinesterase activity in whole bovine blood be adopted first action.

Table 1. Method performance for pH method determination of cholinesterase activity (ΔpH/h) in whole blood[a]

	Not inhibited		Partially inhibited			Totally inhibited		
Coll.	1	2	3	4	5	6	7	8
A	0.60	0.64	0.44	0.43	0.44	<0.1	<0.1	<0.1
B	0.65	0.67	0.42	0.43	0.44	<0.1	<0.1	<0.1
C	0.57	0.56	0.33	0.29	0.29	<0.1	<0.1	<0.1
D	0.47	0.55	0.32	0.32	0.33	<0.1	<0.1	<0.1
E	0.58	0.47	0.41	0.45	0.49	<0.1	<0.1	<0.1
F	0.61	0.58	0.42	0.41	0.42	<0.1	<0.1	<0.1
G	0.68	0.60	0.19[b]	0.38	0.40	<0.1	<0.1	<0.1
H	0.67	0.65	0.36	0.44	0.49	<0.1	<0.1	<0.1
Mean, ΔpH/h	0.60			0.40			—	
s_r	0.04			0.035			—	
RSD_r, %	6.6			8.6			—	
s_R	0.064			0.069			—	
RSD_R, %	10.7			17.3			—	

[a] Samples were distributed as blind replicates: 1 and 2; 3, 4, and 5; 6, 7, and 8.
[b] Dixon outlier; not included in statistical analysis.

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VITAMINS AND OTHER NUTRIENTS

Rapid Determination of Cholesterol in Single and Multicomponent Prepared Foods

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A rapid method has been developed for cholesterol determination in single and multicomponent foods. The method involves alcoholic KOH saponification of the samples, extraction of the nonsaponifiable fraction with hexane, and injection of concentrated extract into the gas chromatograph without derivatizations. It has been applied to a wide variety of frozen and refrigerated foods. More than 300 samples were analyzed with a coefficient of variation (CV) ranging from 0.5 to 8.6%. The average recoveries of cholesterol from spiked oil and tomato vegetable soup samples were 100 ±1.5% and 99.7 ±1.6% and the CVs were 1.5 and 1.6%, respectively. This method reduces labor by >70%, eliminates dangerous chemicals, and minimizes solvent use, compared to the AOAC method and other methods cited in the manuscript. The method was used successfully on a wide variety of multicomponent foods. We recommend this method for collaborative study under the AOAC guidelines for method approval.

Recent escalation in cholesterol awareness and the implication of cholesterol's role in atherosclerosis have increased the demand for a rapid, accurate, and precise methodology to measure cholesterol in foods. Furthermore, the need to comply with food labeling regulations and to facilitate nutritional planning for low cholesterol diets emphasizes the need for such a method (1–4). The controversy over the cholesterol content of eggs has prompted poultry scientists to examine the accuracy and precision of different analytical methods used in cholesterol determination (5, 6). In clinical and medical areas, rapid, precise, and accurate measurement of cholesterol is required for identification and subsequent treatment of individuals with high serum cholesterol levels (7–9).

Little information is available in the literature on the cholesterol value of multicomponent food products. Punwar and Derse (10) reported the cholesterol content of selected consumer products, but the data are far from complete.

Most methods developed recently use direct saponification; however, cholesterol determinations remain laborious and costly and use hazardous reagents for purifications, concentrations, and derivatizations (11–16). Extraction of lipid with chloroform and harsh manipulation of the extract often produce cholesterol oxidation products which in most cases have resulted in erroneous values (17–19). The multistep extraction of lipid material from the matrixes results not only in solvent disposal problems but also in health hazards and negative environmental impact.

In 1991, methodology was developed for the determination of cholesterol in selected frozen foods (20). The method required the derivatization of cholesterol with trimethylsilyl chloride and hexamethyldisilazane, which are undesirable reagents because of their toxicity and the negative effect on the FID detector. The present paper describes a method involving direct saponification with direct injection of the concentrated hexane extract of the nonsaponifiable fraction. The method can be applied to a wide variety of matrixes of foods including frozen prepared foods, meat, fish, and cheese.

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Experimental

Apparatus

(a) *Gas chromatograph (GC).*—Equipped with flame ionization detector (FID) and on-column injection system (Varian Instrument Group, Sunnyvale, CA); 1.8 m × 3 mm id coiled glass column packed with 1.0% SE-30 on Gas Chrom Q (80–100 mesh). Nitrogen carrier gas set at 50 mL/min, hydrogen at 30 mL/min, and air at 300 mL/min. Column temperature 235°C, injection port 280°C, and detector 290°C.

(b) *Standard taper flat-bottom flask.*—250 mL (Fisher Scientific, Pittsburgh, PA).

(c) *Standard ground joint condenser.*—(Fisher Scientific).

(d) *Magnetic stirrer/hot plate combination.*—Equipped with variable speed and heat controls (Fisher Scientific).

Reagents

(a) *Cholesterol standard.*—99% + purity (Sigma Chemical Co., St. Louis, MO). Prepare 1 mg/mL solution of cholesterol in hexane. Prepare 0.25 and 0.50 mg/mL working standard solutions in hexane.

(b) *Cholestane internal standard.*—>99% purity (Sigma). Prepare 1 mg/mL stock solution of 5α-cholestane in hexane. Prepare 0.2 mg/mL working standard solution in hexane.

Table 1. Cholesterol content of entrees analyzed by direct saponification and direct injection

Type of entrees[a]	No. samples analyzed	Mean, mg/100 g	CV, %
Chicken a la orange	15	19.3 ± 0.8	4.3
Chicken chow mein	15	14.9 ± 0.8	5.4
Fettucini Alfredo	15	19.7 ± 0.8	4.1
Glazed chicken	15	18.7 ± 0.8	4.4
Seafood Newburg	15	23.3 ± 2.0	8.6
Oriental pepper beef	15	15.6 ± 0.7	4.7
Rigatoni w/meat	15	7.4 ± 0.6	8.6
Chicken w/vegetables	15	10.2 ± 0.7	6.6
Roasted turkey	15	16.3 ± 1.0	6.4
Mandarin chicken	15	15.1 ± 0.8	5.3
Beef fajita	15	15.9 ± 1.3	8.0
Chicken fajita	15	18.8 ± 0.8	4.1
Cheese manicotti	15	8.7 ± 0.5	5.6
Baked cheese ravioli	15	8.4 ± 0.5	6.0
Chicken fettucini	15	19.5 ± 1.3	6.7

[a] Consist of protein source (chicken, beef, seafood, and cheese), carbohydrate source (pasta, rice, and potato), vegetables, spices, and flavoring.

Table 2. Cholesterol content of selected frozen dinners analyzed by direct saponification and direct injection

Type of dinners[a]	No. samples analyzed	Mean, mg/100 g	CV, %
Salisbury steak	4	13.7 ± 0.2	1.6
Breast of turkey	4	13.0 ± 0.1	1.0
Oriental chicken	4	15.4 ± 0.7	4.3
Sirloin tips	4	17.6 ± 0.4	2.2
Shrimp creole	4	18.3 ± 0.2	1.3
Pot roast	4	14.5 ± 0.4	2.4
Shrimp marinara	4	20.0 ± 0.9	4.7
Pasta primavera	4	4.2 ± 0.2	4.1

[a] Consist of protein source (beef, chicken, and seafood), vegetables (beans, carrots, zucchini, broccoli, vegetable medley), carbohydrate source (rice, potato), spices, flavoring, and fruit dessert.

Table 3. Cholesterol content of selected canned soups[a] and breakfast[b] products

Type of product	No. samples analyzed	Mean, mg/100 g	CV, %
Chicken noodle	4	21.3 ± 0.6	2.9
Beef stew	4	10.4 ± 0.1	1.0
Chunky beef vegetable	4	7.4 ± 0.3	6.0
Turkey chili w/bean	4	15.2 ± 0.2	1.2
Spaghetti	4	9.3 ± 0.3	3.3
Muffin sandwich	6	21.1 ± 0.7	3.4
Western style omelet	6	14.8 ± 0.6	3.8

[a] Consist of meat, vegetables, carbohydrate sources, spice, and flavoring.
[b] Consist of turkey sausages, egg, bread, and some vegetables.

Table 4. Cholesterol content of selected ingredients

Type of product	No. samples analyzed	Mean, mg/100 g	CV, %
Breaded fish fillets	10	27.4 ± 0.7	2.6
Cooked beef	10	60.7 ± 1.1	1.8
Cheese	10	54.5 ± 1.2	2.2
Turkey sausage	10	74.3 ± 1.9	2.5
Chicken chunk	10	56.9 ± 0.3	0.5
Turkey ham	10	68.2 ± 2.2	3.2
Turkey meat	10	40.2 ± 1.4	3.4

(**c**) *Reagent alcohol.*—Specially denatured anhydrous ethyl alcohol, 90% with 5% methyl alcohol and 5% isopropyl alcohol (Fisher Scientific Co., Cat. No. A962–4).

(**d**) *Potassium hydroxide solution.*—Dissolve 60 g KOH pellets in 40 mL deionized water.

(**e**) *Hexane.*—Nanograde (Fisher Scientific).

Determination

Accurately weigh 5–10 g (±0.01 g) well-ground food sample (depending on components of sample matrixes) into 250 mL flat-bottom flask. Disperse sample thoroughly in reagent alcohol in amount equivalent to 4 mL/g sample. Add 1 mL 60% KOH/g sample. Add stirring bar, connect flask to water-cooled condenser, and reflux 30 min. Shake occasionally to loosen debris from sides of flask (16, 20–22). Cool digest to room temperature by placing flask in cool water bath. Add exactly 100 mL hexane from a volumetric pipet. Stopper flask and stir magnetically 10 min. Add 25 mL deionized water, stopper, and stir an additional 15 min. Allow layers to separate, decant, and pass the hexane through anhydrous sodium sulfate to remove moisture, collecting in another flask. Remove 25 mL hexane and evaporate to dryness under a stream of nitrogen. Dissolve residue in 2 mL hexane containing 0.2 mg 5α-cholestane internal standard/mL, transfer to sample vial, and inject 5 μL onto gas chromatograph. Keep injecting same volume for sample and standard throughout run. Adjust GC parameters so that retention times for 5α-cholestane and cholesterol are ca 4 and 7 min, respectively. If gas chromatographic response of sample is greater than that of standard, dilute sample solution or take less extract for evaporation.

Calculation

Calculate amount of cholesterol in mg/100 g sample, V_{cl}, as follows:

$$V_{cl} = \frac{RF \times A_{cl} \times DF \times 100}{A_{ce} \times SW}$$

$$RF = \frac{A_{ce_{is}} \times 0.25 \text{ mg/mL (or } 0.50 \text{ mg/mL)}}{A_{cl_{is}}}$$

where RF = response factor, A_{cl} = cholesterol peak area, DF = dilution factor, A_{ce} = cholestane peak area, SW = sample weight, $A_{ce_{is}}$ = cholestane peak area in standard, $A_{cl_{is}}$ = cholesterol peak area in standard.

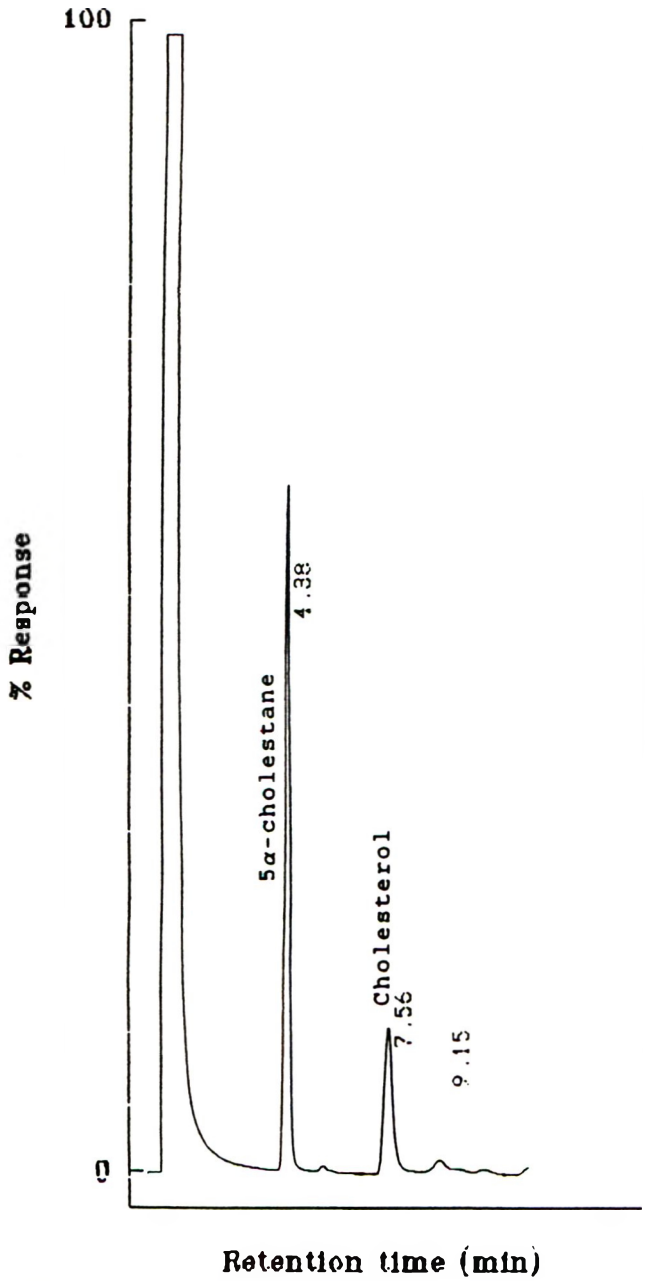

Figure 1. Gas chromatographic separation of cholestane and cholesterol in multicomponent foods (chicken w/vegetables and pasta). Ordinate: FID response percent full scale. Abscissa: Retention time (min).

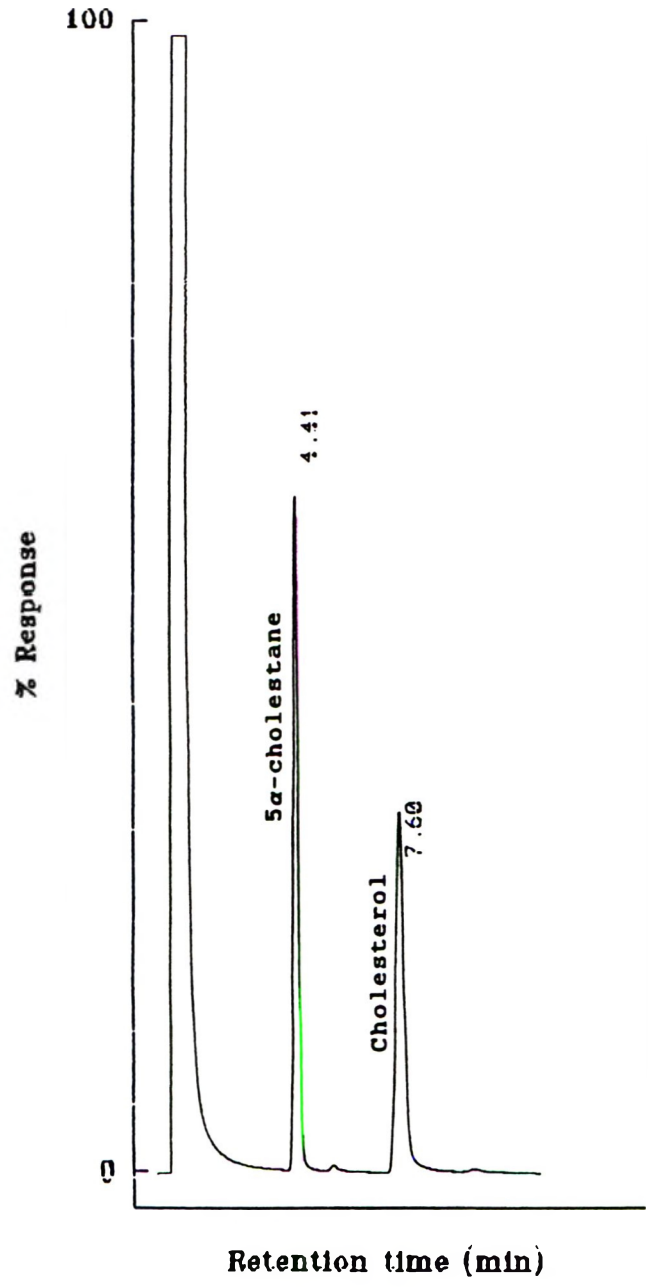

Figure 2. Gas chromatographic separation of cholestane and cholesterol standards. (Deflection represents 1.0μg cholestane and 1.25 μg cholesterol, respectively. Ordinate: FID response percent full scale. Abscissa: Retention time (min).

Detection limit of this method is 0.5 μg cholesterol/g sample.

Add same amount of internal standard to unknown sample as the amount of calibration standard.

Results and Discussion

As nutritional regulations are promulgated, the demand for rapid, accurate, and precise methods becomes imperative. In this study, a method was developed using direct saponification (16, 20–22) and direct injection of the extracted and concentrated nonsaponifiable fraction into the GC. To examine the aforementioned criteria, cooked beef sample was analyzed 10 times to measure precision. The results for 10 assays ranged from 58.7 to 61.9 mg/100 g (average 60.7 ±1.1; coefficient of variation (CV) 1.8%). The CV of 1.8% shows better precision of the method than the CV of 11.5–13.9% of blind samples reported in an AOAC collaborative study (3). Accuracy was measured by spiking tomato vegetable soup with 2 levels of cholesterol, 25 and 50 mg/100 g. The soup was chosen because

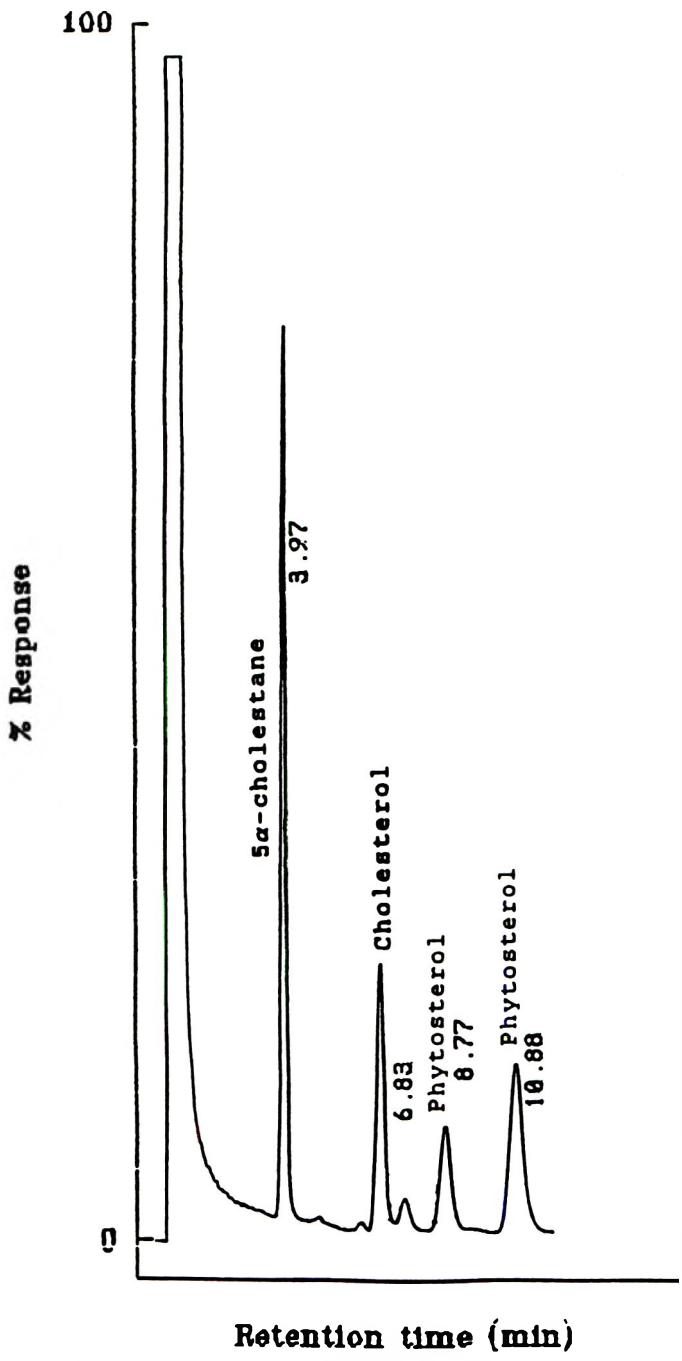

Figure 3. Chromatographic separation of 5α-cholestane, cholesterol, and phytosterols in breaded fish fillets. Ordinate: FID response percent full scale. Abscissa: Retention time (min).

of its multicomponent matrix and because it is cholesterol-free. Each level was analyzed 5 times. The average recovery was 99.7 ±1.6% (CV 1.60%). Also, vegetable oil was spiked with 2 levels of cholesterol, 25 and 50 mg/100 g. Each level was analyzed 5 times. The average recovery was 100.0 ±1.50% (CV 1.50%). The average recovery reported for the AOAC method (3) was 95.4% (10).

