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JOURNAL·OF AOAC INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence

AOAC INTERNATIONAL

JAINEE 76(2)257-460 (1993)
ISSN 1060-3271

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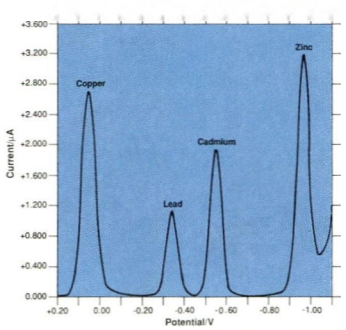

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Not all procedures in this manual have achieved official AOAC status through collaborative testing; but all represent the methodology currently in use in FDA laboratories.

CONTENTS

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

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AOAC INTERNATIONAL

JOURNAL OF AOAC INTERNATIONAL

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

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The *Journal of AOAC International* (ISSN 1060–3271) is published bimonthly by AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 76 (1993) are: Members, $120.00 in U.S., $158.00 outside U.S.; Nonmembers, $175.00 in U.S., $213.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.
POSTMASTER: Send address changes to AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA.

For Your Information

Meetings

March 23–25, 1993: 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.

March 24, 1993: AOAC MidAtlantic USA Section Meeting. Contact: David B. MacLean, 6422 Alloway Ct, Springfield, VA 22152, telephone 703/451-1578.

March 29–30, 1993: AOAC Europe Section Meeting, Barcelona, Spain. Contact: Juan Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres 6, 08029 Barcelona, Spain, telephone 343-322 88 06.

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

May 21–22, 1993: AOAC Official Methods Board Meeting, AOAC, Arlington, VA. Contact: Nancy Palmer, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

June 7–9, 1993: AOAC Midwest Section Meeting, West Des Moines, IA. Contact: P. Frank Ross, USDA National Veterinary Services Laboratories, Toxicology Laboratory, PO Box 844, 1800 Dayton Rd. Ames, IA 50010, telephone 515/239-8542.

June 23–25, 1993: AOAC Pacific Northwest Section Meeting, Evergreen State College, Olympia, WA. Contact: Andrew Held, MEI Charlton, Inc., 2233 SW Canyon Rd, Portland, OR 97301, telephone 503/228-9663.

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

July 25–29, 1993: 107th AOAC International Annual Meeting and Exposition, Washington, DC. Contact: Marga-

ret Ridgell, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

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

November 3–5, 1993: AOAC Central Section Meeting, West Lafayette, IN. Contact: Hussein S. Ragheb, Purdue University, Dept of Biochemistry, West Lafayette, IN 47904, telephone 317/494-4331.

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

AOAC and A2LA Form Partnership for Expanded Scientific Education Program

AOAC International and the American Association for Laboratory Accreditation (A2LA) have joined forces to provide members and constituents of both organizations with an expanded program of quality education for the scientific professional. Both associations are leaders in developing quality training for the analytical scientist and the analytical science laboratory. Together they will offer a variety of courses designed to meet the needs of a wide range of scientists in government, industry, and academia.

A2LA is a nonprofit, nongovernmental, public service, membership society dedicated to the formal recognition of competent laboratories and related activities. Accreditation is available to any type of laboratory, be it private or government. Accreditation is based on internationally accepted criteria for competence for laboratories, specifically ISO/IEC Guide 25–1990. A2LA has also established a program for quality system registration, based on ISO 9000 series or ANSI/ASQC Q90 series standards, of reference material suppliers

and certification of specific lots of registered supplier material. A2LA's 281 members represent individuals, institutions, and corporations interested in the recognition of competent testing services.

The goals of A2LA are to: (*1*) provide a comprehensive national laboratory accreditation system that establishes widespread recognition of the competence of accredited laboratories; (*2*) provide publicity for accredited laboratories and a service to their users by publishing an annual directory of accredited laboratories, newsletters, press releases, articles, and other related documents; (*3*) improve testing within the United States and, thereby, enhance the quality and reputation of U.S. goods in markets at home and overseas; (*4*) negotiate agreements on mutual recognition with other laboratory accreditation systems and, thereby, strive for national and international acceptance of the competence of accredited laboratories; and (*5*) eliminate unnecessary multiple assessment of laboratories.

Currently, A2LA's current short courses are being phased into the AOAC short course schedule. By October 1993, AOAC will be managing all A2LA short courses.

