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The
Scientific Association
Dedicated
to
Analytical Excellence

AOAC
INTERNATIONAL

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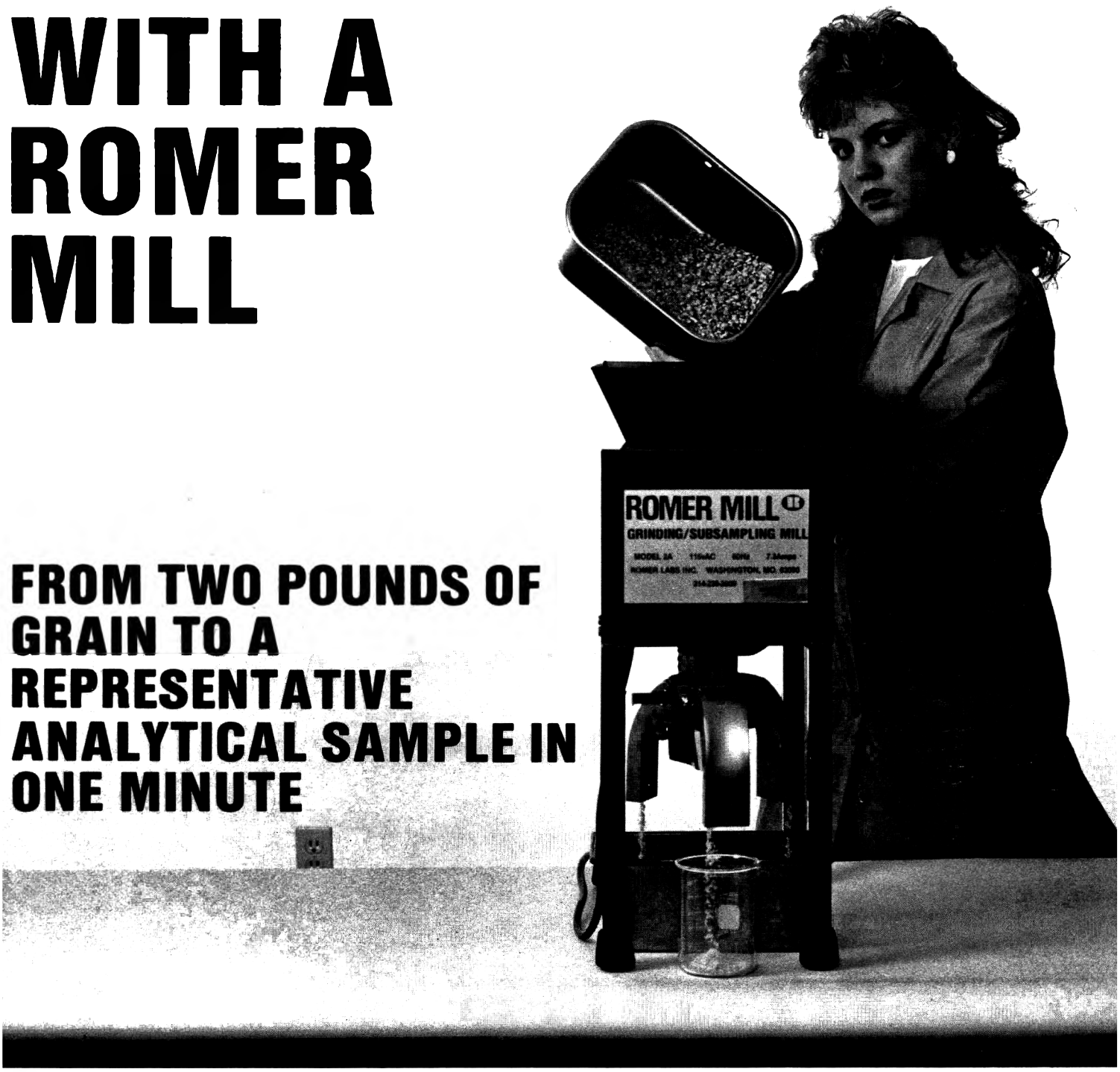

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JOURNAL OF AOAC INTERNATIONAL

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

Table of Contents

March/April 1992 VOL. 75, No. 2 JANCA 75(2)227–374 (1992)

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

Meetings

March 25, 1992: MidAtlantic Regional Section Meeting, College Park, MD. Contact: Charles P. Lattuada, USDA-FSIS, Bldg 322, Barc-E, Beltsville, MD 20705, telephone 301/504-8514.

May 11–12, 1992: Northeast Regional Section Meeting, Nova Scotia, Canada. Contact: Ben Harnish, Nova Scotia Dept of Agriculture and Marketing, Box 550, Truro, NS, B2N 5E3, Canada, telephone 902/893-6562.

May 14, 1992: New York/New Jersey Regional Section Meeting, Cook Campus Center, Biel Rd, New Brunswick, NJ. Contact: Alex MacDonald, Hoffmann La Roche, Inc., 340 Kingsland St, Nutley, NJ 07110, telephone 201/235-4641

June 2–4, 1992: AOAC Board of Directors Meeting, AOAC, Arlington, VA. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

June 8–10, 1992: Midwest Regional Section Meeting, Champaign, IL. Contact: Karen Harlin, University of Illinois, Department of Veterinary Bioscience, 2001 S. Lincoln, Urbana, IL 61801, telephone 217/244-1569.

June 24–26, 1992: Pacific Northwest Regional Section Meeting, Olympia, WA. Contact: Norma J. Corristan, Oregon Dept of Agriculture, 635 Capitol St NE, Salem, OR 97310-0110, telephone 503/378-3793.

August 30–September 3, 1992: 106th AOAC Annual International Meeting and Exposition. Cincinnati, OH. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

August 30 and September 4, 1992: AOAC Board of Directors Meeting, Cincinnati, OH. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

March 29–30, 1993: Europe Regional Section Meeting, Barcelona, Spain. Contact: J. Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres 6, 08029 Barcelona, Spain, telephone 34/3-410-9343

July 26–29, 1993: 107th AOAC Annual International Meeting and Exposition, Washington, DC. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

New Corporation Formed to Administer Test Kit Confirmation Program

A new corporation, the AOAC Research Institute, has been formed. The program for third-party review of proprietary, commercial test kits to confirm manufacturers' claims for performance, originally initiated under the auspices of AOAC International, will be administered by the Institute.

The Institute, incorporated in the state of Virginia in the United States, is a nonprofit scientific and educational membership association with AOAC International as its only member.

The Institute will have a 5-member Board of Directors separate from AOAC International, but will lease facilities and employees from AOAC International. It will also operate under a license from AOAC International for use of the AOAC name in its research activities. The Institute Board will provide administrative, budgetary, and policy oversight. The staff Program Administrator, under supervision of the Program Director and Executive Director, will have budgetary and policy implementation responsibility, including recruiting and tracking technical reviewers, recruiting and contracting independent testing laboratories, granting confirmation status on the basis of recommendations, granting renewals for unchanged kits or kits with minor changes, and submitting documented appeals.

Application and Testing for Kit Confirmation.—The general operating principles of the test kit confirmation program were developed by the AOAC test kit task force and received the support of AOAC International's Board of Directors prior to transfer of the program to the AOAC Research Institute. As proposed, applicants will submit a defined data package; an application, including indemnification of the AOAC Research Institute; and an application fee. Technical reviewers will be chosen to determine the adequacy of the data package, develop the independent laboratory test protocol, evaluate the test results, and make a recommendation to the Program Administrator.

Independent testing laboratories will be chosen according to predetermined qualifications, including appropriate accreditation, adherence to good laboratory practice requirements, and the like.

The independent testing laboratories will test production lot kits for critical performance claims, according to the predetermined protocol that has been accepted by all parties.

Confirmation status will be granted on the basis of the reviewers' evaluation and laboratory data that meet the limits of the confirmation protocol.

Fees and Payments.—Applicants seeking the AOAC Research Institute's independent confirmation of their kit's performance specifications will pay a fee for the initial application for kit confirmation; a discounted fee will be available for multiple kit applications of a similar nature. Holders of existing certificates of confirmation will pay an annual renewal fee for each renewal of confirmation status for unchanged kits or kits with minor changes.

Separate testing laboratory fees will be set as part of the protocol to be ac-

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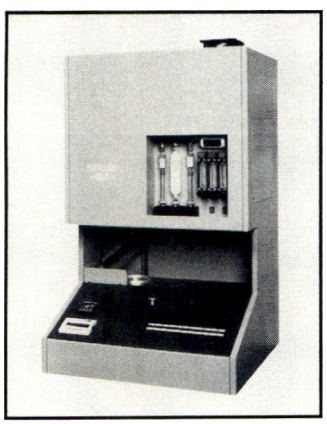

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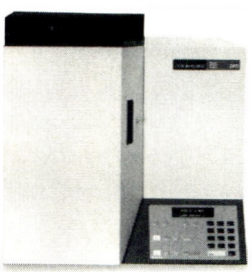

The PE 2410 Series II meets AOAC requirements.

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For more information on the PE 2410 Series II Nitrogen Analyzer, contact your local Perkin-Elmer office. For product literature in the U.S., call **1-800-762-4000.**

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cepted by all parties. This fee will be billed back to the applicant firm.

Technical reviewers will receive an honorarium.

Confirmation and Licensing.—A certificate of confirmation, with a 1-year expiration date, will be issued to the applicant of each kit confirmed. A licensing agreement between the applicant and the AOAC Research Institute will spell out rules, rights, and obligations, including right to use the Institute certification mark and specified language in advertising, kit inserts, and the like.

Certificates of confirmation will be documented and numbered to permit control and tracking.

Renewals, Changes, Complaints.—Holders of certificates of confirmation must submit annual renewal applications, either certifying no changes in the original tested kit, or identifying minor changes with supporting data. Kits changed in a major way, in the opinions of the manufacturer or Program Administrator, must be retested.

Adverse comments and complaints regarding kit performance will be required to be submitted to the manufacturer for initial resolution. Only unresolved complaints will be permitted to be brought to the Institute for final resolution, under standardized procedures.

Information and Publication.—The AOAC newsletter, *The Referee*, under agreement between AOAC and the AOAC Research Institute, is designated as the initial medium for information dissemination. Notices will be published regarding test kits received for confirmation, test kits granted confirmation status, and test kits for which confirmation status has been revoked or expired.

A compendium will be also be published periodically, listing all successfully tested kits and a summary of the testing protocol and data developed on each kit.

Conflicts of Interest, Antitrust, and Due Process.—In so far as possible, technical reviewers and testing laboratories will be selected which do not have a competing interest with the applicant. Possible conflicts will be required to be disclosed. The AOAC Research Institute will operate so as not to favor or exclude any segment of possible applicants. Protocols for the process will be written and followed, all parties to the process will be notified of pending actions and actions taken, and a process for appeals, separate from review and confirmation, will be followed.

Further Information.—Anyone wanting further information, and persons or laboratories interested in serving as technical reviewers or testing laboratories, contact the AOAC Research Institute, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-2529.

Three Pre-Meeting Workshops Planned for 1992

AOAC will offer 3 workshops just prior to the start of the 1992 AOAC Annual International Meeting in Cincinnati, OH, on August 30–September 3, 1992.

In addition to the Workshop on Antibiotics and Drugs in Feeds, a "hands-on" Workshop on Methods of Analysis for the Determination of Juice and Flavor Composition and Authenticity is being organized by the Technical Committee for Juice and Juice Products, and a Workshop on Quality Assurance of Benchtop Mass Spectrometric Data is also planned. All workshops are scheduled the weekend preceding the meeting.

AOAC introduced "pre-meeting" workshops with the first Antibiotic and Drug Workshop at the 1989 AOAC Annual International Meeting in St. Louis, MO. The Association of American Feed Control Officials (AAFCO) and the U.S. Food and Drug Administration were co-sponsors. The success of this

first workshop, organized by Mary Lee Hasselberger of the Nebraska Department of Agriculture, launched this AOAC program, which has become an integral part of the AOAC annual international meetings.

The Antibiotic and Drug Workshop was initiated by the Laboratory Methods and Services Committee of AAFCO to review, update, and perhaps supplant older official AOAC methods. The slate of assays selected for the initial workshop will continue to appear on the program until problems with the assays are resolved or new analyses are adopted to replace them. Other topics for the program are selected by canvassing previous attendees. These have included presentations on technique, QA/QC, and safety.

The program organizer, Mary Lee Hasselberger, says, "I have been very gratified by the enthusiastic response of the attendees, the cooperation of the presenters, and the support of AAFCO, AOAC, FDA, and the Nebraska Department of Agriculture."

Allan Brause, organizer of the Juice and Flavor Composition Workshop, reports that this workshop will include 14 training stations where participants will have the opportunity to perform chemical analyses or view simulations of techniques for the determination of isotopic compositions. The analytical methods to be conducted include LC determinations of sugars (including the Low method), organic acids, anthocyanins, flavonoids, d,l-malic acid, sodium benzoate, and polyphenols; atomic absorption spectroscopic determination of metals; spectroscopic determination of pulpwash in orange juice; and GC/MS analyses of flavors. The simulation stations will include SNIF/NMR, CSIRA, and OSIRA, and computer pattern recognition analyses. The instructors will be experts in these techniques. Early registration is advised because the number of participants must be limited to permit the "hands-on" aspects of this workshop.

"Allan Brause is well-known throughout AOAC International for his ability to organize an excellent scientific program. I believe this workshop will be the first of many because I am confident that attendees will be impressed not only by the quality of the instruction but also with the care and attention to detail for which Brause is well-known," enthused Margaret R. Ridgell, AOAC Director of Administration and Meetings.

The Workshop on Quality Assurance of Benchtop Mass Spectrometric Data is being organized by Joan Fisk and James Baron of the U.S. Environmental Protection Agency, Washington, DC. It will emphasize "benchtop" applications. The ease of operation of mass spectrometers permits their use by analysts without extensive experience in mass spectrometry. Their use for regulatory and other applications with legal considerations necessitates careful consideration of criteria for confirmation of analyte identity and quantitation. Data comparability among the several types of commercially available benchtop instruments will also be discussed.

For additional information on any of these workshops, call the AOAC International Meetings and Education Department at 703/522-3032.

New Task Force to Address Methods Needs In Nutrient Labeling Area

The Board of Directors at its December 5, 1991, meeting authorized formation of a new Task Force on Methods for Nutrient Labeling Analyses. The group is charged with developing a strategy for identifying methodology deficiencies, improving existing validated methods, recruiting interlaboratory studies leading to additional validated methods, and incorporating standard reference materials in nutrient methods use. A focused effort is needed in this area because of activities in Europe in setting standards and methods for the European Community common market, and activities in the United States related to the proposed Nutritional Labeling and Education Act.

It is expected that the task force will identify deficiencies in current methodology, including the lack of any adequate method; identify and publicize validated methods available for nutrient analyses; identify what revisions are needed in existing methods, and what additional methods are needed; develop an approach for recruiting researchers and getting necessary studies under way; identify and publicize available standard reference materials; identify needs for standard reference materials; and develop an approach for incorporating the use of reference materials in nutrient analytical methods.

Anyone interested in serving on the task force or receiving informational materials as the group proceeds with its work should contact AOAC Technical Services at the AOAC International address.

New Sustaining Members

AOAC welcomes the following new sustaining members: Central Science Laboratory, Ministry of Agriculture, Fisheries and Food, Berkshire, England; NIR Systems, Inc., Silver Spring, MD; and Laboratoria Tecnologico Del Uruguay (LATU), Montevideo, Uruguay.

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 23–25, 1992, in San Diego, CA, and became official at that time. These methods will be published in the fourth supplement (1993) to the 15th edition (1990) of *Official Methods of Analysis*.

■ *Pesticide Formulations and Disinfectants*: Methamidophos in Technical Products and Pesticide Formulations, Liquid Chromatographic Method, CIPAC-AOAC Method.

Methazole in Technical and Pesticide Formulations, Liquid Chromatographic Method.

■ *Foods I:* Sampling of Milk from Bulk Tanks and Other Storage Equipment, Automated Method (Modification of **970.26**).

■ *Foods II:* Analysis of Milk-Based Infant Formula, Phase V (Folic Acid, Pantothenic Acid, Vitamin E, and Vitamin A).

Corn-Derived Acetic Acid in Apple Cider Vinegar, Detection by Carbon Stable Isotope Ratio Analysis.

Sugar Beet-Derived Syrups in Frozen Concentrated Orange Juice, $\delta^{18}O$ Measurements in Water, Stable Isotope Ratio Mass Spectrometric Method.

■ *Microbiology and Extraneous Materials:* Light Filth from Fish Products Containing Spice, Flotation Method.

Motile and Non-Motile *Salmonella* in Foods, Polyclonal Enzyme Immunoassay Method.

Light Filth in Sauces Containing Soy Sauce, Thickeners, and Spices, Flotation Method.

Light Filth in Tofu, Sieving Method.

■ *Environmental Quality:*Pesticides in Water, Liquid Chromatographic Method with Ultraviolet Detector (National Pesticide Survey Method 4).

Books in Brief

Chromatography, 5th edition: Fundamentals and Applications of Chromatography and Related Differential Migration Methods. Edited by E. Heftmann. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. Part A: Fundamentals and Techniques: 552 pp. Price: US $179.50/Dfl. 350.00. ISBN 0-444-88236-7. Part B: Applications: 630 pp. Price: US $189.50/Dfl. 370.00. ISBN 0-444-88404-1.

This is a completely new book, organized according to the successful plan of the previous 4 editions. Part A covers the theory and fundamentals of such methods as column and planar chromatography, countercurrent chromatography, field-flow fractionation, and electrophoresis. Affinity chromatography and supercritical-fluid chromatography are covered for the first time. Part B presents various applications of these methods. New developments in the analysis and separation of inorganic compounds, amino acids, peptides, proteins, lipids, carbohydrates, nucleic acids, their constituents and analogs, porphyrins, phenols, drugs, and pesticides are reviewed and summarized. Important topics, such as environmental analysis and the determination of synthetic polymers and fossil fuels, are covered for the first time.

Trace and Ultratrace Analysis by HPLC. By Satinder Ahuja. Published by John Wiley & Sons, Inc., 1 Wiley Dr., Somerset, NJ 08875-1272, 1992. 419 pp. Price: $75.00. ISBN 0-471-51419-5.

Of the various separation techniques used for trace and ultratrace analyses, high performance liquid chromatography is by far the most popular and commonly used. HPLC's heightened capacity for analyzing and discovering new compounds has been essential to the success of trace and ultratrace analyses. This new monograph provides the first definitive, technically up-to-date look at the theory, equipment, and applications of what is becoming chemistry's most powerfully reliable analytical technique. *Trace and Ultratrace Analysis by HPLC* outlines in a clear, progressive format the state of the art of the technique, covering the scope of trace and ultratrace analysis by HPLC; considerations for HPLC equipment; sensitive detectors in HPLC; sample preparation; method development for trade and ultratrace analysis; selectivity optimization for trace and ultratrace analysis; computer-based optimization; and optimizing detectability.

Atomic Absorption Spectrometry: Theory, Design, and Applications. Edited by S.J. Haswell. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 530 pp. Price: US $177.00/Dfl. 345.00. ISBN 0-444-88217-0.

Atomic absorption spectroscopy is now a well-established technique for the determination of trace elements covering a wide range of analyte types. The early theory and instrumentation chapters incorporate recent trends in instrumental design and methodology, in particular those associated with electrothermal techniques and background correction. The major thrust of the book is represented by 14 application chapters that give an extensive well-referenced review of the practical use of the techniques written by experts drawn from their own specialty areas. These include the determination of trace elements in areas as diverse as environmental, chemical, and industrial analysis.

The National Toxicology Program's Chemical Data Compendium. By Lawrence H. Keith and Douglas B. Walters. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1991. Eight volumes, 10 297 pp. Price: US $1,800.00/Outside US $2,472.00. ISBN 0-87371-723-6.

This compendium provides a vast amount of information about potentially toxic chemicals to regulatory agencies, consultants, academics, and libraries. *The National Toxicology Program's Chemical Data Compendium* consists of 8 volumes containing 50 fields that present detailed information on 2270 different chemicals. The data are obtained from the literature or experimentally determined. Each compound is listed in every volume even when there is no information available for it in some volumes. Information in the NTP Compendium was gathered and updated as compounds were used throughout a 12-year period from 1979 to 1991. Throughout the 8 volumes, the primary chemical name and the Chemical Abstracts Service Registry Number (CAS No.) remain constant and all 2270 chemicals are listed alphabetically in each volume.

Analytical Applications of Spectroscopy II. By A.M.C. Davies and C.S. Creaser. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1991. 324 pp. Price: $124.95. ISBN 0-85186-403-1.

Analytical Applications of Spectroscopy II provides a broad coverage of recent developments in analytical spectroscopy and particularly the common themes of chromatography-spectroscopy combinations, new techniques, and data handling. These themes have played an increasingly important role in advances in spectroscopic techniques and emphasize the multidisciplinary approach of present research. Each section reviews key areas of current research, as well as short reports of new developments in those areas.

Important Peak Index of the Registry of Mass Spectral Data. Edited by Fred W. McLafferty and Douglas B. Stauffer. Published by John Wiley & Sons, Inc., 1 Wiley Dr., Somerset, NJ 08875-1272, 1991. 4000 pp. Price: $750.00.

The largest collection of unit electron ionization mass spectra in the world was made available in 1989 with the groundbreaking print edition of *The Wiley/NBS Registry of Mass Spectral Data* (Wiley/NIST 1989). The Registry gives the complete spectra and is organized for use by people who want to look up the mass spectrum of a known chemical. In contrast to this, a derivative work of the Registry, the *Important Peak Index of the Registry of Mass Spectral Data* selects the most important peaks from each spectrum to identify the chemical by its spectrum. It then organizes this peak information for easy use in identifying unknown chemicals by mass spectroscopy. This index now contains over 400 000 entries, more than twice the coverage of both spectra and compounds found in any other source. The index peaks have been chosen according to their statistical importance rather than just their abundance; this gives substantially higher reliability and recall in matching. Arranged according to the mass of their first, second, and third most important peaks—within each listing, the entries are ordered by the mass of the other most important peaks—each of the spectra appear in the index 3 times. Each entry lists the mass and percent abundance for the 6 most important peaks.

Food Safety. By Julie Miller Jones. Published by Eagan Press, 3340 Pilot Knob Rd, St. Paul, MN 55121, 1992. 380 pp. Price: US $48/Outside US $58.00.

Food Safety enables the reader to access in one readily available volume the vast range of information on today's most pressing food safety issues — from microorganisms to naturally occurring food toxicants to chemical additives and preservatives. The goal of the book is to present a balanced look at current food safety data, detailing both the risks and benefits associated with the way we produce food today.

New Products

Dedicated System for Determination of Aromatic Content in Petroleum Fuels

The Aromatic analyzer provides excellent quantitation of the total aromatic content of diesel fuels, jet fuels, gasoline, and other types of petroleum products. The analysis methodology is based on ASTM method D 5186 for the analysis of the aromatic content in diesel fuel, which has proven round-robin, intralaboratory reproducibilities over a wide range of aromatic content values. The Aromatic analyzer methodology replaces the D 1319 fluorescent indicator adsorption method with a method that can be automated and produces consistent, reliable data with reproducibilities in the 1 to 3% range. Suprex Corp.
Circle No. 313 on reader service card.

Low Volume FTIR Gas Cell—Model LV7

A new FTIR gas cell designed for use with Nicolet FTIR analyzers measure gas concentrations in small volume samples. It has been configured specifically for use with Nicolet Models 205, 821E, and 60SX, as well as the 500, 700, and 800 series. The LV7 gas cell is exceptionally compact, easy to use, and has a high pathlength-to-volume ratio. Cell volume is 222 cc, with a maximum optical pathlength of 7.25 m. Pathlength is adjusted with a direct reading dial. A solid aluminum housing permits application of vacuum, pressure, and heat. The Foxboro Co.
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Sedimentograph Offers Faster Scanning

A sedimentograph reduces analysis time through computer-controlled, variable-speed vertical movement of the unit's photometer light system. The Fritsch A-20 scanning photo sedimentograph determines size distribution for particles ranging from 0.5 to 500 μm. Particle size is determined by measuring changes in the blockage of transmitted light as the same particles settle from a sedimentation liquid. The Fritsch A-20 offers precise, cost-effective analysis for applications where the density of sample particles is constant and known, where the density and viscosity of the sedimentation liquid are known, and where the sample does not dissolve, swell, or flocculate in the liquid. It features a reliable optical system and precise-scanning components that assure high reproducibility. All electrical and electronic components are modular for easy access and replacement. Aside from cleaning the cuvette after each use, no routine maintenance is required. Gilson Co., Inc.
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CLP Forms Templates for Windows 3.0

CLP Forms Templates are designed to ease you through the tedious task of filling out CLP reports. All of the formatting of these reports is already done for you using Microsoft's Excel 3.0 templates. You start with the main menu that lists all forms and then choose the appropriate form by clicking with a mouse. Simply tab from one field to the next and save the completed form as an Excel Worksheet. The different fields are color coded to inform the analyst if the data is automatically calculated, is common header information, or if it is a data input field. Automatic calculation is executed within the spreadsheet for calculated values and the number of decimal places is adhered to as per the "Statement of Work." Notes are attached to column headers and cells to guide the analyst to input the exact format required. Completed forms can be printed or archived for later retrieval. WindowChem Software, Inc.
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High Capacity Precision Toploaders

Three new high capacity precision toploading balances are available in 8, 12, or 15 kg capacities at 0.1 g readability. Their stainless steel pans are the largest of any high capacity toploader and all have weigh below capability. The versatile new balances feature 4 levels of stability, 3 averaging levels, and an indicator head that controls all functions, whether attached, detached, or tower mounted. The balances are designed specifically to fulfill the high capacity requirements of manufacturing, quality control, and testing applications. Their simple operation, push button calibration, and solid construction make them more than suitable for years of trouble-free use in less than ideal environments. Ohaus Corp.
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Database Offers FDA and DEA Regulations On-Line

Drug, medical device, food, and cosmetic executives now can use their computers to find U.S. Food and Drug Administration and Drug Enforcement Agency rules in Title 21 of the Code of Federal Regulations. The electronic database, titled "21CFR Online," is available through the Data-Star Service and the BRS Search System. "21 CFR Online" is a new service of DIOGENES, a partnership of Washington Business Information, Inc. (WBI), publisher of newsletters covering regulations of drugs and medical devices, and FOI Services, Inc., leading document retrieval service in the FDA area. "21CFR Online" contains Title 21's full text—with over 7000 entries—and is updated monthly. It can be searched by date, titles of chapters or subchapters, or other key words. DIOGENES.
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CElect Capillary Electrophoresis Column Kits Offer Four Bonded Phases in One Package

The CElect family of capillary electrophoresis columns offers a choice of 4 bonded phases, spanning a range of functionalities. Each phase has a unique effect on the separation mechanisms. Now you can obtain the entire range of functionalities in a convenient kit containing 4 CElect columns of the same ID (50 or 75μm). Each kit contains a weakly hydrophobic CElect-H column, a moderately hydrophobic CElect-H1 column, a strongly hydrophobic CElect-H2 column, and a neutral hydrophilic CElect-P1 column. The kit also includes a neutral marker standard. Each column is shipped ready to install, with an optical window and mounted on a convenient storage spool. Supelco, Inc.
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Electronically Operated Distribution Valve

The chemically inert 12 V valve features simple or complex flow combinations, 4-port positioning, synchronized output, and simple TTL control. It is bi-directional and tubing can be connected instantly. The Omnifit range of chromatography columns, accessories, and fittings is claimed to be the most comprehensive currently available for microbore, analytical, and preparative work. The high performance range is designed for chromatography with both aqueous and organic solvent capability. Included are accessories such as in-line valves, septum injectors, adjustable endpieces, and water jackets. Applications include peptide synthesis, and biomodule purification. Omnifit Ltd.
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Full Power Statistical Package for Windows 3

Unistat for Windows is one of the first full power statistical packages for Windows 3 and offers complete data handling, analysis, and presentation

capabilities. It includes a fully featured spreadsheet allowing import and export of data in most industry standard file formats, a wide range of statistics and powerful 2D and 3D graphics. With Unistat's alpha-numeric spreadsheet, the researcher can handle the process data sets with ease. Unistat also offers powerful statistical data handling procedures such as factorize, defactorize, aggregate, and rank. Aston Scientific Ltd.
Circle No. 321 on reader service card.

Easy To Use Plotting Software

Techni-Curve, a new Windows 3 version of the Techni-Curve graph plotting and curve fitting software, provides a complete range of technical graph formats such as X-Y, bar chart, and histograms, and allows large amounts of independent data to be loaded, superimposed, and manipulated. Data can be entered manually or be imported from spreadsheets, databases, or even directly from instruments. Complete control is provided over all graph parameters such as asix limits, scaling, line thickness, labels, fonts, symbols, and colors with the easy to use Windows "point-and-click" approach. Aston Scientific Ltd.
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Automated GPC Sample Cleanup

The AUTOVAP AS-2000 provides true unattended automation, featuring GPC cleanup with on-line evaporation and transfer of the concentrated sample to a sealed vial, ready for further analysis. It meets all EPA, FDA, USDA, and USDI requirements for GPC cleanup; incorporates new Envirosep-ABC high performance columns for complete sample cleanup in less than 30 min; operates in 3 modes including GPC cleanup with on-line evaporation/concentration, stand alone GPC cleanup, and stand alone evaporation/concentration only; and the AUTOVAP evaporation technique uses liquid level sensing to achieve excellent recoveries of environmental organic compounds. It has a 23 sample capacity. ABC Laboratories.
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Standard Reference Materials Feature Laser Diffraction Reticle

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CEREALS AND CEREAL PRODUCTS

■ Interlaboratory Study of Decreasing the Number of Standard Points in the Official Iron Standard Curve—J.I. Martin and A.I. Soliman

CHEMICAL CONTAMINANTS MONITORING

■ Total Diet Study of Lead and Cadmium in Food Composites: Preliminary Investigations—R.W. Dabeka and A.D. McKenzie

MICROBIOLOGICAL METHODS

■ Quantitation of Microorganisms in Raw Minced Meat Using the Direct Epifluorescent Filter Technique: NMKL Collaborative Study—F. Boisen, N. Skovgaard, S. Ewald, G. Olsson, and G. Wirtanen

MYCOTOXINS

■ Bioassay, Extraction, and Purification Procedures for Wortmannin, the Hemorrhagic Factor Produced by *Fusarium oxysporum* N17B Grown on Rice—H.K. Abbas, C.J. Mirocha, W.T. Shier, and R. Gunther

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General Information

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

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2. To Managing Editor, AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm] of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgements, references); 4.Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.

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2. **Abstract** (separate sheet, **double spaced**): ≤ 200 words. Provide specific information, not generalized statements.

3. **Text** (consecutive sheets, **double spaced**): *Introduction.* Include information on why work was done, previous work done, use of compound or process being studied.

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

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

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

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

JOURNAL ARTICLE REFERENCE

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

BOOK CHAPTER REFERENCE

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

BOOK REFERENCE

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **29.070–29.072**

4. **Figure captions** (separate sheet(s), **double spaced**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (See **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.

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

1/92

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A $2,500 annual award presented to an outstanding scientist or scientific team for analytical contributions in an area of interest to AOAC International.

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Cumulative Author Index

SPECIAL REPORT

Precision Parameters of Methods of Analysis Required for Nutrition Labeling. Part II. Macro Elements—Calcium, Magnesium, Phosphorus, Potassium, Sodium, and Sulfur

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Nutrition Research Division, Health Protection Branch, Ottawa, ON, K1A 0L2, Canada

A previous paper reviewed the precision of analyses for the major macronutrients—fat, protein, and carbohydrates—as well as moisture/solids, ash, and "fiber." A similar review is now presented for the macro inorganic nutrients—calcium, magnesium, phosphorus, potassium, sodium, and sulfur. The precision parameters among laboratories (standard deviations, S; relative standard deviations, RSD; and repeatability, r, and reproducibility, R, values) are not characterized by any conventional distribution. The typical precision of the methods of analysis for these elements in food can be expressed solely as a logarithmic function of concentration, independent of analyte, matrix, and method. The average RSD_R value from each collaborative assay found in the literature is used as the numerator in a ratio containing, as the denominator, the value calculated from the logarithmic function:

$$RSD_R\,(\%) = 2^{(1\,-\,0.5\log C)}$$

where C is the concentration as a decimal fraction. If this ratio, designated as HORRAT, is above 2, the method is probably unacceptable with respect to precision. About 20% of the 465 interlaboratory data sets studied for this paper show an RSD_R exceeding the acceptable limit, with an overall average HORRAT of 1.2 at C ranging from about 20×10^{-6} (20 ppm) to about 10^{-1} (10%). The variability, although high, may be acceptable for the purpose of nutrition labeling.

The Codex Committee on Food Labeling is attempting to develop a list of appropriate methods of analysis and sampling for use in the enforcement of Codex Guidelines on nutrition labeling (1). The first paper in this series (2) examined the precision parameters of the macrocomponents of food: moisture/solids, ash, protein, carbohydrates, fat, and fiber. This second paper extends the examination of the precision parameters to the macro inorganic elements of importance in nutrition: calcium, magnesium, phosphorus, potassium, sodium, and sulfur. Nutrients are designated here as "macro" if they typically exist in food at a concentration above about 100 ppm (0.01 g/100 g).

The purpose of this paper is to provide a reasonable approximation of the analytical error to be expected in the analyses by different laboratories of these elements that are typically present in foods at concentrations of 0.01 to 10 g/100 g. The methods of analysis commonly used are those adopted and published by AOAC (3) after validation by method-performance (collaborative) studies and publication of the results in the *Journal of AOAC International*. Results from interlaboratory studies of methods published elsewhere are also included.

Procedure

The procedure used by Peeler et al. (4) to develop a database of the precision parameters of methods of analysis for milk and milk products was followed for the macro inorganic elements in foods. The data contained in the collaborative studies referenced in *Official Methods of Analysis* (3) were entered into a computer workspace in the APL language for recalculation of the mean and the within-laboratory (RSD_r) and among-laboratories (RSD_R) relative standard deviations by the harmonized IUPAC-1987 (5) procedure. The RSD_R values were then used as numerators in ratios containing, as denominators, the corresponding values predicted by the Horwitz equation (6):

$$RSD_R\,(\%)\ (\text{predicted}) = 2^{1\,-\,0.5\log_{10} C} = 2C^{-0.1505}$$

where C is expressed as a fractional concentration (i.e., 100% = 1.00; 1% = 0.01). An RSD_R ratio (HORRAT[1]) of 1 indicates a value corresponding exactly to the Horwitz equation; a series of HORRAT values near or above 2 (representing RSD_R values (found) that are twice as large as those calculated from the Horwitz equation) usually indicates an unacceptable method with respect to precision. A series of HORRAT values bracketing 1.0, or consistently smaller than 2.0, indicates acceptable precision.

Received August 9, 1991. Accepted September 9, 1991.

[1] HORRAT = RSD_R (found)/RSD_R (predicted)

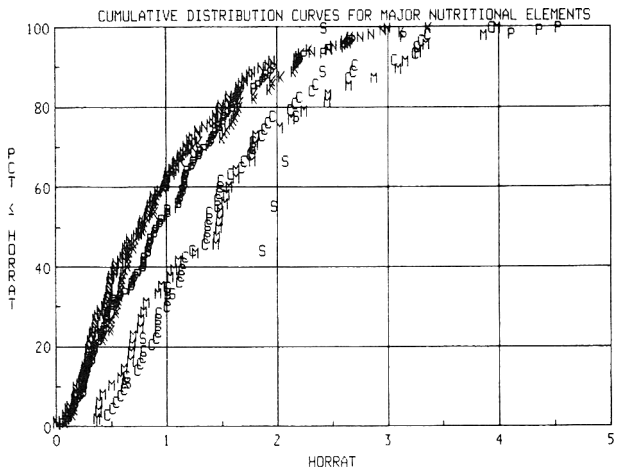

Figure 1. The cumulative distribution curves for the 6 major inorganic elements in food. Legend: C, calcium; M, magnesium; P, phosphorus; K, potassium; N, sodium; and S, sulfur. For example, approximately 60% (y-axis) of the HORRAT values for calcium (C) are equal to 1.5 or less (x-axis). The 90% HORRAT interval is 0.6–3.2 (values on the x-axis corresponding to the 5 and 95% points, respectively, on the y-axis for the C curve).

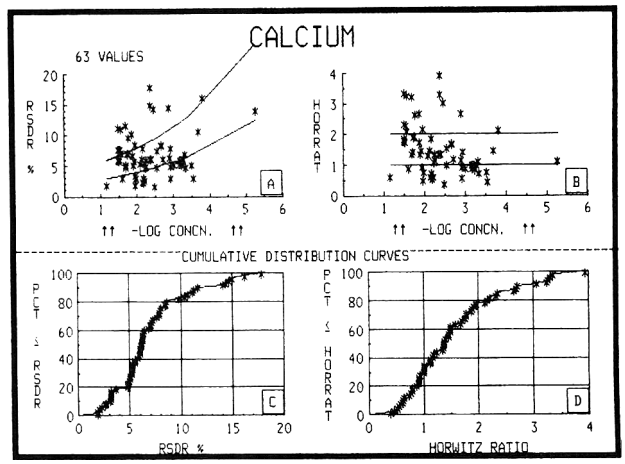

Figure 2. Precision parameters RSD_R and HORRAT of calcium as a function of concentration (–log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against –log C. (B) Scatterplot of HORRAT against –log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

Because RSD_R and HORRAT values are not characterized by any standard statistical distribution, they are plotted as empirical cumulative distribution curves (CDCs) (Figure 1). Each curve provides the 90% interval as a characterizing precision parameter for a specific analyte-matrix-method combination over all studies. The 90% interval contains the center 90% (i.e., the range between the 5th and 95th percentile points) of the values (either HORRAT or RSD_R). RSD_R values, particularly when normalized with respect to concentration by calculation of the corresponding HORRAT values, permit direct comparison of precision characteristics across commodities, analytes, and methods with considerably different analyte concentrations. Such comparison is not possible with the primary statistical function, standard deviation, S, or with the ISO maximum tolerable difference functions, repeatability and reproducibility, r and R, respectively (7).

Original collaborative data from other sources, such as the *Journal of the Association of Public Analysts*, and documents from the International Dairy Federation (IDF), the International Organization for Standardization (ISO), and the International Union of Pure and Applied Chemistry (IUPAC), are also included in the database.

Results for analytes originally reported as oxides or salts were recalculated to the element, except when the analysis was interpreted for a non-nutritional purpose, e.g., lipid phosphorus for egg content and salt reported as part of the analytical residue of "salt and curd" of butter. Analyses for non-nutritional purposes are not included in this survey.

Specific problems encountered in reconstructing results from older collaborative studies and in dealing with studies showing very poor precision, i.e., an RSD_R greater than twice the RSD_R calculated from the Horwitz equation (6), are discussed under the individual analytes.

Each data set showing a HORRAT value above 2.0 (above twice the RSD_R calculated from the Horwitz equation) was examined in detail by consulting the original paper and the authors, if available, to determine if an explanation existed for the high variability. As a result of that review, invalid data were removed, and the precision parameters were recalculated. The criteria used for classification as "invalid data" were discussed in a previous paper (2). The outlier designations of the original papers were ignored in all cases. Data were subsequently removed only by the harmonized IUPAC-1987 procedure (Cochran, Grubbs, paired Grubbs at "alpha" = 0.01) (6) as extended by Kelly (8).

The precision estimates based on the valid data, purged of outliers, were then plotted as RSD_R and HORRAT values against –log C and as CDCs of RSD_R and HORRAT values, as exemplified in Figure 2 for calcium. Clusters of high values in the scatterplots and CDCs were further examined for common characteristics, such as low residue weights, same or similar matrixes or methods, explanations by the authors, or other features. Such clusters are discussed under the individual analytes. Unexplained high RSD_R values were sometimes designated as "unaccepted assays" for reasons given in the text and were removed from the database.

Assay Acceptability Criteria

A single numerical limit can not be selected, either for RSD_R or for HORRAT values, that will differentiate acceptable from unacceptable assays. Our examination of over 6000 collaborative assays has shown that a HORRAT of 2.0 is the general

dividing line between acceptable and unacceptable assays because it compensates in large part for the increase in RSD_R with a decrease in concentration. However, the use of any limit must be tempered by the realization that in a series of HORRAT values in the upper acceptable region, an occasional value will exceed 2.0 merely because of ordinary statistical fluctuations. The data set with such a value should be kept with the bulk of its cluster. Similarly, a series of data sets with HORRAT values above 2.0 will have an occasional data set with a HORRAT below 2.0 that nevertheless should be rejected along with the other unacceptable data sets in the series. Fortunately, in a series of more than about 50 data sets, an occasional controversial decision that results in a misclassification will rarely affect a conclusion as to the acceptability or nonacceptability of a method. Therefore, an occasional inconsistency in judgment between reviewers, and even sometimes by the same reviewer, can be expected and must be tolerated.

Results

The macro constituents typically are present in food at a concentration above about 100 ppm (0.01 g/100 g). This class includes important inorganic elements such as calcium, phosphorus, and sodium, and organic constituents such as cholesterol and vitamin C. However, the classification is merely for convenience; similar analytes are grouped together even though they may be somewhat outside the typical classification limits. Ash, for example, is classified as a major nutrient because it is required as a correction factor for the calculation of carbohydrates by difference. However, the determination of ash also provides a valuable internal quality control feature because it should approximate the sum of the major inorganic constituents. In this second paper, macro elements include only calcium, magnesium, phosphorus, potassium, sodium, and sulfur. The complete list of accepted data sets arranged by analyte is shown in Database 1. In the printout of this database, RSD0 was substituted for RSD_r, within-laboratory relative standard deviation, because of printer limitations.

Calcium

Calcium was originally determined by the classical titrimetric (VOLU) methods using permanganate or EDTA, and is currently determined by emission (EMSP), atomic absorption (ATAB), and inductively coupled plasma emission (ICPE) spectroscopy. Margosis et al. (9) pointed out that the precision of the classical titrimetric and gravimetric methods begins to degrade at an analyte concentration below about 1 g/100 g. Four of the 10 data sets for calcium in meat and poultry products, determined by an EDTA titration method, (1983)-0989AEJ, have HORRATs of over 2. (**NOTE**: To avoid a long reference list, a nonredundant citation form is used for references to database entries discussed in the text: (year), first page of article in 4 digit format, and the 3-letter key to computer retrieval of the raw data from APL workspace $WAH/BERT. Citations refer to the *Journal of AOAC International* unless a different source is specified.) The HORRAT values for the other 6 products are between 1 and 2. Two of the assays measured a calcium content of 0.035 g/100 g (HORRATs of 7 and 9); the other 2 assays with high HORRAT values contained calcium at 0.1 and 0.2 g/100 g. The method, when used as a measure of bone content, requires a correction factor for natural calcium present in tissue of 0.015 g/100 g, a substantial fraction of the low values. Application of a limiting HORRAT value of 2 would require a lower limit of determination of 0.25 g calcium/100 g, which appears reasonable. Therefore, assays of calcium in meat and poultry products by titrimetric methods showing less than 0.25 g calcium/100 g were removed from the database as below the limit of determination. The corresponding methods should be revised to include a minimum specification for calcium, such as "at least 4 mg Ca with a 1 g test portion," which is equivalent to a titration volume of 1.0 mL 0.02N $KMnO_4$. The permanganate titration method for calcium in enriched flour, (1944)0402ACI, was acceptable down to 0.1 mg calcium/100 g, but this method required a larger quantity of analyte in the test solution.

Four of 12 determinations of calcium in the collaborative study of infant formula by ICPE, (1984)0985AFK, show unexplained high HORRAT values above 2.0. Almost all the other HORRAT values are between 1 and 2. Other elements in the same study also showed similar sporadically high HORRAT values. Because no explanation for the high values was offered in the report of the study, it may be surmised that the contamination problem was not emphasized sufficiently in the instructions. The 3 assays for calcium in infant formula by the atomic absorption method, (1985)0514AFO, are acceptable. Two substantial method-performance studies of calcium in cheese (10) had to be discarded by the organizer because of erratic results until instructions were added to the method emphasizing the necessity to avoid P, Ca, and Mg contamination.

The IUPAC-1987 protocol requires removal of outliers in the following order: Cochran, Grubbs, and multiple Grubbs. Such absolute rules occasionally produce an anomaly when fewer than 9 laboratories participate in the study. Atomic absorption spectroscopic analysis of a wheat flour by 8 laboratories, *Z. Lebensm. Forsch.* 190,0199(1990)AIO03CA, produced very extreme Cochran and Grubbs outliers from different laboratories, resulting in an RSD_R of 66% at 0.02 g calcium/100 g (HORRAT = 9). Because of the rule that stops outlier removal whenever 22.2% of the laboratories have been rejected, only the Cochran outlier could be removed. Removing the Cochran outlier hardly affected RSD_R and left an obvious "sore-thumb" outlying laboratory in the data set that would have been removed had one more satisfactory laboratory been present. Consequently, the IUPAC-1987 rule was overridden, and the obviously out-of-line pair of values from the second extreme laboratory was removed.

When examined by emission spectroscopy, (1983)-0764AHM, 5 test samples of plant materials that were candidates for the National Institute of Standards and Technology (NIST) standard reference materials (SRMs) all had HORRAT values of 2–4 at calcium concentrations of 0.4–3 g/100 g. Examination of the same and similar plant materials by atomic absorption spectroscopy, (1975)0436AHM, and inductively coupled plasma emission spectroscopy, (1985)0499AHL, also

Database 1. Identification and precision parameters of accepted assays for calcium, magnesium, phosphorus, potassium, sodium, and sulfur.

ANALYTE=CALCIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
ALFALFA	EMSP	1.686	PCT.	-	9.81	0/11	AHM01C	JONES 58,0764(1975)	<953.01>...	2.65
ALFALFA-LEA	ATAB	1.803	PCT.	-	7.04	0/ 7	AHN01A	ISAAC 58,0436(1975)	<975.03B(A)	1.92
ALFALFA-LEA	ATAB	1.787	PCT.	-	7.19	0/ 7	AHN01B	ISAAC 58,0436(1975)	<975.03B(B)	1.96
BEANS-LIMA-	VOLU	0.020	PCT.	6.54	10.68	2/13	AHP03	STEAGALL 51,0796(1968)	<968.31>...	1.48
BEEF	VOLU	0.583	PCT.	2.23	4.86	0/ 6	AEJ08A	CORRAO 66,0989(1983)	<24.062[84]	1.12
BEEF	VOLU	0.811	PCT.	3.32	5.58	0/ 6	AEJ10A	CORRAO 66,0989(1983)	<24.062[84]	1.35
BEEF	VOLU	0.429	PCT.	5.16	6.24	0/ 6	AEJ09A	CORRAO 66,0989(1983)	<24.062[84]	1.37
BERMUDA-GRA	ICPE	0.281	PCT.	-	4.91	0/12	AHL06C	ISAAC 68,0499(1985)	<975.03>...	1.01
BREAD	VOLU	0.118	PCT.	1.88	3.23	1/14	ACI3A	MUNSEY 27,0402(1944)	<14.014[84]	0.59
CHEESE-CHED	ATAB	0.698	PCT.	1.58	2.61	0/12	AIU03A	POLLMAN 74,0027(1991)	<991.25>...	0.62
CHEESE-MOZZ	ATAB	0.651	PCT.	1.52	2.32	0/12	AIU02A	POLLMAN 74,0027(1991)	<991.25>...	0.54
CHEESE-PARM	ATAB	1.107	PCT.	1.13	2.03	1/12	AIU05A	POLLMAN 74,0027(1991)	<991.25>...	0.52
CHEESE-PROC	ATAB	0.608	PCT.	1.63	2.71	1/12	AIU01A	POLLMAN 74,0027(1991)	<991.25>...	0.63
CHEESE-ROMA	ATAB	1.027	PCT.	1.74	3.17	0/12	AIU04A	POLLMAN 74,0027(1991)	<991.25>...	0.80
CITRUS-LEAV	ATAB	2.985	PCT.	1.19	5.98	1/ 9	AIO01C	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.76
CITRUS-LEAV	ATAB	3.143	PCT.	-	6.03	1/ 7	AHN02A	ISAAC 58,0436(1975)	<975.03B(A)	1.79
CITRUS-LEAV	ATAB	3.070	PCT.	-	6.32	0/ 7	AHN02B	ISAAC 58,0436(1975)	<975.03B(B)	1.87
CITRUS-LEAV	ICPE	3.122	PCT.	-	7.92	0/12	AHL07C	ISAAC 68,0499(1985)	<975.03>...	2.35
CITRUS-LEAV	EMSP	3.156	PCT.	-	11.31	0/10	AHM02C	JONES 58,0764(1975)	<953.01>...	3.36
CORN	ICPE	0.662	PCT.	-	3.15	0/12	AHL05C	ISAAC 68,0499(1985)	<975.03>...	0.74
CORN-LEAVES	ICPE	0.561	PCT.	-	6.06	0/12	AHL01C	ISAAC 68,0499(1985)	<975.03>...	1.39
CORN-STALK	ICPE	0.209	PCT.	-	8.52	0/12	AHL03C	ISAAC 68,0499(1985)	<975.03>...	1.68
FLOUR	VOLU	0.332	PCT.	1.09	1.86	1/14	ACI4A	MUNSEY 27,0402(1944)	<14.014[84]	0.39
FLOUR	VOLU	0.096	PCT.	1.46	5.27	0/14	ACI1A	MUNSEY 27,0402(1944)	<14.014[84]	0.93
FLOUR	VOLU	0.052	PCT.	3.05	6.34	0/ 9	ACI2A	MUNSEY 27,0402(1944)	<14.014[84]	1.02
INFANT-FORM	ATAB	0.046	PCT.	3.11	4.85	0/ 9	AFO1A	TANNER 68,0514(1985)	<43.A37[84]	0.76
INFANT-FORM	ATAB	0.059	PCT.	1.05	5.31	1/ 9	AFO2A	TANNER 68,0514(1985)	<43.A37[84]	0.87
INFANT-FORM	ICPE	0.118	PCT.	1.74	5.12	0/ 6	AFK06A	SUDDENDORF 67,0985(1984)	<984.27>...	0.93
INFANT-FORM	ATAB	0.060	PCT.	3.37	6.14	0/ 9	AFO3A	TANNER 68,0514(1985)	<43.A37[84]	1.01
INFANT-FORM	ICPE	0.066	PCT.	4.37	6.10	0/ 6	AFK03A	SUDDENDORF 67,0985(1984)	<984.27>...	1.01
INFANT-FORM	ICPE	0.048	PCT.	1.12	7.16	1/ 6	AFK02A	SUDDENDORF 67,0985(1984)	<984.27>...	1.13
INFANT-FORM	ICPE	0.190	PCT.	3.51	6.36	0/ 6	AFK01A	SUDDENDORF 67,0985(1984)	<984.27>...	1.24
INFANT-FORM	ICPE	0.403	PCT.	1.15	6.32	0/ 6	AFK05A	SUDDENDORF 67,0985(1984)	<984.27>...	1.38
INFANT-FORM	ICPE	0.115	PCT.	2.83	8.10	0/ 5	AFK02B	SUDDENDORF 67,0985(1984)	<984.27>...	1.46
INFANT-FORM	ICPE	0.211	PCT.	3.94	8.63	0/ 6	AFK06B	SUDDENDORF 67,0985(1984)	<984.27>...	1.71
INFANT-FORM	ICPE	0.675	PCT.	1.99	8.04	0/ 6	AFK04B	SUDDENDORF 67,0985(1984)	<984.27>...	1.89
INFANT-FORM	ICPE	1.105	PCT.	2.62	8.71	0/ 6	AFK05B	SUDDENDORF 67,0985(1984)	<984.27>...	2.21
INFANT-FORM	ICPE	0.127	PCT.	8.02	14.69	0/ 6	AFK03B	SUDDENDORF 67,0985(1984)	<984.27>...	2.69
INFANT-FORM	ICPE	0.343	PCT.	10.71	14.39	0/ 5	AFK04A	SUDDENDORF 67,0985(1984)	<984.27>...	3.06
INFANT-FORM	ICPE	0.424	PCT.	2.82	17.92	0/ 6	AFK01B	SUDDENDORF 67,0985(1984)	<984.27>...	3.94
MILK-POWDER	ATAB	1.236	PCT.	1.94	5.30	0/ 9	AIO04C	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.37
MILK-POWDER	ATAB	1.186	PCT.	1.41	5.84	0/ 9	AIO05C	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.50
MUSSEL-FLOU	ATAB	0.124	PCT.	3.56	6.45	0/ 9	AIO02C	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.18
ORCHARD-LEA	ATAB	2.020	PCT.	-	5.01	1/ 7	AHN03A	ISAAC 58,0436(1975)	<975.03B(A)	1.39
ORCHARD-LEA	ATAB	1.963	PCT.	-	5.28	0/ 7	AHN03B	ISAAC 58,0436(1975)	<975.03B(B)	1.46
ORCHARD-LEA	EMSP	2.008	PCT.	-	11.69	0/11	AHM03C	JONES 58,0764(1975)	<953.01>...	3.25
PEACH-LEAVE	ICPE	1.110	PCT.	-	3.65	1/12	AHL04C	ISAAC 68,0499(1985)	<975.03>...	0.93
PECAN-LEAVE	ATAB	1.285	PCT.	-	6.87	1/ 7	AHN04A	ISAAC 58,0436(1975)	<975.03B(A)	1.78
PINE-NEEDLE	EMSP	0.422	PCT.	-	15.06	0/10	AHM04C	JONES 58,0764(1975)	<953.01>...	3.31
POTATOES-CA	VOLU	0.067	PCT.	4.90	5.53	0/13	AHP01	STEAGALL 51,0796(1968)	<968.31>...	0.92
POULTRY	VOLU	0.553	PCT.	4.42	5.08	0/ 6	AEJ04A	CORRAO 66,0989(1983)	<24.062[84]	1.16
POULTRY	VOLU	0.723	PCT.	3.59	6.26	0/ 6	AEJ05A	CORRAO 66,0989(1983)	<24.062[84]	1.49
POULTRY	VOLU	0.280	PCT.	3.09	7.70	0/ 6	AEJ03A	CORRAO 66,0989(1983)	<24.062[84]	1.59
POWDER	VOLU	6.857	PCT.	0.84	1.91	1/13	AHQ02A	STEAGALL 50,0787(1967)	<968.31>...	0.64
POWDER	VOLU	2.399	PCT.	0.86	3.17	0/13	AHQ01A	STEAGALL 50,0787(1967)	<968.31>...	0.90
STANDARD-SO	ATAB	5.5	PPM	-	14.14	0/ 7	AHN06C	ISAAC 58,0436(1975)	<975.03C>..	1.14
TOMATO-JUIC	VOLU	0.028	PCT.	1.21	3.17	0/13	AHQ04A	STEAGALL 50,0787(1967)	<968.31>...	0.46
TOMATO-LEAV	ATAB	2.749	PCT.	-	7.30	0/ 7	AHN05B	ISAAC 58,0436(1975)	<975.03B(B)	2.12
TOMATO-LEAV	ATAB	2.819	PCT.	-	7.93	0/ 7	AHN05A	ISAAC 58,0436(1975)	<975.03B(A)	2.32
TOMATO-LEAV	ATAB	1.329	PCT.	-	10.38	0/ 7	AHN04B	ISAAC 58,0436(1975)	<975.03B(B)	2.71
TOMATO-LEAV	EMSP	2.740	PCT.	-	11.23	0/11	AHM05C	JONES 58,0764(1975)	<953.01>...	3.27
TOMATOES-CA	VOLU	0.030	PCT.	-	5.35	2/18	AHO01	STEAGALL 53,0720(1970)	<968.31>...	0.79
WHEAT-FLOUR	ATAB	0.015	PCT.	4.56	16.26	1/ 8	AIO03C	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	2.17

ANALYTE=MAGNESIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
ALFALFA	EMSP	0.357	PCT.	-	13.43	0/11	AHM01M	JONES 58,0764(1975)	<953.01>...	2.88
ALFALFA-LEA	ATAB	0.359	PCT.	-	5.90	0/ 7	AHN01A	ISAAC 58,0436(1975)	<975.03B(A)	1.26
ALFALFA-LEA	ATAB	0.363	PCT.	-	6.88	0/ 7	AHN01B	ISAAC 58,0436(1975)	<975.03B(B)	1.48
BERMUDA-GRA	ICPE	0.164	PCT.	-	12.90	1/12	AHL06M	ISAAC 68,0499(1985)	<975.03>...	2.46
CHEESE-CHED	ATAB	0.025	PCT.	3.92	4.32	0/11	AIU03C	POLLMAN 74,0027(1991)	<991.25>...	0.62
CHEESE-MOZZ	ATAB	0.024	PCT.	3.85	4.75	0/11	AIU02C	POLLMAN 74,0027(1991)	<991.25>...	0.68
CHEESE-PARM	ATAB	0.040	PCT.	0.73	2.54	1/11	AIU05C	POLLMAN 74,0027(1991)	<991.25>...	0.39
CHEESE-PROC	ATAB	0.024	PCT.	3.85	5.34	1/11	AIU01C	POLLMAN 74,0027(1991)	<991.25>...	0.76
CHEESE-ROMA	ATAB	0.051	PCT.	1.11	2.32	0/11	AIU04C	POLLMAN 74,0027(1991)	<991.25>...	0.37
CITRUS-LEAV	ATAB	0.533	PCT.	1.25	4.55	1/ 9	AIO01M	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.03
CITRUS-LEAV	ATAB	0.570	PCT.	-	8.83	0/ 7	AHN02B	ISAAC 58,0436(1975)	<975.03B(B)	2.03
CITRUS-LEAV	EMSP	0.593	PCT.	-	9.74	0/10	AHM02M	JONES 58,0764(1975)	<953.01>...	2.25
CITRUS-LEAV	ICPE	0.555	PCT.	-	10.77	0/12	AHL07M	ISAAC 68,0499(1985)	<975.03>...	2.46
CITRUS-LEAV	ATAB	0.537	PCT.	-	13.58	0/ 7	AHN02A	ISAAC 58,0436(1975)	<975.03B(A)	3.09
CORN-LEAVES	ICPE	0.410	PCT.	-	9.76	0/12	AHL01M	ISAAC 68,0499(1985)	<975.03>...	2.13
CORN-LEAVES	ICPE	0.152	PCT.	-	14.07	1/12	AHL05M	ISAAC 68,0499(1985)	<975.03>...	2.65
CORN-STALK	ICPE	0.274	PCT.	-	15.35	0/12	AHL03M	ISAAC 68,0499(1985)	<975.03>...	3.16
INFANT-FORM	ICPE	58.6	PPM	1.25	4.35	1/ 6	AFK03I	SUDDENDORF 67,0985(1984)	<984.27>...	0.50
INFANT-FORM	ICPE	74.6	PPM	1.52	4.88	1/ 6	AFK02I	SUDDENDORF 67,0985(1984)	<984.27>...	0.58
INFANT-FORM	ATAB	62.2	PPM	1.86	5.92	0/ 9	AFO2B	TANNER 68,0514(1985)	<43.A37[84]	0.69
INFANT-FORM	ATAB	73.6	PPM	2.22	5.86	0/ 9	AFO1B	TANNER 68,0514(1985)	<43.A37[84]	0.70
INFANT-FORM	ICPE	121.1	PPM	1.41	5.97	0/ 6	AFK06I	SUDDENDORF 67,0985(1984)	<984.27>...	0.77
INFANT-FORM	ICPE	418.8	PPM	0.72	5.10	1/ 6	AFK05I	SUDDENDORF 67,0985(1984)	<984.27>...	0.79
INFANT-FORM	ICPE	203.3	PPM	4.84	7.67	0/ 6	AFK06J	SUDDENDORF 67,0985(1984)	<984.27>...	1.07
INFANT-FORM	ATAB	61.6	PPM	2.67	9.55	0/ 9	AFO3B	TANNER 68,0514(1985)	<43.A37[84]	1.11
INFANT-FORM	ICPE	733.2	PPM	1.57	8.57	0/ 6	AFK05J	SUDDENDORF 67,0985(1984)	<984.27>...	1.45
INFANT-FORM	ICPE	415.0	PPM	2.96	9.43	0/ 6	AFK01J	SUDDENDORF 67,0985(1984)	<984.27>...	1.46
INFANT-FORM	ICPE	114.9	PPM	7.68	11.60	0/ 6	AFK03J	SUDDENDORF 67,0985(1984)	<984.27>...	1.48
INFANT-FORM	ICPE	139.1	PPM	3.33	11.79	0/ 5	AFK02J	SUDDENDORF 67,0985(1984)	<984.27>...	1.55
INFANT-FORM	ICPE	221.0	PPM	2.67	11.61	0/ 6	AFK01I	SUDDENDORF 67,0985(1984)	<984.27>...	1.64
INFANT-FORM	ICPE	1062.6	PPM	1.75	9.97	0/ 6	AFK04J	SUDDENDORF 67,0985(1984)	<984.27>...	1.78
INFANT-FORM	ICPE	457.5	PPM	22.98	25.33	0/ 5	AFK04I	SUDDENDORF 67,0985(1984)	<984.27>...	3.98

Database 1. ***Continued***

ANALYTE=MAGNESIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
MILK-POWDER	ATAB	0.106	PCT.	1.33	3.84	1/ 9	AIO05M	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	0.69
MILK-POWDER	ATAB	0.104	PCT.	2.75	4.60	1/ 9	AIO04M	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	0.82
MUSSEL-FLOU	ATAB	0.019	PCT.	1.59	3.07	1/ 9	AIO02M	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	0.42
ORCHARD-LEA	ATAB	0.590	PCT.	-	4.01	1/ 7	AHN03A	ISAAC 58,0436(1975)	<975.03B(A)	0.93
ORCHARD-LEA	EMSP	0.636	PCT.	-	6.71	0/11	AHM03M	JONES 58,0764(1975)	<953.01>...	1.57
ORCHARD-LEA	ATAB	0.600	PCT.	-	7.07	0/ 7	AHN03B	ISAAC 58,0436(1975)	<975.03B(B)	1.64
PEACH-LEAVE	ICPE	0.412	PCT.	-	15.29	0/12	AHL04M	ISAAC 68,0499(1985)	<975.03>...	3.34
PECAN-LEAVE	ATAB	0.404	PCT.	-	8.31	0/ 7	AHN04B	ISAAC 58,0436(1975)	<975.03B(B)	1.81
PECAN-LEAVE	ATAB	0.370	PCT.	-	17.93	0/ 7	AHN04A	ISAAC 58,0436(1975)	<975.03B(A)	3.86
PINE-NEEDLE	EMSP	0.132	PCT.	-	17.78	0/10	AHM04M	JONES 58,0764(1975)	<953.01>...	3.28
POWDER	VOLU	3.064	PCT.	0.64	1.29	0/13	AHQ01B	STEAGALL 50,0787(1967)	<968.31>...	0.38
POWDER	VOLU	1.785	PCT.	2.09	3.51	0/13	AHQ02B	STEAGALL 50,0787(1967)	<968.31>...	0.96
TOMATO-LEAV	ATAB	0.671	PCT.	-	6.34	0/ 7	AHN05A	ISAAC 58,0436(1975)	<975.03B(A)	1.49
TOMATO-LEAV	ATAB	0.673	PCT.	-	7.56	0/ 7	AHN05B	ISAAC 58,0436(1975)	<975.03B(B)	1.78
TOMATO-LEAV	EMSP	0.697	PCT.	-	7.62	0/11	AHM05M	JONES 58,0764(1975)	<953.01>...	1.80
WHEAT-FLOUR	ATAB	0.034	PCT.	4.37	10.16	0/ 9	AIO03M	NSMUNTERFORSCH 190,0199(1990)	<IUPAC>....	1.53

ANALYTE=PHOSPHORUS

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
APRICOT-PRE	SPEC	103.7	PPM	0.74	2.81	1/ 9	AIJ02	GERRITZ 24,0393(1941)	<22.037[70]	0.35
BOLOGNA	AUTO	0.184	PCT.	-	2.39	0/ 6	AIE06A	GANTENBEIN 55,0123(1972)	<972.22>...	0.46
BOLOGNA	AUTO	0.265	PCT.	-	2.28	0/ 6	AIE05A	GANTENBEIN 55,0123(1972)	<972.22>...	0.47
BREAD	VOLU	0.157	PCT.	2.31	12.81	1/12	AIB01B	MUNSEY 31,0269(1948)	<948.09BB>.	2.42
BREAD	VOLU	0.165	PCT.	2.04	14.01	1/12	AIB01A	MUNSEY 31,0269(1948)	<948.09BA>.	2.67
CEREAL-INFA	VOLU	0.668	PCT.	1.16	2.22	1/12	AIB03B	MUNSEY 31,0269(1948)	<948.09BB>.	0.52
CEREAL-INFA	VOLU	0.668	PCT.	1.77	2.64	1/12	AIB03A	MUNSEY 31,0269(1948)	<948.09BA>.	0.62
CHEESE-CHED	SPEC	0.468	PCT.	1.47	2.05	1/12	AIU03B	POLLMAN 74,0027(1991)	<991.25>...	0.46
CHEESE-MOZZ	SPEC	0.444	PCT.	1.32	1.85	1/12	AIU02B	POLLMAN 74,0027(1991)	<991.25>...	0.41
CHEESE-PARM	SPEC	0.698	PCT.	1.17	2.11	2/12	AIU05B	POLLMAN 74,0027(1991)	<991.25>...	0.50
CHEESE-ROMA	SPEC	0.662	PCT.	1.15	1.15	2/12	AIU04B	POLLMAN 74,0027(1991)	<991.25>...	0.27
CHERRY-PRES	SPEC	102.5	PPM	0.78	2.80	1/ 9	AIJ01	GERRITZ 24,0393(1941)	<22.037[70]	0.35
CITRUS-LEAV	ICPE	0.130	PCT.	-	8.88	0/13	AHL07P	ISAAC 68,0499(1985)	<985.01>...	1.63
CORN-LEAVES	ICPE	0.271	PCT.	-	5.78	1/13	AHL05P	ISAAC 68,0499(1985)	<985.01>...	1.19
CORN-LEAVES	ICPE	0.279	PCT.	-	7.38	0/13	AHL01P	ISAAC 68,0499(1985)	<985.01>...	1.52
CORN-STALK	ICPE	0.162	PCT.	-	11.84	0/13	AHL03P	ISAAC 68,0499(1985)	<985.01>...	2.25
EGGS-DRIED	VOLU	0.863	PCT.	0.45	1.70	0/ 5	AHZ01C	MITCHELL 16,0298(1933)	<923.07>...	0.42
FLOUR-SELF-	VOLU	0.521	PCT.	1.64	3.04	2/12	AIB04A	MUNSEY 31,0269(1948)	<948.09BA>.	0.69
FLOUR-SELF-	VOLU	0.515	PCT.	0.49	5.79	1/12	AIB04B	MUNSEY 31,0269(1948)	<948.09BB>.	1.31
FRANKFURTER	AUTO	0.094	PCT.	-	1.73	0/ 6	AIE01A	GANTENBEIN 55,0123(1972)	<972.22>...	0.30
FRANKFURTER	AUTO	0.111	PCT.	-	2.88	0/ 6	AIE02A	GANTENBEIN 55,0123(1972)	<972.22>...	0.52
GELATIN	GRAV	0.119	PCT.	-	1.81	0/ 6	AIF01A	BURKEPILE 55,0581(1972)	<972.21>...	0.33
GELATIN-DES	GRAV	0.246	PCT.	-	2.12	0/ 6	AIF02A	BURKEPILE 55,0581(1972)	<972.21>...	0.43
GELATIN-DES	GRAV	0.027	PCT.	-	10.19	0/ 6	AIF04A	BURKEPILE 55,0581(1972)	<972.21>...	1.48
GELATIN-DES	GRAV	0.048	PCT.	-	13.93	0/ 6	AIF03A	BURKEPILE 55,0581(1972)	<972.21>...	2.21
GRAPE-JUICE	SPEC	113.5	PPM	0.75	1.46	1/ 9	AIJ03	GERRITZ 24,0393(1941)	<22.037[70]	0.19
GRAPE-JUICE	SPEC	113.6	PPM	0.43	1.96	0/ 9	AIJ04	GERRITZ 24,0393(1941)	<22.037[70]	0.25
GRASS-BERMU	ICPE	0.145	PCT.	-	6.69	0/13	AHL06P	ISAAC 68,0499(1985)	<985.01>...	1.25
HAM	AUTO	0.248	PCT.	-	2.56	0/ 6	AIE08A	GANTENBEIN 55,0123(1972)	<972.22>...	0.52
HAM	AUTO	0.276	PCT.	-	3.36	0/ 6	AIE07A	GANTENBEIN 55,0123(1972)	<972.22>...	0.69
HAM	GRAV	0.302	PCT.	2.19	3.76	1/ 9	AID03A	OKAMOTO 52,0634(1969)	<962.31A>..	0.78
HAM	GRAV	0.303	PCT.	3.11	3.79	0/ 9	AID02B	OKAMOTO 52,0634(1969)	<962.31B>..	0.79
HAM	GRAV	0.354	PCT.	2.30	3.72	0/ 9	AID04B	OKAMOTO 52,0634(1969)	<962.31B>..	0.80
HAM	GRAV	0.316	PCT.	3.07	3.82	0/ 9	AID06B	OKAMOTO 52,0634(1969)	<962.31B>..	0.80
HAM	GRAV	0.283	PCT.	3.72	4.03	0/ 9	AID05B	OKAMOTO 52,0634(1969)	<962.31B>..	0.83
HAM	GRAV	0.299	PCT.	2.23	4.04	0/ 9	A1D03B	OKAMOTO 52,0634(1969)	<962.31B>..	0.84
HAM	GRAV	0.354	PCT.	1.22	3.97	1/ 9	AID04A	OKAMOTO 52,0634(1969)	<962.31A>..	0.85
HAM	GRAV	0.307	PCT.	2.88	4.09	0/ 9	AID02A	OKAMOTO 52,0634(1969)	<962.31A>..	0.86
HAM	GRAV	0.273	PCT.	2.73	4.43	0/ 9	AID01B	OKAMOTO 52,0634(1969)	<962.31A>..	0.91
HAM	GRAV	0.274	PCT.	3.55	4.94	0/ 9	AID01A	OKAMOTO 52,0634(1969)	<962.31A>..	1.02
HAM	GRAV	0.310	PCT.	4.43	7.99	0/ 9	AID06A	OKAMOTO 52,0634(1969)	<962.31A>..	1.67
HAM	GRAV	0.281	PCT.	6.27	9.18	0/ 9	AID05A	OKAMOTO 52,0634(1969)	<962.31A>..	1.90
HAM-CANNED	GRAV	0.236	PCT.	1.89	4.29	0/ 5	AIT15B	CHRISTIANS 74,0022(1991)	<969.31B>..	0.86
HAM-CANNED	SPEC	0.237	PCT.	2.25	5.52	0/ 7	AIT13A	CHRISTIANS 74,0022(1991)	<991.27>...	1.11
HAM-CANNED	SPEC	0.233	PCT.	4.29	5.80	0/ 7	AIT14A	CHRISTIANS 74,0022(1991)	<991.27>...	1.16
HAM-CANNED	SPEC	0.237	PCT.	3.56	6.68	0/ 7	AIT15A	CHRISTIANS 74,0022(1991)	<991.27>...	1.34
HAM-CANNED	GRAV	0.234	PCT.	5.06	6.92	0/ 5	AIT14B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.39
HAM-SMOKED	GRAV	0.299	PCT.	1.18	3.55	1/ 5	AIT01B	CHRISTIANS 74,0022(1991)	<969.31B>..	0.74
HAM-SMOKED	SPEC	0.221	PCT.	2.41	4.00	0/ 7	AIT02A	CHRISTIANS 74,0022(1991)	<991.27>...	0.80
HAM-SMOKED	SPEC	0.296	PCT.	2.56	4.01	0/ 7	AIT01A	CHRISTIANS 74,0022(1991)	<991.27>...	0.83
HAM-SMOKED	SPEC	0.221	PCT.	4.52	4.52	0/ 7	AIT03A	CHRISTIANS 74,0022(1991)	<991.27>...	0.90
HAM-SMOKED	GRAV	0.214	PCT.	3.62	6.57	0/ 5	AIT02B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.30
HAM-SMOKED	GRAV	0.213	PCT.	4.92	11.13	0/ 5	AIT03B	CHRISTIANS 74,0022(1991)	<969.31B>..	2.20
HAM-WATER-A	GRAV	0.244	PCT.	2.59	3.55	0/ 5	AIT05B	CHRISTIANS 74,0022(1991)	<969.31B>..	0.72
HAM-WATER-A	SPEC	0.257	PCT.	4.65	4.69	0/ 7	AIT06A	CHRISTIANS 74,0022(1991)	<991.27>...	0.96
HAM-WATER-A	SPEC	0.234	PCT.	3.43	5.03	0/ 7	AIT04A	CHRISTIANS 74,0022(1991)	<991.27>...	1.01
HAM-WATER-A	GRAV	0.254	PCT.	3.05	5.53	0/ 5	AIT06B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.12
HAM-WATER-A	SPEC	0.241	PCT.	2.71	5.77	0/ 7	AIT05A	CHRISTIANS 74,0022(1991)	<991.27>...	1.16
HAM-WATER-A	GRAV	0.231	PCT.	2.37	5.85	0/ 5	AIT04B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.17
HAMBURGER	GRAV	0.170	PCT.	5.88	7.50	0/ 5	AIT16B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.44
HAMBURGER	SPEC	0.175	PCT.	4.58	7.53	0/ 7	AIT16A	CHRISTIANS 74,0022(1991)	<991.27>...	1.45
HAMBURGER-7	SPEC	0.135	PCT.	7.67	7.67	0/ 7	AIT18A	CHRISTIANS 74,0022(1991)	<991.27>...	1.42
HAMBURGER-7	GRAV	0.129	PCT.	2.75	8.24	1/ 5	AIT18B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.51
HAMBURGER-8	GRAV	0.139	PCT.	2.55	2.55	1/ 5	AIT17B	CHRISTIANS 74,0022(1991)	<969.31B>..	0.47
HAMBURGER-8	SPEC	0.141	PCT.	2.67	6.94	0/ 7	AIT17A	CHRISTIANS 74,0022(1991)	<991.27>...	1.29
INFANT-FORM	SPEC	449.3	PPM	0.62	0.73	1/ 8	AFP2B	TANNER 69,0777(1986)	<965.17>...	0.11
INFANT-FORM	SPEC	472.9	PPM	0.84	1.53	0/ 8	AFP3B	TANNER 69,0777(1986)	<965.17>...	0.24
INFANT-FORM	SPEC	339.3	PPM	2.04	3.90	0/ 8	AFP1B	TANNER 69,0777(1986)	<965.17>...	0.59
INFANT-FORM	ICPE	0.094	PCT.	1.31	5.11	0/ 6	AFK06O	SUDDENDORF 67,0985(1984)	<984.27>...	0.90
INFANT-FORM	ICPE	0.042	PCT.	5.47	7.43	0/ 6	AFK03O	SUDDENDORF 67,0985(1984)	<984.27>...	1.15
INFANT-FORM	ICPE	0.034	PCT.	1.58	8.22	1/ 6	AFK02O	SUDDENDORF 67,0985(1984)	<984.27>...	1.23
INFANT-FORM	ICPE	0.068	PCT.	3.25	8.47	0/ 5	AFK02P	SUDDENDORF 67,0985(1984)	<984.27>...	1.41
INFANT-FORM	ICPE	0.327	PCT.	1.22	7.40	1/ 6	AFK05O	SUDDENDORF 67,0985(1984)	<984.27>...	1.56
INFANT-FORM	ICPE	0.138	PCT.	0.45	9.89	1/ 6	AFK01O	SUDDENDORF 67,0985(1984)	<984.27>...	1.83
INFANT-FORM	ICPE	0.237	PCT.	1.45	9.46	1/ 6	AFK01P	SUDDENDORF 67,0985(1984)	<984.27>...	1.90
INFANT-FORM	ICPE	0.585	PCT.	2.00	8.55	0/ 6	AFK04P	SUDDENDORF 67,0985(1984)	<984.27>...	1.97
INFANT-FORM	ICPE	0.190	PCT.	4.60	11.18	0/ 6	AFK06P	SUDDENDORF 67,0985(1984)	<984.27>...	2.18
INFANT-FORM	ICPE	0.676	PCT.	1.80	10.46	0/ 6	AFK05P	SUDDENDORF 67,0985(1984)	<984.27>...	2.47
INFANT-FORM	ICPE	0.104	PCT.	7.57	15.10	0/ 6	AFK03P	SUDDENDORF 67,0985(1984)	<984.27>...	2.68

Database 1. *Continued*

ANALYTE=PHOSPHORUS

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
INFANT-FORM	ICPE	0.245	PCT.	10.77	15.55	0/ 5	AFK04O	SUDDENDORF 67,0985(1984)	<984.27>...	3.15
OATMEAL	VOLU	0.460	PCT.	1.16	3.18	1/12	AIB02B	MUNSEY 31,0269(1948)	<948.09BB>.	0.71
OATMEAL	VOLU	0.474	PCT.	2.56	5.13	1/12	AIB02A	MUNSEY 31,0269(1948)	<948.09BB>.	1.15
OIL	ATAB	11.9	PPM	7.66	10.33	0/18	AIV06A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	0.94
OIL	ATAB	8.5	PPM	8.07	11.70	1/18	AIV05A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	1.01
OIL	ATAB	21.1	PPM	7.97	11.47	0/18	AIV03A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	1.13
OIL	ATAB	19.3	PPM	8.40	11.92	1/18	AIV04A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	1.16
OIL	ATAB	30.4	PPM	9.01	14.14	1/18	AIV01A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	1.48
OIL	ATAB	28.8	PPM	14.72	14.72	0/18	AIV02A	HENDRIKSE PACO0,0000(1991)	<PURE.APPLI	1.53
ORCHARD-LEA	EMSP	0.203	PCT.	-	23.04	0/11	AHM03P	JONES 58,0764(1975)	<953.01>...	4.53
PEACH-LEAVE	ICPE	0.167	PCT.	-	13.70	0/13	AHL04P	ISAAC 68,0499(1985)	<985.01>...	2.62
PINE-NEEDLE	EMSP	0.142	PCT.	-	23.43	0/10	AHM04P	JONES 58,0764(1975)	<953.01>...	4.37
PORK-SMOKED	AUTO	0.181	PCT.	-	1.46	0/ 6	AIE04A	GANTENBEIN 55,0123(1972)	<972.22>...	0.28
PORK-SMOKED	AUTO	0.207	PCT.	-	1.78	0/ 6	AIE03A	GANTENBEIN 55,0123(1972)	<972.22>...	0.35
SAUSAGE-COO	SPEC	0.127	PCT.	5.15	5.15	0/ 7	AIT11A	CHRISTIANS 74,0022(1991)	<991.27>...	0.94
SAUSAGE-COO	GRAV	0.107	PCT.	-	8.91	1/ 5	AIT12B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.59
SAUSAGE-COO	GRAV	0.124	PCT.	7.21	8.83	0/ 5	AIT11B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.61
SAUSAGE-COO	SPEC	0.114	PCT.	3.31	9.21	0/ 7	AIT12A	CHRISTIANS 74,0022(1991)	<991.27>...	1.66
SAUSAGE-COO	SPEC	0.133	PCT.	6.36	9.75	0/ 7	AIT10A	CHRISTIANS 74,0022(1991)	<991.27>...	1.80
SAUSAGE-COO	GRAV	0.125	PCT.	6.32	10.25	0/ 5	AIT10B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.87
SAUSAGE-POR	SPEC	0.126	PCT.	3.66	6.04	0/ 7	AIT07A	CHRISTIANS 74,0022(1991)	<991.27>...	1.11
SAUSAGE-POR	GRAV	0.122	PCT.	6.35	6.48	0/ 5	AIT07B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.18
SAUSAGE-POR	GRAV	0.109	PCT.	5.02	6.96	0/ 5	AIT09B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.25
SAUSAGE-POR	GRAV	0.099	PCT.	7.14	9.03	0/ 5	AIT08B	CHRISTIANS 74,0022(1991)	<969.31B>..	1.59
SAUSAGE-POR	SPEC	0.103	PCT.	3.67	9.19	0/ 7	AIT08A	CHRISTIANS 74,0022(1991)	<991.27>...	1.63
SAUSAGE-POR	SPEC	0.111	PCT.	3.39	10.86	0/ 7	AIT09A	CHRISTIANS 74,0022(1991)	<991.27>...	1.95
SOLN-STD	VOLU	219.9	PPM	0.39	1.85	1/ 8	AEL05B	MUNSEY 36,0760(1953)	<14.139[84]	0.26
SOLN-STD	VOLU	222.3	PPM	0.88	3.11	0/ 8	AEL05A	MUNSEY 36,0760(1953)	<14.139[84]	0.44
TOMATO-LEAV	EMSP	0.335	PCT.	-	19.38	0/11	AHM05P	JONES 58,0764(1975)	<953.01>...	4.11
VINEGAR-CID	VOLU	0.006	PCT.	0.32	2.13	1/ 9	AIQ01A	SANDERS 24,0684(1941)	<930.35I>..	0.25
VINEGAR-COR	VOLU	0.003	PCT.	0.53	6.62	0/ 9	AIQ05A	SANDERS 24,0684(1941)	<930.35I>..	0.68
VINEGAR-DIS	VOLU	0.001	PCT.	1.30	3.26	0/ 9	AIQ02A	SANDERS 24,0684(1941)	<930.35I>..	0.29
VINEGAR-MAL	VOLU	0.041	PCT.	1.03	2.94	0/ 9	AIQ03A	SANDERS 24,0684(1941)	<930.35I>..	0.46
VINEGAR-MOL	VOLU	0.003	PCT.	0.70	3.94	2/ 9	AIQ04A	SANDERS 24,0684(1941)	<930.35I>..	0.40
WINE	SPEC	112.2	PPM	-	0.45	1/ 8	AIC06A	NELSON 45,0624(1962)	<962.11CA>.	0.06
WINE	SPEC	135.8	PPM	-	0.90	0/ 8	AIC08A	NELSON 45,0624(1962)	<962.11CA>.	0.12
WINE	SPEC	130.0	PPM	-	1.13	0/ 8	AIC07B	NELSON 45,0624(1962)	<962.11CB>.	0.15
WINE	SPEC	113.4	PPM	-	1.17	0/ 8	AIC06B	NELSON 45,0624(1962)	<962.11CB>.	0.15
WINE	SPEC	168.4	PPM	-	1.22	0/ 7	AIC02A	NELSON 45,0624(1962)	<962.11>...	0.16
WINE	SPEC	69.0	PPM	-	1.41	0/ 8	AIC05A	NELSON 45,0624(1962)	<962.11CA>.	0.17
WINE	SPEC	69.4	PPM	-	1.88	0/ 8	AIC05B	NELSON 45,0624(1962)	<962.11CB>.	0.22
WINE	SPEC	135.5	PPM	-	1.91	0/ 8	AIC08B	NELSON 45,0624(1962)	<962.11CB>.	0.25
WINE	SPEC	130.3	PPM	-	2.14	0/ 8	AIC07A	NELSON 45,0624(1962)	<962.11CA>.	0.28
WINE	SPEC	106.9	PPM	-	2.56	0/ 7	AIC03A	NELSON 45,0624(1962)	<962.11>...	0.32
WINE	SPEC	110.5	PPM	-	2.64	0/ 7	AIC04A	NELSON 45,0624(1962)	<962.11>...	0.34
WINE	SPEC	148.9	PPM	-	2.95	0/ 7	AIC01A	NELSON 45,0624(1962)	<962.11>...	0.39