The method was applied to a wide variety of multicomponent frozen foods (dinners, entrees, breakfast items) and single component products (e.g., cooked beef, chicken, fish, turkey ham, sausage, meat, and cheese). The CVs ranged from 0.5 to 8.6% for entrees, dinners, soups and breakfasts, and single component ingredients (Tables 1–4, respectively). The high CV might be attributed to sample heterogeneity and the presence of noncholesterol-contributing materials such as vegetables. Very good baselines for all samples and standards were maintained throughout the run (Figures 1 and 2, respectively); however, the chromatogram of fish fillet showed not only cholestane and cholesterol peaks but also some phytosterols due to the breading component and possibly soybean oil (Figure 3).

The above described method has several advantages over the AOAC method and other methods in the literature (11–16, 21, 23, 24).

(*1*) Because it saves 70% labor and uses less solvents, it is more economical and provides results more quickly.

(*2*) It eliminates the use of toxic reagents, e.g., trimethysilylchloride, hexamethyldisilazane, dimethylformamide, chloroform, and methanol.

(*3*) Because it does not create disposal problems, it is environmentally compatible.

(*4*) It eliminates the need for expensive glassware, e.g., rotary evaporator.

(*5*) It eliminates silicone dioxide deposits on FID, thus reducing maintenance.

We believe that this method should be tested collaboratively with a view to its adoption as an official method of the AOAC.

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TECHNICAL COMMUNICATIONS

Extension of a Liquid Chromatographic Method for *N*-Methylcarbamate Pesticides in Cattle, Swine, and Poultry Liver

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Liver samples of bovine, swine, and poultry were fortified with 0, 5, 10, and 20 ppb mixed standards of an additional 6 carbamates as part of a method extension study. Each of the 3 species in this study was analyzed 4 times at 0, 5, 10, and 20 ppb fortification levels. The average of 12 recoveries of 6 carbamates at all 3 fortification levels was greater than 90%, with a maximum coefficient of variation less than 18%.

The mission of the Technical Services Laboratory, U.S. Department of Agriculture (USDA), is to support inspection operations that ensure safe, wholesome, and accurately labeled meat and poultry products for American consumers. As a part of this mission, our laboratory has analyzed carbamate residues in liver tissues of beef, swine, and poultry since 1988 for the Food Safety and Inspection Service (FSIS) National Residue Plan. The determination of *N*-methylcarbamate pesticide residues in liver tissue by liquid chromatography (LC) (1) was extended to 6 additional carbamates because of their structural similarities with the 10 carbamates in the previous study (1). These additional carbamates are propoxur, bendiocarb, oxamyl, dioxacarb, isoprocarb, and promecarb. The extension study of these additional carbamates was an abbreviated form of the original recovery studies (1).

Each of the 3 species in this study (bovine, porcine, and poultry) was analyzed 4 times at 0, 5, 10, and 20 ppb fortification levels, which resulted in 12 recoveries altogether. The protocol for developing or extending a regulatory residue method at FSIS requires monitoring the compound at 3 different levels: tolerance level, double the tolerance level, and one-half the tolerance level. In a multiresidue method, these 3 levels are chosen on the basis of one compound that has the lowest tolerance in the group. The tolerance of aldicarb in drinking water and in edible animal tissues and foods was established at 10 ppb by the U.S. Environmental Protection Agency (EPA). Because this is the extension of a previously published method (1), the same 3 levels (5, 10, and 20 ppb) based on the tolerance of aldicarb in edible animal tissues were used for this study.

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Experimental

Reagents and Apparatus

Reagents and apparatus used were as described in the LC method (1). Six additional carbamate standards were added: propoxur, bendiocarb, oxamyl, dioxacarb, isoprocarb, and promecarb (EPA/FDA Reference Standards, EPA Pesticides and Industrial Chemicals Repository, MD-8, Research Triangle Park, NC 27711).

Preparation of LC Standard Solution

(a) *Stock solutions.*—Stock solutions of all 16 carbamates, including the 6 additional carbamates, were made as described previously (1).

(b) *Working solutions (2.0 ng/μL).*—Two working solutions were made, one with 6 additional carbamates and the other with 16 carbamates including the previous 10 (1). The working standard of all 16 carbamates was used, with further dilution, to generate a 20 ppb chromatogram of the mixed standards. The working standard of 6 additional carbamates was used in sample fortification for recoveries and, with further dilution, to prepare a standard curve each day.

Sample Extraction and Determination

The experimental part of the method extension involving sample extraction and determination was performed following the published LC procedure (1). A set of recoveries consisting of 4 liver samples of one species was fortified with 0, 5, 10, and 20 ppb mixed standards of 6 carbamates. Four sets of recoveries of each species were analyzed on 4 different days. These repeatability studies for method extension were performed on 3 different species: bovine, swine, and duck.

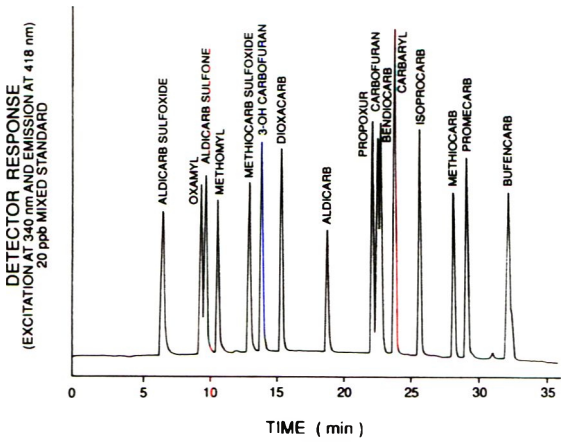

Figure 1. Liquid chromatograms of mixed standards of 16 carbamates at the highest levels (20 ppb) used in the method: postcolumn OPA fluorescence excitation and emission at 340 and 418 nm, respectively.

Identification

Using conditions of the LC method (1), the relative retention times (min) of the 6 additional carbamates are as follows: oxamyl, 9.3; dioxacarb, 15.3; propoxur, 22.1; bendiocarb, 22.7; isoprocarb, 25.6; and promecarb, 29.7.

Table 1. Repeatability studies of 6 carbamates in beef, pork, and duck livers (n = 4 for all spikes)

Carbamate	Added, ppb	Beef av. rec., ppb	Pork av. rec., ppb	Duck av. rec., ppb
Oxamyl	5	4.5	4.8	4.4
	10	9.0	9.6	10.1
	20	17.7	18.3	18.6
Dioxacarb	5	4.5	4.9	4.9
	10	9.4	9.4	10.1
	20	19.1	18.8	19.9
Propoxur	5	4.5	4.9	4.8
	10	9.2	9.6	10.9
	20	18.9	18.8	19.5
Bendiocarb	5	4.4	4.8	4.6
	10	9.3	9.9	10.9
	20	19.0	18.9	19.1
Isoprocarb	5	4.4	4.7	4.7
	10	9.3	9.1	10.5
	20	18.6	18.1	18.7
Promecarb	5	4.4	4.7	4.6
	10	9.2	9.4	10.5
	20	18.6	18.4	18.9

Results and Discussion

The method extension study was performed following the previously published method (1) using 6 additional *N*-methylcarbamates (oxamyl, dioxacarb, propoxur, bendiocarb, isoprocarb, and promecarb) because of their structural similarities with the original 10 carbamates. Reversed-phase LC was used to separate the pesticides.

Figure 1 shows chromatograms of mixed standards of all 16 carbamates at the highest level (20 ppb) of interest used in the method. The resolution (R values) between oxamyl and aldicarb sulfone peaks and bendiocarb and carbofuran peaks are commonly 0.5 min or less (especially as the column ages). If the presence of any one of these 4 compounds in the sample is indicated, 2 recoveries are run for identification and quantitation purposes, one with oxamyl and carbofuran and the other with the remaining 14 carbamates.

Figures 2A–2C show chromatograms of mixed standards, mixed recoveries of the 6 additional carbamates in beef liver at 20 ppb, and a beef liver blank. Figures 2A′–2C′ show chromatograms of mixed standards, mixed recoveries in duck liver of the same 6 carbamates at 5 ppb, and a duck liver blank. Figure 3 shows chromatograms of official samples collected by USDA inspectors and submitted for analysis. Beef liver sample was found to contain methiocarb and methiocarb sulfoxide residues; duck liver sample was found to contain aldicarb, aldicarb sulfoxide, and aldicarb sulfone residues.

Table 2. Overall performance summary for 6 carbamates at 5, 10, and 20 ppb fortifications in beef, pork, and duck livers

Carbamate	Added, ppb	No. rec.	Av. found, ppb	Mean rec., %	SD, %	CV, %
Bendiocarb	5	12	4.6	92.5	17.0	18.4
	10	11	10.1	100.5	11.7	11.6
	20	12	19.0	95.0	12.2	12.8
Dioxacarb	5	12	4.7	94.2	17.9	19.0
	10	11	10.1	101.2	12.5	12.4
	20	12	19.5	97.7	12.7	13.0
Isoprocarb	5	12	4.6	91.0	15.2	16.7
	10	11	9.5	95.1	12.8	13.5
	20	12	18.2	90.9	13.4	14.7
Oxamyl	5	12	4.6	92.7	17.3	18.7
	10	11	9.6	95.9	12.2	12.7
	20	12	18.2	91.0	14.1	15.5
Promecarb	5	12	4.6	92.0	14.8	16.1
	10	11	9.7	97.3	11.5	11.8
	20	12	18.7	93.3	13.7	14.7
Propoxur	5	12	4.7	94.8	16.3	17.2
	10	11	10.0	99.5	12.9	13.0
	20	12	19.1	95.4	13.1	13.7

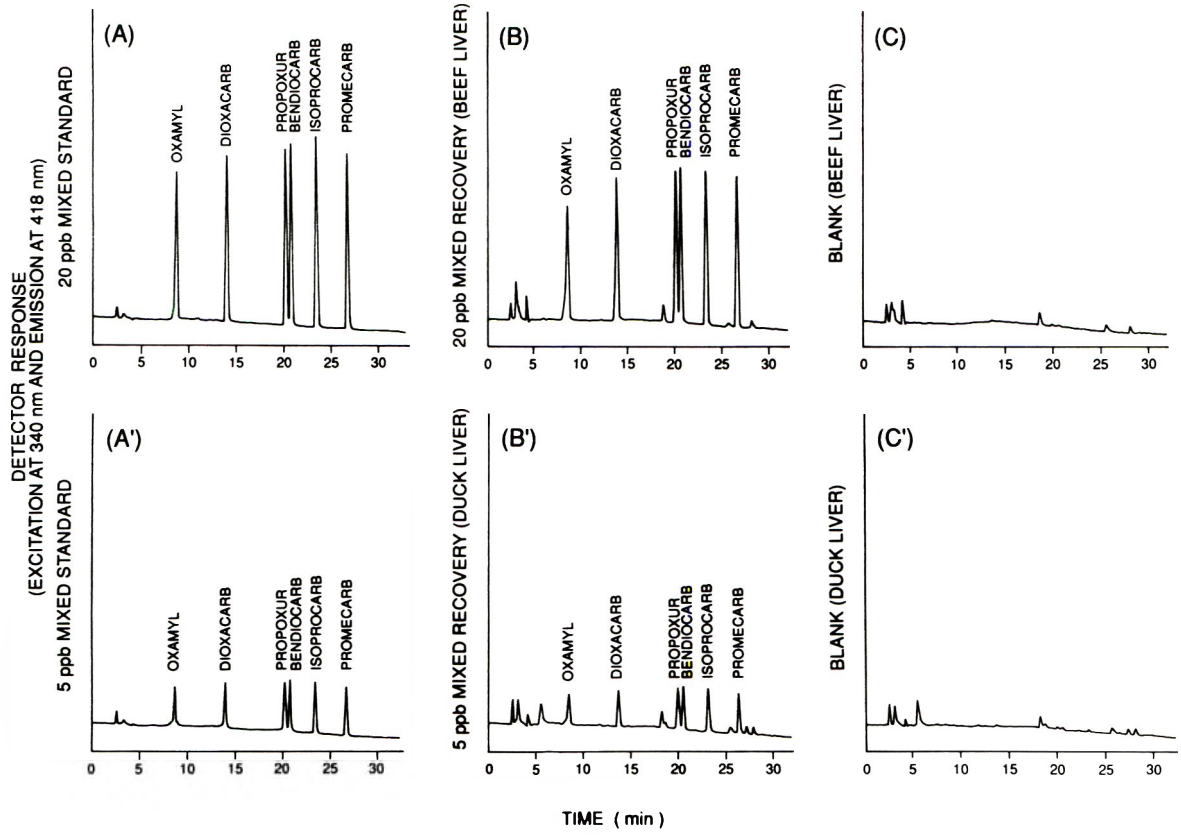

Figure 2. (A–C) Liquid chromatograms of mixed standards and mixed recoveries in beef liver of 6 carbamates at the highest levels (20 ppb) used in the method and a blank beef liver. (A′–C′) Liquid chromatograms of mixed standards and mixed recoveries of 6 carbamates in duck liver at the lowest levels (5 ppb) used in the method and a blank duck liver: postcolumn OPA fluorescence excitation and emission at 340 and 418 nm, respectively.

Except for oxamyl, which has 8 known metabolites (2), metabolites of the remaining 5 carbamates have not been reported, although carbamates are known to form conjugates with glucose in tissues (3). Our data from fortified liver do not show the presence of any breakdown product having an *N*-methylcarbamate structure resulting from extraction.

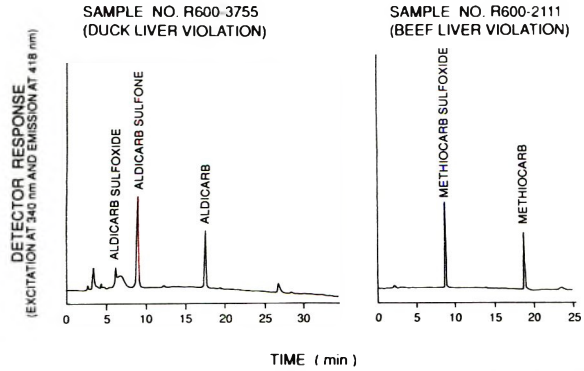

Figure 3. Liquid chromatograms of official samples of methiocarb sulfoxide and methiocarb found in beef liver and aldicarb sulfoxide, aldicarb sulfone, and aldicarb found in duck liver: postcolumn OPA fluorescence excitation and emission at 340 nm and 418 nm, respectively.

Recovery data were based on analysis of liver samples fortified with each of the 6 carbamates at 0, 5, 10, and 20 ppb. This is an abbreviated study of the previous method performance study of 10 carbamates (1). Each of the 3 species (bovine, porcine, and poultry) was analyzed only 4 times on 4 different days at 0, 5, 10, and 20 ppb fortification levels, which resulted in 12 recoveries altogether.

The repeatability studies of 6 carbamates in beef, pork, and duck liver are summarized in Table 1. Statistical analysis of the method recovery data is summarized in Table 2 for oxamyl, dioxacarb, propoxur, bendiocarb, isoprocarb, and promecarb. Data shown are calculated as overall recovery for the compounds listed in beef, pork, and poultry liver. The combined average recoveries of all 6 carbamates in beef, pork, and poultry liver tissues at all 3 levels of fortification was greater than 90%, with a coefficient of variation <18%. The general FSIS guidelines for a regulatory method are that it meet established criteria for concentration of analyte, expected recovery range, and %CV. If the concentrations of an analyte vary from 1 to 400 ppb, the expected recovery range should be 60–110% and the repeatability coefficient of variation should be <20%. Data obtained for the method extension studies are well within the limits of acceptable criteria for a regulatory method.

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TECHNICAL COMMUNICATIONS

Determination of Selenium in Feeds, Premixes, Supplements, and Injectable Solutions by Hydride-Generated Inductively Coupled Plasma Atomic Emission Spectrometry

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A simple method is described for the determination of 0.01–30 000 μg selenium/g sample. Selenium is present in feed supplements, premixes, mineral mixes, and injectable solution as either selenite or selenate. High concentrations of other common minerals present in these supplements are tolerated by the method. The samples are initially digested by heating with nitric acid and then boiled in a mixture of sulfuric and perchloric acids to convert all selenium species to selenate. The selenate is reduced to selenite, Se (IV), with hydrochloric acid at 95°C. The selenite in turn is then reduced by acidic sodium borohydride to hydrogen selenide, which is measured by hydride-generated inductively coupled plasma atomic emission spectrometry at 196.026 nm. The instrument detection limit for this method is 0.0005 μg Se/g sample.

Selenium, an essential animal nutrient, is toxic at high concentrations (1). Selenium enters the food chain almost exclusively through plants, primarily in the form of selenates. In acidic soils, selenium is typically unavailable, whereas in alkaline soils, selenium may accumulate at high levels in plants (2, 3). Because natural feeds contain <10 ppm (μg/g), selenium is a common feed additive in the form of sodium selenate (Na_2SeO_4) and selenite (Na_2SeO_3). Numerous reports have been published on the determination of selenium in various matrixes by different techniques, e.g., colorimetry (4), graphite furnace atomic absorption spectrometry (GFAAS) (5–9), flame AAS (10), ion chromatography (11), hydride-generated AAS (12–14), stripping voltametric analysis (15), gas chromatography (16–18), fluorometry (19–22), and neutron activation analysis (23). Methods reported for feeds, mineral mixes, and supplements involve flame (10) and hydride-generated AAS (12, 23–26) and colorimetric analysis (4).

All of these methods for selenium determination in supplements and feeds present various problems. The flame and hydride-generated AAS methods suffer from matrix effects, poor precision, and lengthy standard addition procedures (4). The colorimetric method has the disadvantages of interferences from ions common to mineral mixes, lengthy and involved digestion/extraction procedures, multiple manipulations to remove interfering ions, and purification of reagents, which also absorb at the same wavelength as the analyte. The colorimetric method also uses the carcinogen diaminobenzidine (DAB) and the toxic organic solvent toluene (4).

In this paper, we report the use of hydride-generated inductively coupled plasma atomic emission spectrometry (ICP AES) for the determination of selenium in feeds, mineral mixes, and injectable solutions. A slightly modified version of this method for the analysis of serum and biological tissues (27) showed no chemical interferences from iron (150 ppm), copper (5 ppm), zinc (150 ppm), lead (5 ppm), and cadmium (5 ppm) (27), which is consistent with previous work (24). A working range of 3 orders of magnitude was determined for the slightly modified version of this method (27). We have not observed any interferences from sodium, potassium, magnesium, chromium, manganese, iron, cobalt, zinc, or calcium. Interference studies were conducted with a concentration of 2% (20 000 μg/g) for each metal. Common elements in mixes, at levels typical in supplements and mineral mixes, gave no evidence of interference. Copper showed no interference below 0.3%, but at levels above 2%, it suppressed the selenium signal. Nickel did not show interfence at 0.3%, but above 2%, it enhanced the selenium signal.

This method requires only modest samples sizes, needs infrequent supervision during digestion, requires no further extractions or quantitative liquid transfers, and is amenable to autosampling on the ICP AES.

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Experimental

Reagents

(a) *Concentrated sulfuric, nitric, perchloric, and hydrochloric acids.*—Trace metal analysis grade (J.T. Baker, St. Louis, MO).

(b) *Sodium borohydride solution.*—6% $NaBH_4$ (J.T. Baker; 98% purity) in 5% NaOH (J.T. Baker). Dissolve 3 g $NaBH_4$ and 2.5 g NaOH in 500 mL 18 MΩ water. Prepare solution fresh daily.

(c) *Selenium stock standard.*—10.00 mg/L, 1 mg Se as SeO_2/L. Prepare 10.00 mg/L standard by pipetting 0.100 mL of a 10 000 mg/L stock selenium solution (J.T. Baker) into 100 mL volumetric flask. Prepare tertiary standards at 100 ng/mL by pipetting 0.1 mL of a 10 mg/L stock standard solution into 10 mL digestion tube. Prepare standards of 300, 100, and 10.0 µg/L in the same manner. Digest blanks and standards in the same manner as samples.

(d) *Reference materials.*—Bovine Liver NIST 1577a, Rice Flour NIST 1568a, Oyster Tissue NIST 1566a (National Institute of Standards and Technology, Gaithersburg, MD).

(e) *Sodium selenate.*—Anhydrous (Sigma Chemical, St. Louis, MO).

(f) *Sodium selenite.*—(Sigma).

(g) *Mineral mixes.*—Prepared by University of Idaho Feeds from Selenium "2600" Premix (Lecco).