Members of both AOAC and A2LA will receive the member discount for all courses of both organizations. The two associations will market their short courses jointly, except for specialized courses with a very narrow application, which require a more targeted approach. In addition, market research and needs assessment costs will be shared by both associations to reduce the cost of development. New educational programs of both associations will be designed to be complementary and avoid duplication.

Courses will be offered in:

- Jacksonville, FL: May 24–28
- Washington, DC: July 24–30
- Manhattan Beach, CA: October 4–9

AOAC Courses:

- Quality Assurance for Analytical Laboratories
- Quality Assurance for Microbiological Laboratories
- Statistics for Methodology
- Laboratory Waste Disposal, Environmental Compliance and Safety
- How to Testify as an Effective Witness
- Improving Your Technical Writing Skills

A2LA Courses:

- Laboratory Accreditation vs Registration
- Environmental Laboratory Quality Assurance and Assessment
- Environmental Lead Laboratory Accreditation
- How to Design a Quality Manual
- Achieving Accreditation for Your Laboratory
- Laboratory Equipment Calibration Practices
- National Association of Testing Authorities Lead Assessor Quality Training
- Statistical Measurement Control
- Laboratory Information Management Systems
- Laboratory Occupational Safety and Health Standards

All courses will not be offered at each location. For a course catalog containing additional information on course scheduling, duration, price, and availability, contact the Meetings and Education Department, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA, telephone +1 (703) 522-3032, fax +1 (703) 522-5468.

Reference Materials Group Organizes to Form Technical Division

A number of scientists met at AOAC headquarters on December 8, 1992, to organize a technical division within AOAC focused on reference materials. The group voted to petition the AOAC

Board of Directors to form the Technical Division on Reference Materials, with the purpose to improve the quality of analytical measurements through the use of reference materials in validation and use of AOAC methods.

The following interim Executive Committee was elected, to serve until annual elections during the AOAC International Annual Meeting in July 1993, if the Board grants technical division status: Chairman: Wayne Wolf, U.S. Dept of Agriculture and the National Institutes for Science and Technology; Vice Chairman: James T. Tanner, U.S. Food and Drug Administration; Secretary: Jon DeVries, General Mills; Treasurer: Sungsoo Lee, Kellogg Co.; Members-at-Large: Norman E. Fraley, Armour Swift Eckrich, Inc.; Philip J. Oles, Lancaster Laboratories, Inc.; and Darryl M. Sullivan, Hazleton Laboratories.

A Nominating Committee, headed by Vice Chairman James Tanner, will begin work immediately to prepare a slate of candidates for the July election.

The group agreed on a set of draft bylaws to present to the AOAC Board of Directors, and identified initial activities that should be pursued. Because the idea for a technical division evolved from work of a subcommittee on reference materials of the AOAC Task Force on Nutrient Labeling Analyses, the Division plans to concentrate its activities in the nutrition area, defining appropriate food matrixes, identifying suitable reference materials, and facilitating by various means the development and characterization of additional reference materials needed for valid nutrition analyses.

The Division will expand to cover reference materials needs in all areas of AOAC methods development and validation. The Division will also be responsible for organizing and conducting the BERM (Biological and Environmental Reference Materials) symposium series. The BERM-5 symposium was held in 1992 in Aachen, Germany; the next meeting is scheduled for April 17–21, 1994, in Kona, Hawaii.

If you are interested in joining and influencing the AOAC activities in the reference materials area, as carried out by the Technical Division, AOAC will supply you with additional information and membership materials as they become available. For specific information about the Technical Division, contact Wayne Wolf, U.S. Dept of Agriculture, Nutrient Composition Laboratory, 10300 Baltimore Ave, Beltsville, MD 20705-2350, telephone +1 (301) 504-8927, fax +1 (301) 504-8314.

Quality Assurance Checklist for Small Laboratories

The AOAC Laboratory Quality Assurance Committee recently developed the following quality assurance (QA) checklist to serve as a guide to analysts in the laboratory, and to laboratory managers for a quick review of the status of quality assurance practices in their laboratories. Also, auditors can use the checklist for a preliminary evaluation of a laboratory to determine if enough of the basic issues have been addressed for a productive audit.