ANALYTE=POTASSIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
ALFALFA-LEA	ATAB	1.738	PCT.	-	2.04	0/ 6	AHN01B	ISAAC 58,0436(1975)	<975.03B(B)	0.55
ALFALFA-LEA	ATAB	1.678	PCT.	-	4.83	0/ 6	AHN01A	ISAAC 58,0436(1975)	<975.03B(A)	1.31
APRICOT-FRU	GRAV	345.9	PPM	0.31	1.14	0/ 6	AII01A	WOOD 25,0429(1942)	<22.028[80]	0.17
APRICOT-FRU	GRAV	3487.8	PPM	0.55	2.10	0/ 6	AII01B	WOOD 25,0429(1942)	<22.026[70]	0.45
CHERRY-FRUI	GRAV	4570.8	PPM	0.45	1.62	0/ 5	AIK02A	WOOD 24,0391(1941)	<22.028[80]	0.36
CHERRY-FRUI	GRAV	1638.1	PPM	0.85	2.65	0/ 7	AIK01A	WOOD 24,0391(1941)	<22.028[80]	0.50
CHERRY-FRUI	GRAV	1635.9	PPM	1.47	4.24	0/ 8	AIK01B	WOOD 24,0391(1941)	<22.026[70]	0.81
CHERRY-FRUI	GRAV	4432.1	PPM	2.08	4.05	0/ 7	AIK02B	WOOD 24,0391(1941)	<22.026[70]	0.90
CITRUS-LEAV	ATAB	1.782	PCT.	-	1.28	1/ 6	AHN02A	ISAAC 58,0436(1975)	<975.03B(A)	0.35
CITRUS-LEAV	ICPE	1.834	PCT.	-	1.88	2/ 9	AHL07K	ISAAC 68,0499(1985)	<985.01>...	0.51
CITRUS-LEAV	ATAB	1.803	PCT.	-	3.84	0/ 6	AHN02B	ISAAC 58,0436(1975)	<975.03B(B)	1.05
COD	ATAB	3374.0	PPM	1.72	7.70	0/ 8	ADJ6C	THOMPSON 52,0055(1969)	<969.23>...	1.63
COD	ATAB	3657.5	PPM	4.34	14.56	0/ 8	ADJ6D	THOMPSON 52,0055(1969)	<969.23>...	3.13
CORN-LEAVES	ICPE	2.079	PCT.	-	8.23	0/ 9	AHL01K	ISAAC 68,0499(1985)	<985.01>...	2.30
CORN-LEAVES	ICPE	2.127	PCT.	-	9.32	0/ 9	AHL05K	ISAAC 68,0499(1985)	<985.01>...	2.61
CORN-STALK	ICPE	1.139	PCT.	-	8.52	0/ 9	AHL03K	ISAAC 68,0499(1985)	<985.01>...	2.17
CRAB	ATAB	1597.8	PPM	4.35	6.60	0/ 8	ADJ8C	THOMPSON 52,0055(1969)	<969.23>...	1.25
CRAB	ATAB	1691.0	PPM	2.85	11.62	0/ 8	ADJ8D	THOMPSON 52,0055(1969)	<969.23>...	2.22
CREAMER-COF	ATAB	0.993	PCT.	2.65	6.72	0/10	AGY03B	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.68
CURRANT-JUI	GRAV	2140.0	PPM	0.70	0.80	1/ 6	AII03B	WOOD 25,0429(1942)	<22.026[70]	0.16
CURRANT-JUI	GRAV	2133.9	PPM	0.66	0.88	0/ 8	AIL01A	GERRITZ 25,0433(1942)	<22.028[80]	0.17
CURRANT-JUI	GRAV	2129.2	PPM	0.68	0.96	0/ 6	AII03A	WOOD 25,0429(1942)	<22.028[80]	0.19
CURRANT-JUI	VOLU	2131.8	PPM	0.43	1.21	0/ 8	AIL01B	GERRITZ 25,0433(1942)	.<...>.....	0.24
DIET-TOTAL	ATAB	0.639	PCT.	1.90	7.73	0/10	AGY02B	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.81
DIET-TOTAL	ATAB	0.648	PCT.	3.09	8.38	1/10	AGY01B	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.96
FRUIT	FLME	1917.2	PPM	1.30	2.21	0/ 9	ACB5	BOLAND 48,0521(1965)	<22.031[84]	0.43
FRUIT	FLME	1796.1	PPM	0.99	2.61	0/ 9	ACB4	BOLAND 48,0521(1965)	<22.031[84]	0.50
FRUIT	FLME	2100.6	PPM	1.24	3.04	0/ 9	ACB3	BOLAND 48,0521(1965)	<22.031[84]	0.60
FRUIT	FLME	2480.6	PPM	1.57	3.12	0/ 9	ACB1	BOLAND 48,0521(1965)	<22.031[84]	0.63
FRUIT	FLME	1667.8	PPM	3.44	3.44	0/ 9	ACB6	BOLAND 48,0521(1965)	<22.031[84]	0.66
FRUIT	FLME	2140.6	PPM	1.95	4.23	0/ 9	ACB2	BOLAND 48,0521(1965)	<22.031[84]	0.84
FRUIT-ASH	GRAV	1257.7	PPM	1.17	3.02	0/ 7	AIK04A	WOOD 24,0391(1941)	<22.028[80]	0.55
FRUIT-ASH	GRAV	786.9	PPM	1.49	3.61	0/ 5	AII02A	WOOD 25,0429(1942)	<22.028[80]	0.62
FRUIT-ASH	GRAV	808.7	PPM	2.71	3.93	0/ 4	AII02B	WOOD 25,0429(1942)	<22.026[70]	0.67
FRUIT-ASH	GRAV	1237.8	PPM	0.82	4.38	0/ 8	AIK04B	WOOD 24,0391(1941)	<22.026[70]	0.80
GRASS-BERMU	ICPE	0.850	PCT.	-	8.84	0/ 9	AHL06K	ISAAC 68,0499(1985)	<985.01>...	2.16
HADDOCK	ATAB	2836.3	PPM	2.24	3.45	0/ 8	ADJ5D	THOMPSON 52,0055(1969)	<969.23>...	0.71
HADDOCK	ATAB	2716.3	PPM	3.09	4.39	0/ 8	ADJ5C	THOMPSON 52,0055(1969)	<969.23>...	0.90
INFANT-FORM	ATAB	933.0	PPM	2.92	3.28	1/ 9	AFO3D	TANNER 68,0514(1985)	<43.A37[84]	0.57
INFANT-FORM	ICPE	0.071	PCT.	1.31	3.51	1/ 5	AFK02G	SUDDENDORF 67,0985(1984)	<984.27>...	0.59
INFANT-FORM	ICPE	0.079	PCT.	4.65	4.65	0/ 5	AFK03G	SUDDENDORF 67,0985(1984)	<984.27>...	0.79
INFANT-FORM	ICPE	0.949	PCT.	1.22	3.81	1/ 5	AFK05G	SUDDENDORF 67,0985(1984)	<984.27>...	0.95
INFANT-FORM	ATAB	754.0	PPM	2.23	5.95	1/ 9	AFO2D	TANNER 68,0514(1985)	<43.A37[84]	1.01
INFANT-FORM	ICPE	0.174	PCT.	1.95	5.25	0/ 5	AFK06G	SUDDENDORF 67,0985(1984)	<984.27>...	1.01
INFANT-FORM	ICPE	0.136	PCT.	3.15	5.86	0/ 5	AFK02H	SUDDENDORF 67,0985(1984)	<984.27>...	1.09
INFANT-FORM	ATAB	761.6	PPM	3.07	6.82	0/ 9	AFO1D	TANNER 68,0514(1985)	<43.A37[84]	1.16
INFANT-FORM	ICPE	1.887	PCT.	3.73	5.51	0/ 5	AFK01G	SUDDENDORF 67,0985(1984)	<984.27>...	1.52
INFANT-FORM	ICPE	0.257	PCT.	6.63	7.85	0/ 6	AFK06H	SUDDENDORF 67,0985(1984)	<984.27>...	1.60
INFANT-FORM	ICPE	0.483	PCT.	4.35	7.23	0/ 4	AFK04G	SUDDENDORF 67,0985(1984)	<984.27>...	1.62
INFANT-FORM	ICPE	0.136	PCT.	3.96	9.02	0/ 6	AFK03H	SUDDENDORF 67,0985(1984)	<984.27>...	1.67
INFANT-FORM	ICPE	0.294	PCT.	8.67	9.32	0/ 6	AFK01H	SUDDENDORF 67,0985(1984)	<984.27>...	1.94

Database 1. *Continued*

ANALYTE=POTASSIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
INFANT-FORM	ICPE	1.150	PCT.	1.32	8.63	0/ 5	AFK04H	SUDDENDORF 67,0985(1984)	<43.292[84]	2.20
INFANT-FORM	ICPE	1.552	PCT.	2.79	12.61	0/ 6	AFK05H	SUDDENDORF 67,0985(1984)	<984.27>...	3.37
KALE-POWDER	ATAB	2.340	PCT.	2.82	9.16	0/10	AGY04B	P-A-CHEM 61,0114(1989)	<GIVEN>....	2.60
LIVER-BOVIN	ATAB	0.942	PCT.	2.36	6.31	0/10	AGY05B	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.56
ORCHARD-LEA	ATAB	1.454	PCT.	-	0.38	1/ 6	AHN03B	ISAAC 58,0436(1975)	<975.03B(B)	0.10
ORCHARD-LEA	ATAB	1.438	PCT.	-	2.48	1/ 6	AHN03A	ISAAC 58,0436(1975)	<975.03B(A)	0.65
PEACH-LEAVE	ICPE	2.081	PCT.	-	6.45	1/ 9	AHL04K	ISAAC 68,0499(1985)	<985.01>...	1.80
PECAN-LEAVE	ATAB	1.002	PCT.	-	2.38	1/ 6	AHN04A	ISAAC 58,0436(1975)	<975.03B(A)	0.60
PECAN-LEAVE	ATAB	1.090	PCT.	-	3.62	0/ 6	AHN04B	ISAAC 58,0436(1975)	<975.03B(B)	0.92
PINEAPPLE-J	GRAV	147.8	PPM	0.49	1.62	0/ 6	AII05A	WOOD 25,0429(1942)	<22.028[80]	0.21
PINEAPPLE-J	GRAV	148.8	PPM	0.34	1.81	0/ 6	AII05B	WOOD 25,0429(1942)	<22.026[70]	0.24
PINEAPPLE-J	VOLU	1471.4	PPM	0.37	1.31	0/ 8	AIL03B	GERRITZ 25,0433(1942)	.<...>.....	0.25
PINEAPPLE-J	GRAV	1474.5	PPM	0.33	1.36	0/ 8	AIL03A	GERRITZ 25,0433(1942)	<22.028[80]	0.25
RASPBERRY-J	VOLU	1412.6	PPM	-	0.46	1/ 7	AIL02B	GERRITZ 25,0433(1942)	.<...>.....	0.09
RASPBERRY-J	GRAV	1408.1	PPM	0.96	0.96	0/ 8	AIL02A	GERRITZ 25,0433(1942)	<22.028[80]	0.18
RASPBERRY-J	GRAV	1405.6	PPM	1.14	1.14	0/ 6	AII04A	WOOD 25,0429(1942)	<22.028[80]	0.21
RASPBERRY-J	GRAV	1431.9	PPM	0.77	1.42	0/ 6	AII04B	WOOD 25,0429(1942)	<22.026[70]	0.26
SHERRY	FLEM	904.5	PPM	-	1.85	2/10	AIH02B	MEURON 47,0720(1964)	.<...>.....	0.32
SHRIMP	ATAB	2361.5	PPM	2.10	9.77	0/ 8	ADJ7C	THOMPSON 52,0055(1969)	<969.23>...	1.97
SHRIMP	ATAB	2539.5	PPM	1.75	10.04	0/ 8	ADJ7D	THOMPSON 52,0055(1969)	<969.23>...	2.04
SNAPPER	ATAB	4320.3	PPM	4.19	6.99	0/ 8	ADJ4D	THOMPSON 52,0055(1969)	<969.23>...	1.54
SNAPPER	ATAB	4148.3	PPM	3.23	9.97	0/ 8	ADJ4C	THOMPSON 52,0055(1969)	<969.23>...	2.18
SOLN	ATAB	943.8	PPM	-	3.25	0/ 8	ADJ1C	THOMPSON 52,0055(1969)	<969.23>...	0.57
SOLN	ATAB	615.0	PPM	-	4.35	0/ 8	ADJ2C	THOMPSON 52,0055(1969)	<969.23>...	0.71
SOLN	ATAB	468.8	PPM	-	4.62	0/ 8	ADJ3C	THOMPSON 52,0055(1969)	<969.23>...	0.73
SUGAR-BLANK	GRAV	83.3	PPM	2.04	9.37	0/ 5	AIK03B	WOOD 24,0391(194[illegible]	<22.026[70]	1.14
SUGAR-BLANK	GRAV	82.2	PPM	2.37	10.06	0/ 7	AIK03A	WOOD 24,0391(1941)	<22.028[80]	1.22
TOMATO-LEAV	ATAB	4.658	PCT.	-	1.92	0/ 6	AHN05A	ISAAC 58,0436(1975)	<975.03B(A)	0.61
TOMATO-LEAV	ATAB	4.583	PCT.	-	2.43	0/ 6	AHN05B	ISAAC 58,0436(1975)	<975.03B(B)	0.76
TUNA	ATAB	3020.0	PPM	5.83	7.91	0/ 8	ADJ9C	THOMPSON 52,0055(1969)	<969.23>...	1.65
TUNA	ATAB	3210.0	PPM	3.28	12.66	0/ 8	ADJ9D	THOMPSON 52,0055(1969)	<969.23>...	2.67
WINE	FLEM	925.0	PPM	-	2.87	0/ 7	AIG06B	MEURON 46,0299(1963)	<963.13>...	0.50
WINE	FLEM	464.4	PPM	-	4.20	0/ 7	AIG07B	MEURON 46,0299(1963)	<963.13>...	0.66
WINE	FLEM	711.0	PPM	-	6.10	0/ 7	AIG08B	MEURON 46,0299(1963)	<963.13>...	1.02
WINE-APPLE	FLEM	1140.6	PPM	-	3.26	0/10	AIH04B	MEURON 47,0720(1964)	.<...>.....	0.59
WINE-BLACKB	FLEM	892.8	PPM	-	1.84	2/10	AIH05B	MEURON 47,0720(1964)	.<...>.....	0.32
WINE-DRY-RE	FLEM	1320.9	PPM	-	4.88	0/10	AIG03B	MEURON 46,0299(1963)	<963.13>...	0.90
WINE-DRY-RE	FLEM	553.1	PPM	-	5.70	0/10	AIG04B	MEURON 46,0299(1963)	<963.13>...	0.92
WINE-LOGANB	FLEM	718.4	PPM	-	1.86	1/10	AIH03B	MEURON 47,0720(1964)	.<...>.....	0.31
WINE-PORT	FLEM	1191.7	PPM	-	5.43	0/10	AIG05B	MEURON 46,0299(1963)	<963.13>...	0.99

ANALYTE=SODIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
APPLE-JUICE	ATAB	14.8	PPM	-	0.00	1/ 9	ADI2A	BOLAND 49,0617(1966)	<22.032[84]	0.00
APPLE-JUICE	ATAB	573.1	PPM	-	1.80	1/ 9	ADI5A	BOLAND 49,0617(1966)	<22.032[84]	0.29
APRICOTS	ATAB	3667.5	PPM	-	2.32	0/ 9	ADI6A	BOLAND 49,0617(1966)	<22.032[84]	0.50
APRICOTS	ATAB	20.6	PPM	-	30.00	0/ 9	ADI3A	BOLAND 49,0617(1966)	<22.032[84]	2.96
BEEF-BROTH	POTC	0.583	PCT.	0.22	0.38	1/10	AEH01A	BRAMMELL 57,1209(1974)	<32.034[84]	0.09
BEEF-ROAST	KIT	0.401	PCT.	4.31	6.79	0/11	AHS02A	WERF 54,0587(1971)	<971.19>..	1.48
BEETS	IONE	0.068	PCT.	2.62	4.59	0/ 6	AAC4	MCNERNEY 59,1131(1976)	<43.271[84]	0.77
BRANDY-APPL	FLEM	20.0	PPM	-	11.18	0/ 9	AIH01A	MEURON 47,0720(1964)	<963.09>...	1.10
BREAD	POTC	0.605	PCT.	0.22	0.45	1/10	AEH07A	BRAMMELL 57,1209(1974)	<32.034[84]	0.10
BUTTER	VOLU	1.143	PCT.	0.24	0.64	1/ 8	AGS05A	IDF 235,0020(1988)	<IDF12A[69]	0.16
BUTTER	VOLU	0.825	PCT.	0.32	0.70	0/ 8	AGS04A	IDF 235,0020(1988)	<IDF12A[69]	0.17
BUTTER	VOLU	1.153	PCT.	0.27	0.70	0/ 8	AGS06A	IDF 235,0020(1988)	<IDF12A[69]	0.18
BUTTER	VOLU	0.197	PCT.	0.56	0.92	0/ 8	AGS01A	IDF 235,0020(1988)	<IDF12A[69]	0.18
BUTTER	VOLU	1.138	PCT.	0.39	0.85	0/ 8	AGS06B	IDF 235,0020(1988)	<IDF12A[69]	0.22
BUTTER	VOLU	1.128	PCT.	0.53	0.96	0/ 8	AGS05B	IDF 235,0020(1988)	<IDF12A[69]	0.24
BUTTER	VOLU	0.195	PCT.	0.52	1.50	0/ 8	AGS01B	IDF 235,0020(1988)	<IDF12A[69]	0.29
BUTTER	VOLU	0.578	PCT.	1.05	1.28	0/ 8	AGS02A	IDF 235,0020(1988)	<IDF12A[69]	0.29
BUTTER	VOLU	0.573	PCT.	0.89	1.43	0/ 8	AGS02B	IDF 235,0020(1988)	<IDF12A[69]	0.33
BUTTER	VOLU	0.805	PCT.	0.29	1.39	0/ 7	ADH1B	WEIK 49,0518(1966)	<16.235[84]	0.34
BUTTER	VOLU	0.608	PCT.	1.25	1.51	0/ 8	AGS03A	IDF 235,0020(1988)	<IDF12A[69]	0.35
BUTTER	VOLU	0.813	PCT.	0.37	1.48	1/ 8	AGS04B	IDF 235,0020(1988)	<IDF12A[69]	0.36
BUTTER	VOLU	0.944	PCT.	0.40	1.78	1/ 7	ADH4B	WEIK 49,0518(1966)	<16.235[84]	0.44
BUTTER	VOLU	0.601	PCT.	0.80	1.95	1/ 8	AGS03B	IDF 235,0020(1988)	<IDF12A[69]	0.45
BUTTER	VOLU	0.593	PCT.	0.71	2.12	0/ 7	ADH7B	WEIK 49,0518(1966)	<16.235[84]	0.49
BUTTER	VOLU	0.883	PCT.	0.41	2.38	1/ 7	ADH3B	WEIK 49,0518(1966)	<16.235[84]	0.58
BUTTER	VOLU	0.508	PCT.	1.01	2.77	0/ 7	ADH5B	WEIK 49,0518(1966)	<16.235[84]	0.63
BUTTER	VOLU	0.392	PCT.	2.46	3.36	0/ 7	ADH2B	WEIK 49,0518(1966)	<16.235[84]	0.73
BUTTER	VOLU	0.822	PCT.	2.20	3.44	0/ 7	ADH1A	WEIK 49,0518(1966)	<15.135[65]	0.84
BUTTER	VOLU	0.604	PCT.	0.46	4.23	1/ 7	ADH7A	WEIK 49,0518(1966)	<15.135[65]	0.98
BUTTER	VOLU	0.506	PCT.	1.31	4.47	0/ 7	ADH5A	WEIK 49,0518(1966)	<15.135[65]	1.01
BUTTER	VOLU	0.389	PCT.	4.58	6.23	0/ 7	ADH2A	WEIK 49,0518(1966)	<15.135[65]	1.35
BUTTER	VOLU	0.939	PCT.	0.50	5.75	1/ 7	ADH4A	WEIK 49,0518(1966)	<15.135[65]	1.42
BUTTER	VOLU	0.887	PCT.	4.55	6.38	0/ 7	ADH3A	WEIK 49,0518(1966)	<15.135[65]	1.57
CARROTS	IONE	0.015	PCT.	1.56	4.79	0/ 6	AAC1	MCNERNEY 59,1131(1976)	<43.271[84]	0.64
CARROTS-BAB	POTC	0.089	PCT.	0.55	1.78	1/10	AEH06A	BRAMMELL 57,1209(1974)	<32.034[84]	0.31
CHEESE	VOLU	1.203	PCT.	0.33	0.97	0/ 8	ACS03C	POORTVLIET 65,1350(1982)	<16.268[84]	0.25
CHEESE	VOLU	0.642	PCT.	0.51	1.20	0/ 8	ACS01B	POORTVLIET 65,1350(1982)	<16.272[84]	0.28
CHEESE	VOLU	1.171	PCT.	0.48	1.47	0/ 8	ACS03B	POORTVLIET 65,1350(1982)	<16.272[84]	0.38
CHEESE	VOLU	0.657	PCT.	0.43	1.69	0/ 8	ACS01C	POORTVLIET 65,1350(1982)	<16.268[84]	0.40
CHEESE	VOLU	0.975	PCT.	0.47	1.60	0/ 8	ACS02C	POORTVLIET 65,1350(1982)	<16.268[84]	0.40
CHEESE	VOLU	0.959	PCT.	0.72	1.85	0/ 8	ACS02A	POORTVLIET 65,1350(1982)	<16.272[84]	0.46
CHEESE	VOLU	0.650	PCT.	1.16	2.02	1/ 8	ACS01A	POORTVLIET 65,1350(1982)	<16.272[84]	0.47
CHEESE	VOLU	0.957	PCT.	0.31	1.95	1/ 8	ACS02B	POORTVLIET 65,1350(1982)	<16.272[84]	0.48
CHEESE	VOLU	0.552	PCT.	1.60	2.21	0/11	ABJ1B	STONE 20,0339(1937)	<16.272[84]	0.51
CHEESE	VOLU	1.173	PCT.	0.50	2.54	0/ 8	ACS03A	POORTVLIET 65,1350(1982)	<16.272[84]	0.65
CHEESE-BLUE	KIT	1.846	PCT.	2.80	6.05	0/12	AHS06A	WERF 54,0587(1971)	<971.19>..	1.66
CHEESE-CHED	KIT	0.638	PCT.	2.28	3.38	0/12	AHS05A	WERF 54,0587(1971)	<971.19>..	0.79
CHEESE-PARM	POTC	1.458	PCT.	0.33	1.01	0/10	AEH09A	BRAMMELL 57,1209(1974)	<32.034[84]	0.27
COD-DRIED-S	VOLU	12.608	PCT.	0.24	0.82	0/ 6	ACQ2B	GRIGSBY 23,0589(1940)	<18.034[84]	0.30
CRAB	ATAB	2448.8	PPM	2.33	3.30	0/ 8	ADJ8A	THOMPSON 52,0055(1969)	<18.038[84]	0.67
CRAB	ATAB	2530.5	PPM	4.70	5.19	0/ 8	ADJ8B	THOMPSON 52,0055(1969)	<18.038[84]	1.06

Database 1. *Continued*

ANALYTE=SODIUM

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
CREAMER-COF	ATAB	0.246	PCT.	3.24	8.32	0/10	AGY03A	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.68
DIET-TOTAL	ATAB	0.668	PCT.	1.88	3.71	1/10	AGY02A	P-A-CHEM 61,0114(1989)	<GIVEN>....	0.87
DIET-TOTAL	ATAB	0.642	PCT.	1.52	4.57	2/10	AGY01A	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.07
DINNER-PREP	POTC	0.320	PCT.	0.33	0.86	0/10	AEH08A	BRAMMELL 57,1209(1974)	<32.034[84]	0.18
DISTD-SPIRI	VOLU	23.3	PPM	-	2.88	0/ 9	AHR02A	HUBACH 49,0498(1966)	<966.09>...	0.29
EGG-WHITE	VOLU	3.931	PCT.	0.10	0.28	1/ 5	AHW01A	MITCHELL 22,0302(1939)	<17.025[84]	0.09
EGGS-DRIED	VOLU	0.409	PCT.	1.71	2.44	0/ 5	AHZ01E	MITCHELL 16,0298(1933)	<17.026[84]	0.53
EGGS-SALTED	POTC	4.062	PCT.	0.34	5.38	1/10	AEH03A	BRAMMELL 57,1209(1974)	<32.034[84]	1.66
FISH	ATAB	3282.0	PPM	1.90	3.57	0/ 8	ADJ5A	THOMPSON 52,0055(1969)	<18.038[84]	0.75
FISH	ATAB	3344.3	PPM	1.71	3.89	0/ 8	ADJ5B	THOMPSON 52,0055(1969)	<18.038[84]	0.82
FISH	ATAB	930.5	PPM	4.20	6.94	0/ 8	ADJ4A	THOMPSON 52,0055(1969)	<18.038[84]	1.21
FISH	ATAB	2166.8	PPM	4.43	7.05	0/ 8	ADJ6B	THOMPSON 52,0055(1969)	<18.038[84]	1.40
FISH	ATAB	1014.3	PPM	9.32	9.32	0/ 8	ADJ4B	THOMPSON 52,0055(1969)	<18.038[84]	1.65
FISH	ATAB	2044.5	PPM	3.78	9.02	0/ 8	ADJ6A	THOMPSON 52,0055(1969)	<18.038[84]	1.78
GREEN-BEANS	IONE	0.007	PCT.	3.32	10.92	0/ 6	AAC5	MCNERNEY 59,1131(1976)	<43.271[84]	1.28
HAM	POTC	1.236	PCT.	0.27	0.88	0/10	AEH11A	BRAMMELL 57,1209(1974)	<32.034[84]	0.23
INFANT-FORM	ICPE	0.032	PCT.	1.37	4.07	0/ 6	AFK03M	SUDDENDORF 67,0985(1984)	<984.27>...	0.60
INFANT-FORM	ICPE	0.232	PCT.	3.32	3.82	0/ 6	AFK04N	SUDDENDORF 67,0985(1984)	<984.27>...	0.77
INFANT-FORM	VOLU	0.038	PCT.	0.37	5.53	1/ 8	AFP2A	TANNER 69,0777(1986)	<43.B06[84]	0.85
INFANT-FORM	VOLU	0.029	PCT.	0.46	6.04	1/ 8	AFP1A	TANNER 69,0777(1986)	<43.B06[84]	0.88
INFANT-FORM	ICPE	0.048	PCT.	2.66	5.82	0/ 5	AFK02N	SUDDENDORF 67,0985(1984)	<984.27>...	0.92
INFANT-FORM	VOLU	0.035	PCT.	0.52	6.60	1/ 8	AFP3A	TANNER 69,0777(1986)	<43.B06[84]	1.00
INFANT-FORM	ICPE	0.053	PCT.	3.36	6.81	0/ 6	AFK06M	SUDDENDORF 67,0985(1984)	<984.27>...	1.10
INFANT-FORM	ATAB	232.3	PPM	2.30	8.17	1/ 9	AFO3C	TANNER 68,0514(1985)	<43.A37[84]	1.16
INFANT-FORM	ICPE	0.072	PCT.	3.56	7.03	0/ 6	AFK01M	SUDDENDORF 67,0985(1984)	<984.27>...	1.18
INFANT-FORM	ATAB	241.3	PPM	4.16	8.52	0/ 9	AFO2C	TANNER 68,0514(1985)	<43.A37[84]	1.22
INFANT-FORM	ICPE	0.230	PCT.	3.36	6.43	0/ 6	AFK05M	SUDDENDORF 67,0985(1984)	<984.27>...	1.29
INFANT-FORM	ATAB	175.2	PPM	5.22	10.94	0/ 9	AFO1C	TANNER 68,0514(1985)	<43.A37[84]	1.49
INFANT-FORM	ICPE	0.176	PCT.	4.21	8.92	0/ 6	AFK01N	SUDDENDORF 67,0985(1984)	<984.27>...	1.72
INFANT-FORM	ICPE	0.529	PCT.	1.92	7.56	0/ 6	AFK05N	SUDDENDORF 67,0985(1984)	<984.27>...	1.72
INFANT-FORM	ICPE	0.118	PCT.	4.36	10.25	0/ 4	AFK04M	SUDDENDORF 67,0985(1984)	<984.27>...	1.86
INFANT-FORM	ICPE	0.059	PCT.	5.37	11.79	0/ 6	AFK03N	SUDDENDORF 67,0985(1984)	<984.27>...	1.92
INFANT-FORM	ICPE	0.098	PCT.	2.66	11.11	0/ 6	AFK06N	SUDDENDORF 67,0985(1984)	<984.27>...	1.96
INFANT-FORM	ICPE	0.017	PCT.	8.80	17.11	0/ 6	AFK02M	SUDDENDORF 67,0985(1984)	<984.27>...	2.32
KALE-POWDER	ATAB	0.040	PCT.	3.68	17.23	0/10	AGY04A	P-A-CHEM 61,0114(1989)	<GIVEN>....	2.66
LIMA-BEANS	IONE	0.005	PCT.	2.90	2.90	1/ 6	AAC3	MCNERNEY 59,1131(1976)	<43.271[84]	0.32
LIMA-BEANS	IONE	0.069	PCT.	2.10	6.72	0/ 6	AAC9	MCNERNEY 59,1131(1976)	<43.271[84]	1.12
LIVER-BOVIN	ATAB	0.234	PCT.	1.88	6.17	0/10	AGY05A	P-A-CHEM 61,0114(1989)	<GIVEN>....	1.24
MARGARINE	POTC	0.779	PCT.	2.13	6.01	0/10	AEH04A	BRAMMELL 57,1209(1974)	<32.034[84]	1.45
MEAT-LUNCHE	POTC	0.236	PCT.	1.48	1.53	1/ 8	AGP02B	BELJAARS 68,0480(1985)	<32.032[84]	0.31
MEAT-LUNCHE	POTC	0.425	PCT.	1.13	2.19	0/ 8	AGP03B	BELJAARS 68,0480(1985)	<32.032[84]	0.48
MEAT-LUNCHE	POTC	0.143	PCT.	1.47	2.82	1/ 8	AGP01B	BELJAARS 68,0480(1985)	<32.032[84]	0.53
MEAT-LUNCHE	POTC	0.809	PCT.	1.76	2.62	0/ 8	AGP04B	BELJAARS 68,0480(1985)	<32.032[84]	0.63
MEAT-LUNCHE	VOLU	0.761	PCT.	1.38	9.18	1/11	AGP04A	BELJAARS 68,0480(1985)	<ISO1841>..	2.20
MEAT-LUNCHE	VOLU	0.383	PCT.	4.26	12.73	0/11	AGP03A	BELJAARS 68,0480(1985)	<ISO1841>..	2.75
MUSTARD-PRE	POTC	1.235	PCT.	0.28	0.49	0/10	AEH05A	BRAMMELL 57,1209(1974)	<32.034[84]	0.13
PEANUT-BUTT	POTC	0.561	PCT.	0.74	1.25	2/10	AEH12A	BRAMMELL 57,1209(1974)	<32.034[84]	0.29
PEAS	IONE	0.002	PCT.	9.06	30.01	0/ 6	AAC7	MCNERNEY 59,1131(1976)	<43.271[84]	3.00
SALMON-CANN	POTC	0.581	PCT.	0.40	1.04	0/10	AEH10A	BRAMMELL 57,1209(1974)	<32.034[84]	0.24
SALMON-CANN	VOLU	0.540	PCT.	0.30	2.13	1/ 9	ACQ1B	GRIGSBY 23,0589(1940)	<18.034[84]	0.49
SARDINES-CA	KIT	0.715	PCT.	2.87	9.17	0/12	AHS04A	WERF 54,0587(1971)	<971.19>..	2.18
SHERRY	FLEM	48.6	PPM	-	13.93	0/10	AIH02A	MEURON 47,0720(1964)	<963.13>...	1.56
SHRIMP	ATAB	1855.3	PPM	7.73	7.73	0/ 8	ADJ7A	THOMPSON 52,0055(1969)	<18.038[84]	1.50
SHRIMP	ATAB	1958.5	PPM	6.01	7.99	0/ 8	ADJ7B	THOMPSON 52,0055(1969)	<18.038[84]	1.56
SOLN	ATAB	205.7	PPM	-	2.60	1/ 8	ADJ1A	THOMPSON 52,0055(1969)	<18.038[84]	0.36
SOLN	ATAB	340.0	PPM	-	6.67	0/ 8	ADJ2A	THOMPSON 52,0055(1969)	<18.038[84]	1.00
SOLN	ATAB	340.0	PPM	-	6.67	0/ 8	ADJ3A	THOMPSON 52,0055(1969)	<18.038[84]	1.00
SOY-SAUCE	POTC	6.926	PCT.	0.65	1.07	0/ 8	AEH02A	BRAMMELL 57,1209(1974)	<32.034[84]	0.36
TOMATO	ATAB	22.3	PPM	-	0.00	1/ 9	ADI1A	BOLAND 49,0617(1966)	<22.032[84]	0.00
TOMATO-JUIC	ATAB	2190.3	PPM	-	4.43	0/ 9	ADI4A	BOLAND 49,0617(1966)	<22.032[84]	0.88
TOMATO-PAST	VOLU	0.147	PCT.	2.81	7.72	0/10	AHY01A	BEACHAM 24,0424(1941)	<32.032[84]	1.45
TOMATO-PAST	VOLU	0.371	PCT.	1.36	12.26	1/15	AHX01B	BEACHAM 23,0353(1940)	<32.033[84]	2.64
TOMATO-PAST	VOLU	0.361	PCT.	0.84	13.29	1/13	AHX01A	BEACHAM 23,0353(1940)	<XXXV.22[35	2.85
TOMATO-PURE	VOLU	0.326	PCT.	1.05	2.59	2/10	AHY02A	BEACHAM 24,0424(1941)	<32.032[84]	0.55
TUNA	ATAB	4980.3	PPM	1.44	2.38	0/ 8	ADJ9A	THOMPSON 52,0055(1969)	<18.038[84]	0.54
TUNA	ATAB	5177.1	PPM	2.91	7.77	0/ 7	ADJ9B	THOMPSON 52,0055(1969)	<18.038[84]	1.76
TUNA-CANNED	KIT	0.311	PCT.	-	11.79	1/12	AHS03A	WERF 54,0587(1971)	<971.19>..	2.47
WAX-BEANS	IONE	0.009	PCT.	4.18	5.36	1/ 6	AAC8	MCNERNEY 59,1131(1976)	<43.271[84]	0.66
WAX-BEANS	IONE	0.005	PCT.	2.95	7.03	0/ 6	AAC2	MCNERNEY 59,1131(1976)	<43.271[84]	0.78
WIENERS	KIT	1.003	PCT.	2.51	4.73	0/11	AHS01A	WERF 54,0587(1971)	<971.19>..	1.18
WINE	VOLU	49.2	PPM	-	0.99	0/ 9	AHR04A	HUBACH 49,0498(1966)	<966.09>...	0.11
WINE	VOLU	36.3	PPM	-	1.55	0/ 9	AHR03A	HUBACH 49,0498(1966)	<966.09>...	0.17
WINE-APPLE	FLEM	21.0	PPM	-	25.40	0/10	AIH04A	MEURON 47,0720(1964)	<963.13>...	2.51
WINE-BLACKB	FLEM	161.5	PPM	-	6.35	0/10	AIH05A	MEURON 47,0720(1964)	<963.13>...	0.85
WINE-DRY-RE	FLEM	795.5	PPM	-	2.82	0/10	AIG04A	MEURON 46,0299(1963)	<963.13>...	0.48
WINE-DRY-RE	FLEM	93.6	PPM	-	8.84	0/10	AIG03A	MEURON 46,0299(1963)	<963.13>...	1.09
WINE-LOGANB	FLEM	84.6	PPM	-	9.33	0/10	AIH03A	MEURON 47,0720(1964)	<963.13>...	1.14
WINE-PORT	FLEM	141.2	PPM	-	6.53	0/10	AIG05A	MEURON 46,0299(1963)	<963.13>...	0.86

ANALYTE=SULFUR

MATRIX	METHOD	CONCN	UNIT	RSDO	RSDR	DROP/ #LABS	CODE	REFERENCE	METHOD NO.	HORRAT
COTTONSEED-	GRAV	0.510	PCT.	3.41	8.27	1/ 6	AIP01B	06,0414(1923)	<923.01>...	1.87
COTTONSEED-	GRAV	0.516	PCT.	5.52	9.20	0/ 5	AIP01A	06,0414(1923)	<923.01>...	2.08
COTTONSEED-	GRAV	0.514	PCT.	7.87	9.61	0/ 5	AIP01C	06,0414(1923)	<923.01>...	2.17
MUSTARDSEED	GRAV	0.863	PCT.	1.90	2.67	1/ 5	AIP03C	06,0414(1923)	<923.01>...	0.65
MUSTARDSEED	GRAV	0.866	PCT.	0.76	3.23	0/ 6	AIP03B	06,0414(1923)	<923.01>...	0.79
MUSTARDSEED	GRAV	0.867	PCT.	4.10	4.31	0/ 5	AIP03A	06,0414(1923)	<923.01>...	1.05
SOYBEAN-MEA	GRAV	0.405	PCT.	9.04	9.04	0/ 5	AIP02C	06,0414(1923)	<923.01>...	1.97
SOYBEAN-MEA	GRAV	0.385	PCT.	5.73	11.17	0/ 5	AIP02A	06,0414(1923)	<923.01>...	2.42
SOYBEAN-MEA	GRAV	0.392	PCT.	6.32	11.16	0/ 6	AIP02B	06,0414(1923)	<923.01>...	2.42

Database 1. ***Continued***

OBS	ANALYT	NO	NR	CONC_AV	RSDO_AV	RSDR_AV	RSDO_05	RSDO_95	RSDR_05	RSDR_95	HORR_05	HORR_95
1	CALCIUM	40	63	0.0103063	2.90625	6.97683	0.8695	7.9460	2.088	14.9860	0.521016	3.29869
2	MAGNESIUM	27	48	0.0032811	3.21889	8.49417	0.6720	16.8600	2.419	17.8625	0.385940	3.62773
3	PHOSPHORUS	91	125	0.0019054	3.33824	6.13640	0.4500	8.2020	1.185	14.5460	0.165463	2.65334
4	POTASSIUM	61	91	0.0059270	2.25262	4.88637	0.3430	5.7120	0.928	10.6840	0.173323	2.60570
5	SODIUM	108	129	0.0061135	2.06509	5.33419	0.2535	5.7220	0.565	13.6100	0.118834	2.57515
6	SULFUR	9	9	0.0059088	4.96111	7.62889	0.7600	9.0400	2.670	11.1700	0.652860	2.42298
7	GRAND	336	465	0.0052175	2.71437	6.05535	0.3300	7.6875	0.960	14.1400	0.178711	2.74090

showed high HORRAT values. Because the methods were adopted for the analysis of plant materials, the assays were retained in Database 1. The same type of variability was exhibited when the same test samples were analyzed for other elements in the same study. Analysts should be aware of the propensity of emission spectroscopic methods to produce such highly variable results, although they may be acceptable for the intended application of monitoring soil nutrient levels.

The RSD_R and HORRAT values for the 63 accepted calcium data sets are plotted directly as functions of –log concentration in Figures 2A and 2B, and as CDCs in Figures 2C and 2D. The CDCs show on the y-axis the percentage of values (RSD_R or HORRAT) equal to or less than any given value on the x-axis. In Figure 2D, for example, about 75% of the plotted HORRAT values are at or below 2.0. Figures 2A and 2B show that the many poor precision (high RSD_R) assays are at the higher concentrations, between 0.1 and 10 g calcium/100 g. The high overall variability could arise from heterogeneity of the test material, interference by other elements present, or from unsuspected contamination. A summary statement of the distribution of the HORRAT values for calcium is incorporated in Table 1. Certified reference materials (e.g., nonfat dry milk and various biological tissues) are available to provide quality control and proficiency checks. House and formulated standards for this analyte can be easily prepared and stored to maintain acceptable levels of analytical proficiency.

Magnesium

Forty-eight data sets are available for magnesium; almost all are results by various spectroscopic methods. Only 2 collaborative data sets involving a titrimetric method were found. The data are plotted as scatterplots and CDCs in Figure 3, and summarized in Table 1. The CDC of the HORRAT values for magnesium, Figure 1 (symbol M), is almost identical to that for calcium (symbol C), with the same percentage of values (about 30%) over a HORRAT of 2.0.

Phosphorus

Because the published data for the automated method for phosphorus in meats, (1972)0123AIE, did not give the individual results, the averages of duplicates were treated as single values. About a 10% correction (to higher RSD_R values) would be required to adjust the values to single results. Such a correction would hardly alter the already exceptionally good (0.3–0.5) HORRAT values.

Some of the collaborative studies for phosphorus and potassium in fruit products were performed on ash solutions, resulting in abnormally low HORRAT values. Because all steps of the method were not included in the studies, the results from these abbreviated studies were not included in Database 1.

A few studies of standard solutions were also omitted because the results were reported on a percent recovery basis, making the denominator of the HORRAT unrecoverable. One data set from a recent study of meat products, (1991)0022AIT, had to be omitted because of a computer artifact. Identical blind duplicates from each of the 5 participating laboratories caused a 0/0 problem that the computer program could not handle.

Overall, 90% of the official AOAC assays for phosphorus had HORRAT values of 2.0 or smaller, but here again the poorest precision shown in Figure 4 was at the higher concentrations. The high overall precision is also shown by the position of the "P" curve in the left part of Figure 1.

Potassium

Both potassium and sodium added to distilled spirits as nonnutritional markers at the 20–30 ppm level, (1963)0299AIG and (1964)0720AIH, were designated as impurities and not included in the database of acceptable assays. In the review of precision parameters for pesticide formulations (11), it was found that methods for minor ingredients with maximum specification limits were not as well-optimized as methods developed for composition specifications. The parameters from assays by the similar flame emission spectroscopic method for wines, which was used to determine about 0.1 g potassium/100 g, were satisfactory.

Although emission spectroscopic methods, (1975)-0764AHM, produced very high HORRAT values of 4–7 for potassium in many of the candidate plant tissue SRMs, these values are acceptable for plant tissue analysis; however, they do not appear to be good enough for nutritional purposes. Therefore, they were removed from the database of acceptable assays. In the corresponding studies of the same plant tissues by atomic absorption spectroscopy, (1975)0436AHN, and inductively coupled plasma emission spectroscopy, (1985)-0499AHL, results from 1 of 7 and 1 of 10 laboratories, respectively, were consistently low, but not sufficiently low to be rejected by the Grubbs test for a single study. Results for a synthetic standard analyzed simultaneously by the laboratory giving the low values were also low for this element, indicating an improperly prepared working standard solution. The

Table 1. Summary of statistical precision parameters from the database of interlaboratory determinations of inorganic elements of nutritional interest in food

		HORRAT			90% interval	
Analyte	No. data sets	Av.	Median	Concn range, g/100 g	RSD_R, %	HORRAT
Calcium	63	1.54	1.38	0.015–6.9	2.1–15	0.5–3.3
Magnesium	48	1.57	1.48	0.006–3.0	2.4–18	0.4–3.6
Phosphorus	125	1.10	0.94	0.001–0.9	1.2–15	0.2–2.7
Potassium	91	1.03	0.80	0.008–4.7	0.9–11	0.2–2.6
Sodium	129	0.94	0.77	0.002–13	0.6–14	0.1–2.6
Sulfur	9	1.7	2.0	0.4–0.9	2.7–11	0.7–2.4
Overall	465				1.0–14	0.2–2.7

IUPAC-1987 rules were overridden to remove the data from this laboratory from all the assays for potassium.

The scatterplots and CDCs for 91 assays for potassium are shown in Figure 5, and summaries of the precision and CDC parameters are given in Tables 1 and 2, respectively.

Sodium, Sodium Chloride

Forty years ago the direct determination of sodium was very difficult, but the introduction of emission and absorption spectroscopic methods and the sodium ion electrode have greatly simplified the methodology. Most natural foods have a relatively low sodium content, to which salt may be added for enhanced flavor at levels of about 1–4 g sodium/100 g. At these levels, sodium was and still is estimated indirectly by the relatively simple titrimetric methods for the chloride ion. Assay results initially reported as chloride, sodium chloride, and salt as surrogates for sodium were recalculated and labeled as sodium in the database.

The nonspecific titrimetric method for the determination of parts-per-million chlorine, (1939)0539ACY, which serves as an indicator of the use of chlorine-bleaching agents in flour, was removed from Database 1 as unacceptable methodology for current requirements. Similarly, the applications of the titrimetric methods to nuts, (1951)0357ADT, and to unsalted butter, (1966)0518ADH, at sodium levels below 0.1 g/100 g, were removed from Database 1 as below the practical limit of determination. Titrimetric methods for salt should be restricted to levels greater than 0.1 g sodium (0.2 g as NaCl)/100 g.

The extremely poor precision of a 4-assay study of several elements including sodium, by atomic absorption spectroscopy, *Cereal Foods World* (1987)0443AEZ, even for a cereal product containing 1 g sodium/100 g (HORRAT = 2.6), indicates inhomogeneity. The entire study was not accepted for Database 1. Several other assays using atomic absorption spectroscopy, (1966)0617ADI, and the selective-ion electrode, (1976)1131AAC, in studies of fruits and vegetables containing sodium at the 10–20 ppm level also showed very poor precision, supporting a lower limit of determination of about 25 ppm for this element by these methods. Because many different methods exhibit sporadic high RSD_R values, the variability probably arises from external factors such as contamination. Until about 20 years ago, the role of contamination from airborne dust and residual detergent components on glassware was not appreciated for such common elements as aluminum, calcium, iron, magnesium, and sodium.

The series of 5 candidate NIST standard reference materials examined by emission spectroscopy, (1975)0764AHM, showed extremely high HORRAT values for sodium. The author had stated that ". . . ac spark excitation is not well-suited for analysis for sodium at these low concentrations (less than 1000 ppm)." Such a note should also appear in the applicability statement of the official method.

After the removal of the invalid data sets for the reasons given, 129 remain for the interpretation of the reliability of the determination of sodium in foods; 11 (8%) of these have

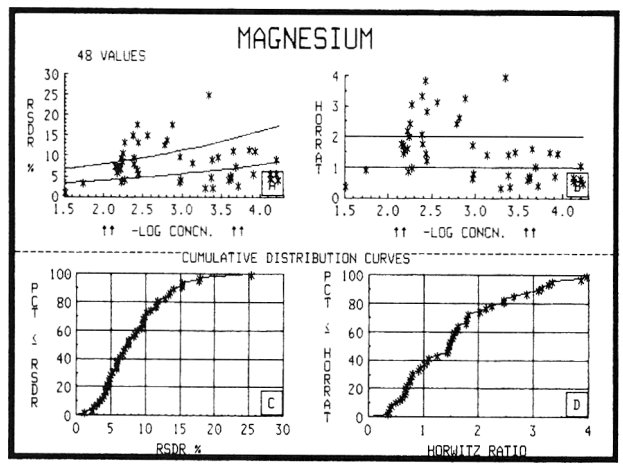

Figure 3. Precision parameters RSD_R and HORRAT of magnesium as a function of concentration (-log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against -log C. (B) Scatterplot of HORRAT against -log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

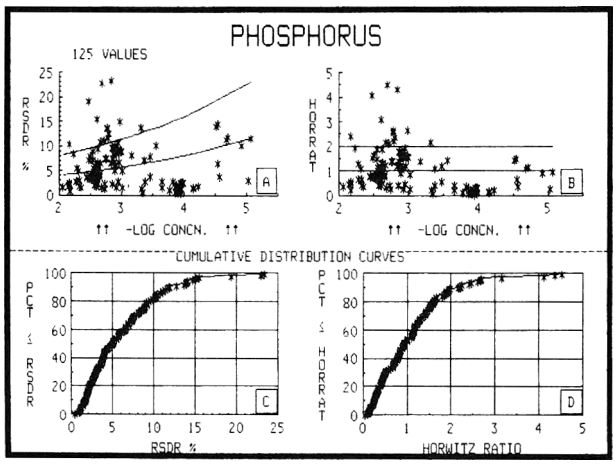

Figure 4. Precision parameters RSD_R and HORRAT of phosphorus as a function of concentration (-log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against -log C. (B) Scatterplot of HORRAT against -log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

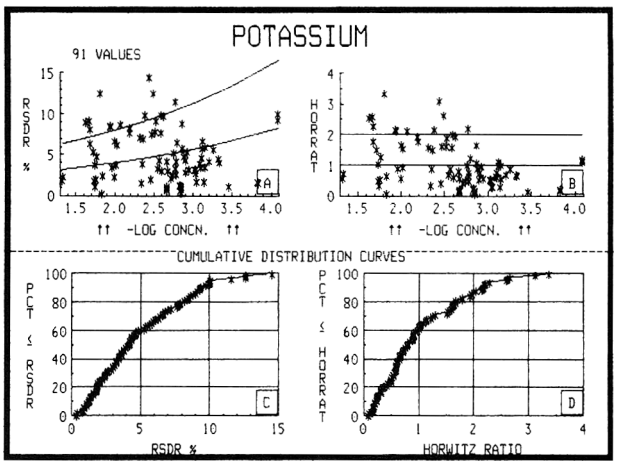

Figure 5. Precision parameters RSD_R and HORRAT of potassium as a function of concentration (-log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against -log C. (B) Scatterplot of HORRAT against -log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

HORRAT values above 2.0. The 90% interval for HORRAT is 0.1–2.6. All of the high HORRAT values are for materials with sodium concentrations of less than about 0.8 g/100 g (2.0 g NaCl/100 g), for numerous methods—inductively coupled plasma emission, atomic absorption, flame emission, selective ion electrode, and titrimetry—and for many different commodities. Such a pattern is undoubtedly the result of contamination; analysts should be alerted to this potential source of error. The data are plotted in Figure 6, and the precision and CDC parameters are summarized in Tables 1 and 2, respectively.

Sulfur

Only 9 data sets exist for sulfur, a number that is insufficient for comparison with the other larger databases. They are plotted in Figure 7 and summarized in Tables 1 and 2, pending amplification by other studies.

Discussion

Originally, the inorganic components of food were determined by the classical titrimetric and gravimetric methods. As shown by Margosis et al. (9) for drugs, the precision of the classical methods of analysis begins to degenerate at analyte concentrations below about 1 g/100 g. Except for the titrimetric determination of chloride, calculated to sodium, these methods have practically disappeared from the modern food laboratory.

All of the methods of analysis used to determine the 6 inorganic elements discussed in this paper are Type II, Reference Methods, or Type III, Alternative Approved Methods, in the Codex scheme of types of methods (12). These methods, in theory at least, can be validated using known quantities of standard salts formulated into a simulated or actual food. The Codex classification has nothing to do with the quality of a method or the magnitude of its precision errors, except in the case of Type II reference methods, which are usually, but not necessarily, chosen on the basis of minimum analytical errors. The bias of Type I methods is zero, by definition. The bias of other methods may be positive, negative, or zero, but generally at the higher concentrations considered here (i.e., 1% ±1 order of magnitude) the bias is negligible. Codex methods are accepted on the basis of an elaborate approval process involving several committees that take into consideration wide usage, acceptable performance characteristics, and general applicability.

In Part I, it was shown that only nitrogen by the Kjeldahl method had acceptable precision characteristics. Acceptable recovery could be built in by requiring the simultaneous performance of internal quality control determinations of ammonium salts and amino acids. Methods based on the measurement of volatiles or residues—moisture, solids, ash—could be made more precise by including a requirement of weighing at least 50 mg residue in the case of solids and ash, and 50 mg volatiles in the case of moisture. A minimum analyte quantity requirement also applies to the titrimetric methods for calcium and chloride (calculated to sodium).

Some of the methods used for the macro nutrient elements are about as simple as can be devised, such as aspirating a liquid into a flame or inserting an electrode into a solution; others have multiple steps, such as precipitating a phosphorus complex and titrating the separated precipitate to a somewhat indefinite endpoint. Yet, simplicity or complexity is not the factor

Table 2. Summary of the cumulative distribution curves from the database of interlaboratory determinations of inorganic elements in foods

Analyte	No. data sets	HORRAT		% HORRAT equal to or less than				
		Av.	Median	0.5	1.0	1.5	2.0	3.0
Calcium	63	1.54	1.38	3	29	62	78	90
Magnesium	48	1.57	1.48	8	35	54	73	88
Phosphorus	125	1.10	0.94	30	52	75	90	97
Potassium	91	1.03	0.80	23	60	71	87	98
Sodium	129	0.94	0.77	37	60	81	91	99
Sulfur	9	1.7	2.0	0	22	33	56	100

governing the distribution of the precision of interlaboratory assays, even when normalized for concentration. Some inductively coupled plasma emission studies contained instructions for using standards before and after each set of 10 tests and a test for drift. These methods also included a standard solution of multiple elements, yet the reported precisions are at the poor end of the scale. Obviously, analysts are unfamiliar with the sources of error in analytical determinations and with the principles of quality assurance. Methods must incorporate instructions that will assist the analyst in determining if the analytical operations are in statistical control. Independently prepared standard solutions or standard reference materials, from certifying or commercial organizations, can be the first safeguard against error. However, the occurrence of outright blunders, such as the failure to act on flagrant discrepancies, is not only a professional oversight but also a management deficiency.

A summary of the precision parameters for the macro elements of nutritional interest is given in Table 1. None of these elements approaches the precision exhibited by nitrogen reported in the previous paper (2).

The significance of contamination and the importance of incorporating internal quality control indicators are illustrated in the very recent study of calcium, magnesium, and phosphorus in cheese (10). The resources invested in 2 substantial studies were wasted because of high and "unusual" results between laboratories. Additional work led to the conclusion that calcium contamination was the source of the problem. The instructions now require special cleaning of glassware and crucibles. Furthermore, the section on the AA spectrophotometer indicates that the "Linear range and detector response must be comparable with manufacturer's specifications." Responses of working standard solutions must be checked periodically, and new calibration curves should be prepared if the response differs by more than 1% from the original calibration curve. With these instructions for avoiding dirty glassware and with requirements for internal quality control, excellent results were obtained. The

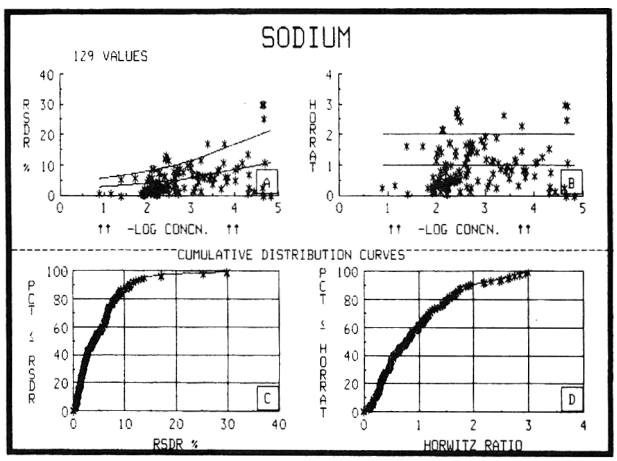

Figure 6. Precision parameters RSD_R and HORRAT of sodium as a function of concentration (-log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against -log C. (B) Scatterplot of HORRAT against -log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

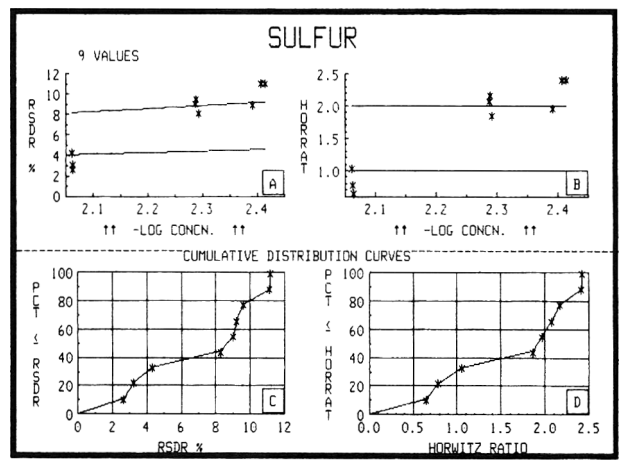

Figure 7. Precision parameters RSD_R and HORRAT of sulfur as a function of concentration (-log C) and the cumulative distribution curves (CDC) of these parameters. (A) Scatterplot of RSD_R (%) against -log C. (B) Scatterplot of HORRAT against -log C. The lower curve of A is a plot of the Horwitz equation and the upper curve is twice that equation. The corresponding HORRAT values at 1 and 2, respectively, are shown in B as horizontal lines. (C) CDC of RSD_R. (D) CDC of HORRAT.

ranges of HORRAT values for 5 products (4 for P) were Ca, 0.5–0.8; Mg, 0.4–0.8; and P, 0.3–0.5. These ranges are considerably better than those for most of the other studies of these elements.

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DRUG FORMULATIONS

Liquid Chromatographic Determination of Flurazepam Hydrochloride in Bulk Drug and Dosage Forms: Collaborative Study

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Eleven laboratories collaboratively studied the liquid chromatographic method for determining flurazepam hydrochloride in capsules and bulk drug. The method uses an octadecylsilane reversed-phase column, a mobile phase of methanol–1% ammonium acetate (80 + 20), and photometric detection at 239 nm. Each collaborator received 8 samples: powdered composites of 5 commercial capsule preparations, each as a blind duplicate pair, and 3 bulk drug samples (1 blind duplicate). The ranges of repeatability and reproducibility relative standard deviations were 0.93–2.46 and 2.42–3.86% for the capsule samples and 0.97 and 1.76–2.27% for the bulk drug samples, respectively. The method was adopted first action by AOAC International.

Flurazepam hydrochloride is an off-white to yellow benzodiazepine compound, which is used to treat various types of insomnia (1). The USP XXII methods for flurazepam hydrochloride bulk drug and capsules (2) involve either titration with perchloric acid or determination by ultraviolet (UV) spectrophotometry after dilution with methanolic sulfuric acid.

A reversed-phase liquid chromatographic (LC) method (3, 4; personal communication, E. Hamza, Barr Pharmaceuticals, Inc., Pamona, NY) was investigated as a specific, stability-indicating assay for flurazepam hydrochloride. That method was modified to form the basis of the method that was collaboratively studied. It involves the use of a C18 reversed-phase column, a mobile phase mixture of methanol and 1% ammonium acetate, and a UV detector operated at 239 nm (5).

The study reported here was initiated as a follow-up to the Compendial Monograph Evaluation and Development Program of the U.S. Food and Drug Administration. The program was designed to evaluate, develop, or improve analytical methods to ensure that they are suitable for regulatory use.

Collaborative Study

Eleven collaborators received a standard, 8 samples, and a resolution mixture. Samples 1–5 were powdered composites of 5 commercial capsule preparations, each as a blind duplicate pair. Samples 1–3 were composites of capsules labeled to contain 15 mg; Samples 4 and 5 were composites of capsules labeled to contain 30 mg. Sample 6 was a bulk drug material as a blind duplicate pair. Samples 7 and 8 were samples of bulk material. The resolution mixture consisted of flurazepam-related Compound F, 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one; flurazepam-related Compound C, 5-chloro-2-(2-diethylaminoethylamino)-2′-fluorobenzophenone hydrochloride; flurazepam hydrochloride; and 2-amino-5-chlorobenzophenone. Each collaborator was requested to submit all data, chromatograms, summary of results, and comments to the Associate Referee.

991.35 Flurazepam Hydrochloride in Bulk Drug and Capsules—LC Method

First Action 1991

Method Performance:
15 mg/capsule
s_r = 0.91–2.28; s_R = 2.40–2.55; RSD_r = 0.93–2.34%;
RSD_R = 2.42–2.60%
30 mg/capsule
s_r = 1.81–2.37; s_R = 2.77–3.73; RSD_r = 1.85–2.46%;
RSD_R = 2.84–3.86%

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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 AOAC. See "Changes in Official Methods of Analysis," *J. AOAC Int.* (1992) **75**, 223–225
Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the U.S. Food and Drug Administration.

Table 1. Raw data for collaborative study of LC determination of flurazepam HCl in capsule and bulk preparations

	Capsule, mg/capsule					Bulk, % found		
Coll.	1	2	3	4	5	6	7	8
1	18.60	17.23	18.64	35.00	35.96	100.3	99.0	94.8
	18.32	17.49	18.19	33.92	36.77	99.9	—	—
2	15.00	14.71	14.97	29.51	29.46	100.2	97.3	92.2
	14.92	14.36	14.73	29.31	29.96	99.0	—	—
3	14.78	14.24	14.95	28.34	29.83	96.5	101.7	94.2
	14.77	14.37	14.70	27.94	28.78	99.3	—	—
4	14.68	13.64	14.41	29.65	28.65	101.1	98.3	91.8
	14.61	14.50	14.24	29.09	28.05	99.3	—	—
5	14.85	14.33	14.51	28.85	28.70	99.6	96.3	94.0
	14.65	14.59	14.40	27.72	28.52	98.5	—	—
6	14.54	15.01	14.31	26.67	29.83	103.7	102.4	98.4
	14.41	14.26	14.41	28.26	29.83	104.8	—	—
7	15.52	15.04	15.38	30.00	30.13	100.8	98.4	95.3
	15.61	15.37	15.71	30.86	29.90	100.9	—	—
8	15.22	14.76	14.89	28.69	28.45	100.7	98.8	94.9
	15.29	15.32	14.82	27.46	27.90	99.6	—	—
9	14.47	14.42	14.48	29.06	29.43	99.4	97.2	98.1
	14.92	14.88	14.68	29.74	29.19	100.8	—	—
10	13.91	13.67	13.66	26.89	27.88	101.4	99.0	90.3
	13.97	13.72	15.47	27.75	29.02	100.0	—	—
11	14.48	14.29	14.62	29.28	29.36	99.8	98.9	95.8
	14.78	14.26	14.68	30.71	31.14	100.6	—	—

Bulk material

$s_r = 0.97$; $s_R = 1.77–2.27$; $RSD_r = 0.97\%$;

$RSD_R = 1.76–2.27\%$

A. Principle

Flurazepam HCl is dissolved in methanol or extracted into methanol–1% ammonium acetate solution and determined by LC using a reversed-phase C18 column, mobile phase of methanol–1% ammonium acetate (80 + 20), and photometric detection at 239 nm.

B. Apparatus

(a) *Liquid chromatograph.*—Equipped with sampling valve capable of 20 μL injections, UV detector capable of operating at 239 nm, and recorder/integrator, or equivalent.

(b) *LC column.*—25 cm × 4.6 mm id, packed with reversed-phase, octadecylsilane, 5 μm spherical particles (e.g., Altex Ultrasphere ODS, Beckman Instruments, or equivalent).

(c) *Filters.*—Polyvinylidene difluoride, 0.45 μm porosity, 25 mm and 47 mm diameter, and appropriate filter apparatus (e.g., Millipore Corp., type HVLP, or equivalent).

(d) *Ultrasonic bath.*

C. Reagents

(a) *Solvent.*—LC grade methanol.

(b) *2-Amino-5-chlorobenzophenone.*—Reagent grade.

(c) *Ammonium acetate solution.*—1% solution of ammonium acetate (LC grade) in LC grade water.

(d) *Mobile phase.*—Methanol–1% ammonium acetate (80 + 20). Filter and degas.

(e) *Flurazepam HCl stock solution.*—Accurately weigh ca 25 mg USP Reference Standard flurazepam hydrochloride into 25 mL volumetric flask. Dissolve and dilute to volume with methanol. Solution is stable 1 week when refrigerated and kept in dark.

(f) *Flurazepam HCl standard solutions.*—(*1*) *Bulk material.*—Pipet 15.0 mL stock solution into 100 mL volumetric flask. Dilute to volume with methanol. Use within 24 h. (*2*) *Capsules.*—Pipet 15.0 mL stock solution into 100 mL volumetric flask. Dilute to volume with mobile phase. Use within 24 h.

(g) *System suitability standard.*—Pipet 15.0 mL flurazepam HCl stock solution into 100 mL volumetric flask containing ca 6 mg 2-amino-5-chlorobenzophenone. Dilute to volume with methanol.

D. Sample Preparation

(a) *Bulk drug.*—Accurately weigh ca 30 mg bulk drug and transfer to 200 mL volumetric flask. Dissolve drug and dilute to volume in methanol. Use within 24 h.

(b) *Capsules.*—Determine average weight of capsule contents. Transfer accurately weighed portion of composited contents containing 30 mg flurazepam HCl to 200 mL volumetric flask. Add 40 mL methanol and shake 10 min. Add 10 mL 1% ammonium acetate and shake 5 min. Dilute to volume with mobile phase, sonicate 2 min, and filter. Use within 24 h.

E. System Suitability Test

(a) *Resolution and tailing factors.*—Set mobile phase flow rate at ca 1.5 mL/min. Inject 10 μL system suitability standard. Retention times for 2-amino-5-chlorobenzophenone and flurazepam HCl should be ca 3 and 5.5 min, respectively. Resolution factor, R, should be ≥2; and tailing factor, T, for flurazepam HCl should be ≤2.

Calculate resolution factor, R, as follows:

$$R = 2(t_2 - t_1)/(W_1 + W_2)$$

Table 2. Collaborative results for LC determination of flurazepam HCl in capsule and bulk preparations

	Capsule, % of declared					Bulk, % found		
Coll.	1	2	3	4	5	6	7[a]	8[a]
1[b]	124.0	114.8	124.2	116.7	119.9	100.3	100.6	99.6
	122.2	116.6	121.2	113.1	122.5	99.9	—	—
2	100.0	98.0	99.8	98.4	98.2	100.2	98.9	96.9
	99.5	96.1	98.2	97.7	99.9	99.0	—	—
3	98.6	94.9	99.6	94.5	99.4	96.5	103.4	99.0
	98.45	95.8	98.0	93.1	95.9	99.3	—	—
4	97.9	91.0	96.1	98.8	95.5	101.1	99.9	96.4
	97.4	96.7	94.9	97.0	93.5	99.3	—	—
5	99.0	95.6	96.7	96.2	96.0	99.6	97.9	98.8
	97.7	97.3	96.0	92.4	95.1	98.5	—	—
6	97.0	100.1	95.4	88.9	99.4	103.7	104.1	103.4
	96.1	95.1	96.1	94.2	99.4	104.8	—	—
7	103.5	100.3	102.5	100.0	100.4	100.8	100.0	100.1
	104.1	102.4	104.7	102.9	99.7	100.9	—	—
8	101.5	98.4	99.3	95.6	94.9	100.7	100.4	99.7
	101.8	102.2	98.8	91.5	93.2	99.6	—	—
9	96.5	96.1	96.5	96.9	98.1	99.4	98.7	103.1
	99.5	99.2	97.9	99.1	97.3	100.8	—	—
10[c]	92.7	91.1	91.1	89.6	92.9	101.4	100.6	94.9
	93.2	91.5	103.1	92.5	96.7	100.0	—	—
11	96.5	95.3	97.4	97.6	97.9	99.8	100.5	100.6
	98.5	95.1	97.9	102.4	103.8	100.6	—	—
Mean, %	99.1	97.2	98.1	96.5	97.6	100.2	100.4	99.8
s_r	0.96	2.28	0.91	2.37	1.81	0.97	—[d]	—[d]
s_R	2.40	2.90	2.55	3.73	2.77	1.77	1.96	2.27
RSD_r, %	0.97	2.34	0.93	2.46	1.85	0.97	—[d]	—[d]
RSD_R, %	2.42	2.98	2.90	3.86	2.84	1.76	1.95	2.27

[a] Sample 7 was adjusted for 1.6% impurity level; sample 8 was adjusted for 4.82% level.
[b] Samples 1–5 are outliers by Grubbs test. Data are not included in statistical analysis.
[c] Equipment problems. Data are not included in statistical analysis.
[d] Repeatability cannot be estimated. Single determinations.

Table 3. Results for resolution mixture for LC determination of flurazepam HCl in capsule and bulk preparations[a]

Coll.	T	R	Column size, mm	Retention time, min			
				Cmpd F	2-Am-5C	FLZPM	Cmpd C
1	1.10	1.65	Ultrasphere 4.6 × 150	3.94	5.70	5.04	8.10
2	1.70	2.62	Ultrasphere 4.6 × 250	2.78	3.89	5.11	8.91
3	1.61	4.0	Ultrasphere 4.6 × 250	2.77	4.12	5.32	9.07
4	2.20	6.6	Econosphere 4.6 × 150	2.03	2.73	5.80	9.32
5	1.50	—[b]	Hypersil 4.6 × 250	—[b]	—[b]	5.45	10.76
6	1.22	6.10	Ultrasphere 4.6 × 250	2.43	3.20	5.12	8.03
7	1.00	1.68	Ultrasphere 4.6 × 250	4.19	6.49	5.74	9.48
8	2.70	10.8	Econosphere 4.6 × 250	2.28	2.79	5.67	11.30
9	1.95	4.2	Econosphere 4.6 × 250	1.92	2.69	5.21	11.8
10	1.50	4.0	Ultrasphere 4.6 × 250	3.88	5.70	7.18	12.55
11	1.10	9.0	Ultrasphere 4.6 × 250	2.9	4.4	5.3	10.1

[a] T = tailing factor for flurazepam HCl; R = resolution factor between flurazepam HCl and 2-amino-5-chlorobenzophenone (2-Am-5C). See text for chemical names of Compounds F and C.
[b] Compound F and 2-amino-5-chlorobenzophenone coeluted on this system.

where t_2 and t_1 = retention times of the 2 components, and W_1 and W_2 = corresponding widths of bases of peaks, obtained by extrapolating relatively straight sides of peaks to base line. If resolution is unsatisfactory, adjust methanol concentration.

Calculate tailing factor, T, as follows:

$$T = W_{0.05}/2f$$

where $W_{0.05}$ = distance from leading edge to trailing edge of peak, and f = distance from peak maximum to leading edge of peak, both measured at point 5% of peak height from base line.

(b) *Reproducibility.*—Inject five 20 µL portions of flurazepam HCl standard solution and measure peak responses. Relative standard deviation should be ≤2.0%.

F. Liquid Chromatography

Inject 20 µL standard and sample solutions. Calculate quantity of flurazepam HCl in sample as follows:

Bulk drug:

$$\text{Flurazepam HCl, mg} = (P/P') \times C \; 200$$

Capsules:

$$\text{Flurazepam HCl, mg/capsule} = (P/P') \times C \times 200 \times (T/S)$$

where P and P' = peak responses (area or height) for sample and standard solutions, respectively; C = mg flurazepam HCl/mL in standard solution; T = average weight of capsule contents, g; and S = sample weight, g.

Ref.: *J. AOAC Int.* **75,** March/April issue (1992)

CAS-1172-18-5 (flurazepam hydrochloride)

Results and Discussion

The raw analytical results obtained by the 11 collaborators are summarized in Table 1. The results for the capsule samples in Table 2 are listed in terms of percent declared for Samples 1–3 at the 15 mg level and Samples 4–5 at the 30 mg level. The results for the bulk drug samples were corrected for the presence of impurities in the material. For Sample 7, a total of 1.6% impurities (6) were found: 1.4% was attributed to flurazepam-related Compound C, 0.1% to flurazepam-related Compound F, and 0.1% to unidentified impurities. For Sample 8, a total of 4.82% impurities were found: 4.5% was attributed to flurazepam-related Compound C, 0.1% to flurazepam-related Compound F, and 0.22% to unidentified impurities. The results were corrected by dividing the raw data value by (100 – the impurity value) and multiplying that value by 100.

Statistical evaluation was performed on data from 10 collaborators. Data from collaborator 10 were excluded because of equipment problems. Results for Samples 1–5 from collaborator 1 were identified as Grubbs outliers and were not used. The reason for the high results could not be determined by either the collaborator or the Associate Referee.

The method (5) sent to collaborators did not include a tailing factor or a resolution factor in the system suitability test. A resolution mixture consisting of flurazepam hydrochloride, 2-amino-5-chlorobenzophenone, and flurazepam-related Compounds C and F was included as part of this collaborative study to gather additional data for the inclusion of 2-amino-5-chlorobenzophenone in a resolution standard. The collaborators were asked to inject this mixture after the samples were completed and to calculate the resolution factor between flurazepam hydrochloride and 2-amino-5-chlorobenzophenone and the tailing factor for flurazepam hydrochloride. These results are listed in Table 3. As a result of the data collected, 2-amino-5-chlorobenzophenone was included in the method as part of a resolution standard and a tailing factor was added for flurazepam hydrochloride.

Collaborators' Comments

None of the collaborating laboratories reported any difficulty with the performance of the method.

Collaborators 6 and 8 reported a late eluting peak in the chromatogram for vial 14 (Sample 8). This peak is attributed to flurazepam-related Compound C, which was present in the

bulk material used for this sample. Collaborators 3, 10, and 11 had a late eluting peak in their chromatograms but did not report it. The other collaborators did not have a late eluting peak in their chromatograms because of the stop time used on each of their integrators.

Collaborators 1 and 7 commented on injecting the resolution mixture last instead of first. A resolution mixture was incorporated into the method.

The suggestion of collaborator 5 to specify use of reagent or higher grade ammonium acetate was accepted.

Recommendation

We recommend that the reversed-phase LC method for the determination of flurazepam hydrochloride in bulk material and capsules be adopted first action.

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DRUG RESIDUES IN ANIMAL TISSUES

Analytical Strategy for the Regulatory Control of Residues of Chloramphenicol in Meat: Preliminary Studies in Milk

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An analytical strategy is described for the regulatory control of residues of the veterinary drug chloramphenicol (CAP) in meat. Screening is performed directly in meat by a simple immunochemical card test with a limit of detection of about 2 μg/kg. Statistical evaluation of a collaborative study involving 13 laboratories showed that at CAP concentrations exceeding 8 μg/kg, no false negatives are found (N = 554). In positive samples, CAP is quantitated with a routinely applicable, collaboratively tested column liquid chromatographic method with a limit of detection of about 1 μg/kg. At concentrations exceeding 10 μg/kg, the identity of CAP is established by its UV spectrum obtained by using diode-array UV/VIS detection. A further confirmation can be obtained by the combination of gas chromatography/mass selective detection in the electron impact mode. Using 2 diagnostic ions (m/z 225 and 208), the limit of identification is about 5 μg/kg. The combination of the different analytical principles ensures reliable quantitation and identification of CAP in positive samples, as established experimentally in incurred samples and spiked samples (n > 100), and theoretically by the estimation of the uncertainty factor. The proposed set-up makes a regulatory program possible in which screening can be performed in a simple laboratory environment, followed by quantitation and identification under more sophisticated conditions. Preliminary experiments indicate that the analytical strategy is also applicable to the control of CAP in milk. Application of mass spectrometry with negative chemical ionization permits the confirmation of CAP concentrations as low as 0.2 μg/L.

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Chloramphenicol (CAP) is a very effective broad-spectrum antibiotic that is used by veterinary practice. Because of its toxicity (1) and the possible occurrence of residues, the drug is banned for food producing animals in the United States. In the European Community (EC), CAP is not approved for laying birds and lactating cows, and its use in other large animals is restricted. In The Netherlands, CAP is also banned for pigs. Withdrawal periods based on a proposed maximum residue level (MRL) of 10 μg CAP/kg were established. To monitor the extra-label use of CAP and to ensure compliance with withdrawal periods, appropriate regulatory analytical methods are required.

In 1985, chromatographic methods for the determination of residues of CAP were reviewed by Allen (1). Since then, a number of chromatographic methods using gas chromatography (GC) (2–5) or liquid chromatography (LC) (6–8) were developed for the quantitation of CAP. For screening purposes, radio-immunoassay (RIA) (4, 9), enzyme-linked immunosorbent assay (ELISA) (10–12), or test kit-based (13–15) methods were proposed.

Regulatory control programs are often performed in 2 stages: a simple, cheap, and routine screening that acts as a first sieve, followed by an often more time-consuming and expensive quantitation and confirmation. A screening method should not produce false negatives, although a limited percentage of false positives is acceptable; yet the confirmation method must not give false positives and must be able to reliably quantitate the amount of analyte when an MRL has been set. With regard to the latter type of method, both the EC and the Codex Committee on Residues of Veterinary Drugs in Food (CC-RVDF), have proposed a number of criteria to ensure the validity of the results obtained with these methods (16, 17).

A screening method either can be directed toward a group of drugs that have a number of common features or can be selectively aimed at a single compound. An example of the first approach is the microbiological screening test for antimicrobials developed in The Netherlands (18). The test is sensitive for many groups of antimicrobials; unfortunately, it is too insensitive for CAP.

Immunochemical techniques, however, are very well-suited for screening a specific compound. A number of immunochemical screening methods for CAP have been published and, to some extent, applied in practice (4, 10). Unfortunately, none of the antibodies used is commercially available thus far. On the other hand, commercially available antisera are not always very well-characterized. This is a serious drawback for a regulatory method. Moreover, RIA and, to a lesser extent, ELISA methods require special instrumentation and are economically attractive only when applied on a large scale. In the organization structure of The Netherlands meat inspection service, this would imply that 12 regional laboratories would have to be equipped with qualified personnel and instrumentation for this purpose. At present, this option was not considered feasible for a decentralized meat inspection.

Recently, the use of a commercially available ELISA-based card test was described for the control of CAP in urine and renal pelvis fluid (13). The test is quick, relatively cheap, and very sensitive, and does not need special instrumentation; therefore, a flexible control system could be set up. The La Carte™ test for the qualitative determination of residues of CAP in biological samples contains polyclonal antibodies directed against CAP that are immobilized in wells on the card. The test kit further contains a solution of enzyme-labeled CAP that is added to compete for the available CAP binding sites, a negative control buffer solution, and a color substrate solution. Limit of detection for urine is about 5 μg/L (13); a procedure for meat with a detection limit of 20 μg/kg is described in the directions for use. Only free, nonconjugated CAP can be determined with the test kit (13). For meat, no deconjugation step is necessary, as no conjugated CAP has been observed in naturally incurred meat samples (7).

A simple LC method for the quantitative determination of CAP in meat at levels exceeding 5 μg/kg has been used for a number of years in State Institute for Quality Control of Agricultural Products (RIKILT) laboratories (7). The method was tested in an international collaborative study (19) and was found to meet the above-mentioned EC and CC-RVDF criteria.

After quantitation (to assess whether the MRL has been exceeded) and identification (by retention time and UV spectrum), additional confirmation may be necessary in case of a dispute. For that purpose, analysis by GC coupled with mass spectrometric detection (GC/MS) was investigated. Although sophisticated mass spectrometers having various sample introduction and ionization modes are available within the RIKILT laboratories, a relatively simple and cheap mass-selective detector with electron impact (EI) ionization was selected. The wider availability of this type of detector will make the analytical strategy applicable to more laboratories.

Experimental

The analytical method for the LC determination of CAP in meat, including reagents and materials, was previously described in detail (19). Therefore, only the analytical procedures for the immunochemical card test and the GC/MS confirmation will be described here.

Reagents

All reagents and solvents are analytical grade, unless stated otherwise. Demineralized water is purified with a purification kit (e.g., Milli-Q, Millipore Corp., Milford, MA 01730).

Immunochemical card test

(a) *Chemicals.*—Disodium hydrogen phosphate·$2H_2O$, potassium dihydrogen phosphate, sodium hydroxide, phosphoric acid.

(b) *Extractant.*—0.1M phosphate buffer, pH 5.0.

(c) *CAP standard solutions.*—(*1*) *Stock solution.*—Accurately weigh 10.0 mg CAP (Sigma Chemical Co., St. Louis, MO 14508) in 100 mL volumetric flask, dissolve in methanol, and dilute to volume with methanol. (*2*) *Working solutions.*—Dilute 5.0 mL stock solution to 100 mL with water in 100 mL volumetric flask (CAP concentration, 5 μg/mL). Pipet 2.0 mL of this solution into 100 mL volumetric flask and dilute to volume with water. This solution contains 0.1 μg CAP/mL.

Confirmation GC/MS

(a) *Solvents.*—Ethyl acetate (Uvasol grade); isooctane; *n*-decane.

(b) *Chemicals.*—*N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA) (Pierce Chemical Co., Rockford, IL 61105); trimethylchlorosilane (TMCS) (Pierce).

(c) *Derivatization reagent.*—BSTFA + 1% TMCS. Prepare solution daily.

(d) *Internal standard solutions.*—Prepare 0.2 μg/mL solution of 2, 3, 4, 2′, 4′, 5′-hexachlorobiphenyl (PCB-138) (Promochem, Wesel, Germany) in isooctane–*n*-decane (4 + 1, v/v).

Also, prepare 1 μg/mL solution of thiamphenicol (Pierce) in ethyl acetate.

Instrumentation and Materials

Immunochemical test kit

(a) *La Carte test kit.*—(Transia-Biocontrol, Waddinxveen, The Netherlands) or Quik™ card (Environmental Diagnostics, Burlington, VT).

(b) *Filters.*—Acrodisc, 0.45 μm.

(c) *Syringes.*—2 mL, disposable.

(d) *Vials.*—4 mL, with screw cap.

(e) *Stomacher.*—Laboratory blender Model 400 (Lameris, Utrecht, The Netherlands).

(f) *Kitchen mixer.*

(h) *Filter paper.*—15 cm diameter.

Confirmation GC/MS

(a) *Thermostatted heating module.*—Equipped with nitrogen evaporation unit (Pierce).

(b) *Mass selective detector.*—Type HP-MS mass spectrometer (Hewlett-Packard Co., Palo Alto, CA 94202), equipped with an HP 5890A gas chromatograph, a model HP 5970B mass selective detector, a model HP 7673A autosampler, and a model HP 59970C chemstation. Experimental conditions are given in Table 1.

Table 1. Experimental conditions used in GC/MS confirmation

	Gas chromatography
Carrier gas	helium, linear velocity 30 cm/s
Split	20 mL/min, opened 4 min after injection
Septum flush	3 mL/min, opened 4 min after injection
Injection volume	3 μL splitless
Temperature, injector	260°C
Temperature, oven	130–280°C
Rate	15°C/min
Initial hold	2 min
Final hold	4 min
MS-interface	direct coupling of fused silica column to ion source
Temperature, interface	260°C
	Mass spectrometry[a]
Chloramphenicol	208/225 *m/z* (base peak)
PCB-138	360 *m/z*
Thiamphenicol	257 *m/z*

[a] In all cases, the measuring period was 0.1 s and EI ionization was used.

(c) *Fused silica capillary column.*—25 m × 0.22 mm id, coated with a 0.12 μm layer of CP Sil-5 (Chrompack, Middelburg, The Netherlands).

METHODS

Immunochemical Test Kit

Weigh 20.0 g finely cut and subsequently homogenized muscle tissue in stomacher bag. Add 20.0 mL 0.1M phosphate buffer, pH 5. Stomach bag 3 min. Filter contents of bag through filter paper and collect filtrate in reagent tube. Filter aliquot of filtrate through Acrodisc filter, collect in 4 mL vial, and use 50 μL of this clear solution for La Carte test. (Procedure can be made less sensitive by diluting final extract with phosphate buffer or blending with different meat-to-buffer ratio.)

With each series, analyze blank and 5 μg/kg spiked sample. Prepare spiked sample by adding 1 mL 0.1 μg/mL standard CAP solution to 20.0 g blank sample, homogenize, and wait 15 min.

La Carte test procedure is described in directions for use included in each test kit. Kit consists of set of 2 cards, each containing 2 wells with immobilized polyclonal antibodies. The kit must be used at room temperature and stored at 4–6°C. Furthermore, each card should be tested for its applicability by using 1 well for a reagent blank. Presence of CAP is indicated by absence of color formation 5–15 min after addition of color substrate. Negative samples show distinct blue color.

Confirmation with GC/MS

Inject 200 μL final aqueous extract from 10 g meat sample subjected to procedure in Reference 19 into reversed-phase LC system. Collect 1.5 mL LC eluate fraction containing CAP. Extract fraction with three 1 mL portions ethyl acetate. Add 25 μL 1 ng/μL thiamphenicol internal standard solution to combined ethyl acetate phases and evaporate to dryness at 40°C with gentle stream of nitrogen.

Dissolve residue in 100 μL derivatization reagent and heat 1 h at 60°C in closed vial with occasional shaking. Carefully evaporate to dryness at 40°C with gentle stream of nitrogen. Dissolve residue in 25 μL PCB-138 internal standard solution. Inject 3 μL of this solution into GC/MS system operated under conditions summarized in Table 1.

Results and Discussion

Immunochemical Card Test

Sample treatment.—The La Carte test procedure was modified to lower the limit of detection. Full details of this optimization are presented in Reference 20. The sample was first homogenized in a kitchen grinder. Then, a 20 g portion was stomached with 20 mL 0.1M phosphate buffer, giving roughly a 2-fold dilution. Because the presence of particles and bacterial and blood cells will interfere with the immunoresponse (13), the aqueous extract was subsequently cleaned by filtration through filter paper and then through a 0.45 μm Acrodisc filter. Then, 50 μL of the final solution was applied to the card.

Limit of detection.—A number of experiments were performed to establish the limit of detection of the modified method. A RIKILT reference sample obtained from a dosed animal (swine), containing about 17.5 μg CAP/kg, was diluted with blank meat to concentrations ranging from 0.5 to 5.0 μg/kg. A negative response was always obtained for CAP concentrations below 1 μg/kg; the color break-point was found to be near 3 μg/kg. Above 4 μg/kg, the response was always positive ($N = 3$).

To investigate the influence of the sample matrix, an extract of a 10 μg/kg spiked sample was diluted with phosphate buffer to concentrations as low as 1 μg/kg. Again, the same break-point at 3 μg/kg was observed, indicating that the immunochemical response is not adversely affected by residual matrix components; that is, the sample cleanup is satisfactory.