(h) *Injectable solutions.*—Sodium selenite (ICN Biochemical) mixed into vitamin A and D desiccated injectable solution (ROCAVIT AD3, Roche Vitamins, Nutley, NJ).

(i) *Corn meals.*—Quaker yellow corn meal.

Apparatus

(a) *Inductively coupled argon plasma atomic emission spectrometer.*—Perkin-Elmer Model P-40 ICP with AS-50 autosampler. Measurements were made at 196.026 nm, with single baseline correction offset at 0.028 nm.

(b) *Nebulizer.*—Meinhard high solids tip, 2 mL/min, 31 psi, type C (Precision Glassblowing, Englewood, CO).

(c) *Mixing manifold.*—Two-channel peristaltic pump (Buchler, Saddle Brook, NJ) with Viton tubing, 0.03 mm diameter for 10M HCl and 0.02 mm diameter for $NaBH_4$. Acid flexible tubing leading to sample and PE autosampler. Sample tubing on the Perkin-Elmer P-40, 1.42 mm diameter. Small "T" pieces connect each tubing to other (Figure 1). Pump rate: sodium borohydride, 0.67 mL/min; HCl, 0.60 mL/min; sample, 3.0 mL/min.

(d) *Temperature controller/digester.*—Digestion system 40, 1016 Digester, and Autostep 1012 Controller (Tecator, Sweden). Fitted with aluminum adapter plate 3 mm thick with forty 17 mm holes on top overlaid on heater block.

(e) *Digestion tubes.*—Kimax 10 mL graduated culture tubes, 16 × 150 mm (No. 60824–568, VWR).

(f) *Perchloric fume hood.*—Water wash-down hood.

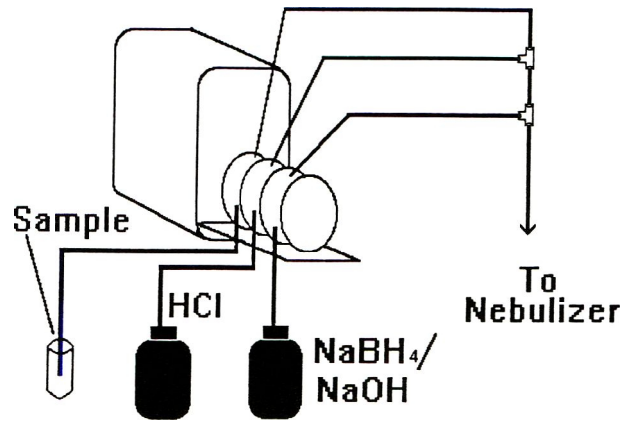

Figure 1. Manifold flow diagram for selenium hydride generation.

Determination

Weigh 0.25 g dried feed or mineral mix, or dissolve 1 mL injectable solution in 1 L water and weigh 1 mL into 10 mL digestion tube. Prepare mineral mixes with high selenium levels (above standards) by adding 10 g mineral mix to 1 L water, dissolving, and subsampling. Add 3.0 mL concentrated nitric acid to each tube. Add 1 ultrapure Teflon boiling chip to each tube. Allow samples to react for ca 1 h in hood. Use digestion system heat ramp given in Table 1. After completing first digestion, add 1 mL each of concentrated perchloric and sulfuric acids and complete second phase of digestion. (Perchloric acid fumes can react explosively with oxidizable material; use perchloric acid fume hood with water wash-down.) The samples may be stored at this stage for several days at 4°C without any adverse effects. Cool samples to ambient temperature and slowly add 8 mL 5M hydrochloric acid. Mix on vortex mixer and return to digester block at 95°C for 15 min. Cool to ambient temperature and dilute to volume with deionized water; analyze within 24 h.

Interference Study

A set of selenium standards, 50 µg/L, and spiked corn meal samples were prepared by pipetting 0.100 mL of a 10.0 mg/L Se standard and weighing 0.25 g corn meal into a series of digestion tubes. Appropriate volumes of stock metal solution at 10 000 mg/L were pipetted to yield desired concentrations of potentially interfering ions. Interference study samples were

Table 1. Digester block temperature ramp

Stage	Initial temp.	°C/min	Final temp., °C	Final temp. hold, min
First stage	Ambient	60	70	30
		30	175	90
Second stage	Ambient	30	190	60
		10	210	20
		20	250	20
		20	310	25

Table 2. Recovery of selenium from injectable solutions, feeds, and supplements

Matrix	No. detns[a]	Se concn, μg/g	Se rec. av., μg/g ± SD	Rec., %
Mineral mix C[b]	14	91	70.3 ± 2.8	77
Mineral mix S[b]	14	89	106 ± 5.0	120
Injectable soln 1[c]	14	0.3%	0.34 ± 0.0026%	113
Injectable soln 2[c]	14	0.3%	0.30 ± 0.0043%	100
Corn mix A[d]	17	91.3	72.0 ± 4.2	79
Corn mix B[d]	17	50.2	40.0 ± 2.0	80
Corn mix C[d]	17	50.2	38.5 ± 2.1	77
Corn mix D[d]	17	102.4	76.0 ± 5.3	74
Bovine liver	100	0.71 ± 0.14	0.73 ± 0.06	103
Oyster tissue	100	2.21 ± 0.48	2.15 ± 0.15	97
Rice flour	42	0.38 ± 0.08	0.33 ± 0.05	87

[a] Number of determinations over a 10 month period.
[b] Mineral mix C = cattle mix, S = sheep mix, contained the following metals: Ca, Na, Mg, P, Co, Cu, Mn, Fe, Zn >100 mg/kg.
[c] Unit for injectable solution is %.
[d] Corn mixed with sodium selenite and selenate. A 100 g sample of corn was mixed in a slurry of either sodium selenite or selenate (corn mix A, 0.0200 g sodium selenite; corn mix B, 0.0110 g sodium selenite; corn mix C, 0.0120 g sodium selenate; corn mix D, 0.0245 g sodium selenate) for 24 h and then dried in an oven at 40°C for 96 h. The samples were then analyzed for selenium content by using 10 individual sample weights varying from 0.1 to 0.35 g.

digested as described above, calibrated, and analyzed in the normal fashion. Metals tested in this way were calcium, magnesium, chromium, manganese, iron, cobalt, nickel, copper, and zinc. Sodium was not tested because sodium is always present in high concentrations with this method; i.e., sodium borohydride solution is 0.65% sodium. All metals were tested at an original sample concentration of 2% (20 000 μg/g). Actual concentrations in the samples did reflect the typical dilution factor of 40 (0.25 g in 10 mL) yielding a 500 μg/g concentration of the metals in the analyzed samples.

Results and Discussion

Recoveries of selenium from different matrixes are listed in Table 2. Recoveries ranged from 74 to 120% with an average of 91.5%. The levels of selenium ranged from 0.3% to 0.38 μg/g. The samples were analyzed over a 10 month period. The instrument detection limit was calculated as 3 times the standard deviation of the intensity of the blank standard. The blank was digested and contained all of the reagents used in the analysis. The values ranged from 0.00015 to 0.00073 μg/g, averaging 0.00058 μg/g over the duration of the study. The method was linear over 3 orders of magnitude; typically, 300, 100, 10.0, and 0.0 μg/L standards are used for a calibration curve.

Selenium in feeds and supplements can occur as both selenite and selenate; therefore, any analytical method must address this problem. Regardless of the form of selenium, this method accounts for the different oxidation states through the initial acidic digestions, which convert all forms of selenium to selenate. The samples are reduced to selenite by the addition of HCl and analyzed within 24 h. Slow oxidation of the samples does occur. The selenite is then reduced with acidic sodium borohydride to hydrogen selenide, which is measured by ICP AES. Unlike other methods, high concentrations of other metals do not interfere in this method (27).

No chemical interferences were observed in the process for calcium, magnesium, chromium, manganese, iron, cobalt, nickel, copper, and zinc up to 2% (20 000 μg/g), the highest levels tested. Selenium recoveries for both standard solutions and spiked corn meal samples were >92%. Copper at 2% suppressed 100% of the selenium signal. At levels typically found in feeds (0.3%), copper did not interfere with the selenium signal. Nickel at 2% enhanced the selenium signal by 131%. Nickel at 0.3% did not interfere with selenium.

Flexibility of this method accommodates both quick turnaround time and multiple day scheduling. For example, 40 samples may be analyzed in 1 day. Only minimal supervision is required through most of the analyses.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Fast Atom Bombardment Mass Spectrometric Analysis of Base Hydrolyzed γ-Lactone

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Fast atom bombardment (FAB) mass spectrometry (MS) is used to characterize the γ-hydroxy carboxylic acid (γ-HCa) formed from base hydrolyzed γ-lactone at room temperature. Acidification of this base hydrolyzed solution reconverts the γ-HCa back to γ-lactone. Mass shift differences between tetramethylammonium (TMA^+) and Na^+ ion-pairs with the anionic γ-HCarboxylate$^{(-)}$ (γ-$HC^{(-)}$) are used to confirm molecular ion identifications. FAB ionization was required to provide mass characterization of the γ-HCa. Electron ionization (EI) MS of the γ-HCa was unsuccessful and reconverted γ-HCa back to γ-lactone because of complete thermally induced dehydration. The present paper suggests structures for EI fragmentation that support the molecular structure of γ-lactone.

The γ-lactone, (R)-5-[1-(2-isopropylimidazol)methyl]-3,3-diphenyl-2(3H) furanone [137415-67-9], is a muscarinic antagonist useful for the treatment of neurogenic bladder and chronic obstructive pulmonary disease (1). Spectroscopic identification of the primary hydrolytic degradation product of this γ-lactone provides information that is important to the synthesis of standard materials. The standard material can then be used for direct quantitation of low level hydrolysis with common chromatographic methods. Several studies used FAB for the MS characterization of lactones (2–13). Egge et al. found characterization of the negative ion FAB ($^-$FAB) molecular ion region of gangliosides to be difficult because of Na, K, and Ca salt formation with multiple sialic acid functions. They added a strong acid to the sample target to convert these acids to 6 membered lactones, which stopped the salt formation and simplified recognition of the molecular ions (2). Riboni et al. used $^-$FAB to find a ganglioside in lactonic form ($G_{D1b\text{-}L}$) in the human brain. They deliberately avoided $G_{D1b\text{-}L}$'s exposure to NaOH during its isolation to guard against the lactone being converted to an acid (3). Levery et al. used positive ion FAB ($^+$FAB) to specifically locate sialic acid carboxyl residues internally esterified to other sugars in gangliosides with ammonolysis and permethylation of these inner lactones (4). Continuous flow $^+$FAB has previously been used to identify a γ-hydroxy acid metabolite (γ-HaM) of the furan alfuzosin (14). The solution equilibrium kinetics for the conversion of the δ-lactone [(S)-9-Dimethylaminomethyl-10-hydroxy-camptothecin hydrochloride] to a δ-hydroxy acid has been measured by using liquid chromatography (15).

None of the above studies used FAB to demonstrate that a lactone could be first hydrolyzed to an hydroxy acid with base

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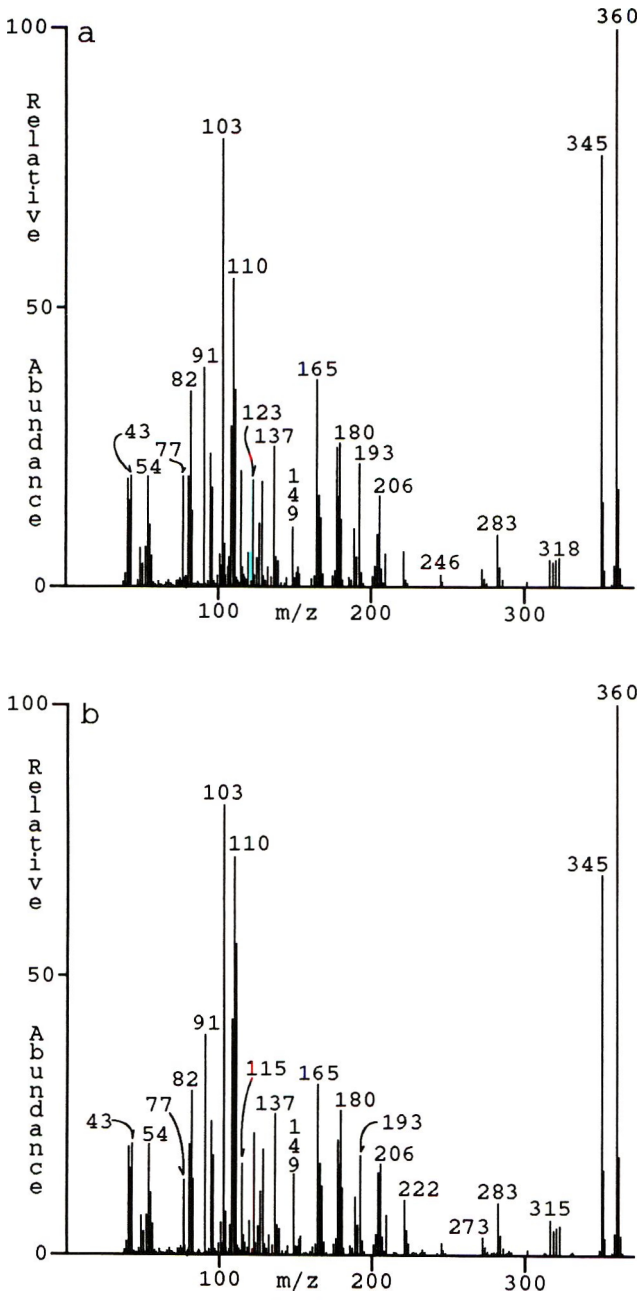

Figure 1. Comparative EI probe spectra: γ-HCa isolate (a), reference γ-lactone (b). Identical spectra indicate γ-HCa's thermally induced dehydration to γ-lactone.

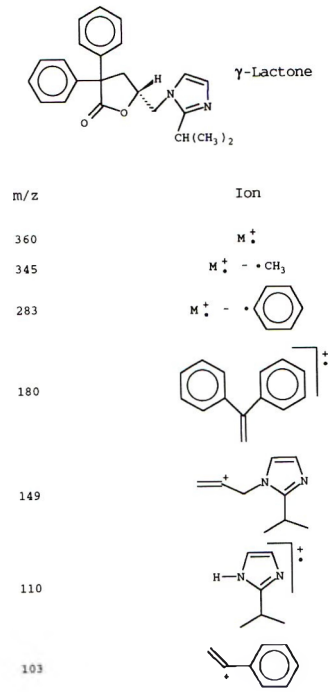

Figure 2. Molecular structure of γ-lactone and proposed mass to ion structure correlations for its EI spectrum (Figure 1).

and then recondensed back to original lactone with acid. In the present paper, the $^{+}$FAB MS identification of γ-hydroxy carboxylic acid (γ-HCa) is done directly from a base catalyzed hydrolysis solution of the starting 5 membered γ-lactone and the γ-HCa is then reconverted back to γ-lactone upon acidification of this same solution.

γ-HC acids form lactones by internal esterification when heated (16). In fact, when a γ-HCa (378 g/mole) isolate was first analyzed by using EI, the MS results were identical to those produced by the parent γ-lactone (360 g/mole); no γ-HCa was detected. See Figure 1a and b for comparative EI spectra. Figure 2 proposes several mass to ion structure correlations for these EI spectra that correspond with γ-lactone. Before ionization in the EI ion source, γ-HCa isolate could undergo thermally induced internal esterification, and regenerate the γ-lactone. A comparable marked dehydration (base peak ion) was also reported for the γ-HaM of the furan alfuzosin with direct exposure chemical ionization (14), although some (2% relative abundance) molecular ions were formed. Application of heat to the sample and ion source during the recording of these EI spectra (see *Experimental*) is generally a characteristic requirement of EI to achieve and maintain vaporization of the sample (17). To provide mass spectral verification for the hydrolysis of the γ-lactone to a γ-HCa, FAB ionization was used directly on a base catalyzed hydrolysis solution of the γ-lactone. FAB ionization does not require probe or source heat to obtain gas phase molecular ions of many thermally labile compounds (18, 19).

Experimental

Mass Spectrometry

EI samples were analyzed by using direct probe induction (>150°C probe heat) on a Kratos Concept S double focusing mass spectrometer with Nier-Johnson geometry. The spectrometer was operated in the positive ion mode at 8 kV acceleration. The ionization voltage was 70 eV with an emission stabilized beam current of 300 μA and a source temperature of 220°C. The magnet was previously calibrated from 39 to 868 daltons by using perfluorokerosene and a sweep rate of 3 s/decade with a 1 s residence at 900 daltons. The static resolution on the mass peak of *m/z* 381 was 2000. FAB ionization was achieved by using a beam of xenon atoms produced by a saddle field gun operated at 7 kV and 1 mA. The heatable source was

set at 30°C. Using reference ions from a mixture of poly(ethylene glycols) 400 and 600 in a ratio of 1:2 (20), calibration at 58–812 daltons was obtained at a scan rate of 10 s/decade with a 2 s residence at 950 daltons. The static resolution on the mass peak of *m/z* 371 was 1500. Samples analyzed by using FAB were preceded in the same run with an analogously prepared matrix blank acquired at slightly higher gain than the subsequent sample. Matrix ions could, thereby, be subtracted from all FAB spectra shown, with only a residual mass spectrum of sample remaining.

Sample Preparation

γ-Lactone hydrochloride (25 mg) was dissolved in 5 mL glycerol–water (1 + 1). FAB analyses were conducted on both the blank and sample in the same run with different probe loadings.

Tetramethylammonium (TMA^+) hydroxide·$5H_2O$ (115 mg) was dissolved, and 3 mL methanol was added (to achieve solubility of the free base γ-lactone that precipitated upon base addition) in both the blank and sample. The measured pH of the sample solution was 12.5. FAB analyses were obtained at 3 and 24 h after base addition.

Hydrochloric acid (12N, 1 mL) was added to both the blank and sample. The measured pH of the sample solution was 0.5. FAB spectra were recorded 2 h after acidification.

Results and Discussion

Figure 3 presents the FAB mass spectrum of γ-lactone in neat glycerol–water (1 + 1). A base peak $[M+H]^+$ *m/z* 361 for the γ-lactone was obtained. Fragment ions *m/z* 319 and 284 can be assigned to $[M+H]^+$ releasing $H_3CCH{=}CH_2$ and ·phenyl,

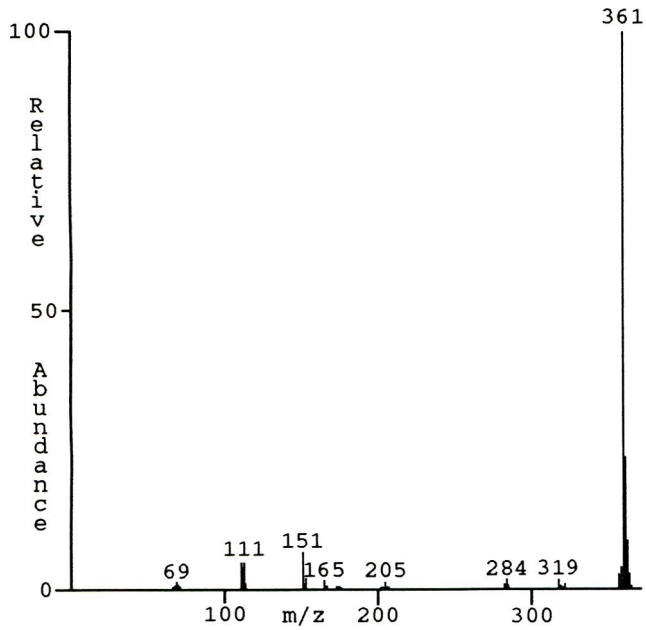

Figure 3. FAB MS of γ-lactone in neat glycerol/water: $[M+H]^+$ *m/z* 361.

respectively. Other ions (not shown) that indicate molecular species were observed at $[2M+H]^+$ *m/z* 721 (2%) and [M+glycerol (G) +H]$^+$ *m/z* 453 (5%).

Figure 4 shows the FAB mass spectrum 3 h after the addition of TMA^+ hydroxide and methanol. TMA^+ ionized molecular ions of the γ-$HC^{(-)}$+TMA^+ ion-pair were observed at *m/z* 525. Typical $[M+H]^+$ molecular ions for the free acid γ-HCa were obtained at *m/z* 379. Molecular ions were also observed at *m/z* 452 that can be represented as either [γ-$HC^{(-)}$ +TMA^++H]$^+$ or [γ-HCa+TMA]$^+$. $[M+H]^+$ molecular ions of parent γ-lactone were still observed at *m/z* 361 and indicate that hydrolysis is incomplete. Figure 5 represents structures for these molecular ions.