Further details in developing a quality assurance program can be obtained from the AOAC publication *Quality Assurance Principles for Analytical Laboratories.*

Laboratory Quality Assurance Checklist

I. Information on Standards

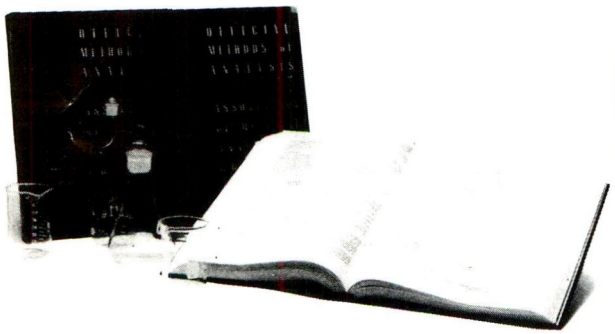
A. Source
B. Purity or activity
C. Preparation
D. Adequate labeling
E. Storage
F. Date received
G. Date prepared
H. Lot number
I. Identity of preparer
J. Certification information

II. Methods and Methods Validation

A. Source (AOAC, AWWA, AOCS, etc.)
B. Standard operating procedures
   - ✔ 1. Calibration of equipment and glassware
   - ✔ 2. Preparation of reagents and media
   - ✔ 3. Identification of safety procedures
   - ✔ 4. Frequency of positive and negative control analyses
   - ✔ 5. Documentation of sample receipt, tracking, storage, and final disposition
   - ✔ 6. Scheme for data and document storage, archiving, and retrieval

C. Validation and Reporting of Results
   - ✔ 1. Scheme for analysis validation: must be prepared prior to analyzing samples for reporting purposes; should be in laboratory notebook or separate report.
   - ✔ 2. Calculation checks: must include spike and recovery and replicate analysis scheme; must be uniform for all samples of a given type and method.
   - ✔ 3. Systematic series of negatives controls: blanks must be specified as to reagent blanks, method blanks, etc.
   - ✔ 4. Acceptance/rejection criteria: must be defined for recovery samples replicates and blanks.
   - ✔ 5. Managerial review and approval of results.

Be Sure Your Laboratory Uses the Best, Most Current Analytical Methods Available...



Official Methods of Analysis of the AOAC, 15th Edition

Save Time and Money, Achieve More Accurate Analysis, and Improve Your Laboratory's Reputation

The demonstrated reliability of AOAC methods prompts their use whenever a need for analysis arises in regulatory, research, and quality control activities. Chemists, microbiologists, and other scientists depend upon Official Methods of Analysis of the AOAC because they know the reliability and reproducibility of AOAC methods have been demonstrated through thorough testing by interlaboratory collaborative studies in government, industry, and university laboratories.

The authority of the methods is further enhanced by AOAC collaboration with other worldwide organizations.

Take Advantage of AOAC Methods Availability and Ease-of-Use

Over 1,800 AOAC methods are ready for your immediate use. The easy-to-follow, step-by-step format specifies all reagents and apparatus. And alternative methods are often provided to accomodate a wide range of laboratory capabilities.

Each method is referenced for background and development material, and contains Chemical Abstracts Service (CAS) numbers where applicable. Both chemical and common names are provided for all drugs and pesticides.

Comply More Easily with Government Regulations!

Since their inception in 1884, AOAC methods have been accorded preferred status in the courts and are often referred to in national, state, and provincial regulations and governmental and commercial specifications.

Be Confident You're Using the Best, Most Current Methods Available

As analytical needs change and as knowledge and techniques advance, new and revised methods are continually being validated by AOAC. Between editions, these are published in annual supplements which are sent, at no additional cost to purchasers of the most recent edition.

Thus, AOAC's OFFICIAL METHODS OF ANALYSIS not only brings you up to date, it keeps you there!

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Volume II—Food Composition

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Flavors
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Gelatin, Dessert Preparations
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Nuts & Nut Products
Oils & Fats
Vegetable Products
Spices & Other Condiments
Sugars & Sugar Products
Vitamins
Color Additives
Food Additives, Direct & Indirect
Natural Poisons

In Volumes I & II:

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

AOAC—For over 100 years, the mission of the Association of Official Analytical Chemists has been to provide the analytical science community with fully validated standard methods for the chemical and biological analysis of foods, drugs, cosmetics, pesticides, fertilizers, feeds, hazardous substances, air, water, soil, and any other products and substances affecting the public health and safety, the economic protection of the consumer, or the quality of the environment.

1200 Pages. 1990. 2 Volumes. Hardbound, 185 illus. ISBN 0-935584-42-0. Binders for supplements are shipped with the first supplement. $281.00 in continental North America (USA, Canada, Mexico). $312.00 outside continental North America. Members: subtract 10% discount. Includes 5 annual supplements plus a looseleaf binder for their storage.

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III. Data Quality Assurance

A. Check samples: Recommended for method, matrix, and analyte customarily performed; these are not substitutes for regular positive controls but are used in addition to regular spikes and/or reference materials.