The performance of the test was further studied by blind analysis of a number of samples obtained from a monitoring program for suspected animals. The samples were analyzed at RIKILT laboratories or the Central Laboratory of the National Inspection Service of Livestock and Meat (CL-RVV, Wageningen, The Netherlands). The CAP content of the samples was screened with the La Carte test and quantitated by the LC method. A number of these samples were also analyzed by the GC/MS confirmation procedure. The extracts of the samples found positive in the La Carte screening test were also diluted 10- or 3-fold, and screened again. On the basis of the actual CAP concentrations as established by the LC method, we concluded that a negative response is observed for samples containing <1–2 μg/kg (Table 2). Although the number of results was limited, all experiments indicated that meat samples containing more than 4 μg/kg CAP would be positive in the

Table 2. Comparison of results obtained with real samples by using an immunochemical screening (La Carte test), LC quantitation, and confirmation with GC/MS for CAP content >1 μg/kg[a]

	Result of La Carte test[b]			GC/MS confirmation		
		Diluted				
LC CAP content, μg/kg	Undiluted	1 + 9	1 + 2	*m/z* 225	*m/z* 208	Ratio[c]
10	+[d]	–		+	+	+
8	+	–		+	+	+
3	–	ND[e]		–	–	–
2	–	ND		–	–	–
36	+	+		+	+	+
21	+	–		+	+	+
14	+	–		+	+	+
1	–	ND		–	–	–
2	–	ND		–	–	–
22	+	–		+	+	+
74	+	+		+	+	+
90	+	+		+	+	+
8	+	ND	+	ND	ND	ND
6	+	ND	+	ND	ND	ND
9	+	ND	+	ND	ND	ND
42	+	ND	+	ND	ND	ND
3	+	ND	–	ND	ND	ND
5	+	ND	–	ND	ND	ND
37	+	ND	+	ND	ND	ND
13	+	ND	+	ND	ND	ND
14	+	ND	+	ND	ND	ND
20	+	ND	+	ND	ND	ND
32	+	ND	+	ND	ND	ND
5	+	ND	±	ND	ND	ND
1	±	ND	–	ND	ND	ND
6	+	ND	±	ND	ND	ND
4	+	ND	–	ND	ND	ND
7	+	ND	±	ND	ND	ND
10	+	ND	±	ND	ND	ND
2	±	ND	–	ND	ND	ND
5	+	ND	–	ND	ND	ND
3	±	ND	–	ND	ND	ND
3	+	ND	–	ND	ND	ND
3	±	ND	–	ND	ND	ND
3	±	ND	–	ND	ND	ND
2	+	ND	–	ND	ND	ND

[a] For CAP content < 1 μg/kg, established by LC (n = 46): Positive La Carte test (n = 46), none; GC/MS confirmation (n = 20) *m/z* 225 and *m/z* 208 and ratio, all negative.
[b] Extracts that gave a positive response in the screening test were diluted 10- or 3-fold with pH 5 buffer and tested again.
[c] This is the qualifier ratio of *m/z* 208 vs *m/z* 225, as established from the corresponding standard curve. It ranges from 35 to 40%.
[d] + = positive finding, colorless sample spot. ± = not colorless but clearly different from blue control spot. In statistical evaluation and meat control, ± is considered to be negative. – = negative finding blue sample spot.
[e] Not determined.

test. The absolute limit of detection is about 100 pg. No false negatives were observed above this level. Likewise, no false positives occurred in samples studied so far.

The performance of the test was further evaluated in a collaborative study involving the regional livestock and meat inspection laboratories of The Netherlands. This study will be described in detail elsewhere, but in the section on *Validation of the Analytical Strategy* below, the results are used to estimate the level at which no false negatives occur.

Cross-reactivity.—Information on the cross-reactivity of a number of CAP analogs is available from the La Carte test kit producer. Additionally, we tested 2 CAP analogs, viz., the metabolite CAP-base, and the related veterinary drug thiamphenicol. Both compounds, which have structural modi-

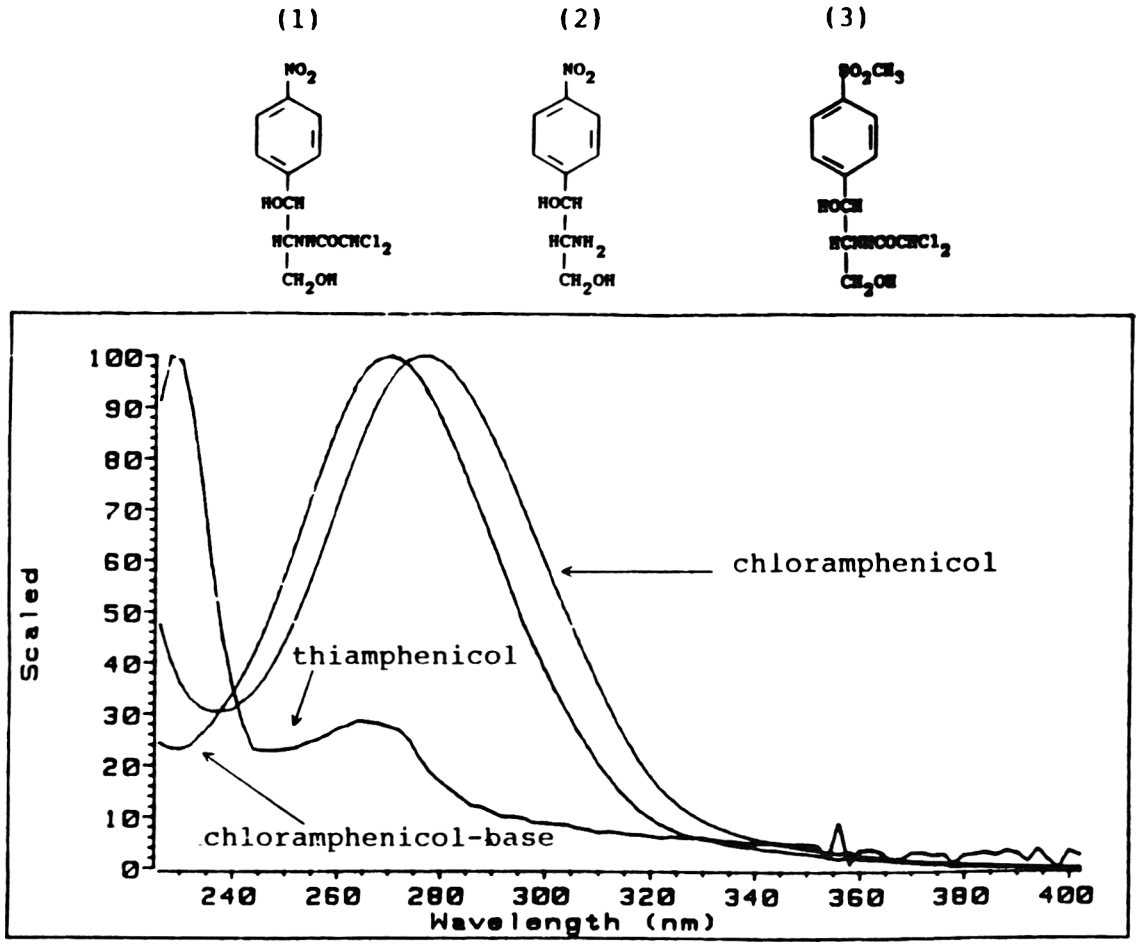

Figure 1. Structures of (1) chloramphenicol, (2) chloramphenicol-base, and (3) thiamphenicol, with their UV/VIS spectra as obtained by diode-array detection.

fications on very different locations in the CAP molecule (Figure 1), showed no cross-reactivity at a concentration of 100 times the CAP limit of detection.

LC Quantitation

The LC method (7, 19) consists of an aqueous extraction followed by cleanup on a cartridge filled with diatomaceous earth (Extrelut™) and elution with dichloromethane. The final aqueous extract is partitioned with toluene before injection into the LC system. The method has a medium but reproducible recovery of about 55%, was collaboratively tested (19), and has been used routinely within RIKILT and other laboratories for a number of years. Examples of chromatograms from a blank and a 10 μg/kg spiked sample, including the diode-array UV/VIS spectrum obtained, are shown in Figure 2.

The method is used to establish whether meat contains violative levels of CAP. For CAP, an MRL of 10 μg/kg has been set in The Netherlands (15). On the basis of hundreds of blank chromatograms obtained during routine use, the limit of detection is about 1 μg/kg for the various meat species (lamb, chicken, veal, cattle, and pork). The absolute limit of detection with the diode-array detector is about 1 ng.

Several hundred samples from suspected animals were recently analyzed by the method. A number of these negatives and positives in the range 1–100 μg/kg were also analyzed by the La Carte screening test and the GC/MS confirmation procedure. As shown in Table 2, the screening and quantitation results show complete correlation, with the LC method having a slightly lower limit of detection. All the quality assurance blank and spiked samples that were included in the monitoring series gave satisfactory results, showing no false positives and reproducible recoveries of about 55%.

Confirmation by GC/MS

The GC/MS method in the proposed analytical strategy does not necessarily provide quantitative information. The method should positively identify CAP in samples that actually contain >10 μg CAP/kg, as established by LC analysis, and should eliminate any false-positive result obtained with the combination of immunochemical screening and LC/diode-array UV/VIS quantitation/confirmation. Unequivocal criteria for positive identification and a limit of identification low enough to compensate for the methods variability are, therefore, essential.

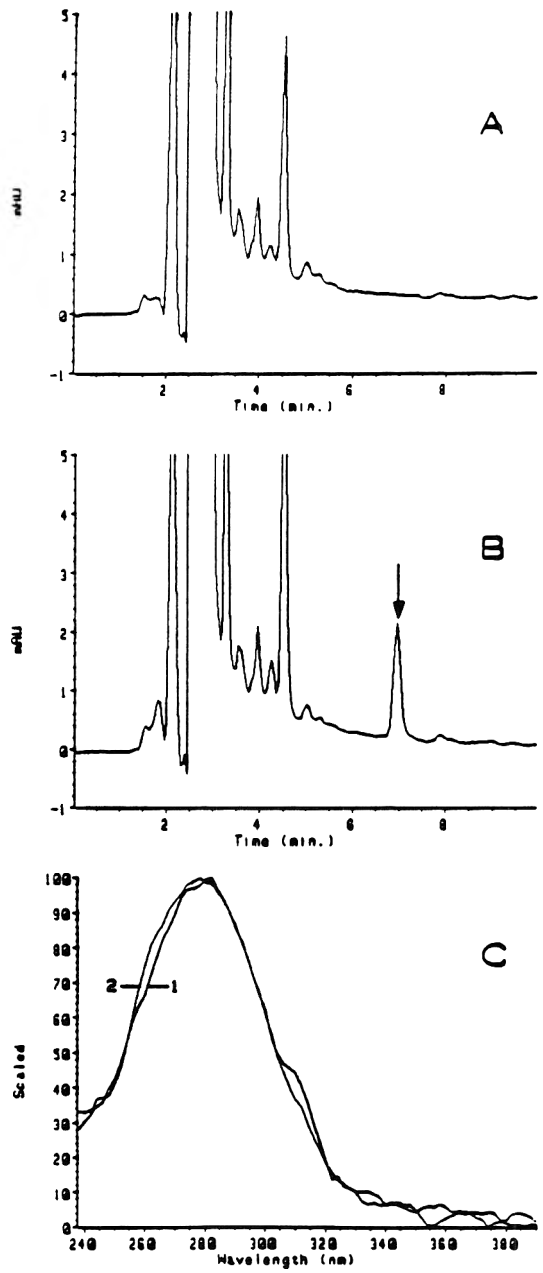

Figure 2. LC traces of (A) blank pork meat and (B) spiked meat containing 10 μg CAP/kg. (C) UV/VIS spectra of CAP in the spiked sample (1) and of standard CAP (2). Samples are analyzed according to procedure previously described (7, 19).

As described in the introductory section, an MS confirmation method using electron impact (EI) ionization was selected. The advantages of this ionization mode, compared to a technique such as chemical ionization (CI), are easy automatability, decreased ion source pollution, and less complex equipment required. The main drawback of EI is the strong fragmentation of the analyte, which may result in a decreased number of selective diagnostic ions. The 2 chlorine atoms present in CAP make negative CI (NCI) a more efficient process than positive CI, and for a disilylated (di-TMS) CAP derivative, an absolute limit of detection as low as 0.6 pg was reported with the use of 5 ion fragments and methane as reaction gas (5). For comparison, with EI and 2 diagnostic ions, a limit of detection of 200 pg was reported (3). Fortunately, the latter sensitivity is sufficient for the confirmation of meat samples analyzed by the quantitative LC method and containing more than 10 μg CAP/kg, as shown below. In Figure 3, an example of an EI mass spectrum of the di-TMS-CAP derivative, is shown.

The main diagnostic ions that can be monitored in the selected ion monitoring (SIM) mode are, in order of decreasing intensity, *m/z* 225 (base peak), 208, and 194.

Sample cleanup.—In the experimental set-up chosen, a 200 μL aliquot of the 400 μL extract obtained in the procedure for the quantitative determination of CAP was injected into the LC system and used for GC/MS confirmation after extraction of the CAP-containing eluate fraction. In this way, a very thorough cleanup was accomplished by using a combination of solid-phase and liquid-liquid extractions and a selective LC separation. The overall recovery of the LC method is about 55%, as mentioned before. The ethyl acetate extraction of CAP from the LC eluate fraction has an efficiency of more than 95%.

Given a sample containing 10 μg CAP/kg and assuming that the derivatization step is quantitative, the theoretical concentration of CAP in the 25 μL final extract, from which a 3 μL portion is injected into the GC/MS system, is about 0.5 μg/mL.

Derivatization.—Four published silylation methods (2, 4, 21, 22) were examined with regard to their response and variability. Table 3 shows the mean results of each 10 replicate derivatizations of CAP standard solutions containing 1 μg CAP/kg. The intensity of the *m/z* 225 base peak was measured. For a number of cases, the repeatability of CAP and PCB-138 retention times on the CP-Sil-5 capillary column is also shown. In our hands, optimum results were obtained with Method 3, using a (BSTFA + 1% TMCS) mixture.

This reagent yields the disilylated product only. Therefore, this procedure, described in detail under *Experimental*, was used further in the experiments. The CAP analog thiamphenicol was tested for its use as an internal standard for the derivatization step. As the repeatability of its derivatization (CV = 22%, N = 26) was inferior to that of CAP, it cannot be considered an ideal internal standard. However, the response of thiamphenicol provides a good check on the derivatization efficiency. In the future, *m*-CAP and, even more ideally, deuterated CAP will be tested as internal standards.

Working range.—The linearity, repeatability, and limit of identification of the confirmation procedure were tested by evaluating the results of 5 standard curves obtained on different days, with each sample injected in duplicate on each day. Table 4 shows the mean calculated CAP concentrations and the corresponding CV values. The results show an adequate linearity and repeatability in view of the qualitative purpose of this method. The CAP concentrations were calculated by using the *m/z* 225 base peak. The ratio of *m/z* 225 vs *m/z* 208 was also calculated. This ratio, which can vary somewhat from day to day depending on instrumentation variability, but should be constant within a series of analyses, is used as qualifier for identifying the presence of CAP in samples. In all standard curves, this qualifier ratio was constant over the full range, 0.2–

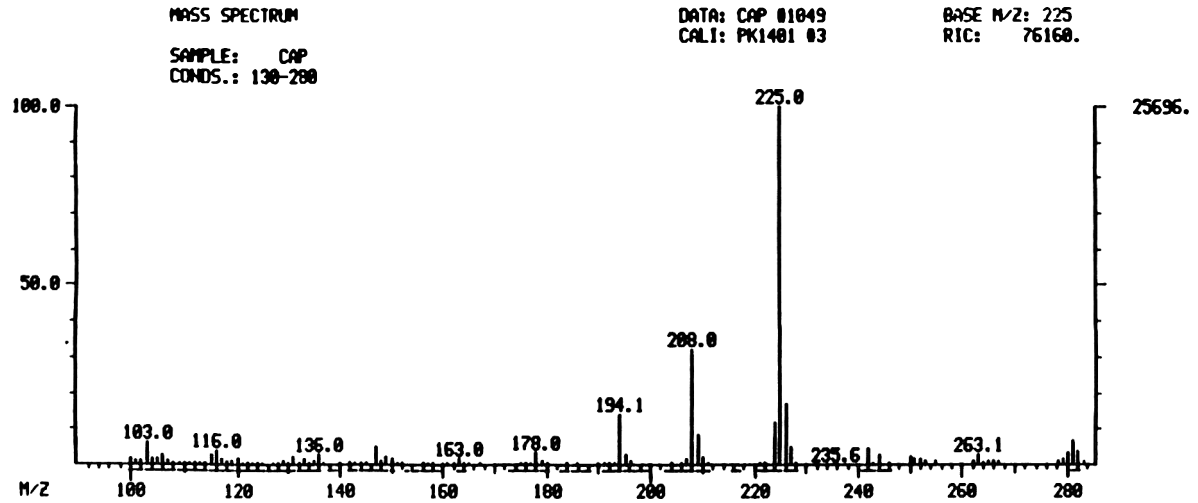

Figure 3. EI mass spectrum (70 eV) obtained for the disilylated CAP derivative.

4 μg/mL. Below 0.2 μg/mL, the qualifier ratio was not always correct because of too low intensity of the *m/z* 208 diagnostic ion. Therefore, the limit of identification based on standards can be set at 0.2 μg/mL, or 600 pg absolute. This value is 0.4 of the theoretical CAP concentration in the final extract resulting from a sample containing 10 μg CAP/kg.

Application of muscle samples.—To test the repeatability and practicality of the method with muscle samples, series of meat samples spiked with 10 μg CAP/kg were analyzed on different days. The concentration of CAP in the final GC/MS extract of these samples was calculated by using the *m/z* 225 response and the corresponding standard curve. These values were used to calculate the concentration of CAP in the sample, corrected for LC recovery. The mean CAP concentration thus found was 9.8 μg/kg (CV 31.5%, n = 10). Without correction for recovery, the CV value was essentially the same (CV 32.0%), indicating that the observed variability is mainly governed by the back extraction and derivatization steps. For all samples, the correct qualifier ratio was found, and the relative retention times of CAP and the GC/MS internal standard PCB-138 were within 0.3% of the values obtained for standard CAP. In all cases, a suitable UV spectrum was obtained in the LC step.

Under the correct experimental conditions, when contamination of the analytical system with CAP is prevented, no false positives have been observed in the series of real samples thus far analyzed (n > 100). Also, no false negatives at a CAP concentration level above 10 μg/kg have been observed (n = 40). On the basis of these results, we concluded that there is an excellent correlation between the results obtained with the LC and the GC/MS methods.

Quality Assurance and Method Validation Criteria

Immunochemical screening.—In commercial immunochemical test kits, slight batch-to-batch differences may occur with regard to quality of the antibodies and the available binding sites. Also, the stability of the kit is limited, usually about 6 months. It is essential that a card not be used after the expiration date. To avoid false positives, the control site on each card is tested with negative control reagent. After addition of the substrate, a distinct blue color should form, indicating that binding capacity and the enzymatic conversion are adequate. In addition, in each sample series a blank control sample, a 5 or 10 μg/kg spiked sample to indicate false negatives, and, preferably, a blind control sample are included. If cards from several batches are used within a series, these quality assurance

Table 3. Comparison of various silylation procedures (n = 10)

Reagent	Reference	t_R CAP, min[a]	t_R PCB, min	*m/z* 225 response, (×10^5)[b]
MSTFA–CH_3CN (3 + 7) 30 min, 25°C	2	10.60 (0.02)	ND[c]	42.2 (21)
HMDS–TMCS–Pyr (3 + 1 + 9) evaporate at 50°C	4	10.60 (0.02)	9.91 (0.02)	47.4 (70)
BSTFA–TMCS (99 + 1) 60 min, 60°C	21	10.60 (0.02)	9.91 (0.02)	66.0 (15)
BSTFA 30 min, 70°C	22	10.61 (0.01)	9.91 (0.02)	22.9 (44)

[a] The numbers in parentheses are the observed coefficients of variation (CV, %). In all experiments, the CV of the PCB-138 *m/z* 360 signal was between 3 and 7%.
[b] Response in arbitrary units.
[c] Not determined.

Table 4. Repeatability of CAP standard curves for GC/MS confirmation

CAP concentration		
Added, μg/mL	Calculated, μg/mL[a]	CV, %
0.20	0.22	26.5
0.50	0.57	28.5
1.00	0.91	22.0
1.50	1.62	15.5
2.00	2.30	18.0
4.00	4.14	5.0

[a] Results obtained, expressed as *m/z* 225 responses, are the average of 10 curves on 5 different days and corrected for the response of the PCB-138 internal standard.

samples should be included for each batch. A sample series is considered to give valid results if the control reagent sites and the blank control sample give a blue color, whereas the spiked sample gives a colorless reading 5–15 min after addition of the substrate. Samples found positive in the screening test are confirmed by LC analysis.

Quantitation by LC.—Contamination of reagents or instrumentation with CAP is strictly avoided, as it may result in false positives. To this end, glassware is cleaned thoroughly and disposable glassware is used as much as possible. The LC eluant should be prepared fresh every day and should never be recirculated. The analytical column is used only for CAP analysis and, if an autosampler is used, the needle system is purged after each injection. If a highly positive sample is found and the next sample is also found positive, the latter sample should be analyzed again to exclude cross-contamination.

At least 2 standard solutions are included in each series, and their responses are compared with the preceding series. A blank meat sample, a 10 μg/kg spiked sample, a naturally incurred reference sample, or a blind sample is co-analyzed. The results for the spiked and reference samples should fall within preset margins, and the diode-array UV/VIS spectra should be acceptable. This latter condition means that the maximum absorption wavelengths in the spectra of the analyte and a CAP standard should fall within ±2 nm. Furthermore, the difference between parts of the 2 normalized spectra having a relative absorbance of over 10% should never be more than 10% of the absorbance of the standard CAP (16). Additionally, the identity of CAP in a positive sample is tested by adding standard CAP to the remaining sample extract and re-injecting this solution (co-chromatography). Only the intensity of the peak assumed to be CAP should increase.

The LC method meets the general criteria for reference methods as proposed by the EC and CC-RVDF (16, 17), previously described (19). In case of a dispute, additional GC/MS confirmation can be performed.

Identification by GC/MS.—A number of quality assurance checks are included in each series of analyses. To prevent false negatives, a 10 μg/kg spiked sample and the internal standards thiamphenicol and PCB-138 are added. Additionally, before an analytical series is started, a standard curve ranging from 0.2 to

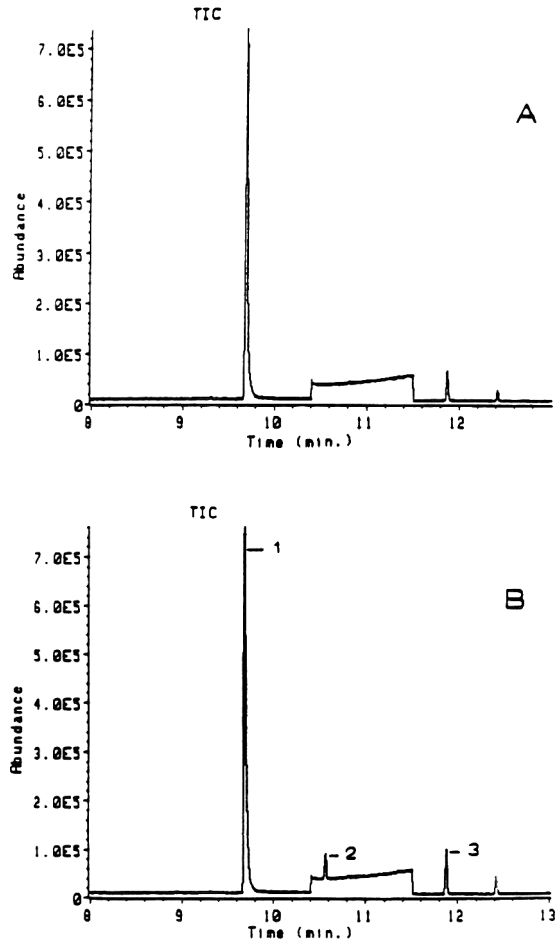

Figure 4. Selected ion monitoring chromatograms of (A) a blank meat sample; and (B) a meat sample spiked with 10 μg CAP/kg: 1 = PCB, 2 = CAP TMS, and 3 = TAP TMS. Samples are analyzed according to procedure described in text.

4 μg CAP/mL is injected. The sample extracts are not analyzed unless the 0.2 μg/mL solution gives a positive *m/z* 225/208 qualifier ratio. The standard curve is injected again after the series of analyses is finished.

To prevent false positives, a vial containing pure injection solvent is placed after each CAP-containing sample vial. In addition, a blank meat sample and a blank reagent sample are included with each series. In Figure 4, samples of a SIM chromatogram of a blank and a positive meat sample (10 μg/kg) are shown.

The criteria for GC/MS identification are as follows: (*1*) The relative retention time of the analyte based on internal standard PCB-138 should be the same as the relative retention time of a CAP standard, within a margin of 0.5%; and (2) the relative intensities of the *m/z* 208 and *m/z* 225 diagnostic ions monitored from the analyte (the qualifier ratio) should be identical to the intensity for standard CAP within a margin of 10%.

Validation of the Analytical Strategy

The analytical strategy proposed in this paper consists of a 3-step procedure, with each step having its own acceptability criteria.

Table 5. Probability of acceptance in relation to the CAP concentration

Level, μg/kg	No. samples investigated	No. samples found negative	Probability of acceptance, %
>0–1	136	134	98
>1–2	90	85	94
>2–3	87	69	79
>3–4	55	38	69
>4–6	58	23	38
>6–8	31	8	26
>8–10	5	0	0
>10–15	31	0	0
>15–20	1	0	0
>20–25	28	0	0
>25–30	26	1	4
>30	6	0	0

Screening.—The aim of a screening technique is to filter out negative samples and identify suspected samples. A limited number of false-positive results can be tolerated, but false-negative results should not occur. The probability that false-negative results will occur can be derived from the probability of acceptance at various concentration levels according to an approach described by de Ruig and Dijkstra (23). In this context, the probability of acceptance at a distinct level is calculated as the percentage of negative results at that level. This approach was applied to the combined data obtained from RIKILT and CLRVV laboratories analyses (Table 2) and results obtained for samples analyzed by 13 laboratories in a collaborative study (24). The actual CAP content of these latter samples was established with the LC method. The 5 collaborative study samples, containing between 0 and 27 μg CAP/kg, were analyzed in duplicate both undiluted (after blending with an equal volume of buffer), and after 4-, 7-, and 10-fold dilution. Altogether, 554 samples were analyzed. The results are summarized in Table 5.

We concluded that in 554 analyses, with 1 accidental exception, no false-negative results were obtained above 8 μg/kg. This concentration is higher than the detection limit found by the RIKILT and CL-RVV laboratories. The discrepancy can be explained by difference in experience with the method, and this is attributed to a learning effect.

Confirmation.—In general, the confirmation criteria described in this study are fully in line with criteria proposed for reference methods within the EC. However, this is not the case for GC/MS identification criteria. The EC states that a minimum of 4 diagnostic ions must be measured or, if the analyte does not yield 4 such ions, 2 independent GC-low resolution MS methods with different ionization techniques and/or derivatives should be used.

Identification based on the GC/MS detection of only 2 diagnostic ions, not including the molecular ion, certainly is not acceptable for a reference method, as such. However, the GC/MS method in the proposed analytical strategy is not a stand-alone method. In our case, the confirmation procedure consists of a combination of the following techniques: LC/UV, UV spectrum detection by diode-array detection (DAD), and capillary gas chromatography with mass selective detection, after derivatization.

For such a method, the ability to estimate the uncertainty of the identity of a compound remaining after application of the sequence of analytical techniques would be highly desirable. A rough estimate can be made by using so-called "uncertainty factors" of the separate steps, as postulated by de Ruig et al. (25). Such a factor is defined as the ratio of the number of indistinguishable items to the total number of items in a set. Although the approach is a gross simplification of the actual situation and it is not very accurate, the concept is interesting in that it offers the possibility of more or less quantitatively comparing methods with regard to their reliability. If the analytical techniques used in a tandem are considered to be independent, which is certainly not always the case, the remaining uncertainty can be calculated by multiplication of the individual uncertainty factors of the techniques used. In this approach, with uncertainty factors 50^{-1} (LC), 50^{-1} (DAD), 200^{-1} (capillary GC), and between 300^{-1} and $45\,000^{-1}$ (MS, in the case of 2 diagnostic ions that cannot be considered totally independent), the uncertainty factor for the whole method will then lie between 6×10^{-9} and 4.4×10^{-11}. These values should be used with caution, however, because the assumption of independence of the of the analytical techniques used can be questioned.

As an illustration of the roughness of the postulated procedure, the more detailed approach of Peysna et al. (26) for estimating the uniqueness of a combination of mass fragments can be mentioned. The intensity of the fragment compared to the base peak and the statistical occurrence of fragments in specified mass regions are the key parameter to calculate the uniqueness. With the diagnostic ions m/z 208 and 225, a factor of 1000^{-1} is then obtained for CAP.

For reference, the acceptable probability of error for evidence in criminal proceedings has been put tentatively at 10^{-6}; for indicative evidence, 10^{-2}–10^{-3} is considered to be acceptable (25). We feel that for routine control, the combination of immunochemical screening and LC/DAD quantitation/confirmation will provide sufficient certainty about the identity and quantity of CAP in the sample. If a re-examination is necessary, the procedure is extended by a GC/MS confirmation step. In this specific analytical framework, to finally produce a false-positive result, a sample would have to contain an analyte, or a combination of analytes, that had the following characteristics: (*1*) a good affinity constant to a highly selective antibody raised against CAP; (*2*) similar solubility in water, dichloromethane, toluene, and ethyl acetate as CAP; (*3*) the same retention time as CAP on a LC column; (*4*) a 285 nm UV response corresponding to >10 μg/kg CAP; (*5*) a UV spectrum identical to that of CAP; (*6*) extractable with ethyl acetate from the pH 4.3 LC eluant; (*7*) probably as a derivative, has exactly the same retention time as the di-TMS-CAP derivative on a highly efficient capillary GC column; (*8*) 2 ion fragments characteristic of the fragmentation pattern of CAP under EI/MS conditions; and (*9*) a relative intensity of these ions identical to that of CAP.

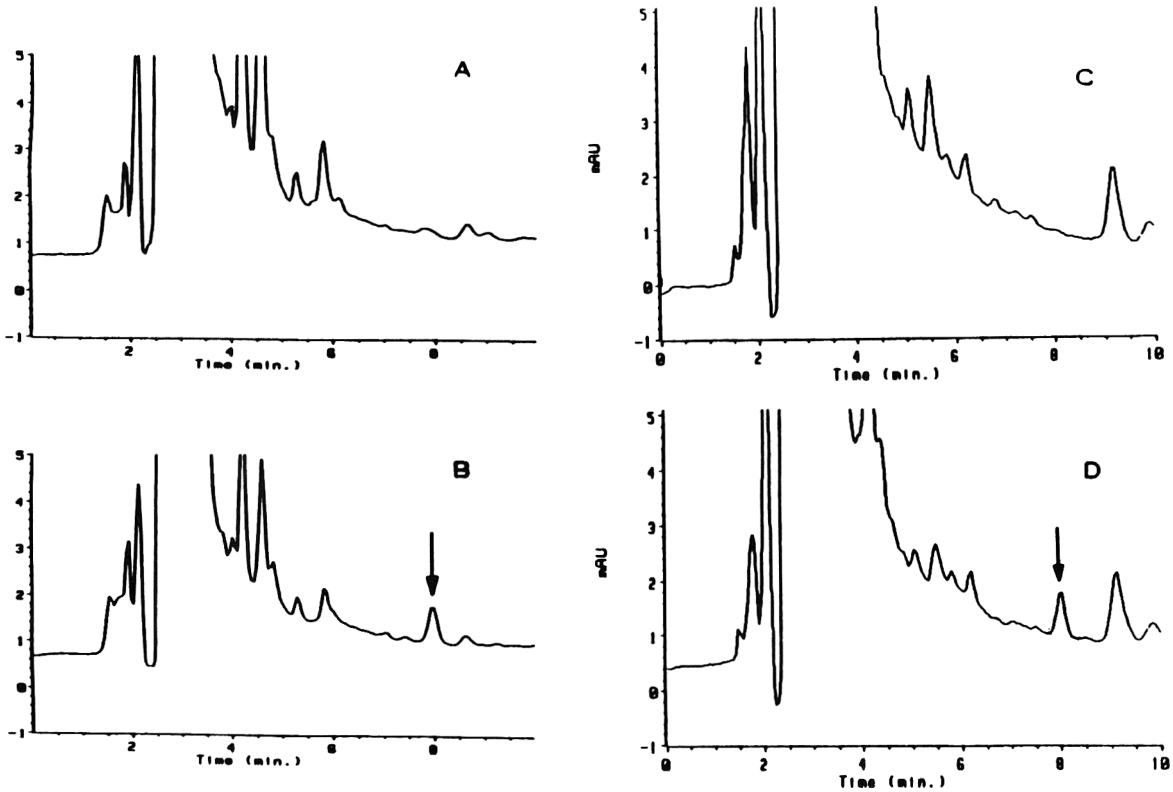

Figure 5. LC chromatograms of (A) blank milk, (B) milk spiked with 1 μg CAP/L, (C) blank pork meat, and (D) meat sample spiked with 1 μg CAP/kg. Samples are analyzed according to modified procedure described in text. Pilot signal is 285 nm.

In summary, this means that the physicochemical, immunochemical, and spectroscopic behavior of such an analyte should be the same as that of CAP. In that respect, we are confident that GC/MS in combination with the other techniques provides conclusive evidence in case of a dispute.

Recent Developments: Application to Milk Control and Improved Sensitivity

Milk.—The analytical approach described for the control of CAP in meat can also be applied to milk. However, because CAP is banned for use in lactating cows, a low MRL of 1 μg/L has been set in many EC member states. This implies that the screening, quantitation, and confirmation should be validated at that level. Results from recent investigations have shown immunochemical screening in milk is possible at the 0.5–1 μg/L level by applying 200 μL deproteinated milk (15) to the La Carte test. The quantitative method used is very similar to the method used for meat. Decreamed milk is directly applied to an Extrelut cartridge and processed according to the method used for meat. The limit of detection obtained is about 0.5 μg/L. Figure 5 shows examples of a blank and a milk sample spiked at 1 μg/kg. The validation of the milk control system, including evaluation of GC/MS confirmation, is currently under investigation. Preliminary experiments show that a limit of identification <0.5 μg/L can be easily achieved when NCI/GC/MS is used with 4 diagnostic ions. Examples are shown in Figures 6 and 7; the blank milk sample depicted contains traces of CAP, corresponding to about 20 ng/L. As can be seen, all 4 diagnostic ions are present, albeit not with proper qualifier ratios.

Meat.—The sensitivity of the LC quantitation and identification of CAP in meat can be improved by blending a 20 g sample with 80 mL water and mixing 60 mL aqueous phase with Extrelut material. This improves the distribution of the meat extract over the diatomaceous earth material, giving a better recovery (27). The meat extract/Extrelut mixture is then transferred into an empty glass column and eluted with dichloromethane. After evaporation, the residue is dissolved in 400 μL water, and 200 μL of this solution is injected into the LC system. Figure 5 shows examples of chromatcgrams of blanks and 1 μg/kg spiked meat samples. Preliminary experiments show that, similar to milk, a limit of identification <0.5 μg/kg can be achieved with NCI/GC/MS.

An attractive alternative approach for obtaining reliable quantitation and confirmation at the 1 μg/kg level may be the use of antibody-loaded (28) or metal-loaded (29) affinity columns to purify and enrich the biological sample. These options will be tested in future research.

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We thank U.A.Th. Brinkman, Free University, Amsterdam, and A. Ruiter, University of Utrecht, for their critical evalua-

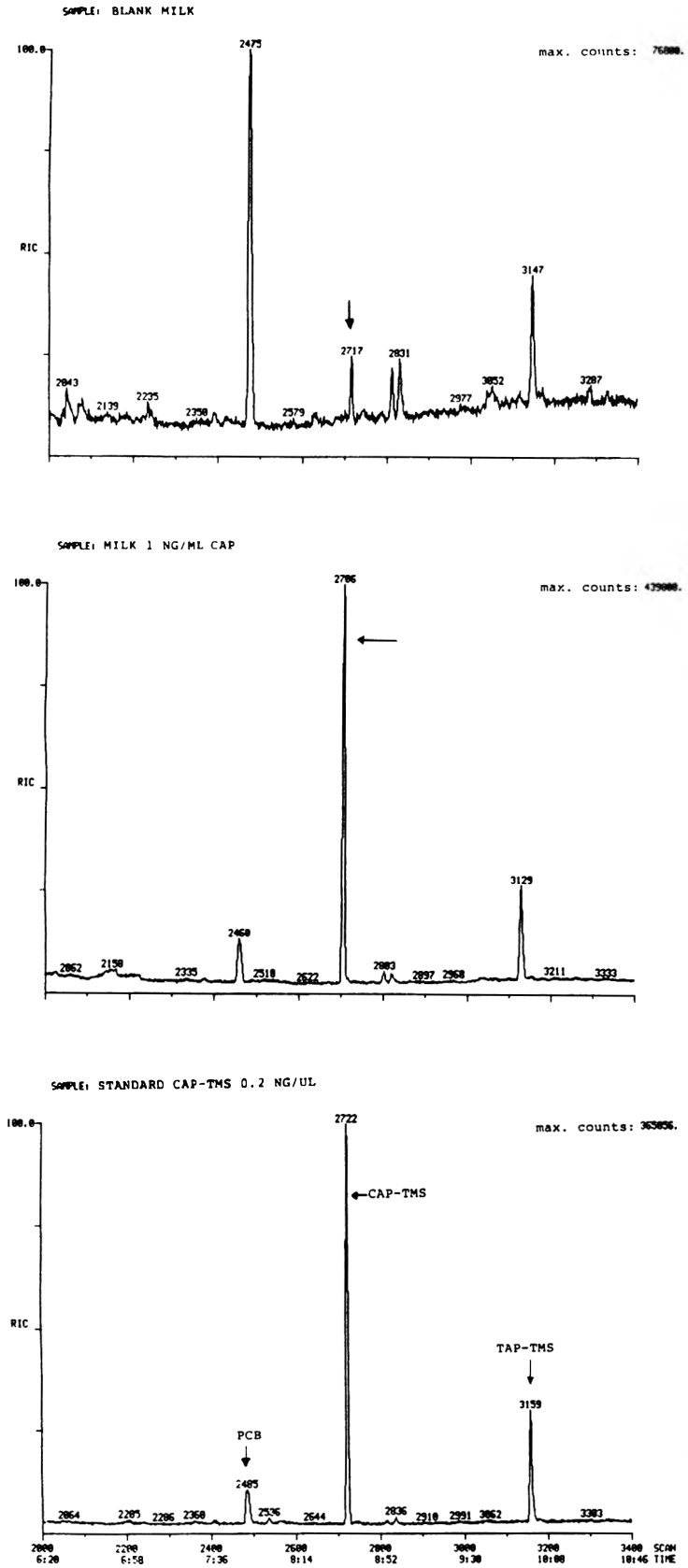

Figure 6. Multiple-ion detection chromatograms of (from the top down) blank milk; 1 μg/L spiked milk; and 0.2 ng/μL standard solution of disilylated CAP. Fragment ions monitored are *m/z* 304/322/466, and 468 (CAP), *m/z* 409 (TAP), and *m/z* 360 (PCB-138). GC/NCI/MS with methane reaction gas was used.

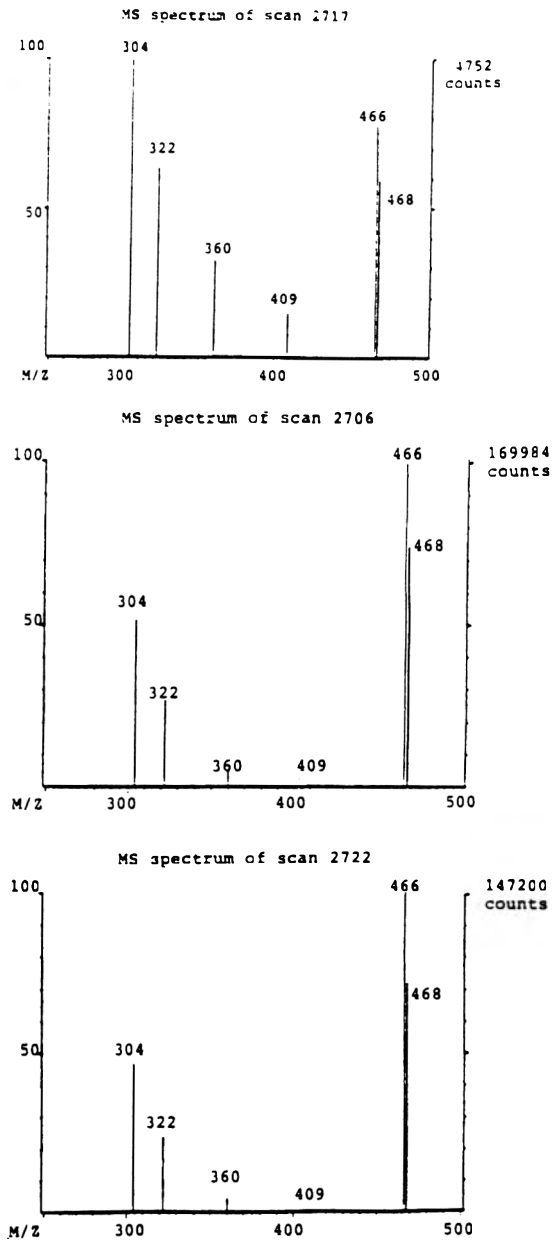

Figure 7. MS spectra of peaks with scan numbers corresponding to standard of disilylated CAP (see Figure 6) for (A) blank milk, (B) 1 ng/mL spiked milk, and (C) standard 0.2 ng/mL.

tion of the results and their suggestions. We also thank L.M.H. Frijns, Central Laboratory of the National Inspection Service for Livestock and Meat, for kindly supplying a number of data on the comparison of the La Carte test and the LC method. The technical assistance of G.M. Binnendijk, M.J.B. de Reuver, P. Kienhuis, and Th.H.G. Polman is greatly appreciated.

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DRUG RESIDUES IN ANIMAL TISSUES

Determination of Cloxacillin and Penicillin V in Milk Using an Automated Liquid Chromatography Cleanup

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A number of screening tests will detect β-lactam antibiotics at levels of less than 10 ng/mL in milk. However, confirmatory procedures of comparable sensitivity are not available. A method using an automated liquid chromatography (LC) cleanup sensitive to 1 ng/mL was developed for penicillin V and cloxacillin. Milk was deproteinized with 2 volumes of acetonitrile. Methylene chloride and hexane were added to the filtrate to separate the water layer containing the antibiotics. The filtrate could also be evaporated directly. The water layer was concentrated and loaded onto a polymeric LC column in 0.01M pH 7 buffer with the aid of an autosampler. The penicillins were eluted with an acetonitrile gradient from pH 7 buffer (100%, 0–3 min) to pH 7 buffer–acetonitrile (40 + 60, 25 min). Fractions containing each compound were collected and rechromatographed isocratically on the same column type in 0.01M H3PO4–acetonitrile (58 + 42 for cloxacillin; 62 + 38 for penicillin V). The cleanup can be fully automated. The approach is applicable to other penicillins, but suitable conditions for analysis of fractions for each compound must be developed. Recoveries for penicillin V were 88 ±2% at 1 ppm, 89 ±10% at 0.1 ppm, and 87 ±13% at 0.01 ppm. For cloxacillin, recoveries were 97 ±5% at 1 ppm, 90 ±4% at 0.1 ppm, and 89 ±8% at 0.01 ppm.

β-lactam antibiotics can be detected in milk at levels of as low as 2–5 ppb by a variety of screening tests (1, 2). Screening tests, with the possible exception of some immunoassays, cannot distinguish β-lactams from one another. They may produce false positives. Therefore, there is a need for specific physico-chemical confirmatory tests of comparable sensitivity. These tests should be able to establish the presence or absence of all possible β-lactam antibiotics at or below levels of sensitivity detected by screening tests. They should also be capable of measuring residues at or below levels of concern to regulatory agencies. Reported methods are deficient in one or more respects. Some do not achieve the needed sensitivity (3–5). A method using gas chromatography described by Meetschen and Petz (6), and methods using liquid chromatography (LC) described by Wiese and Martin (7), Berger and Petz (8), and our laboratory (9) are adequately sensitive but are suitable only for monobasic penicillins. These all use lengthy partitioning cleanup procedures and are unsuitable for determination of amphoteric β-lactams such as ampicillin, amoxicillin, and cephapirin.

A more general method was clearly required for determination of all possible β-lactam antibiotic residues. After exploring a number of approaches, we concluded that direct concentration and cleanup using an LC system was theoretically applicable to any β-lactam antibiotic, and that the method was relatively simple, effective, and reproducible. A deproteinized extract in water was loaded directly onto an LC column and eluted with a solvent gradient. Each compound was eluted as a narrow band. Fractions of the appropriate retention time were collected and rechromatographed under different conditions for analysis. However, optimum conditions must be determined for analysis of each fraction so that the compound of interest can be eluted in a reasonable length of time and separated from interferences in the fraction. This approach was successfully applied to the determination of penicillin G with markedly improved sensitivity (<2 ppb) and excellent separation from interferences (10). The LC cleanup was also applied to determination of ampicillin in milk (11). The present paper describes the application of this approach to the determination of cloxacillin and penicillin V in milk.

Materials and Methods

Reagents

(a) *Acetonitrile.*—LC grade (absorbance <0.02 at 210 nm).

(b) *Methylene chloride and hexane.*—LC grades.

(c) *Cloxacillin and penicillin V standards.*—Reagent grade (Sigma Chemical Co., St. Louis, MO 63178). Prepare β-lactam stock solutions of 1 mg/mL in distilled water and prepare working dilutions as required. Store refrigerated. Prepare fresh solutions monthly or more often if deterioration is noted.

(d) *pH 7.0 buffer, 0.01M.*—Dissolve 1.39 g KH2PO4 and 2.81 g Na2HPO4 in 3 L distilled water, or mix 2 volumes 0.01M Na2HPO4 with 1 volume 0.01M KH2PO4.

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Apparatus

(a) *Glassware.*—Graduated cylinders, 50 and 100 mL, 15 mL conical graduated centrifuge tubes (calibrated to contain), 250 mL glass-stoppered side-arm flasks, 250 mL separatory funnels with Teflon stopcocks, and 125 mL conical flasks. Clean all glassware in special detergent, MICRO (International Products, Trenton, NJ), or equivalent. Rinse in distilled water, ca 0.01N HCl or H2SO4, and distilled water.

(b) *LC equipment cleanup system.*—Pump, Model 9010 (Varian Instrument Group, Sugarland, TX); autosampler, WISP Model 712 with 2000 μL sample loop (Waters Chromatography Division, Milford, MA 01757); fraction collector, FOXY (Isco, Inc., Lincoln, NE 68505); detector/data system, 990 diode array detector (Waters); column, PLRP-S, 4.6 × 150 mm, 5 μm particle size, 100 Å pore diameter (Polymer Laboratories, Amherst, MA 01002).

(c) *LC analysis equipment.*—Pump, LC5000 (Varian); autosampler, 9090 (Varian); detector, Model 481 UV-VIS (Waters); data system, Model 650 (Varian); column, same as cleanup.

(d) *Buchler rotary Evapomix.*—Buchler Instruments, Ft. Lee, NJ.

(e) *Hot plate.*—Thermostatted, with shallow tray.

(f) *Plastic coated lead rings.*—To weight side-arm flasks during evaporation.

(g) *Vortex mixer.*

Determination

Measure 15 mL milk into 125 mL conical flask. Add 30 mL acetonitrile slowly in several small portions with vigorous swirling of flask during addition. (Failure to stir during addition can lead to uneven precipitation and variable recoveries.) Let stand 5–10 min until supernatant clears. Pour supernatant through loose plug of glass wool in stem of funnel and collect 30 mL filtrate in graduated cylinder. Filtrate should be water-clear. If not, pour filtrate back through glass wool plug until clear.

Concentration, Method A.—Transfer filtrate to 250 mL separatory funnel, add 30 mL methylene chloride and 60 mL hexane, shake vigorously, and let layers separate. Collect bottom (water) layer in glass-stoppered side-arm flask. Wash organic layer with 5 mL water and add to side-arm flask. Evaporate liquid in stoppered side-arm flask under reduced pressure (water pump) in a shallow water bath, starting at room temperature, and warming to 40–50°C. A lead ring may be used to weight side-arm flask. After initial boiling, liquid will evaporate quietly. Concentrate to ca 2 mL and transfer liquid to 15 mL conical graduated centrifuge tube. Rinse flask with several small portions of water and add rinsings to tube to final volume of 4 mL. Transfer clear liquid to 4 mL autosampler vials. If visible particles are present, filter through small plug of glass wool into autosampler vials.

Concentration, Method B.—Transfer filtrate to 250 mL side-arm flask with rinse of 10 mL *t*-butanol (to suppress foaming). Evaporate as in *Method A* to ca 2 mL and rinse into graduated centrifuge tubes to give final volume of 4 mL. Add 1 mL acetonitrile and 3 mL hexane, and mix 10 s on Vortex mixer. Centrifuge 1 min at low speed and draw off hexane with pipet. Repeat wash with 3 mL hexane. Evaporate cautiously in Evapomix to less than 4 mL to remove residual solvent. Adjust volume to 4 mL and transfer to 4 mL autosampler vials.

LC Cleanup

(a) *Solvent.*—0.01M pH 7.0 buffer–acetonitrile.

(b) *Gradient.*—0.01M pH 7 buffer (100%, 0–3 min) to 0.01M pH 7 buffer–acetonitrile (40 + 60, 25–30 min) to 0.01M pH 7 buffer (100%, 31 min). Loading of the next sample is started at 40 min. Flow, 1 mL/min.

Determine retention times of compounds of interest using standards. Set autosampler to inject 2000 μL. Set fraction collector to collect 1.2 mL time windows centered on retention times, allowing a 0.1–0.2 min delay for flow from detector to fraction collector. Before each sample set, run 2 μg standards loaded, in successive determinations, in 200 and 2000 μL solvent. Retention times, peak shape, and peak size should be identical. Autosampler starts gradient program, fraction collector, and data system each time sample is injected. If system is run unattended overnight, program pump to flush system with water–acetonitrile (50 + 50) when final sample (water) is injected.

Prepare fractions for analysis by removing acetonitrile under reduced pressure in rotary Evapomix. Adjust volume to 1 mL and transfer to 2 mL autosampler vials (or inserts, if WISP is used for analysis).

Analysis

Injection volume, 200 μL. Isocratic: 0.01M H3PO4–acetonitrile; flow, 1 mL/min. Penicillin V, 62 + 38. Cloxacillin, 58 + 42. Detection: UV, 210 nm. Peak height is linear with concentration to at least 5 μg of each compound.

Recovery and Confirmation

For recovery experiments, add 0.15 mL 100, 10, and 1 μg/mL stock solutions to 15 mL milk to give final concentrations of 1, 0.1, and 0.01 μg/mL. For confirmation, add 0.2 mL penase concentrate (Difco Laboratories, Detroit, MI 48232) to 15 mL milk and let stand 3 h (or as required to destroy cloxacillin standard) at room temperature (ca 22°C) before performing method. Milk from cows treated with cloxacillin was obtained from the U.S. Food and Drug Administration, Center for Veterinary Medicine, Beltsville, MD.

Results and Discussion

Of several monobasic penicillins, only penicillin G and cloxacillin are of major concern as residues in the United States. However, penicillin V is of concern in some other countries, including Germany. The extraction procedure was modified slightly, because cloxacillin is more soluble than penicillin G in organic solvents. The proportion of hexane added to the filtrate was increased to ensure that cloxacillin remained in the water layer formed after addition of methylene chloride and

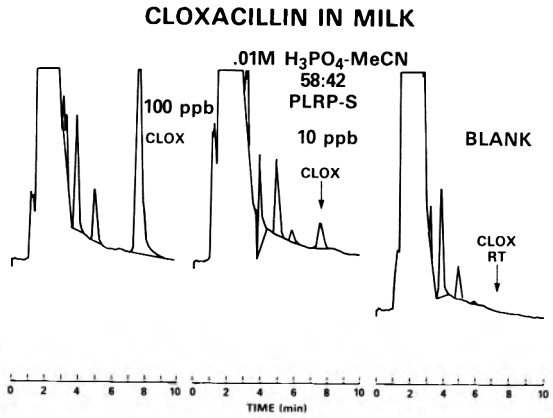

Figure 1. Determination of cloxacillin in milk.

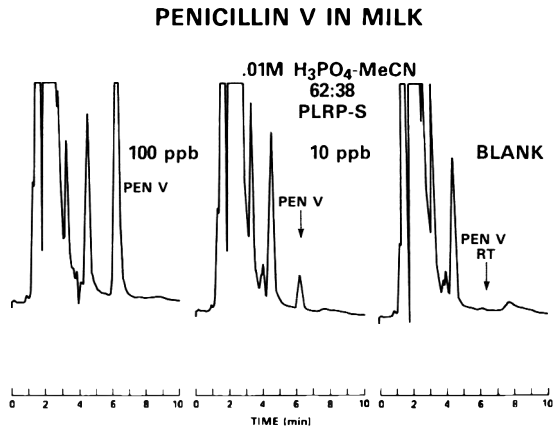

Figure 2. Determination of penicillin V in milk.

hexane. The filtrate could also be concentrated directly (Method B). A prototype centrifugal evaporator manufactured by the Christ Co., Osterode, Germany, was tested with Method B and proved satisfactory. A wash with hexane–acetonitrile was required to clear the concentrated filtrate. The direct concentration procedure saved substantially on solvent. The fraction collection procedure was as previously described for penicillin G (10).

Although the equipment used in our laboratory for cleanup is indicated, any combination of a pump capable of generating a gradient, a fraction collector capable of collecting time windows, a UV detector and data system, and an autosampler capable of injecting 1 mL or more should be suitable. WISP will inject a larger volume (2 mL) than other commercial autosamplers. We would prefer to use even larger volumes of more dilute sample extract if autosamplers of suitable capacity were available. The procedure did not work as satisfactorily when bonded C18 columns were used in place of the polymeric columns.

The strategy for separation of cloxacillin and penicillin V from interferences was, as with penicillin G, to rechromatograph at acid pH the fractions collected at pH 7. This converted the compounds from the salt to the acid forms, which were far more strongly retained on reversed-phase packings. They were thus effectively separated from interference in the fractions as shown in Figures 1 and 2. The limit at which a detectable peak could be observed was about 1 ppb. Because of the widely differing retentions of penicillins on reversed-phase columns, each compound requires a different solvent system for isocratic analysis. The recoveries from spiked samples are shown in Table 1. Mean recoveries were slightly below 90% for penicillin V and 89–97% for cloxacillin. Precision was better at 1 ppm than at lower concentrations, especially 0.01 ppm, as would be expected. No detectable interference was noted in the cloxacillin chromatograms. A minor peak, equivalent to 1.5 ng/mL, eluted near penicillin V. This peak was not present in some milk samples but could be subtracted after the sample was treated with β-lactamase. Table 2 shows results with some milk samples from treated cows. Cloxacillin was present after 48 h but was below detectable levels (1 ppb) at 96 h.

Screening methods are markedly less sensitive for cloxacillin than for other β-lactam antibiotics. Reported detection limits range from 20 ppb for the Angenics spot test (an immunoassay) to 150 ppb for the Penzyme test (1). The present method thus far exceeds the sensitivities of screening tests for cloxacillin and gives better separations from interferences than the LC method previously described by our laboratory (9). These methods also meet the stringent regulatory requirements of Germany, with tolerances for cloxacillin and penicillin V of 10 and 3 ppb, respectively (8).

The method required about 4 h for a single sample. Because the LC cleanup was sequential, about 1 h additional was required per sample. The automated cleanup was usually run overnight so that time was not ordinarily a factor. The presence of cloxacillin and penicillin V can be confirmed by treating duplicate samples with β-lactamase as described by Terada and Sakabe (4). Several hours were required to decompose cloxacillin as compared to a few minutes for penicillin G.

Table 1. Recoveries of cloxacillin and penicillin V from spiked milk samples

Added, μg/mL	Penicillin V, rec., %	Mean ± SD	Cloxacillin, rec., %	Mean ± SD
1.0	88, 84, 87, 91, 90	88 ± 2	95, 89, 104, 100, 97	97 ± 5
0.1	90, 75, 105, 92, 84	89 ± 10	92, 87, 96, 86, 90	90 ± 4
0.01	84, 111, 75, 80, 85	87 ± 13	98, 85, 97, 76, 87	89 ± 8
0	0–0.0015 μg/mL	—	ND[a]	—

[a] Not detectable.

Table 2. Cloxacillin in milk from treated cows

Time after treatment, h	Cloxacillin found, ppb
36	15
48	10
96	None (<1)

The method described is relatively simple because only a single cleanup step is used and derivatization is not required. This undoubtedly contributes to the excellent recoveries observed. The approach should be applicable to determination of any β-lactam antibiotic as well as other compounds. However, conditions must be developed for further separation of analytes from interferences. This has proved especially difficult with amphoteric β-lactams, as shown by studies with ampicillin (11).

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Incurred Nitrofurazone Residues in Chicken Tissues

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One-day-old chicks were raised to maturity on a diet fortified with 0.0055% nitrofurazone. Analyses of tissue extracts by a liquid chromatography/electrochemical detection method revealed that the distribution of residues between liver, breast, and thigh muscle differed significantly from that previously reported in birds that were dosed with the drug before sacrifice. Differences between ground and unground tissues were also observed, suggesting that residues are not distributed evenly throughout the same tissue.

Nitrofurazone (5-nitro-2-furaldehydesemicarbazone), when fed continuously, is effective in preventing coccidiosis in chickens (1). However, information is lacking on residues of the drug in the tissues of treated birds, as a result of the rapid metabolism of the nitrofuran and previous absence of sensitive methods of detection. Recently, we reported a liquid chromatographic (LC) method capable of detecting nitrofurazone residues in fortified chicken tissues at the low ppb level (2). This method was applied to the tissues of birds that had been placed on a diet fortified with 0.0055% nitrofurazone as 1-day-old chicks and continued on the diet to maturity. This communication reports the levels of the drug found in the liver, breast, and thigh muscle, as well as other observations.

METHOD

Reagents and Materials

(a) *Solvents, tissue homogenizer, centrifuge, sand, neutral alumina.*—Same as in Reference 2.

(b) *Drugs.*—Nitrofurazone (Norwich-Eaton Pharmaceuticals, Norwich, NY 13815); Amifur™ medicated premix containing 50 g nitrofurazone/lb (SmithKline Animal Health Products, West Chester, PA 19380).

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(c) *Liquid chromatography/electrochemical detection.*—LC-5000 precision pump (Isco Inc., Lincoln, NE 68505) connected to Model LC-4B amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN 47905); glassy carbon electrode –0.8 V vs Ag/AgCl, 5–10 μA full scale. Altex Model 210A sampling valve with 50 μL loop. Recorder: Fisher Recordall Series 5000 at 10 mV full scale; chart speed 1 cm/min. Column: 25 cm × 4.6 mm id 5 μm Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA 16823). Mobile phase: pH 6.0 phosphate buffer (0.05M monobasic potassium phosphate solution containing 0.001M EDTA adjusted to pH 6.0 with 1N NaOH)–methanol (57.5 + 42.5) purged with helium. Elute samples isocratically at 1.0 mL/min.

Feeding Trials

One-day-old male broiler chicks were placed on a commercial-type starter-grower diet fortified with 0.0055% nitrofurazone. At 42 days of age, 9 birds were sacrificed. An additional 9 medicated birds were removed to a control feed for 2 days and then sacrificed. Immediately after sacrifice, livers were removed, placed in a plastic bag, and frozen in liquid nitrogen to limit postmortem metabolism of the drug. Breast and thigh muscle were removed as quickly as possible and frozen to less than –50°C. All tissues were maintained at less than –50° to –20°C before analyses. Tissues removed from 9 birds raised for 42 days on a nonmedicated feed served as controls.

Tissue Preparation

Frozen tissues from 2 birds were partially thawed, cubed, and blended together in a chilled (4°C) Waring blender. The blended tissues were quickly packed in plastic bags and refrozen in dry ice. The tissues of a single bird were left unground and frozen.

Determination and Quantitation

Analyses were conducted as previously described (2). Daily standard solutions containing ca 2.0, 5.0, 10.0, 84.0, and 168.0 ng/2 mL pH 6.0 phosphate buffer–methanol (1 + 1) were prepared from stock solutions of nitrofurazone in dimethylformamide. Average detector response factor (*R.F.*) was determined by relating concentration to measured peak

Table 1. Concentration of nitrofurazone in tissues of zero birds

Bird No.	Concn, ppb		
	Liver	Thigh	Breast
1024 1028	146.2 ± 3.8	2.22 ± 0.11	2.64[a]
1033 1040	120.1 ± 8.0	2.20 ± 0.22	1.39[a]
1047 1053	87.4 ± 13.2	1.17 ± 0.00	0.69[b]
1058 1065	147.8 ± 5.27	2.30 ± 0.05	2.72 ± 0.14
1067[c]	63.1 ± 14.5	9.11 ± 4.91	5.36 ± 2.31

[a] Single determination.
[b] Detected in 1 sample only.
[c] Unground tissues.

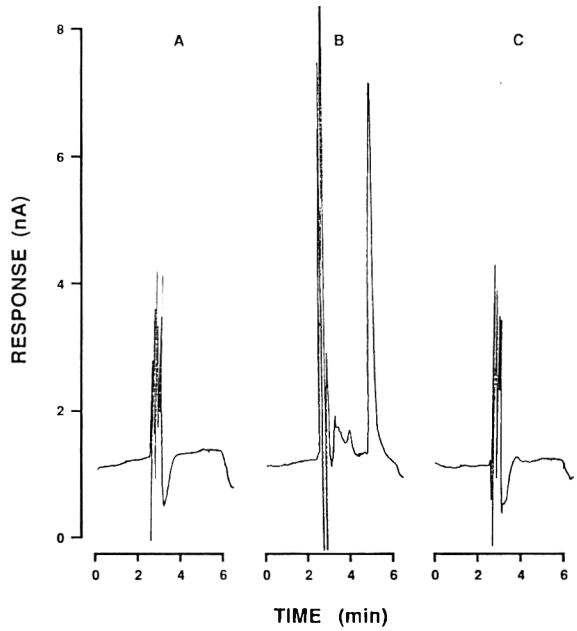

Figure 1. Chromatograms of liver extracts of (A) birds maintained on nonmedicated control feed, (B) birds fed nitrofurazone-fortified feed continuously until sacrifice, and (C) birds fed nitrofurazone-fortified feed continuously, then moved to control feed 2 days before sacrifice. Electrochemical detection: potential −0.8 V; attenuation 10 nA full scale.

heights (ng/mm). Concentration of nitrofurazone in incurred tissue was determined by the following formula:

$$\text{Concentration, ppb} = \frac{R.F.\ (\text{ng/mm}) \times \text{peak height (mm)}}{1.875\ \text{g tissue} \times 0.75}$$

where 0.75 represents percent recovery from fortified tissues (2). Duplicate samples were analyzed on different days.

Results and Discussion

Table 1 summarizes the results of analyses of tissue samples from birds sacrificed while on the nitrofurazone-fortified feed (zero time birds). With the exception of Liver 1047-1053, duplicate determinations were generally in good agreement among the blended samples. Concentrations determined in duplicate unground tissues (Bird 1067) varied considerably, suggesting that the residues were not uniformly distributed throughout the tissue. Furthermore, the ratios of total residues in the breast and thigh muscle to liver tissue in the unground samples were 7–8 times as great as the ratios determined in the ground tissues. The reason for this is unclear but may be a reflection of the uneven distribution of residues in tissues, differences between birds, or poor extraction of residues from unground liver tissues. Nitrofurazone was not detected in the tissues of birds removed from the medicated feed 2 days before sacrifice (minimum level of detection, 0.5 ppb).

In addition to the parent drug (R_t 5.1 min), a small peak, presumably a metabolite, was present in all chromatograms of extracts of incurred liver tissues (Figure 1). The peak had the same retention time (4.0 min) as that previously observed in studies on incurred furazolidone tissues (3). This small peak could be produced in control liver tissues fortified with 200 ppb nitrofurazone by incubating 30 min at 37°C. Stability of the unidentified metabolite on further incubation and/or frozen storage was similar to that observed in the furazolidone studies (3).

The distribution of residues in the tissues in this study differs significantly from that reported by Sugden et al. (4). The latter observed average concentrations (870 ppb) 15 times as great in muscle tissue as in liver tissue 8 h after birds were dosed with 50 mg nitrofurazone. The distribution approached equality (6 ppb) after 24 h. The birds in this study consumed, on the average, approximately 8 mg nitrofurazone (150 g feed) in the 24 h period before sacrifice. These observations suggest that differences occur in drug uptake and/or metabolism between dosing and normal feeding practices.

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EXTRANEOUS MATERIALS

Extraction of Light Filth from Fish Paste and Sauce (Bagoong) Not Containing Spice: Collaborative Study

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A collaborative study was conducted to validate a new method for the extraction of light filth from fish paste and sauce (Bagoong) not containing spice. A 225 g test portion is digested by boiling in a mixture of acid and emulsifying agents. Light filth is isolated by wet sieving on a No. 230 plain weave sieve with Tergitol, a deaeration boil in 40% isopropanol, and flotation with mineral oil and 40% isopropanol in a Wildman trap flask. Three spiking levels were used in the study for rat hairs and insect fragments; 1 level was used for whole or equivalent insects. For rat hairs, recoveries at the low, medium, and high levels averaged 77, 94, and 76%, respectively. Recoveries of insect fragments for these levels averaged 92, 88, and 93%, respectively; recoveries of whole or equivalent insects averaged 85, 70, and 80%, respectively. The method was adopted first action by AOAC International for the extraction of light filth from fish paste and sauce (Bagoong) not containing spice.

The quantity of ethnic food products imported into the United States continues to increase on an annual basis. Fermented fish pastes and sauces comprise a large proportion of the market. These products, prepared from several species of salt water and fresh water fishes, such as anchovy, goby, scad, corvina, and ziganid, are imported from several Asian and European countries, including Thailand, the Philippines, Korea, Spain, and Portugal.

There is no official method for determination of light filth in fish pastes and sauces. A new method has been developed that involves a digestion step that uses a combination of emulsifying agents and acid, a wet sieving procedure with Tergitol, extraction of light filth with mineral oil from a hot 40% isopropanol solution, and trapping in a Wildman trap flask.

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Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the U.S. Food and Drug Administration.

Collaborative Study

The collaborative study was performed as a new method for the extraction of light filth from fish paste and sauce (Bagoong) not containing spice. The product was spiked at 3 different levels. The low spike level consisted of 5 insect fragments (elytral squares of *Tribolium confusum*, approximately 0.5 mm sq) 5 rat hairs (1–3 mm), and 5 whole insects (adult *Drosophila*). The middle spike level consisted of 15 insect fragments, 10 rat hairs, and 5 whole insects. The high spike level consisted of 30 insect fragments, 15 rat hairs, and 5 whole insects. Eight test samples of product in glass containers together with 2 vials of each spike level were sent to each of 6 collaborators. The test samples were numbered from 1 to 6; spike vials were numbered to correspond to the appropriate test sample. Two nonspiked test samples (P1 and P2) were designated for use as "practice" portions. The collaborators were instructed to report these results and analysis times and to return the extract papers so that their results could be checked by the Associate Referee.

991.37 Light Filth from Fish Paste and Sauce (Bagoong) Not Containing Spice—Flotation Method

First Action 1991

Method Performance:
Rat hairs, 5 added
$s_r = 0.5$; $s_R = 1.1$; $RSD_r = 15.0\%$; $RSD_R = 30.1\%$
Rat hairs, 10 added
$s_r = 0.7$; $s_R = 0.7$; $RSD_r = 8.1\%$; $RSD_R = 8.4\%$
Rat hairs, 15 added
$s_r = 2.8$; $s_R = 4.2$; $RSD_r = 24.6\%$; $RSD_R = 36.8\%$
Insect fragments, 5 added
$s_r = 0.3$; $s_R = 0.3$; $RSD_r = 6.4\%$; $RSD_R = 6.4\%$
Insect fragments, 15 added
$s_r = 0.6$; $s_R = 1.5$; $RSD_r = 4.8\%$; $RSD_R = 11.6\%$
Insect fragments, 30 added
$s_r = 1.8$; $s_R = 2.2$; $RSD_r = 6.7\%$; $RSD_R = 8.1\%$

Table 1. Collaborative results for recovery of rat hairs (blind duplicates)

Coll.	Spike level 5		10		15	
A	5	5	15 (10)	10	15	16 (15)
B	7 (4)[a]	4	11 (8)	12 (10)	18 (13)	9
C	2	3	13 (10)	12 (9)	30 (14)	9
D	2	3	7 (9)	5 (8)	1 (2)	7
E	6 (5)	4	24 (10)	15 (9)	15	10
F	9 (5)	4	13 (10)	13 (10)	15	13
$\bar{X}$	4.5 (3.8)		12.5 (9.4)		13.2 (11.4)	
$\bar{X}$, %	90.0 (76.6)		125.0 (94.2)		88.0 (76.1)	
s_r	1.83 (0.57)		3.05 (0.76)		7.00 (2.81)	
s_R	2.09 (1.15)		4.81 (0.79)		7.12 (4.20)	
RSD_r	40.6 (15.0)		24.4 (8.1)		53.2 (24.6)	
RSD_R	46.4 (30.1)		38.5 (8.4)		54.1 (36.8)	

[a] Associate Referee counts in parentheses if different from those of collaborator.

Whole insects, 5 added

$s_r = 0.8$; $s_R = 0.8$; $RSD_r = 19.8\%$; $RSD_R = 20.4\%$

A. Pretreatment

Weigh 225 g sample into 2 L beaker. Add 800 mL 5% HCl (760 mL water + 40 mL HCl) and 15 mL Igepal [5 mL DM-710 and 10 mL CO-730, **945.75C(j)**]. Cover beaker with watch glass, bring contents to full boil, and boil gently with magnetic stirring on stirrer-hot plate, **945.75B(n)**, until homogeneous slurry is obtained, typically 60–90 min. (*Note*: Be sure contents do not boil over during digestion.)

B. Isolation

Transfer slurry portionwise onto No. 230 plain weave sieve, **945.75B(r)**, and wet sieve with forceful stream of hot tap water (55–60°C) from aerator, **945.75B(a)**, until rinse is clear. (*Note*: Use rubber policeman or spatula to remove residue that adheres to sides of beaker.) Add 10 mL Tergitol Anionic 7, **945.75C(bb)**, if substantial residue (>15 cc) remains on sieve. Wet sieve until all foam washes through and residue appearance remains constant. (*Note*: Soaking residue in Tergitol on sieve 2–3 min aids in dispersing clumped material.) Repeat Tergitol procedure twice.

Wet residue on sieve with 40% isopropanol, and quantitatively transfer residue to 2 L Wildman trap flask, **945.75B(h)**(*4*), using 40% isopropanol. Dilute to 800 mL with 40% isopropanol. Place on stirrer-hot plate in fume hood and boil gently 10 min while stirring. Remove from heat, add 50 mL mineral oil, **945.75C(p)**, and stir magnetically 3 min. Fill flask with 40% isopropanol and let stand 30 min with intermittent stirring. Spin wafer disc or stopper to remove sediment and trap off, rinsing neck of flask with 40% isopropanol. Add 35 mL mineral oil. Hand-stir sediment with gentle rotary motion. Fill flask with 40% isopropanol, let stand 20 min, and trap off as before, rinsing neck with isopropanol. Filter onto ruled filter paper, **945.75B(i)**, and examine at 30×, with stereoscopic microscope, **945.75B(o)**(*2*).

Ref.: *J. AOAC Int.* **75**, March/April issue (1992)

Results and Discussion

Most collaborators obtained good recoveries of rat hairs (Table 1). However, Collaborators C and D reported 40–60% recoveries for the low spike level. Collaborator D also obtained low recoveries in both replicates of the high spike level (13 and 47%). None of these results qualified as outliers by the Cochran and Grubbs tests. Both collaborators had good recoveries at the middle spike level. Background striated hairs within the specified size range of the spike rat hairs were counted in several test samples, but this problem was resolved by having an expert third party microanalyst review the extraction plates and record the number of spike hairs (cut, blunted ends, 1–3 mm) found. The background hair fragments were generally longer than 3 mm and all had tapered ends. Third party counts were required on 17 of 36 (47%) rat hair results.

As shown in Table 2, recoveries of insect fragments were good. The mean recovery for insect fragments was 93.2% at the low spike level, 88.3% at the middle spike level, and 93.1% at the high spike level. Collaborator B reported low recoveries (40%) in 1 replicate at the low spike level. Collaborator D counted an insect fragment that was part of the intrinsic filth and of the approximate size and shape of the spike material. One replicate of low spike insect fragment data for Collaborator B was classified as an outlier by the Grubbs test.

Recoveries of whole or equivalent insects were generally acceptable (Table 3). The results for whole insects were analyzed for each replicate pair and as a combined grouping of all test samples on the basis of 6 analyses per laboratory. Collaborator D reported low recoveries for each of the replicate pairs (20%, 0, 0) while Collaborator E had low recoveries in 2 rep-

Table 2. Collaborative results for recovery of insect fragments (blind duplicates)

	Spike level					
Coll.	5		15		30	
A	5	4	15	14	29	30
B	4[a]	2[a]	11	12	28	28
C	5	5	13	14	26	27
D	6 (5)[b]	5	12	11	28	22
E	5	5	15	15	30	29
F	5	5	13	14	28	30
$\bar{X}$	4.67 (4.9)		13.2		27.2	
$\bar{X}$, %	93.0 (91.6)		88.3		93.0	
s_r	0.71 (0.31)		0.64		1.89	
s_R	1.01 (0.31)		1.54		2.26	
RSD_r	15.25 (6.4)		4.8		6.7	
RSD_R	21.6 (6.4)		11.6		8.1	

[a] Outlier by Grubbs test: not included in calculations.
[b] Associate Referee counts in parentheses if different from those of collaborator.

Table 3. Collaborative results for recovery of whole or equivalent insects (blind duplicates)

	Spike level					
Coll.	5		5		5	
A	5	5	5	5	5	5
B	5	4	4	5	5	4
C	4	5	4	3	4	4
D	1[a]	2[a]	0	3	0	3
E	5	5	2	2	5	5
F	5	5	5	4	5	3
$\bar{X}$	4.2		3.5		4.0	
$\bar{X}$, %	85.0		70.0		80.0	
s_r	0.44		1.00		1.08	
s_R	0.44		1.61		1.51	
RSD_r	9.3		28.5		27.0	
RSD_R	9.3		46.0		37.7	

[a] Outlier by Grubbs test; not included in calculations.

licates (both at 40%). Statistical analysis revealed that Collaborator D had 1 replicate determined to be an outlier in both replicate and combined grouping data. Mean recoveries for each set of replicate test samples were 85, 70, and 80%, respectively.

Three collaborators reported no problems with the method. Collaborators A, D, and E experienced difficulty in transferring residue from the digestion beaker to the sieve because material adhered to the sides of the beaker. Filter papers that were returned to the Associate Referee had light to medium product debris. The collaborators took an average of 4 h (range, 2–7 h) per test sample to perform the method.

Two important procedures routinely used in filth extraction methods can affect recovery: the wet sieving operation and quantitative transfer of residue during analysis. Failure to perform either of these operations correctly can result in loss of filth.

The sieving operation is performed a minimum of 1 and a maximum of 4 times in the method. The first sieving step removes matter resulting from product digestion as well as the acid and emulsifying agents used in the digestion process. Subsequent sieving with Tergitol reduces the volume of sieve retainings by further breakdown of undispersed product. The correct sieving procedure, **970.66B(a)**, is crucial. Using too forceful a spray during the sieving operation or holding the sieve at an improper angle during sieving will cause splashing, which can result in low filth recoveries. The use of water at the proper sieving temperature (55–60°C) is also important. If the proper temperature is not used, a more forceful spray is required to "push" the debris through the sieve. One brand of fish product used in the study tended to congeal on the sieve. This was corrected by wet sieving with water at the proper temperature.

Transferring residue from one vessel to another can also result in the loss of filth. As previously mentioned, some collaborators reported that residue collected on the sides of the beakers during the digestion process. Several hot water rinses and the use of a rubber policeman were required to transfer the residue to the sieve.

Recommendation

Recoveries were generally satisfactory, and the filter papers obtained were usually free of interfering debris. We recommend that the proposed method for the extraction of light filth from fish paste and sauce (Bagoong) not containing spice be adopted first action.

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Gary Dzidowski, U.S. Food and Drug Administration (FDA), Detroit, MI

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Deborah Floyd, FDA, Dallas, TX

Beverly Kent, FDA, Baltimore, MD

Joseph Nagy, FDA, Philadelphia, PA

W.T. Van Velzen, FDA, Seattle, WA

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EXTRANEOUS MATERIALS

Extraction of Light Filth from Dried Bean Curd: Collaborative Study

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Results are reported for a collaborative study of a method for the extraction of light filth from dried bean curd. A 100 g test portion is dispersed in a 5.7% HCl solution. Residue from the No. 230 sieve is defatted in isopropanol, and the sieved residue is transferred to a Wildman trap flask. Light filth is isolated from 40% isopropanol by using Na_4EDTA and mineral oil–heptane (70 + 30). Average recoveries by 6 collaborators for 3 spike levels of rat hairs (5, 10, and 15) were 85, 81, and 70%, respectively; for insect fragments (5, 15, and 30), recoveries were 72, 83, and 72%, respectively. The method was adopted by AOAC International as first action.

Dried bean curd is a soybean product made by heating soybean milk to near boiling and recovering the protein–oil film that forms on the surface. The film is formed into sheets or sticks for drying. Another soybean product, often labeled as wet bean curd, is tofu, which is precipitated from boiling soybean milk with $CaSO_4$. Dried bean curd resembles pasta in appearance and is never refrigerated, whereas tofu is packed in water and refrigerated. When tofu is canned, freeze-dried, or preserved, it is not refrigerated.

The generally open system in which dried bean curd is produced can expose it to rodent and insect contamination; therefore, a method was developed to extract light filth from dried bean curd. The method consists of dispersing 100 g of crumbled or broken dried bean curd in dilute HCl solution, wet sieving on a No. 230 screen, defatting in isopropanol, and wet sieving again. The residue is transferred to a 2 L Wildman trap flask with 40% isopropanol, and light filth is extracted by using Na_4EDTA and mineral oil–heptane (70 + 30). The mineral oil–heptane mixture is trapped and filtered, and the filth is counted.

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Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the U.S. Food and Drug Administration.

Collaborative Study

Each collaborator received six 100 g test portions of dried bean curd sticks and 6 vials of spike. The vials consisted of duplicates of 3 spike levels: 5 rat hairs (2.5–3.5 mm long) and 5 insect fragments (elytral squares of *Tribolium confusum*, about 0.5 mm sq) for the low level, 10 rat hairs and 15 insect fragments for the intermediate level, and 15 rat hairs and 30 insect fragments for the high level. The vials were numbered randomly, 1–6. Collaborators were instructed to report their analytical times and to return the extraction papers so the Associate Referee could check their results.

991.40 Light Filth in Dried Bean Curd—Flotation Method

First Action 1991

Method Performance (expert's counts in parentheses):
Rat hairs, 5 added
s_r = 1.8 (1.5); s_R = 1.8 (1.5); RSD_r = 34.6% (35.7%); RSD_R = 34.6% (35.7%)
Rat hairs, 10 added
s_r = 2.4 (1); s_R = 2.6 (1.3); RSD_r = 27.3% (12.3%); RSD_R = 29.5% (16%)
Rat hairs, 15 added
s_r = 2.4 (2.1); s_R = 2.4 (2.1); RSD_r = 22.2% (20%); RSD_R = 22.2% (20%)
Insect fragments, 5 added
s_r = 1.6; s_R = 1.6; RSD_r = 43.2% (44.4%); RSD_R = 43.2% (44.4%)
Insect fragments, 15 added
s_r = 2; s_R = 2.2; RSD_r = 16%; RSD_R = 17.6%
Insect fragments, 30 added
s_r = 4.1; s_R = 8.2; RSD_r = 19.2% (18.9%); RSD_R = 38.5% (37.8%)

Table 1. Collaborative results for recovery of rat hair spikes (blind duplicates) from dried bean curd

Coll.	Spike level											
	5				10				15			
A	6		2		10		8		12		9	
B	4		8	(5)[a]	8		10	(9)	9		12	
C	5	(4)	4		12	(10)	9		11		7	
D	6	(5)	5		15	(9)	8	(7)	14	(12)	9	
E	6	(5)	4		6	(7)	7		14		11	
F	6	(2)	6	(5)	7		6		11	(10)	10	
$\bar{X}$	5.2	(4.2)			8.8	(8.1)			10.8	(10.5)		
$\bar{X}$, %	103.3	(85)			88.3	(80.8)			71.7	(70)		
s_r	1.8	(1.5)			2.4	(1)			2.4	(2.1)		
s_R	1.8	(1.5)			2.6	(1.3)			2.4	(2.1)		
RSD_r	34.6	(35.7)			27.3	(12.3)			22.2	(20)		
RSD_R	34.6	(35.7)			29.5	(16)			22.2	(20)		

[a] Third party counts are in parentheses if different from those of collaborator.

Add 100 g crumbled or broken product to 2 L beaker. (*Note*: Easily crumbled or broken pieces should be <12 mm; thick or hard pieces must be broken to ≤5 mm for adequate digestion.) Add 1 L hot tap water and 60 mL HCl (37%). Cover beaker with watch glass and bring contents to full boil with magnetic stirring, **945.75B(n)**. Remove watch glass and boil 30 min with magnetic stirring so that top of stirring bar is visible at bottom of vortex.

Wet sieve (No. 230), **970.66B(a)**, portionwise with a forceful stream of hot tap water (>50°C). Use of sieve handle, or equivalent, is recommended. Reserve beaker. Wet residue on sieve with isopropanol and transfer quantitatively to reserved beaker with isopropanol. Fill beaker to 500 mL with isopropanol. With hot plate at medium setting, bring mixture to start of boiling with magnetic stirring and boil gently 10 min, using reflux apparatus, **975.49A(e)** (*Caution*: Isopropanol is flammable. Perform boiling procedure in fume hood). Wet sieve (No. 230) with hot tap water. Wet residue on sieve with 40% isopropanol and transfer quantitatively to 2 L trap flask, **945.75B(h)**(*4*).

Dilute to 800 mL with 40% isopropanol. Add 20 mL Na_4EDTA–40% isopropanol solution, **945.75C(z)**, slowly down stirring rod. Hand stir 1 min with gentle rotary motion. Let stand undisturbed 5 min.

Add 75 mL mineral oil–heptane (70 + 30) down stirring rod. Stir magnetically, **970.66B(c)**, 5 min. Let stand 5 min.

Fill flask with 40% isopropanol down stirring rod. Let stand 30 min. (Use intermittent stirring of interface every 3–6 min of first 20 min if trapped product is noted.) Trap off into 400 mL beaker, rinsing neck, wafer, and rod with 40% isopropanol. Add 35 mL mineral oil–heptane (70 + 30). Stir flotation liquid for 5 s to suspend product without incorporating air. Fill flask with 40% isopropanol, if necessary. Let stand 20 min. (Use intermittent stirring at interface every 3–6 min of first 10 min if trapped product is noted.)

Trap off into 400 mL beaker and rinse neck, wafer, and rod with isopropanol. Filter onto ruled filter paper, **945.75B(i)**, washing 400 mL beaker with isopropanol, and filter washings. Examine microscopically at 30X, **945,75B**(o)(*2*).

Ref.: *J. AOAC Int.* **75**, March/April issue (1992).

Results and Discussion

Initially, a laboratory participating in this study experienced difficulties with digesting the product of 2 test portions. The collaborator was unable to obtain counts because of heavy amounts of trapped product. Because no reason for this difficulty could be ascertained after discussions with the collaborator, it was decided that a replacement laboratory should be used. The substitute laboratory received the 4 unused test portions plus 2 replacement test portions from the Associate Referee. No digestion problems were noted by this or the other collaborators.