Figure 6 shows the FAB mass spectrum 24 h after the addition of TMA^+ hydroxide and methanol. Molecular ions specific for γ-$HC^{(-)}$ and γ-HCa, as described for Figure 4 above, are again observed at *m/z* 525, 452, and 379. The molecular ion $[M+H]^+$ *m/z* 361 of the starting γ-lactone is absent lending evidence for its exhaustive hydrolysis to γ-HCa. Ion *m/z* 335 may arise through decarboxylation (– CO_2) of γ-HCa at $[M+H]^+$ *m/z* 379 and indicates the presence of the carboxylic acid. The scarcity of other meaningful high mass ions suggests that under these conditions, the conversion of the γ-lactone to γ-HCa is essentially exclusive.

Figure 7 presents the FAB mass spectrum recorded 2 h after the addition of HCl. Recondensation of the γ-HCa back to original γ-lactone is obvious by the reappearance of γ-lactone's base peak molecular ion, $[M+H]^+$ *m/z* 361. All γ-HC(¯) and γ-HCa molecular ions (*m/z* 525, 452, and 379) are now absent from the mass spectrum and suggest that the conversion back to γ-lactone is complete and exclusive. Ions observed at *m/z* 434 and 453 can be attributed to $[M+TMA]^+$ and $[M+G+H]^+$, respectively, for the γ-lactone. A low abundance ion (not shown) at $[2M+H]^+$ *m/z* 721 (2%) was also observed for the γ-lactone. To add support for the molecular ion identifications of the γ-$HC^{(-)}$/TMA^+ ion-pairs (Figure 5), a second base catalyzed hydrolysis of γ-lactone was performed by using 25 mg NaOH (measured at pH 12.7) instead of 115 mg TMA^+ hydroxide·$5H_2O$. Mass shifts decreased by 51 amu, per TMA^+ replaced by Na^+, should be observed for γ-$HC^{(-)}$/Na^+ ion-pairs when compared to analogous γ-$HC^{(-)}$/TMA^+ ion-pairs.

Figure 8 gives the FAB mass spectrum after the addition of NaOH and 4 days of sample storage at 5°C. Na^+ ionized molecular ions were observed for the γ-$HC^{(-)}$+Na^+ ion-pair at *m/z* 423, or 102 amu less than the corresponding TMA^+ ionized ion-pair of γ-$HC^{(-)}$+TMA^+ at *m/z* 525. A more comparable mass shift was observed at the Na^+ ionized γ-HCa (or H^+ ionized γ-$HC^{(-)}$+Na^+ ion-pair) at *m/z* 401 relative to *m/z* 452 with TMA^+. These mass shifts indicate that TMA^+ was replaced with Na^+, and they support the ion-pair assignments in Figure 5. An additional ion-paired molecular ion (not observed with TMA^+ analogy at *m/z* 598) is found at *m/z* 445. Like the Na^+ ionized γ-$HC^{(-)}$+Na^+ ion-pair, this molecular ion's carboxylic acid proton is replaced with Na^+, *and* the γ-hydroxyl proton is also replaced (γ-ONa). Typical $[M+H]^+$ molecular ions for the γ-HCa were observed at *m/z* 379. Parent γ-lactone was again exhaustively hydrolyzed with NaOH because no signal

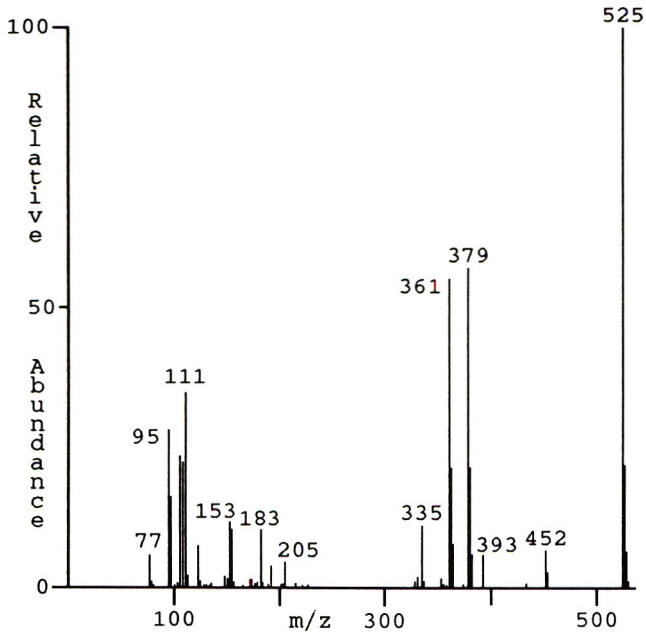

Figure 4. FAB MS at 3 h after base addition to the γ-lactone solution shown in Figure 3. Molecular ions of γ-HC$^{(-)}$; [+2TMA]$^+$ *m/z* 525 and [+TMA$^+$+H]$^+$ *m/z* 452, with molecular ions of γ-HCa; [+TMA]$^+$ *m/z* 452 and [+H]$^+$ *m/z* 379. Molecular ions at [M+H]$^+$ *m/z* 361 shows the γ-lactone is not yet fully hydrolyzed.

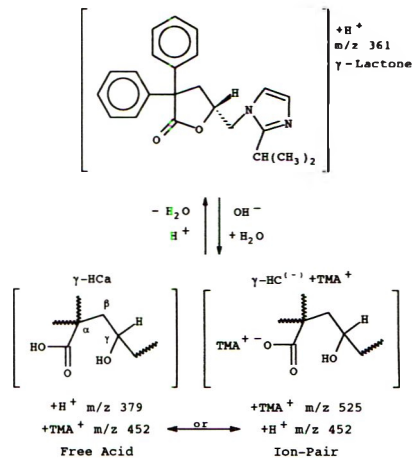

Figure 5. Molecular ion structures for the FAB mass spectrum shown in Figure 4.

is observed at [M+H]$^+$ *m/z* 361. Other molecular ions (not shown) were also observed: *m/z* 493 [γ-HC$^{(-)}$+Na$^+$+G+H]$^+$ (5%), *m/z* 515 [γ-HC$^{(-)}$+Na$^+$+G+Na]$^+$ (20%), *m/z* 537 [γ-HC$^{(-)}$+Na$^+$+γ-ONa+G+Na]$^+$ (10%), *m/z* 559 [γ-HC$^{(-)}$+Na$^+$+γ-ONa+(G-H+Na$^+$)+Na]$^+$ (2%), *m/z* 607 [γ-HC$^{(-)}$+Na$^+$+2G+Na]$^+$ (4%), and *m/z* 801 [2(γ-HC$^{(-)}$+Na$^+$)+H]+ (8%).

Conclusions

EI MS produced spectra consistent with γ-lactone but failed to produce molecular ions of γ-HCa because of its thermal esterification back to γ-lactone. FAB MS produced rapid identification of γ-HCa as the primary degradation product directly from the aqueous hydrolysis of γ-lactone catalyzed by base. Acidifying this same solution showed the γ-HCa fully condensed back to γ-lactone. Conducting the base hydrolysis separately in both TMA$^+$ and Na$^+$ hydroxides revealed mass shift differences between the γ-HC$^{(-)}$/TMA$^+$ and Na$^+$ ion pairs and verified molecular ion identifications. Of particular interest is the observation of the highly abundant molecular ion *m/z* 445 [γ-HC$^{(-)}$+Na$^+$+(γ-*ONa*)+Na]$^+$ in the sodium hydroxide hydrolysis. Na$^+$ evidently replaced the γ-hydroxy proton when under similar conditions with TMA$^+$ hydroxide as base catalyst the expected analogous ion at *m/z* 598 [γ-HC$^{(-)}$+TMA$^+$+(γ-*OTMA*)+TMA]$^+$ was not observed. This noticeable characteristic of Na$^+$, whereby both alcohol and carboxylic acid protons were replaced, might be valuable in differentiating and determining the number of alcohol and acid moieties in other such multiply functionalized compounds. Under the same conditions, TMA$^+$ apparently only replaced the carboxylic acid protons. FAB MS, by virtue of its mass resolution, simultaneously detects both the remaining γ-lactone during its degradation and the generation of its γ-HCa degradation product (see Figure 4). This ability would appear to have quantitative application in the determination of equilibrium kinetics by proportioning the sum of the abundances of the molecular ions of generated γ-HCa (ions *m/z* 379, 452, and 525) to the molecular

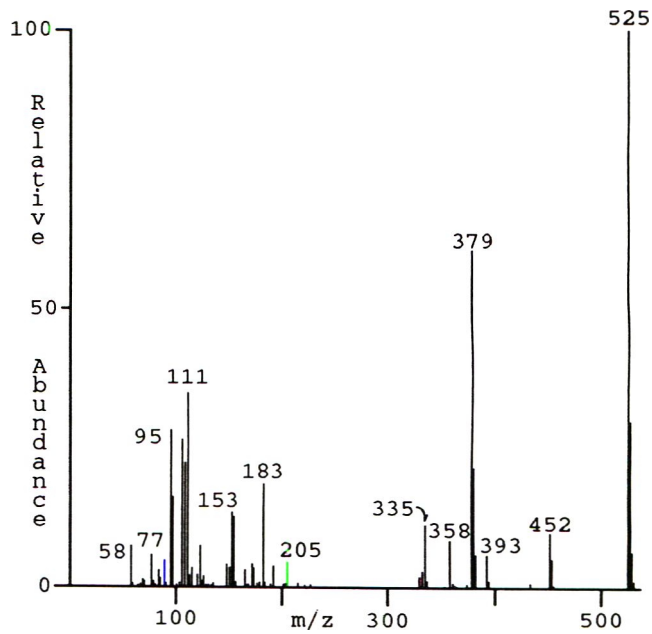

Figure 6. FAB MS at 24 h after base addition to the γ-lactone solution shown in Figure 3. Molecular ions of γ-HC$^{(-)}$ and γ-HCa are at *m/z*'s 525, 452, and 379 (see Figure 5. No [M+H]$^+$ *m/z* 361 molecular ions of γ-lactone indicate it is exhaustively hydrolyzed.

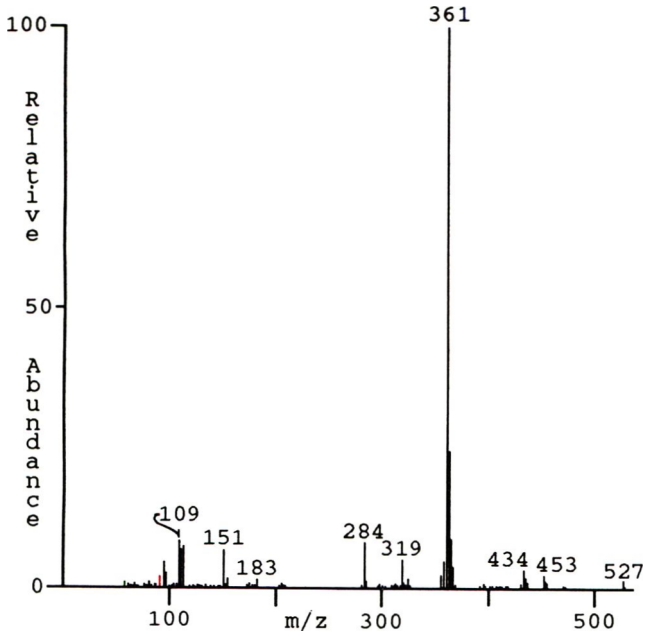

Figure 7. FAB MS of the exhaustively hydrolyzed solution (see Figure 6) 2 h after its HCl acidification. Reformation of the γ-lactone is evident by reappearance of its $[M+H]^+$ molecular ion at *m/z* 361. All molecular ions due to γ-$HC^{(-)}$ and γ-HCa (Figures 4, 5, and 6) are now gone, supporting complete reversion back to γ-lactone.

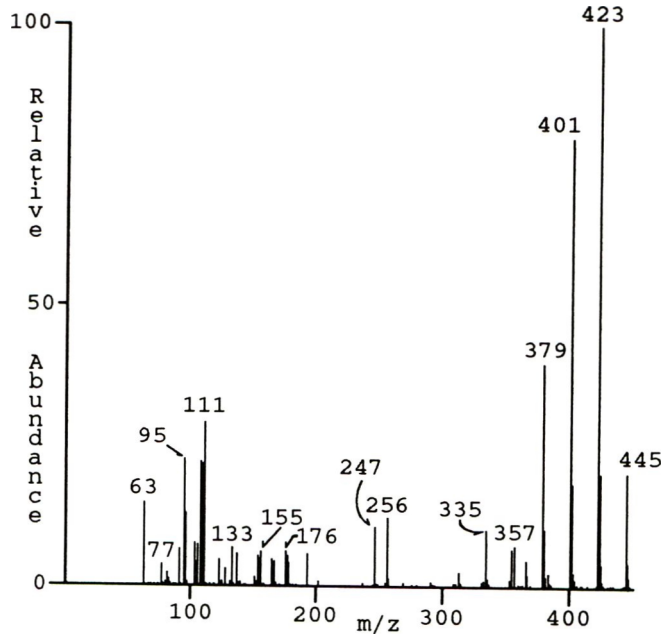

Figure 8. FAB MS of a separate NaOH degradation. Mass shift declinations from TMA^+ ion-pairs (see Figure 6) of 51 amu per TMA^+ replaced by Na^+ are observed at γ-$HC^{(-)}$; $[+2Na]^+$ *m/z* 423 and $[+Na^+ + H]^+$ *m/z* 401. Free acid $[\gamma\text{-HCa}+H]^+$ is at *m/z* 379. The molecular ion *m/z* 445 $[\gamma\text{-}HC^{(-)} + Na^+ + (\gamma\text{-ONa}) + Na]^+$ shows γ-OH proton replaced by Na^+, analogous replacement not observed with TMA^+. No γ-lactone remains, its $[M+H]^+$ *m/z* 361 is absent.

ion abundance value of remaining γ-lactone (*m/z* 361) over time.

Acknowledgment

Sincere appreciation goes to Paul Brown for preparing the γ-HCa isolate analyzed by E.I.

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TECHNICAL COMMUNICATIONS

Automated Beer Analyzer Method for Determination of Alcohol Content and Original Gravity of Beer: Summary of Collaborative Study

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Two collaborative studies were conducted by the American Society of Brewing Chemists (ASBC) to evaluate use of an automatic beer analyzer for determination of alcohol content and original gravity of beer. Repeatability and reproducibility values obtained in the first study were satisfactory. The second study compared the automated method with the ASBC distillation method. Results by the 2 methods were similar; however, repeatability and reproducibility were better for the automated method. The automated method was adopted first action by AOAC International as an ASBC–AOAC method.

The amount of alcohol in beer is of interest to brewers and regulatory agencies. A method to produce quick and reliable results for determination of alcohol and density was evaluated in collaborative studies conducted by the American Society of Brewing Chemists (ASBC).

In the method, an automatic beer analyzer (Tecator SCABA Automatic Beer Analyzer, or equivalent) measures alcohol and density. Alcohol is determined by the catalytic combustion of alcohol in an air stream as it passes over a sensor surface. Density is measured by density meter. Real extract is calculated from alcohol and density by using the Tabarie formula (1) with a small correction constant, which was included to account for distillation losses. The instrument is also programmed to calculate original gravity, apparent extract, real degree of fermentation, and calories by using formulas in AOAC methods **935.20**, **970.90**, **950.06A**, and **971.10**, respectively (2). A complete analysis requires only 3 min, which represents a great time savings over other methods.

Collaborative studies (3, 4) were conducted in 1987 and 1988. In the first year, 21 collaborators each received 4 samples and a detailed method of analysis. Table 1 provides the mean values of the results obtained from the study and the AOAC statistical analysis of the data. Repeatability and reproducibility values were acceptable.

The first collaborative study verified basic performance parameters. The second collaboration focused on comparing the automated method to an established, yet very tedious and time-consuming, distillation procedure (5). Nine collaborators each received 3 sample pairs and were requested to analyze the samples by using both the automated method and the distillation procedure. The statistical summary of this method comparison is shown in Table 2. Even though both methods were determined to be good and comparable, the automated method was better in terms of repeatability and reproducibility.

Submitted for publication September 12, 1991.
The recommendation was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, Jan/Feb issue.

992.29 Ethanol and Original Gravity Content in Beer, SCABA Method, ASBC–AOAC Method

Final Action 1992

(Applicable to determination of original gravity, 3–15, and ethanol, 1–6% w/w, in beer.)

Method Performance

Ethanol, % w/w

$s_r = 0.006$; $s_R = 0.036$; $RSD_r = 0.156\%$; $RSD_R = 0.939\%$

Original gravity, °P

$s_r = 0.018$; $s_R = 0.091$; $RSD_r = 0.164\%$; $RSD_R = 0.828\%$

A. Principle

The instrument calculates original gravity, °Plato, from measurement of density with density meter and measures ethanol, % w/w, by catalytic combustion of ethanol vapor, from evaporated beer sample, in air stream passing over sensor surface.

B. Reagents and Apparatus

(a) *Automatic beer analyzer.*—Capable of calculating original gravity from measuring density of beer (Anton Paar density meter is suitable). Capable of calculating % (w/w) ethanol in beer; equipped with peristaltic pump (to pass samples

Table 1. Statistical evaluation of collaborative mean results for alcohol and original gravity determined by automatic beer analyzer

Analysis	No. samples	No. labs.	Grand mean	s_r	s_R	RSD_r	RSD_R
Alcohol, % w/w							
Beers A/B	42	21	3.835	0.006	0.036	0.156	0.939
Beers C/D	42	21	3.246	0.005	0.031	0.154	0.955
Original gravity, °P							
Beers A/B	42	21	10.987	0.018	0.091	0.164	0.828
Beers C/D	42	21	8.678	0.014	0.075	0.161	0.864

through analyzer), thermostatically controlled coil (to evaporate ethanol in sample), tubing (to mix stream of air with ethanol vapor), and oven with ethanol sensor (to combust and measure ethanol). Microcomputer control unit evaluates and prints out results. (Tecator SCABA Automatic Beer Analyzer, PO Box 110, S-162 12, Stockholm, Sweden, is suitable).

(**b**) *Ethanol standard solutions.*—Prepare 3.5 and 7.0% w/w ethanol standard solutions by diluting 46.4 and 92.3 mL of ethanol (99.5% minimum purity), respectively, to volume with H_2O in separate 1 L volumetric flasks. Discard after 2 weeks.

For system suitability test, determine percent ethanol to nearest 0.01% by pycnometer, **942.06**, or refractometer, **950.04**.

(**c**) *Conditioning solution.*—Dissolve ca 7 g chlorine-free detergent (Tecator Cat. No. 5000-1928 is suitable) in ca 500 mL H_2O in 1 L volumetric flask. Add 75 mL ethanol, and dilute to volume with H_2O.

C. Instrument Conditioning and Calibration

Turn on analyzer and control unit. Select microcomputer program for desired analysis, adjust air flow to analyzer requirements, and connect tubing for peristaltic pump.

To condition analyzer, place almost full vial of conditioning solution one space behind sampling position. When oven and thermostat indicate run temperatures have been reached, start pump and recirculate conditioning solution for >20 min by placing end of drainage tube in vial.

Calibrate analyzer by placing vial with H_2O one space behind sampling position, directly followed by vial with 3.5% then vial with 7.0% ethanol standard solutions, **B(b)**. Select calibration program and process vials.

Table 2. Statistical evaluation of collaborative results for alcohol and original gravity determined by automatic beer analyzer and distillation methods

Analysis	No. samples	No. labs.	Grand mean	s_r	s_R	RSD_r	RSD_R
SCABA method							
Alcohol, % w/w							
Beers A/B	18	9	5.619	0.006	0.041	0.11	0.73
Beers C/D	18	9	3.400	0.014	0.035	0.41	1.03
Beers E/F	18	9	1.144	0.012	0.018	1.05	1.57
Original gravity, °P							
Beers A/B	18	9	14.540	0.018	0.087	0.12	0.60
Beers C/D	18	9	9.022	0.029	0.076	0.32	0.84
Beers E/F	18	9	3.143	0.027	0.039	0.86	1.24
Distillation method							
Alcohol, % w/w							
Beers A/B	18	9	5.594	0.044	0.099	0.79	1.77
Beers C/D	18	9	3.360	0.020	0.073	0.60	2.17
Beers E/F	18	9	1.120	0.034	0.049	3.04	4.38
Original gravity, °P							
Beers A/B	18	9	14.468	0.094	0.146	0.65	1.01
Beers C/D	18	9	8.933	0.036	0.118	0.40	1.32
Beers E/F	18	9	3.093	0.079	0.096	2.55	3.09

D. Determination

Degas beer samples by **920.49**. Turbid samples must be filtered. Fill sample vials at least 2/3 full and load instrument turntable. Select desired analysis program and process vials. Read calculated values for original gravity and ethanol (% w/w and v/v) from control unit printout.

To maintain instrument in standby mode (>10 min between measurements), place almost full vial of conditioning solution behind sample vials with one empty space between them. When instrument begins to pump conditioning solution, recirculate conditioning solution by placing end of drainage tube in conditioning solution vial. Replace vial with fresh conditioning solution every 1–2 h.