B. Control charts and limits: Should be established for the analyte, matrix, and concentration of interest; Results should be readily available.

C. Records of analytical data: Must be kept in a laboratory notebook or other "hard copy" format. Raw data and sample identification should be readily available for reconstruction of analysis. Repetitive information for each sample can be referenced in each case to a laboratory quality assurance notebook or earlier laboratory notebook.

D. Reporting of results
- ✔ 1. Units must be specified
- ✔ 2. Records must be kept for a specified time
- ✔ 3. Timeliness: Dates and times of analysis must be recorded and available
- ✔ 4. Significant figures must be correct
- ✔ 5. Form for data reporting must be uniform
- ✔ 6. Report must be reviewed for correctness

IV. Personnel

A. Resumes of analytical personnel should be on record. These should include educational and professional experience that bears on the training and competence of the analyst.

B. On-the-job training should be listed for each analyst.

V. Implementation of Quality Assurance

A. No undocumented standards should be permitted for analysis. Such standards indicate reanalysis of samples.

B. No undocumented methods are permitted for reporting purposes.

C. When control limits are exceeded, a record must be made of the corrective measures taken, such as re-analysis, recalculation, or other means of bringing the analysis under control.

D. Laboratory environment (control of contamination): Blanks must be "blank" for the parameter of interest.

AOAC International Official Methods Board News

Methods Adopted First Action

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

Pesticide Formulations and Disinfectants

Phosphamidon in Technical and Formulated Products, Liquid Chromatographic Method

Bentazon in Pesticide Formulations, Liquid Chromatographic Method

Additives, Beverages, and Food Process Related Analytes

Nitrate in Baby Food, Spectrophotometric Method

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

Commodity Foods and Products

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

Microbiology and Extraneous Materials

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

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

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

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

Clostridium perfringens from Seafood, Iron Milk Method

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

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

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

Feeds, Fertilizers, and Related Topics

Nitrogen (Total) in Fertilizers, Combustion Method

Environmental Quality

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

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

New Products

OHS Announces Electronic Adaptation to ANSI Z400.1 MSDS Format

Occupational Health Services, Inc., announces the completion of converting 95,000 hazardous substances to the new ANSI Z400.1 format for its OHM MSDS database. ANSI Z400.1 allows expansion of the regulatory and environmental fields, delivering a powerful tool to industrial hygienists, environmentalists, and DOT professionals, working in both the public and private sectors. This format, in addition to providing complete occupational hazards information, includes comprehensive environmental data, expanded SARA hazard category ratings and List-of-Lists. Occupational Health Services, Inc.
Circle No. 320 on reader service card.

Ohmicron Introduces Chlorothalonil Assay

Food samples containing chlorothalonil, a Group B_2 carcinogen, can now be detected quickly, accurately, and cost-effectively with a RaPID Assay kit. Chlorothalonil contamination is also a concern because it is highly toxic to fish. It is used as a fungicide on a wide variety of commodities such as tomatoes, celery, and soybeans. Residues form chlorothalonil may be detected in foods, wells, and streams due to runoff, spills, or normal use. Ohmicron Corp.
Circle No. 321 on reader service card.

Confined Space Entry Monitor Detects Ammonia

The Triple Plus is a portable, multi-gas monitor that can simultaneously monitor up to 4 different gases, including ammonia. The unit can be fitted with any combination of oxygen, combustible gas, and toxic gas sensors for H_2S, CO, Cl_2, SO_2, NO_2, NO, H_2, HCl, HCN, HC_3, and others. The digital display provides readings both instantaneously and as time weighted averages. CEA Instruments, Inc.
Circle No. 322 on reader service card.

Fully Automated System Replaces Large Soxhlet Arrays

The automated SFX 3560 supercritical fluid extractor performs unattended, sequential extraction and collection of up to 24 samples for environmental, food/flavor, pharmaceutical, and polymer analyses. Using safe and inexpensive CO_2, the system provides typical extraction cycles of 15 to 30 min and throughput equivalent to dozens of Soxhlet extractors. Samples up to 10 mL are extracted in exclusive finger-tight cartridges. Static and dynamic extraction steps at up to 10,000 psi and 200°C, fractional collection of extracts, on-line modifier addition, and other parameters are programmable for individual samples or groups. Extracts are collected by bubbling into pre-cooled solvent to assure efficient trapping of analytes. Additional capabilities include bar-code scanning of sample cartridges and collection vials, and printed reports documenting actual extraction conditions for each sample. Isco, Inc.
Circle No. 323 on reader service card.