Collaborators' counts or recoveries of rat hairs and insect fragments (Tables 1 and 2, respectively) were checked by the Associate Referee and verified by an expert microanalyst when the Associate Referee's counts differed from the collaborator's. Statistical outliers were not excluded for the analysis of variance. The data showed good rat hair recoveries with a trend toward lower recoveries as the number of spiked rat hairs was increased. An analysis of variance showed that average percent recoveries are significantly different ($p<0.05$) between the low and high levels. The insect fragment recovery data were slightly lower but showed consistency between spike levels. Variability measures were acceptable except at the low spike levels, where variability increased with small losses of filth elements. High variability for insect fragments at the high spike level was caused by Collaborator D's low replicate results.

The collaborators took an average of 0.6 h (range, 0.2–1.75 h) to break and crumble the dried bean curd sticks, 2.7 h (range, 1.25–4.5 h) for extraction, and 0.4 h (range, 0.1–1 h) to count the plates. All collaborators used only 1 paper per test portion, and only Collaborator A had plates with more trapped product than was expected.

Table 2. Collaborative results for recovery of insect fragment spikes (blind duplicates) from dried bean curd

	Spike level					
Coll.	5		15		30	
A	5	4	15	13	22	23
B	0	5	14	15	29	29 (30)[a]
C	3	3	9	13	24[b]	10[b]
D	3	2	14	10	7 (8)	9
E	6 (5)	4	14	12	26	25 (26)
F	4	5	9	12	27	25 (26)
$\bar{X}$	3.7 (3.6)		12.5		21.3 (21.7)	
$\bar{X}$, %	73.3 (71.7)		83.3		71.1 (72.2)	
s_r	1.6		2		4.1	
s_R	1.6		2.2		8.2	
RSD_r	43.2 (44.4)		16		19.2 (18.9)	
RSD_R	43.2 (44.4)		17.6		38.5 (37.8)	

[a] Third party counts are in parentheses if different from those of collaborator.
[b] Outlier by Cochran test, but included in calculations.

The mandatory use of the sieve handle, or equivalent, in this method was changed to recommended use because most collaborators either did not have a sieve handle or found its use unwieldy in small sinks.

Recommendation

On the basis of acceptable analyte recoveries and precision measurements, we recommend that the proposed method for extraction of light filth from dried bean curd be adopted as first action.

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FEEDS

Colorimetric Determination of Selenium in Premixes and Supplements

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A colorimetric method is described for the determination of 100–10 000 μg selenium/g premix. Selenium is present in premixes as either selenite or selenate. Moderate to high concentrations of several common minerals are tolerated. The premix is briefly digested in a sulfuric acid–perchloric acid–sodium molybdate mixture, boiled in 10% NaCl solution, made basic, and centrifuged. Aliquots are taken if necessary. The mixture is then acidified with formic acid, treated with hydroxylamine hydrochloride–EDTA, and complexed with 3,3'-diaminobenzidine. The pH is adjusted, and an extraction is performed with toluene. The absorbance is measured at 420 nm. Recoveries for 100–10 000 μg selenium/g premix ranged from 88 to 104%, with an average of 97%. The coefficient of variation ranged from 1.6 to 6.9%, with an average of 4.2%.

Selenium (Se) is a toxic element as well as an essential trace element for animals and humans (1). Concentrations of Se in feed >10 ppm or <0.1 ppm can cause problems in animals (2–4). Natural feeds containing >10 ppm Se are rare; however, low concentrations of Se in feed are common (2), and, therefore, Se is a feed additive. Selenium is added to feed as either sodium selenite or sodium selenate, and it is generally not permitted to exceed 0.3 ppm in feeds for chickens, swine, turkeys, sheep, cattle, and ducks. Added as a premix, Se concentrations can range from 90 to 9 900 ppm (5). There are many reported methods for the determination of Se in various matrixes. Some of these methods use graphite furnace atomic absorption spectrometry (AAS) (6), stripping voltametric analysis (7), flame AAS (8), ion chromatography (9), gas chromatography (10), liquid chromatography (11), fluorometric analysis (12), colorimetric analysis (13), hydride-generated AAS (14), x-ray spectrometric analysis (15), inductively coupled plasma atomic emission spectrometry (16), and neutron activation analysis (17). For premixes, the methods reported use flame AAS (8, 18), hydride-generated AAS (19), and colorimetric analysis (20).

The methods for Se determination in feed premixes had several problems. The flame AAS and the hydride-generated AAS methods suffered from matrix effects, high blanks, poor precision, and lengthy standard additions procedures (8, 19). The colorimetric method with an HCl extraction (20) gave very low recoveries; however, a colorimetric procedure appeared to be the most inexpensive, rapid, and convenient method to use for Se determination in premixes (18). Therefore, we modified the colorimetric method of Cummins et al. (13). The interferences caused by various ions (20–22), by the easy interconversion of elemental Se, selenate, and selenite (23), and by digestion problems (24) were all solved. This modified colorimetric method for determination of Se in feed premixes is described.

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METHOD

Reagents

All chemicals were reagent grade. Deionized water was used.

(a) *Digestion mixture.*—Dissolve 10 g sodium molybdate in 150 mL water and slowly add 150 mL 18M sulfuric acid (CAUTION: heat is generated). Allow solution to cool. After cooling, add 200 mL 70% perchloric acid. Use in wash-down hood to catch the reactive perchloric acid fumes.

(b) *50% Sodium hydroxide.*—CAUTION: Corrosive to flesh; heat is generated when making.

(c) *90% Formic acid.*—CAUTION: Corrosive to flesh; use in hood.

(d) *0.10M EDTA–25% hydroxylamine hydrochloride.*—Warm to dissolve the EDTA.

(e) *0.50% Diaminobenzidine (DAB).*—Add 0.50 g 3.3'-diaminobenzidine to 100 mL water. Prepare just before use. CAUTION: DAB is carcinogenic.

(f) *Toluene.*—CAUTION: Flammable and toxic; use in hood.

(g) *Concentrated ammonium hydroxide.*— CAUTION: Corrosive; use in hood.

(h) *Stock Se solution (1000 μg/mL).*— Dissolve 1.000 g Se (99.99%, Aldrich Chemical Co., Inc., Milwaukee, WI 53201)

Table 1. Recovery of Se from premixes and supplements containing Na_2SeO_3 and Na_2SeO_4

Premix	No. detns	Se content, μg/g	Se rec., μg/g	Rec., %	CV, %	Form of Se
Corn	10	100	90.4	90.4	6.9	SeO_3^{-2}
Corn	11	200	198	99.2	3.6	SeO_3^{-2}
Corn	11	200	198	99.0	3.9	SeO_4^{-2}
Corn[a]	5	200	176	88.0	2.8	SeO_3^{-2}
Range 1[b]	5	200	199	99.5	2.9	SeO_3^{-2}
Range 2[c]	5	200	208	104	1.6	SeO_3^{-2}
Hog[d]	5	200	185	92.5	3.1	SeO_3^{-2}
Hog[d]	5	200	190	95.0	6.3	SeO_4^{-2}
Corn	10	600	588	98.0	4.9	SeO_3^{-2}
Corn	10	10250	10290	100	4.1	SeO_3^{-2}

[a] Fe at 10 000 μg/g; added as ferrous ammonium sulfate.
[b] An alfalfa base containing 11 minerals including 300 μg Fe/g, 70 μg Mn/g, and 30 μg Cu/g.
[c] An alfalfa base containing 11 minerals including 400 μg Fe/g, 80 μg Mn/g, and 60 μg Cu/g.
[d] Mix containing 14 minerals including salts of Ca, Mg, Zn, Mn, Cu, Fe, and Co.

in 10 mL concentrated nitric acid in 1000 mL volumetric flask; bring to volume with water. Solution is stable for years. Standards can also be made from reagent-grade sodium selenite. CAUTION: Toxic.

(i) *Premixes (for method validation).*—Grind feed to pass through 0.2 mm sieve; let ground feed set until particles no longer adhere to one another; add appropriate amount of ground sodium selenite or sodium selenate (grind in hood); tumble in mixer 4 days.

Apparatus

(a) *Pipets.*—Use Eppendorf pipets for volumes <2 mL and Class A volumetric pipets for larger volumes. Use Mohr pipets for approximate volumes.

(b) *Heating bath.*—Use covered water bath at 60°C.

(c) *Filter paper.*—Use medium porosity.

(d) *Spectrophotometer.*—Perkin-Elmer Model 320 set at 420 nm; use 1.00 cm cuvettes; spectral bandpass = 6 nm.

(e) *Boiling chips.*—Preheated with digestion mixture, then washed with water.

(f) *pH meter.*—Orion 601A calibrated at pH 2 and 7.

(g) *Hot plate.*—Corning PC-351; 2–4 settings.

Procedure

Weigh 1.00 g premix (supplement) or measure appropriate amount of Se standard into 250 mL Erlenmeyer flask. Add several boiling chips and 10 mL digestion mixture. Boil on hot plate 15–20 min at high setting in wash-down hood. Let cool; add 10 mL water and then 30 mL 10% NaCl. Gently boil 1 h. Cool with 10–20 g ice, and cautiously add 50% NaOH (ca 7 mL) until pH is a little above pH 9. If a brown ferric hydroxide precipitate forms, centrifuge and discard precipitate. Add 5.0 mL formic acid, 10 mL 0.10M EDTA–25% hydroxylamine hydrochloride, and 10 mL 0.50% DAB. Adjust pH to 2–3 with aid of pH meter and either 90% formic acid or 50% NaOH. Heat 20–30 min at 60°C in the absence of light, and then bring pH to >8 with concentrated ammonium hydroxide (ca 15 mL). Verify pH with either pH meter or pH paper. Transfer to 250 mL separatory funnel with water wash, and extract by vigorously shaking 1 min with 50.0 mL toluene. Gravity-filter toluene through 15 g anhydrous sodium sulfate and measure absorbance at 420 nm, using 1 cm cuvette.

This procedure is adequate for test samples containing between 100 and 300 ppm Se. If <100 ppm Se is present, use <50.0 mL toluene for extraction. If >300 ppm Se is present, dilute test solution in appropriate volumetric flask just after the 10% NaCl boil; take appropriate aliquot from this volumetric flask and proceed with addition of 50% NaOH.

Results

Recoveries of Se from 10 different premixes (supplements) made with either sodium selenite or sodium selenate are given in Table 1. Five to 11 trials were made with each test sample, and the spikes ranged from a low of 100 μg Se/g test sample to a high of 10 250 μg/g. Recoveries ranged from 88 to 104%, with an average of 97%. The coefficients of variation (CVs) ranged from 1.6 to 6.9%, with an average of 4.2%. The presence of Fe at up to 10 000 μg/g did not cause any serious loss in Se recovery, and less than 20 μg/g of other common ions from Cu, Mn, Zn, Ca, Mg, and Co also did not interfere.

Discussion

Typical Se premixes contain large amounts of either sodium selenate or sodium selenite, a feed base, and various other inorganic additives such as calcium phosphate, manganese (II) oxide, magnesium oxide, zinc oxide, copper (II) sulfate, cobalt carbonate, and iron (II) salts. The high Se concentrations, the difficult matrix, the potential interferences from several anions and cations, the different Se oxidation states, and the necessity of taking a representative sample make Se analyses in premixes difficult. This colorimetric method coupled with the mild acid digestion address all of these problems.

The short acid digestion destroys the organic matrix and has several benefits. One benefit is that any manganese (II) ion will

not be oxidized to permanganate. Our studies revealed that as little as 20 μg permanganate in the digested test sample would drastically reduce the recovery of Se. Another benefit is that the Se will stay in the selenite form and not be oxidized to selenate. There is little splattering, and there are few perchloric acid fumes formed (perchloric acid fumes react explosively with oxidizable material). Digestion times of up to 1 h are tolerated; however, the standard deviation increases. An attempt to eliminate the digestion by extracting the selenite and selenate with dilute HCl yielded very low recoveries with some premixes. The low recoveries were probably caused by the reduction of selenite to elemental Se by iodide and possibly by iron (II) ions (25).

Boiling 1 h with 10% NaCl is necessary; in the hot acidic chloride solution, any selenate is converted to selenite (26), and only selenite complexes with DAB. Boiling times of <30 min resulted in low recoveries when Se was in the selenate form, and elimination of this step gave recoveries as low as 5% with selenate premixes. Selenite can be converted to selenate by perchlorate ion, and some premixes are made with sodium selenate.

The use of 50% NaOH had 2 purposes: First, it raised the pH; the complex formed with DAB is pH dependent. Second, it removed any large concentrations of iron (III) ion; the iron (III) hydroxide precipitate was easily removed by centrifugation. The removal of the iron (III) ion is necessary because it complexes with DAB and reduces the recovery of Se to 5% if 10 000 μg iron (III) is present. Cation exchange chromatography with a sulfonic acid column also eliminated this interference; however, the ion exchange step added several hours to the analysis, and some brands of the strong cation exchange resin were not effective. Less than 10% of the Se coprecipitated with the iron (III) hydroxide, and the recoveries were still near 90% when ≤10 000 μg iron as ferric hydroxide was precipitated.

The formic acid, EDTA, hydroxylamine, and ammonium hydroxide were all necessary. Formic acid buffers the solution between pH 2 and 3, which is necessary for complex formation between DAB and selenite. EDTA helps to complex potentially interfering cations, such as copper (II). Hydroxylamine helps to prevent oxidation of DAB. Ammonium hydroxide brings the pH of the final solution to pH >8, which lets toluene extract the neutral complex.

DAB reacts with selenite, yielding a yellow complex that absorbs strongly at 420 nm. This complex quantitatively partitions into toluene with 1 extraction; the unreacted DAB is also extracted. However, DAB absorbs weakly at 420 nm. Some DAB does polymerize, and sodium sulfate effectively removes this orange, toluene-insoluble polymer along with water. Other denser extractants, such as dicholoromethane and chloroform, were tested in place of toluene; however, they were not useful. The calibration curve was linear between 50 and 300 μg Se/50 mL toluene. The correlation coefficient was 0.9992, and the linear equation was as follows:

$$Y(\text{abs}) = 0.00205X(\mu\text{g Se}/50\text{ mL}) + 0.00114.$$

One drawback of the method is that the analysis should be completed in 1 working day. However, this is not a serious drawback because most of the reagents and standards are stable and can be made up in advance (only DAB is unstable). Also, up to 20 test samples and standards can be analyzed in 1 day.

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FEEDS

Determination of Monensin in Raw Material, Premix, and Animal Feeds by Liquid Chromatography with Correlation to Microbiological Assay

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A method is described for the detection and quantitation of monensin in raw material, premix, and feeds by liquid chromatography (LC) with postcolumn derivatization with vanillin. Monensin was mixed with vanillin under acidic conditions and heated, and the resulting products were measured by a variable wavelength visible detector operating at 520 nm. The LC response of monensin and monensin-like factors was determined and correlated to the microbiological response of each factor as determined with *Streptococcus faecium*. Monensin reference standard was characterized in the same manner as the individual factors. The chemical composition of the reference standard and the relative microbiological potency values were used in combination to calculate the biopotency contribution of each of the monensin factors. A formula was used to transform chemical composition values of the reference standard to total microbiological activity as obtained directly from a microbiological assay. The formula was tested by comparing samples assayed by LC using the formula to report microbiological potency with samples assayed by the Autoturb method. Finally, the LC method was validated with raw material, premix, cattle rations (including liquid supplements), and poultry rations.

Monensin (Figure 1) is a polyether monocarboxylic acid ionophore (1, 2). Sodium monensin is marketed as a feed additive in chickens and turkeys for the prevention of coccidiosis (Coban™) and as a growth promoter in beef cattle (Rumensin™). Monensin is typically incorporated into feeds at levels ranging from 20 to 100 g/ton.

Several analytical methods were developed for the determination of monensin in premixes, feeds, and animal tissues. In 1970, Kline et al. (3) described a microbiological plate method for monensin in poultry feeds and premixes. Kavanagh and Willis (4) described an automated turbidimetric microbiological method for feeds and premixes. These methods are reliable and relatively sensitive but are not specific for monensin. AOAC currently recognizes 2 microbiological methods (plate and Autoturb) as official methods for the assay of monensin (5). These methods have been used extensively by government and private laboratories.

In 1973, Golab et al. (6) described a colorimetric method for the assay of monensin in feeds and premixes. This method, which is based upon the chemical reaction of monensin with vanillin, was applicable at higher feed concentrations. However, there was potential for interference from feed ingredients at lower monensin concentrations. Macy and Loh (7) developed a liquid chromatographic (LC) assay for monensin in premixes with refractive index detection. The standard procedure for assay of monensin in animal tissues is the thin-layer bioautographic method of Donoho and Kline (8).

At the Eighth Annual Spring Workshop (1983) of AOAC, we described a method for the determination of monensin by LC using postcolumn derivatization (PCD). This method was capable of separating monensin from 2 structurally similar ionophores, narasin and salinomycin (Figure 2). In 1984, Goras and LaCourse (9) described a similar system for the determination of salinomycin, an ionophore that is structurally similar to monensin. Blanchflower et al. (10) published a method for the simultaneous LC determination of monensin, narasin, and salinomycin in feeds using PCD.

Historically, monensin in raw material, premix, and final feeds was determined quantitatively by microbiological methods. Therefore, Coban and Rumensin are sold on the basis of microbiological activity and not by LC response. The reporting of assay results by LC required the correlation of the LC response to the microbiological response. The correlation study was performed in a systematic manner according to the following sequence of events. (*1*) Purified monensin factors were prepared and characterized by LC and turbidimetric assays. (*2*) The monensin reference standard was characterized to identify the various factors in the standard that are detectable by LC/PCD, and the LC response was correlated to the microbiological response of each factor. (*3*) The monensin biopotency was determined in raw material, premixes, and feeds by LC. (*4*) The monensin LC assay with biopotency calculations was validated.

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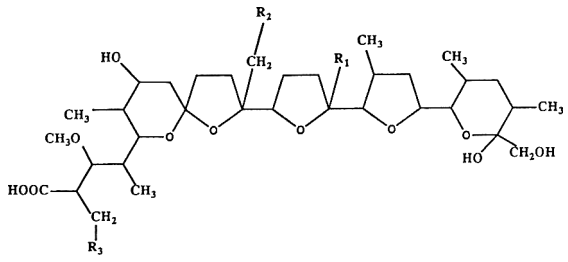


Factor	R_1	R_2	R_3
A	C_2H_5	H	H
B	CH_3	H	H
C	C_2H_5	H	CH_3

Figure 1. Structures for monensin factors.

The LC method is presented as an alternative to the microbiological assay. This method represents improvements over the existing microbiological methods in specificity, analysis time, labor, and overall efficiency.

METHOD

Reagents

(a) *Solvents.*—Reagent and LC grade methanol.

(b) *Water.*—Distilled and deionized or LC grade.

(c) *Acids.*—Sulfuric acid (G.R. reagent); glacial acetic acid, reagent grade.

(d) *Vanillin.*—99% (e.g., Aldrich Cat. No. V110-4).

(e) *Mobile phase.*—Methanol–water–acetic acid (940 + 60 + 1). Filter under vacuum through 0.45 μm Nylon-66 filter (Cat. No. 38-114, Rainin Instrument Co., Woburn, MA 01801). Degas by stirring 10–15 min under vacuum. Prepare fresh as required.

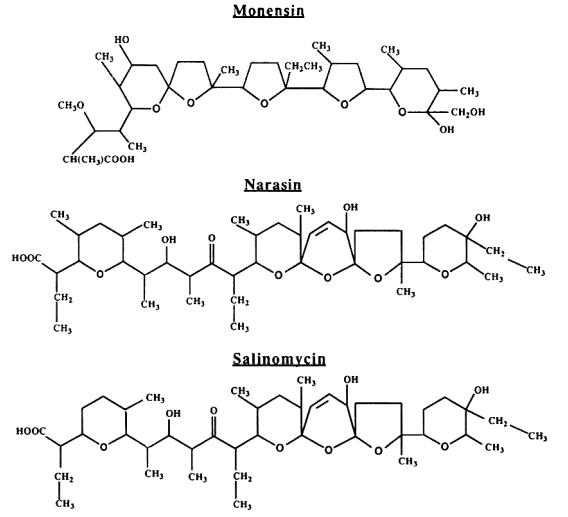


Figure 2. Structures for monensin, narasin, and salinomycin.

(f) *Vanillin reagent.*—While stirring slowly and carefully, add 20 mL concentrated sulfuric acid to 950 mL methanol. Let methanol–acid solution cool to room temperature. Add 30.0 g vanillin while stirring gently. Degas by stirring 10–15 min under vacuum. Protect from light. Prepare fresh daily.

(g) *Extraction solution.*—Methanol–water (9 + 1, v/v).

(h) *Monensin reference standard for LC.*—Accurately weigh enough standard into volumetric flask to obtain 1 mg monensin sodium reference standard/mL. Dissolve and dilute to volume with methanol. Make quantitative dilutions to obtain 5.0, 20.0, and 40.0 μg/mL working standard solutions. These standard solutions may be stored 2 weeks at room temperature protected from direct sunlight or in refrigerator.

(i) *Monensin reference standard for microbiological assays.*—Prepare according to *Official Methods of Analysis* (5), section **976.37(d)**.

(j) *Narasin reference standard.*—Dissolve 100 mg narasin reference standard in methanol and dilute to 100 mL.

(k) *System suitability solution.*—Dilute monensin and narasin standard solutions (1 mg/mL) with methanol to obtain 20 μg/mL solution.

(l) *Monensin Factors A, B, C, and D.*—Dissolve 10 mg in methanol and dilute to 100 mL (100 μg/mL).

Apparatus

(a) *Liquid chromatograph.*—With postcolumn reactor (Figure 3). Pulse dampened pump used to deliver mobile phase (Model 110B, Beckman Instruments, Fullerton, CA 92634); LDC minipump used to deliver vanillin reagent, both at 0.7 mL/min. Autosampler equipped with 200 μL injection loop (Model 8055, Varian Analytical Instruments, Sunnyvale, CA 94034). Place mixing tee (SSI 01-0165) into system so that inlet flows directly oppose one another.

(b) *Chromatographic column.*—25 cm × 4.6 mm id Whatman Partisil 5 ODS-3 25 HPLC column. C18 guard column may be used to extend life of analytical column.

(c) *Autoturb™ system.*—Microbiological assay system (Mitchum-Schaefer, Inc.) including diluter module, water bath (37°C), and reader module.

(d) *Spectrophotometer.*—Spectronic 20, or equivalent.

(e) *Water baths.*—80°C, or steam sterilizer for inactivation of microbial growth.

(f) *Feed grinder.*—Centrifugal grinding mill (3 mm screen, Brinkmann Instruments, Inc., Westbury, NY 11590), or equivalent.

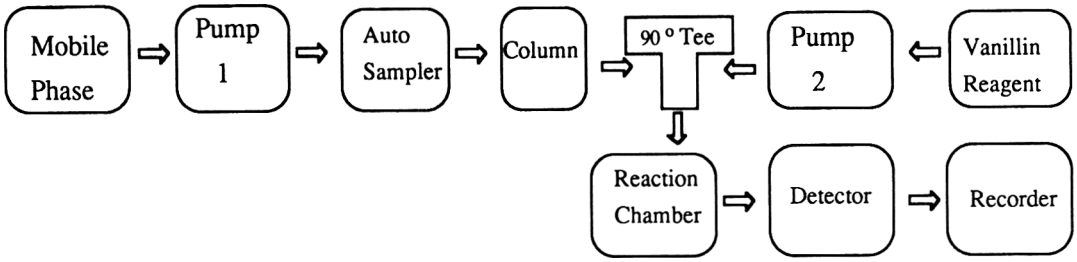
(g) *Feed mixer.*—Hobart Model C100T, or equivalent.

(h) *Gyratory shaker.*—Model G-33 (New Brunswick Scientific, Edison, NJ 08818), or equivalent.

(i) *Balances.*—Top loading and analytical.

Feed Sampling and Sample Preparation

Collect composite sample from bulk feed (bag, feed bunker, or feed bin). Use feed scoop, probe, or other suitable sampling device to collect subsample (at least 500 g) in at least 3 locations within bulk feed. Submit entire sample to assay laboratory for analysis.



Mobile Phase = methanol/water/acetic acid 94/6/0.1 (v/v/v)

Pump 1 = Beckman 110B pulse-dampened pump (0.7 mL/min)

Autosampler = Varian Model 8055 with 200 µL injection loop

Column = C18 - Whatman Partisil 5 ODS-3 (4.6 mm x 25 cm)

90° Tee = SSI 01-0165 - inlet flows directly oppose one another

Pump 2 = LDC minipump (0.7 mL/min)

Vanillin reagent = methanol/H_2SO_4/vanillin, 95/2/3 (v/v/w)

Warning: Special care should be taken when adding concentrated H_2SO_4 to methanol, as it will splatter if added improperly, or too rapidly. Add H_2SO_4 slowly and carefully with a pipette; do not pour. Allow methanol/H_2SO_4 solution to cool to room temperature before adding vanillin.

Reaction chamber = 2-mL stainless steel reaction chamber* enclosed in a 98°C Kratos URA 200 heater (*Applied Biosystems Model 520 Post Column Reactor, part number 9000-5201-reactor, part number 1400-1326-coil)

Detector = Kratos Model 757 variable wavelength absorbance (520 nm)

Recorder = Varian Model 9176

Figure 3. Diagram of monensin LC/PCD system.

Finely mill all feed samples and thoroughly mix before assay. Grind feed samples in grinding mill with 3 mm screen. Mix entire feed sample 10 min in mixer. (Raw material and premix samples generally require no grinding and mixing before assay.)

Extraction of Raw Material, Premixes, and Feeds for LC

Raw material and premix.—Extract 5 g sample with 200 mL extraction solution in suitable container, such as mason jar, by mixing 1 h on gyratory shaker. Let solids settle. Dilute to ca 20 µg/mL with extraction solution, and filter aliquot for LC analysis with Gelman Acrodisc CR filter. Proceed to LC measurement.

Feeds.—(1) Monensin levels ≥ 200 g/ton.—Extract 5 g feed samples with 200 mL extraction solution by mixing 1 h on gyratory shaker. *(2) Monensin levels <200 g/ton.*—Extract 50 g feed samples with 200 mL extraction solution by mixing 1 h on gyratory shaker. Let solids settle and filter aliquot for LC analysis with Gelman Acrodisc CR filter. Proceed to LC measurement.

Liquid supplements.—Because settling of insoluble matter in fortified liquid feed supplements presents sampling problems, use techniques that minimize settling. Thoroughly mix sample by agitation with gyratory shaker, propeller mixer, magnetic stir bar, or equivalent apparatus, depending upon viscous nature of sample. While continuing to mix, transfer 40 g aliquot to 200 mL volumetric flask. Dilute samples to 200 mL with extraction solution and mix thoroughly. Let particulates settle and dilute aliquot of extract to final monensin concentration of ca 20 µg/mL with extraction solution. Filter aliquot of final diluent with Gelman Acrodisc CR filter for analysis by LC.

Determination

Inject 200 µL LC standard solutions and analytical samples into liquid chromatograph (Figure 3). Measure peak area response (PR) at retention volume of monensin Factor A and monensin Factor B for each sample. Using measured responses, construct linear regression plot of standard curve to determine concentration of monensin Factor A and monensin Factor B in experimental samples.

Calculation of Monensin Microbiological Activity

Monensin biopotency (µg/mL) = biopotency of monensin Factors A + B

where, for each factor:

$$Biopotency = \frac{PR\ sample}{PR\ standard} \times [Std] \times \frac{V}{wt} \times BCF$$

where PR = peak area response, V = extraction volume (200 mL), wt = sample weight (g), $[Std]$ = concentration of each factor in reference standard (µg/mL).

[Factor A] = µg/mL Ref. Std. × % Factor A in Ref. Std./100

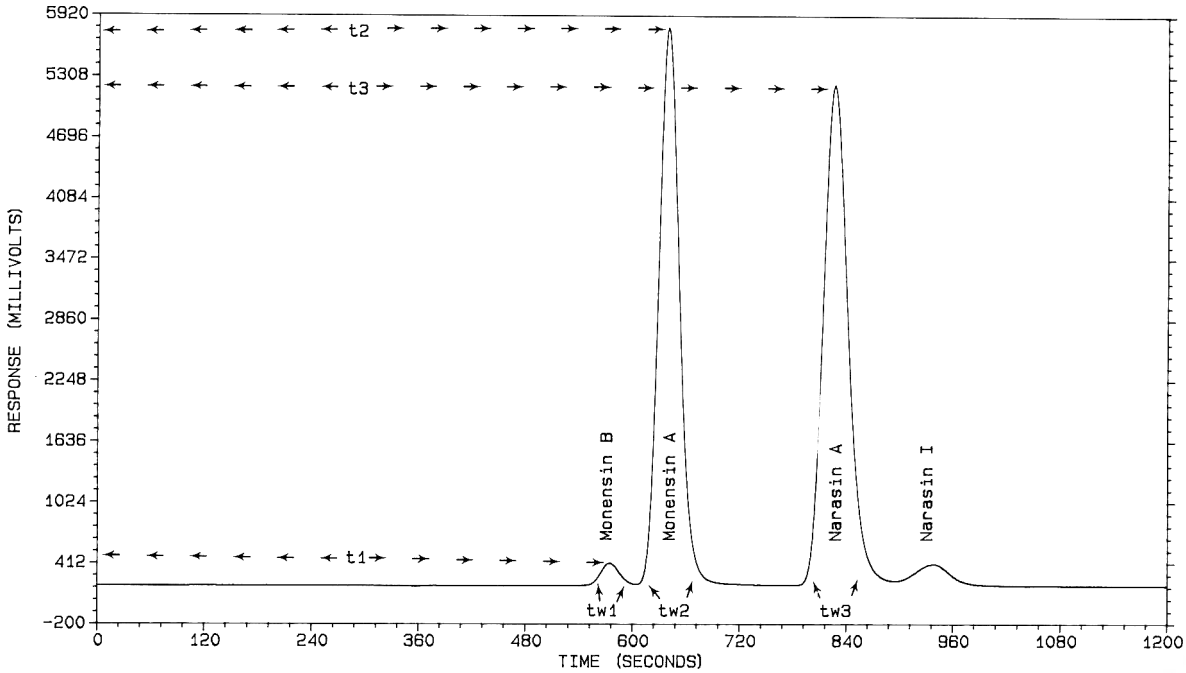


Figure 4. Resolution mixture of monensin and narasin, and parameters used to calculate resolution factors.

[Factor B] = μg/mL Ref. Std. × % Factor B in Ref. Std./100

The relative factor composition must be determined for each new reference standard.

$$BCF \text{ (Factor A)} = 1.000$$

$$BCF \text{ (Factor B)} = 0.280$$

where *BCF* = biopotency conversion factor

LC System Control Parameters

Resolution.—Prepare resolution mixture and analyze daily to ensure that the LC system is performing in an acceptable fashion and that monensin can be separated from other ionophores. Inject resolution mixture and adjust instrumentation so that peak response for major peaks is 60–90% of full-scale deflection. Calculate resolution factor (R_s for each pair of adjacent peaks (Figure 4) according to following formula:

$$R_s = \frac{(t_2 - t_1)}{½(tw_1 + tw_2)}$$

where t_n = retention volume of peak maximum (measured in cm); tw_n = triangular peak width at base line (measured in cm).

Monensin B – Monensin A, example:

$$R_s = \frac{11.85 - 10.65}{½(0.85 + 0.95)} = 1.33 \text{ (must be } \geq 1.25)$$

Monensin A – Narasin A:

$$R_s = \frac{15.55 - 11.85}{½(0.95 + 1.00)} = 3.79 \text{ (must be } \geq 3.50)$$

If R_s does not meet requirements specified adjacent to calculations, adjust LC conditions to improve resolution.

The retention time for monensin Factor A should be between 600 and 700 s. The tailing factor for monensin Factor A of the reference standards should be <1.4. If any of these parameters are not met, LC conditions may need to be adjusted. After adjustment, specification for resolution, retention time, and tailing must be met before any samples are analyzed.

Monensin Turbidimetric Assay

(a) *Microorganism.*—*Streptococcus faecium* ATCC 8043. Maintain and prepare inoculum as described in *Official Methods of Analysis*, **976.37B** (5).

(b) *Assay broth.*—Prepare according to *Official Methods of Analysis*, **976.37B** (5).

(c) *Quantitation.*—The Autoturb automated turbidimetric system was previously described (11). Calculations for sample results can be performed by computer, using appropriate programming, or can be determined according to *Official Methods of Analysis*, **976.37B** (5).

Results and Discussion

Chemical Purity of Monensin Factors

The chemical purity of major monensin factors (Figure 1) was determined by LC/PCD and quantitated by peak area normalization, as summarized in Table 1. Factor D was determined to contain 2 major components, designated D1 and D2, and was not of sufficient purity for additional LC evaluations.

Table 1. Chemical purity of monensin Factors A, B, and C

Monensin factor	Chemical purity, %
A	99.10
B	99.02
C	95.14

Relative Microbiological Activity of Monensin Factors

The relative microbiological activity of the monensin factors was determined by assaying Factors B, C, and D vs Factor A with an assigned potency of 1000 μg monensin activity/mg Factor A. Each factor was diluted to 3 levels calculated to fall within the Factor A standard curve for the purpose of determining linearity of response and parallelism between the factors. The microbiological activities of each factor vs Factor A are listed in Table 2. Factor B had approximately 28% of the activity of Factor A, whereas Factors C and D were 156 and 148.4% as active as Factor A, respectively. The responses for the 3 dilutions of each factor were linear through the range tested and parallel between factors when the log of the concentration was plotted vs the turbidimetric response, as indicated in Figure 5.

Microbiological Potency of Monensin Reference Standard

Monensin reference standard (Lot P-61722) was characterized by LC in the same manner as the individual monensin factors. The peak area normalization for the reference standard, as depicted in Figure 6, was used to calculate the factor composition of this lot by using external standard comparison, and mass balance was determined as listed in Table 3.

Table 2. Relative microbiological activity of monensin Factors B, C, and D compared to monensin Factor A

		Activity of monensin vs monensin A, μg/mg				
Monensin factor	Concn, μg/mg	Day 1	Day 2	Day 3	Mean	RSD, %
B	1.25	283	286	284		
	2.50	265	282	283	280	2.32
	3.33	281	274	279		
C	0.20	1498	1604	1549		
	0.40	1525	1654	1568	1560	3.66
	0.50	1474	1611	1559		
D1/D2	0.20	1433	1579	1491		
	0.40	1470	1483	1468	1484	3.09
	0.50	1433	1529	1474		
A	0.40	993	1031	1002		
	0.60	969	1030	983	1004	2.45
	0.80	985	1040	1002		

Correlation of LC/PCD to Microbiological Potency

The chemical composition of monensin reference standard P-61722 from Table 3 was used with the relative microbiological potency values obtained from Table 2 to calculate the

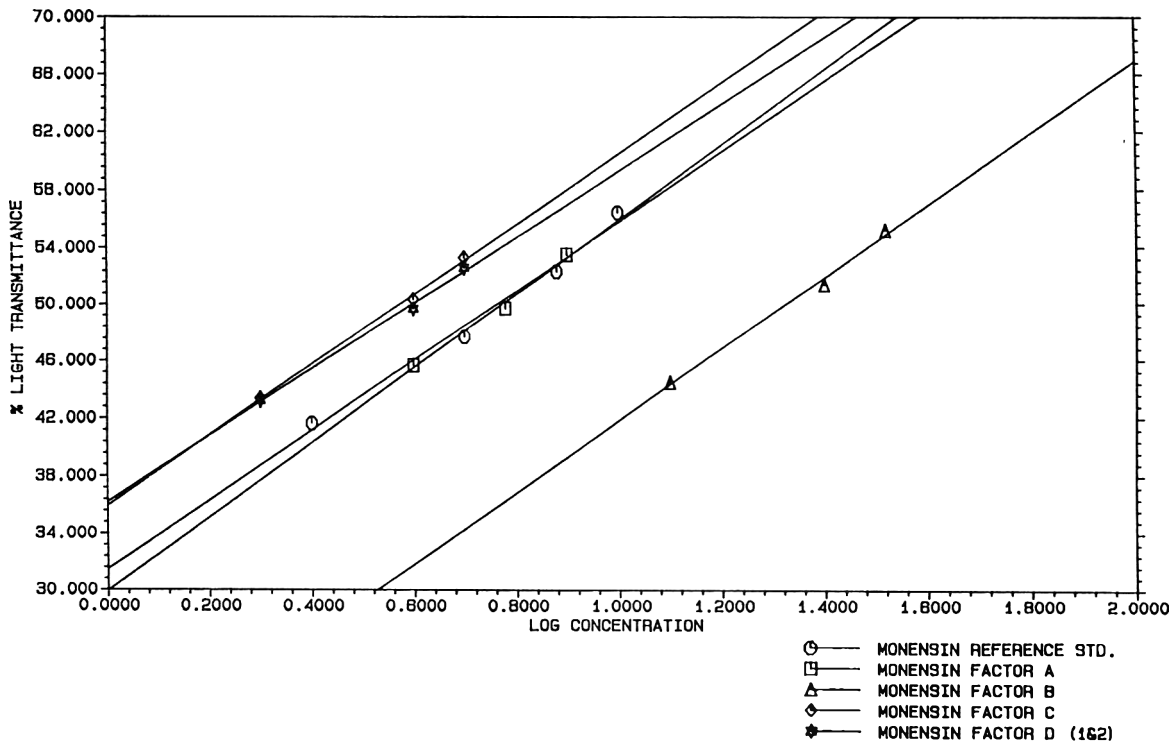


Figure 5. Comparison of the turbidimetric response for monensin Factors A, B, C, and D, and monensin reference standard.

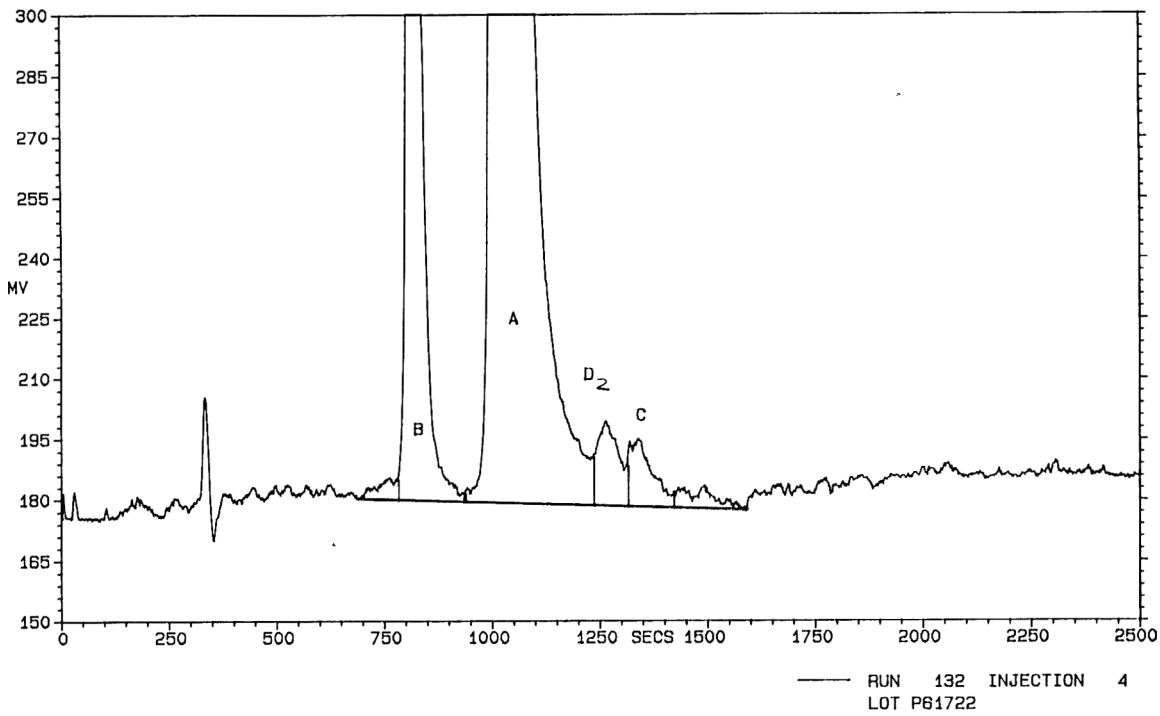


Figure 6. Peak area normalization for monensin reference standard.

Table 3. Factor composition and mass balance of monensin reference standard

Monensin factor	Monensin reference standard factor composition, %
A	94.67
B	3.98
C	0.26
D	0.57
Subtotal	99.5
Water	0.5
Total	100.0

biopotency contribution of each of the monensin factors. A simple mathematical formula was used to transform chemical composition values of the reference standard to total microbiological activity or biopotency as indicated:

Biopotency (μg/mg) = chemical composition (%) × relative microbiological response × 0.01

Example: Monensin Factor A Biopotency = 94.67% × 1000 μg/mg × 0.01 = 946.7 μg/mg

The total biopotency of a given sample is the sum of the biopotency values for Factors A + B + C + D. The complete correlation of the chemical composition with the microbiological responses and the percent contribution of each factor to the total biopotency of a given sample are listed in Table 4. The biopotency calculated by LC/PCD analyses differs from the assigned microbiological potency of the monensin reference standard by 1.1%. The assigned microbiological potency for the reference standard is 960 μg monensin acid activity/mg on an "as is" basis and is the weighted average of the potency values obtained from 3 laboratories. Therefore, the difference of only 1% was considered insignificant, and these values were considered equivalent.

Table 4. Correlation of the chemical factor composition of monensin standard to the microbiological potency of each factor

Monensin factor	Chemical composition, %	Relative microbiological activity, μg/mL	Biopotency, μg/mg	Contribution to biopotency, %
A	94.67	1000	946.7	97.6
B	3.98	280	11.1	1.1
C	0.26	1560	4.1	0.4
D	0.57	1484	8.5	0.9
Total			970.4	100.0

Table 5. Comparison of the monensin biopotency for bulk and raw material lots determined by LC/PCD and turbidimetric assay

Lot	LC/PCD, μg/mg	Turbidimetric assay, μg/mg	LC/PCD, % of turb.
A	943.6	943.3	100.0
B	929.0	928.4	100.1
C	185.9	174.0	106.8
D	170.9	162.0	105.5
E	178.5	170.0	105.0
F	173.2	171.0	101.3
G	173.8	206.0	84.4
H	183.7	195.0	94.2
I	215.7	227.0	95.0
Av.			99.1

Table 6. Recovery of monensin from feeds and liquid feed supplements[a]

Ration	Monensin theory, ppm	Monensin assayed[b] Rec., %	RSD, %
Cattle	10	102.1	1.5
	10	101.9	1.8
	10	102.3	2.4
	30	102.0	1.4
	30	101.8	1.2
	30	101.9	2.3
	100	103.3	3.0
	100	102.0	3.7
	100	103.0	4.8
Poultry	50	100.3	5.1
	50	102.0	5.1
	50	102.6	4.9
	100	98.0	5.5
	100	100.4	5.5
	100	100.5	4.7
	150	98.2	5.5
	150	101.2	5.2
	150	99.7	4.2
LFS	100	100.0	3.0
	200	97.5	2.3

[a] RSD = relative standard deviation; LFS = liquid feed supplement.
[b] $n = 9$.

Table 7. Precision of monensin LC/PCD for premix[a]

Premix	Monensin, g/lb	RSD, %
A	62.4	2.6
B	46.5	3.6

[a] $n = 9$.

Ideally, to obtain the optimum correlative data between LC/PCD and turbidimetric assays, the LC/PCD quantitation should be used to determine monensin Factors A, B, C, and D in the reference standard and samples. Additionally, a mathematical factor greater than 1.0 should be applied to the calculation to account for the possibility that not all monensin factors are vanillin-positive or that not all factors elute from the column. However, at the concentration of monensin in final feeds, Factors C and D are not detectable in the reference standard. Therefore, under practical use conditions, the LC method calculations are based on the detection of monensin Factors A and B, and the calculations for biopotency are as listed in the section on calculation of monensin microbiological activity.

A comparison of the biopotency obtained by turbidimetric assay to that calculated from LC/PCD for several technical and premix lots is summarized in Table 5. These data demonstrate that the results, except for Lot G, agree very closely between methods with an average of 99.1% when LC is compared to turbidimetric assays. If the results for Lot G were not included in the evaluation, the average result, when LC is compared to the turbidimetric results, would be 101%.

Validation of LC/PCD

The linearity of this method was determined by analyzing monensin reference standard concentrations of 1, 5, 20, and 40 μg/mL. These concentrations resulted in a standard curve with a correlation coefficient of 0.9999.

This method was evaluated for the determination of monensin in cattle feeds (10–100 ppm), poultry feeds (50–150 ppm), and liquid supplements (100–200 ppm). Recovery data are listed in Table 6. Recoveries ranged from 97.5 to 103.3% across the different feed types. The precision of the method was determined in monensin premix samples. Two lots of premix were assayed in triplicate over 3 days. These results are listed in Table 7. The relative standard deviations for the 2 premixes were 2.6 and 3.6%, respectively.

The LC system used in this method separates the significant factors of monensin and has the capability of detecting 2–3 ppm narasin (a closely related ionophore) when the procedure is used to assay 100 ppm monensin. The specificity of the method can be further demonstrated by the resolution of monensin, narasin, and salinomycin (Figure 7). These 3 compounds are structurally very similar, as Figure 2 demonstrates.

The limit of quantitation was determined to be 5 g/ton, and the limit of detection was estimated (3× noise of system blank) to be 0.3 g/ton.

Acknowledgments

The authors thank David A. Dickson and Amy J. Gilmore for their technical assistance.

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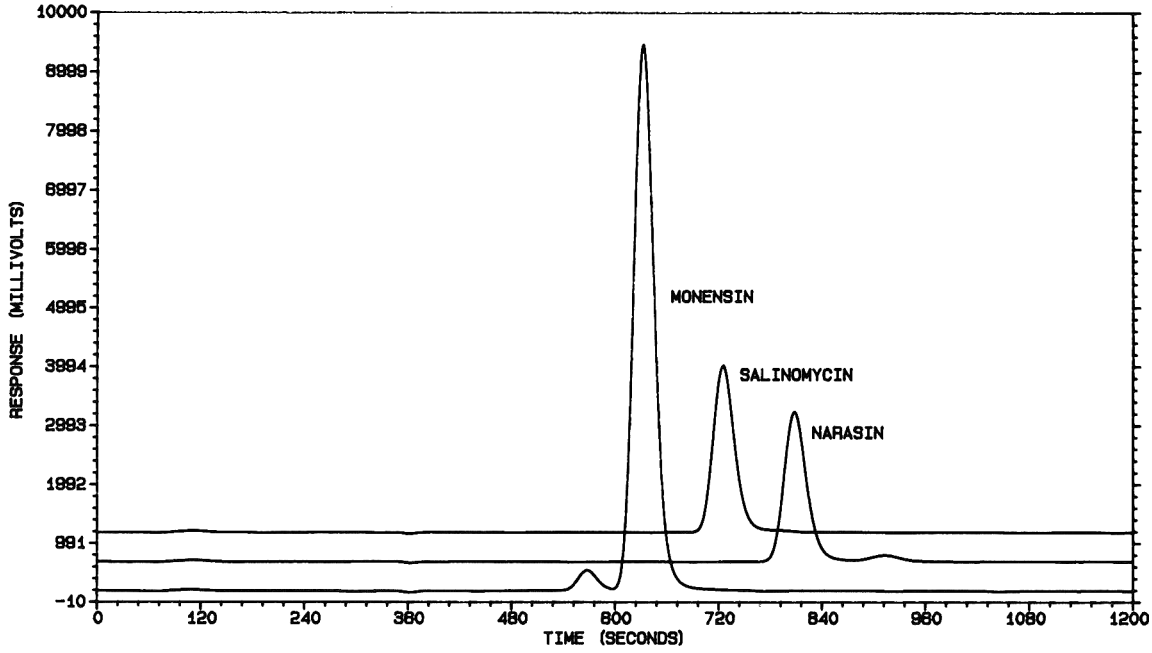

Figure 7. Resolution of monensin, salinomycin, and narasin.

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FOOD COMPOSITION

Composition of Pineapple Juice

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Major and some minor constituents were determined for a series of fresh pineapple juices. Results include: soluble solids 11.2–16.2 g/100 g, acidity (reported as citric acid) 0.46–1.21 g citric acid/100 mL, fructose 1.72–4.75 g/100 mL, glucose 1.21–4.52 g/100 mL, sucrose 2.47–9.73 g/100 mL, citric acid 0.439–1.151 g/100 mL, malic acid 0.073–0.391, isocitric acid 80–265 mg/L, potassium 830–1410 mg/L, formol value 0.74–1.69 meq/100 mL, proline 11–44 mg/L, and carbon isotope ratio 13.5–11.2‰ PDB. Use of these compositional values in the detection of adulterated pineapple juice is discussed.

Pineapple juice has recently undergone a significant increase in price due to a decline in production and strong demand for the juice. This situation raises concern about the possibility that certain suppliers may adulterate their pineapple juice with added sugar and acids. Such adulteration has been frequently observed with other fruit juices.

The literature on the composition of pineapple juice is meager. Gebhart et al. (1) reported analytical data relating to proximate analysis, nutritional parameters, and amino acids for a variety of commercial pineapple products. Sale (2) presented some data on pineapple composition directed toward the goal of estimating fruit content of jams and jellies. These data include soluble solids, acidity, phosphorus, and potassium values. Matthews et al. (3) presented data on the mono- and disaccharide content of commercial pineapple products. Wallrauch and Faethe (4) gave an excellent account of the amino acid content of pineapple juice. Elkins et al. (5) presented some data on the sugar and organic acid content of several commercial pineapple juice concentrates. Doner et al. (6), Parker (7), and Krueger et al. (8) reported results of carbon stable isotope ratio analysis (SIRA) for a few samples of pineapple juice. The French AFNOR values (9) list a wide range of broad analytical specifications for pineapple juice, although no data are presented.

This paper presents some analytical data on the composition of pineapple juice. The data will help form the basis for detecting pineapple juice adulteration.

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Experimental

Samples

Fresh pineapples were purchased from Boston, MA, area stores; the flesh was pressed and filtered through cheesecloth to extract the juice, which was then frozen until analyzed. The samples were from various geographical origins and of varying degrees of ripeness.

Determination

Brix.—Soluble solids by refractometer without correction for acidity, *Official Methods of Analysis*, Method **932.14C** (10).

Fructose, glucose, and sucrose.—*Official Methods of Analysis*, Method **977.20** (10). Samples are filtered and injected undiluted.

Sorbitol.—*RSK Values: The Complete Manual* (11), pp. 142–144.

Acidity.—*Official Methods of Analysis*, Method **942.15B**, reported as citric acid (10).

Citric and malic acids.—*Official Methods of Analysis*, Method **986.13** (10).

L-Malic acid.—*Methods of Biochemical Analysis and Food Analysis Using Test Combinations*, pp. 78–79 (12).

D-Malic acid.—*D-Malic Acid Test Combination* (13).

Isocitric acid.—*Methods of Biochemical Analysis and Food Analysis Using Test Combinations*, pp. 60–61 (12). Samples are pretreated by the alkaline hydrolysis procedure described in the appendix to the method.

Potassium.—*Official Methods of Analysis*, Method **965.30** (10).

Formol value.—*Official Methods of Analysis*, Method **965.31B** (10).

Proline.—*Official Methods of Analysis*, Method **979.20** (10), except that samples were run undiluted.

Carbon stable isotope ratio analysis (SIRA).—*Official Methods of Analysis*, Method **978.17** (10).

Results and Discussion

The results of this study are presented in Tables 1–3. The total soluble solids levels in the pineapple juices reported here ranged from 11.2 to 16.2 g/100 g, averaging 13.8. These results compare with a mean value of 14.4 for 40 samples of pineapple fruit reported by Sale (2), and 14.46 ±0.13 for the total solids content of 19 samples of canned pineapple juice reported by

Table 1. Soluble solids and sugars in pineapple[a]

Sample	BRIX	Fructose, g/100 mL	Glucose, g/100 mL	Sucrose, g/100 mL	Sorbitol, g/100 mL
H1	15.5	2.12	1.81	9.73	—
H2	11.7	1.72	1.44	6.32	—
H3	13.5	2.23	1.21	8.14	—
H4	11.2	2.41	1.33	5.58	—
H5	13.0	3.30	2.53	5.56	—
H6	14.4	3.50	2.52	6.30	0.00
C1	14.9	2.57	2.29	8.63	—
C2	14.1	5.05	4.52	3.10	—
C3	14.6	3.26	2.92	6.51	—
C4	16.2	3.73	3.49	7.56	—
C5	13.6	2.46	1.96	6.11	0.00
D	12.4	4.37	4.04	2.47	—
P	13.2	4.14	4.18	2.81	0.00
T	14.1	3.36	3.71	6.53	0.00
U	14.1	2.23	2.62	8.39	0.00
Mean	13.8	3.10	2.70	6.25	—
Minimum	11.2	1.72	1.21	2.47	—
Maximum	16.2	5.05	4.52	9.73	—

[a] Sample origins: H–Hawaii; C–Costa Rica; D–Dominican Republic; P–Philippines; T–Thailand; U–Unknown origin.

Table 3. Potassium, amino acids, and carbon isotope ratio of pineapple[a]

Sample	Potassium, mg/L	Formol, meq/100 mL	Proline, mg/L	δ13°C, ‰ vs PDB
H1	1410	1.00	36	-12.1
H2	890	0.93	44	-11.2
H3	—	1.08	23	-11.3
H4	—	0.98	15	-11.2
H5	1350	1.40	33	-11.4
H6	830	0.93	36	-11.5
C1	1110	0.74	26	-12.8
C2	—	0.88	14	-12.7
C3	—	1.26	20	-12.4
C4	1320	1.58	30	-12.0
C5	1390	0.92	25	-13.1
D	1350	1.69	28	-13.0
P	1150	1.41	24	-12.5
T	1340	0.97	11	-12.4
U	1020	1.14	26	-13.5
Mean	1100	1.13	26	-12.2
Minimum	830	0.74	11	-13.5
Maximum	1410	1.69	44	-11.2

[a] Sample origins: H–Hawaii; C–Costa Rica; D–Dominican Republic; P–Philippines; T–Thailand; U–Unknown origin.

Gebhart et al. (1). The AFNOR handbook (9) specifies that pineapple juice should have a minimum of 11.0 g/100 g soluble solids. From these results, it would seem that pineapple juice should have an average level of soluble solids of approximately 14 g/100 g. For purposes of comparison, data from commercial pineapple concentrate or reconstituted pineapple juice should probably be normalized to 14 Brix.

Table 2. Acidity and organic acids in pineapple[a]

Sample	Acidity, g/100 mL	Citric, g/100 mL	Malic, g/100 mL	L-Malic, g/100 mL	Isocitric, g/L
H1	1.14	1.151	0.291	—	219
H2	0.89	0.876	0.210	—	158
H3	1.21	1.103	0.199	0.221	265
H4	0.96	0.907	0.253	0.239	231
H5	0.53	0.535	0.102	—	165
H6	0.66	0.597	0.073	0.072	156
C1	1.00	0.825	0.391	—	165
C2	1.21	0.796	0.172	0.182	196
C3	1.01	0.929	0.234	0.250	170
C4	0.46	0.505	0.086	—	112
C5	1.06	0.955	0.222	0.207	181
D	0.49	0.456	0.151	—	127
P	0.68	0.439	0.182	0.204	94
T	0.46	0.557	0.143	0.150	108
U	0.72	0.540	0.297	0.367	80
Mean	0.83	0.745	0.200	—	162
Minimum	0.46	0.439	0.073	—	80
Maximum	1.21	1.151	0.391	—	265

[a] Sample origins: H–Hawaii; C–Costa Rica; D–Dominican Republic; P–Philippines; T–Thailand; U–Unknown origin.

Analysis of the simple sugars of pineapple juice indicates that they consist primarily of fructose, glucose, and sucrose. In most samples, the dominant sugar was sucrose, accounting for up to 71% of total sugars. However, the sucrose content was highly variable, falling below fructose and glucose in some samples. There appears to be a correlation between sucrose content and acidity; high acid samples had higher sucrose values than low acid samples. This correlation may be related to the relative ripeness of the samples; very ripe, low acid samples may undergo some sucrose hydrolysis. The fructose and glucose levels were similar to each other, with fructose usually slightly higher than glucose. These results are similar to those for pineapple juice concentrate observed by Elkins et al. (5), who observed an average sucrose content of 56% of total sugars and a fructose/glucose ratio of 1.05. Matthews et al. (3) also indicate that sucrose predominates in the sugars of raw pineapple fruit.

Analysis of some of the samples for sorbitol content yielded no detectable sorbitol in any of the samples. This observation is hard to reconcile with the findings of Elkins et al. (5), who report finding considerable sorbitol in all of the commercial pineapple juice concentrates that they analyzed. Because analysis of pure pineapple juice spiked with sorbitol yielded a quantitative recovery by our procedure, it seems clear that pure pineapple juice contains no sorbitol. From discussions with J.R. Heuser (personal communication, 1990), it is apparent that the samples reported by Elkins et al. (5) may have in fact been blends of pineapple juice and apple juice. If so, it would account for their finding of sorbitol in the samples.

However, it may cast some doubt upon the validity of their results for other parameters.

The total acidity of fresh pineapple juice, expressed as percent citric acid, is quite variable. The values ranged from 0.46 to 1.21, averaging 0.83. The Brix/acid ratios were similarly variable. These results are very similar to those of Sale (2), who found an average value for 39 samples of 0.73. Organic acid analysis by liquid chromatography indicates that most of the acidity is due to citric acid, with a significant contribution made by malic acid. Enzymatic analysis of the L-malic acid content of some samples indicates that the malic acid is all L-malic acid. More recent enzymatic analysis of the D-malic acid content of some commercial pineapple juices yielded no detectable D-malic acid. The ratio of citric acid to malic acid ranged from 1.8 to 8.2. Elkins et al. (5) also observed that citric acid is the dominant acid in pineapple juice, with the ratio of citric acid to malic acid averaging 2.4. Enzymatic analysis of the concentration of isocitric acid yielded results ranging from 80 to 265 mg/L. The isocitric acid level was correlated to the citric acid content; the ratio of citric acid to isocitric acid ranged from 32 to 68, averaging 47. Elkins et al. (5) found an average citric/isocitric ratio of 65; they did not perform an alkaline hydrolysis of the samples before analysis, unlike the analyses in this study. This may account for the somewhat higher and more variable ratios that they observed.

The potassium content of pure pineapple juice ranged from 830 to 1410 mg/L, averaging 1100 mg/L, compared with an average value for 19 samples of 1220 mg/kg for canned pineapple juice reported by Gebhart et al. (1) and an average for 21 samples of 1640 mg/kg for raw pineapple fruit reported by Sale (2). The data in this study agree well with the data of Gebhart et al., but not with the data of Sale, which were determined by a gravimetric procedure, and may not be comparable with data obtained by flame photometry.

The formol value of pineapple juice ranged from 0.74 to 1.69 meq/100 mL, with an average value of 1.13. The proline concentration ranged from 11 to 44 mg/L, with an average value of 26. Wallrauch and Faethe (4) report an average proline value for 113 samples of 36 mg/L, with a range of 8–80 mg/L.

One property of pineapple juice that is unique among commercially important fruit juices is that the pineapple plant uses the Crassulacean Acid Metabolism (CAM) pathway for photosynthesis. Unlike most other fruit juices, whose source plants use the Calvin Cycle (C3) pathway, the carbon in pineapple juice contains a relatively higher proportion of the minor carbon isotope ^{13}C. Although most plants have carbon SIRA values in the vicinity of −25‰, the carbon SIRA values of pineapple juices are found to be clustered narrowly about −12.2‰ (range −11.2 to −13.5). These results are consistent with single values reported by Doner et al. (6) and Parker (7). The 3 values reported by Krueger et al. (8) are included in this study.

Additions of other fruit juices to pineapple juice can be detected by determining the carbon SIRA value. A more important point is that commercial beet sugar also has a carbon SIRA value of about −25‰. Hence, carbon SIRA determination will also detect additions of beet sugar to pineapple juice. This differs from the application of carbon SIRA to other fruit juices, where cane and corn syrup sugars are detected; these latter will not be easily detected in pineapple juice by carbon SIRA.

Adulteration of pineapple juice with sugars from corn syrup or cane sugar can be detected by changes in the pattern of other analytical values. The presence of normal corn syrup will increase the glucose content, as well as introduce small amounts of maltose. The presence of cane sugar or corn syrup will result in reduced values of potassium and formol, and reduced levels of organic acids. If citric acid is also added to maintain the acidity, the ratio of citric acid to malic acid and to isocitric acid will be increased; additions of malic acid will result in detectable quantities of D-malic acid. We have found this approach to be useful in the detection of adulteration in commercial pineapple juice.

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FOOD PACKAGING

Batching Oils on Sisal Bags Used for Packaging Foods: Analysis by Coupled LC/GC

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Sisal fibers are treated with a batching oil before spinning. Such oils usually consist of mineral oil products and cause considerable contamination of the packed foods (typically 10–100 mg/kg). Batching oils recovered from sisal bags previously used for transporting cocoa and coffee beans were analyzed for total concentrations on the bag as well as the composition of the paraffins and aromatics. Concentrations of total hydrocarbons ranged between 0.3 and 39 g/kg sisal bag; concentrations of aromatics ranged between <0.1 and 2.7 g/kg. The applied batching oils varied between raw mineral oil fractions, somewhat purified fractions, and a crystallized product.

Sisal bags, usually sized for 50–80 kg packings, e.g., of coffee beans, consist of a coarse fabric made of rough, rather hard sisal fibers. Sisal is obtained from the long leaves of an agave originating in Central America. Sisal bags are straw-colored (in contrast to the more brownish jute bags used for similar purposes) and usually of less dense fabrics. They are primarily used for storing and transporting coffee and cocoa beans. Of these products imported into Switzerland, about one third are packed in sisal bags, the rest in jute bags.

Batching Oil for Jute Bags

Recently, mineral oil material from jute bags was found to be an important source of contamination for foodstuffs stored and transported in such bags (1). Before spinning, jute fibers are treated with a batching oil to make them more elastic. The batching oil used for jute bags usually consists of a raw mineral oil fraction and is applied to represent 5–7% of the weight of the jute. A 4–5% concentration remains on the fabric when foods are packed into the sacks. More than 50 used jute bags (normally bags are used only once) were analyzed; the jute contained 1.5–4% mineral oil components. Often *n*-alkanes disappeared (by microbial degradation), leaving behind only the branched alkanes and aromatics, which form a "hump" of largely unresolved components in the gas chromatogram.

Food contamination by such batching oil is considerable (1); the 200 or so samples of hazelnuts analyzed contained 5–500 mg batching oil residues/kg, with typical concentrations in the range of 20–50 mg/kg. Coffee beans were contaminated at about 100 mg/kg. Kernels of cocoa beans contained 5–20 mg mineral oil components/kg; the contained shells, 100–1000 mg/kg. Surprisingly high concentrations were found in cocoa butter (2), causing chocolate to be contaminated at 10–50 mg/kg concentrations.

The composition of a typical batching oil sample for jute was determined by coupled liquid chromatography/gas chromatography (LC/GC) (3). Results showed that 23% of the oil consisted of aromatics, including 8.2% naphthalenes, 3.1% dibenzothiophenes, 2.2% anthracenes and phenanthrenes, 1.0% fluoranthenes and pyrenes, and 8 ppm benzo(e)pyrene; however, the oil consisted of at most 0.5 ppm benzo(a)pyrene. Over 99% of these aromatics were alkylated. At present, considerable efforts are expended to find a more acceptable replacement for the jute batching oil, primarily for the jute bags used for hazelnuts. At least in Switzerland, food contamination with batching oil is tolerated for a limited amount of time in the interest of keeping the jute bag production alive (jute products are the most important export articles of some third-world countries).

Sisal Bags

This paper draws attention to an analogous problem: the batching oils used for sisal bags. Of course, sisal fibers differ from jute fibers, but before spinning, sisal fibers also must be rendered more elastic by a batching oil. Although the production of sisal bags is clearly inferior to that of jute bags, the batching oil should also be under closer control and replaced where necessary. Concentrating on the batching oil for jute bags, while forgetting sisal bags, would be a mistake.

Analyses revealed some interesting results: It appears that some of the sisal fibers were treated with batching oil more carefully than were jute. Less batching oil was applied, and the

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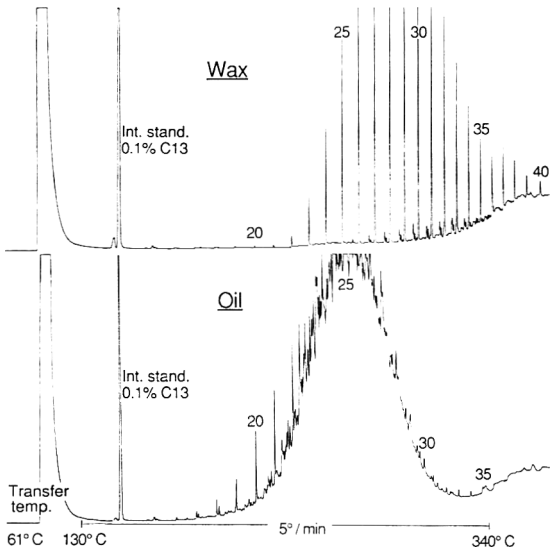

Figure 1. Two typical batching oils recovered from used sisal bags. Upper chromatogram: paraffin wax, 0.1% concentration on the bag. Some *n*-alkanes are labeled by the number of carbon atoms. Lower chromatogram: mixture primarily of isoalkanes, representing 3.9% concentration on the bag.

batching oil often contained clearly reduced concentrations of aromatics. This demonstrates that a more acceptable treatment of the raw fibers is possible and might serve as an example for jute bags.

Experimental

Determination of Paraffins

Samples of 1 g were taken from various parts of the sisal bags and immersed overnight in 50 mL redistilled *n*-pentane. These extracts were analyzed by on-line coupled LC/GC as previously described (1), using the Carlo Erba LC/GC Dualchrom 3000. From the raw extracts, the alkanes were isolated by LC on a 10 cm × 2 mm id silica gel column with *n*-pentane as mobile phase at 200 μL/min. Fractions of 150–250 μL were transferred to the GC system by concurrent eluant evaporation at 60°C column temperature and 1 bar inlet pressure. GC separation was achieved on a 25 m × 0.32 mm id capillary column coated with a PS-255 (methylsilicone) film 0.6 μm thick.

The accuracy of the data was primarily determined by the setting of the base line in the chromatograms and the measurement of the area of the broad hump of unresolved isoalkanes. The accuracy was within 15%. On some bags, residue concentrations at different sites varied by less than 20%; the most extreme variation found on a bag corresponded to a factor of 2. Presumably, bags were stocked in piles and different parts were exposed to air to different extents, causing the losses of batching oil material by evaporation to vary.

Cocoa beans were deshelled. Coffee beans, as well as the kernels and shells of the cocoa beans, were ground and immersed overnight in pentane, as described in Reference 1.

Determination of Aromatics

Concentrations of the sum of all aromatics were determined by the same LC and GC columns used for the paraffins. The beginning of the LC fraction of the aromatics was determined by LC/GC transfers, and it was positioned such that no squalane, added in large quantity to a sample, was transferred to the GC system. Branched alkanes, such as squalane, are slightly more retained than *n*-alkanes. This ensured that no paraffins were included in the fraction of the aromatics, but it does not rule out the possibility that highly alkylated benzenes were lost into the fraction of the paraffins. The end of the transferred LC fraction corresponded to the end of the benzo(a)pyrene peak (as detected by the UV detector), considering that alkylated benzopyrenes are eluted earlier.

The fraction of the aromatics had a volume of 1000 μL and was transferred under the same conditions as the alkanes, except that the GC oven began to increase the temperature 7 min, instead of 5 min, after starting the transfer. Because a 1 mm id sample loop was used, mixing (band broadening) in the loop was a problem (4), and filling of the sample loop was performed in an unusual way. The sample valve was left in "transfer" for the whole GC analysis, i.e., the loop remained empty and the LC effluant passed directly to waste. The valve was switched to "stand-by" (i.e., the sample loop was inserted into the LC flow line) only at the beginning of the LC fraction of interest and returned to "transfer" at the end. Because this required switching of the sample and the gas valve at different times, a 10-port valve on the LC side of the instrument (ISS-300) was used as the sample valve.

Results

We must emphasize that only used sisal bags were analyzed, i.e., the batching oil analyzed was previously subject to partial evaporation and microbial degradation. As documented for the batching oils found on jute bags (1), such losses easily reach a factor of 4; the original concentration and the composition of the oil may have differed substantially, and data on concentrations on the bags can only be interpreted semiquantitatively.

In contrast to the jute bags, the batching oils found on sisal bags (14 samples) strongly varied not only in their concentration on the bag, but also in their paraffin composition and in the concentration of aromatics. The batching oils found can be grouped as follows.

Waxes

Figure 1 shows 2 very different gas chromatograms of the paraffins extracted from sisal bags. Four bags (No. 3–6 in Table 1) contained crystallized mineral oil fractions, i.e., waxes, of the type shown in the upper chromatogram. Waxes almost exclusively consist of *n*-alkanes, and the small amounts of branched alkanes present form peaks of a repetitive pattern. The distribution of the *n*-alkanes was the same for all 4 bags,

ranging between C_{23} and C_{40}. This suggests that these bags were from the same producer or that the same batching oil was applied.

Concentrations of the wax on the bag ranged between 0.2 and 2.1 g/kg, or only 1/10 to 1/100 of those found on jute bags (microbial degradation may have substantially reduced the content of *n*-alkanes; on jute bags, up to 95% of the *n*-alkanes were removed). No aromatics were detected; concentrations of aromatics in crystallized mineral oil are generally low.

Concentrations of batching oil residues in the cocoa beans packed in those sisal bags were higher than expected; concentrations varied between 35 and 100 ppm in the shells of the beans from bags No. 3–5, and between 3 and 10 ppm in the nibs (the kernels after roasting). These values are barely a factor of 2 below those found for cocoa beans from jute bags. The residues contained far larger proportions of isoalkanes than the waxes on the bags, and the molecular weight distribution was shifted toward the more volatiles. We repeatedly observed that the material transferred to the foods corresponded to a small portion of the chromatogram of the batching oil, not only because the volatile components are most efficiently transferred, but also because the transfer of isoalkanes appears to be more rapid than that of the *n*-alkanes. However, there also remains the possibility that the cocoa beans were previously stored in another bag.

Oil

The lower chromatogram in Figure 1 shows a totally different batching oil extracted from bag No. 7. The oil forms a relatively narrow hump of unresolved peaks, indicating that it consists primarily of branched alkanes with 21–32 carbon atoms. Such mixtures are commonly used as a basis for hydraulic oils or lubricating oils and are characterized by low melting points. The concentration on the bag amounted to 3.9%, i.e., 30 times that of the wax. The concentration of the aromatics in this oil, some 3%, was far below that of a raw mineral oil, about 1/10 that of the jute batching oil analyzed (3). This suggests that the batching oil consisted of a processed, technical grade oil. Of course, 3% aromatics is still above what is considered "food-grade" (where concentrations below about 0.1 ppm are required), and 1.25 g aromatics/kg on the bag is considered problematic.

Raw Mineral Oils

Several bags (No. 1, 2, 10, 11, and 13 in Table 1) appeared to contain a batching oil similar to that used for jute bags. The oils were composed of hydrocarbons of a wide range of molecular weights, reaching from about C_{17} to at least C_{35}, with a (broad) maximum between C_{25} and C_{30}. A corresponding chromatogram was shown in Reference 5. The paraffins of an oil with a somewhat more narrow molecular weight distribution are shown in the upper chromatogram of Figure 2. They corresponded to a 2.1% concentration on the bag. The chromatogram primarily shows a broad hump of unresolved components, indicating that the mixture consisted of isoalkanes under nearly complete absence of *n*-alkanes. This may be due to the application of a corresponding oil or, more probably, to microbial degradation of the *n*-alkanes.

The aromatics, shown in the lower chromatogram of the same figure, also formed a hump of unresolved peaks, with a distribution of the retention times corresponding to that of the alkanes. Because an apolar column was used, retention times approximately correspond to volatility, and the correlation of the retention times for aromatics and paraffins are the result of a distillative cut of the fraction. The distribution of the aromatics on this sisal bag differs from that of the aromatics found in

Table 1. Type and concentration of batching oil found on sisal bags analyzed

Bag No.	Origin	Batching oil, g/kg	Aromatics, g/kg	Type of batching oil
		Cocoa		
1	?	13	?	broad hump, strongly degraded
2	?	21	?	broad hump, strongly degraded
3	Ecuador	1.8	low	crystallized
4	Ecuador	0.2	low	crystallized
5	Ecuador	1.4	low	crystallized
6	Venezuela	2.1	< 0.1	crystallized
7	Ecuador	39	1.2	narrow hump, no *n*-alkanes
		Coffee		
8	Costa Rica	27	1.2	narrow hump, no *n*-alkanes
9	Columbia	18	2.2	broad hump, some early *n*-alkanes
10	Columbia	7	1.2	broad hump, strongly degraded
11	Kenya	22	2.1	broad hump, strongly degraded
12	Kenya	0.3	—	broad hump, no *n*-alkanes
13	Kenya	21	2.7	broad hump, strongly degraded
14	Kenya	0.5	—	broad hump, no *n*-alkanes

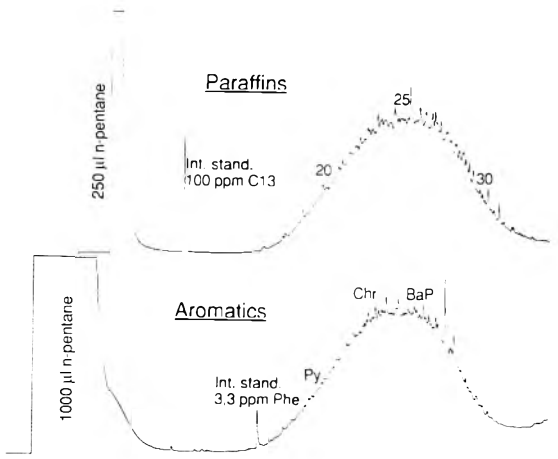

Figure 2. Paraffins and aromatics from a sisal bag used for Kenyan coffee beans. The aromatics corresponded to about 9% of the batching oil (differing attenuations). Phe, phenanthrene; Py, pyrene; Chr, chrysene; BaP, benzo(a)pyrene.

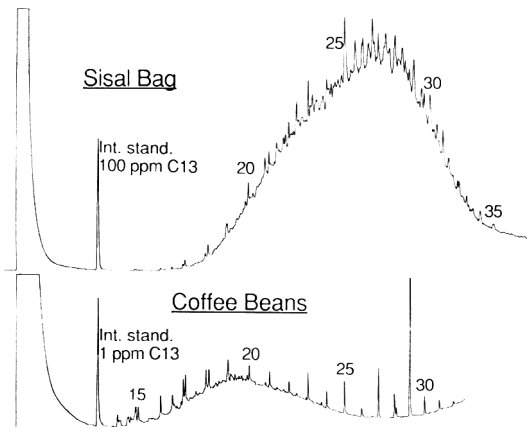

Figure 3. Paraffins from sisal bag and from coffee beans packed in bag. Only the volatile part of the batching oil is efficiently transferred. *n*-Alkanes C_{23}–C_{30} are primarily from the natural waxes on the coffee bean surface.

the jute batching oil (3) by the nearly complete absence of dominating components forming peaks, which is probably due to a higher degree of alkylation, resulting in a larger number of isomers of an aromatic system. This might be linked with the other difference observed: The distribution is shifted toward higher molecular weights. The position of the benzopyrenes was at the end of the hump in the jute batching oil, and it is now a little behind the center of the hump. Higher molecular weights may be due to a higher degree of alkylation.

The aromatics represented a concentration of 2.7 g/kg related to the bag and of some 13.5% related to the batching oil. This is little more than half of that found in the jute bag batching oil, which is primarily due to a lower concentration of early eluted aromatics, possibly of alkylbenzenes. However, if the shift toward higher elution temperatures is at least partly related to a shift toward aromatics with more rings, this is not necessarily an improvement.

Figure 3 shows the paraffins from a sisal bag (No. 11, 2.2% batching oil) and from the coffee beans transported in this bag. Although the batching oil concentration on the bag was barely below that commonly found on jute bags, the 42 mg/kg concentration of batching oil residues on the coffee corresponded to only about half of what was usually found after transport in jute bags (1). This is explained by the molecular weight distribution of the batching oil. Because transfer to the packed foods primarily occurs through the gas phase, the proportion of low molecular weight material is just as important as the total concentration on the bag. In fact, the typical jute bag batching oils contained substantially more volatile material than the oil recovered from bag No. 11.

Bags with Low Batching Oil Concentrations

Two bags (No. 12 and 13) contained low concentrations of a mineral batching oil (as low as found on bag No. 4 with the wax). However, the oils were markedly different: There are again virtually no *n*-alkane peaks, and from the molecular weight distribution, the oils resembled the crude batching oil of jute sacks, except that concentrations were 100-fold lower.

Discussion

Mineral Oil Products for Batching Fibers?

In 1990, intensive studies were started with the aim of finding an acceptable replacement for the batching oil softening jute fibers. Discussions center on radical solutions, such as completely avoiding products based on mineral oil, because it is assumed that the use of mineral oil products will inevitably be strongly restricted in the long run. The hazelnuts of the 1991 harvest imported into Switzerland will be transported in jute bags with a batching oil consisting of partially sulfonated plant oil, applied at a concentration about 1/10 of that for the conventional mineral batching oils. Such triglycerides are practically nonvolatile and are barely transferred to the packed foods. Of course, such a solution should be welcome. However, taking into account that many other packaging materials also contaminate foods with mineral oil material, less radical solutions must also be accepted, at least for the time being.

Which batching oil is acceptable for packaging foods? Other packaging materials may serve as a precedent: Paper, cardboard, and plastics often contain 0.1% of mineral oil products (5), although usually with low concentrations of aromatics. For cardboard and jute, the mass ratio of packaging material and packed food may be similar. Seen from this angle,

0.1% of a mineral oil with a low concentration of aromatics could hardly be refused as a batching oil.

Present Batching Oil for Sisal Bags

The discussion about improved jute batching oils should be broadened, taking into consideration the batching oils used for sisal bags. First, conclusions should also apply to sisal bags; secondly, some batching oils applied to sisal bags seem to indicate that more acceptable batching oils on the basis of mineral oil products are already is use.

We know comparatively little about the production of sisal fabrics. However, a considerable proportion of sisal bags were not treated with a cheap, raw, mineral oil fraction, and this creates the impression that some producers of sisal bags more carefully selected the batching oil than did the jute manufacturers. The crystallized fraction of mineral oil (wax) is relatively clean as far as aromatics are concerned, and since the concentrations on the fabrics are in the range of 0.1%, this is clearly more acceptable than what is commonly used for jute. In fact, concentration and composition seem comparable to the products often found in cardboard. It remains puzzling, however, that a relatively hard wax is used for softening fibers.

The 2 bags containing extremely low concentrations of mineral oil material might be even more interesting. It is hard to believe that some producers achieve equivalent softening of the fibers with 1/100 the amount of oil others use. The mineral oil found could just as well be a residue from another source, e.g., from a lubrication oil or grease applied during spinning. Then, however, the question remains as to what was used as batching oil for softening these fibers.

Conclusions

Completely stopping the use of mineral oils for batching jute and sisal fibers is neither possible nor justified. However, some guidelines must be established to rule out food contamination as severe as that observed today. Such guidelines could involve the following requirements: (*1*) The total concentration of mineral batching oil on the bag must be reduced by a factor of about 10 (to <0.5%) to approach the concentrations also found in other packing materials. (*2*) The mineral batching oil must be free of aromatics (in accordance with regulations on food-grade mineral oil products). (*3*) Because transfer to foods occurs primarily through the gas phase, most of the contamination finally found in the foods involves molecules with up to 25 carbon atoms. Therefore, it is important to use batching oils not containing material more volatile than the alkane C_{25}.

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MEAT AND MEAT PRODUCTS

Soxtec Fat Analyzer for Determination of Total Fat in Meat: Collaborative Study

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A new method for determination of total fat in meat and meat products, which uses a commercially available extraction system, was collaboratively studied by 11 laboratories. The study compared the new Soxtec solvent extraction with the Soxhlet method. The new method reduces extraction time, enables recovery of 60–70% of the solvent, and improves safety through external heating. Each laboratory received 6 samples: canned ham, ground beef, frankfurters, fresh pork sausage, hard salami, and beef patties with added soy. Laboratories were instructed to analyze each sample in duplicate on each of 2 days by both the Soxhlet and Soxtec methods. In general, results for the Soxtec system showed improved performance. For all samples used in Soxtec analysis, ranges of values for repeatability and reproducibility were s_r, 0.106–0.764; RSD_r, 1.01–2.44%; s_R, 0.112–0.799; and RSD_R, 1.53–2.84%. The method was adopted first action by AOAC International.

The determination of total fat content in meat is traditionally based on the Soxhlet principle because its accuracy and reproducibility are accepted worldwide. However, conventional Soxhlet analysis involves tedious and time-consuming manual work and explosion risks.

A new procedure for determination of total fat in meat has been developed. This method uses a commercially available extraction system that is an adaptation of the Randall application of the Soxhlet procedure. With this method, extractions using a wide range of solvents can be performed in a quicker, safer, and more economical way than Soxhlet extraction. Thus, the technique reduces extraction times to less that 20% of Soxhlet times and recovers 60–70% of the solvent; external heating ensures greater safety.

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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 AOAC. See "Changes in Official Methods of Analysis," *J. AOAC Int.* (1992) 75, 223–225.

Samples to be analyzed are weighed into thimbles and inserted into the extraction unit. After solvent addition to the extraction cups, the soluble material is extracted into the solvent in a 2-stage process followed by a solvent recovery cycle. Finally, the extraction cups are dried and weighed.

The service unit supplies the extraction unit with hot oil to achieve solvent evaporation. The tubing connecting the extraction and service units is designed to withstand the use of hot oil and to allow constant temperatures. The length of the tubing permits placing the units in separate rooms. The service unit is also equipped with an air pump for evaporation of the last traces of solvent from the extraction cups (1).

The Soxtec system manufactured by Perstorp Analytical/Tecator meets the described specifications. The collaborative study reported here compares analytical data obtained using classical Soxhlet extraction techniques with data obtained using the Soxtec system.

Collaborative Study Design

We decided that the study should include not only a wide range of fat concentrations but also a varied assortment of meat products. Therefore, a total of 6 meat samples were prepared and submitted to 11 collaborating laboratories. The meat samples chosen were canned ham, ground beef, frankfurters, fresh pork sausage, hard salami, and beef patties (soy added).

Each of the participating laboratories was instructed to analyze each sample in duplicate by conventional Soxhlet analysis and by the Soxtec system. The analytical procedure was then repeated on a separate day. Therefore, each laboratory was required to analyze each sample a total of 8 times, 4 times by conventional Soxhlet analysis and 4 times using the Soxtec system.

Experimental

Soxhlet Procedure

Apparatus

(a) *Thimbles.*—Fat extracted 25 × 80 mm.

(b) *Soxhlet extraction apparatus.*—Interior dimension of extraction tube, 30 mm.

(c) *Glass beads.*—3–4 mm diameter.

Table 1. Collaborative results for determination of total fat content, %, of sample 1 (canned ham) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	4.29	4.49	4.53	4.42
2[a]	8.45	7.45	5.80	5.94
3	4.53	4.46	4.56	4.51
5	4.63	4.72	4.65	4.61
6	4.57	4.54	4.95	4.77
7	4.56	4.71	4.43	4.73
8	4.48	4.58	4.71	4.50
9	4.44	4.52	4.64	4.81
10	4.83	4.65	4.53	4.71
11	4.80	4.70	4.80	4.80
12	4.53	4.61	4.80	4.85
Soxtec analysis				
Coll.	Day 1		Day 2	
1	4.23	4.40	4.08	4.33
2	4.36	4.31	4.23	4.16
3	4.34	4.39	4.38	4.44
5	4.54	4.54	4.37	4.35
6	4.35	4.30	4.32	4.35
7	4.27	4.38	4.32	4.14
8	4.26	4.40	4.42	4.42
9	4.11	4.35	4.45	4.43
10[b]	4.34	5.16	4.37	5.80
11	4.20	4.40	4.30	4.50
12	4.32	4.31	4.55	4.51

[a] All results outliers by Cochran test and single Grubbs test.
[b] All results outliers by single Grubbs test.