At completion of analysis, pump 1 vial of conditioning solution through instrument then 1 vial of H_2O. When all H_2O has been pumped out, turn off pump, control unit, and then analyzer. Unhook pump tubes.

Ref.: *J. Am. Soc. Brew. Chem.* **47**, 130(1989). *J. AOAC Int.* (1993) **76**, July/August issue.

Recommendation

We recommend that the automated method be adopted first action by AOAC International as an ASBC–AOAC method.

References

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TECHNICAL COMMUNICATIONS

Citrate Insoluble P_2O_5: Problems with Ammoniated Fertilizers

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An AOAC collaborative study of a new direct method for available P_2O_5 in fertilizer was conducted last year. Results on a monoammonium phosphate sample showed more variability than other sample types, and the sample also had the largest absolute difference among 3 methods (960.02, 960.03, and a new direct method). Problems with the AOAC method for citrate insoluble P_2O_5 were discovered, and an editorial change in the method is recommended.

An AOAC collaborative study of a new direct method for available P_2O_5 in fertilizer, conducted last year, generated highly variable results on a monoammonium phosphate sample. Results also showed the largest absolute difference among 3 methods compared in the study.

A brief study by Lynch (1) indicated that the AOAC citrate insoluble P_2O_5 method was sensitive to the oven temperature used during the ignition step of the procedure for monoammonium phosphates. This sensitivity is also noted by the Association of Florida Phosphate Chemists (AFPC) in its method for citrate insoluble P_2O_5 (2). In the AFPC method, a note is included stating that the citrate insoluble P_2O_5 residues from some monoammonium phosphates are resistant to acid attack after ignition at 600°C. For such cases, the AFPC suggests an aqua-regia digestion.

Figures 1a–1g show the effect of ignition temperature on the reported citrate insoluble P_2O_5 for a variety of fertilizer ingredients. All data points represent an average of 10 separate analyses by AOAC method **963.03**. The data indicate that at high ignition temperatures, the citrate insoluble (CI) residues from ammoniated phosphates become acid insoluble. The granular triple super phosphate sample did not show this effect. Of particular interest is Figure 1f, which shows results on a sample of monoammonium phosphate from a 1991 collaborative study on available P_2O_5 (3). The CI P_2O_5 varied by ±0.5% over the ignition temperature range of the study.

These results may help answer questions about the high variability of available P_2O_5 analytical data for this type of sample and why results obtained by the AOAC indirect method are often higher than those obtained by the AOAC direct method. This phenomenon was reported in 1977 by Johnson and Farley (4) in an evaluation of the AOAC methods for the determination of available P_2O_5. Figures also show that this

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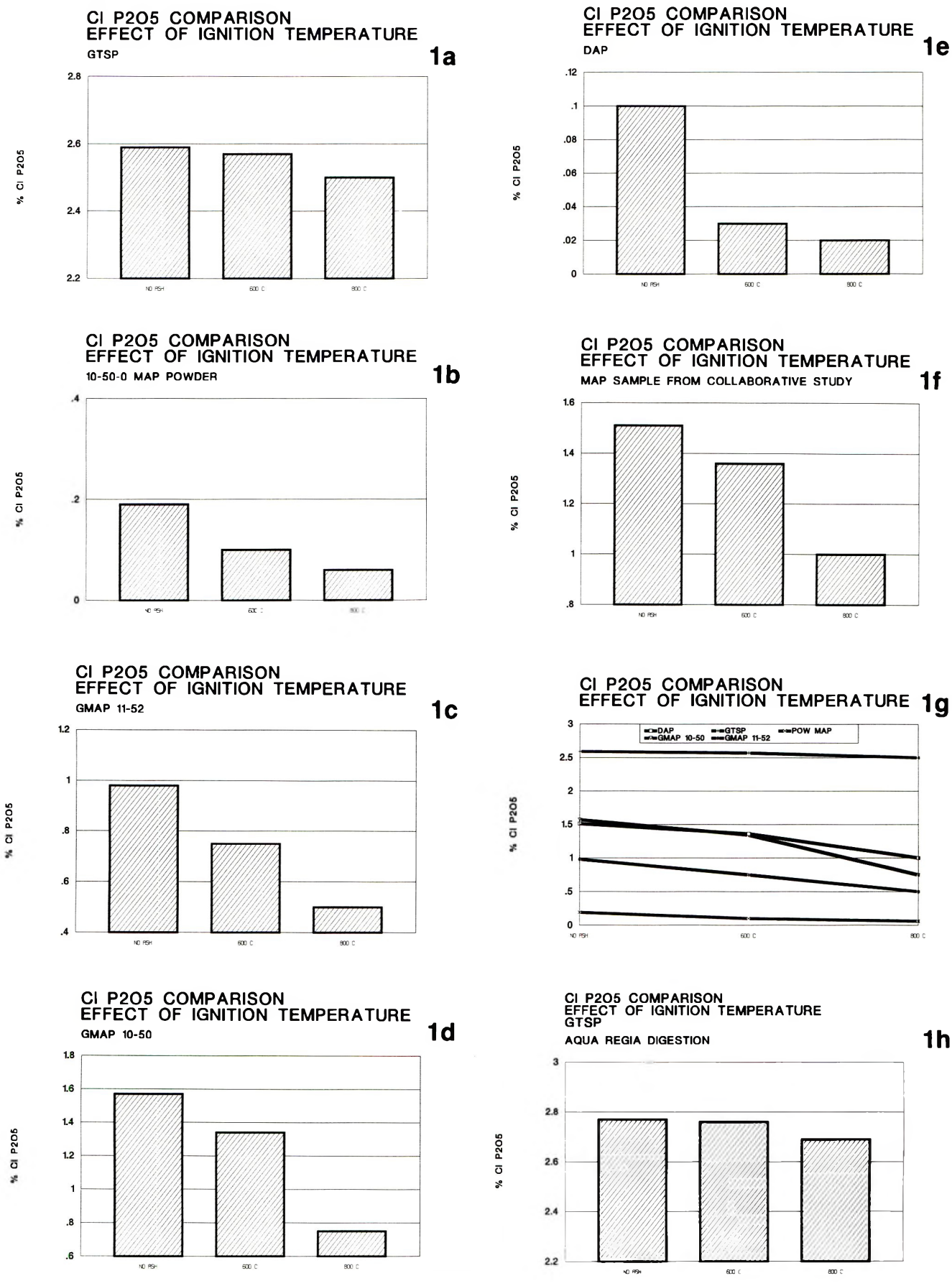
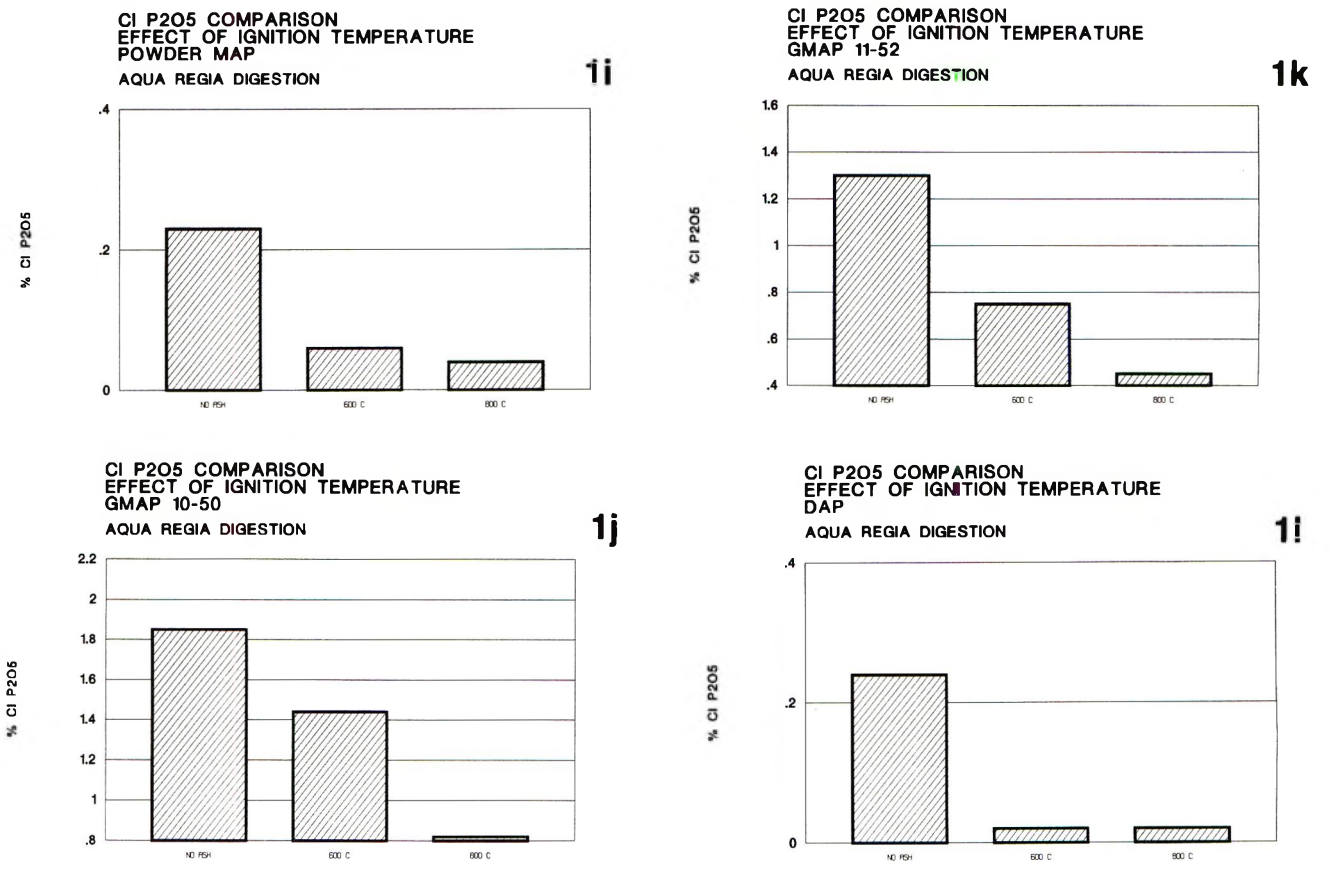


Figures 1a – 1h. Cl P_2O_5 comparison effect of ignition temperature.



Figures 1i – 1l. CI P_2O_5 comparison effect of ignition temperature.

effect can be seen to a lesser degree on samples of diammonium phosphate.

The same samples were run again, this time using the aqua-regia digestion suggested by AFPC method XI 4 and AOAC method **957.02(B)**. Results are shown in Figures 1h–1l. Again, all data points represent an average of 10 separate analyses. Surprisingly, the results were similar when the more rigorous digestion was used. These results indicate that for ammoniated phosphates, an aqua-regia digestion does not hydrolyze the citrate insoluble P_2O_5.

A note should be included in the AOAC method indicating that citrate insoluble residues of ammoniated phosphates become acid insoluble during the ignition step. Further study may prove that an alternative digestion method, such as alkali fusion, will hydrolyze the P_2O_5 after ignition. Until such time, an editorial change is recommended for AOAC method **963.03B(a)** as follows: "For ammoniated fertilizers, use method (2)." This refers to digestion method 2, which avoids an ignition step.

References

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TECHNICAL COMMUNICATIONS

Enzymatic-Gravimetric Determination in Foods of Dietary Fiber as Sum of Insoluble and Soluble Fiber Fractions: Summary of Collaborative Study

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A collaborative study was conducted on an enzymatic-gravimetric method for determination of total dietary fiber in foods, in which soluble fiber and insoluble fiber are determined separately. Ten collaborators analyzed blind duplicate test samples from 5 food products: turnip, wheat bran, beans canned with tomato sauce, rice, and whole wheat bread. Repeatability and reproducibility relative standard deviations ranged from 1.48 to 14.73% and from 4.13 to 17.94%, respectively. The method was adopted first action by AOAC International.

A collaborative study was performed to establish precision of an enzymatic-gravimetric method for determination of total dietary fiber in foods (1). The study was designed according to AOAC guidelines for collaborative studies (2).

The method measures soluble and insoluble fiber separately. To determine soluble fiber, a portion of sample is autoclaved, heat-stable amylase is added, and the mixture is filtered. Amyloglucosidase and protease are added to remove starch and protein, and soluble fiber in the filtrate is precipitated by ethanol and filtered. To determine insoluble fiber, a portion of sample is refluxed with neutral detergent, and the residue is treated with α-amylase from porcine pancreas to remove water-soluble carbohydrates and protein. In each determination, the residue is dried, weighed, ashed, and reweighed. Total dietary fiber is the sum of the 2 residues.

992.16 Total Dietary Fiber Enzymatic-Gravimetric Method

First Action 1992

(Applicable to determination of total dietary fiber in cereals, beans, vegetables, and fruits)

Method Performance (dry wt. basis):

Turnip, 20.71%
$s_r = 1.01$; $s_R = 1.37$; $RSD_r = 4.85\%$; $RSD_R = 6.60\%$

Wheat bran, 46.30%
$s_r = 0.69$; $s_R = 1.91$; $RSD_r = 1.48\%$; $RSD_R = 4.13\%$

Bean, 18.19%
$s_r = 0.90$; $s_R = 2.06$; $RSD_r = 4.93\%$; $RSD_R = 11.30\%$

Rice, 1.21%
$s_r = 0.18$; $s_R = 0.22$; $RSD_r = 14.73\%$; $RSD_R = 17.94\%$

Whole wheat bread, 10.29%
$s_r = 0.74$; $s_R = 0.80$; $RSD_r = 7.18\%$; $RSD_R = 7.81\%$

A. Principle

Food samples, dried and ground, are fat extracted if containing >5% fat. A portion of sample is treated in autoclave with heat-stable amylase, amyloglucosidase, and protease to remove starch and protein. Enzymatically undigested fiber is precipitated by ethanol and filtered. Residue is dried, weighed, ashed, and reweighed. A second portion of sample is refluxed with neutral detergent and treated with α-amylase from porcine pancreas to remove water soluble carbohydrates and protein. Residue is dried, weighed, ashed, and reweighed. Total dietary fiber is calculated as sum of the 2 residues.

B. Apparatus

(a) *Autoclave or pressure cooker.*—Capable of 15 psi.

(b) *Tubes.*—50 mL, heavy duty, with screw caps (Pyrex, Fisher No. 05–558–5B, Fisher Scientific, Pittsburgh, PA 15219, USA, or Corning No. 8422, Corning, Inc., Corning, NY 14831, USA, is suitable).

(c) *Ovens.*— (*1*) Forced draft, capable of maintaining 105 ± 1°. (*2*) Capable of maintaining 55 ± 0.5°.

(d) *Water baths.*—(*1*) Boiling. (*2*) Capable of maintaining 60 ± 0.5°.

(e) *Balance.*—Analytical, sensitive to 0.1 mg.

(f) *Muffle furnace.*—With temperature regulator capable of 525 ± 1° (Fisher Isotemp, Model 497, or Thermoline equipped with Furnatrol controller is suitable).

Submitted for publication November 31, 1992.

The recommendation was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76** Jan/Feb issue.

(**g**) *Neutral detergent fiber extraction system.*—Extraction apparatus with (*1*) condenser to fit 600 mL tall-form beaker without spout, (*2*) hot plate capable of bringing 100 mL neutral detergent to boiling in 5–10 min, and (*3*) filtering device equipped with suitable holder for crucible (Fibertec system 1, Tecator, Fisher No. TC 1010-001 is suitable).

(**h**) *Filtering system.*—Gooch crucible with suitable holder and suction flask (Fibertec-E, with incubation flasks, Tecator, Fisher No. TC-1023-002 is suitable).

(**i**) *Fritted (sintered) glass crucibles.*—(*1*) Gooch type, 50 mL, coarse, ASTM 40–60 μm; or P_2 crucibles 40–90 μm (Tecator No. 1000 1172). (*2*) Gooch type, 50 mL, medium ASTM, 10–15 μm (Fisher No. 08-237-1B) with rubber ring adaptors. Heat 2 h at 525° before use if not used regularly.

(**j**) *Freeze-dryer.*—For drying food samples with minimum heat damage (Virtis freeze mobile, with drying chamber, No. 10-MR-TR, is suitable).

(**k**) *Cutting mill.*—Low-speed rotating blades in chamber bottomed with interchangeable 20 mesh screen (Wiley, intermediate model, Fisher No. 08-338 is suitable). (*Note*: High-speed rotary mills are not suitable, because they produce fine particles that may pass through fritted glass crucible.)

(**l**) *Desiccator.*

C. Reagents

(**a**) *Neutral detergent solution.*—Dissolve 148.5 g sodium lauryl sulfate in 3 L H_2O. Dissolve 92.12 g disodium EDTA and 33.70 g sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) in 1 L H_2O by stirring and heating and add to sodium lauryl sulfate solution. Dissolve 22.57 g sodium phosphate dibasic anhydrous (Na_2HPO_4) in 1 L H_2O by stirring and heating and add to other solution. Mix well. pH must be 6.9–7.1; adjust with NaOH or HCl.

(**b**) *Phosphate buffer.*—0.1M, pH 7.0 ± 0.1. Mix 610 mL 0.1M Na_2HPO_4 with 390 mL 0.1M sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$).

(**c**) *Sodium acetate solution.*—2.0M. Dissolve 164.06 g sodium acetate in H_2O and dilute to 1 L.

(**d**) *Acetate buffer.*—2.0M, pH 4.5 ± 0.1. Mix 200 mL 2.0M sodium acetate, (**c**), with 300 mL 2.0M acetic acid. Adjust pH, if needed, by adding sodium acetate or acetic acid.

(**e**) *Ethanol solution.*—80%. Dilute 800 mL anhydrous ethanol to 1 L with H_2O.

(**f**) *Acetone.*—Glass distilled.

(**g**) *Filter aid.*—Celite (No. C-211, Fisher Scientific). No known suitable substitute. (*Caution*: Celite is a lung, skin, and eye irritant; avoid inhalation and contact with skin and eyes.)

(**h**) *Glass wool.*—Borosilicate fiber glass, 8 μm diameter, free from fluorine, alumina, and heavy metals (Pyrex is suitable).

(**i**) *α-Amylase solution.*—Stir 5.0 g α-amylase, Type VI-B (No. A-3176, Sigma Chemical Co., St. Louis, MO 63178, USA, is suitable source), with 100 mL phosphate buffer, (**b**), 15 min. Centrifuge 10 min at 1500 × *g*, and filter through coarse sintered glass crucible containing glass wool. Prepare daily and store at 4° when not in use.

(**j**) *Amyloglucosidase solution.*—Amyloglucosidase from *Aspergillus niger* (No. A9913, Sigma Chemical Co. is suitable source). Store at 4°.

(**k**) *Protease solution.*—Protease for total dietary fiber assay (No. P3910, Sigma Chemical Co. is suitable source). Store at 4°. Prepare 50 mg/mL H_2O just before use.

(**l**) *Heat-stable amylase.*—α-Amylase for total dietary fiber assay (No. A3306, Sigma Chemical Co. is suitable source). Store at 4°.

(*Caution*: Dried enzymes may cause allergic reaction. Handle in fume hood and avoid inhalation.)

D. Enzyme Suitability Test

Every 3 months or each time enzyme lot changes, verify full enzyme activity and absence of undesirable enzymatic activities by running the standards listed in Table **992.16**.

E. Sample Preparation

Freeze-dry wet samples. Grind samples with cutting mill fitted with 20 mesh screen at bottom of cutting chamber. If fat content is ≥5%, defat dried sample by adding 4 volumes acetone, stirring 1 h at room temperature, filtering on tarred coarse crucible, and evaporating residual acetone 2 h at 55°. Record weight loss due to fat and/or H_2O removal, and make appropriate correction to sample weight in calculation of percent dietary fiber.

F. Fiber Determination

(*1*) Accurately weigh (S_1) duplicate 0.5 g portions of sample to nearest 0.1 mg into 50 mL screw cap tubes. Run duplicate reagent blanks. Add 20 mL H_2O and 2 mL acetate buffer and mix. Autoclave 60 min at 120° and 15 psi (with cap loosened). Decrease autoclave pressure slowly before removing tube from autoclave. Add 0.1 mL heat-stable amylase, mix, and incubate in boiling H_2O bath 30 min. Filter through coarse Gooch (or P_2 with Fibertec) crucible, containing ca 0.5 g Celite, on filtering apparatus equipped with suction flask (or Fibertec E equipped with incubation flask) to receive filtrate. Rinse tube with 10 mL hot H_2O and add rinse to crucible (adding to filtrate). Remove crucible and rinse the filtering device, or Fibertec E tubing, with 5 mL hot (95–100°) H_2O (adding to filtrate). To combined filtrate, add 4 mL sodium acetate solution and mix. Add 0.3 mL amyloglucosidase solution, cover, mix, and incubate 30 min in 60° H_2O bath. Add 0.1 mL protease solution, and continue 60° incubation for 30 min.