L&R Ultrasonics Revolutionizes Laboratory/Scientific Industries

Ultrasonics is generally known for its critical cleaning ability of a wide variety of items. In laboratories, ultrasonics is being hailed for its ability to clean fragile objects such as Buchner funnels. Safety committees at top pharmaceutical companies are recommending L&R ultrasonic machines and its full line of solutions because they eliminate the health hazards associated with cleaning with caustic chemicals and acids. In addition to health risks, use of caustic chemicals means expensive and time-consuming disposal methods. L&R Manufacturing Co.
Circle No. 324 on reader service card.

Modified Pyroprobe Interfaces to Gas Chromatograph for High Temperature Desorption without Pretrapping

The HTD 2000 has been modified for use as a mini thermal desorber for samples in the 100 mg range, the size commonly used for soil desportion work such as EPA Methods 8245, Thermal Chromatography/Mass Spectroscopy for Screening Semi-Volatile Organic Chemicals, and 8440, Total Extractable Petroleum Hydrocarbons (TPH) by IR. The HTD 2000 may be connected to the injection port of the gas chromatograph using a ½ in. interface, which has its own heater to 325°C, and samples can be rapidly desorbed onto the GC column without pretrapping. Suitable for use with soils and other environmental materials, polymers, pharmaceutical, mini air monitoring tubes, and foods, the modified HTD 2000 may be used for both thermal desportion and pyrolysis. CDS Analytical, Inc.
Circle No. 325 on reader service card.

Micro-Punch Designed for Microsampling Materials Resting on Surface or Thin Substrate

The Micro-Punch is designed for microsampling forensic size samples of inks, toner, correction fluid, adhesive, body fluids, or any other media resting on a thin substrate. The uniform dimension and sharp cutting edge of the tip allows for quick, repetitive sampling without risk of damage to the substrate, producing samples of uniform size for quantitative studies. Approximately the size of a fountain pen, the Micro-Punch can be carried in a breast pocket for sample collecting at crime scenes or from specific evidence sites. The forensic size

samples produced with the Micro-Punch are suitable for placement in PGC sample holders, mounting on SEM stages, sandwiching between diamonds on anvil cells or other FTIR micro-sampling devices. Premier Scientific.
Circle No. 326 on reader service card.

Recycling Preparative HPLC System

The Model LC-908 represents the state of the art in preparative HPLC systems. It features a unique sample recycling capability that enables the chemist to achieve optimum separation with high sample loading. After passing through the column and the detector, the partially separated components are returned to the top of the column and recycled until adequate separation is attained. In addition, the mobile phase is recycled resulting in a tremendous decrease in solvent consumption. The system is compatible with all types of column packing, silica based or polymer based, and can be used with reversed-phase, normal phase, or SEC columns. DyChrom.
Circle No. 327 on reader service card.

New Analytical Laboratory Training Plan Offered

Savant Audiovisuals, Inc. has announced a frequent viewers club for analytical laboratory training. This plan offers an opportunity to view an unlimited number of professional analytical training programs for a one-time fixed fee. Viewers can borrow as many programs as desired, one at a time, during the year-long membership period. No additional payments are required. Programs come in a wide range of analytical techniques, including AA, ICP, GC, HPLC, IR, NMR, MS, UV-VIS, RIA, EIA, and laboratory safety. Most are available in either slide/tape or video format and run 20 to 45 min. Savant Audiovisuals, Inc.
Circle No. 328 on reader service card.

CEM Zero/Dilution Air for Use with Continuous Emissions Monitoring Systems

CEM Zero/Dilution Air for use with dilution extractive and standard extractive continuous emissions monitoring systems limits the concentrations of sulfur dioxides, oxides of nitrogen, carbon dioxide, carbon monoxide, water, and total hydrocarbons to very low levels for use by CEM end users. Oxides of nitrogen and sulfur dioxide are each limited to 0.1 ppm, and carbon monoxide is limited to 0.5 ppm. Carbon dioxide is below 1 ppm. This new product is useful as a source of Zero Air, free of critical impurities; a means of checking the quality of house air used in the probes of dilution extractive CEMS; and as a backup source of air, free of critical impurities for dilution of air samples at the CEMS probe. Scott Specialty Gases.
Circle No. 329 on reader service card.