Table 2. Collaborative results for determination of total fat content, %, of sample 2 (ground beef) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	27.57	27.74	27.69	27.86
2[a]	36.24	38.16	30.22	30.57
3[a]	27.50	27.50	41.39	29.82
5	26.86	27.51	27.71	27.79
6	27.39	28.17	28.58	28.28
7	26.90	26.90	27.30	27.20
8	27.96	27.23	27.27	27.54
9[b]	28.16	25.05	27.28	22.43
10	27.38	27.37	27.66	27.49
11	27.90	27.00	28.00	27.60
12	27.72	27.51	27.81	28.15
Soxtec analysis				
Coll.	Day 1		Day 2	
1	27.67	27.27	27.20	26.17
2	27.23	27.76	27.42	27.51
3	26.41	26.87	26.65	26.68
5	26.77	27.02	27.18	25.88
6	27.20	26.94	28.99	29.72
7	26.60	26.90	27.40	27.60
8	27.40	27.20	26.82	27.48
9	27.95	27.49	27.37	27.60
10	27.32	27.15	26.99	27.39
11	27.00	27.30	27.50	27.30
12	27.66	27.42	27.52	27.89

[a] All results outliers by paired Grubbs test.
[b] All results outliers by single Grubbs test.

Reagents

(a) *Petroleum ether.*—AOCS specification H2-41 or AOAC **10.118**, 12th Ed.

(b) *Sand.*—0.004 g extractable/5 g.

(c) *Cotton.*—Defatted.

Extraction and Analysis (2)

Accurately weigh, by difference, 3 g sample into thimble containing small amount of sand. Mix sand and sample with glass rod. Add more sand if necessary. Place thimble in 50 mL beaker and dry in mechanical convection oven for 1.5 h at 125°C. Remove from oven and let cool. Loosen sample–sand mixture, wipe glass rod with small amount of cotton, and place cotton in top of thimble.

Accurately weigh extraction flask containing a few glass beads. Extract sample 4 h with 80 mL petroleum ether at condensation rate of at least 5–6 drops/s in Soxhlet extraction apparatus. (Rinse 50 mL beaker with petroleum ether and add rinsings to extraction tube.) At completion of extraction, evaporate petroleum ether until no odor is detected. Dry flask and contents 2 h in mechanical convection oven at 125°C. Cool and weigh.

Calculation

$$\text{Fat content, \%} = [100 \times (B-C)]/A$$

where A = sample weight, B = weight of flask after extraction, and C = weight of flask before extraction.

991.36 Fat (Crude) In Meat and Meat Products—Solvent Extraction (Submersion) Method

Final Action 1991

(Applicable to meat and meat food products that can be analyzed using **960.39**, **976.21**, and **985.15**.)

Method Performance:
Mean, 4.34% fat
$s_r = 0.106$; $s_R = 0.112$; $RSD_r = 2.44\%$; $RSD_R = 2.59\%$
Mean, 27.29% fat
$s_r = 0.534$; $s_R = 0.637$; $RSD_r = 1.95\%$; $RSD_R = 2.33\%$

Table 3. Collaborative results for determination of total fat content, %, of sample 3 (frankfurters) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	28.45	28.64	27.98	27.87
2	30.06	29.79	29.07	29.50
3	27.32	23.31	27.70	27.77
5	28.18	28.26	28.52	27.79
6	26.78	27.50	28.09	27.40
7	29.30	28.70	28.70	28.30
8	28.28	28.31	28.28	28.43
9[a]	28.25	23.72	20.07	20.76
10	28.17	28.28	27.98	28.30
11	28.10	28.40	28.40	28.10
12	28.15	28.66	28.94	28.55
Soxtec analysis				
Coll.	Day 1		Day 2	
1	28.09	28.05	28.14	28.05
2	27.92	28.04	26.61	27.56
3	27.62	27.94	27.41	27.91
5	27.39	27.71	27.31	28.21
6	27.82	27.66	26.74	26.68
7	28.40	27.90	28.20	27.80
8	28.61	27.95	28.15	27.93
9	27.76	28.01	30.76	30.76
10	28.24	28.17	27.59	28.29
11	27.60	26.50	27.70	27.90
12	28.07	27.70	28.19	28.70

[a] All results outliers by single Grubbs test.

Table 4. Collaborative results for determination of total fat content, %, of sample 4 (fresh pork sausage) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	34.59	34.55	34.13	33.98
2	37.73	36.21	35.83	36.27
3	31.95	35.64	35.09	34.91
5	34.76	35.47	35.00	35.09
6	34.72	35.84	35.92	34.70
7[a]	34.40	34.50	34.40	34.50
8	36.14	34.98	35.23	34.09
9	31.46	29.97	30.39	—
10	35.14	34.72	34.83	34.60
11	34.40	34.40	34.70	34.80
12	34.61	34.79	35.67	36.07
Soxtec analysis				
Coll.	Day 1		Day 2	
1	35.02	34.32	34.77	34.12
2	35.56	33.38	34.19	34.95
3	35.13	35.30	35.57	34.87
5	35.76	34.47	34.20	33.76
6	35.01	34.63	34.44	34.56
7	34.70	34.60	34.70	34.60
8	35.49	33.21	34.23	34.52
9	35.06	34.07	31.26	33.62
10[a]	24.93	30.76	32.94	32.15
11	34.20	34.60	34.30	33.80
12	34.71	35.65	34.35	34.87

[a] All results outliers by single Grubbs test and Cochran test.

Mean, 27.95% fat
$s_r = 0.648$; $s_R = 0.793$; $RSD_r = 2.32\%$; $RSD_R = 2.84\%$
Mean, 34.51% fat
$s_r = 0.764$; $s_R = 0.799$; $RSD_r = 2.21\%$; $RSD_R = 2.31\%$
Mean, 33.57% fat
$s_r = 0.340$; $s_R = 0.516$; $RSD_r = 1.01\%$; $RSD_R = 1.53\%$
Mean, 26.20% fat
$s_r = 0.406$; $s_R = 0.613$; $RSD_r = 1.55\%$; $RSD_R = 2.34\%$

A. Principle

Soluble material is extracted from dried samples of meat and meat food products by 2-step treatment with petroleum ether solvent. Solvent is recovered by condensation, leaving extracted soluble material, which is determined by weight after drying.

B. Apparatus

(a) *Extraction system.*—Capable of simultaneous extraction of 6 samples. Extraction unit for solvent addition to cups, 2-stage extraction process, and solvent recovery cycles. Service unit to supply hot oil through insulated tubing to extraction unit and to pump air for evaporation of last traces of solvent from cups (Soxtec system meets these specifications).

(b) *Thimbles and stand.*—26 × 60 mm, cellulose thimbles and stand to hold 6 thimbles.

(c) *Extraction cups.*—44 mm id, 60 mm height, aluminum.

(d) *Glass beads.*—3–4 mm diameter.

(e) *Mechanical convection oven.*—Capable of maintaining 125 ± 1°C.

Items (a)–(c) are available as Soxtec system from Perstorp Analytical/Tecator Inc., 2875 C Towerview Rd, Herndon, VA 22071

C. Reagents

(a) *Petroleum ether.*—To meet specifications in **945.16A**.

(b) *Sand.*—0.004 g extractables/5 g.

(c) *Cotton.*—Defatted.

D. Determination

Accurately weigh ca 3 g sample into thimble. Add sand to sample and mix with glass rod. Place thimble in thimble stand and dry 1 h in 125°C oven. Remove from oven and let cool. Loosen sample/sand mixture using glass rod. Wipe glass rod

Table 5. Collaborative results for determination of total fat content, %, of sample 5 (hard salami) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	34.17	34.91	33.44	33.32
2	34.84	34.58	34.68	34.74
3	33.70	34.64	33.61	34.13
5	33.80	34.07	33.74	34.10
6	35.06	31.95	34.04	32.69
7	33.90	32.06	34.50	34.20
8	34.35	34.72	34.11	33.33
9[a]	26.98	28.29	34.60	34.31
10	33.70	34.60	35.42	34.45
11	33.80	34.90	34.20	33.90
12	35.37	35.06	33.75	33.46
Soxtec analysis				
Coll.	Day 1		Day 2	
1	33.79	32.94	33.21	32.98
2	33.07	33.87	32.56	33.23
3	33.30	33.32	33.37	33.35
5	33.33	33.38	32.82	33.11
6	33.10	32.83	33.41	33.56
7	33.20	33.90	33.40	33.50
8	33.69	33.66	32.96	33.22
9	34.19	34.32	34.76	34.36
10	34.03	33.82	34.09	33.50
11	34.40	33.60	33.90	33.80
12	34.21	33.41	34.14	34.45

[a] All results outliers by single Grubbs test.

Table 6. Collaborative results for determination of total fat content, %, of sample 6 (beef patties with soy) by Soxhlet and Soxtec analyses

Soxhlet analysis				
Coll.	Day 1		Day 2	
1	26.56	26.37	26.04	26.36
2	28.45	28.40	27.72	27.91
3	26.69	27.34	26.65	26.90
5	26.46	26.81	25.77	26.28
6	25.45	26.73	25.52	27.04
7	28.00	28.40	26.70	26.80
8	26.69	26.33	26.46	26.60
9[a]	20.25	19.72	26.95	27.14
10	26.78	26.92	26.74	25.93
11	27.10	27.10	26.90	26.60
12	26.57	26.90	26.92	26.74
Soxtec analysis				
Coll.	Day 1		Day 2	
1	25.92	26.31	25.79	25.91
2	25.68	26.58	26.09	25.00
3	26.30	26.41	26.31	26.28
5	26.17	26.08	25.81	27.05
6	25.45	24.94	25.33	24.64
7	27.10	27.20	26.30	26.30
8	26.56	26.31	26.36	26.02
9	26.84	27.11	26.85	26.82
10	25.53	26.29	26.22	26.73
11	26.40	25.90	26.90	26.50
12[a]	26.42	20.09	26.80	19.19

[a] All results outliers by single Grubbs test.

with small amount of cotton and place cotton in top of thimble. Transfer thimble to extraction unit.

Accurately weigh extraction cup containing a few glass beads. Extract sample with 40 mL petroleum ether in boiling position for 25 min and in rinsing position for 30 min. Adjust temperature of service unit to ensure condensation rate ≥5 drops/s. At completion of extraction, close condenser valves and recover ether.

Dry cup and contents 30 min in 125°C oven. Cool and weigh.

E. Calculation

Calculate percent fat in sample as follows:

$$\text{Fat content, \%} = [(B - C) \times 100]/A$$

where A = sample weight, B = weight of extraction cup after drying, and C = weight of extraction cup before extraction.

Ref.: *J. AOAC Int.* **75**, March/April issue (1992)

Results and Discussion

The major difference between the 2 methods is the type of extraction apparatus used and the resulting difference in extraction time. In the Soxtec method, both the pre-extraction and postextraction drying times are reduced. All other sample handling and preparation steps, including the mixing of sand and meat in the extraction thimble, should be performed in an identical manner. The differences between the methods are as follows: (*1*) Pre-extraction drying: Soxhlet, 1.5 h at 125°C; Soxtec, 1 h at 125°C. (*2*) Extraction: Soxhlet, 4 h; Soxtec, 55 min total—25 min "boiling step" and 30 min "rinsing step." (*3*) Postextraction drying: Soxhlet, 2 h at 125°C; Soxtec, 30 min at 125°C.

Analytical results obtained by the participating laboratories are shown in Tables 1–6. Outliers were determined using the Cochran test, the single Grubbs test, and the paired Grubbs test. In summary, outliers by Soxhlet analysis were Collaborator 2, Samples 1 and 2; Collaborator 3, Sample 2; and Collaborator 9, Samples 2, 3, 4, 5, and 6. For Soxtec analysis, outliers were Collaborator 10, Samples 1 and 4, and Collaborator 12, Sample 6. The summary shows that Collaborator 9 had considerable difficulty with the Soxhlet method but not with the Soxtec method.

Table 7 summarizes the statistical evaluation of the data calculated using the "balanced or unbalanced replicate" method.

Table 7. Summary of statistical evaluation of data by "balanced or unbalanced replicate" method

		Repeatability		Reproducibility		
Sample	% Total fat, mean	s_r	RSD_r, %	s_R	RSD_R, %	Method
1	4.62	0.122	2.63	0.146	3.15	Soxhlet
1	4.34	0.106	2.44	0.112	2.59	Soxtec
2[a]	28.35	2.481	8.75	3.133	11.05	Soxhlet
2	27.29	0.534	1.95	0.637	2.33	Soxtec
3	28.21	1.555	5.52	1.568	5.57	Soxhlet
3	27.95	0.648	2.32	0.793	2.84	Soxtec
4	34.98	0.734	2.09	0.910	2.60	Soxhlet
4	34.51	0.764	2.21	0.799	2.31	Soxtec
5	34.10	0.769	2.25	0.771	2.26	Soxhlet
5	33.57	0.340	1.01	0.516	1.53	Soxtec
6	26.81	0.466	1.74	0.716	2.67	Soxhlet
6	26.20	0.406	1.55	0.613	2.34	Soxtec

[a] All data included in statistical calculations. *See* text.

In calculating RSD_r, s_r, RSD_R, and s_R for the 2 methods, all results for Soxhlet analysis of Sample 2 had to be used. Because there were 3 outliers (out of a total of 11 laboratories) for the Soxhlet analysis of Sample 2, elimination of all of these outliers would have been greater than 2/9ths of the total laboratories. Therefore, according to AOAC guidelines, all of these results had to be incorporated into the calculations for repeatability and reproducibility for the Soxhlet analysis of Sample 2.

Outliers were significantly fewer for the Soxtec technique. Results by Soxtec analysis had a slightly negative bias compared to Soxhlet results obtained on the identical samples. In general, the Soxtec method generated results that had lower RSD_r, s_r, RSD_R, and s_R values than those for the Soxhlet method.

Recommendation

The Soxtec system gives analytical results that are equivalent to results obtained using the conventional Soxhlet analytical technique. We recommend that the method using the Soxtec system be adopted first action for determination of total fat in all meat and meat food products that can be currently analyzed using AOAC methods **960.39**, **976.21**, and **985.15**.

This analysis will be used as an optional method to those currently used to determine the total fat content in meat and meat food products by ether extraction.

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(1) Operating Manual Tecator Soxtec System HT

(2) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **24.005.a**

MICROBIOLOGICAL METHODS

Automated Conductance Method for the Detection of *Salmonella* in Foods: Collaborative Study

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Seventeen laboratories participated in a collaborative study to validate an automated conductance method for the rapid detection of *Salmonella* in food. The conductance method was compared with the standard BAM/AOAC method for the isolation of *Salmonella*. Media for the conductance method were supplied in ready-to-use, disposable, single-use cells of a novel, unique design. Samples of coconut, fish meal, prawns, nonfat dried milk, liquid egg, and minced beef were artificially contaminated with different *Salmonella* serotypes to 2 target levels of 1–5 cells/25 g and 10–50 cells/25 g. Each participating laboratory tested 10 contaminated and 5 noncontaminated samples per product. Results showed no significant difference between BAM/AOAC and conductance methods. The conductance method was adopted by AOAC International as a first action method.

Conventional methods for isolating *Salmonella* from food are laborious, time-consuming, and require at least 4 days to give a result (1, 2). More rapid methods based on measurement of the changes in electrical conductance of media caused by microbial growth were described (3, 4). Commercially available automated instruments can perform hundreds of assays simultaneously and, being computer-based, can produce data continuously. The use of such systems was reviewed (5).

The conductance method consists of pre-enrichment of food samples followed by an assay in selective media. Changes in the electrical conductance of these media, due solely to the growth and metabolism of the microbes in the sample, are measured through a pair of electrodes in the growth medium. When conductance values reach a certain magnitude, the test is regarded as positive.

The protocol for the detection of *Salmonella* consists of pre-enrichment in buffered peptone water (BPW) containing lysine and glucose (6) and then subculture into 2 selenite-based media (3, 4) containing trimethylamine-*N*-oxide and dulcitol (*Salmonella* Medium 1) and lysine (*Salmonella* Medium 2) held in disposable cells. The cells are connected to a Malthus analyzer and automatically monitored. Results can be displayed as presumptive pass/fail, as conductance data, or graphically. The method was evaluated in a precollaborative trial with 24 foods, and the results indicate that it was as sensitive as the standard culture methods (7).

A collaborative study has been performed to compare the conductance method with conventional culture methods. The study was designed and performed in accordance with AOAC requirements.

Collaborative Study

Food Products

The food products selected for evaluation were representative of those implicated in *Salmonella* food poisoning and included coconut, fish meal, prawns, nonfat dried milk (NFDM), liquid egg, and minced beef. Samples (ca 50 or 75 g aliquots) were prepared at Malthus Instruments Laboratory, Crawley, UK, under the supervision of the Associate Referee and following guidelines proposed by Andrews (8). Samples were distributed by carrier to the 17 collaborators in the United Kingdom and the United States.

Collaborators each received a set of 15 samples per product: 5 uninoculated controls, 5 samples inoculated with a low target level of *Salmonella* (1–5 cells/25 g), and 5 with a high target level (10–50 cells/25 g) (Table 1). *Salmonella* spray-dried in milk was used to inoculate the low water activity (a_W) foods,

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[1] Present address: Radiometer America, Inc., 811 Sharon Dr, Westlake, OH 44145
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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 AOAC. See "Changes in Official Methods of Analysis," *J. AOAC Int.* (1992) 75, 223–225.

Table 1. Test products, test organisms, and inoculation levels

Product	*Salmonella* serotype	Target inoculation level[a]	Most probable No./ g
Coconut	*S. montevideo*	low	0.09
	S. agona	high	0.09
Fish meal	*S. agona*	low	0.03
	S. montevideo	high	0.03
Prawns	*S. enteritidis*	low	< 0.01
	S. typhimurium	high	< 0.01
Nonfat dried milk	*S. agona*	low	< 0.03
	S. montevideo	high	1.3
Liquid egg	*S. typhimurium*	low	0.09
	S. enteritidis	high	0.09
Minced beef	*S. typhimurium*	low	2.3
	S. typhimurium	high	> 120

[a] Low = 1–5 cells/25 g; high = 10–50 cells/ 25 g.

e.g., coconut, fish meal, and NFDM. Collaborators were required to set up positive product controls at the time of testing. For low a_W products, 5 g NFDM containing 10^3 *Salmonella* was reconstituted in BPW, added to the product, and tested according to the conductance protocol. For high a_W products, a frozen culture of 10^7 *Salmonella*/mL in tryptone soy broth (TSB)–glycerol was defrosted, diluted in BPW, and added to product that was then tested according to the conductance protocol.

For the conductance protocol, the conductance responses had to meet the respective criteria for the 2 media (see below). Media negative controls were also performed by adding 0.1 mL BPW–lysine–glucose (BPW–L–G) to each conductance medium. The conductance responses for the negative controls had to be less than the criteria for a presumptive positive *Salmonella* result.

Media Preparation

All media for the BAM/AOAC method and confirmatory tests were supplied to the collaborating laboratories in dehydrated form and prepared according to AOAC instructions (*see* AOAC method **967.25**). Each medium used throughout the trial came from the same production batch. All media for the conductance method were provided in hydrated form. Batches (1 L) of BPW–L–G were prepared, the pH was adjusted to 7.2, and the batches were autoclaved 20 min at 115°C. The sterile medium was dispensed aseptically in 225 mL volumes in pots (Medfor, Fleet, UK) and stored at 20°C before dispatch to collaborating laboratories.

The conductance media were supplied in ready-to-use disposable cells containing *Salmonella* Media 1 and 2. Each medium was prepared, the pH was adjusted, and they were filtered through a 0.22 μm membrane filter and dispensed aseptically into disposable cells. These media were stored at 10°C for up to 2 weeks before dispatch to collaborating laboratories.

Quality Control of Conductance Media

All pre-enrichment broths (BPW–L–G) were incubated to confirm sterility. Portions of these broths were also tested with a pure culture of *S. enteritidis* and with products spiked with the respective *Salmonella* strains listed in Table 1. These were subcultured to *Salmonella* Media 1 and 2 to confirm that they gave the expected conductance responses.

Most Probable Number Determination

Most probable number (MPN) determinations were performed on 2 samples selected at random from each of the negative, low, and high inoculum products to establish the levels of *Salmonella* (9).

Sample Analysis

From each sample and control, a 25 g test portion was analyzed by the BAM/AOAC culture method; a separate 25 g test portion was analyzed by the conductance method. Pre-enrichment, enrichment, isolation, and confirmation of isolates for the BAM/AOAC method were performed according to AOAC method **967.25–967.28**. For the conductance method, test portions were pre-enriched in BPW–L–G for 16 h at 35°C. Then, 0.1 mL volumes of pre-enrichment culture were inoculated into disposable cells containing *Salmonella* Media 1 and 2, placed in the instrument, and incubated up to 30 h at 35°C. Tests were considered positive if they exceeded selected criteria for either or both media. Positive tests were confirmed according to BAM/AOAC procedures.

Analysis of Data

The specificity rates (p_-) and their standard errors (s.e.(p_-)) were calculated as percents using the following relationships:

$$p_- = 1000\ (\Sigma\ a_i\ /\ \Sigma m_i)$$

$$s.e.(p_-) = 100^2[(\Sigma a_i^2 - (\Sigma a_i)^2/L)/m^2L(L-1)]^{1/2}$$

where a_i = number of analyzed negative test portions among "known" negative test portions per laboratory; m_i = number of "known" negative test portions per laboratory; m = average of m_i values; and L = number of laboratories.

The sensitivity rates (p_+) and their standard errors (s.e.(p_+)) were calculated as percents, using the relationships:

$$p_+ = 100\ (\Sigma a_i / \Sigma m_i)$$

$$s.e.(p_+) = 100^2[(\Sigma a_i^2 - (\Sigma a_i)^2/L)/m^2L(L-1)]^{1/2}$$

where a_i = number of analyzed positive test portions among "known" positive test portions per laboratory; m_i = number of "known" positive test portions per laboratory; m = average of m_i values; and L = number of laboratories.

The data, percentages of samples that were positive (*Salmonella* present), were transformed to arcsin angles [angle = (percentage)$^{1/2}$] for each laboratory and method at a given level, and an analysis of variance was used to compare the method means.

Table 991.38. Method performance for 991.38 automated conductance method for *Salmonella* in foods[a]

Food type	Level	Method[b]	Performance rate[c]	
Coconut	low	B	72.00	(9.32)
		M	72.00	(7.25)
	high	B	77.33	(9.33)
		M	86.67	(5.04)
Fish meal	low	B	27.50	(5.74)
		M	36.25	(7.12)
	high	B	38.75	(8.05)
		M	80.75	(8.56)
Prawns	low	B	1.54	(2.54)
		M	3.07	(2.08)
	high	B	26.15	(8.28)
		M	23.08	(7.79)
Nonfat dried milk	low	B	27.06	(6.17)
		M	29.41	(6.67)
	high	B	92.94	(5.93)
		M	95.29	(4.72)
Liquid egg	low	B	87.69	(4.82)
		M	92.30	(2.82)
	high	B	95.38	(2.43)
		M	93.85	(4.17)
Minced beef	low	B	100.00	(0.00)
		M	100.00	(0.00)
	high	B	100.00	(0.00)
		M	100.00	(0.00)

[a] An analysis of variance (ANOVA) technique applied to the data indicated that the method means were not significantly different ($P > 0.05$) at each level of inoculum for each food type.
[b] B = BAM/AOAC method; M = automated conductance method.
[c] Performance rate is shown as sensitivity (standard error in parentheses). Performance rate for specificity was 100.00 (0.00) at both levels for each method for all food types listed.

991.38 *Salmonella* in Foods—Automated Conductance Method

First Action 1991

Method is test procedure for presumptive presence of *Salmonella* in all foods. Positive assays must be confirmed by standard culture methods (*see J*).

A. Principle

Samples are pre-enriched in buffered peptone water–lysine–glucose broth followed by 2-tube conductance assay in selenite-based media containing trimethylamine-*N*-oxide and dulcitol (*Salmonella* Medium 1) and lysine (*Salmonella* Medium 2). *Salmonella* spp. typically give large conductance changes in these 2 media compared to those for non-salmonellae. Presumptive positive result is obtained within 48 h.

B. Method Performance

See Table **991.38** for method performance data.

C. Apparatus

(**a**) *Microbiological analyzer.*—Analytical instrument operating at 35°C that measures microbial growth based on conductance changes recorded at frequency of 10 kHz. Changes are detected by 2 electrodes, capable of detecting a conductance change of <1.00 μS (microsiemens), inserted into growth medium at 35°C. (Equipment meeting these specifications is available from Malthus Instruments Ltd, The Manor, Manor Royal, Crawley, West Sussex, RH10 2PY, UK.) The Malthus system consists of 1 or 2 analyzers with an IBM-compatible personal computer (PC). The system has 240 test capacity with automatic data collection.

(**b**) *Automatic pipet.*—Capable of delivering 100 μL.

D. Media and Reagents

Items (**a**) and (**b**) are available from Malthus Instruments Ltd. Equivalent media may be used.

(**a**) *Pre-enrichment broth.*—10.0 g bacteriological peptone, 5.0 g sodium chloride, 3.5 g disodium phosphate·2H_2O, 1.5 g potassium dihydrogen phosphate·H_2O, 5 g L-lysine, and 5 g D-glucose. Suspend ingredients in 1 L water and dispense 225 mL volumes into screw-cap containers. Autoclave 20 min at 115°C. Final pH should be 7.2 ± 0.2.

(**b**) *Selective conductance media.*—(*Caution*: Media contain sodium biselenite. Avoid contact with skin.) (*1*) *Salmonella Medium 1 (Easter and Gibson).*—Suspend 5.0 g bacteriological peptone, 10.0 g disodium hydrogen phosphate (Sorenson), 5.0 g dulcitol, 5.6 g trimethylamine oxide·HCl, and 4.0 g sodium hydrogen selenite in 1 L water. Add 1 mL L-cystine solution prepared by dissolving 0.1 g L-cystine in 15 mL 1N NaOH and diluting to 100 mL with sterile water. Final pH should be 7.2 ± 0.2.

(*2*) *Salmonella Medium 2.*—Suspend 5.0 g lactalbumin hydrolysate, 10.0 g D-glucose, 10.0 g L-lysine, and 4.0 g sodium hydrogen selenite in 1 L water. Add 10 mL L-cystine solution as in (**b**)(*1*). Final pH should be 6.5 ± 0.2.

Sterilize conductance media, (**b**), by filtration through a bacteriological filter. Aseptically dispense filtered media into sterile growth chambers or cells compatible with automated conductance analyzer.

(**c**) *Sodium hydroxide solution.*—1N.

(**d**) *Hydrochloric acid solution.*—1N.

E. General Instructions

Selective conductance media in cells must be stored at 4–6°C. Media are stable at 4–6°C for 6 months from date of manufacture. Let media equilibrate to room temperature before inoculation.

Include positive [*Salmonella typhimurium*, NCIMB 13034 (National Collections of Industrial and Marine Bacteria, 23 St Machar Dr, Aberdeen AB2 1RY, Scotland)] and negative controls (*Escherichia coli*, ATCC 25922) to ensure correct functioning of both media and analyzer.

F. Preparation of Samples

(**a**) *Pre-enrichment.*—Aseptically weigh 25 g sample into 225 mL pre-enrichment broth, (**D**)(**a**), in suitable container. Mixing and blending procedure will vary according to product. If product is liquid, powdered, ground, or comminuted, blending may be omitted. Blend noncomminuted and whole products 2 min. Cap container and let stand 60 min at room temperature. Mix well, and determine pH with test paper. If necessary, adjust pH to 7.2 ± 0.2 using sterile 1N NaOH or HCl. Mix well before determining final pH. Aseptically transfer sample to a sterile screw-cap 500 mL container. Loosen cap 1/4 turn and incubate 16–24 h at 35°C.

(**b**) *Selective enrichment.*—Transfer 0.1 mL incubated pre-enrichment culture to *Salmonella* Media 1 and 2 in disposable cells. Incubate at 35°C until criteria for a presumptive positive result are met, for maximum of 30 h.

(**c**) *Subculture.*—Any cell that gives presumptive positive result must be subcultured immediately by streaking a loop-full of broth onto Hektoen enteric agar (HE), xylose lysine desoxycholate agar (XLD), and bismuth sulfite agar (BE), as in **967.26**.

G. Installation of Analyzer

(*1*) Connect analyzer to an IBM AT compatible PC via RS232 ports using cable provided.

(*2*) Connect analyzer and PC to main power supply.

(*3*) Connect cooler tubes to ports at rear of cooler and analyzer.

(*4*) Connect cooler interface cable to DIN sockets at rear of cooler and incubator. If only 1 analyzer is in use, connections labeled "Incubator 1" must be used.

(*5*) Check that analyzer is level, switch on and fill with distilled or deionized water through front compartment. Continue filling until "STOP FILLING" message appears on display. Once filled, analyzer can be topped through port on top of instrument.

(*6*) Connect cooler to main power supply but do not switch on. Fill cooler with 3 L 33% v/v ethylene glycol-based antifreeze solution through port on top of unit. Top with coolant until "STOP ADDING COOLANT" message appears on analyzer display.

(*7*) Ensure that MS-DOS is installed on PC. Switch on PC and when C> prompt appears, place software installation Disc 1 (Malthus) in drive A, type A:install, and then follow on-screen prompts.

(*8*) When installation is complete, load program by typing C:\MALTHUS <ENTER> and then MALTHUS, or reboot PC if automatic loading option was selected during installation.

(*9*) Before analyzer can be used, configuration must be set. Select "SYSTEM" from Main Menu and press <ENTER>. Select "change config" (F6) option from program initialization menu and follow on-screen prompts. Return to initialization menu by pressing F10.

(*10*) Set analyzer temperature to 10°C from "change temperature" option (F5). Return to initialization menu and select "Start new test" (F1).

(*11*) Cooler must now be bled to prime pump and remove air from system. After temperature is set, wait 10 min then identify cooler tube connected to lower port on cooler (marked "OUT"). Disconnect this tube from port on analyzer and place end in suitable container at level below that of cooler. Let coolant discharge from tube until no bubbles appear. Reconnect tube.

(*12*) Set analyzer temperature to 35°C by selecting "UTILITIES" option from Main Menu and then selecting "change temp" (F7). Temperature of water in analyzer is indicated on display panel. Ensure that temperature is stable before starting a test.

H. Operation of Analyzer

(*1*) Ensure that all components of system are connected and operating in accordance with manufacturer's user guide.

(*2*) Check that system software is loaded and that analyzer display indicates correct incubation temperature (35°C). If not, use "Utilities" option to set correct temperature.

(*3*) Inoculate cell and tighten cap securely.

(*4*) Locate cell electrode contacts in cell connector cap and push to connect. Avoid excessive force.

(*5*) Place cell in one of holes in analyzer and locate connector in adjacent slot on printed circuit board (PCB). Push down firmly without twisting.

(*6*) Note position of cell in incubator and record for future reference.

(*7*) Once sample is placed in analyzer, data are collected and processed automatically. Presumptive positive *Salmonella* results are highlighted and should be confirmed as described in *J*.

I. Interpretation

Criteria for presumptive positive result for *Salmonella* Medium 1 are an overall conductance change ≥200 μS and maximum rate of change ≥25 μS/h and for *Salmonella* Medium 2,

Table 2. Number of test portions

Product	N_n[a]	N_p (low)[b]	N_p (high)[c]	Total
Coconut	85	85	85	255
Fish meal	85	85	85	255
Prawns	65	65	65	195
Nonfat dried milk	85	85	85	255
Liquid egg	65	65	65	195
Minced beef	65	65	65	195

[a] N_n = number of negative test portions.
[b] N_p (low) = number of positive test portions with low target inoculum level.
[c] N_p (high) = number of positive test portions with high target inoculum level.

overall conductance change ≥100 μS and maximum rate of change ≥25 μS/h.

Overall conductance change and rate of change are measured from detection time, which is defined as time at which differences between consecutive conductance readings from a test cell exceed a preset threshold value (0.8 μS).

J. Confirmation of Presumptive Positive Conductance Results

Presumptive positive conductance assay indicates that *Salmonella* may be present. All presumptive positive results must be confirmed as in **967.25–967.28**.

Ref.: *J. AOAC Int.* **75**, March/April issue (1992)

Results

Thirteen laboratories tested all 6 products, and 4 laboratories tested only coconut, fish meal, and NFDM. Test products, organisms, target inoculation levels, and MPNs are shown in Table 1. The numbers of test portions per product are shown in Table 2. Results submitted by the collaborators were checked against the data on floppy discs and are summarized in Tables 3–8. A comparison of data for both BAM/AOAC and conductance methods for all products is presented in Table 9.

Coconut

All 17 collaborators tested samples of coconut. The *Salmonella* MPNs at the time of testing were 0.09 cells/g and 0.09 cells/g for the low and high target inoculum levels, respectively (Table 1). Trial data for coconut are shown in Table 3. Laboratories 1 and 13 detected *Salmonella* in an uninoculated control sample, so their data were excluded from the statistical analysis. The total numbers of positive samples by both methods and for each inoculum level are shown in Table 9. One laboratory (Table 3) failed to detect any of the positive samples by the BAM/AOAC method, and another found only 1 sample to be positive. However, in an earlier study, the BAM/AOAC and conductance methods gave similar recovery rates for *Salmonella* in coconut (9). Also, in a pilot exercise before the collaborative study, all laboratories received coconut samples and their results were equivalent for both BAM/AOAC and con-

Table 3. Detection of *Salmonella* in coconut by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1[d]	–	+	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	+	–	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
6	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	+	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+
9	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	–	–	+	+	+	+	+
10	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	+	–	+	+	–	+	+	+	+	+
11	–	–	–	–	–	+	–	+	–	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	–	+	+	+
12	–	–	–	–	–	–	–	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	–
13[e]	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+
14	–	–	–	–	–	–	+	–	–	–	+	+	–	+	+	–	–	–	–	–	+	+	–	–	+	–	+	+	+	+
15	–	–	–	–	–	+	+	+	+	+	–	+	–	+	–	–	–	–	–	–	+	+	+	+	+	+	+	–	–	+
16	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	–	+
17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	+

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.
[d] Positive result on a negative sample by BAM/AOAC. Data excluded from statistical analysis.
[e] Positive result on a negative sample by conductance. Data excluded from statistical analysis.

Table 4. Detection of *Salmonella* in fish meal by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	–
3	–	–	–	–	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–
4[d]	–	–	–	–	–	+	+	+	+	+	+	–	+	–	–	–	–	+	–	–	+	+	+	+	+	+	–	+	–	–
5	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	+	–	–	–
7	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
8	–	–	–	–	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	–	+	–	+	+	–	+	+	+	+	+
9	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
10	–	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
11	–	–	–	–	–	–	–	+	+	–	–	+	–	–	+	–	–	–	–	–	–	–	+	+	–	–	+	–	–	+
12	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
13	–	–	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
14	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
15	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	–	–	–	–	–	–	+	–	–	–	+	+	+	–	–
16	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
17	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.
[d] Positive result on a negative sample by conductance. Data excluded from statistical analysis.

ductance methods (unpublished data). Therefore, all of the laboratories were capable of detecting positive samples by the BAM/AOAC method. Because all laboratories used the same media from the same batches, the reason for the failures by the BAM/AOAC method in Laboratories 16 and 17 is unclear.

Sensitivity and specificity rates at the low and high target inoculum levels were the same for both methods (72.0 and 100.0, respectively). Statistical analysis of the data (ANOVA) indicated that there was no significant difference ($P>0.05$) between the 2 methods.

Table 5. Detection of *Salmonella* in prawns by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
2	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+
4	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
11	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	
13	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	+				

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.

Table 6. Detection of *Salmonella* in nonfat dried milk by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+
2	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
3	−	−	−	−	−	−	−	−	+	−	+	−	+	+	+	−	−	−	−	−	+	−	−	−	−	+	+	+	+	+
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
5	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−
6	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
7	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
8	−	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+
9	−	−	−	−	−	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
10	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+
11	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
12	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
13	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−	+	+	+	+	+
14	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	+
15	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
16	−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
17	−	−	−	−	−	−	−	+	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.

Fish Meal

Seventeen laboratories tested samples of fish meal. The Salmonella MPNs at the time of analysis were 0.03 cells/g for both low and high target inoculum levels (Table 1). Trial data for fish meal are presented in Table 4. Laboratory 4 detected *Salmonella* in an uninoculated control sample by conductance, so its data were excluded from the statistical analysis. At the low target inoculum level, 22 samples were positive by the BAM/AOAC method and 29 by the conductance method. At the high target inoculum level, 31 samples were positive by the BAM/AOAC method and 23 by the conductance method (Table 9). The sensitivity rates for conductance were 36.3 and 28.8% for the low and high target inoculum levels, respectively. For BAM/AOAC, the sensitivity rates were 27.5 and 38.8% for the low and high target inoculum levels, respectively. The specificity rate at the low target inoculum level was 100% for both methods. Statistical analysis of the data (ANOVA) indicated that there was no significant difference ($P>0.05$) between the 2 methods.

Prawns

Thirteen laboratories tested samples of prawns. The Salmonella MPNs at the time of analysis were <0.01 cells/g for both the low and high target inoculum levels (Table 1).

In a precollaborative study with prawns, the desired target inoculum levels were achieved (unpublished data). However, the prawns used in the collaborative trial were of exceptional freshness and may have shown antimicrobial activity toward the inocula, resulting in low numbers of positive samples. Trial data for prawns are shown in Table 5. Three positive samples were recorded at the low target inoculum level, 1 by the BAM/AOAC method and 2 by the conductance method. At the high target inoculum level, 17 samples were positive by BAM/AOAC and 15 by conductance (Table 9). The sensitivity rates for the conductance method were 3.1 and 23.1% for the low and high target inoculum levels, respectively, and 1.5 and 26.2%, respectively, for the low and high target inoculum levels for BAM/AOAC. Specificity rates were 100% for both methods. Statistical analysis of the data (ANOVA) indicated that there was no significant difference ($P>0.05$) between the 2 methods.

Nonfat Dried Milk

Seventeen laboratories tested samples of NFDM. The MPNs at the time of analysis were <0.03 and 1.3 cells/g for the low and high target inoculum levels, respectively (Table 1).

Trial data for NFDM are presented in Table 6. At the low target inoculum level, 23 samples were positive by the BAM/AOAC method and 25 by the conductance method. At the high target inoculum level, 79 samples were positive by BAM/AOAC and 81 by conductance (Table 9). Sensitivity rates for the conductance method were 29.4 and 95.3% for the low and high target inoculum levels, respectively. For BAM/AOAC, the sensitivity rates were 27.1 and 92.9% for the low and high target inoculum levels, respectively. Specificity

Table 7. Detection of *Salmonella* in liquid egg by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	+	–	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	+	+	+	–	+	–
3	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	+	+	+	+	+	+	+	+
7	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	–	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
9	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
10	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
11	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	+	–	+	–
13	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.

rates were 100% for both methods. Statistical analysis of the data (ANOVA) indicated that there was no significant difference (P>0.05) between the 2 methods.

Liquid Egg

Thirteen laboratories tested samples of liquid egg. The MPNs at the time of analysis were 0.09 cells/g for both low and high target inoculum levels (Table 1). Trial data for liquid egg are presented in Table 7. At the low target inoculum level, 57 samples were positive by the BAM/AOAC method and 60 by the conductance method. At the high target inoculum level, 62 samples were positive by BAM/AOAC and 61 by conductance (Table 9). Sensitivity rates for the conductance method were 92.3 and 93.9% for the low and high target inoculum levels, respectively. For BAM/AOAC, the sensitivity rates were 87.7 and 95.4% for the low and high target inoculum levels, respectively. Specificity rates were 100% for both methods. Statistical analysis of the data (ANOVA) indicated that there was no significant difference (P>0.05) between the 2 methods.

Minced Beef

Thirteen laboratories tested samples of minced beef. The MPNs at the time of analysis were 2.3 and 120 cells/g for low and high target inoculum levels, respectively (Table 2). The beef used in the trial had a high fat content, and this may have protected the salmonellas from die-off during preparation and shipment of the samples, resulting in higher than expected levels of Salmonella. Trial data for minced beef are presented in Table 8. Laboratories 7, 8, 9, and 13 detected *Salmonella* in uninoculated control samples, so their data were excluded from the statistical analysis. The BAM/AOAC and conductance methods both gave the same isolation rates at both inoculum levels (Table 9), so there was perfect agreement between them. Sensitivity rates and specificity rates were 100% for both methods. Statistical analysis of the data (ANOVA) indicated that there was no significant difference (P>0.05) between the 2 methods.

Discussion

Seventeen laboratories participated in all or part of the study; the 4 U.S. laboratories did not analyze the perishable commodities, prawns, liquid egg, and minced beef, because of transportation and delivery problems. A total of 1245 samples were analyzed by both procedures. Of these, 496 were confirmed positive by the BAM/AOAC method and 503 were positive by the conductance method (confirmed by BAM/AOAC). The agreement, specificity, and sensitivity rates are similar for each product. Statistical analysis of the data (ANOVA) confirm that the 2 test methods gave the same response rates and are, therefore, equivalent.

In previous AOAC collaborative studies, calculation of false-negative rates were straightforward because both the new and the reference methods were performed on the same test portion. However, in the present study, 2 separate test portions were tested because the BAM/AOAC and conductance methods each required different pre-enrichment broths. Thus, because of the low numbers of Salmonella present in some products, there is the distinct possibility that salmonellas were present in one test portion and not the other. Therefore, meaningful false-negative rates can not be determined with this kind of study.

The *Salmonella* MPN values given in Table 1 indicate that for some products there was no apparent difference between the 2 target inoculum levels. Because separate *Salmonella* preparations and dilutions were used for each target level and

Table 8. Detection of *Salmonella* in minced beef by BAM/AOAC and conductance methods

	BAM/AOAC[a]															Conductance[b]													
	Sample No.[c]																												
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7[d]	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+
8[d]	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
9[d,e]	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
10	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
11	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
12	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
13[e]	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+

[a] Samples confirmed positive by BAM/AOAC.
[b] Samples confirmed positive by conductance.
[c] Samples 1–5 were uninoculated controls; samples 6–10 were inoculated at a low level; samples 11–15 were inoculated at a high level.
[d] Positive result on negative sample by conductance. Data excluded from statistical analysis.
[e] Positive result on negative sample by BAM/AOAC. Data excluded from statistical analysis.

product, there was no direct numerical relationship between the numbers of *Salmonella* inoculated at each target level. However, the trial data showed that for all products except minced beef there were more *Salmonella*-positive samples at the higher target level than at the lower target level, indicating that different inoculum levels were achieved in these products.

A Malthus system comprises 1 or 2 analyzers and an IBM compatible PC, giving a maximum capacity of 240 tests per system. Data collection is automatic so that the instrument can be operated while unattended. Data processing is performed by the PC, and results can be displayed as required with automatic highlighting of samples that fail the test. All sample data may be stored on disc for future reference, if required. The automated conductance method for the detection of *Salmonella* is based on 2 selective conductance media. *Salmonella* Medium 1 (3) may fail to detect dulcitol negative salmonellas, but because the conductance signal is mainly derived from the reduction of trimethylamine-*N*-oxide to trimethylamine, this may not be of importance. *Salmonella* Medium 2 (4) relies on the decarboxylation of lysine for the conductance signal. This is such a general property of salmonellas that it is likely to be present even in dulcitol-negative strains. There are no reports in the literature of *Salmonella* from foods negative in both dulcitol fermentation and lysine decarboxylase. Separate pre-enrichment steps were proposed for each medium, but Smith et al. (6) performed a detailed comparison of the pre-enrichment media and concluded that the broth used here was satisfactory for both selective enrichment steps. This is expected; reduced oxygen conditions are sufficient to induce trimethylamine-*N*-oxide reductase, and glucose induces most enzymes and permeases for carbohydrate metabolism.

Recommendation

From the data presented, the automated conductance assay for the detection of *Salmonella* spp. in foods has been shown

Table 9. Detection of *Salmonella* in foods by BAM/AOAC and conductance methods

			Samples positive	
Food type	Level[a]	Samples analyzed	BAM/AOAC	Conductance
Coconut	O	85	1	1
	L	75	54	54
	H	75	60	63
Fish meal	O	85	0	1
	L	80	22	29
	H	80	31	23
Prawns	O	65	0	0
	L	65	1	2
	H	65	17	15
Nonfat dried milk	O	85	0	0
	L	85	23	25
	H	85	79	81
Liquid egg	O	65	0	0
	L	65	51	60
	H	65	62	61
Minced beef	O	65	2	3
	L	45	45	45
	H	45	45	45

[a] O = Negative control, L = low target level, H = high target level.

to be equivalent to the AOAC official BAM/AOAC method. The Associate Referee recommends that the method be adopted by AOAC as first action.

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MICROBIOLOGICAL METHODS

Evaluation of Method for Enumeration of *Brochothrix thermosphacta* in Foods

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A method for enumeration of *Brochothrix thermosphacta* in foods was evaluated in a collaborative study. Freeze-dried mixtures of bacteria were used as simulated food samples. Fifteen laboratories analyzed 2 negative control samples and 4 blind duplicate samples containing different levels of *B. thermosphacta*. The freeze-dried samples were reconstituted and spread on the surface of streptomycin sulphate–thallous acetate–actidione agar and incubated 2–3 days at 20–25°C. The method shows good repeatability (0.13) and reproducibility (0.21). Reproducibility might be improved by specifying a more exact incubation temperature. The number of false-positive results was rather high, but could be diminished if a catalase test was included and the oxidase test performed by using commercial strips instead of by allowing a solution of an oxidase reagent (tetramethyl-*p*-phenylenediamine dihydrochloride) to flow over the agar surface.

Brochothrix thermosphacta is often found in meat and fish products. Because it produces several nasty-smelling products and may grow at relatively low temperatures, it can influence shelf life of several food products (1).

Streptomycin sulphate–thallous acetate–actidione agar (STAA) was described by Gardner (2) for the isolation and enumeration of *B. thermosphacta* in foods. The selectivity is built on a relatively high concentration of streptomycin sulphate, which inhibits many Gram-negative and some Gram-positive bacteria (3). Thallous acetate is included at a much lower concentration, mainly to inhibit yeasts and, to some extent, bacteria. Actidione is added to inhibit yeasts that can grow in the presence of thallous acetate. Some oxidase-positive, Gram-negative bacteria are able to grow on STAA, but they can be differentiated from *B. thermosphacta*, which is oxidase-negative.

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In the collaborative study presented here, the STAA medium was evaluated for its efficiency to enumerate *B. thermosphacta* in foods. Suspected colonies were confirmed by allowing a solution of an oxidase reagent to flow over the STAA plates.

Collaborative Study

Fifteen laboratories took part in the study. Each collaborator received a complete set of instructions, data sheets, STAA base medium and vials containing antibiotic supplements, and a set of simulated food samples in the shape of glass vials containing freeze-dried mixtures of bacteria (Table 1). Four samples were sent as blind duplicates containing different levels of *B. thermosphacta*, and 2 samples were negative controls. The contents of the vials were reconstituted in peptone water before dilution and inoculation on the ready-to-use medium.

Test Samples

Natural bacterial flora were extracted from 10 g food (fish, heat treated meat, and raw meat, respectively, Table 1) by homogenization in 90 mL peptone water for 60 s using a Stomacher. After filtration through a thin cloth, liquid was centrifuged 10 min at 3000 rpm. The bacterial pellet was harvested and resuspended in brain-heart infusion broth (BHI)–glycerol (5 + 1.25 mL) and stored at −70°C before freeze-drying.

Strains of *B. thermosphacta* were inoculated into BHI and incubated 20–24 h at 23°C. The concentration of *B. thermosphacta* was then ca 1×10^8 cfu/mL. After dilution, suitable amounts of the *Brochothrix* culture were mixed with the natural bacterial flora in inositol serum broth (4).

Portions of 0.5 mL were distributed in glass vials and frozen at −70°C. Material was freeze-dried ca 18 h using an Edwards freeze-dryer, Modulyo EF4. Vials were sealed with rubber bungs under vacuum and stored in the dark at room temperature.

Test samples were coded and shipped to the collaborators by mail. Each collaborator received 10 samples. Eight samples (4 blind duplicates, Samples A–D) contained *B. thermosphacta* mixed with natural bacterial flora from meat or fish (Table 1). In Samples A and B, *B. thermosphacta* was present as a part of

Table 1. Test samples, bacterial background flora, and test organisms added

Sample	Natural flora	Test organisms (strain)
A:1, A:2	Raw meat	—
B:1, B:2	Raw fish	—
C:1, C:2	Raw meat	*Brochothrix thermosphacta* (SLV-220)
D:1, D:2	Heat-treated meat	*Brochothrix thermosphacta* (SLV-220)
E	—	*Enterococcus faecium* (SLV-78)
		Escherichia coli (SLV-289)
		Lactobacillus sp.
F	—	*Enterococcus faecium* (SLV-78)
		Escherichia coli (SLV-289)
		Flavobacterium sp.

the natural flora, whereas in Samples C and D, the natural bacterial flora were mixed with dilutions of an outgrown culture of *B. thermosphacta*. Two negative control samples (E and F) contained *Enterococcus faecium*, *Escherichia coli*, and *Lactobacillus* sp. and *Flavobacterium* sp., respectively (Table 1). The latter 2 strains were isolated from meat products using the STAA medium.

Sample Preparation

Test sample vials were rinsed with four 1 mL portions of peptone water. Peptone water (6 mL) was added, and the mixture was blended carefully. The total volume of the samples was 10 mL.

Method

Using a Stomacher, homogenize 5–10 g food 2 min in 45–90 mL peptone water (1 + 9). Make dilutions in 1:10 steps and inoculate 0.1 mL amounts on prepared STAA agar plates. Spread sample over agar surface using glass hockey stick. Incubate plates upside-down in plastic bags 2–3 days at 20–25°C.

Brochothrix thermosphacta grows as distinct, circular, glossy, and whitish colonies with sharp edges, 1–2 mm in diameter. Count plates with 20–200 colonies.

Let fresh oxidase reagent (1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride) flow over the STAA plates. Within 15–20 s, oxidase-positive colonies become blue, whereas oxidase-negative *Brochothrix* colonies remain uncolored. Occasionally, further biochemical tests are needed to confirm typical or atypical *B. thermosphacta*.

Table 2. Bacterial background flora in test samples (log_{10} cfu/mL)

Sample	Aerobic plate count	*Enterobacteriaceae*
A	2.6	< 1
B	4.5	1.6
C	4.0	1.7
D	3.9	< 1
E	5.0	4.1
F	5.2	4.7

For preparation of STAA, suspend 20.0 g peptone, 2.0 g yeast extract, 15.0 g glycerol, 1.0 g K_2HPO_4, 1.0 g Mg_2SO_4, and 13.0 g agar in 1 L distilled water. Dissolve ingredients by heating and sterilize 15 min at 121°C. Before use, add to each 200 mL melted medium cooled to 50°C, 1 mL 10% dihydrostreptomycin (final concentration in the medium: 500 mg/L), 1 mL 1% thallous acetate (final concentration: 50 mg/L), and 1 mL 1% actidione (final concentration: 50 mg/L). Ready-to-use plates can be stored 1–2 weeks at 5°C.

In the collaborative study, reconstituted samples were examined without the homogenization step, and dry base medium and vials containing the antibiotic supplements (Oxoid) were supplied by the organizing laboratory.

Analysis of Data

Before statistical calculations, colony counts were converted to log_{10}. Outlying results were identified using Cochran test and Grubbs test (5). Precision estimates for each sample were calculated using a 2-way ANOVA (6).

Test Sample Homogeneity

Homogeneity of test samples was checked at the organizing laboratory by analyzing 5 vials from each sample. Each vial was reconstituted as described above, and 2 parallel dilution series were prepared. STAA plates were inoculated with suitable dilutions and incubated 48 h at 23°C. An *F*-test was used to investigate whether or not the variance in cfu of *B. thermosphacta* in parallel determinations on the same test sample (method error) differed from the variance in cfu of *B. thermosphacta* in parallel determinations on different test samples (method error + inhomogeneity). If the test samples are homogeneous, these 2 variances should be equal.

Results and Discussion

Homogeneity of Samples

The variations among different test samples were very low. Standard deviations for determinations on 5 test samples ranged from 0.05 to 0.07 log units. None of the Samples A–D had a variance for parallel determinations of cfu of *B. thermo-*

Table 3. Collaborative study results for *Brochothrix thermosphacta* in simulated food samples (log_{10} cfu/mL)

	Sample[a]										Incubation	
Coll.	A:1	A:2	B:1	B:2	C:1	C:2	D:1	D:2	E	F	Temp, °C	Time, h
1	2.91	2.71	3.45	3.52	6.18	5.94	4.42	4.64	< 2	< 2	23	72
2	2.30	2.41	3.13	3.22	5.50	5.55	4.27	4.18	< 2	< 2	20	72
3	2.45	2.61	3.93	3.94	5.90	6.00	4.63	4.70	3.51	3.53	24	48
4	2.18[b]	2.78[b]	3.00[b]	2.15[b]	5.52	5.89	4.04	4.32	< 1	< 1	21	48
5	2.36	2.28	3.34	3.45	5.58	5.34	4.32	4.04	3.00	< 1	21	72
6	2.20	2.08	3.52	3.43	5.70	5.73	4.11	4.11	3.23	< 1	21	72
7	2.49	2.48	3.30	3.46	5.83	5.80	4.62	4.67	< 1	< 1	25	72
8	2.15	2.54	3.18	3.64	6.04	5.89	3.89[b]	4.76[b]	3.69	3.89	25	72
9	2.32	2.40	3.18	3.08	5.79	5.79	4.40	4.48	3.48	< 1	20	72
10	2.45	2.49	3.70	3.70	5.77	5.92	4.54	4.45	< 1	< 1	22	72
11	2.59	2.43	3.82	3.73	5.76	5.70	4.57	4.54	2.65	< 1	25	48
12	2.50	2.34	3.04	3.17	5.95	5.60	4.26	4.03	< 1	< 1	22	72
13	2.50	2.57	3.41	3.01	5.87	5.83	4.38	4.30	< 1	< 1	21	72
14	2.62	2.20	3.36	3.30	5.63	5.71	4.36	4.38	< 1	< 1	22–23	48

[a] See Table 1.
[b] Data excluded from statistical evaluation.

sphacta in the same test sample significantly different ($p > 0.05$) from the variance between different test samples. Therefore, samples could be regarded as homogeneous.

Background Flora

The organizing laboratory determined the cfu numbers of aerobic bacteria (plate count agar, 30°C, 72 h) and *Enterobacteriaceae* (violet red bile agar with glucose, 37°C, 24 h) in the test samples at the time of the collaborative study (Table 2).

Elimination of Outliers

All results, incubation temperature, and incubation time for each collaborator are shown in Table 3. One collaborator found growth on only 2 of the agar inoculated plates; therefore, this collaborator was excluded from the statistical analyses.

Collaborator 4 had a large difference between duplicate determinations on Sample B, and Collaborator 8 had a large difference on Sample D. Both differences were significant (Cochran test, $p < 0.01$). Collaborator 4 also had a large difference on duplicate determinations on Sample A, although this was not significant. However, because Collaborator 4 may have confused a Sample A with a Sample B (samples were coded 369 and 396, respectively), results on both samples were excluded from the statistical analyses.

Precision Estimates

Precision estimates for each test sample are reported in Table 4. The repeatability standard deviation ranged from 0.11 to 0.14. The range for the reproducibility standard deviation is somewhat higher, 0.18–0.27, mainly because of a high value for Sample B. There was no correlation between the level of *B. thermosphacta* in the samples and repeatability or reproducibility. Therefore, the overall repeatability and reproducibility were calculated to be 0.13 and 0.21, respectively.

Effect of Time and Temperature

The method states that STAA should be incubated 2–3 days at 20–25°C. As a result, many different incubation times and temperatures were used. Four collaborators read their plates after 2 days of incubation; 10 read their plates after 3 days. Also, almost all incubation temperatures between 20 and 25°C were used (Table 3).

The effect of incubation conditions on the results was studied using regression analyses. In Samples B, C, and D, there

Table 4. Means and precision estimates by sample for *Brochothrix thermosphacta* in simulated food samples

	Sample			
	A	B	C	D
Mean (log_{10} cfu/mL)	2.44	3.42	5.78	4.38
No. of duplicate determinations	13	13	14	13
Reproducibility standard deviation (S_R)	0.18	0.27	0.19	0.21
Repeatability standard deviation (S_r)	0.14	0.13	0.13	0.11

was a significant ($p < 0.05$) positive correlation between incubation temperature and the number of cfu. The correlation was also positive for Sample A, but not significant. Some of the between laboratory variation can be explained by differences in incubation temperature. If each result is adjusted using the overall temperature coefficient (0.06) to give values for a common temperature, the overall reproducibility standard deviation becomes 0.19, decreased from an overall reproducibility of 0.21.

The effect of incubation time was significant only for Sample B, where the log mean cfu values for 2 and 3 days of incubation were 3.68 and 3.35, respectively. In the other samples, differences were below 0.1 log units.

False-Positive Results

The relatively high number of false-positive results was the main problem in the study. In Sample E, 6 false-positive results were obtained; in Sample F, 2 were obtained. According to the method, confirmation should be done by allowing an oxidase reagent to flow over the plates. At times, the blueing of oxidase-positive colonies is difficult to discern. Some laboratories also stated that the colonies loosened from the agar surface. Furthermore, the oxidase reagent has an extremely short shelf life. Both of the false-positive results from Sample F, which contained a *Flavobacterium* sp. that is oxidase-positive and able to grow on STAA, were probably misidentifications because of an incorrect oxidase test.

However, most of the false-positive results were reported for Sample E, which contained *Lactobacillus* sp. This organism is oxidase-negative and was, therefore, misidentified as a *B. thermosphacta*. A catalase test would have avoided this because *B. thermosphacta* is catalase-positive. Lactobacilli are catalase-negative.

Most reports on the analyses of *B. thermosphacta* in foods show that STAA is so selective that there are no problems with competing organisms, apart from some *Pseudomonas* (7). However, according to Gardner (2), there can be problems with STAA when analyzing soil or feces. The results from this collaborative study indicate that there can be problems in confirming suspect colonies of *B. thermosphacta* on STAA. The oxidase test would probably be easier and more reliable if commercial oxidase strips were used instead of the liquid reagent. Moreover, it might be wise to include a catalase test to exclude possible strains of lactobacilli.

Colony Size

Several laboratories point out that colonies of *B. thermosphacta* on STAA were bigger than those described in the proposed method (up to 4 mm in diameter, as opposed to 1–2 mm).

Recommendations

The following modifications should be incorporated into the method. (*1*) The selective medium should be incubated 48 ± 3 h at 25 ± 1°C. (*2*) In addition to the oxidase test, a catalase test is recommended for confirmation when lactobacilli are suspected. (*3*) Studies have shown that actidione does not improve the selectivity of STAA (Sven Qvist, personal communication); therefore, actidione should be omitted from the medium.

With these modifications, the method has been adopted as an official NMKL method.

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MYCOTOXINS

Comparison of Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay with Thin-Layer Chromatography and Liquid Chromatography for Aflatoxin B_1 Determination in Naturally Contaminated Corn and Mixed Feed

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A 1-step enzyme-linked immunosorbent assay (ELISA) method, using a highly sensitive and specific monoclonal antibody to aflatoxin B_1 (AFB_1), was evaluated by comparison with other methods, including liquid chromatography (LC) and thin-layer chromatography. The detection limit of the ELISA was as low as 100 pg/assay. AFB_1 contents of naturally contaminated corn samples were determined by the 3 methods. The relationships among the methods were investigated, and good correlations were observed. Mixed feeds were also subjected to AFB_1 determination by the 3 methods. For our ELISA system, 3 types of sample preparations were tested. For analysis of mixed feed by ELISA, samples must first be purified by column chromatography. When the relationship between LC and ELISA was also investigated, results were found to have a good correlation coefficient.

Aflatoxin B_1 (AFB_1), the well-known toxic metabolite of fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (1), exhibits extensive toxicity and carcinogenicity (2, 3). Because this compound was found in agricultural commodities such as peanuts, corn, and animal feedstuff as a natural contaminant, there has been increased interest in the development of rapid, sensitive techniques for the detection of AFB_1 in food, feeds, and biological fluids. Thin-layer chromatography (TLC) was first developed and adopted as a standard detection method for determining AFB_1 for regulatory purposes about 20 years ago (4–7). Currently, liquid chromatography (LC), because of its high sensitivity and resolution, is often used instead of TLC (8–11). However, time-consuming chemical cleanup steps after extraction of the samples are essential in both TLC and LC methods. In spite of the high accuracy of LC, this cleanup process is a disadvantage for the examination of many samples. Therefore, an easy, convenient, and rapid detection method is needed. An immunoassay using monoclonal antibody against AFB_1 is preferable for this purpose.

A radioimmunoassay using polyclonal antibody was developed by Lau et al. in 1981 (12). In their method, the sensitivity of AFB_1 was in the range of 30–50 pg/assay. Monoclonal antibodies against AFB_1 were used in the radioimmunoassay of Groopman (13), by which AFB_1 could be detected down to 1 ng/assay. However, radioimmunoassay is not convenient in many laboratories because of a number of limitations.

An enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody was developed by which a sensitivity of 10 pg/assay was attained (1, 14–16). A monoclonal antibody is superior to polyclonals because of its specificity and reproducibility. We have already reported the production of monoclonal antibodies specific for AFB_1 and establishment of ELISA using the antibody (17, 18). Compared to other ELISA systems (19–21), our developed ELISA showed much higher sensitivity and specificity. In this report, the determination of naturally contaminated corn samples and mixed feed is discussed, and results obtained by TLC, LC, and ELISA methods are compared.

Experimental

Reagents and Chemicals

AFB_1 and AFB_1-oxime bovine serum albumin (AFB_1-BSA) were purchased from Sigma Chemical Co. Ltd, St. Louis, MO 63178. The mole ratio of AFB_1 to BSA was claimed to be 10–25. Anti-AFB_1 monoclonal antibody (IgG_1) was purified from ascites fluid of BALB/c mouse by using DEAE-cellulose column chromatography, salting out by ammonium sulfate, and dialysis. Horseradish peroxidase (HRP)-labeled antibody was prepared according to previous reports (17, 18).

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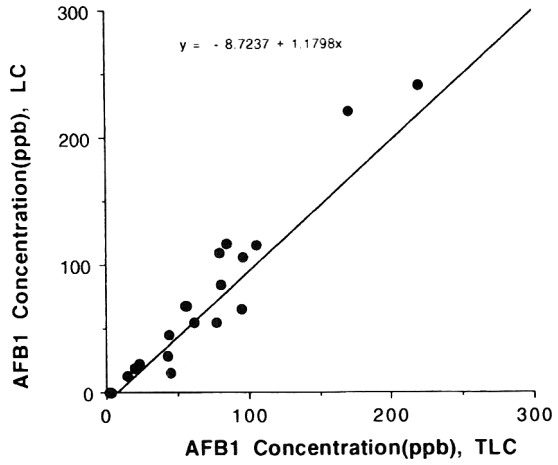

Figure 1. Correlation of LC and TLC data for determination of AFB_1 in naturally contaminated corn samples. Linear regression equation $y = 1.18x - 8.7$ was obtained with correlation coefficient of 0.96. x and y represent AFB_1 concentration (ppb) as determined independently by LC and TLC.

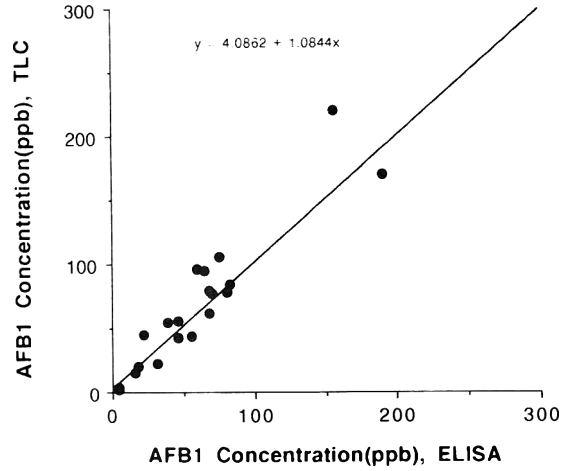

Figure 2. Correlation of TLC and ELISA data for determination of AFB_1 in naturally contaminated corn samples. Linear regression equation $y = 1.08x + 4.1$ was obtained with correlation coefficient of 0.93. x and y represent AFB_1 concentration (ppb) as determined independently by TLC and ELISA.

Enzyme reaction was performed in citrate buffer using *o*-phenylenediamine and hydrogen peroxide as reactants for 3 min at room temperature. The enzyme reaction was stopped by adding 100 μL 3N H_2SO_4.

Apparatus

(a) *Microplate reader.*—Model MTP-32 (Corona Co. Ltd), used as a colorimeter for enzyme immunoassay.

(b) *Fluorescence spectrometer.*—Model F-1050 (Hitachi Co. Ltd, Tokyo, Japan).

(c) *Densitometer.*—Model CS-9200 (Shimazu Corp., Tokyo, Japan).

Sampling and Sample Preparation

Grain samples were ground in an electric grinder, and AFB_1 was determined by the methods described below.

Quantitative Analysis by TLC, LC, and ELISA

TLC.—The American Oil Chemists Society method (22) was used for the TLC analysis. After the sample spots were developed on a thin-layer plate, the concentration of the band was read using a densitometer.

LC.—The analysis was performed according to the method developed at Tokyo Metropolitan Laboratory of Public Health and used for enforcement purposes by the Japanese Ministry of Health and Welfare (23).

ELISA.—The ELISA method used for the analyses was described in the previous report (17). Because certain conditions are different, however, the steps in this method are given in detail as follows: (*1*) A 100 μL portion of AFB_1–BSA conjugate (5 μg/mL) was added to the wells of an immunoplate at 4°C and then blocked 30 min with BSA at room temperature. (This plate-coating process can be performed in advance; the coated plate can be kept at 4°C for a couple of months.) (*2*) After the plate was washed, 50 μL sample solution and the same quantity of enzyme-labeled antibody (ratio of enzyme to antibody is ca 1) were added onto the well of the immunoplate at the same time, followed by incubation 10 min at room temperature. The enzyme-labeled antibody competitively reacted with the AFB_1 in the sample and the AFB_1 fixed to the well of the immunoplate. (*3*) When the well was washed out, only the enzyme-labeled antibody, which was bound with the fixed antigen on the well surface, remained. (*4*) The extent of color development of the solution, using an enzyme reaction, is proportional to the amount of the enzyme-labeled antibody bound to the fixed antigen. By measuring the absorbance of each well after the reaction is stopped, the concentration of AFB_1 is determined. The color produced is inversely proportional to the concentration of AFB_1 in the sample.

Sample preparation for the analysis is already incorporated in both TLC and LC, as mentioned above. However, ELISA systems are now being developed so that the following 3 kinds of preparation for a sample were attempted: (*a*) A sample was homogenized 5 min with 25 mL 55% methanol–water, using an electric mixer for the extraction (17). Each extract was submitted to the procedure. (*b*) The extract obtained was diluted with pure water up to 10% methanol content and assayed. (*c*) A sample was extracted with chloroform and cleaned up by column chromatography according to the method described previously (23). Then, the final, dried sample was dissolved in 55% methanol solution and analyzed by ELISA.

Cross Reactivity of the Monoclonal Antibody

The cross reactivity of the monoclonal antibody used in the ELISA system was investigated (18). If the reactivity of the monoclonal antibody to AFB_1 was defined as unity, the values of other analog chemicals were as follows: $AFB_2 = 0.014$, $AFB_{2a} = 0.008$, $AFG_1 = 0.012$, $AFG_2 = 0.024$, $AFM_1 = 0.017$,

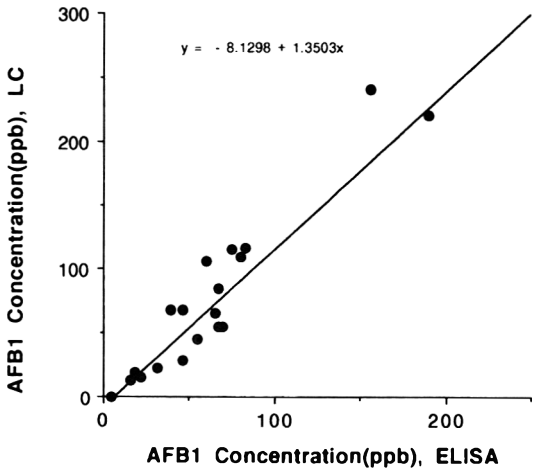

Figure 3. Correlation of LC and ELISA data for determination of AFB_1 in naturally contaminated corn samples. Linear regression equation $y = 1.35x - 8.1$ was obtained with correlation coefficient of 0.95. x and y represent AFB_1 concentration (ppb) as determined independently by LC and ELISA.

$AFQ_1 = 0.072$, $AFP_1 = 0.008$, $AFCol_1 = 0.137$, and $AFCol_2 = 1.25$.

These values concerning AFB_2 and AFG_1 are 10-fold higher than those reported by Candlish et al. (20) and Pestka and Hart (21).

Results

AFB_1 in naturally contaminated corn and mixed feed samples was determined by TLC, LC, and ELISA to investigate the correlation among the 3 analytical methods.

Corn Samples.—Twenty samples of contaminated corn were analyzed by TLC, LC, and ELISA. The values obtained by the 3 techniques were plotted against each other, and the correlation was statistically estimated. The results are shown in Figures 1–3. Figure 1 shows the correlation between TLC and LC; a fairly good relationship was observed, for which the coefficient factor is 0.96. On the basis of examination of the data, about 10 ng/mL may be regarded as a detection limit for TLC and about 1 ng/mL for LC. Taking into account the inhomogeneity of contaminated samples in a lot and the difference between analytical methods, the coefficient factor of 0.96 is considered a good value.

Figure 2 shows the correlation between the TLC and ELISA results. In ELISA, samples were prepared according to procedure (*a*) above. The correlation coefficient is a little smaller (0.93) than in the case of TLC and LC. Because TLC and ELISA are completely different analytical techniques, the coefficient factor of 0.93 must be regarded as satisfactory.

Figure 3 shows the correlation between the LC and ELISA results; the plot gives a good correlation with a coefficient value of 0.96. These results indicate good correlation among the 3 analytical methods.

Table 1. Results of LC, ELISA(A), and ELISA(B) for contaminated corn samples

Sample	AFB_1, ppb		
	LC	ELISA(A)[a]	ELISA(B)[a]
1	ND	5.0	1.5
2	ND	3.5	ND
3	ND	3.5	ND
4	ND	4.5	ND
5	ND	3.3	ND
6	ND	3.5	2.1
7	ND	3.6	1.8
8	ND	2.1	1.5
9	ND	4.0	2.2
10	0.9	5.0	1.5
11	1.3	5.5	1.7
12	1.6	4.3	3.3
13	6.1	8.0	4.4
14	8.2	12.5	14.4
15	9.0	7.5	6.9
16	10.5	9.6	9.6
17	16.0	14.8	14.2
Correlation coefficient (r) with LC		0.94	0.92

[a] ELISA(A) and ELISA(B) represent preparation methods (a) and (b), respectively.

An interesting phenomenon was observed when the calibration curve for AFB_1 was prepared: the lower the methanol concentration, the higher the sensitivity for AFB_1 (data not shown). Moreover, the nonspecific reaction was reduced when the extraction solution (55% methanol solution) was diluted with pure water before ELISA. A 5.5-fold dilution considerably suppressed the nonspecific reaction, which improved the sensitivity.

The nonspecific reaction plays an important role in the ELISA system with respect to the limitation for the measurement at low concentrations (below 10 ng AFB_1/mL). Consequently, corn samples contaminated below 20 ppb AFB_1 were tested for this purpose. First, the samples were extracted with the 55% methanol solution, preparation (*a*), and then diluted to 10% methanol with pure water, preparation (*b*). Both samples were then analyzed by ELISA. Next, the same samples were extracted with chloroform for comparison with results obtained by LC accompanied by the cleanup. For this experiment, 17 slightly contaminated samples (0–20 ppb) were examined; the results are summarized in Table 1.

Figure 4 represents the relationship between LC vs preparation (*a*) and LC vs preparation (*b*). In the case of preparation (*a*) (solid line), the samples over 1 ppb gave LC results in good agreement with ELISA. However, the other points near zero ppb on LC are clustered at around several ppb in ELISA, and the line crosses the abscissa around 3 ppb. This may be ascribed to the occurrence of nonspecific reactions at the low

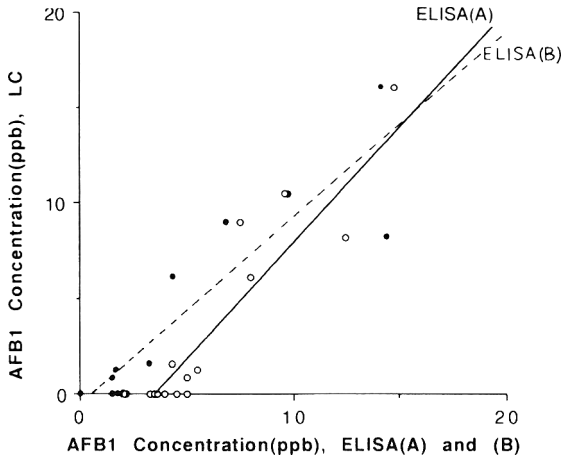

Figure 4. Correlation of LC and ELISA data for determination of AFB_1 in slightly contaminated corn samples. Preparations A and B were applied to the ELISA analysis. Linear regression equation $y = 1.31x - 4.6$ was obtained with correlation coefficient of 0.94 for LC vs preparation A and $y = 0.96x - 0.5$ with 0.92 for LC vs preparation B. x and y represent AFB_1 concentration (ppb) as determined independently by LC and ELISA.

concentration of AFB_1 in ELISA. Therefore, the detection limit for preparation (*a*) seems to be about 3 ppb.

On the other hand, in the case of preparation (*b*), the correlation between LC and ELISA was considerably improved, as seen in the same figure (dotted line). In this case, the points below 3 ppb AFB_1 were linear for both analytical methods. The line crosses the abscissa at near zero ppb and the slope is 0.96. Consequently, the detection limit for preparation (*b*) is ca 1 ppb. From these results, preparation (*b*) is preferable to preparation (*a*) for the low concentration range.

Table 2. Comparison between LC and ELISA for AFB_1 in naturally contaminated mixed feeds

	AFB_1, ppb			
Sample	LC	ELISA(A)[a]	ELISA(B)[a]	ELISA(C)[a]
1	7.3	14.0	10.3	4.3
2	2.8	ND	4.5	2.1
3	5.0	ND	4.7	3.6
4	3.0	10.0	6.1	4.8
5	2.9	10.0	10.3	4.3
6	9.3	10.0	10.7	8.5
7	1.5	ND	9.5	1.2
8	1.4	ND	5.8	1.5
9	1.7	ND	8.5	1.7
10	1.7	ND	10.2	2.0
11	2.2	4.6	10.7	2.2
12	ND	5.8	5.0	0.9
13	0.8	5.1	4.4	1.4
14	0.5	ND	ND	0.8
Correlation coefficient (r) with LC		0.57	0.46	0.90

[a] ELISA(A), ELISA(B), and ELISA(C) represent preparation methods (a), (b), and (c), respectively.

Mixed Feed.—The results are summarized in Table 2. They indicate that preparations (*a*) and (*b*) are not suitable for the AFB_1 assay of mixed feeds, judging from the poor correlation coefficients, namely, 0.57 and 0.46, respectively. Because they are composed of many kinds of cereals, etc., some ingredients seem to interface with the immunochemical reaction. To remove the unknown substances that influence the immunochemical reaction, further purification of the extract was attempted as described for preparation (*c*). The purified samples were analyzed by ELISA and LC methods. The results are shown in Table 2 and Figure 5, where a good correlation (slope = 1.12, r = 0.90) is observed even at the low concentration range of AFB_1. By using preparation (*c*) for mixed feed samples, the detection limit seems to be ca 1 ppb.

Discussion

TLC was first developed about 20 years ago, and since then it has been widely used as a method to detect aflatoxin. On the other hand, LC is often used instead of TLC because of its high sensitivity and resolution. Accordingly, both methods are in general use worldwide for the determination of aflatoxins. However, these methods require cleanup steps that increase the time required to obtain analytical results. The combination is not suitable for the simultaneous analysis of many samples. To overcome these disadvantages, an ELISA system was recently studied.

The good correlation between TLC and ELISA results was confirmed by Chu et al. (16) and Candlish et al. (20). Nonetheless, few studies on the relationship between ELISA results and those obtained by TLC and LC have been performed. In this discussion, the validity of these measurements is described from the viewpoint of both correlation factor and slope.

Figures 1–3 represent the analytical results for 20 corn samples that were naturally contaminated up to about 250 ppb. In this study, a good correlation was observed between the TLC and LC results, as shown in Figure 1. However, the slope of the line is not 1.0, but 1.18. This means that the measured concentration of AFB_1 is a little higher in the LC method than in the TLC method even though good correlation can be observed. In this analysis, the cleanup was performed independently by TLC and LC, according to the instructions for the method in the experimental section. This small difference between the TLC and LC analytical data resulted in the slope of 1.18.

Good correlations were observed for results obtained by using ELISA with TLC, and TLC with LC. The slope of the line in Figure 2 is 1.08 and the abscissa is about 4. Thus, a good correlation obviously exists between ELISA and TLC results. The concentrations of analytical results obtained by TLC are slightly higher than those by ELISA in spite of the good correlation coefficient of 0.93. Chu et al. (16) obtained a correlation coefficient of 0.91 with a slope of 0.99 between TLC and an

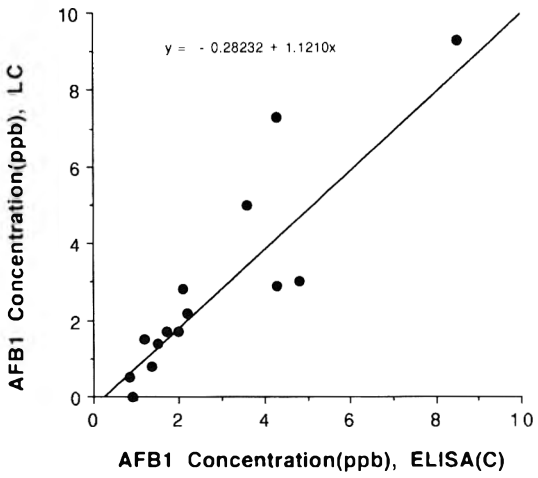

Figure 5. Correlation of LC and ELISA data for determination of AFB_1 in naturally contaminated mixed feeds. Preparation C was used for the ELISA analysis. Linear regression equation $y = 1.12x - 0.3$ was obtained with correlation coefficient of 0.90. x and y represent AFB_1 concentration (ppb) as determined independently by LC and ELISA.

ELISA method in which an AFB_1–POD (peroxidase) conjugate was used for the competitive reaction. Although the conjugate in our experiment is monoclonal antibody–HRP, the results are fairly comparable to each other.

With regard to the correlation between ELISA and LC (Figure 3), the AFB_1 concentration by LC is 1.35-fold higher than that by TLC. This may be because chloroform was used as the extraction solvent for TLC and LC, while 55% aqueous methanol was used as solvent for ELISA. The extraction efficiency of chloroform seems to be slightly higher than that obtained by using 55% aqueous methanol as solvent. The correlation between LC and ELISA results is good, with a correlation coefficient of 0.95. Ram et al. (15) obtained a correlation coefficient of 0.955 with a slope of 1.72 when comparing ELISA and LC. The slope of 1.72 seems high, whereas the correlation coefficient is near 1. The large value of the slope indicates a deviation in the concentration of AFB_1 between the 2 methods. The reason for this deviation is not discussed in the article.

In any event, it must be emphasized that in this study good correlations exist among the TLC, LC, and ELISA methods used. The reason for the good correlation among the 3 methods may be mainly due to the high specificity and reproducibility of the monoclonal antibody. Other factors such as the enzyme-marker, HRP, and optimal experimental conditions may also improve ELISA. Concerning the detection limit of the ELISA using 55% aqueous methanol, 3–4 ng/mL is reasonable, judging from the results obtained.

Throughout these discussions, we emphasize that the ELISA system used in this experiment, as well as TLC and LC, is very reliable for determining the concentration of AFB_1.

Nonspecific reactions are always a big problem in ELISA systems, causing incorrect data. It is a unique phenomenon that about 5-fold dilution with water exerts a huge effect on the elimination of nonspecific reactions from the ELISA system (see Figure 4).

The monoclonal antibody is divided into Fab and Fc portions. In these 2 portions, Fc readily bonds to plastics such as polystyrene and polypropylene because of its hydrophobic property. The Fc portion sometimes causes the nonspecific reaction. Therefore, an antibody is digested into Fab and Fc fragments. To eliminate the nonspecific reaction, only the Fab portion is used in an immunoassay. In this experiment, Fc has not been removed, so that to some extent nonspecific reaction may occur. Organic compounds from the sample can interact with Fc as a result of its hydrophobic properties. This is one possible mechanism for the nonspecific reaction. The dilution of the sample extract with water may reduce this type of nonspecific reaction. In addition, monoclonal antibodies show low reactivity in organic solvents such as methanol, ethanol, etc. Thus, the antigen-antibody reaction is ideally performed in aqueous solution. This may also contribute to the improvement of sensitivity in this ELISA experiment.

TLC and LC methods, which are generally used for the determination of AFB_1, require many time-consuming steps to clean up the sample extract. With regard to the corn samples, the crude sample extract can be applied to the ELISA system described here without further cleanup; all steps, including sample extraction and assay, can be performed in a total of 30 min. In addition, many samples can be assayed at the same time with high accuracy.

For mixed feeds, the crude extract cannot be applied directly to ELISA because of the occurrence of large nonspecific reactions. However, if the same sample preparation procedure used in the LC method is included in ELISA, high sensitivity can be achieved with the mixed feed, as seen in Figure 5. Still another advantage in using ELISA is that many samples can be assayed simultaneously with high accuracy.

The direct ELISA method for AFB_1 described here has high sensitivity and some advantages of both time and economy. In particular, many samples can be assayed in a short time. Therefore, if the samples are chosen in many sections of the lot and every samples is assayed by ELISA, the average and accurate AFB_1 value for the lot can be obtained within a short time; consequently, the AFB_1 contamination of the lot is estimated with high accuracy. The assay system is applicable to the analysis of cereals. The system is very useful for screening foods and feeds.

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MYCOTOXINS

Liquid Chromatographic Determination of Fumonisins B_1, B_2, and B_3 in Foods and Feeds

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Three recently described and toxicologically important *Fusarium* mycotoxins, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), are the major fumonisins produced in cultures of *F. moniliforme*, a fungus that occurs worldwide on corn. Contamination of food and feed with *F. moniliforme* has been associated with a number of diseases in both animals and humans. Aspects of a recently reported liquid chromatographic method for the determination of FB_1 and FB_2 in corn, including initial extraction, extract purification, and stability of derivatives, were investigated and, where necessary, optimized further both to reduce the analysis time and to include the co-determination of FB_3. The method was applied for the determination of FB_3, in a series of U.S. feed samples associated with outbreaks of equine leukoencephalomalacia, which were shown previously to contain both FB_1 and FB_2. Twelve of the 13 feed samples contained FB_3 at levels ranging between 50 and 2650 ng/g, corresponding to 2.2–18% of the total fumonisin concentrations present in the FB_3-positive feed samples. This is the first report of the natural occurrence of FB_3.

The fumonisins (Figure 1), a group of structurally related secondary metabolites, were isolated from corn culture material of *F. moniliforme* strain MRC 826 (1) and their structures elucidated (2). Three of the 6 known fumonisins (1–3), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), are the major fumonisins produced in cultures of *F. moniliforme* (3).

Toxicological investigations to date have resulted in the reproduction of 2 important animal diseases, equine leukoencephalomalacia (LEM) and porcine pulmonary edema (PPE), following the administration of pure FB_1 (4–6). FB_1 has also been shown to be both hepatotoxic and carcinogenic to rats (7). Short-term carcinogenesis studies in a rat liver bioassay have indicated that FB_2 and FB_3 exhibit toxicological and cancer-initiating activities similar to those observed for FB_1 (8).

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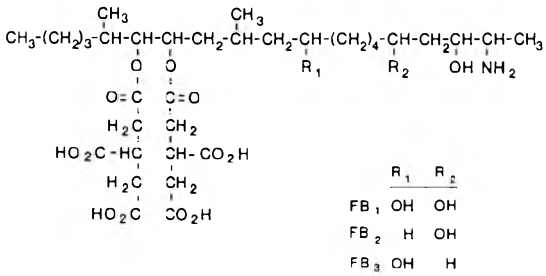
These observations suggest that the fumonisins may pose a threat to human and animal health, especially because *F. moniliforme* and the fumonisins occur worldwide on corn and corn products (9).