Table 992.16. Standards for testing enzyme activity

Standard	Wt of std, g	Activity tested	Expected rec., %
Cornstarch (Sigma S-2388)	0.5	Amylase	0–1
Wheat starch (Sigma S-1514)	0.5	Amylase	0–1
Casein (Sigma C-7906)	0.5	Protease	0–1
β-Glucan (Sigma G-7391)	0.1	β-Glucanase	90–95
Citrus pectin (Sigma P-7536)	0.1	Pectinase	80–85
Arabinogalactan (Sigma A-9788)	0.2	Hemicellulase	95–100

Table 1. Summary of data for collaborative study on determination of total dietary fiber as sum of soluble and insoluble fiber fractions[a]

Material	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %
Turnip	10	20.71	1.01	1.37	4.85	6.60
Wheat bran	10	47.31	1.02	3.70	2.15	7.82
Wheat bran[b]	9	46.30	0.69	1.91	1.48	4.13
Beans with tomato sauce, canned	10	18.19	0.90	2.06	4.93	11.30
Rice	10	1.34	0.31	0.39	22.93	29.23
Rice[c]	8	1.21	0.18	0.22	14.73	17.94
Bread[d]	9	10.58	1.07	1.29	10.13	12.24
Bread[e]	8	10.29	0.74	0.80	7.18	7.81

[a] Samples distributed as blind duplicates: *n* = number of collaborators; mean = material mean (dry weight basis).
[b] Results for collaborator 8 (Grubbs outliers) not included.
[c] Results for collaborator 4 (Cochran outliers) and 5 (Grubbs outliers) not included.
[d] No results reported by collaborator 6.
[e] Results for collaborator 4 (Grubbs outliers) not included.

Add 4 volumes (166 mL) of anhydrous ethanol and mix. Let precipitate form at room temperature ≥60 min. Filter mixture through medium Gooch crucible containing glass wool. Rinse residue successively with two 20 mL portions 80% ethanol and two 20 mL portions of acetone. Discard eluates.

Dry crucible and contents overnight at 105° in forced-draft oven. Cool in desiccator to room temperature and weigh to nearest 0.1 mg (C_{1r}). Ash residue 4 h at 525°. Cool in desiccator to room temperature and weigh to nearest 0.1 mg (C_{1a}).

(*2*) Accurately weigh (S_2) second set of duplicate 0.5 g portions of sample to nearest 0.1 mg into 600 mL tall form beaker or P_2 crucible. Add 100 mL neutral detergent solution to beaker; place on hot plate and fit condenser (or fit P_2 crucible in hot extractor and add 100 mL neutral detergent solution, preheated to 80°, to boiling column). Heat to boiling within 5–10 min, and then reduce heat and reflux 60 min from onset of boiling. Filter through coarse Gooch or P_2 crucible. Wash residue with ca 100 mL hot H_2O. If filtration is difficult, apply any of following procedures to facilitate filtration: (*1*) apply back pressure, (*2*) add 100 µL heat-stable amylase, (*3*) add ca 0.5 g Celite to crucible, (*4*) reduce sample weight to 0.3 g and analyze in triplicate, (*5*) clean crucible, or (*6*) use new crucible.

Add 10 mL cold α-amylase solution and 15 ± 2 mL hot H_2O to crucible and hold 5 min on filtering device or hot extractor without heating. Apply suction to remove enzyme solution and wash residue with ca 20 mL hot H_2O. Stopper bottom of crucible (use No. 8 rubber stopper for Gooch crucible or No. 7 for P_2 crucible) and add 10 mL cold α-amylase solution and 15 ± 2 mL hot H_2O. Incubate 60 min in 55° oven. Filter on filtering device or cold extractor and wash residue successively with ca 100 mL hot H_2O and two 20 mL portions of acetone. Discard eluates.

Dry crucible and contents overnight at 105° in forced-draft oven. Cool in desiccator to room temperature and weigh to nearest 0.1 mg (C_{2r}). Ash residue 4 h at 525°. Cool in desiccator to room temperature and reweigh (C_{2a}).

G. Calculations

Total dietary fiber:

$$TDF, \% = \{[(C_{2r} - C_{2a})/S_2] + [(C_{1r} - C_{1a} - B)/S_1]\} \times 100$$

$$\text{Blank (B)} = C_b - C_a$$

where C_b = wt of crucible with blank; C_a = wt of crucible with blank after ashing; C_{1r} = wt of crucible with residue, **F**(*1*); C_{1a} = wt of crucible with residue after ashing, **F**(*1*); C_{2r} = wt of crucible with residue, **F**(*2*); C_{2a} = wt of crucible with residue after ashing, **F**(*2*); and S_1 and S_2 = wts of dry samples.

Ref.: Cereal Foods World **35**, 319(1990); J. AOAC Int. (1993) **76**, July/August issue.

Results and Recommendation

In the study, 10 collaborators analyzed blind duplicate test samples from 5 products: turnip, wheat bran, beans canned with tomato sauce, rice, and whole wheat bread. The statistical evaluation of the data is summarized in Table 1.

We recommend that the method be adopted first action.

References

(1) Mongeau, R., & Brassard, R. (1990) *Cereal Foods World* **35**, 319–324

(2) "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (1989) *J. Assoc. Off. Anal. Chem.* **72**, 694–704

TECHNICAL COMMUNICATIONS

International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories

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Preface

AOAC International (then the Association of Official Analytical Chemists), the International Organization for Standardization (ISO), and the International Union of Pure and Applied Chemistry (IUPAC) cooperated to produce an agreed "Protocol for the Design, Conduct, and Interpretation of Collaborative Studies" (1). The Working Group that produced this protocol agreed at its April 1989 Washington, DC, meeting to develop a further protocol on proficiency tests, i.e., results generated in interlaboratory test comparisons considered for the purpose of a continuing assessment of the technical competence of participating testing laboratories.

Such a harmonized protocol must outline the minimum requirements for agencies (laboratories or other organizations) that wish to develop and operate proficiency testing schemes, and recommend statistical treatment of the reported data.

A draft harmonized protocol for the organization of proficiency testing schemes was prepared and discussed at the AOAC/ISO/IUPAC meeting on the "Harmonization of Quality Assurance Systems in Chemical Analysis," in Geneva, Switzerland, May 1991, as part of the development process of such a protocol, and finalized at a meeting of the Working Party in Delft, The Netherlands, May 1992.

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International Union of Pure and Applied Chemistry Analytical, Applied, Clinical, Inorganic and Physical Chemistry Divisions Interdivisional Working Party for Harmonisation of Quality Assurance Schemes for Analytical Laboratories. Membership of the Working Party during 1989–1992 was as follows: *Chairman*: M. Parkany; *Members*: P. De Bievre; S.S. Brown; L.E. Coles (deceased); R. Greenhalgh; B. Griepink; A. Head; R.F.M. Herber; W. Horwitz; S.H.H. Olrichs; G. Svehla; M. Thompson; R. Wood.

1. Introduction

For a laboratory to produce consistently reliable data, it must implement an appropriate program of quality assurance procedures.

Analytical methods must be validated as fit for their purpose before use in the laboratory. If possible, validation should be achieved by means of collaborative trials that conform to a recognized protocol (1). These methods must be fully documented, laboratory staff must be trained in their use, and control charts should be established to ensure that the procedures are under statistical control. If possible, all reported data should be traceable to reliable and well-documented reference materials, preferably certified reference materials. When certified reference materials are not available, traceability should be established to a definitive method. Accreditation of the laboratory by the appropriate national accreditation scheme, which should in turn conform to accepted standards (2), indicates that the laboratory is applying sound quality assurance principles. ISO Guide 25(3) describes the general guidelines for assessing a testing laboratory's technical competence. Although proficiency tests can be executed independently, accreditation assessments now use the information produced by proficiency tests (3).

Participation in proficiency testing schemes provides laboratories with an objective means of assessing and demonstrating the reliability of the data they are producing. Although there are several types of proficiency testing schemes, as described in ISO Guide 43(4), they all share a common feature: test results obtained by one testing laboratory are compared with those obtained by one or more other testing laboratories. Schemes may be "open" to any laboratory, or participation may be by invitation only. Schemes may be designed to assess the competence of laboratories undertaking a specific analysis in a specific matrix (i.e., lead in blood, fat in bonemeal) rather than a general type of analysis (i.e., food).

Although various protocols for the design and operation of proficiency testing schemes have been produced to cover particular areas of analytical chemistry, a harmonized protocol that would be universally acceptable is needed for the organization of proficiency testing schemes. The harmonized protocol described in Section 3 contains specific details and does not, therefore, coincide with ISO Guide 43(4). In addition to describing the organization and operation of the practical aspects of proficiency testing schemes, the document prescribes a minimal statistical treatment of the analytical data produced, which are primarily measurements of concentration.

Although various terms may be used to describe a scheme conforming to this protocol (e.g., external quality assessment, performance schemes, etc.), the preferred term is "proficiency test."

For any particular scheme, the aims must be carefully described by the coordinating organization. In addition, the procedure outlined below should be regarded as the minimum that should be undertaken.

Schemes cannot cover all aspects of some areas of activity and must, therefore, be regarded as *representative* of the particular sector of interest.

2. Definitions and Terminology Used in Protocol

2.1 Proficiency Testing Scheme

Methods of checking laboratory testing performance by means of interlaboratory tests.

(To determine trueness, a laboratory's results are compared at intervals with those of other laboratories (5)).

2.2 Internal Quality Control (IQC)

The set of procedures undertaken by the laboratory for continuous monitoring of operations and results. IQC determines whether the results are reliable enough to be released by monitoring the batchwise accuracy of results on quality control materials and precision on independent replicate analysis of test materials.

2.3 Quality Assurance Program/System

The sum total of a laboratory's activities aimed at achieving the required standard of analysis. Although IQC and proficiency tests are very important, a quality assurance program must also include staff training, administrative procedures, management structure, auditing, etc. Accreditation bodies judge laboratories based on their quality assurance program.

2.4 Testing Laboratory

A laboratory that measures, examines, tests, calibrates, or otherwise determines the characteristics or performance of materials or products.

2.5 Reference Material (RM)

A material or substance one or more of whose properties values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials (6).

Further information on reference materials is available in ISO REMCO documentation (7).

2.6 Certified Reference Material (CRM)

A reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence (6).

Further information on certified reference materials is available in ISO REMCO documentation (7).

2.7 True Value

The actual concentration of the analyte in the matrix.

2.8 Assigned Value

The value to be used as the true value by the proficiency testing coordinator in the statistical treatment of results and the best available estimate of the true value of the analyte in the matrix.

2.9 Target Value for Standard Deviation

A numerical value for the standard deviation of a measurement result, which has been designated as a goal for measurement quality.

2.10 Interlaboratory Test Comparisons

Organization, performance, and evaluation of tests on the same items or materials on identical portions of an effectively homogeneous material by 2 or more different laboratories in accordance with predetermined conditions.

2.11 Coordinator

The organization with responsibility for coordinating all of the activities involved in the operation of a proficiency testing scheme.

2.12 Accuracy

The closeness of agreement between a test result and an accepted reference value.

Note: The term accuracy, when applied to a set of test results, describes a combination of random components and a common systematic error or bias component.

2.13 Trueness

The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

Note: The measure of trueness is usually expressed in terms of bias.

2.14 Bias

The difference between the expectation of the test results and an accepted reference value.

Note: Bias is a systematic error as contrasted to random error. One or more systematic error components may be contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value.

2.15 Laboratory Bias

The difference between the expectation of the test results obtained from a particular laboratory and an accepted reference value.

2.16 Bias of the Measurement Method

The difference between the expectation of the test results obtained from all laboratories using that method and an accepted reference value.

Note: The bias of a measurement method is negative, for example, if a method that purports to measure the sulfur content of a compound consistently fails to extract all the sulfur. The bias of a measurement method is measured by the displacement of the average of results from a large number of different laboratories all using the same method. The bias of a measurement method may be different at different analyte concentrations.

2.17 Laboratory Component of Bias

The difference between the laboratory bias and the bias of the measurement method.

Notes: (*1*) The laboratory component of bias is specific to a given laboratory and the conditions of measurement within the laboratory and may also be different at different analyte concentrations. (2) The laboratory component of bias is relative to the overall average result, not the true or reference value.

2.18 Precision

The closeness of agreement between independent test results obtained under prescribed conditions.

Notes: (*1*) Precision depends only on the distribution of random errors and does not relate to the accepted reference value. (*2*) The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Higher imprecision is reflected by a larger standard deviation. (*3*) *Independent test results* are defined as results obtained in a manner not influenced by any previous result on the same or similar material.

3. Organization (Protocol) of Proficiency Testing Schemes

3.1 Framework

Test samples must be distributed on a regular basis to the participants who are required to return results within a given time. The results will be subject to statistical analysis by the coordinator, and participants will be promptly notified of their performance. Advice will be available to poor performers, and all participants will be kept fully informed of the progress of the scheme. Participants will be identified in reports by code only.

The structure of the scheme for any one analyte or round in a series should be as follows:

(*1*) Coordinator organizes preparation, homogeneity testing, and validation of test material; (*2*) coordinator distributes test samples on a regular schedule; (*3*) participants analyze test portions and report results centrally; (*4*) results are subjected to statistical analysis, and performance of laboratories is assessed; (*5*) participants are notified of their performance; (*6*) advice is available for poor performers, on request; (*7*) coordinator reviews performance of scheme; (*8*) next round commences.

Preparation for the next round of the scheme **may** have to be organized while the current round is taking place; details of the next round may have to be adjusted in the light of experience from the current round.

3.2 Organization

The coordinator will be responsible for the day-to-day operation of the scheme, and they must document all practices and procedures in a quality manual (see Appendix I). Test materials will be prepared either by contract laboratories or by the coordinator. The laboratory that prepares the test material should have demonstrable experience in the area of analysis being tested. The coordinator must retain control over the assessment of performance to help maintain the credibility of the scheme. Overall direction of the scheme should be overseen by a small advisory panel with representatives (who should be practicing laboratory scientists) from, for example, the coordinator, contract laboratories (if any), appropriate professional bodies, participants, and end-users of analytical data.

3.3 Test Materials

The test materials distributed in the scheme must be generally similar in type to the materials that are routinely analyzed (in respect of the composition of the matrix and the concentration range or quantity of the analyte). The homogeneity and stability of the test materials must be acceptable. The assigned value will not be disclosed to the participants until after the results have been collated.

The bulk material prepared for the proficiency test must be sufficiently homogeneous for each analyte so that all laboratories will receive test samples that do not differ significantly in analyte concentration. The coordinator must clearly state the procedure used to establish the homogeneity of the test material (see Appendix II). As a guide, the between-sample standard deviation should be less than 0.3 times the target value for the standard deviation.

If possible, the coordinating laboratory should also provide evidence that the test material will be stable throughout the duration of the proficiency tests. Prior to distribution of the test samples, therefore, the matrix must be stored for an appropriate period of time, and the stability of the matrix and its analytes must be determined. During the stability trials, storage conditions, especially time and temperature, must represent those conditions likely to be encountered during the entire proficiency test. Stability trials must, therefore, take account of the transport of the test samples to participating laboratories as well as the conditions encountered purely in a laboratory environment. The concentrations of the various analytes must show no significant changes during the stability tests. The magnitude of a "significant change" would be assessed from the knowledge of the variance expected for replicate analyses of the bulk material. When unstable analytes are to be assessed, the coordinating organization may need to prescribe a date by which time the analysis must be accomplished.

Ideally, the quality checks on the samples referred to above should not be performed by the same laboratory that prepared the sample, although we recognize that this may cause difficulties to the coordinating organization.

The number of test materials to be distributed per round will depend mainly on the range of composition that needs to be covered. Practical considerations will dictate an upper limit of 6 test materials per analyte.

Coordinators should consider any hazards that the test materials might pose and act appropriately to advise any party that might be at risk (e.g., test material distributors, testing laboratories, etc.) of the potential hazard involved.

3.4 Frequency of Test Sample Distribution

The appropriate frequency for the distribution of test samples in any one series depends upon a number of factors, the most important of which are as follows:

(*1*) The difficulty of executing effective analytical quality control; (*2*) the laboratory throughput of test samples; (*3*) the consistency of the results from previous rounds; (4) the cost/benefit of the scheme; (*5*) the availability of suitable material for proficiency testing schemes.

In practice, the frequency will probably fall between once every 2 weeks and once every 4 months.

A frequency greater than once every 2 weeks could lead to problems in the turnaround time of test samples and results. It might also encourage the belief that the proficiency testing scheme can be used as a substitute for internal quality control, an idea that should definitely be discouraged. If the period between distributions extends much beyond 4 months, the delays in identifying and correcting analytical problems will be unacceptable, meaningful trends in a laboratory's performance could be difficult to monitor, and the impact of the scheme on the participants could be small.

When the above factors are considered, a longer time scale between distribution of test samples may be acceptable under certain circumstances. The Advisory Panel should comment on the frequency of distribution appropriate for a particular scheme.

In addition, this panel should also advise on the areas to be covered in any particular sector of analytical chemistry. Establishing priorities is particularly difficult when the sector has a considerable number of diverse analyses.

3.5 Establishing the Assigned Value

The coordinator should explain how the assigned value was obtained where possible with a statement of its traceability and uncertainty.

The assigned value for the concentration of analyte and its uncertainty in a test material can be established by using a number of possible approaches, but only 4 are normally considered.

3.5.1 Consensus Value From Expert Laboratories

This value is the consensus of a group of expert laboratories that achieve agreement by the careful execution of recognized reference methods; it is the best procedure in most circumstances for determining the assigned value in representative materials. When such a value is used, the organizing body should disclose the identities of the laboratories producing the individual results, the method of calculating the consensus value and, if possible, a statement of the traceability and of its uncertainty. The consensus value will normally be a robust mean (8) or the mode.

3.5.2 Formulation

This method comprises the addition of a known amount or concentration of analyte to a base material containing none. The method is especially valuable when it is the amount of analyte added to individual test portions that is subject to testing, as there is no requirement for ensuring a sufficiently homogenous mixture in the bulk test material. In other circumstances, problems might arise with the use of formulation, as follows:

(*1*) The base material must be effectively free from analyte, and the residual analyte concentration must be accurately known; (*2*) the analyte may be difficult to homogenize into the base material when this is required; (*3*) the added analyte may be more loosely bonded than, or in a different chemical form from, that found in the typical materials that the test materials represent.

Unless these problems can be overcome, representative materials (containing the analyte in its normally occurring form in a typical matrix) are usually preferable. When formulation is used, traceability to certified reference materials or reference methods should be cited, if possible.

3.5.3 Direct Comparison with Certified Reference Materials

In this method, the test material is analyzed along with appropriate certified reference materials by a suitable method under repeatability conditions. In effect, the method is calibrated with the CRMs, providing direct traceability and an uncertainty for the value assigned to the test material. The CRMs must have both the appropriate matrix and an analyte concentration range that spans, or is close to, that of the test material. In some areas, the lack of CRMs will restrict the use of this method.

3.5.4 Consensus of Participants

A value often advocated for the assigned value is the consensus (usually a robust mean or the mode) of the results of all of the participants in the round of the test. This value is clearly the cheapest and easiest to obtain. The method usually gives a serviceable value when the analysis is regarded as easy, for example, when a recognized method is applied to a major constituent. In an empirical method (when the method "defines" the content of the analyte), the consensus of a large number of laboratories can be safely regarded as the true value.

There are a number of drawbacks to the consensus of participants. At a fundamental level, a traceability or an uncertainty is difficult to attribute to such a value, unless all participants were using the same reference method. Other objections that can be leveled against the consensus are as follows:

(*1*) There may be no real consensus among the participants and (*2*) the consensus may be biased by the general use of faulty methodology. Neither of these conditions is rare in the determination of trace constituents.

3.5.5 Choice Between Methods

The choice between these methods of evaluating the assigned value depends on circumstances and is the responsibility of the organizing agency. It is usually advisable to have an estimate in addition to the consensus of participants. Any significant deviations observed between the estimates must be carefully considered by the technical panel.

Empirical methods are used when the analyte is ill-defined chemically. In an empirical method, e.g., the determination of "fat," the true result (within the limits of measurement uncertainty) is produced by a correct execution of the method. In these circumstances, the analyte content is clearly defined only if the method is simultaneously specified. Empirical methods can give rise to special problems in proficiency trials when a choice of such methods is available. If the assigned value is obtained from expert laboratories and the participants use a different empirical method, a bias may be apparent in the results even when no fault in execution is present. Likewise, if participants are free to choose between empirical methods, no valid consensus may be evident among them. Several recourses are available to overcome this problem:

(*1*) a separate value of the assigned value is produced for each empirical method used; (*2*) participants are instructed to use a prescribed method; or (*3*) participants are warned that a bias may result from using an empirical method different from that used to obtain the consensus.