The provision of a sensitive, accurate, and reproducible analytical method for the determination of the major fumonisin mycotoxins in foods and feeds is essential for an assessment of potential human and animal exposure to these compounds. The initial thin-layer chromatographic (TLC) method developed during the isolation of the fumonisins (1) was found to be unsuitable for the detection of fumonisin concentrations lower than 500 μg/g (10). Capillary gas chromatographic (GC) procedures have involved the hydrolysis of fumonisin-contaminated sample extracts, followed by esterification of the 1,2,3-propane tricarboxylic acid (10) and/or acylation of the aminopolyol moieties of the fumonisins (10, 11). These procedures have the advantage of being able to combine GC with mass spectrometry (MS) for confirmation purposes (10–12). However, because the GC/MS methods are time-consuming and require expensive instrumentation, they are neither appropriate nor practical for the screening of large numbers of samples.

The fumonisins do not absorb either UV or visible light, nor do they fluoresce. To use conventional spectrophotometric detectors in conjunction with liquid chromatography (LC), either UV-absorbing or fluorescent products must be derived from the fumonisins. Derivatization with maleic anhydride (1), coupled with reversed-phase LC and UV detection, enabled the quantitative determination of FB_1 and FB_2 in culture material of *F. moniliforme*. The sensitivity of this procedure (10 μg/g) was superior to that of the TLC method, but it still lacked the necessary sensitivity for the determination of fumonisins in naturally contaminated foods and feeds (10). The use of several fluorescent derivatives has been investigated, including fluorescamine (10), naphthalene-2,3-dicarboxaldehyde (G. M. Ware, personal communication) and *o*-phthaldialdehyde (OPA) (13). Shephard et al. (13) developed a method that proved to be sensitive, accurate, and reproducible for the simultaneous LC determination of FB_1 and FB_2, as their OPA derivatives, at levels as low as 50 ng/g. This method is currently the subject of an international collaborative study.

The report of the toxicological significance of FB_2 and FB_3 (8) emphasizes the need to quantitatively determine the levels



	R_1	R_2
FB_1	OH	OH
FB_2	H	OH
FB_3	OH	H

Figure 1. Chemical structures of fumonisins B_1, B_2, and B_3.

of these fumonisin mycotoxins, in addition to FB_1. On the basis of comments from participants in the collaborative study of the method of Shephard et al. (13) and on our own observations, this paper describes the alteration of the method to include FB_3. We also address a number of parameters that may influence the performance of the method, including initial fumonisin extraction, sample purification, and fumonisin–OPA derivative stability. The paper reports the application of the method to the determination of FB_3 in a series of feed samples, and describes in detail the optimized method for the co-determination of FB_1, FB_2, and FB_3 in corn-based samples.

Experimental

General reagents and apparatus are listed in the *Method* section. Several factors affecting the performance of the method were investigated separately and are subsequently listed as such.

Optimization of Sample Purification

(a) *Initial extraction.*—A 500 g corn sample, previously shown to be naturally contaminated with FB_1 and FB_2, was prepared by grinding in a laboratory mill to pass a 840 μm sieve and was subsequently well mixed. Three subsamples (25 g each) were extracted with 50 mL methanol–water (3 + 1) for 1, 3, and 5 min, respectively, in a Polytron homogenizer. An additional three 50 g subsamples were extracted with 100 mL of the same extraction solvent blend, for similar periods of time, using a Sorvall Omnimixer. A single 50 g subsample was also extracted with 100 mL extraction solvent for 60 min on a wrist-action shaker. The extracts were examined for the presence of FB_1 and FB_2 according to the method of Shephard et al. (13).

(b) *Application of extracts to strong anion exchange (SAX) cartridges.*—Corn extracts were prepared by using the Sorvall omnimixer as cited in section (a). Aliquots (10 mL) were applied to 4 separate SAX solid-phase extraction cartridges at flow rates of 1, 2.1, 3.1, and 8.2 mL/min. The fumonisins were eluted from the SAX cartridges and determined as previously described (13).

(c) *Elution of fumonisins from SAX cartridges.*—Additional corn extracts were prepared by the Sorvall system as described in section (a), and 10 mL aliquots were applied to 4 separate SAX cartridges at a flow rate of 2 mL/min. The fumonisins were eluted from the cartridges with 14 mL 0.5% acetic acid in methanol at flow rates of 1, 2.5, 4.1, and 6.7 mL/min, and the fumonisin levels were determined as previously described (13). Extracts of a naturally contaminated corn sample containing predominantly FB_1 were similarly prepared, and suitable aliquots were applied to 2 SAX cartridges at a flow rate of 2 mL/min. The fumonisins were eluted from one SAX cartridge with 0.5% acetic acid in methanol and from the second cartridge with 1% acetic acid in methanol, at a flow rate of 0.8 mL/min. For each cartridge, the first 4 mL fraction followed by 5 subsequent 2 mL fractions were collected separately (to give a total volume of 14 mL eluate). Each fraction was evaporated to dryness in separate vials, and the fumonisin concentrations were determined as previously described (13).

Stability of Fumonisin–OPA Derivatives

Fumonisin standards were prepared as described (13) and 50 μL aliquots were used to prepare 8 similar OPA derivatives (see *Method* section). After mixing, each derivative was capped and stored on the bench under laboratory fluorescent lights for either 0.5, 1, 2, 4, 8, 16, 32, or 64 min, and then injected onto the LC system (see *Method* section).

Regeneration and Re-use of SAX Cartridges

A corn extract was prepared by extracting 100 g fumonisin-contaminated corn with 200 mL methanol–water (3 + 1), using a Sorvall Omnimixer as described for the initial extraction (a). Aliquots (10 mL) of the filtered extract were applied to 4 separate SAX cartridges at flow rates of 2 mL/min, and fumonisin concentrations were determined by the method of Shephard et al. (13). The used cartridges were treated with 5 mL 0.1M aqueous hydrochloric acid followed by 8 mL distilled water. Additional 10 mL aliquots of the original sample extract were then re-applied to the regenerated cartridges, and the fumonisin concentrations were again determined as described (13).

FB_3 Recoveries

Fumonisin-free control corn was spiked with FB_3 standard in methanol at a level of 1000 ng/g. A 100 g subsample of the spiked corn was extracted with 200 mL methanol–water (3 + 1) in the Polytron homogenizer as described for the initial extraction (a). Aliquots (10 mL) of the extract were then applied to 5 separate SAX cartridges, and the FB_3 levels were determined by the method detailed below.

The results of the various parameters described in the *Experimental* section and evaluated in the *Results and Discussion* section of this paper were combined into an optimized method for the determination of FB_1, FB_2, and FB_3 in corn-based products, which is described in the following section.

METHOD

Apparatus

(a) *Liquid chromatograph.*—M-45 pump (Waters Corp., Milford, MA 01757), with a Waters U6K injector.

(b) *LC column.*—Stainless steel, 12.5 cm × 4 mm id, packed with Lichrosorb 5 μm C8 reversed-phase material (Merck & Co., Darmstadt, Germany).

(c) *Fluorescence detector.*—650S fitted with an 18 μL flow cell and set at 335 nm (excitation) and 440 nm (emission), and slit widths of 12 nm (Perkin-Elmer Corp., Norwalk, CT 06859).

(d) *Data system.*—Waters Model 745.

(e) *Blender.*—Polytron mixer (Kinematica, Luzern, Switzerland).

(f) *Solid-phase extraction (SPE) columns.*—Bond-Elut strong anion exchange (SAX) cartridges, 3 mL capacity, containing 500 mg sorbent (Analytichem, Harbor City, CA 90710).

(g) *SPE manifold.*—SPE tube manifold (Supelco, Bellefonte, PA 16823).

(h) *Sample evaporator.*—Silli-therm module (Pierce, Rockford, IL 61105).

Reagents

(a) *Solvents and reagents.*—Analytical grade acetonitrile, methanol, *o*-phosphoric acid (>85%), glacial acetic acid, OPA, 2-mercaptoethanol, sodium dihydrogen phosphate, and disodium tetraborate.

(b) *LC mobile phase.*—Methanol–0.1M sodium dihydrogen phosphate (15.6 g $NaH_2PO_4 \cdot 2H2O$ in 1 L distilled water) (68 + 32), adjusted to pH 3.35 with *o*-phosphoric acid. Filter mobile phase through 0.45 μm Waters HV membrane and pump at 1 mL/min flow rate.

(c) *Preparation of OPA.*—Dissolve 40 mg OPA in 1 mL methanol and dilute with 5 mL 0.1M disodium tetraborate (3.8 g in 100 mL distilled water). Add 50 μL 2-mercaptoethanol and mix. Store solution for up to 1 week at room temperature in dark, capped amber vial.

(d) *Fumonisin standard solution.*—Prepare standard solution of FB1, FB_2, and FB_3 (9, 11) in acetonitrile–water (1 + 1) at concentration of 50 μg/mL for each standard. Store solution at 4°C.

Extraction and Cleanup

Place subsample (25 g) of corn or mixed feed, previously ground and blended, into suitable glass tube and homogenize 2 min with 50 mL methanol–water (3 + 1), using Polytron homogenizer, 60% full speed setting. Centrifuge blended extract 10 min at 500 *g* and ca 4°C, and filter supernatant through fluted Whatman No. 4 filter paper. Check pH of eluate and adjust, if necessary, with 0.1M KOH to ≥pH 5.8 (i.e., to 5.8–6.5).

Attach SAX cartridge to SPE manifold and condition by washing successively, first with 5 mL methanol and then with 5 mL methanol–water (3 + 1). While maintaining flow rate of no more than 2 mL/min, apply 10 mL aliquot of filtered sample extract to cartridge. Wash cartridge with 8 mL methanol–water (3 + 1), followed by 3 mL methanol. Elute and collect fumonisins in vial, with 10 mL 1% methanolic acetic acid, at flow rate of no more than 1 mL/min. Evaporate eluate to dryness in 4 mL capacity vial under stream of nitrogen at ca 60°C. Wash collection vial with 1 mL methanol and add to 4 mL vial. Evaporate additional methanol to dryness under nitrogen, cap vial, and retain dried residue at 4°C until LC analysis.

Derivatization and LC Analysis

(a) *Preparation of standard derivative.*—Transfer 25 μL fumonisin standard solution to base of small test tube. Add 225 μL OPA reagent, mix solutions, and inject 10 μL derivatized solution into LC system within 1 min of adding OPA reagent. Adjust sensitivity of fluorescence detector so that FB_1–OPA derivative standard (= 50 ng injected onto LC column) gives at least an 80% recorder response.

(b) *Corn extracts.*—Redissolve purified sample residue in 200 μL methanol. Transfer aliquots (25 or 50 μL) of extract to base of small test tube and add OPA reagent (225 or 200 μL). Mix solutions and inject 10 μL derivatized solution into LC system within 1 min of adding OPA reagent.

Results and Discussion

Optimization of Sample Purification

(a) *Initial extraction.*—Several different ratios of methanol–water and acetonitrile–water were evaluated for the extraction of the fumonisins from naturally fumonisin-contaminated corn. The methanol–water blend (3 + 1), as used for the initial isolation of the fumonisins (1), was found to give marginally higher recoveries than some of the other solvent blends. With this solvent blend as extractant, the use of the Polytron homogenizer for the initial extraction of the fumonisins from corn-based matrixes was evaluated against homogenization with a Sorvall Omnimixer and shaking on a wrist-action shaker. No significant differences in fumonisin recovery were observed in homogenizing 25 g subsamples with 50 mL methanol–water (3 + 1) for either 1, 3, or 5 min with the Polytron homogenizer; relative standard deviations (RSD) of less than 4.0% were recorded. The use of a Sorvall Omnimixer for extraction of a 50 g subsample with 100 mL methanol–water (3 + 1) at 60% full speed for 5 min resulted in fumonisin recoveries of 94–99% of the Polytron results. Extraction on a wrist-action shaker for 60 min resulted in recoveries of 67–74% of the Polytron results. We concluded that the Polytron gave the most consistent and reproducible recoveries of the 3 procedures; Sorvall extraction using larger sample weights and solvent volumes than previously prescribed (13) could give comparable results but required longer extraction times. Because of the low recoveries, the use of a wrist-action shaker for the initial extraction of the fumonisins from corn-based products is not recommended.

(b) *Application to and elution from SAX cartridges.*—In ion exchange chromatography, separation takes place as a result of the competition between ions in the eluant and the solute for oppositely charged sites on the stationary phase. Although this principle would appear to be simple, the separation mechanism on ion exchange materials can be complex, and several types of separation mechanisms can be operating simultaneously (14). Factors such as the pH and the ionic strength of solvents can significantly affect either the retention or elution of solutes.

Although in this laboratory the pH values of naturally contaminated sample extracts have been found to range from 6.0–6.5, fumonisin recoveries from SAX cartridges may be

Table 1. Effect of sample extract application rate to strong anion exchange cartridges

	Fumonisin concn, ng/g	
Application flow rate, mL/min	FB_1	FB_2
1.0	564	213
2.1	563	207
3.1	604	220
8.2	603	232
Mean	584	218
RSD, %	4.0	4.9

Table 2. Effect of elution rate of fumonisins from strong anion exchange cartridges, using 0.5% methanolic acetic acid

	Fumonisin concn, ng/g	
Elution flow rate, mL/min	FB_1	FB_2
1.0	1517	605
2.5	1154	500
4.1	957	428
6.7	858	407

Table 3. Effect of elution solvent strength on elution profiles of fumonisin B_1 from strong anion exchange cartridges at flow rates of 0.8 mL/min

		Percentage FB_1	
Fraction	Volume, mL	0.5% CH_3COOH/CH_3OH	1.0% CH_3COOH/CH_3OH
1	4	91.8	97.9
2	2	4.2	1.6
3	2	2.4	0.5
4	2	1.4	0.0
5	2	0.2	0.0
6	2	0.0	0.0

compromised at pH values below 5.8. Therefore, the pH of sample extracts should be measured before application to SAX cartridges and adjusted if necessary. Table 1 shows the effect of the application rate of sample extracts, contaminated with FB_1 and FB_2, on the recovery of the fumonisins. The data (Table 1) clearly indicate that complete retention of the fumonisins by the SAX stationary phase is not a function of the initial application rate, as no significant differences in fumonisin concentrations were observed for sample extracts applied to the SAX cartridges at flow rates of 1–8.2 mL/min.

Table 2 illustrates the effect of the elution rate from the SAX cartridge on the recovery of the fumonisins using 14 mL 0.5% acetic acid in methanol as prescribed in the method of Shephard et al. (13). Recoveries of the fumonisins from the SAX cartridges decreased substantially at elution flow rates between 2.5 and 6.7 mL/min when compared with the recoveries obtained at an elution flow rate of 1 mL/min (Table 2). The equilibrium between the elution solvent, the fumonisins, and the ion exchange material would appear to be critical. Therefore, the elution flow rate of 2 mL/min previously prescribed by Shephard et al. (13) required further consideration.

Table 3 compares the elution profiles of FB_1 at different elution solvent strengths from SAX cartridges at a constant flow rate of 0.8 mL/min. More than 90% of the FB_1 eluted from the SAX cartridge within the first 4 mL when the elution solvent was 0.5% acetic acid in methanol. However, FB_1 was completely eluted only after 12 mL eluate was collected. In contrast, the use of a solution of 1% acetic acid in methanol resulted in the complete elution of FB_1 within 8 mL; more than 99% was eluted in the first 6 mL. Further studies using corn extracts indicated that the use of the stronger solvent did not result in the co-elution of corn-intrinsic compounds that interfered with the chromatographic determination of the fumonisins. Even though the time required for complete elution of the fumonisins was slightly increased, this alteration improved the method by significantly reducing the volume of eluate, which reduced the time required for the solvent evaporation of the eluate.

Stability of Fumonisin–OPA Derivatives

OPA derivatives of the fumonisins are prepared under alkaline conditions (pH 9–10) in the presence of 2-mercaptoethanol. The stability of OPA derivatives was reviewed (15, 16), and OPA was reported to be less stable than other fluorometric reagents. To assess the stability of OPA–fumonisin derivatives, fumonisin standards were reacted with OPA, and the solutions were capped and left to stand under fluorescent light 0.5–64 min before injection into the LC. No significant difference in fluorescence response was observed in those standards injected within 4 min of addition of the OPA reagent. However, after 8 min, the response was approximately 95% of that previously observed, and after 64 min, it decreased to 48% of the initial readings. Therefore, the data indicated that, provided LC injections are made reproducibly and within at least 4 min following the addition of the OPA reagent, no appreciable derivative stability problems should be encountered.

Re-use of SAX Cartridges

To ascertain whether the SAX cartridges could be re-used, an extract of a fumonisin-contaminated corn sample was prepared and applied to 4 separate SAX cartridges, both before and after regeneration. No significant difference was observed between the main fumonisin concentrations recorded for the 2 series of determinations; RSD values were 0.85% for FB_1 and 0.35% for FB_2 in the first series, and 1.0% for FB_1 and 0.25% for FB_2 in the second series. These results suggest that if necessary, SAX cartridges can be regenerated and reused for fumonisin purification without loss of analyte recovery.

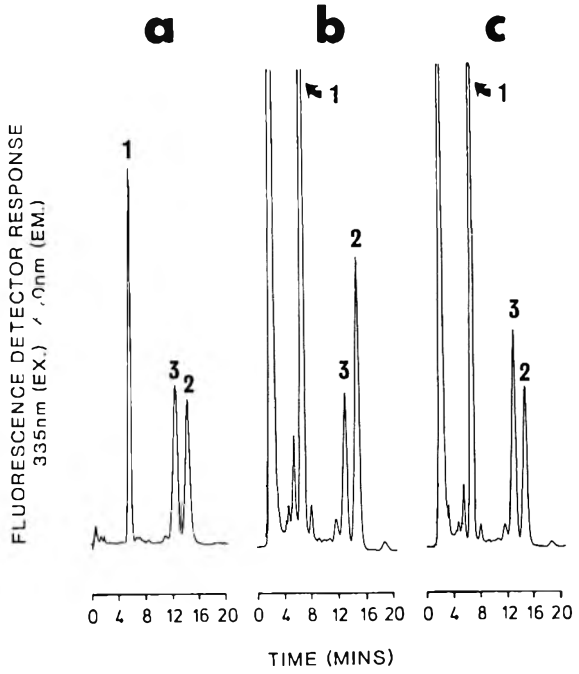


Figure 2. (a) Chromatographic separation of OPA derivatives of 50 ng each of 1 (fumonisin B_1), 2 (fumonisin B_2), and 3 (fumonisin B_3); (b) chromatogram of a similarly prepared derivative of the purified extract of sample number U6205; and (c) the chromatogram of the extract shown in 2b, spiked with authentic fumonisin B_3 standard.

Levels of FB_3 in Corn-Based Mixed Feeds

On the basis of 5 separate determinations, the mean recovery of FB_3 from SAX cartridges (spiked in fumonisin-free control corn at 1000 ng/g) was 96.8%, with an RSD of 2.3%. The limit of detection was found to be similar to that reported for FB_1 and FB_2 (i.e., 50 ng/g). The method was applied to a series of feed samples associated with field outbreaks of equine LEM in the United States over a 4-year period (1983–1986) (17).

Figure 2a shows the LC separation of 50 ng each of FB_1, FB_2, and FB_3 standards as their OPA derivatives. The figure illustrates that baseline separation of the 3 mycotoxins may be achieved within 16 min using an isocratic LC system coupled with fluorescence detection. Figure 2b shows the chromatogram obtained from a similarly prepared derivative of an extract of a feed sample (U6205); Figure 2c shows the chromatogram of the same extract spiked with FB_3 standard. The excellent agreement between the retention times for the peaks corresponding to FB_3 in the standard (Figure 2a) and in the sample extract (Figure 2b), coupled with the successful spike of the sample (Figure 2c), confirmed the presence of FB_3 in the sample extract.

The levels of FB_3 determined in the 13 U.S. feed samples are listed in Table 4. The levels ranged between 0 and 2650 ng/g and were considerably lower than the corresponding

Table 4. Fumonisin concentrations in feed samples associated with confirmed cases of equine leukoencephalomalacia in the United States[a]

Sample	Fumonisin concn, ng/g			
	FB_1	FB_2	FB_3	FB_3, %[b]
U6177	4600	1100	160	2.7
U6178	4400	700	200	3.8
U6179	3700	600	120	2.7
U6205	8000	4100	2650	18.0
U6213	6000	2400	730	8.0
U6256	5800	1600	270	3.5
U6258	4500	1000	160	2.8
U6262	1300	100	50	3.5
U6263	9600	3100	290	2.2
U6264	16800	6500	680	2.8
U6292	7100	6300	920	6.4
U6301	6200	1300	490	6.1
U6302	3200	500	ND[c]	0

[a] Data on FB_1 and FB_2 in feed samples previously published (17).
[b] % FB_3 = 100 × [FB_3/(FB_1 + FB_2 + FB_3)].
[c] Not detected (<50 ng/g).

levels of FB_1 (1300–16 800 ng/g) and FB_2 (100–6500 ng/g) recorded in the same samples (17). The extent to which the FB_3 levels reported in Table 4 may have contributed to the development of equine LEM is as yet unknown; however, the fact that the FB_3 levels corresponded to 2.2–18.0% of total fumonisin concentrations in the 12 positive U.S. equine LEM samples stresses the importance of screening extracts for the presence of FB_3 as well as FB_1 and FB_2.

In summary, the alterations made to the LC method, coupled with the chromatographic observations, resulted in the provision of a method that enables the co-determination of the 3 most abundant and possibly the most toxicologically important fumonisin mycotoxins in corn-based foods and mixed feeds. The analysis times required for sample extraction, prechromatographic purification, and analytical determination were substantially reduced without loss of recovery or resolution. The method was successfully applied to a series of U.S. feed samples associated with field outbreaks of equine LEM, and resulted in the first report of the natural occurrence of FB_3.

Acknowledgments

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MYCOTOXINS

Liquid Chromatographic Determination of Cyclopiazonic Acid in Corn and Peanuts

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A liquid chromatographic (LC) method is described for the determination of cyclopiazonic acid (CPA) in corn and peanuts. CPA was extracted from the test portion with methanol–2% $NaHCO_3$ solution (7 + 3); the extract was defatted with hexane and then acidified. CPA was partitioned into chloroform and applied to a Sep-Pak silica cartridge. CPA was eluted with chloroform–methanol (3 + 1), the solvent was evaporated, and the residue was dissolved in methanol–water (60 + 40). CPA was quantitated by reversed-phase LC with a linear gradient of 0–4mM $ZnSO_4$ in methanol–water (85 + 15) and UV measurement at 279 nm. Recoveries of CPA from corn spiked over the range of 50–500 ng/g and peanuts spiked over the range of 100–500 ng/g were 72–84% and 74–80%, respectively. The limits of quantitation for CPA in corn and peanuts were about 50 and 100 ng/g, respectively. CPA (820 ng/g) was found in corn naturally contaminated with aflatoxin B_1, and CPA identity was confirmed by tandem mass spectrometry.

Cyclopiazonic acid (CPA) is a toxic indole tetramic acid (Figure 1) produced by some species of *Aspergillus* and *Penicillium* (1–3). Some strains of *A. flavus* produce both aflatoxins and CPA (4). Consequently, aflatoxins and CPA can occur together in a single food commodity. Gallagher et al. (5) indicated that CPA might occur with a frequency similar to or greater than that of the aflatoxins. When given experimentally, CPA has caused toxic effects in rodents (6), dogs (7), pigs (8), and chickens (9). Some cases of human and animal poisoning reported in India may be the result of CPA contamination of millet (10). CPA accumulates in the skeletal muscle of rats (11) and, more importantly from a food safety aspect, in chicken meat after oral dosing (12).

Several analytical procedures were developed for the determination of CPA in agricultural commodities. Thin-layer chromatographic separation coupled with postdevelopment derivatization with Ehrlich's reagent under acidic conditions, followed by densitometric analysis, gave a limit of quantitation of 50 ng with the use of standards (13). CPA was also determined in corn at 100 ng/g by normal-phase liquid chromatography (LC) using a mobile phase of ethyl acetate–2-propanol–25% aqueous ammonia (55 + 20 + 5) (14). This solvent, however, may shorten the useful life of the column by dissolving the silica gel packing. A recent study used a ligand-exchange reversed-phase LC method that was a modification of a previously published procedure (15) to determine CPA in the meat of chickens dosed with the toxin (12, 16). In this method, the LC column requires overnight equilibration with the mobile phase. Stack et al. (17) used zinc sulfate as a chelating agent in the mobile phase to separate tenuazonic acid from interferences in tomatoes. We found this chelating agent useful in a gradient LC system (0–4mM $ZnSO_4$ in methanol–water, 85 + 5) for the determination of CPA in corn and peanuts.

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METHOD

Apparatus

(a) *Blender.*—High speed with 1 L blender jar and cover.

(b) *Rotary evaporator.*—Buchi Model RE 120 performs satisfactorily.

(c) *Liquid chromatograph.*—2 Model 510 LC pumps, U6K injector, 490 multiwavelength detector, and 840 chromatography data station (Waters Associates, Inc., Milford, MA 01752).

(d) *LC column.*—150 × 3.9 mm id, packed with Resolve C18, 5 μm (Waters).

(e) *Guard column.*—15 × 3.2 mm id, packed with New-Guard RP-18 (Applied Biosystems, Ramsey, NJ 07446).

(f) *Tandem mass spectrometer.*—Triple Stage Quadrupole (TSQ) 46 (Finnigan MAT, San Jose, CA).

Reagents

(a) *Solvents.*—LC grade chloroform, acetone, methanol, hexane, anhydrous ethyl ether, and distilled water.

(b) *Chemicals.*—Reagent grade $NaHCO_3$, KCl, HCl, and anhydrous Na_2SO_4.

(c) *LC mobile phase.*—Solvent A, methanol–water (85 + 15); Solvent B, methanol–water (85 + 15) with 4mM $ZnSO_4 \cdot 7H2O$.

(d) *Silica cartridge.*—Sep-Pak silica (Waters).

(e) *Standard solution.*—Pure CPA (available from Joe W. Dorner, U.S. Department of Agriculture, National Peanut Research Laboratory, Dawson, GA 31742) dissolved in methanol (100 μg/mL).

(f) *Working standards.*—Transfer 40, 100, and 200 μL stock solution into separate 2 mL volumetric flasks and dilute to volume with methanol. Working standards contain CPA at 2.0, 5.0, and 10 ng/μL, respectively.

Extraction

Grind corn or peanuts and blend to pass U.S. No. 20 sieve. Place 50 g ground corn or peanuts in blender jar. Add 200 mL methanol–2% NaHCO3 solution (7 + 3) and blend 3 min at high speed. Filter extract by gravity through fluted paper. Transfer 100 mL corn filtrate or 50 mL peanut filtrate to 250 mL separatory funnel. Add 100 mL hexane and shake funnel 1 min. Let phases separate and then drain lower phase into another 250 mL separatory funnel. Add 50 mL 10% KCl solution, acidify with 2 mL 6N HCl, and extract with 50 mL chloroform. Repeat extraction with additional 50 mL chloroform. Combine chloroform extracts. Add 50 g anhydrous $Na2SO_4$ and filter into 250 mL round-bottom flask. Evaporate to dryness, using rotary evaporator in 40°C water bath.

Silica Cartridge Cleanup

Place silica gel cartridge on port of vacuum manifold. Condition cartridge by washing with 5 mL chloroform before use. Gently apply suction to generate flow rate of 3 mL/min. Dissolve residue in 10 mL chloroform and add to cartridge. Wash cartridge with 10 mL ethyl ether, 10 mL chloroform–acetone (1 + 1), and 10 mL chloroform–methanol (95 + 5). Do not let column run dry. Place 50 mL round-bottom flask under column and elute CPA with 10 mL chloroform–methanol (3 + 1). Evaporate eluate to dryness with rotary evaporator in 40°C water bath. Dissolve residue in chloroform and transfer quantitatively to 4 mL screw-cap vial. Evaporate and save for LC analysis.

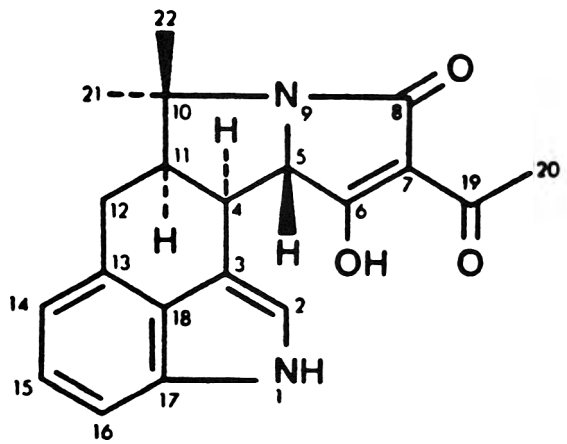

Figure 1. Structure of cyclopiazonic acid.

Liquid Chromatography

The chromatography conditions are as follows: linear gradient from 100% A to 100% B in 10 min, followed by 100% B for 10 min, 1.0 mL/min flow rate, and measurement at 279 nm. Inject 30 μL working standard containing CPA at 1 ng/μL to ensure that 20 μL loop is completely filled. The retention time of CPA is ca 10 min. Adjust sensitivity of UV detector to give reasonable integrator response (signal:noise = 5:1) for 20 ng CPA. If strip chart recorder is used, adjust control to give 10% full-scale deflection with 20 ng CPA. Prepare standard curve daily. Dissolve residue in 1 mL methanol and filter through 0.2 μm filter. Inject 20 μL test solution. Identify CPA peak in chromatogram by comparing retention time with that of reference standard. Determine quantity of CPA in test solution injected from standard curve. Calculate concentration of CPA in corn or peanuts, using the following equation:

$$ng/g = X \times (Tv/Iv) \times (1/W) = 50 \times (X/W)$$

where W = weight of test portion (W = 25 g for corn, W = 12.5 g for peanuts), X = ng CPA in test solution injected, Tv = volume of concentrated eluate (1000 μL), and Iv = volume of test solution injected (20 μL).

Confirmation of CPA Identity by Tandem Mass Spectrometry

Isolation of CPA.—Collect presumed CPA from LC detector outlet. Repeat injections as many times as necessary until a total of 100 ng presumed CPA is collected. Add 1 drop 1N HCl to combined CPA fractions in separatory funnel and mix. Add 3 mL chloroform. Shake funnel and let layers separate. Collect chloroform layer and evaporate to dryness under stream of nitrogen. Save residue for mass spectrometric (MS) analysis.

Mass spectrometric analysis and conditions.—Reconstitute residue in chloroform and apply 2 μL to loop of direct exposure probe for introduction into mass spectrometer. The following instrumental parameters were used: methane positive ion chemical ionization (PICI), source temperature 110°C, pre-amp 10^{-8} A/V, electron energy 70 eV, electron multiplier 1400 eV; emission current 0.35 mA, conversion dynode 5 keV, argon collision gas pressure 1.8 mtorr, and collision energy –14 eV. The first quadrupole was set to pass the ions at *m/z* 337 (the CPA protonated molecular ion), the second quadrupole acted as a collision cell, and the third quadrupole recorded the daughter ion spectra, scanning a range from 60 to 360 daltons.

Results and Discussion

The method described in this paper is an improvement over existing methods. The use of a direct, basic methanol extraction, rather than an acidic methanol extraction followed by basic partition, improves recoveries by 20%. KCl is added before CPA is partitioned into chloroform to eliminate emulsion problems. A time-saving, solvent-efficient cleanup step is provided by the use of the 700 mg silica gel cartridge. The $ZnSO_4$ linear gradient system resolves CPA from an interference that has a retention time close to that of CPA when chromato-

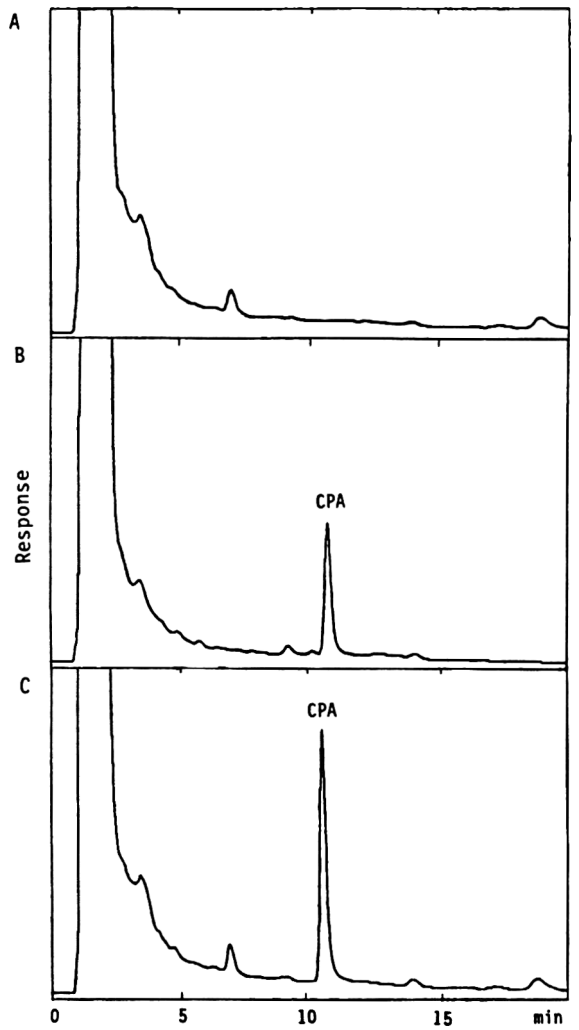

Figure 2. Liquid chromatograms of (A) control corn, (B) naturally contaminated corn (CPA at 230 ng/g), and (C) spiked corn extracts (CPA at 500 ng/g).

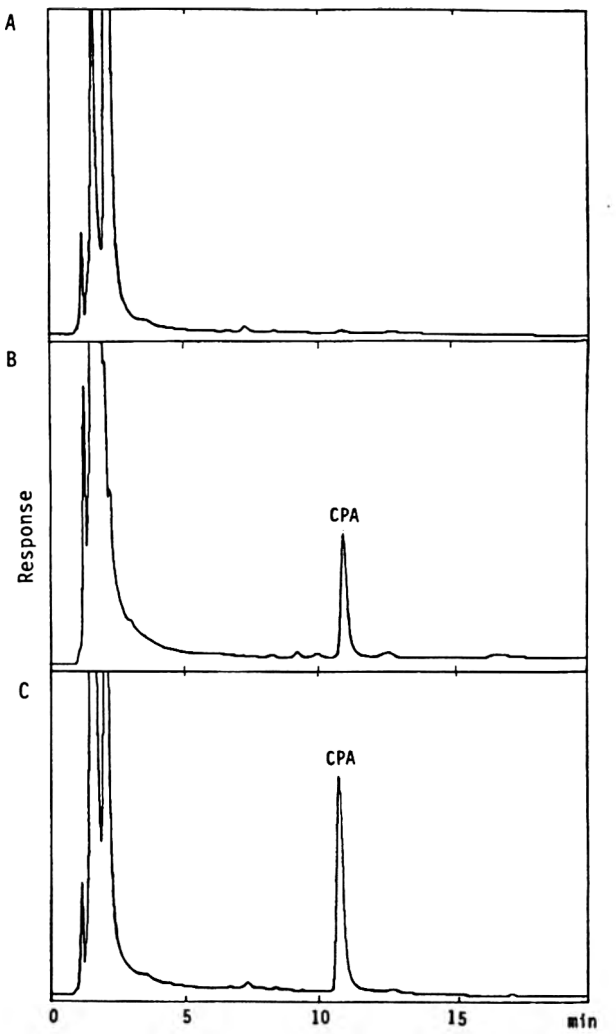

Figure 3. Liquid chromatograms of (A) control peanuts, (B) naturally contaminated peanuts (CPA at 430 ng/g), and (C) spiked peanut extracts (CPA at 1000 ng/g).

graphed isocratically. The limit of quantitation of the CPA standard is about 20 ng (signal:noise > 5:1), and the minimum quantifiable concentrations of CPA in corn and peanuts are 50 and 100 ng/g, respectively. Figures 2A, 2B, and 2C are chromatograms of control corn, naturally contaminated corn (CPA, 230 ng/g), and spiked corn extracts (CPA, 500 ng/g), respectively. Figures 3A, 3B, and 3C are chromatograms of control peanuts, naturally contaminated peanuts (CPA, 430 ng/g), and spiked peanut extracts (CPA, 1000 ng/g), respectively. Figure 4 is a mass spectrum of the presumed CPA fraction from naturally contaminated corn (CPA, 820 ng/g). The protonated molecular ion at *m/z* 337 (M + H) and daughter ions at *m/z* 182 and 196 were observed. The relative intensities of the ions from the test extract agreed with those of the corresponding ions from the CPA standard. Thus, the identity of CPA in the test extract was confirmed. The identity of CPA in naturally contaminated corn at 50 ng/g was confirmed by using the same MS conditions for standard and extracts.

Table 1 gives the average recoveries of CPA added to corn and peanuts. The recoveries from corn containing CPA at 50–500 ng/g were 72–84%, and the recoveries from peanuts containing CPA at 100–500 ng/g were 74–80%. The relative standard deviations were 3.5–7.4%.

Results obtained by this method are equivalent to those obtained by previously reported methods. However, this method offers a time-saving alternative, a simpler LC separation, and a technique for the isolation of CPA for MS. The application of MS/MS for confirmation of CPA identity at 50 ng/g in grain was demonstrated for the first time. Currently, we are using this method to conduct survey studies of CPA in corn and peanuts. The results and co-occurrences of CPA and aflatoxins in these 2 commodities will be reported in a separate publication.

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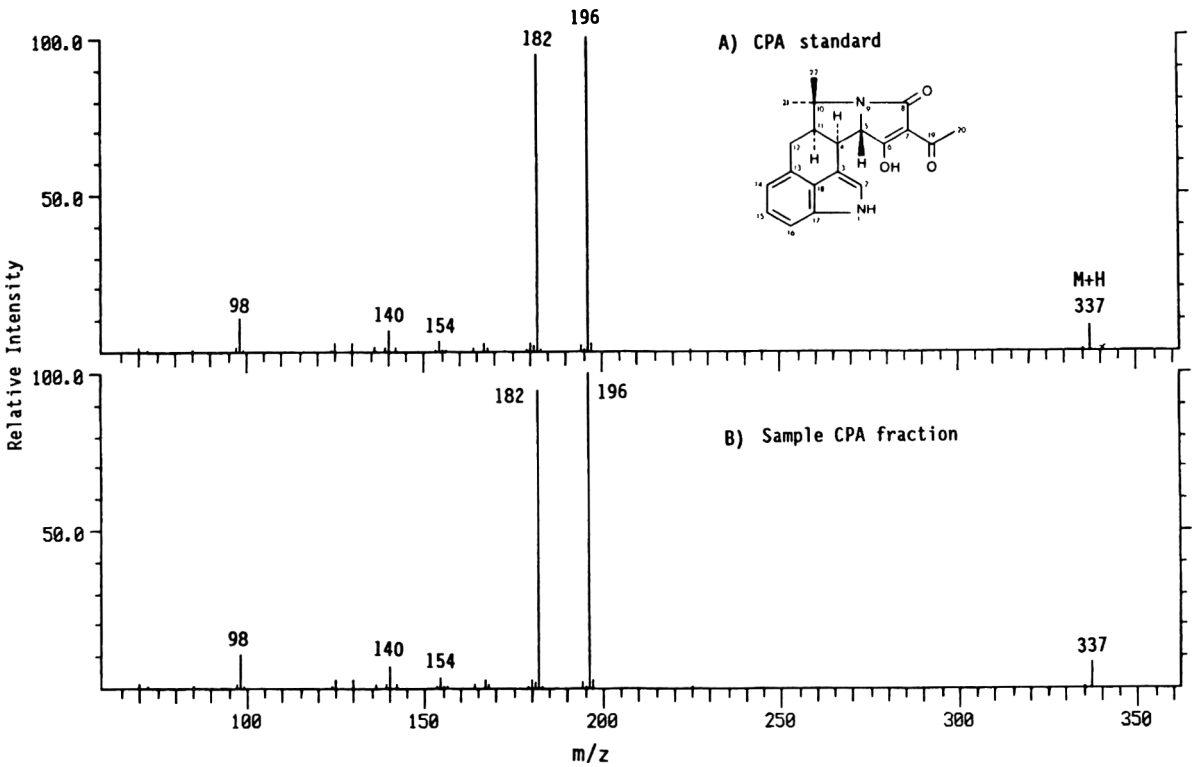

Figure 4. PICI MS/MS spectrum of (A) CPA standard and (B) CPA fraction from naturally contaminated corn (CPA at 820 ng/g).

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Table 1. Recovery of CPA added to corn and peanuts

CPA added, ng/g	Corn, %				Peanuts, %			
	Av.	Range	SD	RSD	Av.	Range	SD	RSD
50	72	69–78	4.9[a]	6.8	—[c]	—	—	—
100	77	72–84	5.7[a]	7.4	74	72–77	2.6[a]	3.5
200	82	78–88	3.7[b]	4.4	76	72–82	4.6[b]	6.0
500	84	80–90	4.5[b]	5.3	80	76–85	3.2[b]	4.0

[a] $n = 3$.
[b] $n = 6$.
[c] Not determined; signal:noise < 5:1.

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Methyl 2-Benzimidazolecarbamate in Blueberries by Competitive Inhibition Enzyme Immunoassay

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A polyclonal enzyme immunoassay (EIA) method was developed that will quantitate methyl 2-benzimidazolecarbamate (MBC), the degradation product of benomyl, in blueberries. The entire analysis is completed within 25 min, and up to 8 samples can be analyzed simultaneously with a detection limit of 18 ppb. The assay response was linear from 0.69 to 22 ppb MBC. Cross-reactivity was confined to the benzimidazole and dicarboximide type pesticides. Intra-assay percent coefficients of variation (% CVs) ranged from 3.4 to 18.4 for the standards, and from 3.2 to 20.6 for the samples. Interassay % CVs varied from 7.5 to 22.3 for the standards, and from 7.5 to 22.0 for the samples. Correlation coefficient between immunoassay and liquid chromatography was 0.87 for the methanol extract and 0.98 for the methylene chloride partition of the methanol extract containing 100 mL 1% sodium chloride. None of the 40 field samples analyzed approached the tolerance of 15 ppm (mean of 20 positive samples was 55 ppb). The ability of the EIA to monitor MBC easily and inexpensively at concentrations far below the tolerance has major implications for current dietary risk assessment methods.

Benomyl, a systemic benzimidazole fungicide, is used as either a preharvest or postharvest treatment on fruit and vegetables to prevent Botrytis and rotting during refrigeration (1). Benomyl degrades rapidly to methyl 2-benzimidazolecarbamate (MBC, or carbendazim) (2, 3). Tolerances, including MBC, range from 0.12 to 35 ppm. Recently, questions have arisen about the safety of benomyl and MBC (4, 5). Because of possible health effects (4, 5), widespread use (6), and insufficient residue data (6), there is a need to monitor benomyl and MBC in food commodities. Accurate and thorough monitoring of pesticide residues in foods is crucial for proper dietary risk assessment.

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Previous methods for benomyl and MBC determination in foods have focused on spectrophotometric and chromatographic procedures (1, 5, 7, 8). Because benomyl decomposes rapidly to MBC in many organic solvents, most methods determine total MBC by converting benomyl to MBC (9, 10). Recently, liquid chromatography (LC) has become the method of choice for benzimidazole fungicides (8). However, enzyme immunoassay technology is beginning to be applied to pesticide residue analysis for reasons recently discussed (11). In fact, many scientists now believe that adequate monitoring of pesticides in our food supply will require that immunoassay play an increasingly key role (11). Previously, 2 EIA methods were developed for MBC in foods, but these were not applicable to blueberries (7, 12).

This paper describes an EIA method for the determination of MBC in blueberries (fresh, frozen, highbush, and lowbush) that is extremely sensitive without the need of a cleanup step. In fact, it was our use of the EIA technique that first gave us the indication that MBC was present in our blueberry samples. A modified LC method was then developed to confirm the results obtained by EIA.

METHOD

Apparatus and Reagents

(a) *Reagents.*—All reagents pertaining to preparation of immunogens for raising antisera to MBC were previously described (7). Methanol used for sample extraction was ACS grade (Fisher Scientific, Fairlawn, NJ 35666). All other solvents were LC grade (VWR, Boston, MA 02101).

(b) *Analytical standards.*—All pesticide standards were obtained from U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

(c) *Pesticide standard stock solutions.*—Weigh 7.0 mg each pesticide into 20 mL scintillation vials, and dissolve and dilute to volume with 20 mL methanol.

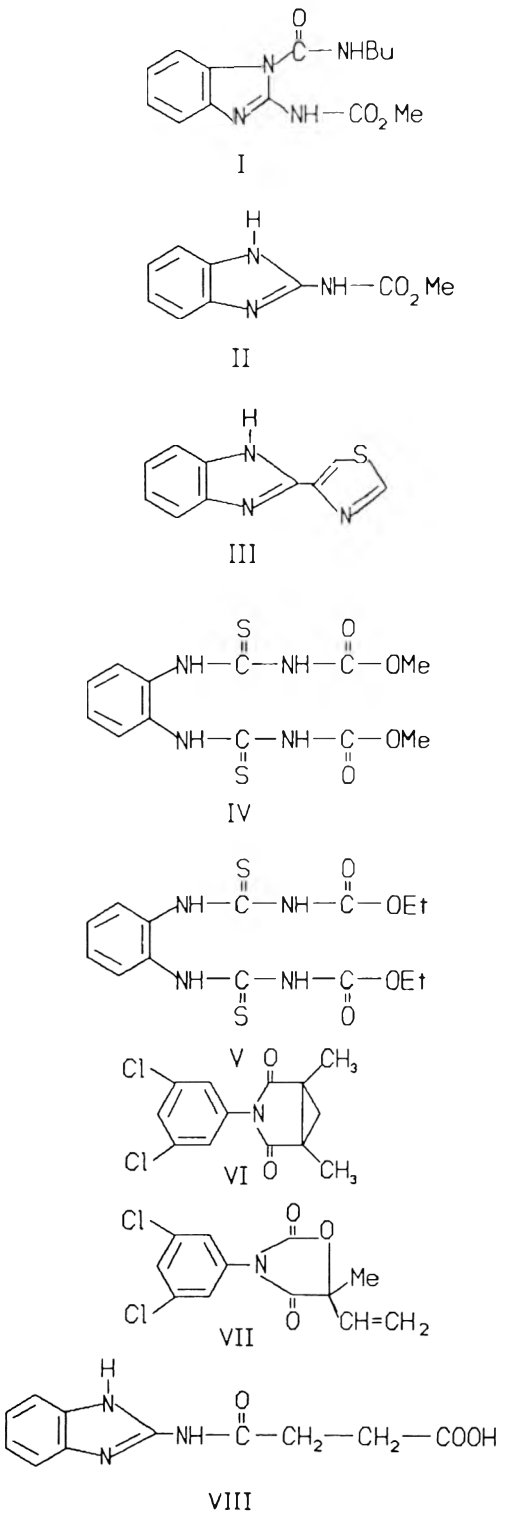

Figure 1. Chemical structures of pesticides that cross-react with the benomyl antibody and the hapten used for the conjugate. Structures: (I) benomyl, (II) MBC, (III) thiabendazole, (IV) thiophanate-methyl, (V) thiophanate, (VI) procymidone, (VII) vinchlozolin, and (VIII) 2-succinamidobenzimidazole (hapten).

(d) *Pesticide intermediate standards for immunoassay.*—Take 0.1 mL aliquot from corresponding stock solution and place into 50 mL volumetric flask. Dilute to volume with water.

(e) *Pesticide working standards for immunoassay.*—Remove 10, 20, 40, 80, 160, and 320 μL aliquots and place each into 20 mL scintillation vial containing 1 mL evaporated MeOH blueberry extract. Reconstitute to original volume with water.

(f) *MBC working standard for LC.*—Remove 0.1 mL stock solution and add 19.9 mL LC mobile phase.

(g) *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 310 nm; UV absorbance 280 nm; absorbance range 0.04 AUFS.

(h) *Chromatographic column.*—Ultracarb 30 ODS, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Torrance, CA 90501).

(i) *Mobile phase.*—Acetonitrile–methanol–water–monoethanolamine (135 + 30 + 235 + 0.05).

(j) *Sample extraction solvent.*—Use methanol for both LC and immunoassay and use methylene chloride as partitioning solvent for LC analysis.

(k) *Sample cleanup.*—Acid alumina (Sigma Chemical Co., St. Louis, MO 63178) and basic alumina (Fisher Scientific).

(l) *Conjugate synthesis and antisera.*—Follow methods described previously (7, 12, 13). See Figure 1 for hapten structure.

Extraction of MBC for Immunoassay

Use method of Gilvydis and Walters (8). Add 50 g blueberries (fresh or frozen) to 1 L blender jar followed by 100 mL ACS grade methanol. (In working with sample that may have free benomyl present, 10 mL 1N HCl can be added to MeOH before extraction.) Blend 5 min before filtering. Record volume of filtrate.

Immunoassay of MBC

Use EnviroGard™ EIA kits (Millipore Corp., Bedford, MA 01730), which consist of polystyrene test tubes coated with MBC antibodies and an enzyme conjugate (horseradish peroxidase bound to MBC). Use hydrogen peroxide as the substrate and tetramethylbenzidine as the chromogen.

Prepare MBC standards as described in (e). In the same way as for MeOH samples, remove 0.1 mL aliquot from methanol extract and place in 7 mL glass scintillation vial. Evaporate to dryness under nitrogen. Add 1 mL water to residue and sonicate.

For methylene chloride partition samples, remove 0.1 mL aliquot from LC sample before alumina cleanup step (for more details on LC samples, see LC determination of MBC in *Methods* section). Upon removal of 0.1 mL aliquot, follow procedure described for MeOH samples.

Analyze standards and samples by adding 160 μL to coated tube followed by 160 μL enzyme conjugate. As many as 8

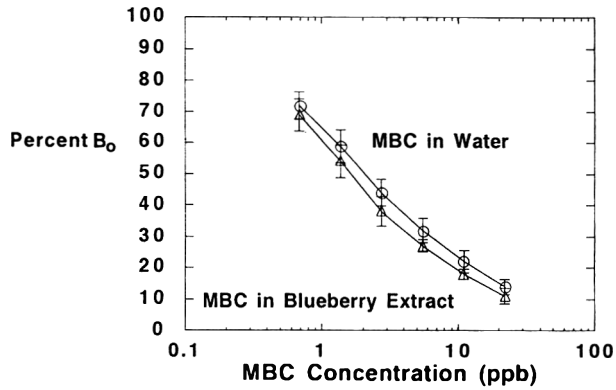

Figure 2. Typical standard curve for MBC in water (0) and MBC in MeOH blueberry extract (Δ). The error bars are standard deviation values between runs.

Table 1. Reproducibility of the MBC immunoassay for standards prepared in a 1:10 dilution of MeOH organic blueberry extract

MBC standard, ppb	CV, % (intraassay)[a]	CV, % (interassay)[b]
0.69	3.4	7.5
1.4	5.0	9.9
2.8	11	12
5.5	9.9	7.6
11	18	9.1
22	18	22

[a] Percent coefficients of variation based on 6 determinations in 1 day.
[b] Percent coefficients of variation based on 9 determinations performed on 9 different days.

tubes can be prepared simultaneously. Incubate tubes for 10 min at room temperature. Rinse tubes 4 times with water to remove unreacted sample and enzyme conjugate. Add 160 μL each of substrate and chromogen. Incubate tubes for 10 min before adding 1 drop 2.5N sulfuric acid to stop reaction. Sulfuric acid causes a blue to yellow color change in tubes.

Determination of MBC by Immunoassay

Measure amount of yellow color formed after reaction is stopped by reading 200 μL aliquots of each tube on Bio-Tek Model EL 301 microwell strip reader (Burlington, VT 05404) at 450 nm. Run control tube with each set of tubes to calculate $\%B_0$ values of standards and samples (absorbance at 450 nm of standard or sample/absorbance at 450 nm of control × 100). Run standard curve at beginning and end of each day. Take average of both runs and use this to quantitate MBC in samples. Prepare curves by analyzing 0.0, 0.69, 1.38, 2.76, 5.52, 11.04, and 22.08 ppb MBC in blueberry matrix. Calculate unknowns, using standard curve.

LC Analysis of MBC

After methanol extract (described in *Methods* section under extraction of MBC for immunoassay), place filtrate in 500 mL separatory funnel and adjust pH by adding 40 mL 2.0M ammonium chloride, pH 9.5. Extract with two 100 mL portions methylene chloride. Combine methylene chloride fractions, dry over sodium sulfate, and evaporate to dryness. Dissolve residue in 2 mL methanol–acetonitrile (50 + 50) followed by 2 mL water. Remove 1 mL aliquot and pass it through an alumina column made from Pasteur pipet containing 5 mm acid alumina and 5 mm basic alumina. Inject 5 μL sample, using LC conditions described in *Methods* section ((g), (h), and (i)). (Ultracarb column is excellent for basic compounds.)

Results and Discussion

The immunoassay standard curve showed a linear relationship (Figure 2) from 0.69 to 22 ng/mL, which was observed between the logarithm of the MBC concentration and $\%B_0$ at 450 nm. For samples having a concentration greater than 22 ng/mL (indicated by $\%B_0 < 11$), a simple dilution must be performed on the sample.

To quantitate MBC in blueberries by EIA, a 1:10 dilution step was used to reduce matrix effects. Figure 2 shows a comparison between MBC standard curves in water and a 1:10 dilution of blueberry MeOH extract. The slopes were identical but the inhibition for the standard curve containing the blueberry extract was slightly greater. The difference between curves results in an error of approximately 3 ppb. For maximum accuracy using EIA, MBC can be prepared in 1:10 dilutions of non-MBC blueberry extracts.

With any analytical technique, precision within and between days is crucial. Reproducibility results of the MBC immunoassay for standards and blueberries is illustrated in Tables 1 and 2. Table 1 shows the consistency data obtained from analyzing MBC standards fortified in a 1:10 dilution of organic blueberry extract, intra- and interassay. Percent coeffi-

Table 2. Reproducibility of the MBC immunoassay for MeOH extracts of blueberries

Blueberry sample	MBC, ppb	CV, % (intraassay)[a]	CV, % (interassay)[b]
1	25	8.7	9.8
2	48	3.2	20
3	55	17	9.6
4	123	6.6	20
5	62	5.4	18
6	63	11	11
7	39	11	21
8	46	4.5	22
9	65	21	12
10	54	9.8	21
11	151	8.8	20
12	63	3.4	7.5
13	63	11	9.9

[a] Percent coefficients of variation based on 6 determinations in 1 day.
[b] Percent coefficients of variation based on 4 determinations performed on 4 different days.

Table 3. Accuracy of MBC immunoassay for blueberries[a]

MBC added, ppb	MBC found, ppb	Mean rec., %	CV, %
27	28	103	9.4
54	56	103	13
108	110	102	19
216	253	117	14
810	980	121	8.9

[a] Mean and percent coefficients of variation based on 5 determinations.

cients of variation (% CV) ranged from 3.4 to 22.3, with most below 20. This range is considered excellent for a residue method.

MBC was determined in 13 blueberry samples (ranging from 25.2 to 151 ppb MBC) by EIA. Intra-assay and interassay precision for field blueberry samples is depicted in Table 2. The % CVs ranged from 3.2 to 22.0, with most % CVs being 20 or lower. Unlike the standard data in Table 1, the interassay data are not as reproducible as the intra-assay results. However, the precision was still good, especially in view of the quickness and simplicity of using MeOH extracts compared to the more complex LC method, which requires extensive cleanup.

Recovery studies were also performed on spiked organic blueberries. Results are given in Table 3. Spiking levels varied from 27 to 810 ppb, with recoveries ranging from 103 to 121% (mean recovery of 109%). Thus, the accuracy was acceptable.

Cross-reactivity of the benomyl antibody is shown in Table 4, with the structures of the cross reactants shown in Figure 1. As might be expected, other benzimidazole and thiophanate types of fungicides (thiophanate-methyl can undergo cyclization to MBC) show some cross-reactivity (8). MBC is the most cross-reactive. In fact, it has the same sensitivity as benomyl. Of the other 5 pesticides that show cross-reactivity, only thiabendazole is sufficiently reactive to be detected at residue levels. Seventy-one pesticides representing the major chemical classes showed no cross-reactivity at a concentration of 1.5 ppm, including 2-aminobenzimidazole (2-AB), a metabolite of benomyl that is not included as part of the tolerance for benomyl.

Table 4. Cross-reactivity of the benomyl antibody[a]

Pesticide	I_{50}, ppb[b]	LDD, ppb[c]
Benomyl	4.0	0.5
MBC	4.0	0.5
Thiabendazole	30.0	5.0
Thiophanate-methyl	200.0	43.0
Thiophanate	200.0	43.0
Procymidone	550.0	—
Vinchlozolin	1500.0	—

[a] The following pesticides showed no cross-reactivity at a concentration of 1.5 ppm: 2-aminobenzimidazole, iprodione, carbofuran, folpet, salithion, phosalone, alachlor, amitrole, chlormephos, bayleton, pronamide, guthion, tetradifon, metolachlor, dinoseb, acifluorfen, atrazine, benefin, diphenylamine, chlorothalonil, aldicarb, diazinon, metalaxyl, asulam, propachlor, thidiazuron, pyrolan, anilazine, bentazone, butylate, fluchloralin, butachlor, diphenamid, oxythioquinox, secbumeton, pyrazophos, metrazole, rubigan, basalin, morestan, pyrene, carbaryl, chlorpropham, acephate, mercarbam, nitralin, iodofenphos, bendiocarb, oryzalin, fluazifop-butyl, linuron, molinate, triforine, dodine, fenarimol, profluralin, monolinuron, methomyl, terbutylazine, barban, captofol, bensulfide, oxamyl, metoxuron, alar, tebuthiuron, terbutryn, diuron, phosmet, propoxur, mefluidide, and carbanolate.

[b] Concentration in ppb giving 50% inhibition.

[c] Least detectable dose in ppb estimated at % B_0 of 80.

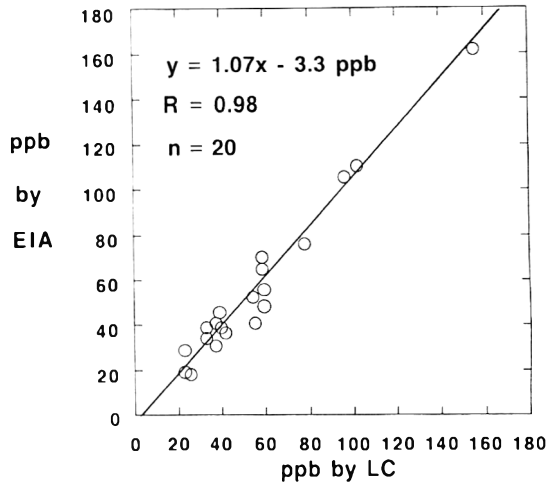

Figure 3. Correlation between MBC concentrations as determined by EIA and LC in MeOH blueberry extracts.

A correlation study between immunoassay and LC was performed on 40 blueberry samples. Twenty samples were negative for MBC by both methods, with a limit of detection of 18 ppb for both methods. The other 20 samples showed detectable levels of MBC by both analytical techniques; their correlation results are shown in Figures 3 and 4.

Figure 3 depicts the correlation for the 20 MBC-positive samples between LC, using the modified method of Gilvydis and Walters (8), and immunoassay, using the initial methanol extract from the LC method. The correlation coefficient, 0.87, is excellent considering that the initial crude extract from the LC method was used for the immunoassay analysis. Therefore, the only limitation on the number of samples that can be analyzed in 1 day is the number of extractions that can be done, because 8 samples can be analyzed simultaneously by immunoassay in 25 min.

Because a correlation was obtained that deviated from 1.0 for the MeOH extracts (r = 0.87), a second sample was taken after the methylene chloride partitioning during the MBC extraction and was analyzed by EIA. The correlation of these results with the LC data is shown in Figure 4. The improvement in correlation suggests some type of matrix effect from these blueberry samples that the methylene chloride partitioning step removes. The increased expense and sample preparation time required may not be warranted for rapid screening. The MBC

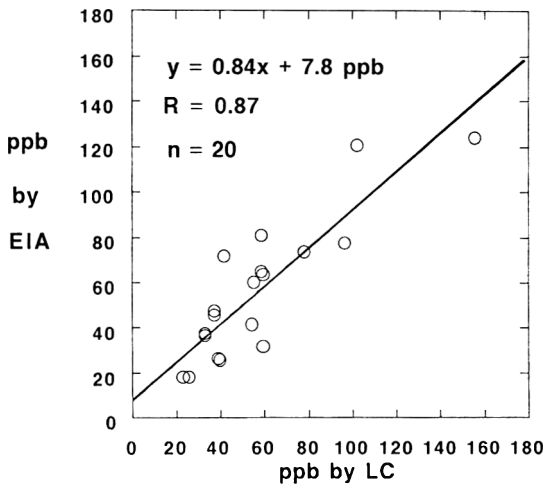

Figure 4. Correlation between MBC concentrations as determined by EIA and LC in MeOH blueberry extracts partitioned into $MeCl_2$.

levels found in the 20 positive blueberry samples by all methods are given in Table 5.

Immunoassay technology makes it possible to analyze a large number of samples with a significant reduction in cost because of less labor, capital equipment, and solvents. The need for testing increased numbers of samples for pesticides has become apparent during the last few years and will become increasingly important for proper risk assessment. Immunoassay technology should allow for the simple measurement of actual pesticide concentrations in foods, which will aid the scientists dealing with the dietary risk assessment process. At present, scientists trying to assess dietary risk from pesticides do not have sufficient residue data and have to make an assumption that the pesticide residue on that particular food is at the tolerance level (4, 14), which is usually not the case.

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Table 5. Comparison of immunoassay and LC for determination of MBC in blueberries

Sample	MBC, ppb		
	Immunoassay–MeOH	Immunoassay–$MeCl_2$	LC
1	32	49	59
2	60	41	55
3	18	18	25
4	42	53	54
5	37	34	33
6	46	31	37
7	48	41	37
8	65	70	58
9	72	37	41
10	64	56	59
11	81	65	58
12	38	39	33
13	18	29	23
14	26	39	40
15	27	46	39
16	121	111	102
17	124	162	155
18	78	106	96
19	74	76	78
20	18	19	23

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Automated Closed-System Headspace Determination of Methyl Bromide in a Variety of Raw and Processed Nuts

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An automatic gas chromatographic method was developed for determining methyl bromide residues in 11 different types of nuts. A 50 g sample is placed in a modified stainless steel blender container and mixed with 200 mL 0.5M sodium sulfate. The blender container is sealed with a screw lid, and the sample is blended 3 min and equilibrated 17 min in a bath-recirculator at 26°C. The headspace gas is automatically sampled and analyzed twice for methyl bromide residues. Recoveries are based on deviation from predetermined partitioning coefficients (*p*-values). The average *p*-values for the different nuts ranged from 0.28 to 0.43, and the CVs for their determinations ranged from 7.7 to 23.5%. The method is sensitive, simple, and reproducible, and it is operated in a totally closed system. Over 1100 samples of assorted nuts were analyzed within a 6-month period.

Methyl bromide (MB, bromomethane) has a registered use as an insecticidal fumigant for a variety of postharvest commodities. The U.S. Environmental Protection Agency (EPA) has expressed tolerances for MB in terms of inorganic bromide (INBR) residues in foodstuff. In recent years, EPA has solicited residue data for MB and INBR from the food industry and the U.S. Department of Agriculture (USDA) because of concern about the following MB toxic properties: time-weighted average (TWA), 5 ppm (20 mg/m^3) on skin;[1] short-term exposure limits (STEL), 15 ppm (60 mg/m^3) on skin; and Occupational Safety and Health Administration (OSHA) ceiling limit, 20 ppm (80 mg/m^3) on skin (1). Because MB is a gas (bp, 3.6°C; vapor pressure, 2 atm at 23.3°C) (2), sample residue integrity is difficult to maintain. Therefore, samples for MB residue analysis must be shipped to a laboratory at dry ice temperatures. Upon receipt, they must be stored in cryogenic freezers until analysis.

Methods of analysis for MB residues were determined in a number of matrixes using several techniques. Initially, wet chemical methods were used, as described by Stenger, Shrader, and Beshgetour (3, 4); MB was converted to INBR and the difference between the total bromide (Br^-) and INBR was measured. These methods used a muffle furnace to ash the sample and determined Br^- by titration. The methods were laborious, and only a few samples could be analyzed at one time. Reeves et al. (5) compared the ashing method to a modification of the Stijve (6) packed column gas chromatographic (GC) method using electron capture detection (ECD); however, the data comparisons varied greatly, and it was obvious that the GC method was superior after GC/mass spectrometric (MS) confirmation of the comparative samples.

Heuser and Scudamore (7, 8) and Scudamore (9) used cold solvent extraction and packed column GC/ECD to determine residues of MB in a variety of commodities. However, some extraction times exceeded 48 h for certain matrixes, which limited the number of samples that could be analyzed in a given time frame. Fairall and Scudamore (10) converted MB to methyl iodide (MI) after the same type of cold solvent extraction of the sample. With this method, they were able to detect most types of samples in the 10 ppb range because MI is more sensitive than MB by ECD. However, they found that certain commodities exhibited an artifact that co-eluted with the MI peak on GC.

Malone (11, 12), using a special glass apparatus, boiled grain in an acid medium and collected the volatile fumigants in a solvent at −86°C. An aliquot was taken for the ensuing GC/ECD analysis with a packed column. Recoveries of spiked samples ranged from 59 to 105%. Also, these methods were limited by the number of samples that could be analyzed in allocated time frames.

King et al. (13), using a headspace technique followed by packed column GC/ECD analysis, determined residues of MB in grapefruit. In this procedure, sample vapors were collected manually from the headspace of a modified blender container after the sample was blended with water. After equilibration time, an aliquot was taken from the headspace with a gas-tight syringe for manual injection on the GC. This method required minimal time for sample preparation and analysis, resulting in rapid turnaround for a large number of samples.

Page and Avon (14) reported a headspace technique using a gas-tight syringe with a modified needle assembly and determined MB residues by capillary GC/ECD and cryofocusing.

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[1] "Skin" notation indicates that there is a potential contribution to the overall exposure by direct contact with MB.

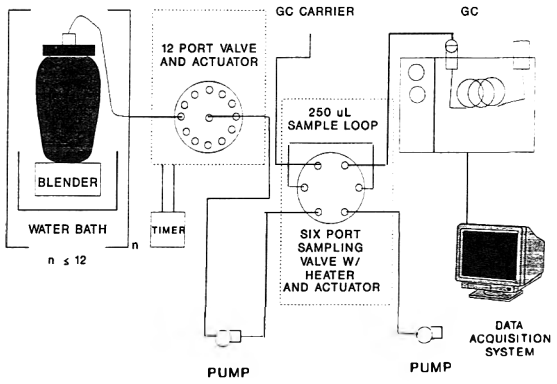

Figure 1. Diagram of automated headspace analyzer with blender/water bath, 12-port actuated valve, 6-port actuated sampling valve, gas chromatograph, and data acquisition system.

This method equilibrated the sample in a sodium sulfate (Na_2SO_4) slurry for 1 h at room temperature. Recovery data from spiked samples for a range of residue levels gave a CV of 6.8%.

DeVries et al. (15) developed a headspace technique using capillary GC/ECD, which could be automated for the analysis of large groups of samples. The analysis was accomplished without cryofocusing or cold-trapping of the sample. This technique, similar to the other headspace methods mentioned, provided rapid analysis and reproducibility of spiked samples.

In 1990, the U.S. Food and Drug Administration (FDA), under an interagency agreement with USDA, Animal and Plant Health Inspection Service (APHIS), Science and Technology (S&T), National Monitoring and Residue Analysis Laboratory (NMRAL), contracted to monitor pesticide residues in certain processed food commodities from various regions of the United States. One of the task assignments in this contract was to determine MB residues in processed nuts. Over 1100 samples of assorted nuts were collected for this survey and analyzed within a 6-month period. To accomplish this task within the allotted time frame, NMRAL developed an automated headspace capillary GC/ECD method. Residues can be confirmed by using photoionization detection (PID) in tandem with ECD. This method required no cryofocusing of the sample nor oven temperatures lower than 40°C.

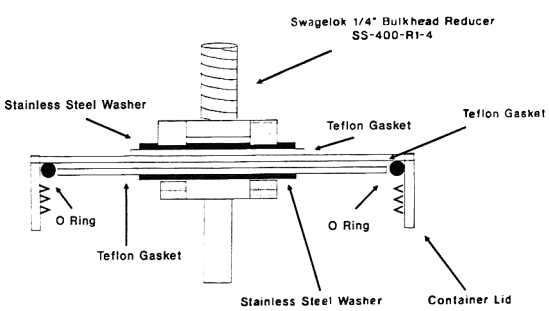

Figure 2. Diagram of modified blender, stainless steel screw lid.

Experimental

Apparatus

(a) *Gas chromatograph.*—Model 5700A (Hewlett-Packard Co., Palo Alto, CA 94303) equipped with a ^{63}Ni ECD system and a PID system, 10.0 eV lamp, 6-port electronically actuated sampling valve (Valco Instruments Co., Inc.) with 250 µL sample loop (Figure 1), and 30 m GS-Q Megabore™ 0.53 mm id capillary column (bonded Porapak Q particles, J & W Scientific, Folsom, CA 95630). Operating conditions: temperatures, injector 150°C, detector 300°C (ECD) and 180°C (PID); oven temperature program, 100°C for 5 min, then to 180°C at 20°C/min, and final hold at 180°C for 1 min. Helium carrier gas at 10 mL/min and 5% methane–argon make-up gas at 35 mL/min. Retention time of methyl bromide is ca 4.6 min on both detectors.

(b) *Headspace blender container assembly.*—Commercial blender (Waring Products Div., New Hartford, CT 06057), 2-speed, No. 8430 power unit. Blender container lid (Eberbach Corp., Ann Arbor, MI 48106) fitted with a 1/4 in. stainless steel Swagelok Bulkhead Reducer (SS-400-R1-4, Swagelok Co., Solon, OH 44139). Reducer is sealed to the screw lid with Teflon gaskets on both sides and tightened with a bulkhead nut (Figure 2).

(c) *Automated headspace sampling assembly.*—12-port electronically actuated valve (Valco). Up to 12 ports connect to each of up to 12 individual headspace blender container assemblies, (b), via lengths of 1/8 in. od, 1.5 mm id Teflon tubing (No. 200-32, Rainin Instrument Co., Inc., Woburn, MA 01801), using an appropriate valve and 1/8 in. Swagelok nuts (Figure 1).

The common port of the 12-port valve is connected to the suction side of a pump (135–200 mL/min 12 V D.C. minipump, Spectrex Corp., Redwood City, CA 94063) by a suitable length of 1/8 in. od, 1.5 mm id Teflon tubing. The discharge side of the pump is connected to the 6-port valve described in (a). The suction side of a second pump (135–200 mL/min 12 V D.C. minipump, Spectrex) is connected to the 6-port valve described in (a). The actuators of both the 12-port and 6-port valves are electrically connected to a digital programmable timer/controller (CD-03, ChronTrol Corp., San Diego, CA 92126) for synchronization and programmability.

(d) *Data acquisition system.*—Gas chromatographic data acquisition and processing are done with a Nelson Analytical Model 2600 chromatography system and AT&T 6386 microcomputer.

(e) *Permeation device.*—Model SC-100 calibrator equipped with a permeation tube rated at 10 958 ng/min, attached to the gas chromatograph to calibrate methyl bromide concentrations (Kin-Tek Laboratories, Inc., Texas City, TX 77590).

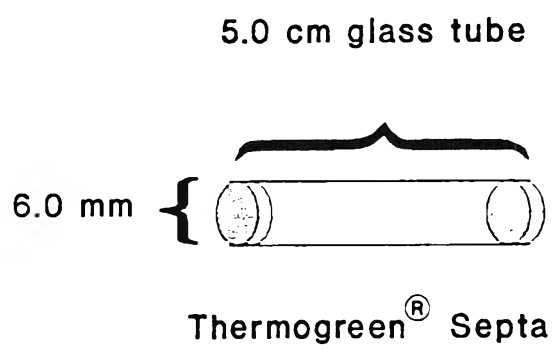

Figure 3. Diagram of septum vial used for control sample fortification.

(f) *Septum vial.*—5 cm glass tube made by cutting a 5.0 cm length from a Pasteur pipet (9 in. borosilicate glass, No. 72050, Kimble). Wash tube with detergent and rinse with water, acetone, and hexane. Reduce the size of septa (Thermogreen LB-2, 9.5 MM, No. 2-0652, Supelco, Inc., Bellefonte, PA 16823) to 6.0 mm, using a No. 4 cork borer. On each end of the 5.0 cm tube, insert a 6.0 mm septum to make a gas-tight vial (Figure 3).

(g) *Water bath/circulator.*—Water bath/circulator (Grant W38, Science/Electronics, Inc., Dayton, OH 45401) is used to maintain the temperature (26°C) of the blender containers with the sampler.

(h) *Syringes.*—50 mL Gastight (No. 1050, Hamilton Co., Reno, NV 89502); 5 µL (No. 160021), 10 µL (No. 160022), 25 µL (No. 160023), 50 µL (No. 160024), and 100 µL (No. 160025), Pressure-Lok (Dynatech Precision Sampling Corp., Baton Rouge, LA 70895).

(i) *Graduated cylinders.*—250 mL (Pyrex No. 3025) and 500 mL (Pyrex No. 3042).

(j) *Gas sampling bags.*—1 and 2 L, aluminum clad, laminated (Calibrated Instruments, Inc., Ardsley, NY 10502).

(k) *Volumetric flask.*—2 L (No. 28014, Kimax).

(l) *Magnetic stirrer.*—Thermolyne Nuova II Model SP 18425 (Barnstead/Thermolyne Corp., Dubuque, IA 52001); stirring bar; 1 1/2 × 3 in. (Cat. No. 37110-1128, Bel-Art Products, Pequannock, NJ 07440).

Reagents

(a) *Methyl bromide gas.*—Scott Specialty Gases, Houston, TX.

(b) *Nitrogen gas.*—Matheson Gas Products, Inc., Secaucus, NJ 07096.

(c) *Sodium sulfate.*—0.5M, prepared by weighing 142 g sodium sulfate (previously conditioned 24 h at 180°C) into a 2 L volumetric flask and filling to the mark with boiling LC grade or deionized water (previously extracted 5 times with isooctane, 100 mL/500 mL water). After solution cools, fill to mark with additional cool water.

(d) *Isooctane.*—Nanograde™ (Mallinckrodt, St. Louis, MO 63134).

(e) *Preparation of standards.*—Pure MB and MB concentrations in high purity nitrogen or air are maintained in compressed gas cylinders. These standards are certified in terms of purity for the neat MB or ppm of MB in the mixtures. They were compared with certified permeation tubes used in a permeation device for the calibration of the GC system, which will be described later in this paper. Working standards were made by transferring desired concentrations of MB from the cylinders to gas sampling bags, using regulators and tubing and further diluting to additional gas sampling bags by milliliter and microliter syringes, as needed.

All gas transfers were performed in a well-ventilated laboratory hood, and all gas sampling bag concentrations were stored under a hood after use for safety purposes. MB boils at 3.6°C. Because the density of MB at 25°C is 3.88 g/L, all dilutions and calibrations were maintained at this temperature. Low levels of standards were transferred from gas sampling bags to septum vials via a microliter syringe for GC calibration and sample spiking.

Calibration of Gas Chromatograph

The permeation device is equipped with a permeation tube rated at 10 958 ng/min at 25°C. Its operation is based on the molecular permeation of vapors emitted from the rated tube through a Teflon wall to provide a precise, controlled flow in µL/min of the pure component gas. This very small flow is mixed with a controlled flow of dilution gas (air or nitrogen) to form the concentration of calibration gas desired. This gas concentration is sampled by the automated sampling valve attached to the GC system. A specific volume is drawn into the fixed sample loop connected to the 6-port valve and then automatically injected into the GC system for calibration. The calibration units can be expressed in ng, ppb, or ppm.[2] The permeation device is operated for 16 h to reach equilibrium. After equilibration, and before it is calibrated, at least 3–4 injections are made onto the GC system to ensure stability of the gas concentration.

If a permeation device is unavailable, the GC system can be calibrated by adding a known amount of MB to a septum vial, then placing it into a headspace blender container, blending, and equilibrating as described in the method. An automated headspace sample can be taken for GC/ECD analysis. The GC calibration is based on the headspace volume, the amount of MB in the blender container, and the fixed sample loop volume attached to the 6-port valve.

Sample Preparation and Analysis

Weigh 50 g nuts into 250 mL graduated cylinder. Note volume and pour nuts into modified blender container. Add 200 mL 0.5M sodium sulfate solution. Install screw cover and hand-tighten. Attach 1/8 in. Teflon tubing to screw cover of Swagelok fitting and to appropriate port on 12-port valve. Headspace is now sealed. Blend 3 min at high speed. After blending, transfer blender container to water bath/circulator and let stand 17 min at 26°C. Once sample equilibrates, start

[2] Calibration in ppb or ppm is expressed on a volume-to-volume basis rather than a weight-to-weight basis and should not be confused with ppb or ppm of residue results.

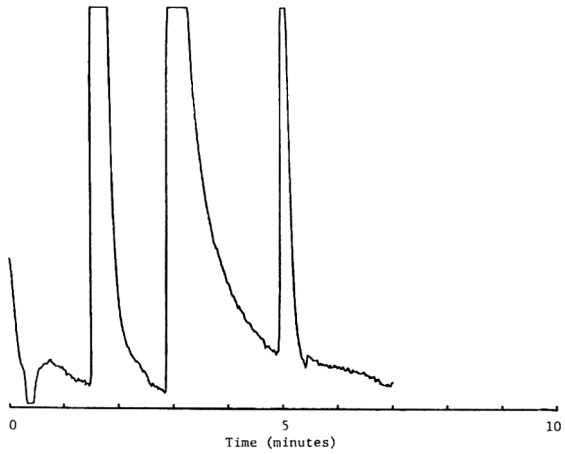

Figure 4. Chromatogram of headspace from walnut sample spiked at 776 ppb MB; MB retention time, 5.05 min. Conditions as listed in text.

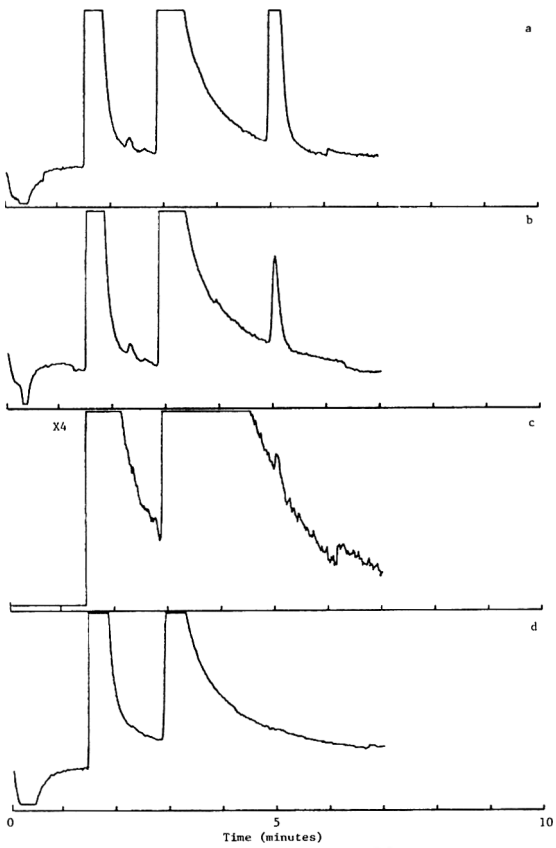

Figure 5. (a) Chromatogram of headspace from pistachio sample spiked at 200 ppb MB; MB peak indicated by arrow. Conditions as listed in text; MB retention time, 5.06 min. (b) Chromatogram of headspace from pistachio sample spiked at 20 ppb MB; MB peak indicated by arrow. Conditions as listed in text. (c) Chromatogram of headspace from pistachio sample spiked at 0.2 ppb MB; MB peak indicated by arrow. Conditions as listed in text. Chromatogram sensitivity expanded ×4. (d) Chromatogram of headspace from blank pistachio sample. Conditions as listed in text.

digital timer/controller. Once activated, timer/controller switches 12-port valve to appropriate sample container and turns on the 2 pumps, thereby connecting sample loop to sample blender of interest. Headspace gas is drawn into sample loop for 5.0 s, at which time pumps are turned off; 6-port valve is switched to inject position, and GC temperature programmer is started. After an additional 5 min (during which time methyl bromide elutes), 6-port valve is switched to "load" position and GC oven begins temperature programming to thermally strip column of extraneous material in preparation for next analysis. Analyze 2 headspace pulls for each sample and average results.

Determination of Partitioning Coefficients

Partitioning coefficients (*p*-values) for the 11 nut matrixes are determined at 8 different spike levels. Spike levels are made by adding the following amounts of methyl bromide to septum vials: 2.5 and 5.0 μL of a mixture of 60 mL pure methyl bromide and 1940 mL pure N_2 gas (v/v), and 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μL pure methyl bromide gas. The corresponding ppm levels are 5.84, 11.68, 38.8, 77.6, 155.2, 388.0, 776.0, and 1552.0, respectively. An appropriate amount of methyl bromide is injected into a clean septum vial with a gas-tight syringe. The fortified septum vial is placed in the bottom of a clean blender container; 50 g nut matrix (previously determined to be free of methyl bromide) is poured into the blender, and 200 mL 0.5M sodium sulfate solution is added. The screw cover is installed and hand-tightened, and the procedure is continued as described in *Sample Preparation and Analysis*. When the jar contents are blended, the septum vial is broken, thereby releasing the methyl bromide to interact with the sample matrix. The GC measures the MB gas in the headspace, and the data acquisition system calculates and prints the amount found in nanograms. This amount of MB is then converted to recovered ppm. The *p*-value is the ratio of MB in the headspace to the total spike amount:

$$p\text{-value} = \text{ppb MB in headspace/ppb MB fortified}$$

Sample Group Control Spikes

A group of samples is composed of 4 controls (a solvent blank, a matrix blank, and 2 levels of fortified matrix) and up to 10 samples. Two levels of fortification (194 and 388 ppb, respectively) are prepared by adding 2.5 or 5.0 μL pure methyl bromide to clean septum vials. Analyses are performed as described in *Determination of Partitioning Coefficients*.

Results and Discussion

Residue determination for MB in treated commodities has been difficult because of the nature of the compound. Because MB is a highly volatile gas with a low boiling point, sample enrichment is impossible unless the sample is maintained in a closed system. Therefore, a headspace method is the most logical approach for the development of reliable residue data. The approach of this method satisfies the need to rapidly analyze and report large numbers of samples within a short turnaround time. It also reduces systematic sample transfer errors that sometimes arise from man-

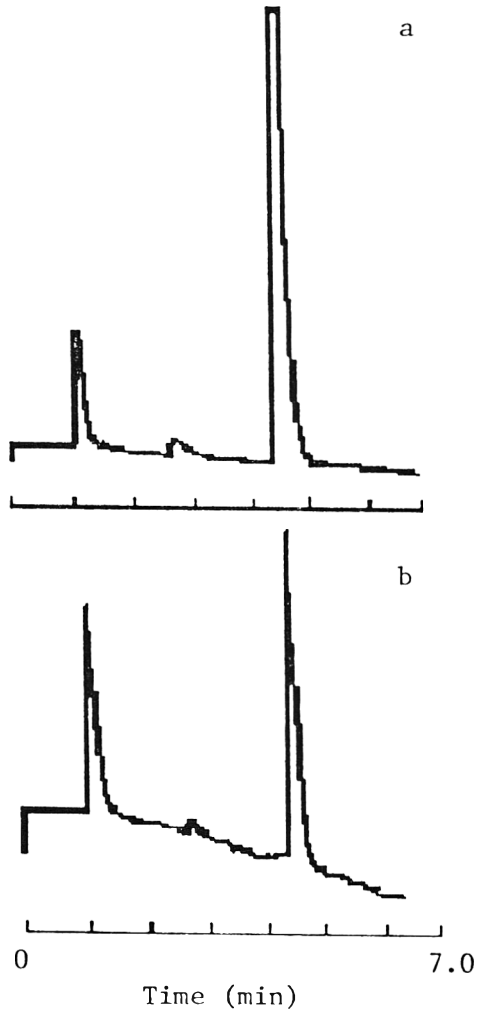

Figure 6. (a) Chromatogram of headspace from peanut sample spiked at 1 ppm MB; MB retention time, 4.63 min with ECD. Conditions as listed in text. (b) Chromatogram of headspace from peanut sample spiked at 1 ppm MB; MB retention time, 4.58 min with PID. Conditions as listed in text.

ual injections of samples, which are drawn from the headspace by a syringe and injected into the GC system.

The GC column used does not require subambient or cryogenic temperatures for MB analysis. Samples can be analyzed at an oven temperature of 80°C or higher, and the resulting chromatogram is well defined without spurious peaks and/or noisy base line (Figure 4).

The main advantage of this method is its simplicity. It is almost a push-button exercise. Once all GC and timer/controller parameters are set and the data system has been properly programmed, samples can be analyzed unattended for 24 h or longer if enough sampling containers, blenders, and water baths are available.

Legendre et al. (16) reported residue levels of MB at 1 ppb using an external, closed-inlet device (ECID) with a 10-port valve, solid-state cooled sample loop, and backflush interfaced to a GC system. Because of the restriction of the sampling vessel used, the maximum sample size was 1 g. The vessel was part of the ECID and was used to purge the MB onto the GC column. Although sensitive, the method suffers from nonhomogeneity and is not representative because of the restricted sample size. The headspace method addressed in this text uses a larger sample (50 g) and can detect 2 ppb MB routinely.

Table 1. *p*-Value determination for pistachios

Fort., ng	Fort., ppb	Rec., ppb	Calc. *p*-value
292	5.84	1.65	0.28
584	11.68	3.37	0.29
1940	38.80	14.35	0.37
3880	77.60	21.29	0.27
7760	155.20	45.60	0.29
19400	388.00	99.57	0.26
38800	776.00	188.85	0.23
77600	1552.00	467.18	0.30

Ford developed a method using portable GC (manufactured by XonTech, Inc., Van Nuys, CA) for determination of MB in spices (17). With this equipment, the stability of MB in the headspace was examined on an oregano sample spiked at 388 ppb. The headspace gas was sampled successively for 24 h, and the data generated gave a mean residue value of 342 ppb and a CV of 12.3% based on 116 analyses. In light of this information, a similar conclusion can be drawn for assorted nut samples fortified at comparable levels.

Initially, this same instrument was used in the analysis of nuts; however, the GC system had a limit of quantitation (LQ) of 100 ppb because its detector used a ^{3}H source. Using ^{63}Ni ECD provides more linearity and a significant increase in sensitivity for the determination of MB in nuts. Figure 5a shows a substantial signal for 200 ppb MB, which elutes at approximately 5.0 min. An LQ of 20 ppb (Figure 5b) can be routinely achieved. By increasing the scale sensitivity by a factor of 4, a level of 0.2 ppb (Figure 5c) can be detected. Figure 5d shows the chromatogram of the pistachio nut matrix blank.

Figure 6 shows 1 ppm of a spiked nut sample detected in tandem with ECD and PID. The sensitivity for MB on the PID appeared to be low; however, its electrometer was not operating at its highest sensitivity level. Further investigations are being made with the PID and other detectors, and these will be addressed in future publications.

Because a *p*-value is a physical constant (in this case for the distribution of MB in a given liquid and the headspace), it is assumed that the *p*-values will be unchanged for the commodity samples once equilibrium has been established. This value (previously defined) was developed for each nut type. Table 1 contains the following data pertaining to the *p*-value determination for pistachio nuts: fortification levels of MB in nanograms, the corresponding ppb (based on 50 g sample), the ppb of MB found in the headspace, and the calculated *p*-values. The *p*-value for the matrix is then calculated by averaging the individual trial *p*-values. Table 2 contains the average *p*-values, standard deviations, CV, and linear regression analy-

Table 2. Statistical analysis of *p*-value data for different nuts

Commodity	Av. *p*-value	SD	CV, %	Linear regression		
				Corr. coeff.	Y-inter.	Slope *p*-value
Blanch almonds	0.36	0.05	14.3	0.997	0.21	0.30
Honey roast P/N	0.33	0.05	14.2	0.999	0.13	0.28
Creamy P/N butter	0.37	0.07	18.8	0.982	0.44	0.24
Pistachios	0.29	0.04	13.0	0.995	0.07	0.29
Cashews	0.32	0.03	7.7	0.998	-0.07	0.34
Walnuts	0.35	0.08	23.5	0.999	0.03	0.33
Port. pine nuts	0.24	0.03	13.6	0.979	0.18	0.20
Chin. pine nuts	0.28	0.05	16.0	0.999	0.01	0.30
Dry roast P/N	0.43	0.05	11.1	0.998	0.17	0.39
Crunchy P/N butter	0.34	0.07	19.4	0.999	0.16	0.27
Pecans	0.28	0.04	14.5	0.994	0.26	0.22

sis for each of the 11 nut types addressed in this paper. The slope *p*-value is the *p*-value based on the entire raw fortification and headspace data for each matrix, and is coupled to the *y*-intercept. There is good agreement between the average *p*-value and the slope *p*-value in most cases.

Once the *p*-values are determined, groups of samples for a particular nut type are analyzed. Each group of samples contains controls, as stated in *Sample Group Control Spikes*. Corrections are calculated for the 2 control spikes by dividing the headspace determination by the *p*-value. The corrected headspace results are then divided by the fortification level and multiplied by 100% to obtain percent recovery. Therefore, recovery values for a particular set of spikes are actually the deviations from the original *p*-value determined.

Methyl bromide residues were determined in 1132 nut samples. Three residues were found: 0.030 ppm in a pistachio, and 0.017 ppm and 0.014 ppm in processed walnuts. This was expected because most of the samples were processed (heat-treated), and any residual MB would have been driven off by the heat treatment.

The method described was used to analyze raw and processed nuts and nut by-products. This technique, which is quite reproducible, was used successfully on other commodities with and without modifications. Some samples of high water content such as fruits and vegetables were found to have higher *p*-values because the MB was not absorbed by the oils as in the nuts. The method is also designed to analyze large groups of samples. After 100+ headspace samplings, the system must be thoroughly cleaned to ensure that there are not any constrictions in the tubing and valves caused by matrix material. These constrictions may cause reduced sensitivity to MB and/or the appearance of spurious GC peaks.

Acknowledgments

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A Survey of Benzene in Fruits and Retail Fruit Juices, Fruit Drinks, and Soft Drinks

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Recent findings of benzene in several fruit-flavored mineral waters at low μg/kg levels, reportedly arising from added benzoate, have prompted a survey of various fruits, juices, and drinks for traces of benzene. Headspace sampling, capillary gas chromatography, and mass spectrometric detection enabled detection with confirmation (full-scan spectrum) of benzene as low as 0.03 μg/kg. With selected ion monitoring, the method detection limit was 0.02 μg/kg, i.e., 3 times the analytical blank. In total, 97 samples were analyzed. Benzene was found at levels ranging from 0.018 to 3.83 μg/kg. Samples labeled to contain added benzoate or believed to contain natural benzoate, such as cranberries, were found to contain benzene at higher levels (n = 41, av. 0.66 μg/kg) than other samples (n = 32, av. 0.082 μg/kg). Average levels of benzene in fruits (as expressed juice), in juices with and without benzoate, in noncarbonated drinks with and without benzoate, and in soft drinks with and without benzoate were 0.042, 0.672, 0.056, 0.395, 0.116, 0.793, and 0.062 μg/kg, respectively.

In early 1990, considerable public and analytical interest was directed toward the occurrence of benzene in bottled mineral waters (1, 2) at levels exceeding the Canadian guideline of 0.005 mg/L as the maximum acceptable concentration (MAC) in drinking water (3). The U.S. Environmental Protection Agency (EPA) maximum contaminant level (MCL) for benzene is also 0.005 mg/L (4). It was reported that the benzene resulted from contaminated filtering equipment (5). Later in the same year, some samples of fruit-flavored mineral waters were also found to contain benzene at levels greater than 5.0 μg/kg (6). In this second instance, the mineral water was not found to be the source of benzene, and initial speculation suggested that the benzene could be a natural constituent of the fruits. It was also suggested that the benzene could arise from the long-term decomposition of sodium benzoate in the presence of ascorbic or erythorbic acid (7). Benzoic acid, or its sodium or potassium salt, is a permitted additive in fruit juices, fruit drinks, and other carbonated and noncarbonated beverages and functions as an antimicrobial agent. In Canada and the United States, the maximum permitted level, as benzoic acid, in these products is 1000 mg/kg (8, 9). The usual amount added to soft drinks ranges from 300 to 500 mg/kg (10). Benzoic acid, however, was also reported as a minor natural constituent in a number of fruits. In a survey for natural benzoic acid, Nagayama et al. (11) found the acid at <5 mg/kg in a variety of fruits and berries. Two exceptions were observed: cranberry-based drinks (20% cranberry juice) and strawberries had 77 and 14 mg/kg concentrations of benzoic acid, respectively. Others have also reported benzoic acid as a natural constituent in cranberries, prunes, plums, and most berries (12, 13). Benzene, as a result of the above-mentioned decomposition of benzoic acid, could possibly be expected to occur in fruits, juices, or drinks whenever the natural or added precursor acid is present. It was decided, therefore, to conduct a survey of freshly expressed fruit juice and retail samples of fruit juices, fruit drinks, and soft drinks to determine the levels of benzene in these products.

Purge-and-trap sample concentration and capillary gas chromatography (GC) with mass spectrometric (MS) detection is probably the most sensitive analytical technique available to determine benzene in fruit juices, fruit drinks, and other beverages. In our laboratories, however, this desired instrumental configuration was not available. We used the headspace (HS) sampling technique with capillary GC and MS detection. With this system, we were able to confirm the presence (full spectrum) and concentration of benzene at levels as low as 0.03 μg/kg in most samples.

This report describes the sample preparation and handling, the analytical methods used, the monitoring of laboratory contamination, and the results of a survey for benzene in fresh fruits and retail fruit juices, fruit drinks, and soft drinks. A study was made of the possible correlation between the levels of benzene found in the various products and the naturally occurring or labeled addition of benzoic acid or its salts.

Experimental

Apparatus and Instrumentation

To minimize contamination, glassware was rinsed with hot tap water and then with acetone and methanol, and blown dry with nitrogen; stirring bars were rinsed with hot water. Both

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were stored in a 60°C oven and removed and cooled to room temperature just before use. Septa were used as received; microliter syringes were rinsed with acetone and methanol and blown dry with nitrogen. Different microliter syringes were used for different concentrations.

(a) *Gas chromatograph.*—Model Vista 6000 with direct interface to the MS system was used (Varian Analytical Instruments, Sunnyvale, CA 94034). The GC system was equipped with cryogenic oven cooling, an on-column injector, and a DB-624 (1.8 μm film thickness) fused silica capillary column, 30 m × 0.32 mm (J&W Scientific Inc., Folsom, CA 95630). Operating conditions: He carrier gas at 1.5 mL/min; oven temperature program, −20°C (hold 1 min) to 200°C at 10°C/min and injector at 100°C.

(b) *Mass spectrometer.*—GC/MS was performed with a VG Analytical model 7070EQ tandem mass spectrometer (hybrid MS/MS with EBQQ configuration) using the conventional section (configuration EB) with direct interface to the GC system. The MS conditions were as follows: electron-impact ionization, 70 eV: source, all re-entrant and transfer line temperatures, 200°C; mass resolution, 1000. For repetitive scanning, the MS system was scanned exponentially down from 350 to 50 at 0.6 s/decade with 0.2 s interscan time. Data were acquired by a VG 11/250 data system. The reconstructed ion chromatogram (RIC) at *m/z* 78 (molecular ion) was examined, and the peak height was used for quantitation. The identification of benzene was confirmed by both retention time and a full spectrum (after background subtraction) down to 0.03 μg/kg. For selected ion monitoring (SIM), a resolution of 5000 (10% valley) was used. The ions monitored were *m/z* 78.0469 for benzene and *m/z* 68.9952 for PFK lock mass.

(c) *Headspace sampling syringe and needles.*—A 1 mL gas-tight locking syringe series A-2 (Precision Sampling Corp.) fitted with a 2 in. sideport needle was used for the headspace sampling. This needle was then replaced by a modified on-column injection needle, as described previously (14), for the on-column injection of the headspace sample.

(d) *Headspace sample vials.*—30 mL Hypovials, actual capacity ca 37 mL, were sealed after sample addition with Teflon-laminated silicon rubber discs (discarded after single use) and aluminum seals. Vials were tightly crimped so that the aluminum seals could not be turned. Magnetic Teflon-coated stirring bars (25 × 7.5 mm) were used in the vials.

Preparation of Samples

Fruits, fruit juices, fruit drinks, and soft drinks were purchased locally. The fruits, analyzed as their expressed juice, included replicate samplings of grapefruit, lemon, lime, orange, clementine (citrus fruit), kiwi, green and red grapes, apple, pear, and pineapple. Fresh cranberries were not available at the time of sampling. Expressed juice was obtained from peeled, diced fruit by using a 125 mL conical flask to press the juice from the fruit into a 400 mL beaker. The juice was decanted, filtered through glass wool, and stored in Teflon-lined screw-cap amber bottles. These samples were stored in the freezer (−20°C) until required. Other juices and drinks were stored unopened under refrigeration until analysis. The retail juices included apple, grape, lemon, lime, orange, pineapple, grapefruit, and a tropical mix (pineapple, orange, passion fruit, and tangerine). Fruit drinks based on apricot, lemon, cranberry, raspberry, grape, cherry, orange, apple, and mixed tropical fruit were sampled. The soft drinks included tonic, ginger ale, cola, root beer, cream soda, orange, lime, lemon, and several mixed tropical fruit-based drinks. Frozen juice and drink concentrates were thawed and opened, and representative aliquots were diluted with boiled liquid chromatographic (LC) grade water (Milli-Q system, Millipore Corp., Bedford, MA 01730) in the proportions recommended on the package. LC grade water was boiled 15 min in an electric kettle to further reduce volatile interferences. Periodically, a 20 g portion of this water was sampled and analyzed by HS/GC/MS, as described below, to monitor the residual level of benzene. A 20 g portion of each juice or drink was sampled for HS/GC/MS.

Standards and Quantitation

The stock solution was prepared by adding 10 μL benzene to a tared 10 mL volumetric flask containing methanol, reweighing to accurately determine the weight of added benzene, and diluting to volume. This stock solution and all other standards were diluted to volume at 4°C and stored under refrigeration at 4°C. Serial dilutions, using chilled (<4°C) pipets or syringes, were made into cold (<4°C) boiled LC grade water to obtain secondary standards of ca 0.8 or 0.08 μg/mL. Standard responses were obtained by adding microliter quantities of these aqueous standards to 20 mL of the boiled LC grade water and sodium sulfate in the headspace vial. Sample peak responses (height or area) were compared to those of similar standards for quantitation. Typical headspace standards were prepared at 0.08, 0.4, and 1.6 μg/kg. Other concentrations were prepared as required. The linearity of benzene response in the headspace procedure was studied by using standards prepared at ca 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 μg/kg.

Sampling

Samples of 20 g thawed, freshly expressed juice, retail fruit juice, fruit drinks, or bottled noncarbonated beverages at 4°C (refrigerator) were decanted into cold, tared 30 mL headspace vials and weighed on a top-loading balance. Before sampling, the vials were kept at 0°C in a water-ice bath and loosely covered with the silicon disc and aluminum cap. Each vial contained a magnetic stirring bar and 4 g anhydrous sodium sulfate, used to increase the headspace partition. Carbonated beverages in cans at 4°C were opened and the 20 g sample was withdrawn by using a cold (≤0°C) 20 mL Luer-lok syringe fitted with a syringe stopcock and a 6 in. needle (14 or 16 gauge). Before the analytical sample was taken, the syringe parts were prewetted by withdrawing a small amount of the cold sample, which was then discarded. The analytical sample was withdrawn slowly to minimize foaming in the syringe barrel. The sample was then slowly dispensed into the headspace vial.

Carbonated bottled drinks were sampled as shown in Figure 1 by using a 6 or 8 in. 18 gauge double needle/gas purge unit (Aldrich Chemicals Co., Inc., Milwaukee, WI 53201) fitted through a small hole in a threaded plastic cap (to fit the

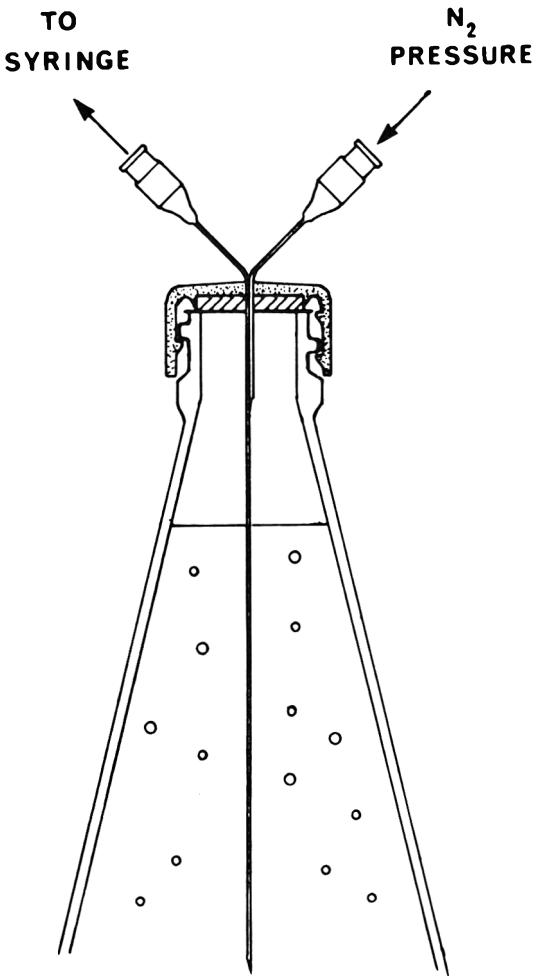

Figure 1. Sampling device for bottled drinks.

bottle). The needle unit was sealed into the cap by using a Teflon liner (next to the sample) and a 20 mm Teflon-lined silicone septum to provide a seal. The needle-cap assembly was screwed onto the cold (4°C) beverage bottle, nitrogen pressure was applied to the short needle, and the sample was forced into a cold (≤0°C) 20 mL Luer-lok syringe. The needle-cap assembly was removed and the sample was slowly dispensed, through the needle, into the headspace vial.

All sample weights were determined immediately after the sample was added to the vial. All vials were immediately sealed and brought to room temperature over a 15 min period with stirring in a 250 mL beaker containing ca 75 mL 45–50°C water. (Stirring bars trapped in the hydrated sodium sulfate can be released by carefully tapping the vial on a hard surface.) The vials were equilibrated 1 h (with stirring) before headspace sampling with the locking 1 mL gas-tight syringe.

Headspace Sampling

With the lock open, the barrel of the A-2 locking syringe and attached sideport needle were flushed by passing nitrogen through the needle. The plunger was replaced, trapping 1 mL nitrogen, the needle was inserted 1–2 cm through the septum of the headspace vial, and the 1 mL nitrogen was injected. The plunger was withdrawn slowly (5 s) to beyond the 1 mL mark and the barrel was allowed to fill (10 s). The plunger tip was aligned with the 1 mL mark, the syringe was locked, and the needle was withdrawn. The sideport needle was removed and replaced with the modified on-column injection assembly. The needle was inserted into the on-column injector, the syringe was unlocked, the headspace sample was injected onto the column, and the temperature program was started. Benzene was identified by its retention time and mass spectrum.

Repeatability and Benzene Partition Studies

To determine the variability (CV) associated with spiking standards in water, 6 separate vials each containing 20 g water, were individually spiked with 20 μL standard to give about 0.094 μg benzene/kg. To determine the CV of benzene as determined from 6 separate aliquots from a single sample of expressed juice, ca 200 mL freshly expressed grapefruit juice was prepared and six 20 g aliquots were taken and analyzed. To determine the CV of benzene as determined in the expressed juice from 6 individual fruits of the same type (purchased from the same store), the expressed juices from 6 lemons and 6 pineapples were separately collected and analyzed.

To compare the sample matrix effect of a particular sample to that of water, benzene partition studies were performed by comparing the peak responses obtained after identical microliter spiking into 20 g water (as standard) or 20 g sample (for recovery) to give about 0.8 or 1.2 μg benzene/kg. To estimate the actual percentage of benzene in the headspace at equilibrium, the response was determined by 1 μL injections of benzene in isopentane (0.938 μg/mL).

Evaluation of Laboratory Contamination

To estimate the possible increase in benzene levels in fresh fruit juice during sample preparation, when the samples are exposed to the laboratory environment (air) for a considerable time, 25 g samples of our boiled LC quality water and 25 g samples of an orange juice were placed in 100 mL beakers, exposed to the laboratory air for 0, 1, or 2 h, and then analyzed. On another day, other water and orange juice samples were exposed to the laboratory environment and the same glassware for the same times as during the juice preparation. The samples were then analyzed as before.

Results and Discussion

Benzene was determined in most of the fruits (as expressed juice), fruit juices, fruit drinks, and soft drinks by HS/GC/MS with the MS system operating in the repetitive scanning mode. It is known that 1,2-dichloroethane (*m/z* 98) coelutes with benzene (*m/z* 78) on the DB-624 column; however, the MS detection selectively determines only benzene. The benzene was quantified from the peak heights of the *m/z* 78 RIC and confirmed by a full-scan spectrum using background subtraction. In this mode, no coeluting peaks interfering with the full-scan spectra of benzene were noted with any of the samples. The detection limit was estimated to be about 0.03 μg/kg for a full-scan spectrum. The repetitive scanning mode was initially used, as screening for other volatiles was also being conducted

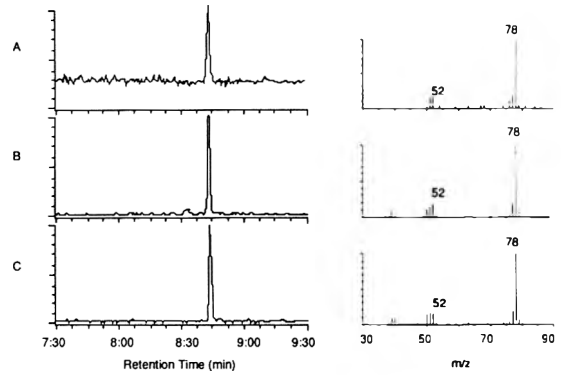

Figure 2. Reconstructed ion chromatograms and corresponding accumulated mass spectra of benzene in: A, freshly expressed grapefruit juice (0.049 μg/kg); B, orange juice labeled to contain benzoate (0.84 μg/kg); and C, standard (0.50 μg/kg).

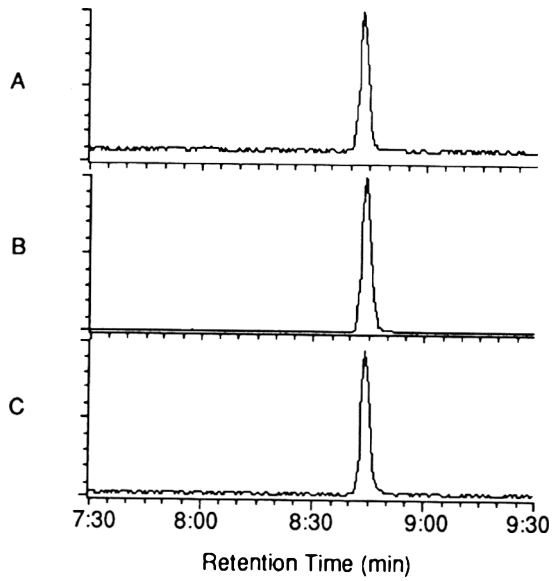

Figure 3. Selected ion chromatograms of benzene in: A, orange juice, benzoate not declared (0.027 μg/kg); B, orange juice labeled to contain benzoate (0.53 μg/kg); and C, benzene standard (0.047 μg/kg).

simultaneously. Later analyses were conducted with the MS system operating in the SIM mode at a resolution of 5000 (10% valley) for increased selectivity. In this mode, the computer storage requirements and data manipulation time are considerably reduced. Although the instrumental sensitivity is capable of detecting 0.001 μg/kg benzene with SIM (signal to noise ratio of 3:1), the overall method sensitivity is limited by the total analytical blank. With the boiled LC grade water, these blank values averaged 0.0066 μg/kg, giving a method detection limit of about 0.02 μg/kg, i.e., 3 times this average analytical blank. No attempt was made to lower this blank.

The differences in sensitivity of the benzene peak compared to baseline noise in the chromatograms obtained in the repetitive scanning and SIM operating modes of the MS system can be observed in Figures 2 and 3, respectively. As shown in Figure 2A, the baseline noise in RIC for the freshly expressed grapefruit juice at 0.049 μg/kg is greater than that in the selected ion chromatogram of the orange juice at 0.027 μg/kg in Figure 3A. A similar trend is observed in a comparison of chromatograms of orange juice at higher levels of benzene in Figures 2B and 3B.

A calibration plot of the benzene standard response over the 50-fold range studied showed a slightly decreasing slope with increasing concentration and a correlation coefficient of 0.9934. For quantitation of samples by comparison to individual standards, therefore, the standard was chosen so that its response did not differ from that of the sample by ±50%. All samples below 0.05 μg/kg, however, were quantified against the 0.05 μg/kg standard.

Repeatability and Benzene Partition Studies

Table 1 gives the repeatabilities and partition data for benzene in standards and samples. The CV for replicate (n = 6) determinations of individually spiked benzene standards in water at 0.094 μg/kg was found to be 6.49% by the SIM mode for detection. This mode may be expected to give better sensitivity and repeatability than that obtained by using RIC. With RIC, the CVs were 37.5% for replicate (n = 6) determinations of "natural" benzene in pooled grapefruit juice at 0.028 μg/kg and 15.9% and 15.3%, respectively, for the juices of 6 individual lemons and 6 pineapples at 0.046 and 0.056 μg/kg.

The relative partition data in Table 1 compare the headspace partition of benzene in the aqueous sample to the water standard and indicate the effect of the aqueous matrix. The samples and water were spiked at levels exceeding the blank levels of benzene to obviate the need to correct for blanks. The typical blank represented less than 1% of the benzene present. The differences between the spiked water and the sample result from a reduced or enhanced partition into the headspace of the lipophilic volatiles. This change in partitions is due to increased (or decreased) solubility in (or affinity to) the sample liquid phase compared to that of the aqueous standard. The lower partition is more apparent in the orange juice, probably because of analyte solubility in the natural orange oils, than in the pineapple juice or the tonic water. In fact, the tonic water partition into the headspace is slightly greater than that of water, possibly because of dissolved sugars. In our survey, all the samples were quantified by comparison to benzene standards in water. Ideally, each analytical sample should be quantified by standard additions or against a spiked identical sample matrix to obtain accurate quantitation. Because of the variety of samples analyzed, however, quantitation by these methods was not practical. Therefore, the reported HS/GC/MS survey results in Table 2 may, in fact, be slightly higher or lower than the actual values as a result of the matrix effect.

The actual percentage of benzene that partitions into the headspace of an equilibrated standard was found to be about 31%. This figure was calculated by using the headspace volume (17 mL) and comparing the benzene peak area of a 1 μL

Table 1. Repeatability and partition (vs water) of benzene in standards, fruit juices, and drinks

Sample	Benzene, μg/kg	Repeatability, CV, %	Partition, %, vs water
Water (6 spikes)[a]	0.094	6.49	—
Grapefruit (6 aliquots)[b]	0.028	37.5	—
Pineapple (6 fruits)[b]	0.056	15.3	—
Lemon (6 fruits)[b]	0.046	15.9	—
Orange juice (spike)[a,c]	1.17	—	65.5
Orange drink (spike)[b,c]	0.83	—	80.6
Pineapple (spike)[a,c]	1.17	—	100.8
Tonic water (spike)[a,c]	1.17	—	103.2

[a] Quantitation by peak areas from SIM chromatograms.
[b] Quantitation by peak heights from RIC.
[c] Average of duplicate spikes.

(0.938 ng) direct injection to that of a 1 mL headspace injection taken over 20 g of a 1.173 μg/kg standard.

Laboratory Contamination

The results of the laboratory contamination study show virtually no contribution from the laboratory environment to the blank water and orange juice samples. The water blank, with an initial benzene level of 0.013 μg/kg, showed no increase when exposed to the laboratory environment or to the juice preparation glassware for up to 2 h. The orange juice blank of 0.026 μg/kg actually decreased to 0.014 μg/kg after a 2 h exposure. These analyses demonstrate that benzene contamination during the analytical method is not a problem in our laboratories.

Sampling Problems

In every analysis for volatile compounds, there is always the problem of analyte loss by volatility when the standard or sample is transferred from one container to another. Steps taken to minimize this loss include cooling the syringes, other equipment contacting the sample, samples, and standards to reduce analyte volatility; minimizing manipulative steps; and transferring samples by using inert gas pressure and syringes rather than reduced pressure and pipets.

In the sampling of carbonated drinks, however, volatile losses can occur as soon as the container is opened; the pressure over the drink falls to atmospheric pressure, and degassing, with possible purging of the analyte, can occur. Furthermore, degassing, and possible volatile loss, can also occur during any subsequent sample manipulation (pouring, pipetting, or other sample transfer). With headspace analysis, it is especially difficult to avoid degassing when the sample is added to the headspace vial containing the sodium sulfate. The sampling device for bottled samples shown in Figure 1 is designed to prevent or reduce degassing during sampling from a bottle. Cold aqueous standards in water, when decanted into the headspace vial, however, give only a slightly (<5%) lower response than equivalent standards prepared by syringe spiking through 20 mL water to the bottom of the vial, which was quickly sealed. Thus, with noncarbonated samples, losses on sample decanting should be minimal; for pressurized products, reported results are probably less than the actual volatile level in the unopened product.

Survey Results

The results of the survey for benzene in freshly expressed fruit juice, retail fruit juices, fruit drinks, and soft drinks are given in Table 2. As noted above, the reported levels of benzene are not enhanced by any background contamination; however, these results may be compromised by the matrix effect and by slight losses of benzene that may occur during sampling, especially with carbonated drinks.

Examples of the chromatograms obtained in the repetitive scanning and the SIM modes for a freshly expressed juice and a retail juice without added benzoate are shown in Figures 2A and 3A, respectively. Similar chromatograms of orange juices with added benzoate are shown in Figures 2B and 3B, respectively.

The occurrence of benzene and the levels reported in Table 2 are not entirely representative of the marketplace. The survey was undertaken in 2 phases. The first sampling was performed without regard to added sodium benzoate. Of the fruit juices selected, only 2 of 15 were labeled to contain benzoate; of the fruit drinks, 1 of 15; and of the soft drinks, 9 of 15. The second sampling was biased toward samples containing added benzoate to investigate the possibility of benzene arising from the use of this additive. Several samples of cranberry drinks were also selected to explore the possibility of naturally occurring benzoic acid as a source of benzene.

In Table 2, the average benzene level found in the freshly expressed juices (0.042 μg/kg) was only slightly less than that found in the retail samples of juice not labeled to contain benzoate. If the single highest juice sample, at 0.236 μg/kg, were excluded, the average benzene level in the retail juice samples would be 0.041 μg/kg, essentially the same as that of the fresh fruit, and the range would become 0.022–0.063 μg/kg, again equivalent to that of the freshly expressed juice (0.018–0.071 μg/kg). With the fruit juices labeled to contain benzoate, the average benzene levels were about 10 times as high. A similar trend is noted with the carbonated soft drinks, the samples labeled as containing benzoate being about 10-fold higher. With the fruit drinks, however, the higher benzene levels are

Table 2. Benzene levels found in fruits (expressed juice), fruit juices, fruit drinks, soft drinks, and other drinks by HS/GC/MS

Sample	No. samples	Benzoate on label	Benzene, μg/kg	
			Range	Av.
Fruit (as juice)	24	—	0.018–0.071	0.042
Juice (retail)	10	Yes	0.14–1.5	0.67
	13	No	0.022–0.24	0.056
Fruit drinks	7	Yes	0.045–0.28	0.14
Include cranberry	16	No	0.011–1.8	0.29
Exclude cranberry	13	No	0.011–0.66	0.12
Cranberry only	3	No	0.46–1.8	0.95
Carbonated soft drinks	6	No	0.029–0.12	0.062
	20	Yes	0.013–3.8	0.79
Iced tea	1	Yes	0.55	0.55

not unequivocally associated with labeled benzoate. This may be due to naturally occurring benzoic acid in a juice, such as that from cranberries or other berries, or to undeclared added benzoate. Similar low levels of benzene were also found in drinks labeled as containing benzoate. Benzoic acid was not determined quantitatively in any of the samples, either to verify benzoate label declarations or to measure naturally occurring benzoate. In addition, no attempt was made to determine the production date of any beverage. With all other factors equal, one would expect the level of benzene in a benzoate-containing beverage to increase with benzoate concentration as well as with time.

The average level of benzene in samples with benzoate declared on the label was 0.63 μg/kg for 38 samples. If the 3 samples of cranberry juice (natural benzoic acid) are included, the average for the 41 samples becomes 0.66 μg/kg. For the 32 retail juices and drinks not labeled to contain benzoate and not including cranberry juice, the average was 0.082 μg/kg. The highest level of benzene found, attributed to added benzoate, was 3.83 μg/kg in a soft drink. Benzene at 1.81 μg/kg was found in a cranberry drink, with no declared benzoate. For the 73 bottled juices and drinks, the average benzene level was 0.40 μg/kg.

Source of Benzene

The decomposition of the added antimicrobial benzoate salts or benzoic acid in the presence of ascorbic or erythorbic acid was proposed as a possible source of benzene in fruit drinks (7). The results of Table 2 support the involvement of benzoate. The listing of benzoate salts as an ingredient appears to be associated with an approximate 10-fold increase in the concentration of benzene in fruit juices and soft drinks compared to those beverages without added or sufficient natural benzoate. Benzoate in the fruit drinks, however, was not necessarily associated with the findings of higher benzene levels. Traces of benzene in all samples containing added benzoate or natural benzoic acid are expected to increase with time.

Clearly, the higher levels of benzene found (>0.1 μg/kg) are associated with benzoate or benzoic acid. The consistent but low concentrations of benzene in the expressed or retail fruit juices, however, suggest that benzene should be considered as a naturally occurring constituent of the fruit but not necessarily arising from traces of benzoic acid. Studies demonstrated that laboratory contamination is minimal. None of the samples was analyzed for benzoic acid to verify label claims or to estimate concentrations of the naturally occurring acid.

Conclusions

A comparison of the benzene levels in the fruit juices, fruit drinks, and soft drinks, with and without label-declared benzoic acid or its salts, strongly suggests that the enhanced levels of this contaminant are due to the benzoate additive. Naturally occurring benzoic acid must also be considered as a possible source of "natural" benzene, as higher levels were found in fruit drinks in which benzoic acid or its esters were reported to occur naturally. The low concentrations of benzene found in freshly expressed fruit juice also suggest that benzene is a natural constituent of the fruit. Other experiments showed contamination from the laboratory environment to be negligible. The highest level of benzene found in the 73 bottled juices and drinks analyzed was 3.8 μg/kg. The average level was 0.40 μg/kg. This level is more than 10-fold lower than the Canadian or EPA guideline of 0.005 mg/L (5 μg/kg) for drinking water, and is not considered to represent a health concern.

Our studies demonstrated the HS/GC/MS method used to be reproducible, sensitive, and selective for the determination and confirmation of benzene at concentrations as low as 0.03 μg/kg in fruit juices, fruit drinks, and soft drinks.

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SEAFOOD TOXINS

Production and Characterization of Antibodies Against Neosaxitoxin

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Antibody against neosaxitoxin (neo-STX) was obtained from rabbits after immunization with neo-STX conjugated to either keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). An indirect enzyme-linked immunosorbent assay (ELISA), in which either neo-STX-BSA or neo-STX-KLH was coated to the microplate, was used to monitor the antibody titer. Although high antibody titers were obtained from rabbits after immunization with both immunogens, only antibody obtained from rabbits immunized with neo-STX-KLH was useful for immunoassay. Competitive indirect ELISA revealed that the antibodies obtained from rabbits immunized with neo-STX-KLH are specific for neo-STX but also have good cross-reactivity with STX. The concentrations causing 50% inhibition binding of neo-STX-BSA to the anti-neo-KLH by neo-STX, STX, and decarbamoyl-STX (DC-STX) were 0.9, 8.0, and 53.1 ng/mL, respectively. Saxitoxin conjugated to polylysine (STX-PLL) was also used as the coating reagent in the indirect ELISA. The concentrations causing 50% inhibition binding of anti-neo-STX-KLH to STX-PLL coated on the microtiter plate by neo-STX, STX, and DC-STX were 1.2, 4.1, and 36.1 ng/mL, respectively. With this newly developed antibody, ELISA could be a very effective method for monitoring seafood for both neo-STX and STX.

Saxitoxin (STX) is one of the most important and potent of a group of toxins involved in paralytical shellfish poisoning (PSP) (1–5). The toxin is produced predominantly by the dinoflagellate *Alexandrium (= Protogonyaulax) catenallum* and *A. tamarense*, and it is primarily encountered in toxic mussels, clams, and other marine animals. In addition to dinoflagellates, fresh-water cyanobacterium (blue-green alga) *Aphanizomenone flos-aquae* is also known to produce STX and neosaxitoxin (neo-STX) (6). The toxins involved in PSP tend to have little adverse effect on the shellfish using the dinoflagellate as a food source. However, human ingestion of toxin-contaminated shellfish may result in PSP, which can be fatal (3, 5).

Because of the potential health hazard, a quick, sensitive, and specific method is needed to determine the presence of toxin in shellfish. Both mouse bioassay and chemical methods were used for the analysis of PSP toxins (7–12). With the availability of antibodies against STX, several immunoassay protocols (13), e.g., hemagglutination (14), radioimmunoassay (15), and enzyme-linked immunosorbent assays (ELISA) (16–18), were developed. Monoclonal antibodies against STX (19, 20) were produced, but these monoclonal antibodies had lower affinity to STX than the polyclonal antibodies. A commercial ELISA kit is also available (Inst. Armand-Frappier, Laval, Quebec, ON, H7N 423, Canada). However, antibodies used in these ELISAs are very specific for STX, with little cross-reaction with other STX-related PSP toxins. The cross-reactivity with neo-STX was only 2% that of STX (16–18). Because neo-STX is also frequently involved in PSP, the use of these antibodies in the analysis of PSP could result in underestimation of the total toxin concentrations in naturally occurring outbreak samples.

In the present study, polyclonal antibodies against neo-STX were developed. We found that these antibodies also have good cross-reactivity with STX. Details for the production of antibody in rabbits and characteristics of the polyclonal antibodies, as well as the use of these antibodies in an indirect ELISA for neo-STX and STX, are described in this paper.

Experimental

Materials

(a) *Saxitoxin.*—All chemicals and organic solvents were reagent grade or better. Purified STX was kindly provided by E.J. Schantz (FRI, UW) and R.W. Wannemacher, Jr (USAMRIID). Neo-STX was prepared in the U.S. Food and Drug Administration laboratory by the method previously described (5). Decarbamoyl (DC)-STX was prepared by hydrolysis of STX in the presence of 6N HCl according to the methods of Ghazarossian et al. (21) and Koehn et al. (22).

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(b) *Tritiated saxitoxinol (STXOH).*—Prepared by reduction of STX with tritiated $NaBH_4$ (50 Ci/mmol, New England Nuclear, Boston, MA) (21, 22) and purified by affinity chromatography using the antibodies against STX.

(c) *Keyhole limpet hemocyanin (KLH).*—Obtained from Pierce Chemical Co., Rockford, IL 61105.

(d) *Bovine serum albumin (BSA, RIA grade), o-phenylenediamine, and Tween 20.*—Sigma Chemical Co., St. Louis, MO 63178.

(e) *Hydrogen peroxide.*—30% solution (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(f) *Freund's adjuvant.*—Complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37 Ra and incomplete Freund's adjuvant were obtained from Difco Laboratories, Detroit, MI 48232.

(g) *Enzymes.*—Goat anti-rabbit IgG-peroxidase conjugate and horseradish peroxidase (HRP) (ELISA grade, Cat. No. 605 220) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

(h) *ELISA microtiter plates and minisorp RIA tubes.*—Nunc, Roskilde, Denmark.

(i) *Rabbits.*—*Pasteurella*-free female New Zealand rabbits were purchased from LSR Industries, Union Grove, WI.

Preparation of Immunogens

Saxitoxin and neo-STX were conjugated to KLH and BSA or polylysine via the Mannisch reaction under the conditions previously described (16). In general, 10 mg protein carrier was reacted with 1 mg neo-STX. Formaldehyde was used as the cross-linking reagent. Because of limited amounts of conjugates available, the amount of neo-STX conjugated to each mole of protein carrier was not determined.

Production of Antibody

The immunization schedule and methods of immunization were essentially the same as those described for T-2 toxin (23) by the multiple injection technique. Two immunogens, neo-STX-KLH and neo-STX-BSA, were tested with 3 rabbits in each group. Each rabbit was injected intradermally with 500 μg immunogen in 1.0 mL 0.1M phosphate buffer (PB) containing 0.85% NaCl (PBS) (pH 7.4), with 2.0 mL complete Freund adjuvant. For booster injections, 300 μg antigen in 1.0 mL PBS and 2.0 mL incomplete Freund adjuvant was used. The collected antisera were precipitated with $(NH_4)_2SO_4$ to a final saturation of 35%. Precipitates were redissolved in water and reprecipitated twice. Finally, precipitates were reconstituted to half the original volume with distilled water and dialyzed 0.5–1 h against distilled water, then against 0.01M PB overnight at 6°C, and lyophilized.

Monitoring Antibody Titers by Indirect ELISA

The protocol for the indirect ELISA is similar to that described for STX (16). Depending on the immunogen used in the immunization, a neo-STX-protein conjugate with a protein carrier that is different from the protein carrier for immunogen was used in each assay. For example, neo-STX-BSA was used for monitoring the antibody titers of rabbits that were immunized with neo-STX-KLH. In general, 0.1 mL neo-STX-protein (1 μg/mL in bicarbonate buffer at pH 9.5, or 100 ng/well) was added to each well of a 96-well ELISA microtiter plate (Nunc plate 2-69620). The plate was kept at 6°C overnight. After the solution was removed, wells were washed 4 times with 0.35 mL PBS–Tween buffer (0.01M phosphate saline buffer, pH 7.4, with 0.5% Tween 20). This was followed by incubation with 0.17 mL 0.1% gelatin in 0.01M PBS (serves as the blocking agent) 30 min at 37°C. The plate was washed 4 times with 0.35 mL PBS–Tween to remove the excess blocking agent. To each well, 0.1 mL portions of various dilutions of anti-neo-STX antiserum were added, and the solutions were gently mixed. The plate was incubated 1 h at 37°C and then washed with 0.35 mL PBS–Tween 4 times. Goat anti-rabbit IgG-HRP conjugate (0.1 mL) at 1:20 000 dilution in 0.01M PBS was added to each well. The plate was incubated 1 h at 37°C and then washed, and 0.1 mL freshly prepared OPD-substrate solution was added. After 10 min of color development in the dark at room temperature, the reaction was terminated by adding 0.1 mL 1N HCl. The absorbance at 490 nm was determined in an automatic ELISA reader (THERMOmax microplate reader, Molecular Devices Co., Menlo Park, CA); samples were run in triplicate.

Competitive Indirect ELISA

For antibody characterization or analysis of STXs, a competitive indirect ELISA was used. The protocol was similar to those for monitoring antibody titers as described above, except that 50 μL antiserum at an appropriate dilution and 50 μL neo-STX (or STX) at different concentrations were added to the neo-STX-protein conjugate-coated microplate well. The optimum dilution of neo-STX-protein coated on the microtiter plate was determined by titration against various serum dilutions.

Radioimmunoassay (RIA)

Protocols for RIA were essentially the same as those described for T-2 toxin, except that ethanol was used as the reagent to separate the free and bound toxin (23) instead of ammonium sulfate. In general, 100 μL radioactive STXOH (ca 11 450 dpm) was incubated with 0.1 mL antiserum solution of various dilutions in phosphate buffer (0.01M, pH 7.5) at room temperature for a minimum of 5 min (5–30 min). For competitive RIA, 50 μL radioactive STXOH (ca 11 450) and 50 μL of various concentrations of different STX derivative were incubated with 100 μL diluted antiserum. The reaction mixture (200 μL) was first precipitated with 400 μL absolute EtOH; the precipitate was dissolved in 100 μL deionized water and precipitated again with 300 μL EtOH. The radioactivity of the pooled, unbound ligand (1 mL solution) was determined in a Beckman model LS-5801 liquid scintillation spectrometer in 5 mL Ready-Solu™ MP (Beckman, Fullerton, CA).

Results and Discussion

Production of Antibody

An indirect ELISA was used to monitor antibody production. Because some nonspecific binding of pre-immune serum

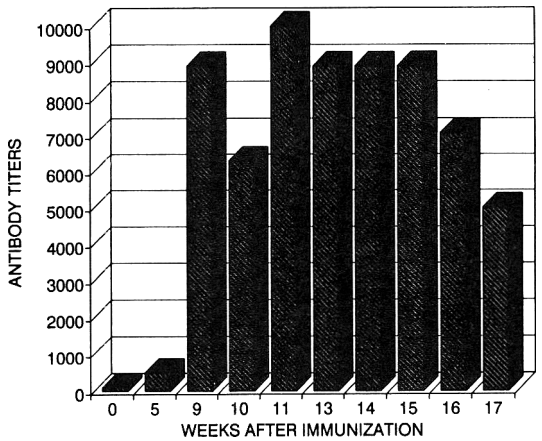

Figure 1. Antibody titers against neo-STX from rabbit No. 28. Neo-STX-BSA at a concentration of 1.0 μg/mL was coated on the ELISA plate. Booster injections were made at the 6th, 10th, and 15th week after initial immunization.

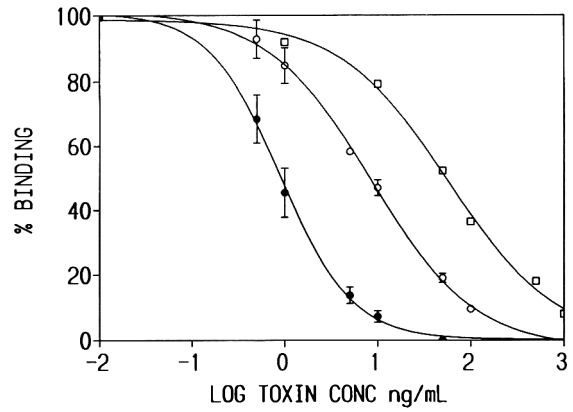

Figure 2. Competitive indirect ELISA of neo-STX using anti-neo-STX-KLH antiserum. Neo-STX-BSA (1.0 μg/mL) was coated on the ELISA plate. The antiserum dilution (rabbit No. 28, 9th week bleeding) was 1:1000. Neo-STX (●), STX (○), and dec-STX (□). Data with no-error bars indicate that the errors were within the size of the symbols. For dec-STX, data for average of 2 experiments are presented; thus, no-error bars are not shown.

to the coated microtiter plate was observed, a preselected absorbance of 0.6 was arbitrarily used in the estimation of antibody titer. Thus, the antibody titer was defined as the reciprocal of the antiserum dilution that gives an absorbance of 0.6 at 490 nm under the indirect ELISA conditions described. The antibody titers for the pre-immune serum varied from 100 to 300. Among 3 rabbits for each immunogen tested, only 2 gave good response. The rabbits started to elicit antibodies as early as 5 weeks after immunization. Antibody titers for one of the best rabbits (No. 28) immunized with neo-STX-KLH are shown in Figure 1. Although good antibody titers were also obtained from rabbits that were immunized with neo-STX-BSA, free-neo-STX could not compete for the binding of the antiserum with solid-phase neo-STX-KLH conjugate. Thus, the antisera obtained from this group of rabbits were not characterized further.

Specificity of Antibody

The specificity of the antibody was determined by a competitive indirect ELISA. The conditions for the analysis were optimized by a "checkerboard" titration. In general, with the minimum amount of antigen coated in the plate, the antiserum dilution that was used in the assay gave an absorbance in the 0.7–1.0 range of the final substrate reaction (70% of the maximum absorbance in the titration). The typical inhibition curves, in which neo-STX, STX, and dec-STX were competing for the binding of the anti-neo-STX-KLH antibody with neo-STX-BSA as the solid phase, are shown in Figure 2. The concentrations causing 50% inhibition of binding of anti-neo-STX-KLH antibodies to the solid-phase antigen neo-STX-BSA (coated on the plate) by neo-STX, STX, and DC-STX were found to be 0.91, 8.0, and 53.1 ng/mL, respectively. Thus, the relative reactivities of neo-STX, STX, and DC-STX to the antibody are 100, 11.4, and 1.7%, respectively.

The linear response of inhibition of binding by neo-STX and STX in the competitive indirect ELISA was in the 0.5–5 ng/mL range (25–250 pg/assay) and 2–20 ng/mL (100–1000 pg/assay). If we assume that the concentration of neo-STX and STX causes a 20% inhibition of binding as the minimum detection level in these immunoassays, the minimum detection levels for neo-STX and STX in indirect ELISA would then be 25 and 100 pg/assay, respectively. The present ELISA is considerably more sensitive than indicated in an earlier study in which anti-STX-BSA antibody was used (16). The concentrations for 50% inhibition binding of the anti-STX to STX-PLL by neo-STX and STX were 180 and 3.2 ng/assay, respectively.

Present results clearly indicate that the antibodies have good cross-reactivity with both STX and neo-STX. In contrast, the anti-STX-BSA antibodies, which are currently used in various immunoassays (15–18), are highly specific for STX with less than 2% cross-reactivity with neo-STX (15–18). Data are consistent with the observations for several other haptens (24). When an immunogen containing an additional hydroxyl group in the side chain of a hapten molecule is used in the antibody production, the antibodies generally have good cross-reactivities with both the hydroxylated and nonhydroxylated haptens. Conversely, the antibodies have weak cross-reactivity with the hydroxylated derivatives when the nonhydroxylated derivative is used (24).

Indirect Competitive ELISA Using STX-PLL as Coating Reagent

Because the data show that the antibody has good cross-reactivity with STX, we also tested whether the STX-polylysine (STX-PLL) could be used as the coating reagent in the indirect ELISA. Results, shown in Figure 3, indicate that STX-PLL could indeed be substituted for neo-STX-BSA in the assay. The

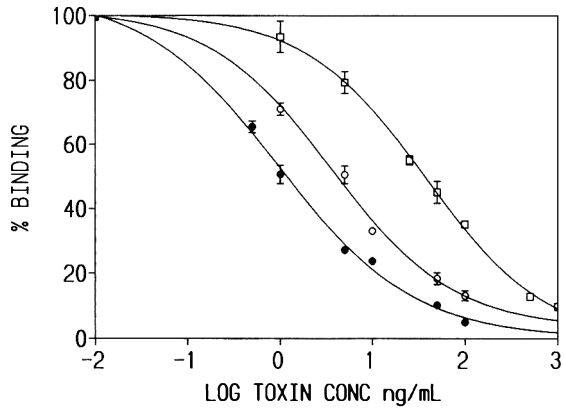

Figure 3. Competitive indirect ELISA of neo-STX using anti-neo-STX-KLH antiserum. STX-polylysine (1.0 μg/mL, or 0.1 μg/well) was coated on the ELISA plate. The antiserum dilution (rabbit No. 28, 9th week bleeding) was 1:1000. Neo-STX (●), STX (○), and dec-STX (□). Data with no-error bars indicate that the errors were within the size of the symbols.

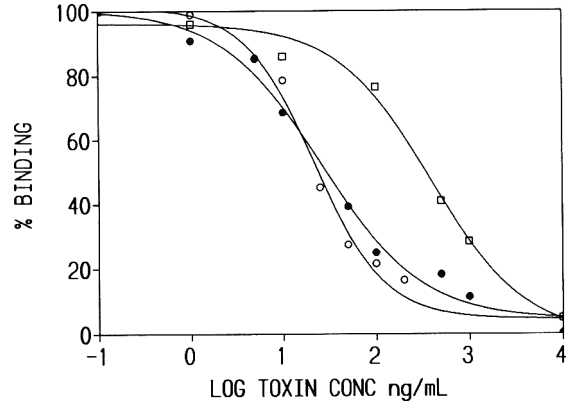

Figure 4. Radioimmunoassay for neo-STX using anti-neo-STX-KLH. The antiserum dilution was 1:60 (0.1 mL) with tritiated STXOH of 11 450 dpm/assay. Neo-STX (●), STX (○), and dec-STX (□). Data of average of 2 experiments are presented; thus, no-error bars are not shown.

concentrations causing 50% inhibition binding of STX-PLL to the antibody by neo-STX, STX, and DC-STX were 1.2, 4.1, and 36.1 ng/mL, respectively. The relative reactivities of neo-STX, STX, and DC-STX to the antibody under this condition are 100, 29.3, and 3.3%, respectively. The sensitivity of this system for monitoring neo-STX is almost the same as that described above when neo-STX-BSA was coated in the wells of microplate (0.9 vs 1.2 ng/mL at 50% inhibition); yet the sensitivity for determination of STX (8.0 vs 4.1) increased almost 2-fold. Thus, this system could be used for monitoring the presence of both toxins in the sample. Another advantage is that a more commonly available toxin, STX, could be used as the starting reagent.

Radioimmunoassay

Results for the radioimmunoassay are shown in Figure 4. A high concentration of antiserum (1:60 dilution) was necessary to give 50% binding of 11 450 dpm tritiated STXOH in the assay system. The concentrations causing 50% inhibition of binding of tritiated STXOH to the antibody by neo-STX, STX, and DC-STX in RIA were found to be about 29.3, 22.3, and 345.7 ng/mL, respectively. The apparent affinity constants as determined by the method of Muller (25) of the antibody for neo-STX, STX, and DC-STX were found to be 1.23×10^8, 1.56×10^8, and 7.9×10^6 L/mol, respectively. Competitive RIA for monitoring STX and neo-STX in this system was also less sensitive than indirect ELISA.

Conclusion

Polyclonal antibodies that have good cross-reactivity to both neo-STX and STX were successfully obtained after immunization of rabbits with neo-STX conjugated to protein carriers. Using such antibodies, an indirect ELISA, which is considerably more sensitive than previously reported methods, was established for simultaneous analysis of neo-STX and STX. For example, 1 mouse unit is equivalent to 185 ng STX or neo-STX (2, 5, 7). In contrast, as little as 0.5 ng/mL of either toxin could be measured in the present system. The sensitivity of the ELISA also compares favorably to that of the membrane assay, in which 1nM (0.372 ng/mL) of PSP toxins could be measured (12). A radioimmunoassay was established in the present study, but it is less sensitive than the indirect ELISA. We also observed a complete protection of the lethal toxic effect in mice when 200 μL of undiluted antisera and 350 ng neo-STX were administered together to the test mice (Huang and Chu, unpublished observation). These data indicate that this antibody not only will be very useful for analytical purpose but could also be used as a therapeutic agent.

Because all the standard curves established in the experiments were prepared in buffer solutions, the matrix interference problems should not be overlooked. In a previous study (16), we found that extracts obtained from clams and mussels greatly interfered with the indirect ELISA. The interference was minimal when the concentrations of clams and mussels were at 50 and 5 mg/mL, respectively (16). Based on these data, the sensitivity of ELISA for neo-STX in clam and mussel extracts would be 20 and 80 ppb, respectively. Nevertheless, applications of present protocols for the analysis of STX and neo-STX in these samples, as well as the cross-reactivity of the antibodies with a battery of STX derivatives, need to be vigorously tested. There is also a need for a monoclonal antibody that could cross-react with other STX derivatives. Research efforts in our laboratory are currently directed to these areas.

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VITAMINS AND OTHER NUTRIENTS

Fluorometric Determination of Thiamine Vitamers in Chicken

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A comparison was made of the direct determination of thiamine in acidified heated chicken extracts, by either flow injection or chromatographic determination, with the standard method (acid and enzyme digestion, adsorption, and elution, followed by the fluorometric determination of thiochrome extracted by isobutanol from $K_3Fe(CN)_6$-treated eluates). Liquid chromatography of extracts, followed by oxidation of thiamine vitamers to thiochromes, showed 1 light scatter emission peak and 2 thiochromes, the latter corresponding to thiamine and thiamine monophosphate. Both forms were determined quantitatively by flow injection determination, the lower detection limit of which was about 60 femtomol. The determination was linear from 0.1 ng to 10 μg thiamine/mL, and the pooled coefficient of variation was 4%. The determination of thiamine in chicken extracts provides a nondestructive method for determining thiamine and its phosphate esters, either *in toto* by flow injection determination or as individual components by chromatography.

During the course of the study of the effects of ionizing radiation on thiamine in chicken, we had occasion to determine the concentrations of the various thiamine vitamers: thiamine (Thmn), thiamine monophosphate (TMP), and thiamine diphosphate (TDP, cocarboxylase). In the standard method of thiamine determination (1, 2), the last 2 vitamers are dephosphorylated by an enzyme digestion step because, for the measurement of the thiochrome formed from the oxidation of thiamine, the thiochrome is extracted into isobutanol in which the thiochrome phosphate esters are not soluble. This method does not distinguish the vitamers from each other. Liquid chromatography obviates the need for cleanup (2–16) and determines the phosphate esters individually, but most of the methods studied (3–12) involve precolumn oxidation to thiochrome, which destroys the vitamers before they are separated.

We determined the thiamine vitamers directly in an aqueous chicken extract, but the solutions were too turbid for fluorescence measurements because of Rayleigh and Raman scattering. Acidification and heating (equivalent to the AOAC rapid method, **953.17** (1)) yielded clear solutions suitable for measuring thiochrome fluorescence, but we consistently obtained higher values for the thiamine content (ca 1.60 μg thiamine/g meat) than those reported in the literature (ca 0.4–0.8 μg/g) (3, 8, 17–20). Therefore, we examined the thiamine content of chicken breast extracts at each step of the sample preparation to ascertain if the lower values obtained from the standard method were due to losses in 1 or more of the steps. We also chromatographed the extracts on different resins to ascertain if we were measuring an artifact. We extended the study to the determination of the thiamine vitamers *in toto* by flow injection determination.

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Mention of any company or product name does not constitute endorsement.

Experimental

Reagents

All thiamine vitamers were obtained from Sigma Chemical Co., St. Louis, MO 63178: thiamine hydrochloride (T4625, lots 125F-0250 and 94F-0334), thiamine monophosphate chloride (T8637, lot 93F-00391), and cocarboxylase (C-8754, lots 106F-0182 and 110G-2420). For accurate determination of the fluorescence intensity, the vitamers were dried over silica gel at 84°C. By chromatography, thiamine was found to be a single component, but both thiamine monophosphate and cocarboxylase contained varying amounts of thiamine, probably due to decomposition of the phosphate esters during storage. All other chemicals were reagent grade and all solutions were prepared in deionized/distilled water. As necessary, eluting solutions were filtered through 45 μm Supor-450 membrane filters and either sonically degassed or purged with helium.

Equipment

Samples were injected into the buffer stream by either an ISIS autoinjector or a Rheodyne 7125 sample injector (Rheodyne, Inc., Cotati, CA 94931), both with 200 μL loops. Two fluorescence detectors were used: a Waters 420 fluorescence detector with a F4T5/BL lamp (Waters Chromatography, Milford, MA 01757), 365 nm excitation filter, and a 425 cut-off emission filter, and a MacPherson FL-750 photofluorometer, $\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm, with either a 400 or a 440 nm cutoff filter. MPF-44E spectrophotofluorometer was used to measure the fluorescence spectra (Perkin-Elmer Corp.,

Norwalk, CT 06859). For scanning the excitation spectra, the emission wavelength was set at 435 nm and the excitation spectrum was scanned from 325 to 400 nm. For the emission spectra, the excitation wavelength was set at 435 nm and the excitation spectrum was scanned from 380 to 500 nm.

Sample Preparation

Figure 1 is a flow diagram of sample preparation by the standard method. Asterisks indicate the steps after which samples are removed for thiamine determination. The double asterisk indicates the point at which samples are removed for the rapid method.

Slurry

Fresh chicken breasts were obtained from a wholesale dealer, usually 1 day after slaughter, although some breasts were obtained the first day. The skin and excess fat were removed, and the meat was separated from the bone and sliced into 1/4–1/2 in. cubes. For homogeneity and ease of handling, 80 g of the meat was blended 15 s with 160 mL water under nitrogen in a glove bag. (We felt the precaution of blending under nitrogen was advisable because the blending process introduces a large amount of gas into the liquid.) The resulting slurry is a highly homogeneous material for the study of sample preparation; it was easily transferred quantitatively by aspiration into a 50 mL irrigation syringe and transferred by weight into appropriate containers.

Sample Sets

For all runs, sample sets consisted of a water blank, a thiamine vitamer standard, a chicken extract, and a chicken extract spiked with the standard.

Sample Preparation, Standard Method

To determine the thiamine concentration at each step of the standard method, the steps had to be modified slightly. For the HCl extracts, 8 mL 1N HCl was added to 90 g slurry to lower the pH to 1.5, and the mixture was stirred vigorously. The slurry was then drawn up into an irrigation syringe and 16.3 g was transferred into 50 mL Erlenmeyer flasks; 2 sample sets were prepared. Then, 15 g water was added to the zero concentration and the standard flasks. For the standard and spiked samples, 0.5 mL of a stock solution of 10 μg thiamine/mL was added to the appropriate tubes. Next, 1.5 mL 1N HCl was added to all flasks in both sets, and each flask was diluted to ca 35 mL. The flasks were stoppered with rubber stoppers covered with Saran wrap and heated 30 min in a boiling water bath. The flasks were cooled and the contents were adjusted to pH 4.5–4.7; one sample set was diluted to volume to serve as the acid digestion sample. Then, 2.5 mL 5% α-amylase was added to each of the flasks in the second sample set, and the set was incubated overnight at 37.5°C. We found that these time and temperature conditions were necessary for complete conversion of TMP to thiamine by the α-amylase preparation we used (cf 2, 7). The samples in this set were transferred to 100 mL volumetric flasks and diluted to volume. A 25 mL aliquot of each sample was placed on a Bio-Rex column prepared as described below, washed with 20 mL 70°C water, and eluted with 70°C acid–KCl into 25 mL volumetric flasks. Next, 2.5 mL of each column eluant was placed in a centrifuge tube (capped type), 2.5 mL 0.04% $K_3Fe(CN)_6$ and 7.5 mL isobutanol were added to each tube, and the tubes were capped and shaken lightly for 2 min. This last operation was performed in subdued light coming from the windows 30 ft away (thiochrome is sensitive to light), and in a hood (isobutanol is mutagenic). After the tubes were allowed to stand 1 min, the isobutanol supernatant cleared and was suitable for thiochrome determination in a fluorometer.

A total of 5 sample sets was obtained: (*1*) TCA/heat treated, (*2*) HCl/heat treated, (*3*) HCl/heat treated after enzyme digestion, (*4*) HCl/heat treated after adsorption/elution on Bio-Rex 70, and (*5*) HCl/heat treated after thiochrome formation/isobutanol extraction.

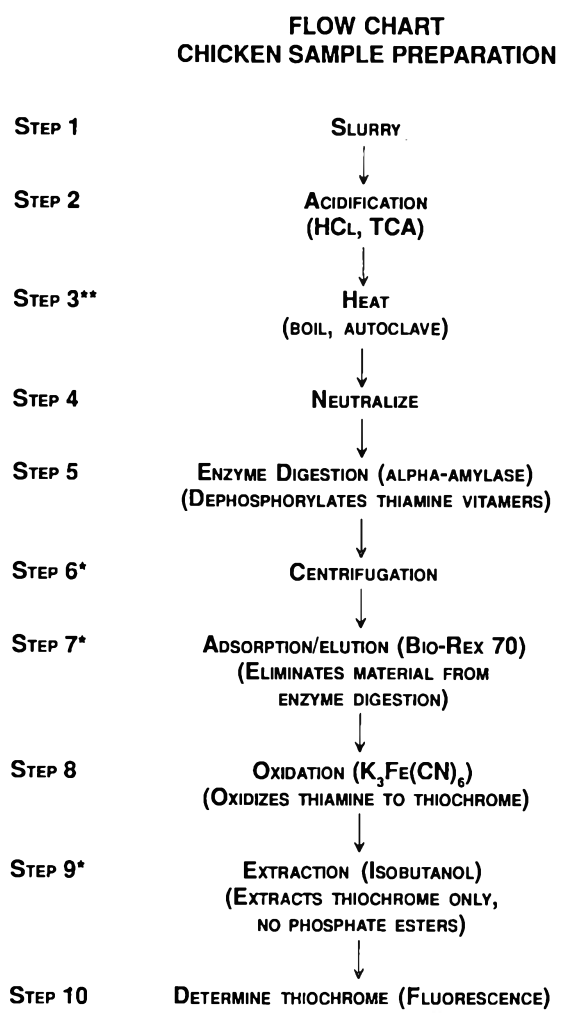

Figure 1. Flow chart for the standard method of thiamine determination. Asterisks indicate the step after which samples were taken for thiamine.

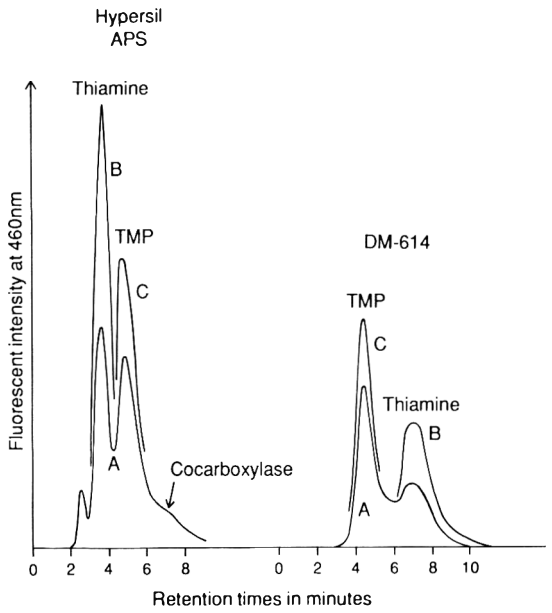

Figure 2. Chromatograms of chicken extracts, with and without spiking with thiamine and thiamine monophosphate, on Hypersil APS and DM-614. Curves A, chicken extract. Curves B, same with thiamine spike. Curves C, same with thiamine monophosphate spike.

Bio-Rex 70 Preparation and Use

Bio-Rex 70 resin, 50–100 mesh, Control No. 33772, was used to purify thiamine in chicken extracts. About 20 g resin was washed with distilled water, and the fines were decanted. The resin was then washed with four 250 mL portions 1N HCl, and the resin was allowed to stand for a short period of time in the acid. The resin was washed until the washings were neutral to pH test papers. The columns consisted of a 50 mL tube sealed to a column 13 cm long by 6 mm id, with a Luer tip sealed to the end. A 3-way valve was placed on the Luer tip, and a 22 g needle (square tip) was attached to the valve. The column was plugged with polypropylene wool and filled to 10 cm with the washed resin. With the mesh used and the 22 g needle, the flow rate was a little over 1 mL/min.

Sample Preparation, Rapid Method

Samples were acidified with either trichloroacetic acid (TCA) (15, 21) or HCl (1, 2). For the TCA extracts, 9 g slurry was weighed into centrifuge tubes (polyallomer, capped type) and 18 mL 2% (w/v) trichloroacetic acid was pipetted into the tube. For the zero thiamine concentration and standard samples, 9 g water was weighed into tubes. For the standard and spiked chicken extracts, 0.3 mL 10 μg/mL standard thiamine was added to the appropriate tubes. The tubes were capped tightly, shaken vigorously, and heated 30 min in a boiling water bath. After cooling, the tubes were shaken again, and centrifuged 15 min at 20 000 × *g* and 5°C.

Columns

For chromatographic separation of the thiamine vitamers, we used either Shodex's DM-614 (equivalent to a C3-4 reversed-phase) (22) or Hypersil APS (anion exchange) columns (Chrompak B.V., Middelburg, The Netherlands). The eluting solution for the DM-614 and Hypersil APS was 0.05M citrate buffer, pH 4.5. The columns were run at room temperature; any variations in retention times were compensated by including standards in all runs.

Determination of Thiamine

Thiamine was determined in either the standard or rapid method extracts by flow injection determination (FID) or after chromatographic separation (CD), except for the isobutanol-extracted thiochrome, for which the fluorescence was measured in a 1 cm sealed cuvette. After sample injection into the buffer stream, either with or without a column, a solution of 0.04% $K_3Fe(CN)_6$ in 2% NaOH was mixed into the stream at the same flow rate as the buffer stream, and allowed to flow through a reaction coil 160 × 0.060 cm id at room temperature. Although this length of coil allows only 0.5 min of reaction time, we found, as Cooper and Matsuda (13) observed, that the oxidation of thiamine by ferricyanide is exceedingly rapid. The thiochrome produced by oxidation of thiamine by ferricyanide was determined fluorometrically in a 12 μL flow cell.

Results

Before we initiated the major study, some preliminary studies were performed. We tried the direct addition of ferricyanide to the chicken extracts, but the results were erratic and generally low. The indicated heating step was found necessary to obtain clear extracts and eliminate reduced yields for both the trichloroacetic and hydrochloric acid extracts. The usual concentration of alkali for the oxidation step is 15% (w/v), but successive dilutions showed no difference in the measured thiochrome until about 1% (w/v) NaOH. The desired alkaline conditions of about pH 13 were produced by choosing 2% (w/v). Concentrations of ferricyanide above 0.1% resulted in decreased yields of thiochrome, probably through further oxidation of the thiochrome formed (23). The only 2 thiamine vitamers found in any great quantity in the chicken breasts we studied were thiamine and thiamine monophosphate; cocarboxylase was present in only very low quantities. The exception to this observation occurred when the chicken was slaughtered the same day, in which case the cocarboxylase content was distinctly greater. Because the first 2 compounds constituted the bulk of the vitamin present, we focused our attention on them.

Thiamine and Thiamine Monophosphate

These vitamers were identified in the column effluents by their retention time on DM-614 and Hypersil APS resins and by their fluorescent spectra. Curves "A" in Figure 2 represent the chicken extracts on the 2 resins; peaks "B," the changes in the curves when the chicken extracts were spiked with thia-

Table 1. Retention times in minutes

Compound	Hypersil APS	Shodex DM-614
Thiamine	3.8	7.1
Thiamine monophosphate	4.8	4.5
Cocarboxylase	7.0	4.5
Light-scattering material	2.5	8.2

mine; peaks "C," TMP–spiked chicken extracts. The thiamine and thiamine monophosphate peaks had the same retention times as the standards (Table 1), and the fluorescence excitation and emission spectra of the thiochromes were identical to the spectra of the thiochrome standards (Figure 3). The peak identified as TMP disappeared after the digestion by crude α-amylase, showing it to be the phosphate ester. The low shoulder below the cocarboxylase arrow had approximately the same retention time as the cocarboxylase standard, but, as shown, was usually present in only very small quantities. A sharp spike is shown in the Hypersil APS column effluent preceding the thiamine peak, but it had neither an excitation nor an emission spectrum; that is, the peak was a scatter peak due to soluble compounds in the extracts. This peak was the only peak present in the alkali blanks, where it was as high as in the ferricyanide-treated effluents. In the effluents from DM-614 columns, this peak appeared in the alkali blanks and came off the column shortly after thiamine. This peak was highly variable from preparation to preparation, as expected, and it was not always observed.

Trichloroacetic Acid Extracts

The TCA/heated extracts gave uniformly clear solutions, with insoluble precipitates that packed well upon centrifugation. The thiochrome spectra of the chicken extracts chromatographed on DM-614 showed the presence of both thiamine and TMP, with retention times of 7.1 and 4.5 min, respectively (Table 1). There was no indication of cleavage of the phosphate esters. The results from the flow injection determination of thiamine in 12 chicken breasts are shown in Table 2. The first 3 values are not significantly different from each other, but after the adsorption/elution step, the determined concentration of thiamine was about half the initial values, which was a significant difference ($P < 0.05$). The precision of the flow injection determination is shown in Table 3, which summarizes the coefficient of variation for the various steps and sample variation. The first 3 rows are for the determination step in standards and chicken slurries. The next 3 rows show the variation due to the sample preparation procedure, the variation between chicken breasts, and the variation from chicken to chicken. The average value for thiamine in the TCA extracts was highest of all the reliable measurements (excluding enzyme-digested samples) and showed the lowest pooled coefficient of variation (Table 2).

Hydrochloric Acid Extraction

The HCl/heated solutions were not always clear when adjusted to pH 4.0–4.3, the pH used for the enzyme digestion

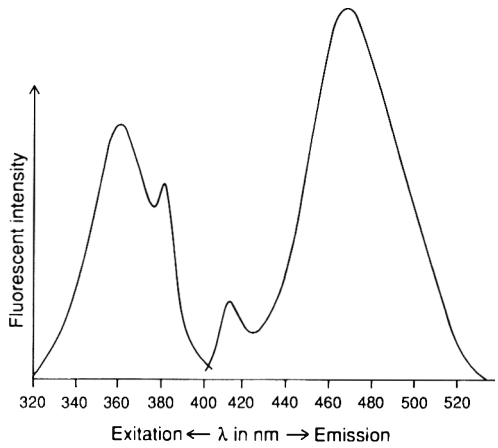

Figure 3. Fluorescent spectra of thiochrome and thiochrome monophosphate, both standards, isolated from chicken extracts by liquid chromatography. All had the same spectra.

(1, 2). This introduced a very high background in the FID or CD separation determinations, and the turbid solutions tended to foul the Bio-Rex 70 columns. Upon investigation, it was found that if the pH of the solutions were brought to pH 4.6–4.8 before centrifugation, clear solutions were obtained (24). The coefficients of variation were quite high: 30.6% for the between-chicken variation and 17.4% for the breast pairs. Of the 2 acids, TCA was preferred over HCl because it produced a clear solution in weak acid solutions, whereas it was necessary to raise the pH of the HCl solutions to a value where thiamine oxidation was a factor.

λ-Amylase Digestion

The enzyme-digested solutions were yellowish, but clear. The chicken samples were generally lighter than the thiamine standards or water solutions, and the spiked chicken samples lighter still, but the differences were not reflected in the fluorescent spectra. That is, the colored compounds did not fluoresce. There was, however, a very large amount of light scattering material, reflected in very high alkali blanks in the FID determination. Chromatography of the enzyme-digested extracts on DM-614 showed that the addition of the enzyme introduced an emission peak that had the same retention time as TMP, but was lower in the thiochrome solutions than in the alkali blanks. That is, the oxidation reduced the fluorescence. When the chicken and spiked chicken extracts were corrected for this difference, no TMP was found in the extracts, as expected after enzyme digestion. The amount of thiamine increased slightly, identifying the peak as TMP, but the increase was not always commensurate with the loss of TMP. When chicken extracts were spiked with TMP, the enzyme digestion eliminated the TMP peak, but there was very little increase in the thiamine peak (Table 4).

Table 2. Determination of thiamine during sample preparation (results of 12 preparations) in μg thiamine/g chicken[a]

	TCA/heat	HCl/heat	Enzyme digestion	Bio-Rex 70 effluent	Thiochrome/ isobutanol
Av.	1.81	1.64	2.26	1.08	0.95
s	0.20	0.40	1.44	1.18	0.30

[a] Values for TCA/heat, HCl/heat, and enzyme digestion are not significantly different; values for Bio-Rex 70 effluent and thiochrome/isobutanol are not significantly different.

After Adsorption/Elution on Bio-Rex 70

The values were uniformly low, averaging 1.21 μg thiamine/mL, about 2/3 of the values were obtained by direct measurement. There was no background fluorescence and only 1 thiamine peak by chromatography.

Thiochrome Formation/Isobutanol Extraction

This is the final step in the usual method of thiamine determination, and it is the principal reason for the enzyme digestion and adsorption/elution steps. Because it is a hand operation, it is inherently less reliable than FID. The coefficient of variation rose to 29%. The average value was 0.95 μg thiamine/mL, which is about the value usually reported for thiamine in chicken but is half the value determined in the acid/heated samples.

Spike Recovery

Spike recovery was the best for the TCA extracts, averaging 99.7% of the standard in preparations 1–12. In a separate experiment, standard solutions of thiamine ranging from 1 to 5 μg/g of chicken were added to portions of a slurry, which was then processed and the thiamine determined. The coefficient of regression was 85.3 units/μg spike/mL, compared to a value of 84.5 units/μg thiamine/mL for the standard. That is, the spike fluorescence was quantitatively the same as that of the standard.

Table 3. Precision of flow injection determination

	Coefficient of variation, %	
Source of variation	TCA	HCl
Thiamine determination		
Standards, $n = 25$		
400 nm cut-off filter	2.10	1.91
440 nm cut-off filter	1.36	0.00
1 slurry, 5 preps., $n = 15$	1.52	0.37
Sample preparation		
1 slurry, 5 preps., $n = 5$	3.37	1.50
Breast pairs, $n = 6$	6.7	17.4
Chicken variation, $n = 12$	15.7	30.6

Attributes of the Method

Specificity

A determinative method is required to be specific, accurate, precise, linear, sensitive, reproducible, repeatable, and rugged. Both the CD and FID measurements were specific for thiamine and/or its vitamers by criteria of retention time and identity of the fluorescent spectra of the standards with those of the vitamers isolated from chicken. As shown by chromatography, the measured emission of the alkali blanks was due to a single light-scatter peak, the magnitude of which was the same in both the alkali blanks and the ferricyanide-treated samples. The sample peaks in FID were, therefore, specific for thiamine and its esters after subtraction of the scatter peak of the alkali blanks.

Linearity and Sensitivity

Linearity was tested in standard solutions and spiked chicken extracts. The determination of thiamine in water or buffer using the MacPherson FL-750 was linear over a range of 0.1 ng thiamine/mL (60 femtomol) to 10 μg/mL. Figure 4 is

Table 4. Thiamine monophosphate loss during enzyme digestion and adsorption/elution

	Thiamine, μg/mL					
	Thiamine		TMP		Total	
Source	Run 16	Run 17	Run 16	Run 17	Run 16	Run 17
TCA extract						
standard (TMP)	0.56	0.00	0.43	1.00	0.99	1.00
chicken	1.68	2.40	0.54	0.00	2.22	2.40
spike (TMP)	1.88	2.40	1.60	1.00	3.48	3.64
Bio-Rex 70 effluent						
standard	1.00	1.00	0.00	0.00	1.00	1.00
chicken	0.93	0.96	0.00	0.00	0.93	0.96
spike	1.20	1.37	0.00	0.00	1.20	1.38

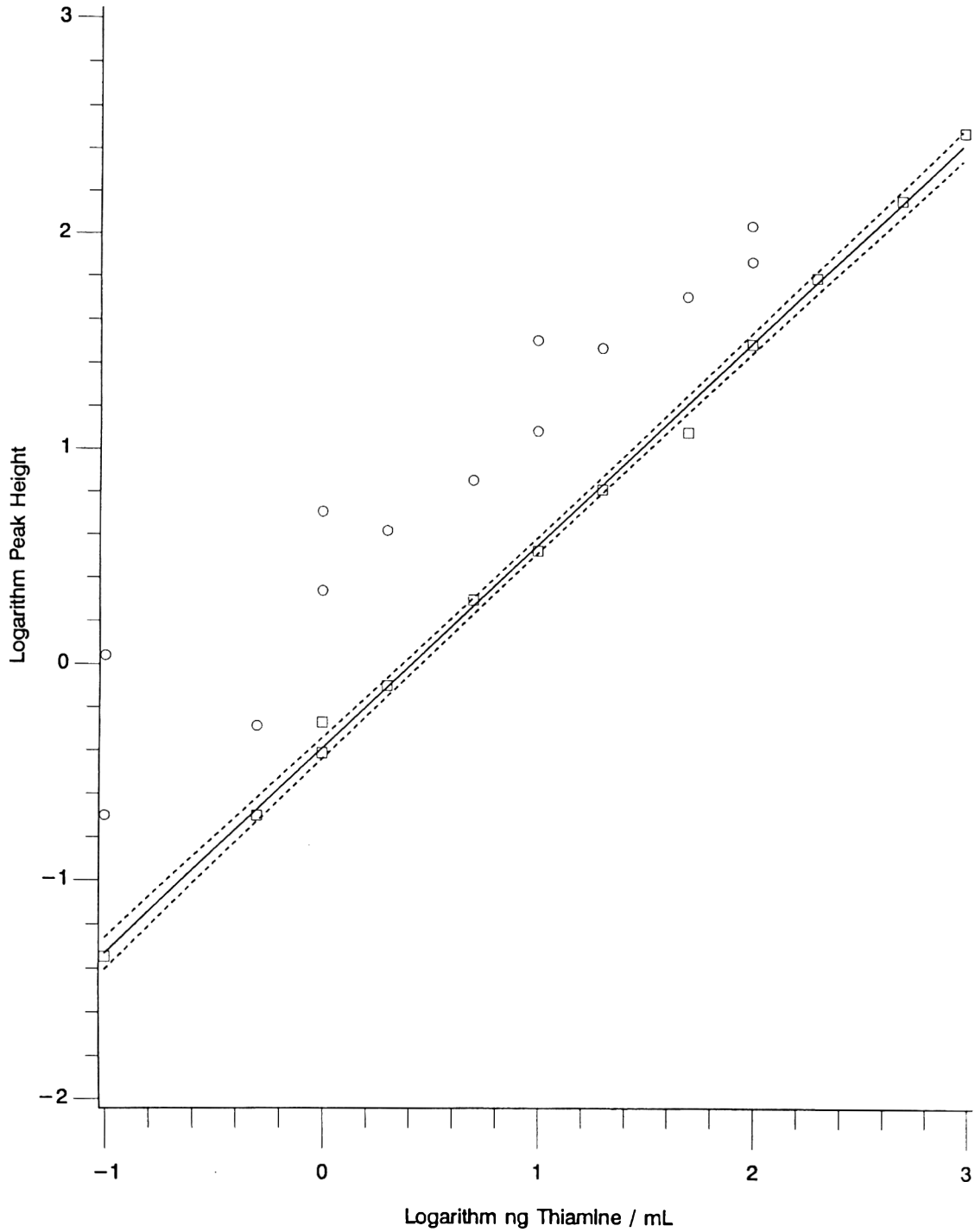

Figure 4. Expression of method sensitivity: square symbols, fluorescence determined with a 440 nm cut-off filter; 95% confidence limits shown are for these data only. Circular symbols, fluorescence determined with a 400 nm cut-off filter in the emission light beam. The latter show the effect of Rayleigh and Raman light scattering at low concentrations of thiamine.

a plot of the lower portion of the curve and shows the effect of using different wavelength cut-off filters. The data represented by the square symbols and the circular symbols were determined by using a 440 nm cut-off filter and a 400 nm filter, respectively, the latter showing the effect of reducing Rayleigh and Raman scattering. Such scattering becomes an important factor at the very low levels of thiochrome fluorescence from the thiamine in chicken. Sensitivity values of 7 and 30 femtomol were reported by Brunnekreeft et al. (6) and Kimura and Itokawa (15) for precolumn and postcolumn oxidation to thiochrome, respectively, compared with our value of 60 femtomol. However, as the sensitivity is equivalent to less than 0.1% of that normally found in the determination of thiamine in chicken, we did not pursue the matter further.

Precision

The results of a quintuplicate preparation/triplicate determination experiment are given in Table 3. The coefficients of variation for the determination of thiamine in a single chicken slurry using 5 preparation samples were 0.37% for the HCl extracts and 1.52% for the TCA extracts. The difference between the HCl and TCA values represents some unknown and erratic stability factor, in either the oxidation process or the output of the fluorometer. At certain times of the day, we did observe great instabilities in the fluorometers, which may have been due to voltage fluctuations.

Accuracy

Confirming our previous observations, the results of the direct addition of ferricyanide to the chicken extracts yielded higher values for the thiamine content of chicken meat than after the adsorption/elution and thiochrome/extraction steps. In Table 2, the first 3 values are not significantly different from each other but are significantly different ($P < 0.01$) from the extracts after adsorption/elution. Because the values after the adsorption/elution step are about the same as reported in the literature (10.8 and 0.95 μg thiamine/g chicken), the modifications made in the method for purposes of the study did not alter the essential character of the procedure.

Repeatability, Reproducibility, and Ruggedness

Repeatability is difficult to determine in the case of thiamine because the vitamin is unstable and varies from chicken to chicken; therefore, a standard concentration sample is impossible to establish. Because the experiment involving the 12 samples was performed over a period of 4 weeks, the measured precision is partly a repeatability measurement. Reproducibility was not tested at this time. Because the method is faster (an important factor with unstable compounds) and contains few steps (consistent with accurate and precise results), the method is as rugged a procedure as can be devised.