3.6 Choice of Analytical Method

Participants will be able to use the analytical method of their choice except when otherwise instructed to adopt a specified method. Methods used must be validated by an appropriate means, e.g., collaborative trial, reference method, etc. As a general principle, procedures used by laboratories participating in proficiency testing schemes should simulate those used in their routine analytical work.

When an empirical method is used, the assigned value will be calculated from results obtained using that defined procedure. If participants use a method not equivalent to the defining method, then an automatic bias in result must be expected when their performance is assessed (see Section 3.5.5).

3.7 Assessment of Performance

Laboratories will be assessed on the difference between their result and the assigned value. A performance score will be calculated for each laboratory, using the statistical scheme detailed in Section 4.

3.8 Performance Criteria

For each analyte in a round, a criterion for the performance score may be set, when appropriate, against which the performance score obtained by a laboratory can be judged. A "running score" could be calculated to give an assessment of performance spread over a longer period of time; this score would be based on results for several rounds.

The performance criterion will be set so that the analytical data routinely produced by the laboratory is adequate for its intended purpose. The performance criterion may not need to be set at the highest level that the method is capable of providing.

3.9 Reporting of Results

Reports issued to participants should be clear and comprehensive and include data on the distribution of results from all laboratories together with participant's performance score. The test results used by the coordinator should also be displayed to let participants check that their data have been correctly entered. Reports should be made available as quickly as possible after the return of results to the coordinating laboratory and, if at all possible, before the next distribution of samples.

Although ideally all results should be reported to participants, this procedure may not be possible in some very extensive schemes (e.g., when there are 700 participants each determining 20 analytes in any one round). Participants should, however, receive at least (*1*) reports in clear and simple format, and (*2*) results of all laboratories in graphical, e.g., histogram form.

3.10 Liaison with Participants

Participants should be provided with a detailed information pack on joining the scheme. Communication with participants should be via a newsletter or annual report together with a periodic open meeting; participants should be advised imme-

diately of any changes in scheme design or operation. Advice should be available to poor performers. Participants who consider that their performance assessment is in error must be able to refer the matter to the coordinator.

Feedback from laboratories should be encouraged so that participants actively contribute to the development of the scheme. Participants should view it as *their* scheme rather than one imposed by a distant bureaucracy.

3.11 Collusion and Falsification of Results

Although proficiency testing schemes are intended primarily to help participants improve their analytical performance, some participants may provide a falsely optimistic impression of their capabilities. For example, collusion may take place between laboratories so that truly independent data are not submitted. Laboratories may also give a false impression of their performance if they routinely carry out single analyses, but report the mean of replicate determinations on the proficiency testing samples. Proficiency testing schemes should be designed to ensure that little collusion and falsification is possible. For example, alternative samples could be distributed within one round, with no identifiable reuse of the materials in succeeding rounds. Also, instructions to participants should make it clear that collusion is contrary to professional scientific conduct and serves only to nullify the benefits of proficiency tests to customers, accreditation bodies, and analysts alike.

Although all reasonable measures should be taken by the coordinators to prevent collusion, the participating laboratories are ultimately responsible.

3.12 Repeatability

Procedures used by laboratories participating in proficiency testing schemes should simulate those used in routine sample analysis. Thus, duplicate determinations on proficiency testing samples should be carried out only if this is the norm for routine work. The result should be reported in the same form (e.g., number of significant figures) as that normally reported to the customer. Some proficiency testing coordinators like to include duplication in the tests to obtain a measure of repeatability proficiency. Duplication should be allowed as a possibility in proficiency tests but is not a requirement of this protocol.

4. Generalized Statistical Procedure for the Analysis of Results

The approach described here is intended to provide a transparent procedure by using accepted statistics without any arbitrary scaling factors.

4.1 Estimates of Assigned Value

The first stage in producing a score from a result x (a single measurement of analyte concentration (or amount) in a test material) is obtaining an estimate of the bias, which is defined as the following:

bias estimate = $x - X$

where X is the true value. In practice the assigned value, $\hat{X}$, which is the best estimate of X, is used. Several methods are available for obtaining the assigned value (see Section 3.5). If x is not a concentration measure, a preliminary transformation may be appropriate.

4.2 Formation of a z-Score

Most proficiency testing schemes proceed by comparing the bias estimate (as defined above) with a target value for standard deviation that forms the criterion of performance. An obvious approach is to form the z-score given by

$$z = (x - \hat{X})/\sigma$$

where σ is the target value for standard deviation.

Although z has the form of a normal standard deviate, there is no presumption that this necessarily will be the case. In some circumstances the technical panel may decide to use an estimate of the actual variation ($\tilde{s}$) encountered in a particular round of a trial in place of a target standard deviation. In that case $\tilde{s}$ should be estimated from the laboratories' results after outlier elimination, or by robust methods (8) for each analyte/material/round combination. A value of $\tilde{s}$ will thus vary from round to round. In consequence, the z-score for a laboratory could not be compared directly from round to round. However, the bias estimate ($x - \hat{X}$) for a single analyte/material combination could be usefully compared round by round for a laboratory, and the corresponding value of $\tilde{s}$ would indicate general improvement in "reproducibility" round by round.

A fixed value for σ is preferable and has the advantage that the z-scores derived from it can be compared from round to round to demonstrate general trends for a laboratory or a group of laboratories. It is suggested that whatever value of σ is chosen is practical and that it is accepted by participants. For some of the tests it is only necessary that the value chosen is sufficient clearly to discriminate in a pass/fail situation.

The value chosen can be arrived at in several ways:

4.2.1 By Perception

The value of σ could be fixed arbitrarily, with a value based on a perception of how laboratories perform. The problem with this criterion is that both perceptions and laboratory performance may change with time. The value of σ, therefore, may need to be changed occasionally, disturbing the continuity of the scoring scheme. However, there is some evidence that laboratory performance responds favorably to a stepwise increase in performance requirements.

4.2.2 By Prescription

The value of σ could be an estimate of the precision required for a specific task of data interpretation. This criterion is the most satisfactory type, if it can be formulated, because it relates directly to the required information content of the data. Unless the concentration range is very small, σ should be specified as a function of concentration.

This is frequently used in legislation when method performance characteristics may be specified.

4.2.3 By Reference to Validated Methodology

When a standard method is prescribed for the analysis, σ could be obtained by interpolation from the standard deviation of reproducibility obtained during appropriate collaborative trials.

4.2.4 By Reference to a Generalized Model

The value of σ could be derived from a general model of precision, such as the "Horwitz Curve" (9). Although this model provides a general picture of reproducibility, substantial deviation from it may be experienced for particular methods. It could be used if no specific information is available.

4.3 Interpretation of z-Scores

If $\hat{X}$ and σ were good estimates of the population mean and standard deviation, and the underlying distribution were normal, then z would be approximately normally distributed with a mean of zero and a unit standard deviation. An analytical system can be described as "well behaved" when it complies with these conditions. Under these circumstances an absolute value of z ($|z|$) greater than 3 suggests poor performance.

Because z is standardized, it can be usefully compared among all analytes, test materials, and analytical methods. Values of z obtained from diverse materials and concentration ranges can, therefore, *with due caution* (see Section 4.5), be combined to give a composite score for a laboratory in one round of a proficiency test. Moreover the meaning of z-scores can be immediately appreciated, i.e., values of $|z| < 2$ would be very common and values of $|z| > 3$ would be very rare in well-behaved systems.

Schemes explicitly based on the z-score method include the "Laboratory Accreditation and Audit Protocol" (10). The z-score method is also implicit in the modified "variance index" method of Whitehead et. al (11), when scaling to a "chosen coefficient of variation" (i.e., relative standard deviation) effectively gives a z-value multiplied by an arbitrary factor.

4.4 Alternative Score

An alternative type of scoring, Q-scoring, is based not on the standardized value but on the relative bias, namely

$$Q = (x - \hat{X})/\hat{X}$$

where x and $\hat{X}$ have their previous meaning. Although not recommended in this protocol, a number of sectors, e.g., occupational hygiene, use this approach. The scoring does have the disadvantage that the significance of any result is not immediately apparent. The alternative type of scoring is described in greater detail in Appendix V.

4.5 Combination of Results of a Laboratory Within a Round of the Trial

It is common for several different analyses to be required within each round of a proficiency test. Although each individual test furnishes useful information, many participants want a single figure of merit that will summarize the overall performance of the laboratory within a round. This approach may be appropriate for the assessment of long-term trends. However, there is a danger that such a combination score will be misinterpreted or abused by nonexperts, especially outside the context of the individual scores. Therefore, the general use of combination scores is not recommended, but it is recognized that they may have specific applications if based on sound statistical principles and used with due caution.

It is especially emphasized that there are limitations and weaknesses in any scheme that combines z-scores from dissimilar analyses. If a single score out of several produced by a laboratory were outlying, the combined score may well be not outlying. In some respects this feature is useful, in that a lapse in a single analysis is downweighted in the combined score. However, there is a danger that a laboratory may be consistently at fault only in a particular analysis and frequently report an unacceptable value for that analysis in successive rounds of the trial. This factor may well be obscured by the combination of scores. See Appendix III.

4.6 Running Scores

Although the combination scores discussed above and detailed in Appendix IV give a numerical account of the performance of a laboratory in a single round of the proficiency test, for some purposes it may be useful to have a more general indicator of the performance of a laboratory over time.

Although the value of such indicators is questionable, they can be constructed simply and give a smoothed impression of the scores over several rounds of the test.

Some procedures that may be used are described in Appendix IV. It must be stressed that, as with combination scores (see Section 4.5), it is difficult to produce running scores that are not prone to misinterpretation, etc.

4.7 Classification, Ranking, and Other Assessment of Proficiency Data

Classification is not the primary aim of proficiency tests. However, it is possible that accreditation agencies will use proficiency testing results for this purpose, so it is essential that any classification used should be statistically well founded.

4.7.1 Classification

If the frequency distribution of a proficiency score is known or presumed, then significance can be attributed to results according to the quantiles of that distribution. In a well-behaved analytical system z-scores would be expected to fall outside the range $-2 < z < 2$ in about 5% of instances, and outside the range $-3 < z < 3$ only in about 0.3%. In the latter case it could be interpreted that the probability is so small for a "well-behaved" system, that it almost certainly represents a poor performance. It would, therefore, be possible to classify scores as the following:

$|z| \leq 2$, Satisfactory

$2 < |z| < 3$, Questionable

$|z| \geq 3$, Unsatisfactory

z-scores are broadly comparable, but the use of any classification must, in practice, be treated with care as the knowledge of the relevant probabilities rests on assumptions that may not be fulfilled: (*1*) that the appropriate values of $\hat{X}$ and σ have been used; and (*2*) that the underlying distribution of analytical errors is normal, apart from outliers. In addition, the division of a continuous measure into a few named classes has little to commend it from the scientific point of view, although it may have a psychological effect on the participants. Consequently, classification is not recommended in proficiency tests. "Decision limits" based on z-scores may be used as an alternative when necessary.

4.7.2 Ranking

Laboratories participating in a round of a proficiency trial are sometimes ranked on their combined score for the round or on a running score. Such a ranked list is used for encouraging better performance in poorly ranked laboratories by providing a comparison among the participants. However, ranking is not recommended as it is an inefficient use of the information available and may be open to misinterpretation. A histogram is a more effective method of presenting the same data.

5. Outline Example of How Assigned Values and Target Values may be Specified and Used

See Appendix VI.

6. References

The references cited throughout this document and its appendices are given below. Additional references are given in ISO Guide 43.

(1) Horwitz, W. (1988) *Pure Appl. Chem.* **60**, 855–864

(2) *Testing Laboratory Accreditation Systems: General Recommendations for the Acceptance of Accreditation Bodies*, ISO Guide 54 (1988) International Organization of Standardization, Geneva, Switzerland

(3) *General Requirements for the Competence of Calibration and Testing Laboratories*, ISO Guide 25, 3rd Ed. (1990) International Organization of Standardization, Geneva, Switzerland

(4) *Development and Operation of Laboratory Proficiency Testing*, ISO Guide 43 (1984) International Organization of Standardization, Geneva, Switzerland

(5) *Accuracy (Trueness and Precision) of Measurement Methods and Results, Part 1: General Principles and Definitions*, ISO DIS-5725-1, International Organization of Standardization, Geneva, Switzerland

(6) *Terms and Definitions used in Connection with Reference Materials*, ISO Guide 30 (1981) International Organization of Standardization, Geneva, Switzerland

(7) *Uses of Certified Reference Materials*, ISO Guide 33 (1989) International Organization of Standardization, Geneva, Switzerland

(8) Analytical Methods Committee Report (1989) *Analyst* **114**, 1693–1697

(9) Horwitz, W. (1982) *Anal. Chem.* **54**, 67A–76A

(10) *Laboratory Accreditation and Audit Protocol* (1986) Food Inspection Directorate, Food Production and Inspection Branch, Agriculture Canada, April 1986

(11) Whitehead, T.P., Browning, D.M., & Gregory, A. (1973) *J. Clin. Pathol.* **26**, 435–445

Appendices

Appendix I.

Suggested Headings in a Quality Manual for Organization of Proficiency Testing Schemes (Not Necessarily in This Order)

1. Quality policy
2. Organization of agency
3. Staff, including responsibilities
4. Documentation control
5. Audit and review procedures
6. Aims, scope, statistical design and format (including frequency) of proficiency testing programs
7. Procedures
   - — sample preparation
   - — testing of sample homogeneity
   - — equipment
   - — suppliers
   - — logistics (e.g., sample mailing)
   - — analysis of data
8. Preparation and issuing of report
9. Action and feedback by participants when required
10. Documentation of records for each program
11. Complaints handling procedures
12. Policies on confidentiality and ethical considerations
13. Computing information, including maintenance of hardware and software
14. Safety and other environmental factors
15. Sub-contracting
16. Fees for participation
17. Scope of availability of program to others

Appendix II.

Recommended Procedure for Testing Material for Sufficient Homogeneity

The procedure to be followed by the laboratory preparing proficiency testing materials is as follows:

1. Use an appropriate method to homogenize the whole of the bulk material.
2. Divide the material into the containers that will be mailed to the participants.
3. Randomly select a minimum (n) of 10 containers.
4. Separately homogenize the contents of each of the n selected containers and take 2 test portions.
5. Use an appropriate method to analyze the $2n$ test portions in a random order under repeatability conditions. The analytical method used must be sufficiently precise to satisfactorily estimate s_s.
6. Estimate the sampling variance (s_s^2) and analytical variance (s_a^2) by using one-way analysis of variance, without exclusion of outliers.
7. Report values of $\bar{x}$, s_s, s_a, n, and the result of the F-test.
8. If σ is the target value for standard deviation for the proficiency tests at analyte concentration = $\bar{x}$, the value of s_s/σ should be less than 0.3 for sufficient homogeneity.

As an example we take the data in Table A.

Table A. Copper in soybean flour ($\mu g\ g^{-1}$)

	Copper content	
Sample No.	1	2
1	10.5	10.4
2	9.6	9.5
3	10.4	9.9
4	9.5	9.9
5	10.0	9.7
6	9.6	10.1
7	9.8	10.4
8	9.8	10.2
9	10.8	10.7
10	10.2	10.0
11	9.8	9.5
12	10.2	10.0
Grand mean		10.02

Analysis of variance gives the following results (Table B).

Table B. Analysis of variance

Source of variation	df	Sum of squares	Mean square	F
Between samples	11	2.54458	0.231326	3.78
Analytical	12	0.735000	0.06125	

Critical value of F ($p = 0.05, \nu_1 = 11, \nu_2 = 12$) is $2.72 < 3.78$

There are significant differences between samples

$s_a = \sqrt{0.0613} = 0.25$

$s_s = [(0.2313 - 0.0613)/2]^{1/2} = 0.29$

$\sigma = 1.1$ (This is an example value of a target value for reference standard deviation and is not derived from the data)

$s_s/\sigma = 0.29 / 1.1 = 0.26 < 0.3$

Although there are significant differences between samples (F – test), the material is sufficiently homogeneous for the purpose of the proficiency trial, as $s_s/\sigma = 0.26$ is less than the maximum recommended value of 0.3.

Appendix III.

Combination of Results of a Laboratory Within One Round of a Trial

The general use of combination scores is not recommended, but it is recognized that they may have specific applications if used with due caution.

1. Introduction

   Several methods by which independent z-scores produced by a laboratory can be combined in one round of the test seem potentially appropriate: (*1*) the sum of scores, $SZ = \Sigma z$; (*2*) the sum of squared scores, $SSZ = \Sigma z^2$; and (*3*) the sum of absolute values of the scores, $SAZ = \Sigma |z|$.

   These statistics fall into 2 classes. The first class (containing only SZ) uses information about the signs of the z-scores while the alternative class (SSZ and SAZ) provides information only about the size of scores, i.e., the magnitude of biases. Of the latter, the sum of the squares is more tractable mathematically and is, therefore, the preferred statistic although

rather sensitive to single outliers. SAZ may be especially useful if outliers are extreme or many laboratories are outliers, but because its distribution is complicated, it is not recommended.

2. Sum of Scores, SZ

The distribution of SZ is zero-centered with variance m, where m is the number of scores being combined. Thus SZ could not be interpreted on the same scale as the z-scores. However, a simple scaling restores the unit variance, giving a rescaled sum of scores $RSZ = \Sigma z/\sqrt{m}$ and harmonizing the scaling. In other words, both z and RSZ can be interpreted as standard normal deviates.

SZ and RSZ have the advantage of using the information in the signs of the biases. Thus, if a set of z-scores were 1.5, 1.5, 1.5, 1.5, the individual results would be regarded as nonsignificant positive scores. However, regarded as a group, the joint probability of observing 4 such deviations together would be small. This finding is reflected in the RSZ value of 3.0, which indicates a significant event. This information would be useful in detecting a small consistent bias in an analytical system but would not be useful in combining results from several different systems, when a consistent bias would not be expected and is unlikely to be meaningful.

Another feature of the RSZ is the tendency for errors of opposite sign to cancel. In a well-behaved situation (i.e., when the laboratory is performing without bias according to the designated σ value), this causes no problems. If the laboratory were badly behaved, however, the possibility arises of the fortuitous cancellation of significantly large z values. Such an occurrence would be very rare by chance.

These restrictions on the use of RSZ serve to emphasize the problems of using combination scores derived from various analytical tests. When such a score is used, it should be considered simultaneously with the individual scores.

3. Sum of Squared Scores, SSZ

This combination score has a chi-squared (χ^2) distribution with m degrees of freedom for a well-behaved laboratory. Hence, the score cannot be simply interpreted on a common scale with the z-scores. However, the quantiles of the χ^2 distribution can be found in most compilations of statistical tables.

SSZ takes no account of the signs of the z-values because of the squared terms. Thus, in the example considered previously, when the z-scores are 1.5, 1.5, 1.5, 1.5, we find SSZ = 9.0, a value that is not significant at the 5% level and does not draw enough attention to the unusual nature of the results as a group. However, in proficiency tests, we are concerned much more with the magnitude of deviations than with their direction, so SSZ seems appropriate for this use. Moreover, the problem of chance cancellation of significant z-scores of opposite sign is eliminated. Thus, the SSZ has advantages as a combination score for diverse analytical tests and is to an extent complementary to RSZ. A related score, SSZ/m, is used in the "Laboratory Audit and Accreditation Scheme."

Appendix IV.

Calculation of Running Scores

The general use of running scores is not recommended, but they may have specific applications if used with due caution. Running scores are usually calculated by forming a "moving window" average. The procedure can be applied to a z or a combination score.

For example, a running score covering the current (n-th) round and the previous k rounds could be constructed as follows:

$$RZ_n = \sum_{j=n-k}^{n} z_j \;/\; (k+1)$$

where z_j is the z-score for the material in the j–th round.

The running score may have an advantage in that instances of poor performance restricted to one round are smoothed out somewhat, allowing an overall appraisal of performance. On the other hand, an isolated serious deviation will have a "memory effect" in a simple moving window average that will persist until $(k + 1)$ more rounds of the trial have passed. This memory effect might cause a laboratory persistently to fail a test based on the running score, long after the problem has been rectified.

Two strategies for avoiding undue emphasis on an isolated bad round are suggested. First, individual or combined scores can be restrained within certain limits. For example, we could apply a rule as follows:

if $|z| > 3$, then $z' = \pm 3$, the sign being the same as that of z

where z is the raw value of a z-score, and the modified value z' is limited to the range ± 3.

The actual limit used could be set so that an isolated event does not raise the running score above a critical decision level for otherwise well-behaved conditions.