Discussion

Accuracy

The question of accuracy was one of the principal reasons for the study, the answer to be found in either the measurement of an artifact in the CD and FID measurements or a loss of the vitamin in one of the steps in the usual purification scheme. From the chromatographic results, the only extraneous interference in the HCl and TCA extracts occurred equally in both the alkali blank and ferricyanide solutions, producing neither positive nor negative interference in either CD or FID measurements. The possibility that some component in the chicken extracts was causing greater conversion of thiamine to thiochrome in the extracts than in the standards was eliminated by the observation that the thiamine concentrations in the spikes were equivalent to the standards. The loss of thiamine in the standard method indicates that the rapid method yields a more accurate and higher value for the thiamine content of the chicken than the standard method does.

Table 5. Spike recovery (average percent of the standard)

	TCA/heat	HCl/heat	Enzyme digest	BR-70 effluent	Isobutanol extract
Av.	99.7	91.1	110.7	99.3	91.8
s	11.9	7.2	18.7	15.8	4.4

The loss of thiamine in the adsorption/elution step was observed by many authors (9, 25–27), and was listed by McRoberts (24) as a specific problem to be addressed in the determination of thiamine in enriched flour. Early in the history of the procedures for determining thiamine, Wang and Harris (28) listed as one of the special advantages of their procedure the elimination of the adsorption/elution step!

The problem is not just one of loss of thiamine in chicken on the Bio-Rex 70 columns, however. The concentration of thiamine in the chicken extracts was calculated by using the fluorescence of a standard that itself was adsorbed and eluted from the resin. That is, there was a loss of thiamine in the chicken extracts not observed in the standards. The problem was not one of incomplete elution. Pippen and Potter (25) found that a larger volume, 50 mL, was frequently required to effect total elution from Decalso. However, when we eluted Bio-Rex 70 with further portions of hot acid–KCl, we obtained no more thiamine. Furthermore, incomplete elution should have affected spike recovery, but no diminution in the spikes was observed (Table 5). The measured concentration of thiamine in a chicken extract appears to be lower simply because it is in the extract, which suggests that the thiamine in chicken differed from free thiamine. It apparently was not bound to any other compound, as its retention times on the Hypersil APS and DM-614 columns were the same as those of free thiamine. Oxidation of the chicken thiamine on the resin seems unlikely, as the oxidation would be expected to extend to the free thiamine in the spike. The nature of the problem of the poor elution of the thiamine in chicken extracts is not clear. Further investigation should yield interesting information on the state of thiamine in this meat.

Fluorescence

The fluorescent spectra of the various thiochrome derivatives and the molar fluorescences were dissimilar from some reports in the literature. Ishii et al. (10) reported excitation and emission spectra that show only 1 maximum in either spectra, but both of their peaks were skewed. Matsuda and Cooper (21) reported 2 emission maxima at 435–440 and 450 nm but only 1 excitation maximum at 365 nm. Mohamed et al. (29) reported spectra with a major excitation peak at 360 nm and a major emission peak at 425 nm, and minor peaks at 415 (excitation) and 360 (emission) nm. All of the thiochrome vitamers, both standards and from chicken, had the spectra shown here. The spectra were not of any compounds extracted from the plastic tubes used for the digestion, because the zero concentration thiamine samples showed no fluorescence. Ishii et al. (10) did not give details of how they measured the spectra, and

it may be that their instrument did not have the resolution of the Perkin-Elmer instrument we used, hence the skewing in their spectra.

Molar fluorescence values are also a problem. We did not observe any difference in the molar fluorescence of thiochrome and its phosphorylated vitamers, thiochrome monophosphate and thiochrome diphosphate. Ishii et al. (10) reported that the molar fluorescences of thiochrome monophosphate and thiochrome diphosphate were lower than that of thiochrome, in the ratio of 63:83:100, respectively. Conversely, Matsuda and Cooper (21) reported that the fluorescences of thiochrome diphosphate and thiochrome triphosphate had to be multiplied by factors of 0.87 and 0.80 because they were high in comparison with thiochrome. Lewin and Wei (30) did not find any difference in the molar fluorescences of the 3.

The problem is not simple. As Risinger and Pell (31) reported, thiamine is readily oxidized to the disulfide, and Ryan and Ingle (32) and Rose and France (33) observed multiple oxidation products during the formation of thiochrome. Furthermore, the reaction does not go completely to thiochrome (34). Barger et al. (35) reported that the reaction, as they ran it, resulted in only 30% conversion of thiamine to thiochrome. Marquez et al. (23), using a kinetic method to determine thiamine, found maximal production of thiochrome at about 10 min in their system, with a gradual decrease of fluorescence after that time. Under these conditions, it is possible that the vitamers might not be oxidized to the same extent depending on the conditions, but taking the literature *in toto*, and in view of our own results, we believe it is correct to assume that the molar fluorescences of the vitamers are all the same.

Precision

Precision values in the literature are limited to total procedures and a limited number of samples. A survey of the articles in which precision values are reported showed a range of 0.5–50% for the coefficients of variation, with an average of about 8%. Coefficients of variation reported for chicken thiamine are 4% for replicate determination (3) (in this study, ca 1.5%); 8.1–21% (17) and 8.6–28.6% (18) for replicate samples (in this study, 3.37%). The higher coefficients of variation in the last 2 may be due to the chicken-to-chicken variation, which was found to be 15.7% in this study. From these comparisons, the precision of the rapid method is better than reported in the literature. One of the major problems in precision comes at the fluorescence measurement step, where a very small amount of light scattering can result in very large deviations in the measured peak. Light scattering results in high and variable peaks; it was observed that the outliers were almost all in the direction of greater values. The use of a cut-off filter as close to the desired emission wavelength as possible reduces or eliminates variation from this source (Figure 3) and increases the precision of the measurements.

Conclusions

The determination of thiamine has to be a balance between elimination of interferences and losses introduced by excessive handling or too many steps in preparation. The results of this study indicate that the AOAC rapid method, **953.17** (1), when modified, yields more precise and accurate values for the thiamine in chicken than does the longer method, **942.23**, principally because of losses in the adsorption/elution and thiochrome formation/extraction steps of the latter. The use of trichloroacetic acid, flow injection determination, and cut-off filters as close to the emission wavelength as possible yields the best results.

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VITAMINS AND OTHER NUTRIENTS

Graphite Furnace Atomic Absorption Method for the Determination of Chromium in Foods and Biological Materials

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A method was developed for the determination of chromium in food samples and other biological materials. Samples are dry ashed in a muffle furnace and are analyzed by graphite furnace atomic absorption spectrometry. Magnesium nitrate is used as a matrix modifier, and samples are quantitated by platform atomization and peak area measurements with direct calibration against aqueous standards. The detection limit (based on 3.29 σ) was 5.6 pg, or 0.28 μg/L for a 20 μL injection. The characteristic mass was 3.2 pg. This method was validated by analyzing a range of reference materials and was subsequently used for the analysis of a variety of food samples. A comparison of analytical results obtained from direct calibration and method of additions was made.

Because chromium is an essential element for sustaining life, it is of interest to nutritionists and health professionals. The estimated safe and adequate daily dietary intake (ESADDI) of chromium is 0.05–0.20 mg/day (1). Adequate methods for diagnosing chromium status do not exist; therefore, ESADDI is based on the absence of signs of chromium deficiency in the majority of the U.S. population, which consumes an average of 50 μg chromium daily. The suggested range is based on the assumption that a varied diet providing an adequate intake of other essential micronutrients will furnish chromium with an average absorbability of 0.5%. If one assumes that any food contributing 5% or more of ESADDI in a typical 100 g portion is significant, then the levels of interest are 25–100 ng/g (dry weight basis). Taking into account the moisture level of foods and any dilution factor associated with the sample preparation step, a very sensitive analytical method is needed to determine chromium at the levels of interest, often ppb and sub-ppb concentrations. Unfortunately, most chromium data presented in the literature are quite variable, and there are very few papers with chromium food composition data (2–6). Many older references that report chromium values are not useful because samples were clearly contaminated. Also, the data for some references were obtained by atomic absorption analyses without adequate background correction or where significant matrix interferences were present. These errors resulted in unreasonably high chromium concentrations. The lack of reliable chromium food composition data is, then, the result of a combination of factors, including insensitive techniques, lack of data, and lack of low-level, commercial certified reference materials that can be used to validate analytical methods.

Graphite furnace atomic absorption spectrometry (GFAAS) is a highly sensitive technique that can be used to determine chromium at sub-ppb concentrations. This analytical technique is used routinely in the Nutrient Composition Laboratory for the determination of very low concentrations of elements in foods and biological fluids (7, 8). In this report, we outline an optimized method for the routine determination of low-level chromium in foods by peak area measurements and calibration against aqueous standards. Zeeman background correction was

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Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the U.S. Department of Agriculture.

Table 1. Dry ashing method validation study

Reference material	(Cr concn, ng/g, dry weight) Certified concn	Determined concn[a]
Mixed Diet RM 8431	102 ± 6	108 ± 4
Oyster Tissue SRM 1566	690 ± 270	588 ± 29
Citrus Leaves SRM 1572	800 ± 200	780 ± 26

[a] Uncertainties represent ±1 standard deviation (n = 9).

used to ensure adequate compensation for background from complex sample matrixes. No time-consuming matrix matching of standards or method of standard additions was required. The method was developed by starting with the evaluation of sample preparation methods. Next, charring and atomization studies were completed and calibration range studies were performed. The final phase was validation of the method and establishment of quality control protocols. Analytical figures of merit for the method are presented and accuracy of validation data are reviewed.

Experimental

Apparatus

A Perkin-Elmer Model 5100PC with Zeeman background correction was used for most of the study (Perkin-Elmer Corp., Norwalk, CT 06859). Preliminary work was done with a Perkin-Elmer Model 3030, but that spectrometer was replaced with a Model 5100PC, and the final method was validated using the newer spectrometer. Zeeman background correction was used to eliminate interferences associated with background, typically due to a complex sample matrix. The spectrometer was equipped with an AS-60 autosampler and an HGA-600 furnace. All analyses were done using platforms (commercial Perkin-Elmer platforms part No. B0109324) inserted into pyrolytically coated graphite tubes (Perkin-Elmer part No. B0109322). A 2% HNO_3 rinse was used for the autosampler to avoid carryover from one injection to the next. A single-element, hollow cathode lamp (Perkin-Elmer part No. N066-1297) was operated at 25 mA, and all data were taken at the 357.9 nm wavelength. The integration time was 8 s for all analyses. The slit width was 0.7 nm. Argon was used as the purge gas.

Reagents

Care was taken to avoid sample contamination by the reagents. Nitric acid used to prepare samples and standards was sub-boiling distilled [National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899, or Seastar Chemicals, Seattle, WA]. The nitric acid is certified as containing 0.03 μg Cr/L. Ultra-pure magnesium nitrate (Johnson Matthey, Materials Technology, UK) was used to prepare a 20% solution that was diluted and used as an ashing aid and matrix modifier.

Sample Blending

Samples for analysis were blended in commercial food processors (typically a Robot Coupe Model R-15) equipped with a plastic bowl or a nylon-coated bowl and titanium blades. Food samples were cut with a titanium knife. Great care was taken to minimize the likelihood of contamination by eliminating the use of stainless steel apparatus. All utensils were nylon or polyethylene, and samples were prepared in a clean room or clean hood (Class 100). Homogenized samples were placed in acid cleaned polyethylene containers and stored at –40°C.

Dry Ashing Procedure

Approximately 0.5–2.0 g sample was weighed into a 25 × 150 mm quartz test tube and 100 μL 0.36% magnesium nitrate was added. Samples were dried on a heating block at 100°C. Dry samples such as cereals and grains were slurried with deionized distilled water before addition of magnesium nitrate to ensure adequate interaction of sample and matrix modifier. Samples were then placed in a muffle furnace at 100°C. Temperature was ramped 50–75°C/h to a final temperature of 480°C. Samples were heated overnight at 480°C; the next morning, they were removed from the muffle furnace, allowed to cool, and 1 mL concentrated sub-boiling distilled nitric acid was added. Samples were again dried on a heating block and then returned to the muffle at 250°C, and the temperature was ramped to a final temperature of 480°C. Samples were heated overnight. This acid treatment step was repeated until the sample ash was white. Finally, samples were cooled and diluted to a final volume of ca 10 mL with 5% nitric acid.

Results and Discussion

Dry Ashing Method Validation

Although we routinely do wet ash (HNO_3/H_2O_2) digestions of food samples, we recognize the benefit of dry ash sample preparation procedures for some elements and applications. Unfortunately, we often have had difficulty with chromium contamination from peroxide and have found that even high purity peroxides contain significant, and highly variable, amounts of chromium. We have had some success with Perone peroxide (Du Pont, Wilmington, DE), which is a product produced for the semiconductor industry, but there is some variability in chromium contaminant levels between batches of this product. As a result, we chose to pursue a modified dry ashing procedure that has reduced likelihood of contamination and that requires no peroxide. In addition, the equipment needed for dry ashing is simple, and sample manipulation is minimized, making this an attractive technique for batch analyses of large numbers of samples.

Three NIST standard reference materials (SRMs) were dry ashed and analyzed to validate this dry ashing procedure. Samples were dry ashed in quartz test tubes or disposable borosilicate tubes that were silanized to help keep samples from creeping up tube walls and to minimize contact with test tube walls. The results appear in Table 1. The first material, Mixed Diet RM 8431, was prepared and characterized by the Nutrient

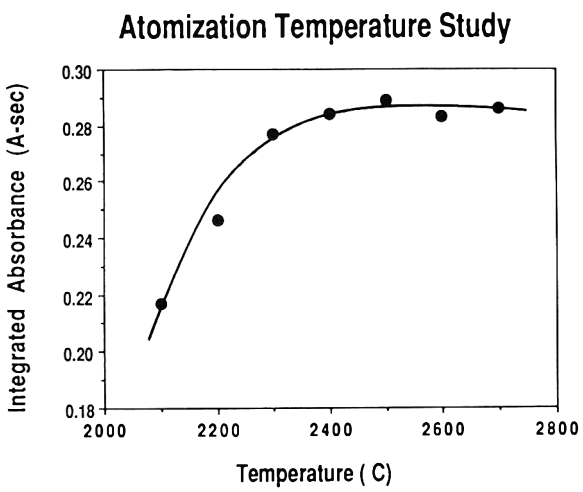

Figure 1. Atomization temperature optimization data.

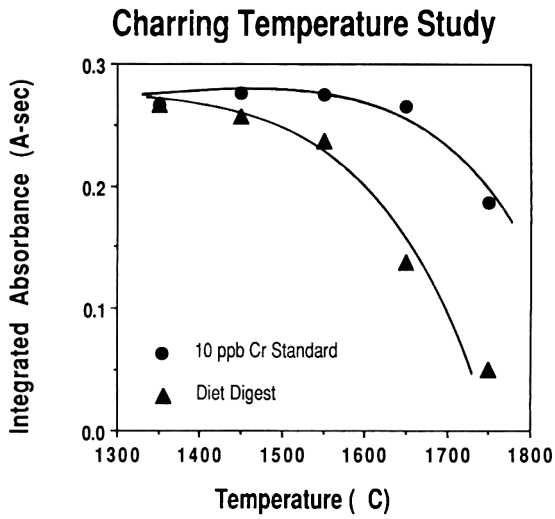

Figure 2. Charring temperature optimization data.

Composition Laboratory and is marketed by NIST (9). This freeze-dried material contains a representative cross section of those foods routinely consumed in an American diet. The determined chromium concentrations (mean ± standard deviation, $n = 9$) for all 3 materials fell within the certified concentration range (mean ± uncertainty) provided by NIST. These data verified the accuracy of the dry ashing sample preparation procedure.

GFAAS Program Optimization

The next step was to optimize the GFAAS method. This was done by performing charring and atomization temperature studies and selecting an appropriate matrix modifier. Magnesium nitrate was selected as a matrix modifier, and 5 µL 1.2% $Mg(NO_3)_2$ was added to provide a total of 0.06 mg/sample. Atomization temperature studies were conducted with temperatures ranging from 2200 to 2700°C. Figure 1 shows the results from the atomization temperature study. The optimum temperature was 2500°C because this was the lowest temperature in the plateau region of the curve. A charring study was performed with temperatures ranging from 1350 to 1750°C (Figure 2). Note that 2 different materials were used to establish the optimum charring temperature. An aqueous standard was used, and the data suggest that a temperature as high as 1650°C could be used without premature loss of analyte. A digest of Mixed Diet RM 8431 was then used, and the data suggest that 1450°C was the maximum allowable charring temperature that could be used without analyte loss, as evidenced by an almost 50% decrease in the chromium integrated absorbance signal. Mixed Diet RM 8431 was selected as being representative of the various food matrixes of interest because of its composition. Clearly, the sample matrix must be considered when determining optimum GFAAS operating conditions. The optimum char temperature was determined to be 1450°C and was used for the balance of this work. The optimized GFAAS program used is summarized in Table 2. Figure 3 contains resultant absorbance vs time plots for these conditions. Minimal background is seen with the 1450°C char, and peak shapes are very reasonable for both an aqueous standard and for the diet sample matrix. Slavin et al. (10) reported that fast furnace analyses are possible with the elimination of the char step. This was investigated and the results are summarized in Figure 4, where absorbance vs time plots are shown for the analysis of a Mixed Diet RM 8431 digest with and without the char step. With no char step, the results are biased low by approximately 13% and the background signal is more than doubled. The conclusion was that the char step could not be eliminated for this work.

Calibration Range Optimization

The identification of an optimum calibration working range was the next phase of this research. The concentration range considered was 1.0–100.0 µg/L (for 20 µL GFAAS injections). Because calibration algorithms are available to handle nonlinear curves, we were not necessarily limited to linear least squares fits. A calibration study was done starting with 0, 5.0, and 10.0 µg/L standards. Subsequently, 20.0, 50.0, and 100.0 µg/L standards were run and calibration curves were constructed. Figure 5 shows the curves with 10.0, 20.0, 50.0, and 100.0 µg/L top standards. For the first 3 curves, slopes are nearly identical and correlation coefficients suggest a good lin-

Table 2. Optimized GFAAS program[a]

Step	Temp., °C	Ramp, s	Hold, s
Dry	180	80	40
Char	1450	20	40
Cool down	20	1	10
Atomize	2500	0	8[b]
Clean out	2700	1	5

[a] Platform atomization used; matrix modifier, 0.06 mg $Mg(NO_3)_2$.
[b] 10 mL/min.

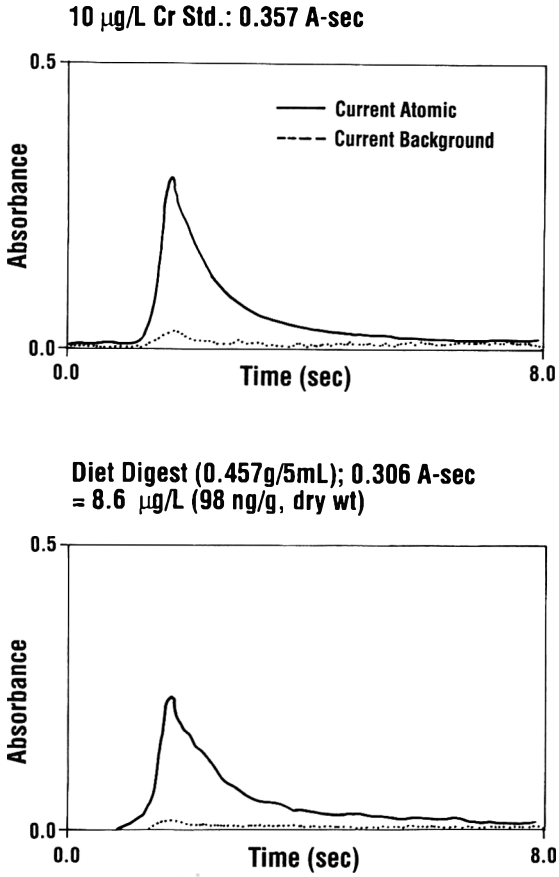

Figure 3. (Top) Absorbance vs time profile for 10 μg/L standard. (Bottom) Absorbance vs time profile for digest of Mixed Diet RM 8431.

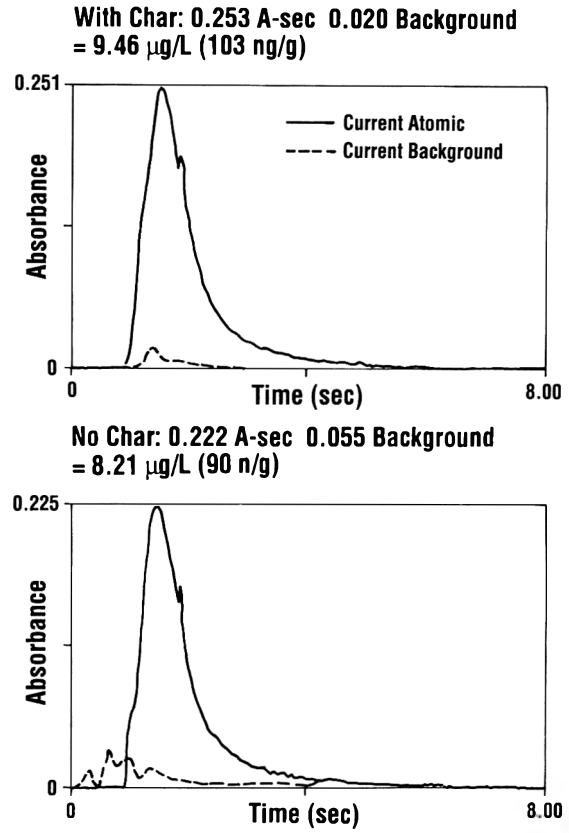

Figure 4. Absorbance vs time profile of digest of Mixed Diet RM 8431 (reference concentration, 102 ±6 ng/g) (top) with a char step, and (bottom) with no char step.

ear least squares fit. Admittedly, correlation coefficients are not necessarily the best measure of goodness-of-fit (11), but the fourth curve shows a poorer correlation coefficient as well as a decreased slope, suggesting that with a 100.0 μg/L top standard, the curve is not linear. Figure 6 compares the linear least squares fit and the nonlinear calibration algorithm provided in the Perkin-Elmer software (12). The nonlinear calibration algorithm provided an even poorer fit. On the basis of these data, the upper limit for calibration was selected to be 50 μg/L (for a 20 μL injection). Concentration precision studies were also conducted to discern where the optimum measurement precision could be obtained. The conclusion was that it is best to work above 20 μg/L whenever possible. Unfortunately, many samples analyzed fell below 20 μg/L. Table 3 contains data for several different dilutions of a Citrus Leaves NIST SRM 1572 digest. A single digest was diluted so that determinations were made in the 4–22 μg/L concentration range. Recoveries, based on comparison to the mean reference concentration value, ranged from 97 to 104%. There was no apparent systematic bias as a function of concentration. Although additional replicates are required at lower concentrations to obtain reasonable uncertainties, these data and similar data for other digests suggested that the optimum working range for analysis was approximately 5.0–25.0 μg/L.

Performance Characteristics

Through the course of the study, characteristic mass (m_0) and detection limits were monitored. The m_0, defined as that amount of analyte required to produce an integrated absorbance measurement of 0.0044, was typically 3.2 pg. This is consistent with the manufacturer's specification for chromium (3.3 pg). The calculation of the detection limit was based on 3.29 σ. A well-characterized blank was used to compute the

Table 3. Citrus leaves digest analyses[a]

Dilution	Approximate Cr concn, μg/L	Determined Cr concn, ng/g
×2	22	834
×5	8.5	772
×10	4.3	807

[a] Citrus leaves digest, 0.55 g/10 mL (ca 44 μg/L); certified concn, 800 ± 200 ng/g.

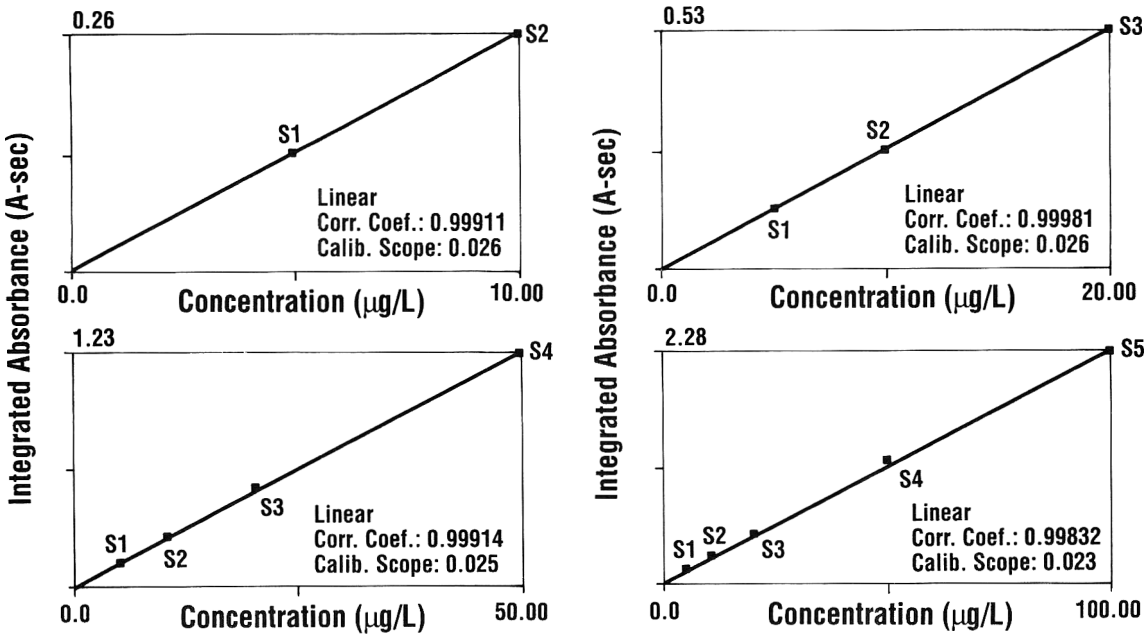

Figure 5. Cr calibration curves with top standards of (top left) 10 μg/L, (top right) 20 μg/L, (bottom left) 50 μg/L, and (bottom right) 100 μg/L.

standard deviation (σ). The average detection limit was 5.6 pg, or approximately 0.3 μg/L for a 20 μL injection.

Aqueous Calibration vs Method of Additions

We have experience in our laboratory with a prototype multielement atomic absorption spectrometer that has helped us to develop a strategy for method development (13). The preference is to avoid the unnecessary use of matrix modifiers, if possible, and to develop methods that are rugged and not matrix specific. As a result, the use of method of additions is avoided when possible. In this study, direct calibration against aqueous standards was compared to the method of standard additions. Table 4 compares data for 7 materials analyzed by both methods. Visual inspection of the data shows excellent agreement between the 2 methods for all materials. These materials represent a range of sample matrixes, and this suggests that direct calibration (with platform atomization and quantitation by integrated absorbance measurements) provides accurate results. Direct calibration was used for the balance of the study.

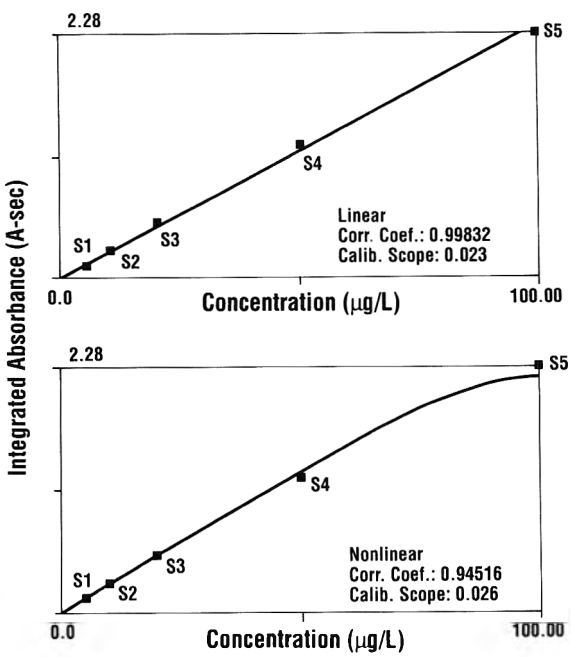

Figure 6. Comparison of curve fitting algorithms of (top) linear least squares, and (bottom) nonlinear fit.

Applications

The GFAAS method described here was used to determine chromium in a wide range of foods (14). This method was also

Table 4. Analytical results comparing straight calibration and method of additions

Food	Determined Cr concn, ng/g, as received	
	Aqueous calibration[a]	Method of additions[b]
Cola	2.00 ± 0.28	2.03 ± 0.04
Vegetable juice	11.9 ± 1.6	12.3 ± 1.4
Pepper	1027 ± 141	1067 ± 248
Corn chips	107 ± 16	100 ± 17
Cereal	100 ± 4	107 ± 2
Grape jelly	23.2 ± 1.5	22.7 ± 1.6
Mixed Diet RM 8431[c]	102 ± 9	107 ± 10

[a] Uncertainties represent ±1 standard deviation (n = 9).
[b] Uncertainties represent ±1 standard deviation (n = 3).
[c] Reference value for Mixed Diet RM 8431, 102 ± 6 ng/g.

Table 5. Beverage analyses

Material	Determined Cr concn, μg/L[a]	
	Dry ashing	Direct
Orange soda	4.63 ± 0.29	4.13 ± 0.50
Fruit punch	3.21 ± 0.26	2.90 ± 0.25
Vegetable juice	10.8 ± 1.1	10.2 ± 2.0

[a] Uncertainties represent ±1 standard deviation (n = 9).

used to provide some data that contributed to the reference values for 7 in-house control materials as well as a wide range of biological samples. A modified version of this method was also used successfully to determine chromium in urine and serum as well as chromium in solids prepared as slurries (15). Also, this method was used to help characterize several solid sampling controls developed by our colleagues in Germany. A wide range of beverages was also analyzed directly (not ashed) using the GFAAS method described. Data appear in Table 5 for 3 beverages analyzed by the direct method compared to the dry ash method. Good agreement is seen between the 2 methods, with determined concentrations (mean ± uncertainty) for the 2 methods agreeing favorably, pointing out the feasibility of doing direct determinations of beverages.

Conclusions

A graphite furnace atomic absorption method was developed for the accurate determination of chromium in foods and biological samples. Analysis of a diet sample digest highlighted the problem with optimizing charring temperatures with an aqueous standard. Magnesium nitrate was found to be a beneficial ashing aid as well as a matrix modifier. The deletion of the charring step of the furnace program was not possible because low chromium values were produced with this procedure. The time-consuming method of standard additions was found to be unnecessary. Samples were successfully quantitated using platform atomization, peak area measurements, and calibration against aqueous standards.

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VITAMINS AND OTHER NUTRIENTS

Determination of Insoluble and Soluble Dietary Fiber in Foods and Food Products: Collaborative Study

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A collaborative study was conducted to validate a method to determine the insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) contents of foods and food products by using a combination of enzymatic and gravimetric procedures. The method was basically the same as that for determining total dietary fiber, which was adopted as final action by AOAC and further modified to include changes in the concentration of buffer and base and substitution of hydrochloric acid for phosphoric acid. Thirty-nine collaborators were each sent 7 test samples in a staggered design for duplicate blind analysis. They were also sent a standard containing 4.3–5.4% IDF and 1.5–2.7% SDF. The 22 foods that were analyzed for IDF and SDF were cabbage, carrots, French beans, kidney beans, butter beans, okra, onions, parsley, chick peas, brussels sprouts, barley, rye flour, turnips, soy bran, wheat germ, raisins, Calimyrna figs, prune powder, Black Mission figs, apple powder, peach powder, and apricot powder. Both IDF and SDF values were calculated as the weight of residue minus the weight of protein and ash reported on a dry weight basis. The reproducibility relative standard deviation (RSD_R) of the IDF results ranged from 3.68 to 19.44% for the foods analyzed; almost half the test samples had an RSD_R <10%. The RSD_R values for the SDF results were somewhat higher. Approximately 50% of the foods analyzed had an RSD_R >20%, and 45% had an RSD_R between 10 and 20%. An RSD_R approaching 45% was calculated for the 2 test samples with the lowest SDF content, 1.35 and 1.90%. Raisins and prune powder had high RSD_R values for both SDF and IDF. A major reason for high RSD_R values seems to be filtration problems, which are avoidable by analyzing 0.5–0.25 g test samples. The method for the determination of SDF requires further study, but the method for the determination of IDF was adopted first action by AOAC International.

The enzymatic-gravimetric determination of total dietary fiber (TDF) in foods, **985.29**, was adopted as final action by AOAC (1). Further modifications, including changes in concentration of buffer and base and the use of hydrochloric acid instead of phosphoric acid, were also adopted as final action (2). A pilot study (2) found that the basic method for the determination of TDF could be modified to measure insoluble dietary fiber (IDF) by filtering out the IDF before precipitating the soluble dietary fiber (SDF) with ethanol, as described earlier in a similar method using physiological enzymes (3). TDF results from independent analysis were similar to TDF values obtained by summing IDF and SDF. However, the soy isolate appeared to have approximately 4 times as much IDF as TDF

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Mention of trade names, commercial firms, or specific products or instrumentation is for identification purposes only and does not constitute endorsement by the U.S. Food and Drug Administration.

(probably from the precipitation of some material other than fiber during the enzyme treatment step) and, therefore, about 4 times as much TDF when calculated by summing IDF and SDF, as compared with the independent determination of TDF. All other values (9 foods) for TDF obtained by summing IDF and SDF were acceptable. In the present study, the number and the variety of foods were extended to include more fruits and vegetables with higher amounts of SDF than those in the previous collaborative study. This study was designed according to the rules of Youden and Steiner (4).

Collaborative Study

The 39 collaborators participating in this study, representing 15 countries, were analysts in food companies, universities, and commercial and government laboratories. Each collaborator was sent 7 test samples for duplicate blind analysis. They were also sent a standard, containing 4.3–5.4% IDF and 1.5–2.7% SDF, and 3 enzymes (Termamyl, amyloglucosidase, and protease) to be used in the procedure. The values for the standard were derived from a previous collaborative study. The collaborators were further instructed to weigh test portions to the nearest 0.1 mg and to calculate % IDF and % SDF to 2 decimal places according to the formulas provided.

The following 22 foods were to be analyzed for SDF and IDF: (a) cabbage, (b) carrots, (c) French beans, (d) kidney beans, (e) butter beans, (f) okra, (g) onions, (h) parsley, (i) chick peas, (j) brussels sprouts, (k) barley, (l) rye flour, (m) turnips, (n) soy bran, (o) wheat germ, (p) raisins, (q) Calimyrna figs, (r) prune powder, (s) Black Mission figs, (t) apple powder, (u) peach powder, and (v) apricot powder. Each food was analyzed by at least 6 laboratories except for q, t, and v. Items a–m were purchased at the local supermarket either fresh, canned, or as the dried product. Item n was supplied by Solnut, Inc., Hudson, IA; item o by Vitamins, Inc., Chicago, IL; item p by the California Raisin Advisory Board, San Francisco, CA; items q and s by the California Fig Advisory Board, Fresno, CA; and items r and t–v by Vacu-Dry, Santa Rosa, CA.

Table 991.42. Method performance for 991.42 Insoluble dietary fiber in food and food products by enzymatic-gravimetric method

Food/food product	No. labs.	IDF, av. %	s_r	s_R	RSD_r, %	RSD_R, %
Beans, butter	10	17.36	0.41	1.96	2.34	11.31
Beans, French	10	25.64	0.83	1.51	3.23	5.87
Beans, kidney	13	16.33	0.74	1.04	4.53	6.39
Brussels sprouts	15	30.23	0.69	2.39	2.27	7.89
Cabbage	9	21.60	0.86	1.68	4.00	7.79
Carrots	12	32.29	1.74	3.68	5.38	11.39
Chick peas	12	16.69	1.73	2.80	10.38	16.80
Okra	14	24.15	1.55	3.28	6.43	13.57
Onions	12	13.32	0.87	1.57	6.51	11.79
Parsley	12	34.39	1.22	4.69	3.56	13.64
Turnips	12	21.38	1.41	3.55	6.60	16.61
Apples	4	55.57	0.51	2.53	0.92	4.55
Apricots	5	44.92	0.39	3.69	0.86	8.22
Figs, Calimyrna	5	43.07	2.41	7.92	5.59	18.40
Figs, Mission	6	33.61	0.93	4.06	2.76	12.09
Peaches	6	39.53	0.86	2.44	2.17	6.16
Prunes	6	46.18	2.82	8.98	6.11	19.44
Raisins	8	49.18	2.71	9.49	5.51	19.30
Barley	12	4.30	0.43	0.62	9.92	14.33
Rye flour	15	11.81	0.58	1.02	4.87	8.62
Soy bran	13	65.24	0.91	2.40	1.40	3.68
Wheat germ	9	15.67	0.71	0.96	4.54	6.13

To prepare test samples, all products were homogenized in a Cuisinart, lyophilized, and ground in a continuous-grinding Microjet 10 Centrifugal Mill (Quartz Technology, Inc., Westbury, NY) to a uniform size of 350 μm. Items b and p–v were extracted 3 times each with 10 volumes of 85% methanol to remove sugars, lyophilized overnight to a dry material, and ground in a Microjet 10 Centrifugal Mill. The test samples were dried at 70°C in a vacuum oven and then stored at room temperature in numbered 25 mL plastic vials. None of the test samples contained >10% fat; therefore, fat extraction was not recommended.

A moisture analysis was performed before the determination of IDF and SDF by the collaborator to report the results on a dry matter basis.

991.42 Insoluble Dietary Fiber in Food and Food Products—Enzymatic–Gravimetric Method, Phosphate Buffer

First Action 1991

(Applicable to determination of insoluble dietary fiber in vegetables, fruit, and cereal grains.)

Method Performance:

See Table **991.42** for method performance data.

A. Principle

Duplicate test portions of dried foods, fat-extracted if they contain >10% fat, are gelatinized with Termamyl (heat-stable α-amylase) and then enzymatically digested with protease and amyloglucosidase to remove protein and starch. SDF is removed by filtering and washing residue with water. Remaining residue, IDF, is washed with 95% ethanol and acetone, dried, and weighed. One duplicate is analyzed for protein, and the other is incinerated at 525°C to determine ash. IDF is weight of residue less weight of protein and ash.

B. Apparatus

See **985.29B.**

C. Reagents

See **985.29C.** (*Note*: Reagent (**e**), Termamyl solution, is available from Novo Nordisk Biolabs, 33 Turner Rd, Danbury, CT 06810.)

(**a**) *85% Methanol.*—Place 105 mL water into 1 L volumetric flask, and dilute to volume with 95% ethanol.

Table 1. Collaborative results (blind duplicates) of determination of IDF and SDF by enzymatic-gravimetric method

Food/food product	Coll.[a]	IDF duplicates, %		SDF duplicates, %	
Beans, butter	2	14.77	15.60	2.76	2.98
	3	16.61	17.04	3.04	3.42
	8	18.25	18.25	3.15	3.66
	12	15.44	15.54	4.33	4.30
	13	16.46	15.87	2.79	2.87
	17	19.52	19.89	3.06	2.75
	21	21.32	20.40	2.76	3.19
	23	15.22	15.66	2.25	1.66
	27	16.57	17.48	2.98	2.14
	30	18.76	18.53	3.97	3.39
Beans, French	2	24.65	24.26	8.74	10.12
	7	25.15	26.76	11.74	11.20
	16	27.20	27.53	11.64	11.28
	17	25.05	27.07	9.69	10.02
	19	25.77	24.91	12.06	11.51
	23	24.29	24.87	11.42	10.49
	25	23.79	24.13	—[b]	—
	26	24.95	25.37	11.65	11.23
	27	25.64	25.20	11.65	11.41
	30	27.40	29.69	9.37	10.43
Beans, kidney	1	16.79	17.59	4.45	3.91
	2	15.93	14.18	3.79	4.26
	4	15.39	16.04	4.55	5.53
	17	20.01	27.52[c]	2.88	3.39
	22	15.33	15.23	3.44	5.46
	23	16.60	15.05	2.90	3.31
	27	16.88	16.99	4.18	3.66
	30	17.30	18.37	3.63	3.69
	31	15.52	15.89	3.80	3.74
	35	17.06	17.84	4.67	3.53
	37	15.47	17.35	3.05	3.29
	38	16.62	16.67	4.02	4.13
	39	16.47	17.55	3.24	3.30
	40	15.25	—	2.13[d]	1.78[d]
Brussels sprouts	1	29.24	29.26	6.38	6.14
	3	33.26	31.28	6.36	7.39
	4	31.01	30.85	7.65	8.49
	8	31.58	32.65	5.84	6.91
	12	29.14	29.14	6.73	6.57
	13	27.89	26.56	8.09	9.54
	21	35.09	34.04	4.93	5.27
	22	29.56	30.70	5.70	—
	31	30.84	32.33	6.29	6.24
	33	32.33	—	5.90	—
	35	32.96	31.95	4.49	4.92
	37	29.36	29.00	4.77	6.50
	38	27.67	27.31	6.40	6.73
	39	26.62	26.11	5.08	4.94
	40	28.65	28.27	5.00	3.92
Cabbage	1	21.16	20.56	7.03	6.52
	4	21.53	19.74	6.99	9.08
	22	23.00	21.08	5.93	5.90
	31	24.00	24.07	5.53	6.85
	35	23.95	22.92	3.80	3.32
	37	23.14	22.25	3.42	3.36
	38	20.55	20.99	5.18	5.21
	39	18.97	19.23	4.42	4.96
	40	19.85	21.86	4.77	5.03
Carrots	2	31.71	30.99	11.03	11.37
	5	27.85	29.85	10.01	—
	9	34.89	35.47	8.56	9.54
	10	37.64	—	8.17	—
	11	30.07	32.71	12.86	13.42
	14	28.34	31.33	—	—
	17	36.12	34.98	10.71	12.38
	23	27.94	23.82	13.68	—
	27	34.95	35.29	11.24	9.45
	28	29.92	—	10.83	—
	30	36.97	35.34	10.73	10.65
	32	28.50	32.81	14.43	10.61
Chick peas	3	15.25	15.57	0.91	1.20
	8	19.05	19.03	1.11	1.36
	9	13.31	16.05	0.97	—
	10	17.00	16.90	0.72	0.45
	11	18.17	14.82	0.72	2.53
	12	12.23	12.46	2.11	2.11
	13	16.35	17.59	1.07	1.31
	14	20.66	18.22	1.38	0.87
	21	24.13	18.23	1.16	1.77
	28	12.60	—	1.35	—
	32	18.32	17.09	1.86	2.64
	33	18.39	16.57	0.77	1.63
Okra	1	22.84	26.19	13.28	12.27
	4	21.74	24.15	14.47	12.76
	5	18.58	20.30	10.86	10.48
	9	23.04	23.66	9.82	12.45
	11	24.57	—	13.91	—
	14	31.50	31.14	—	—
	22	21.66	23.23	—	—
	28	22.43	28.04	15.64	14.49
	31	21.97	23.19	14.90	12.95
	32	28.43	29.34	9.32	9.08
	35	25.19	26.21	12.48	10.36
	37	24.02	22.48	13.87	12.18
	38	22.54	23.40	11.14	11.94
	39	20.23	21.60	10.38	10.08
	40	8.96	16.15[d]	10.23	—
Onions	2	12.42	13.11	3.49	2.23
	5	10.14	12.71	1.51	2.95
	9	14.31	13.66	2.65	5.31
	10	16.83	—	2.30	—
	11	12.07	12.14	1.88	5.07
	14	14.72	12.66	—	—
	17	12.11	12.26	3.91	3.82
	23	11.67	11.53	5.34	5.29

Table 1. ***Continued***

Food/food product	Coll.[a]	IDF duplicates, %		SDF duplicates, %	
	27	15.56	15.30	1.93	1.93
	28	13.19	—	4.23	—
	30	12.55	12.78	4.58	3.95
	32	13.06	14.82	4.85	5.19
Parsley	3	34.31	33.30	4.54	3.21
	7	28.27	27.28	10.17	9.02
	8	39.11	39.21	3.09	3.85
	12	24.46	25.43	10.46	10.43
	13	39.15	37.74	3.40	3.23
	16	39.92	36.46	1.92	4.27
	18	31.51	—	—	—
	19	37.37	38.98	5.82	2.35
	21	36.06	35.50	4.25	4.37
	25	36.91	—	—	—
	26	34.37	36.15	4.55	3.41
	33	33.99	31.34	—	—
Turnips	3	19.84	24.40	11.03	8.25
	7	19.61	19.65	11.40	12.78
	8	24.70	28.44	5.46	3.62
	12	20.32	20.39	9.22	9.71
	13	17.91	17.41	10.22	10.26
	16	26.03	26.67	7.27	6.73
	18	22.42	—	—	—
	19	17.40	17.60	10.25	10.51
	21	25.10	23.39	—	—
	25	18.33	—	—	—
	26	17.26	18.07	10.61	11.77
	33	24.13	23.28	—	—
Apples	7	55.73	55.20	16.25	15.86
	18	59.09	59.16	16.96	18.23
	19	53.89	53.21	20.68	21.11
	26	54.71	53.56	19.18	18.29
Apricots	2	41.14	41.03	25.51	26.48
	17	48.16	48.83	25.92	23.36
	23	42.42	37.49	25.81	24.04
	27	47.77	47.48	22.85	22.78
	30	42.09	42.89	34.10	33.43
Figs, Calimyrna	2	41.54	41.18	17.85	16.66
	17	58.10	54.86	13.37	15.54
	23	43.48	37.37	19.29	20.74
	27	41.67	39.68	17.41	17.62
	30	35.18	37.66	21.76	21.22
Figs, Mission	7	32.22	34.61	11.27	11.44
	16	38.24	38.74	8.43	9.71
	18	38.10	38.34	11.17	11.36
	19	31.63	32.07	12.79	9.78
	25	30.67	—	12.41	14.93
	26	28.24	29.78	12.22	11.07
Peaches	7	40.21	39.38	23.97	23.44
	16	43.71	42.54	25.50	25.89
	18	41.25	41.61	26.62	26.02
	19	37.64	37.24	29.05	31.17
	25	37.84	37.54	29.33	28.47
	26	38.93	36.41	30.12	29.24
Prunes	3	39.61	39.52	39.76	41.46
	8	56.46	55.01	23.19	22.76
	12	37.32	38.02	44.25	44.24
	13	37.72	37.36	39.19	40.23
	21	56.20	50.24	27.11	33.49
	33	49.58	57.14	24.49	20.91
Raisins	5	54.24	—	—	—
	9	63.27	56.34	10.62	8.75
	11	60.06	58.14	9.39	11.49
	14	50.35	51.36	19.75	—
	28	44.45	—	11.69	—
	37	42.01	40.47	23.39	23.39
	39	51.42	53.38	9.36	8.71
	40	35.35	33.51	19.82	16.74
Barley	1	4.42	4.75	4.89	4.13
	3	3.24	4.38	2.41	3.67
	4	3.30	2.92	1.25	1.33
	8	5.47	4.37	3.47	2.34
	12	4.05	4.21	4.49	4.45
	13	4.02	4.85	4.14	4.75
	21	4.62	—	4.39	3.80
	22	4.90	5.20	5.54	6.91
	31	4.02	4.26	3.95	4.50
	33	3.86	4.46	2.18	1.80
	35	4.06	3.91	4.24	4.26
	38	4.69	4.71	4.26	4.84
Rye flour	1	11.70	12.32	3.55	3.30
	4	12.63	12.01	3.51	1.97
	9	11.20	10.24	2.36	2.48
	10	11.51	12.25	3.44	3.52
	11	12.16	11.42	3.88	3.50
	14	12.52	13.42	4.01	2.88
	22	11.19	11.22	3.78	—
	28	12.99	—	3.74	—
	31	11.94	11.84	4.54	4.32
	32	13.75	13.89	2.25	3.29
	35	11.71	—	4.06	—
	37	11.39	11.35	2.89	3.35
	38	11.14	11.17	3.49	3.60
	39	10.99	10.81	2.99	3.16
	40	11.46	9.24	2.62	2.56
Soy bran	5	58.48	60.55	—	—
	7	62.40	61.73	7.40	7.93
	9	66.35	65.93	4.95	5.91
	10	67.43	68.08	5.65	7.96
	11	64.40	66.36	5.84	8.73
	14	65.21	65.01	7.29	8.42

Table 1. ***Continued***

Food/food product	Coll.[a]	IDF duplicates, %		SDF duplicates, %	
	16	68.16	67.25	6.73	6.14
	18	67.35	67.61	—	—
	19	64.99	64.61	7.14	6.84
	25	64.87	65.39	—	—
	26	66.44	63.55	6.55	8.71
	28	66.08	67.75	7.15	7.63
	32	64.98	65.17	7.22	7.43
Wheat germ	1	15.21	17.49	2.45	2.14
	4	16.61	17.35	0.70	0.77
	22	15.33	15.30	3.69	2.35
	31	16.33	15.80	2.18	2.10
	35	14.97	16.26	2.97	2.52
	37	16.23	15.46	1.59	1.93
	38	15.50	15.08	1.67	1.29
	39	15.53	15.52	2.18	1.72
	40	13.63	14.43	1.23	0.67

[a] Collaborators 6, 15, 24, 26, 29, and 30 did not report IDF results and Collaborators 6, 15, 24, 29, and 30 did not report SDF results in time for evaluation. IDF and SDF results from Collaborator 5 are not included because of analyst's concerns.
[b] No results reported.
[c] Cochran and Grubbs outlier.
[d] Grubbs outlier.

D. Enzyme Purity

See **985.29D.**

E. Sample Preparation

Analyze dry samples without pretreatment whenever possible. Mill dry samples to 0.3–0.5 mm mesh. Homogenize and freeze-dry wet foods before milling. If high fat content (>10%) prevents proper milling, defat with petroleum ether (3 times with 25 mL portions/g sample) before milling. Determine residual moisture in milled samples by drying overnight in 70°C vacuum oven, or 5 h in 105°C air oven. Record weight loss due to fat and/or water, and make appropriate correction to final % TDF. (*Note*: For samples high in sugars that cannot be dried by lyophilization, extract 3 times each with 10 volumes of 85% methanol to remove sugars, which may interfere in determination.)

F. Determination

Run blank with samples to measure any contribution from reagents to residue.

Weigh duplicate 1 g samples, accurate to 0.1 mg, into 400 mL tall-form beakers. Duplicate sample weights should not differ by >20 mg. Add 50 mL phosphate buffer to each beaker. Check pH and adjust to pH 6.0 ± 0.2 by adding 0.275N NaOH or 0.325N HCl. Add 0.1 mL Termamyl solution to each beaker. Cover beakers with aluminum foil and place in boiling water bath. Shake beakers gently at 5 min intervals throughout incubation. When thermometer indicates beaker contents have reached 100°C, continue incubation 15 min. Total of 30 min in bath is usually sufficient. Cool solutions to room temperature. Adjust to pH 7.5 ± 0.1 by adding ca 10 mL NaOH solution.

Add 5 mg protease to each solution. Protease sticks to spatula, so it may be preferable to prepare enzyme solution (50 mg in 1 mL phosphate buffer) just before use, and pipet 0.1 mL to each sample.

Cover beakers with aluminum foil. Incubate 30 min at 60°C with continuous agitation. Cool. Check pH and adjust to pH 4.0–4.6 with ca 10 mL HCl solution. Add 0.3 mL amyloglucosidase, cover with aluminum foil, and incubate 30 min at 60°C with continuous agitation.

Weigh crucible containing Celite to nearest 0.1 mg. Wet and redistribute bed of Celite in crucible, using stream of water from wash bottle. Apply suction to draw Celite onto fritted glass as even mat. Apply enzyme mixture from beaker to crucible, filtering into suction flask. Wash residue 2 times with 10 mL water (removing SDF), 2 times with 10 mL 95% EtOH, and 2 times with 10 mL acetone. Break surface film that develops after addition of sample to Celite with spatula, to improve filtration. Careful intermittent suction throughout filtration and back-bubbling with air, if available, will speed up filtrations. Normal suction can be applied at washing.

Dry crucible containing residue overnight in 70°C vacuum oven or 5 h in 105°C air oven. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weights to determine residue weight.

Using 1 of duplicates, scrape sample, Celite, and fiber mat onto filter paper that can be folded shut, and analyze for protein by **960.52**. Use N × 6.25 as conversion factor.

Incinerate second of duplicates 5 h at 525°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weights to determine ash.

G. Calculations

See **985.29G**, calculating IDF as described for TDF.

Ref.: *J. AOAC Int.* **75**, March/April issue (1992)

Results and Discussion

The determination of TDF by an enzymatic-gravimetric procedure was adopted as final action by AOAC in March 1986 (1) and modified by changing the concentration of buffer and base and by using hydrochloric acid instead of phosphoric acid (2). In the collaborative study reported in this paper, the method for measuring IDF and SDF was applied to a wide variety of 22 foods: cabbage, carrots, French beans, kidney beans, butter beans, okra, onions, parsley, chick peas, brussels sprouts, barley, rye flour, turnips, soy bran, wheat germ, raisins, Calimyrna and Black Mission figs, and prune, peach, apple, and apricot powders.

Thirty-two of the laboratories submitted results. Six laboratories did not report any results for the test samples they were sent, despite numerous follow-up letters. Laboratory 5 reported values but indicated dissatisfaction with its data and the procedure. We decided against using its results.

The results of the individual determinations of IDF are shown in Table 1. Only 2 laboratories' values were not used

Table 2. Method performance for SDF in food and food products by enzymatic-gravimetric method

Food/food product	No. labs.	SDF, av. %	s_r	s_R	RSD_r, %	RSD_R, %
Beans, butter	10	3.07	0.33	0.68	10.62	22.17
Beans, French	9	10.85	0.51	0.95	4.71	8.75
Beans, kidney	13	3.48	0.61	0.68	17.43	19.48
Brussels sprouts	15	6.16	0.61	1.29	9.91	20.99
Cabbage	9	5.41	0.62	1.56	11.51	28.88
Carrots	11	11.02	1.25	1.74	11.37	15.76
Chick peas	12	1.35	0.53	0.60	39.06	44.38
Okra	13	12.06	1.05	1.91	8.71	15.85
Onions	11	3.59	1.09	1.36	30.41	37.86
Parsley	9	5.13	1.12	2.92	21.83	56.86
Turnips	8	9.32	0.97	2.53	10.38	27.14
Apples	4	18.56	0.43	2.44	2.34	13.17
Apricots	5	26.43	1.05	4.31	3.98	16.36
Figs, Calimyrna	5	18.15	0.93	2.79	5.10	15.39
Figs, Mission	6	10.84	1.23	1.47	11.32	13.58
Peaches	6	27.30	0.84	3.17	3.09	11.61
Prunes	6	33.42	2.19	9.53	6.56	28.51
Raisins	7	14.61	1.34	6.02	9.14	41.21
Barley	12	3.83	0.53	1.37	13.92	35.87
Rye flour	15	3.35	0.47	0.64	13.96	19.25
Soy bran	10	7.08	1.04	1.04	14.66	14.66
Wheat germ	9	1.90	0.40	0.81	21.12	42.83

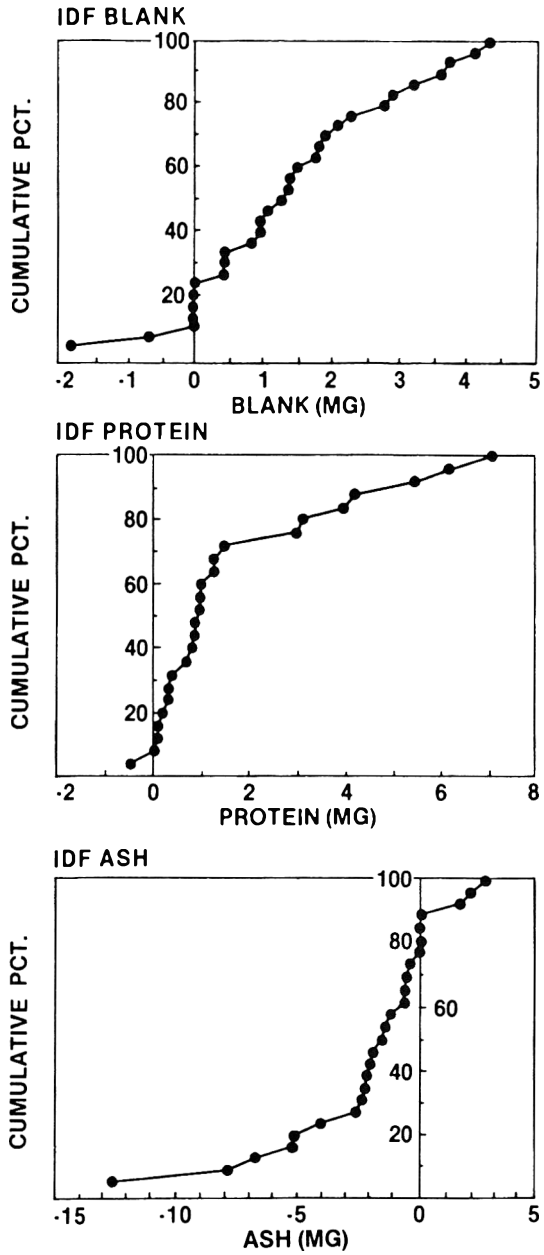

Figure 1. Comparison of cumulative percent for blanks, protein, and ash for IDF.

because of statistical considerations. Data from 1 of 15 laboratories for the determination of IDF in okra, and data from 1 of 14 for the determination of IDF in kidney beans were omitted. All other values were used as reported by the analysts. The measures of precision for IDF are shown in Table **991.42**. The average IDF values ranged from 4.30% for barley to 65.24% for soy bran. The repeatability relative standard deviation (RSD_r) of the determination for IDF of the 22 foods analyzed ranged from 0.86% for apricots to 10.38% for chick peas. These RSD_r values are considered excellent for this concentration range. The reproducibility relative standard deviation (RSD_R) ranged from 3.68% for soy bran to 19.44% for prunes. These RSD_R values are also very good, considering that 10 of the 22 foods analyzed had RSD_R values <10%, and an additional 7 foods had RSD_R values <15%. The highest RSD_R values were those for prunes, raisins, and Calimyrna figs; the lowest was for apples.

The results of individual determinations of SDF are shown in Table 1. Collaborator 40's values represented a Cochran and Grubbs outlier in the determination of SDF in kidney beans and were dropped for statistical considerations. The measures of precision for the determination of SDF are shown in Table 2. The products analyzed had average SDF values that ranged from 1.35% for chick peas to 33.42% for prunes. RSD_r values ranged from 2.34% for apples to 39.06% for chick peas, with approximately 50% of the laboratories having RSD_r values <10%. Of RSD_R values for the 22 foods, approximately 50% were >20%, 45% were between 10 and 20%, and only French beans had an RSD_R <10%. The preliminary results for SDF determinations indicate that a major reason for the differences between the laboratories is long and variable filtration times, both when the IDF fraction is filtered away from the TDF fraction and when the precipitated SDF fraction is separated in the second filtration. Several laboratories suggested that these problems can be overcome by using a 0.5–0.25 g test portion for analyzing materials with high levels of viscous fiber, which hinders the filtration.

Because good results are obtained both for TDF determinations (1) and, as this study has demonstrated, for IDF determinations, it follows that SDF can be determined by the difference between TDF and IDF.

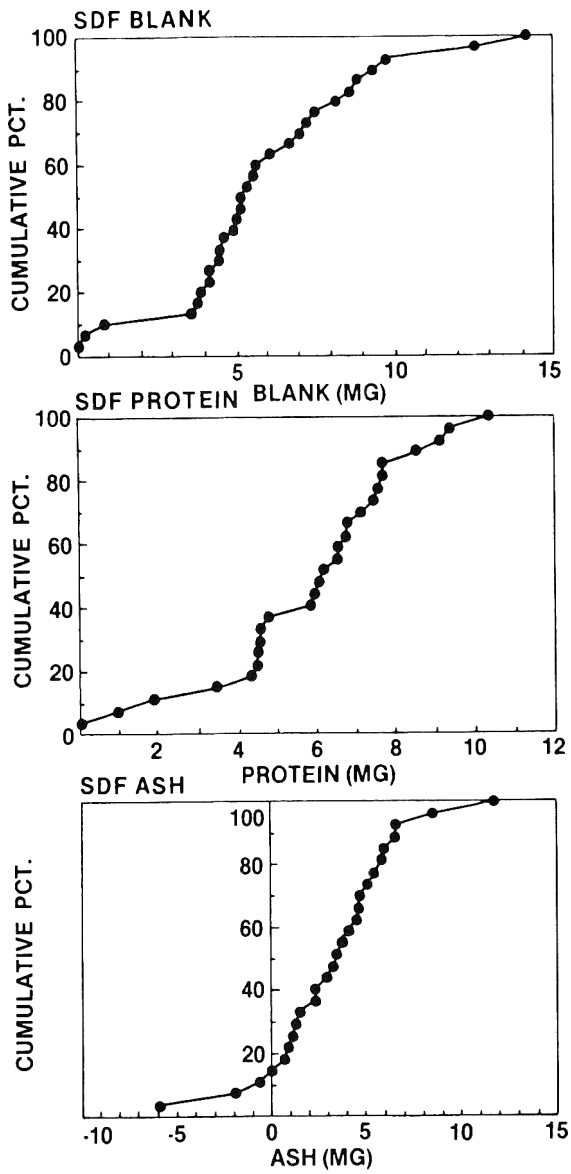

Figure 2. Comparison of cumulative percent for blanks, protein, and ash for SDF.

In previous collaborative studies, we did not report values for blanks, protein, and ash in the determination of dietary fiber. For this study, the individual values for blanks, protein, and ash for IDF are illustrated in Figure 1. If one plots the percentage equal to or greater than (cumulative %) the value for the measurement of blanks, protein, and ash against the individual values for the respective determinations, the following observations can be made. For IDF blanks, the 5–95 percentile range is from –1.2 to 3.9 mg, i.e., 5% of the blank values fall below –1.2 mg and 5% fall above 3.9 mg. The average IDF blank is 1.44 mg. For IDF protein, the 5–95 percentile values range from 0.37 to 6.03 mg. The average IDF protein is 1.75 mg. One negative protein value was reported by a laboratory. Also, the reporting of zero or close to zero values by a few participants suggests that the Kjeldahl procedure should be checked. For IDF ash, the 5–95 percentile values range from –11.25 to 2.05 mg. The average IDF ash value is –2.08 mg. The negative ash values reported by many laboratories are obviously due to losses of Celite through the filter. The quality of both the crucibles and the Celite should be checked when such losses exceed 5 mg. Such results were reported by 3 laboratories.

The individual values for blanks, protein, and ash for SDF are illustrated in Figure 2. For SDF blanks, the 5–95 percentile values range from 0.1 to 11.2 mg. The average SDF blank is 5.95 mg. For SDF protein at the 5–95 percentile, the values range from 0.35 to 9.33 mg. The average SDF protein is 5.95 mg, with a range of 0.0–10.5 mg. For SDF ash at the 5–95 percentile, the values range from –4.65 to 7.9 mg. The average SDF ash is 3.34 mg.

Only 3 laboratories reported negative ash values for the SDF blank determination. This finding indicates less Celite loss in the second filtration with 78% ethanol. The zero or very low protein values reported by some laboratories should also be checked.

Recommendation

We recommend that the method for the determination of IDF be adopted as first action. This method can be used in conjunction with the final action method for the determination of TDF to obtain SDF by difference until a procedure for the determination of SDF is developed. We further recommend that test samples high in sugar be extracted with 3 volumes of 85% methanol to remove sugars, which keep the test samples from drying and may interfere with the fiber determination.

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TECHNICAL COMMUNICATIONS

History of the IUPAC/ISO/AOAC Harmonization Program

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As a result of the preference of the Joint Food and Agriculture Organization/World Health Organization Codex Alimentarius Program to endorse methods of analysis for which interlaboratory performance parameters are available, many international organizations now conduct method-performance (collaborative) studies. International meetings sponsored by the International Union of Pure and Applied Chemistry, the International Organization for Standardization, and AOAC International have produced a harmonized protocol for the design, conduct, and interpretation of method-performance studies, and this protocol is being implemented by many method standardization organizations. The same group of organizations hopes to provide a harmonized protocol for the quality control and quality assurance of laboratory (analyst) performance.

Considerable evidence exists in the literature that few analytical chemists pay attention to the question of the reliability of the analytical results they produce. These chemists believe a natural law exists in measurement science that if the directions for conducting a measurement are followed, the true value necessarily results. This may have been true a generation or two ago, when analytical measurements were based on the laws of mass action, and the analytical system was based on a series of unit operations such as weighing, solution, precipitation, filtration, drying, or titration. Then, only carelessness and inexperience kept the laws of chemistry from producing the correct result.

Today, most analytical operations are based on physical, not chemical, principles. One would think that physical laws would be harder to transgress than chemical laws, but Murphy's law, "If anything can go wrong, it will," is overriding. Unfortunately, the consequences of the action of Murphy's law lie hidden until someone's life or property is affected. Then, experiments are repeated, disparate results occur, and disagreements arise. In the manufacturing of items, where the concept of quality control of repetitive operations arose, mistakes exhibited themselves by items not conforming to specifications, parts not fitting, and machines not operating. However, analytical mistakes do not announce themselves. We must find ways to discover aberrant chemical results and to eliminate them before others find them for us.

Many analytical laboratories appear to believe in the divine right of chemists to analyze everything for anything, and they think that to turn down the opportunity to perform an analysis reflects on their professional credentials. However, part of every analytical report should be a local audit that provides a confidence interval in which the "true value" is expected to lie within a reasonable probability, e.g., 95%. The basis for such a confidence interval is the proper use of validated methods that would give expected results when applied to materials with a known true value. Without such a chain of evidence, how do you know that your results are right? The fact that proper results were obtained yesterday is a necessary, but not a sufficient, condition.

Methods

To obtain valid results, the first line of defense is validated methods. Chemists would never think of using a new instrument before performing exhaustive tests to ensure that the machine is operating properly; that the sensitivity, resolution, and repeatability at least match the specifications on which the order was based. However, chemists seem to have no hesitation in taking a method fresh out of the literature and applying it to the current problem, particularly when they see the opportunity for fine-tuning some of the conditions. By adjusting the pH or modifying a reagent concentration, they can state that the analysis was accomplished by their modification of the method.

The first and probably the easiest way to improve the results of chemical analysis was to standardize the analytical methods. Many organizations, both national and international, had undertaken this task on a commodity-by-commodity basis for more than a century. This was noted by Harold Egan, the Government Chemist of the United Kingdom, in his lively address to AOAC in 1973 (1). Egan pointed out the dangers "of overlap and duplication of effort and the difficulty in deciding which of 2 or more well-authenticated methods, each properly established by collaborative study, should be selected for standardization or referee or arbitration purposes. . . ." Egan continued, "The time is coming, I believe, when we will need to consider the harmonization of collaborative studies, and to call upon the various agencies which organize collaborative studies to study not the methods of analysis themselves but the basis on which

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collaborative studies are made and their management." With his insight he prophesied that it would be helpful "to consider the question of how far methods require standardization and how far the analyst (or rather the standard of his professional training and attainment) should instead be the subject of standardization. Where in a method of analysis does the written description end and the professional analyst begin?"

Egan was the United Kingdom delegate to the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products. This organization was established in 1958 to develop international standards for milk products. It is the predecessor organization to the FAO/WHO Codex Alimentarius ("Codex" for short), which develops international standards for all food products. From its earliest sessions, the Milk Committee bogged down on the question of choice of methods of analysis because the 3 primary organizations in the field were attempting to dominate the method approval process: the International Dairy Federation (IDF), the International Organization for Standardization (ISO) Technical Committee on Milk Products, and AOAC. At the time, the United States was not a participant in either IDF or ISO standardization programs, and both organizations had a strong incentive to have such participation. Largely at the urging of the Codex Secretariat, an IDF/ISO/AOAC technical group was formed with the responsibility of supplying jointly approved methods of analysis for supporting international Codex standards for milk products. In a surprisingly short time, about 12 harmonized methods were approved by the joint group and by its sponsoring organizations for submission to the Committee of Government Experts. This Joint Committee continues to function and sponsors an annual "Chemical Week" at which methods are discussed among representatives of the 3 organizations. The harmonization has been extended even to microbiological methodology.

A similar situation developed in the Codex Committee on Methods of Analysis and Sampling (CCMAS), which had the responsibility to review and endorse methods of analysis required for supporting the now enlarged overall program of developing international standards for all food. Many food standards contained specifications that required enforcement through the application of analytical methods. Many organizations aspired to the role of supplying its methods to the Codex program. Food analysts had a history of 100 years of "improving" their methods of analysis. This resulted in a multitude of procedures for food constituents, some with good reason, such as driving off volatiles from delicate sugars at 70°C to avoid decomposition. However, the conditions of some procedures are arbitrary, such as various methods for loss on drying, using practically every temperature from 98 to 105°C, with and without vacuum. These irritatingly small deviations nevertheless made a significant difference in the reported solids content of a food. Consequently, a set of principles was developed for use by this Committee as a guide for the selection of methods of analysis.

The detailed set of principles for the choice of methods of analysis adopted by the Codex (2) may be summarized as follows: (*1*) Methods of analysis compiled by international organizations should be preferred; (*2*) methods of analysis whose reliability was established through method-performance (collaborative) studies are preferred, speed and simplicity are of secondary importance; (*3*) methods of analysis should measure the entity that they are claimed to measure; (*4*) methods of analysis should be usable in laboratories with the usual modem apparatus; and (*5*) methods of analysis that are applicable uniformly to various groups of materials are preferred to those that can be applied only to individual materials.

As a result of the adoption of these guidelines, organizations realized that methods to be used by many laboratories had to be tested in many laboratories. The imprimatur of a prestigious organization no longer sufficed as the guarantee of satisfactory performance. Even the largest organization, ISO, began encouraging its technical committees to perform interlaboratory method-performance studies so that methods could be submitted for acceptance to the Committees of the Codex Alimentarius. However, such independent activities naturally led to independent guidelines for the design, conduct, and interpretation of collaborative studies. Therefore, Egan recommended that the Analytical, Applied, and Clinical Divisions of the International Union of Pure and Applied Chemistry (IUPAC) "call together representatives of the main international bodies who at present sponsor collaborative analytical studies in order to discuss the philosophy of collaborative analysis and the possible harmonization of the approach to such studies" (3).

The representatives of various organizations that standardize methods of analysis met in London, England, in 1978, and they later participated in a formal meeting in Helsinki, Finland, August 20–21, 1981, under the auspices of IUPAC's Analytical, Applied, and Clinical Divisions. The proceedings (4) show that there was no lack of appreciation for the necessity of providing estimates of reliability of methods of analysis applied to commodities ranging from iron ores to foods and pesticides. Many organizations were already conducting such studies in exactly the same manner but were using different vocabularies and outlier tests, which made the mechanics appear quite different.

A third harmonization meeting was also planned in 1984 in Washington, DC, by Egan in conjunction with AOAC's centennial anniversary. Unfortunately, Egan did not live to see his dreams fulfilled, but his work was carried on by his colleagues from IUPAC. A series of 13 papers was published in the *Journal of the AOAC* (5), which outline in detail how various specialists and organizations viewed method performance studies; their interpretations were remarkably similar.

Production of Harmonized Protocols

Representatives of the international organizations met again in 1987 in Geneva, Switzerland, to agree by consensus on the specifics of a protocol for the design, conduct, and interpretation of method-performance studies (6). The protocol specified the number of laboratories (5 or 8), test samples (3 or 5), and replicates (1 or 2), the special conditions under which the smaller number may be used, and the statistical analysis and outlier removal techniques to be used. In 2 important respects, 1-way analysis of variance and removal of outliers by the

Cochran and Grubbs tests, the protocol complies with the general ISO standard for method-performance studies, ISO 5725-1986, which is currently under revision.

A follow-up meeting entitled "The Second International Workshop on the Harmonization of the Adoption and Presentation of Methods Standardized by Collaborative Study" was held in Washington, DC, April 17–18, 1989. The resulting document (7) provides guidance on acceptability criteria for standard methods, minimum requirements for quality control, use of repeatability and reproducibility limits, and checks on bias. A recommended format is given for reporting the results of interlaboratory tests to avoid overlooking important items of information.

The relationship between what has been accomplished in the harmonization of protocols to study and report method performance and what we wish to accomplish in the future arises from the most controversial aspect of the previous protocol—outlier removal. Some organizations do not permit data removal unless an explanation is available for the aberrant value. At the other extreme, a liberal policy of applying scientific judgment to the data removes all inconsistent values. The first philosophy allows some ludicrous values to remain to contaminate an otherwise "normal" set of data. With the second philosophy, different values would be removed by different judges, destroying the value of the harmonized protocol we worked so hard to achieve. If we provide a means of preventing the production of outlying data to start with, we can avoid the difference in opinion engendered by the problem of outlier removal because there would be no outliers to remove!

Valid Data

We recently provided some concrete evidence showing that analytical chemists produce many more outliers than they are willing to admit to (8). We examined the published data on the biological standard reference materials (SRMs) of the National Institute of Science and Technology (NIST) as compiled by Gladney et al. (9). This compilation contains practical data from that part of the analytical chemistry community that is willing to place their output on public display. As such, this publication shows the best performance that the analytical chemistry profession has to offer. These SRMs were analyzed with full knowledge of the assigned value of the certified analyte. As pointed out by Thompson recently (10) and by Banes many years ago (11), analysts exhibit better precision and less bias when analytical samples are analyzed as knowns rather than as unknowns.

We reviewed the compiled data for the 117 certified analyte-matrix combinations from 11 biologically related SRMs for which at least 8 values from different references were available, representing 28 elements over a concentration range from 5 ng/g (10^{-9}) to 40 mg/g (4%). We looked at the numbers of outliers removed by the compilers, who exercised subjective, critical analytical judgment to eliminate data "clearly beyond the limit of acceptability." From the 117 certified combinations, 16 (14%) had to have more than 22% (maximum 39%) of the values removed; only 1 combination had no outliers removed by the compilers. To put this in perspective, the ISO 5725 criteria for flagging outliers by statistical tests operates at the 1% probability level. Independent application of the Cochran and Grubbs tests should remove only 1% outliers by each (total 2%) merely from the operation of normal distribution statistics. In this case, even the conservative IUPAC protocol removed an average of 5% of the values instead of the expected 2%.

We looked at the data from another point of view. We recalculated the relative standard deviation (RSD_R) of the data (when more than 8 values were present per analyte/matrix combination) in 3 ways: (*1*) considering the associated NIST uncertainty as a standard deviation; (*2*) using the compilers consensus values after their judgmental outlier removal procedure; and (*3*) by the IUPAC-1987 protocol. We then normalized the RSD_R with respect to concentration by taking the ratio to the RSD_R calculated from the Horwitz curve:

$$[RSD_R\,(\%) = 2^{(1-0.5\log_{10} C)}]$$

where C is expressed as a decimal fraction (e.g., 1% = 10^{-2}) because the concentration range covered 7 decades. On the basis of our experience with over 6000 among-laboratories data sets covering numerous commodities, analytes, and methods, we found that the typical normalized ratio is about 1 and that a ratio over 2 is usually unacceptable. By this criterion, only 2 of the 117 analyte/matrix combinations using the consensus value provided by the compilers have a ratio over 2.0. When the IUPAC-1987 protocol is applied, 30 of the 117 (26%) of the data sets show an unacceptably high ratio. In other words, the IUPAC protocol is leaving in too many reported analytical values in the unusually favorable situation of knowing the "true value."

Conclusion

Our conclusion is that the judgment applied by the compilers provides a consensus value that closely matches the values supplied by the certifying organization, but at the expense of removing about 14% of the values. We regard this as excessive. In our opinion, a massive effort is required to bring the techniques of quality control to the attention of analysts so that all results reported in the literature are reported together with a statement of the quality control efforts that support the published data. Our efforts here should be devoted to providing relatively simple guidelines that should be taken as good analytical practices: consistent use of primary or historical reference materials, independent replication under different conditions, and quality control parameters built into the methods of analysis to inform the analyst that his analytical operations are proceeding as expected. The modern computer can memorize calibration curves and compare today's curve with last year's curve and notify us of significant differences. We have performed several million calculations during the preparation of our method performance databases and have yet to find a mistake made by our computers. All the mistakes we discovered were caused by human error.

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TECHNICAL COMMUNICATIONS

Nonenzymatic-Gravimetric Determination of Total Dietary Fiber in Fruits and Vegetables

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Most gravimetric methods for total dietary fiber (TDF) determination require the complete removal of starch and the partial removal of protein with various combination of enzymes in buffers at different pH values and at temperatures much above ambient condition. A hydrolysis step is crucial in dietary fiber analysis of samples, such as cereals and legumes, which contain appreciable amounts of starch. However, many vegetables and most fruits contain very little or no starch, and they are often eaten uncooked. It would be unnecessary to use high temperatures and enzymes on these types of samples. Initially, we found that hexane and dilute alcohol extractions of a few selected fruits and vegetables gave residue weights comparable to those after enzymatic treatments. We were able to show later that simply suspending the samples in deionized water for 90 min at 37°C and then adding 95% ethanol also yields TDF values similar to those obtained from other published methods. Comparison data obtained by using variations of the AOAC/TDF method are presented for 10 fruits and vegetables.

In 1936, McCance et al. (1) described a "simple" method for "dietary fiber" determination. The samples were extracted with 80% ethanol to give an alcohol-insoluble fraction whose starch, protein, and ash contents were determined separately and subtracted from the original residue weight. The value thus obtained was considered to be dietary fiber, which included other insoluble material not accounted for. Sandberg et al. (2) compared the total dietary fiber content of 6 samples obtained by the above method, the neutral detergent fiber (NDF) method, and a direct determination that estimated dietary fiber as the sum of polysaccharide components and Klason lignin minus starch. Their results indicated that NDF gave consistently lower values, while results by the other 2 methods were quite similar, particularly for carrots and white cabbage.

Siddiqui (3) suggested that the total dietary fiber content of a fruit or vegetable may be evaluated precisely by determining its moisture, starch, protein, and ash contents and subtracting the sum of these from the ethanol-insoluble residue. We have gone a step further to recommend that starch determination should be omitted for most fruits and vegetables. The method we propose is a further simplification of a method based on the same principles as the official AOAC method for total dietary fiber determination (4, 5). Unlike most enzymatic gravimetric methods, our 1988 version uses only 1 enzyme, amyloglucosidase, for starch hydrolysis. The method makes no attempt to hydrolyze any of the protein present in the original sample; however, the value for protein that remains in the residue is later corrected based on a Kjeldahl nitrogen determination. A gelatinization temperature of 121°C was incorporated to accommodate samples with relatively high starch content, e.g., rice, potatoes, bread, etc. After we completed a study of the effect of freeze-drying and cooking on some vegetables, and in view of the observations made by others (6, 7), it became clear that the use of high temperatures and enzymes may be entirely unnecessary when analyzing fruits and vegetables that are known to contain little or no starch.

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METHOD

Apparatus

(a) *Analytical balance.*—Capable of weighing to 0.1 mg.

(b) *Filtering flask.*—1 L.

(c) *Fritted crucible.*—Porosity No.2 (coarse ASTM 40–60 μm). Wet and redistribute Celite (0.5 g) with 78% ethanol; then suck dry to form an even mat. Ash crucible containing Celite 1 h at 525°C at least 1 day before use.

(d) *Muffle furnace.*—525°C.

Reagents

(a) *Ethanol.*—95% and 78%. Mix 207 mL deionized water with 95% ethanol to give a final volume of 1 L 78% ethanol.

(b) *Celite.*—Analytical filter aid, No. C211-500 (Fisher Scientific, Fairlawn, NJ).

Determination

Weigh duplicate 500 mg freeze-dried, ground samples (accurate to 0.1 mg) into 250 mL beakers. Add 25 mL deionized water to each beaker; sonicate or stir suspensions until no clumps remain. Rinse insides of beakers with 1–2 mL deion-

$$TDF\ (\%) = \frac{[mg\ residue - \frac{(\%\ protein\ in\ residue + \%\ ash\ in\ residue)}{100} \times mg\ residue]}{mg\ sample} \times 100$$

Figure 1. TDF calculation.

ized water. Cover beakers with aluminum foil and leave 90 min without stirring in incubator or water bath at 37°C. Add 100 mL 95% ethanol to each beaker and let stand 1 h at room temperature. Collect dilute alcohol-insoluble material under vacuum on preweighed crucible containing Celite. Wash residues twice with 20 mL 78% ethanol, twice with 10 mL 95% ethanol, and once with 10 mL acetone. Dry crucibles containing residues in air oven at least 2 h at 105°C. Cool crucibles in desiccator at least 2 h and weigh to nearest 0.1 mg. Ash residues from 1 of the duplicates in a muffle furnace 5 h at 525°C. Analyze residues from remaining duplicate for protein by Kjeldahl nitrogen determination, using N × 6.25 as conversion factor. Calculate TDF as shown in Figure 1.

Results and Discussion

Our initial attempt at extracting some fruit and vegetable samples with hexane and dilute alcohol (either 80% methanol or ethanol) indicated that the alcohol-insoluble residues, after correction for protein and ash, were similar to the TDF values obtained by other enzymatic-gravimetric methods (4, 5). Further experimentation led to even simpler procedures as described above. We determined the total dietary fiber content of 10 different fruits and vegetables (freeze-dried test samples from various collaborative studies or purchased locally) by the following procedures: (*A*) Incubate in deionized water 90 min at 37°C, dilute with 95% ethanol, filter, and dry; (*B*) extract with hexane and 80% ethanol, filter, and dry; and (*C*) simplified TDF method (4).

Contents collected in crucibles by Procedures A and B were corrected for residual protein and ash. Duplicate TDF values were compared with the mean values of the same samples from various collaborative studies or the values obtained in our laboratory by either the AOAC/TDF method or a modified version. An overall comparison is presented in Table 1.

To illustrate the similarities and differences between the various procedures, we include a sample work sheet, shown in Table 2. The TDF values obtained from Procedures A and B are very comparable for all samples, except for onion, and none of these 2 procedures requires a blank determination. However, Procedure A is less labor-intensive, can be easily performed in beakers, and does not require any solvent other than 95% ethanol and water. Further comparisons between the simplified enzymatic-gravimetric method and these 2 procedures indicated that an autoclaving temperature of 121°C may be too drastic for certain samples, e.g., broccoli, resulting in lower TDF values. In general, Procedure A compares favorably with other methods, such as the AOAC/TDF method (5) and the AACC approved method (8). The nonenzymatic-gravimetric method described here is suitable for the determination of the

Table 1. Comparison of total dietary fiber values (g/100 g dry weight) of fruits and vegetables by different methods

Plant food	Procedure[a] A		B		C		Others
Apple	13.2	13.2	11.6	11.4	11.3	11.3	12.2[b]
Apricot	69.3	68.7	66.4	66.7	63.5	61.9	67.2[c]
Broccoli	30.3	30.3	32.9	32.1	30.0	26.2	30.1[b]
Cabbage	21.2	21.1	23.2	22.4	21.0	20.0	21.2[b]
Carrot	22.3	22.2	23.2	22.4	21.6	20.4	20.5[b]
Cucumber	15.9	16.4	17.1	16.8	16.8	16.9	15.4[c]
Green pepper	31.3	31.7	30.1	30.7	29.6	31.3	32.4[c]
Onion	15.2	14.7	19.8	21.2	16.9	16.5	16.6[c]
Prune	31.4	32.3	31.9	32.0	27.9	28.4	30.2[c]
Turnip	20.3	20.7	19.9	20.0	19.1	19.3	22.2[b]

[a] A: incubate in deionized water, add 95% ethanol, filter, and dry.
B: extract with hexane, 80% methanol or ethanol, filter, and dry.
C: Simplified TDF method.
[b] AOAC/TDF method.
[c] AACC/TDF method.

Table 2. Sample worksheet for calculating total dietary fiber of cabbage, methods comparison

Measurements	Procedure									
	A		B		C		AOAC/TDF method		AACC/TDF method	
Sample weight, g	0.4621	0.4679	0.5005	0.5026	0.5100	0.5167	1.0013	1.0002	1.0006	1.0072
Residue, g	0.1302	0.1326	0.1617	0.1587	0.1451	0.1490	0.2720	0.2853	0.2849	0.2850
% of sample	28.2	28.3	32.3	31.6	28.4	28.8	27.2	28.5	28.5	28.3
Protein, mg	25.0	—	37.3	—	33.0	—	16.7	—	44.6	—
% of residue	19.2	—	23.1	—	22.8	—	6.2	—	15.7	—
Ash, mg	—	7.9	—	11.5	—	8.3	—	36.0	—	18.9
% of residue	—	5.9	—	7.3	—	5.6	—	13.2	—	6.6
Blank correction, mg	—		—		3.18		13.5		8.55	
TDF, %	21.2	21.1	22.5	21.9	19.8	20.0	20.8	21.6	21.3	21.1

total dietary fiber content of most fruits and vegetables, especially for quality control and labeling purposes.

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