Second, a strategy can be developed for avoiding memory effects whereby the scores could be "filtered." Results from rounds further in the past would have a smaller effect on the running score. For example, exponential smoothing uses a rule as follows:

$$\hat{z}_n = \sum_{i=0}^{\infty} \alpha^i z_{n-i}(1-\alpha)$$

calculated by

$$\hat{z}_n = (1-\alpha)z_n + \alpha\hat{z}_{n-1}$$

where α is a parameter between zero and one, controlling the degree of smoothing.

Appendix V.

Alternative Scoring Procedure for Proficiency Testing Schemes

An alternative type of scoring, Q-scoring, is not based on the standardized value but on the relative bias:

$$Q = (x - \hat{X})/\hat{X}$$

where x and $\hat{X}$ have their previous meanings. This type of scoring relates directly to the analytical errors, without any reference to a value of σ, which must be derived either from the participants' data or from an imposed performance standard.

The overall distribution of Q is expected to be centered on zero. It must be centered on zero when all-participant means are used as the estimate of the true value, provided that the number of outliers is relatively low, and when expert laboratory consensus means are used, as long as the group of nonexpert laboratories does not show an overall bias relative to the experts. When the true value is defined as a known addition, the Q distribution will be centered on zero provided that this true value is correct and that there is no widespread use of methodology leading to biased results. In many cases, the actual distribution of Q-scores can be used to test the underlying assumptions.

The distribution of Q-scores cannot be predicted. The distribution of scores should be examined when criteria are developed for assessing whether performance is acceptable. In practice, the distribution is usually normal.

Q-scoring has an advantage in that it directly measures the error associated with a determination. This measurement can subsequently be compared with a performance standard judged as appropriate to the determination (1). If different end-users of the determination require different performance standards, the Q-score can be used in comparison with whatever standard is most appropriate. Furthermore, if the organizer of a scheme decides at anytime that a change in performance standard is justified, previously generated results can easily be compared retrospectively with a revised standard.

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Appendix VI.

Outline Example of How Assigned Values and Target Values may be Specified and Used

A hypothetical example of how assigned values and target values may be specified and used has been developed. The values specified are for illustrative purposes only; real schemes must take account of factors specific to their area.

1. Scheme

Test materials must be mailed 4 times per year, on the Monday of the first full working week of January, April, July, and October. Results must reach the organizers by the last day of the respective month. A statistical analysis of the results will be mailed to participants within 2 weeks of the closing dates. In the examples used in this appendix, the results are based on the single distribution of 2 test materials for the determination of 2 analytes.

2. Testing for Sufficient Homogeneity

See Appendix II.

3. Analyses Required

The analyses required in each round will be as follows: (*1*) hexachlorobenzene in oil and (*2*) Kjeldahl nitrogen in a cereal product.

4. Methods of Analysis and Reporting of Results

No method is specified, *but* the target values were determined by using a standard method, and participants must provide an outline of the method actually used or a reference to a documented method. Participants must report a single result, in the same form as would be provided for a client. Individual reported values are given in Table 1.

5. Assigned Values

5.1 Hexachlorobenzene in Oil

Take the estimate of assigned analyte concentration $\hat{X}$ for the batch of material as the robust mean of the results of 6 expert laboratories. Results of reference laboratory 7, 9, 10, 13, 18, and 19 are 115.0, 112.0, 109.0, 117.0, 116.2, and 115.0 µg/kg, respectively.

$\hat{X}$ is 114.23 µg/kg: traceability of the assigned value was established by using a reference method calibrated with in-house reference standards; uncertainty of the assigned value was determined (±10µg/kg) from a detailed assessment of this method by the reference laboratories.

5.2 Kjeldahl Nitrogen in a Cereal Product

Take the assigned value of analyte concentration $\hat{X}$ for the batch of material as the median of the results from all laboratories.

6. Target Values for Standard Deviation

6.1 Hexachlorobenzene in Oil

In the example used in this appendix the $\%RSD_R$ value has been calculated from the Horwitz equation:

$$(RSD_r \text{ in } \% = 2^{(1 - 0.5 \log \hat{X})})$$

The target value for the standard deviation (σ) is as follows:

$$\sigma_1 = 0.222 \hat{x} \text{ µg/kg}$$

6.2 Kjeldahl Nitrogen in a Cereal Product

In the examples used in this appendix, the $\%RSD_R$ value has been calculated from published collaborative trials.

The target value for the standard deviation (σ) is as follows:

$$\sigma_2 = 0.018\hat{X} \text{ g/100g}$$

7. Statistical Analysis of Test Results

7.1 Hexachlorobenzene in Oil: Formation of z-Score

Calculate:

$$z = (x - \hat{X}) / \sigma$$

for each individual result (x) using the values of $\hat{X}$ and σ derived above. These results are shown in Table 1.

7.2 Kjeldahl Nitrogen in a Cereal Product: Formation of z-Score

Calculate:

$$z = (x - \hat{X}) / \sigma$$

for each individual result (x) using the values of $\hat{X}$ and σ derived above. These results are shown in Table 1.

8. Display of Results

8.1 Tables for z-Scores

The individual results for hexachlorobenzene pesticide in oil and for Kjeldahl nitrogen in a cereal product, together with associated z-scores, are displayed in Table 1.

8.2 Histograms for z-Scores

The z-scores for hexachlorobenzene pesticide in oil and for Kjeldahl nitrogen in a cereal product are also displayed as bar charts (Figures 1 and 2).

9. Decision Limits

Results with an absolute value for z of less than 2 will be regarded as satisfactory. Remedial action will be recommended when any z-scores exceed an absolute value of 3.0. In the examples used in this appendix, the results are as follows: Laboratories 005, 008, 012, 014 for hexachlorobenzene pesticide in oil and Laboratory 008 for Kjeldahl nitrogen in a cereal product.

Table 1. Tabular examples

	Hexachlorobenzene in oil, $\hat{X}$ = 114.2 µg/kg		Nitrogen in cereal, $\hat{X}$ = 2.93 g/100 g	
Laboratory	Result	z-Score	Result	z-Score
001	122.6	0.3	2.97	0.9
002	149.8	1.4	2.95	0.5
003	93.4	−0.8	3.00	1.4
004	89.0	−1.0	2.82	−2.0
005	17.4	−3.8	2.88	−0.9
006	156.0	1.7	3.03	2.0
007	115.0	0.0	2.94	0.3
008	203.8	3.5	3.17	4.7
009	112.0	−0.1	3.00	1.4
010	109.0	−0.2	2.82	−2.0
011	40.0	−2.9	2.99	1.2
012	12.0	−4.0	2.84	−1.6
013	117.0	0.1	2.85	−1.4
014	0.0	−4.5	2.93	0.1
015	101.8	−0.5	2.80	−2.4
016	140.0	1.0	2.96	0.7
017	183.5	2.7	2.97	0.9
018	116.2	0.1	2.88	−0.9
019	115.0	0.0	2.92	−0.1
020	42.3	−2.8	2.88	−0.9
021	130.8	0.7	2.78	−2.8
022	150.0	1.4	2.92	−0.1

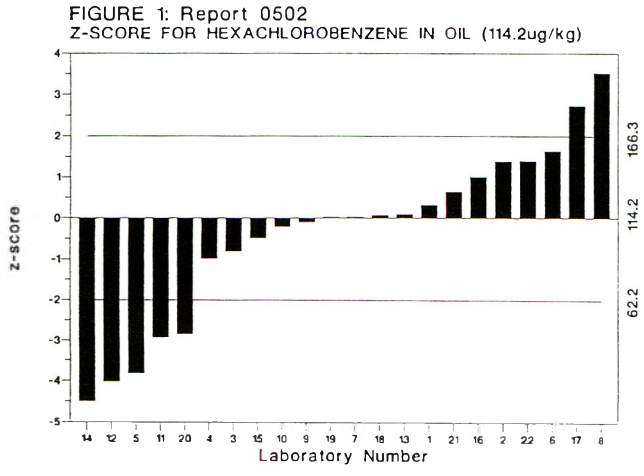

Figure 1. Report 0502 — Z-score for hexachlorobenzene in oil (114.2µg/kg)

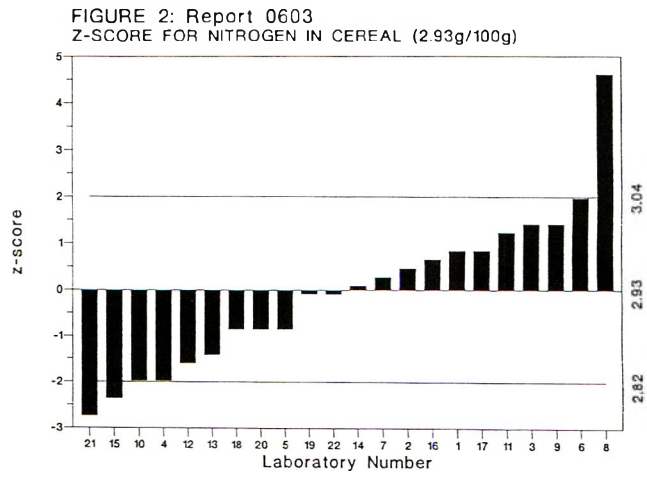

Figure 2. Report 0603 — Z-score for nitrogen in cereal (2.93g/100g)

TECHNICAL COMMUNICATION

Discriminating Between Adult Mandibles of *Oryzaephilus surinamensis* and *Oryzaephilus mercator* Using Setal Brush Length

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The setal brush was identified as a diagnostic characteristic which can be used to distinguish between the mandibles of adult *Oryzaephilus surinamensis* and *O. mercator*. The right and left setal brushes of 50 specimens from each species were measured. Statistical analysis showed that the mean brush lengths of the 2 species were significantly different ($p = 0.0001$). A demarcation value was established to distinguish between the mandibles. By using this value it is possible to reliably classify the mandibles of *O. surinamensis* and *O. mercator*. A mandible with setal brush >0.0296 mm would be classified as *O. mercator*; a mandible with a setal brush <0.0296 mm would be classified as *O. surinamensis*.

When insects are present in a food product during grinding or milling, the finished product will be contaminated with large numbers of microscopic insect fragments. To determine the sanitary significance of insect contamination, the fragments must be identified. The analyst identifies insect fragments associated with food products by their micromorphological characteristics, which have been described in numerous papers (1–12).

As stated in previous papers on fragment identification, the mandibles represent an ideal fragment for insect identification (1, 7). Because of their small size and durable nature, the mandibles usually survive processing undamaged. As an intact structural unit, they provide a variety of distinct characteristics which are a reliable key to the insect contaminant's identity. Unfortunately, the mandibles of some closely related species are very difficult to differentiate. The literature states that some closely related species are without distinct differences. This has been the prevailing opinion regarding the distinction between the mandibles of adult *Oryzaephilus surinamensis* and *O. mercator* (11, 13). Although these mandibles are very similar in appearance, this author discovered that the setal brush of *O. mercator* is longer than the setal brush of *O. surinamensis*. The objective of this study was to demonstrate that the mandibles of these 2 species can be differentiated by the difference in the setal brush length. This objective was accomplished by determining the mean length and range of the setal brushes as well as a demarcation point to use to classify the mandibles.

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Materials and Methods

To ensure the genetic variability of the sample, specimens from 5 isolated sources were examined. They were acquired from the following sources:

U.S. Department of Health and Human Services, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC

U.S. Department of Agriculture, Agricultural Research Service, Stored Product Insect Research and Development Laboratory, Savannah, GA

U.S. Department of Agriculture, Agricultural Research Service, Grain Marketing Research Laboratory, Manhattan, KS

Ministry of Agriculture, Fisheries, and Food, Slough Laboratory, Slough, England

University of Wisconsin, Department of Entomology, Madison, WI

The right and left setal brushes of 10 specimens from each source were measured. Altogether, 50 specimens and 99 mandibles of each species were examined. One mandible of each species broke before the setal brush could be measured. Setal brush measurements on 3 of the 5 sources were replicated on different days to determine the degree of repeatability. When repeating measurements on a sample, the author was unaware of the result from the previous measurement on that sample.

To measure the setal brush, the mandibles were removed from the head and mounted in glycerin jelly on a microscope slide. The mandibles were mounted on the dorsal surface to view the brush. The setal brush was measured from the anterior end of the prostheca to the distal end of the brush at the mesal spike or mesal blade. An Olympus Model FHT Microscope at 40X and an Olympus OSM Ocular Micrometer (total magnification 400X) were used.

The population means, standard deviations, and ranges were calculated. Analysis of variance tests were conducted in which the source of the insect was treated as a random blocking factor. PC/SAS, version 6, was used for the calculations.

To be sure the setal brush was intact and unaffected by the isolation procedure, a light filth flotation was performed according to AOAC method **980.27** (14). Two 50 g samples of

rolled oats were spiked with mandibles of each species. Ten mandibles of each species isolated from the oats were mounted on slides and examined microscopically.

Results and Discussion

The mean lengths of the setal brushes of *O. surinamensis* and *O. mercator* were 0.0246 and 0.0345 mm, respectively. An ANOVA showed that the mean difference between the brush lengths of the 2 species, 0.00989 mm, was significant ($p = 0.0001$). Estimates of the standard deviations for individual setal brush measurements, based on the analysis of variance, were approximately equal to 0.00252 mm for both species. The difference was 4 times the standard deviation, indicating the ability to distinguish the species from a few observations.

For *O. surinamensis* the variation between insects within a source contributed the greatest amount of variation to the total variation. This was not true for *O. mercator*. For this species there existed a significant source effect ($p = 0.001$, df = 4) due primarily to 1 source. In both species, there was a tendency for the left setal brush to be larger than the right brush, and this difference was statistically significant in *O. mercator* ($p = 0.05$, df = 4). The average difference between the left and right setal brush of *O. mercator* was 0.0012 mm. The replication variance was approximately 8% of the total variance within a species, which represents a small fraction.

A bar graph (Figure 1) depicting the distribution of the data for the 2 species reveals 2 distinct populations based on the setal brush length.

The data indicate that 2 populations exist and that the species can be differentiated on the basis of the setal brush length. Because the variation within each species was approximately equal, we can establish a demarcation value at the half-way point between the means, 0.0296 mm. The tendency for the left brush to be larger than the right should not have any practical effect on the classification of the mandibles of these 2 species. Thus, setal brushes >0.0296 mm would be classified as *O. mercator* and those <0.0296 mm would be classified as *O. surinamensis*. The 90% confidence interval of the demarcation point based on a *t* statistic with 4 degrees of freedom is 0.0284 mm, 0.0307 mm.

Based on this demarcation value, and assuming that the above standard deviation and population means are equal to the estimates derived above and that the brush lengths within a species are normally distributed, the false classification rate is calculated to be approximately 3%. In the raw data, there was one false classification for a rate of < 1%.

Microscopic examination of the mandibles isolated from the rolled oats revealed that the setal brushes were intact and unaffected by the isolation procedure.

Conclusion

The results of this study indicate that the mandibular setal brush of *O. mercator* is, statistically, significantly longer than that of *O. surinamensis* and that, with only a few observations,

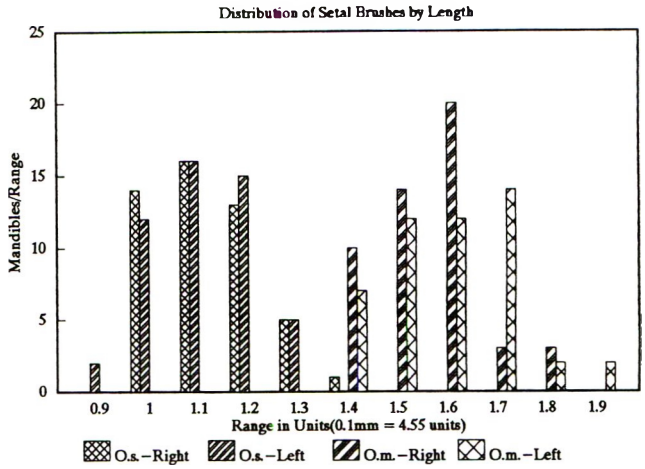

Figure 1. Distribution of the lengths of the right and left setal brushes of *O. surinamensis* (*O. s.*) and *O. mercator* (*O. m.*).

the setal brush can be used to differentiate the mandibles of these 2 species. Using a key to the mandibles of stored product insects (13) and the demarcation point calculated in this paper, a food sanitation entomologist can distinguish between the mandibles of *O. surinamensis* and *O. mercator*.

Because possible source effects do exist, the examination of specimens from additional sources could lead to an adjustment in the demarcation value. The USDA will continue to examine specimens from other sources to expand this data base and to make adjustments to the demarcation value.

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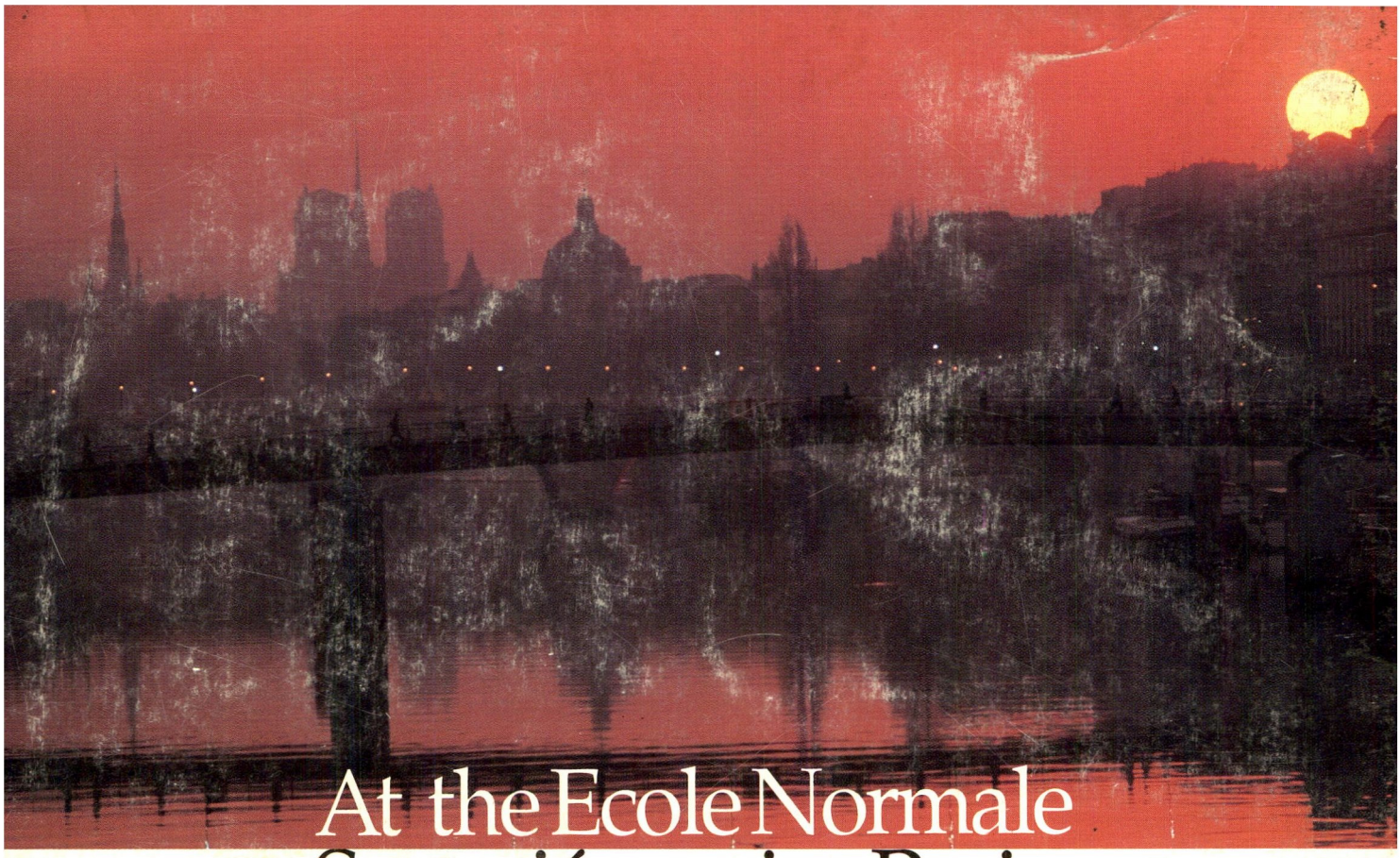

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Step 2: $PhNi^{II}Br(dppe) + e \longrightarrow PhNi^{I}(dppe) + Br^-$

$E_p^3 = -1.75\ V$

Step 3: $PhBr + PhNi^{I}(dppe) \longrightarrow Ph_2Ni^{III}Br(dppe)$

$k_3 = 960\ M^{-1}s^{-1}$

Step 4: $Ph_2Ni^{III}Br(dppe) \longrightarrow Ph\text{-}Ph + Ni^{I}Br(dppe)$

$k_4 = 18\ s^{-1}$

Step 5: $Ni^{I}Br(dppe) + e \longrightarrow Ni^{0}(dppe) + Br^-$

$E_p^5 = -1.32\ V$ [7b]

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