Helium Purifier Improves GC Results

The Model 75-800 helium purifier is designed to be installed in the carrier gas line between the GC supply cylinder and the instrument. By trapping gas stream impurities into heated purifying pellets, the unit purifies 99.999% helium to the 99.99999% level required by many of today's GC applications. Under normal GC operating conditions, the unit will prove over 2 years of purification. GOW-MAC Instrument Co.
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Chromex Introduces New 250PS Parallel Spectrometer

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IHS Regulatory Products, a division of Information Handling Services, is now offering state environmental and safety related state regulations to customers using the Environmental/Safety Library on CD-ROM. Regulations from over 30 states will be made available to customers in phases during 1993; regulations for California and Florida are now ready for shipment. The Environmental/Safety Library on CD-ROM contains federal laws, regulations, and compliance information issued by EPA, OSHA, and the Department of Transportation, as well as related industry standards. Information within this database is automatically updated every 60 days and an "Environmental/Safety Alert" newsletter containing abstracts from the *Federal Register* and state publications is issued weekly to provide access to timely information between updates. Information Handling Services.
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Books in Brief

Japanese/English English/Japanese Glossary of Science and Technology: Glossary of Scientific and Technical Terms. By Louise Watanabe Tung. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1993. Price: $79.95. ISBN 0-471-57463-5.

Today, fluency in the current international language of technology means not only having a rudimentary technical vocabulary but also a command of the foreign equivalent of everyday technical jargon. Areas such as electronics, biotechnology, polymer science, optics, ceramics, and textiles have assumed a distinctly bilingual tone. All over the world, technical specialists in fields of interpretation, science, engineering, and language education found that the available language reference guides were either out-of-date or did not include scientific terms — until now. The Japanese/English English/Japanese Glossary of Scientific and Technical Terms written by Louise Watanabe Tung, a leading translator specializing in patents and technical papers, is the most comprehensive bilingual reference designed specifically for the technical specialist and researcher. The compendium covers 120 different subject ares, from computer and logic terms to the latest in chemistry and engineering.

Determination of Vitamin E: Tocopherols and Tocotrienols. By C. Bourgeois. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 162 pp. Price: $150.00. ISBN 1-85166-754-7.

This book is the only volume completely dedicated to the determination of vitamin E. It covers the determination of the 8 natural homologs of vitamin E in pharmaceutical, biological tissues, oils and fats, and feed and foodstuffs. Vitamin E is a biological antioxidant of paramount importance; it breaks the antioxidant chain by scavenging free radicals before they can damage the cell membranes. However, its determination poses many problems. First, there are 8 natural homologs, and, then, there is the broad range of samples in which it must be determined, sometimes at very low levels. After a short introductory chapter on the structures and properties of tocopherols and tocotrienols, there follows a chapter on the very important sample extraction stage. Chapter 3 is an extensive bibliographic chapter, containing approximately 500 references, which discusses most of the published methods for the determination of vitamin E homologs. The methods covered are spectrophotometry, spectrofluorometry, electrochemistry, the various chromatography methods: TLC, GC, and HPLC. The latest and most modern methods are discussed more comprehensively. For example, in the case of HPLC, methods are grouped by fields of application: pharmacy, blood and biological tissues, oils and fats, cosmetics, and feed and foodstuffs. The last chapter presents automated methods used for biological tissues, food and feed samples.

Emerging Strategies for Pesticide Analysis. Edited by Thomas Cairns and Joseph Sherma. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 352 pp. Price: U.S.: $74.95, Outside U.S.: $90.00. ISBN 0-8493-799-1.

In this first volume of a new series, chapters have been collected together on state-of-the-art analytical technologies in the field of pesticide residue analysis written by international experts in their respective areas. In no other field has such intense scrutiny been focused on the development of analyses to protect the public health and the ecosystem. In several important respects, this book is a major milestone in an effort to come to grips with what is at once an exquisitely intriguing scientific challenge and a public policy issue of profound importance. No one knows how many miles lie ahead of us in either the scientific or the socio-political aspect of pesticide residue analysis, but this much is certain: the work described in this book represents a quantum leap forward from where the field stood just a few years ago.

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- The Fractional Factorial Design Approach for Optimizing Analytical Methods—P.J. Oles

CHEMICAL CONTAMINANTS MONITORING

- Food and Drug Administration Monitoring of Pesticide Residues in Infant Foods and Adult Foods Eaten by Infants/Children—N. Yess, E.L. Gunderson, and R.R. Roy

STATISTICAL ANALYSIS

- Reporting Ongoing Results of Interlaboratory Comparison Programs (ILCPs)—E.A. Viggers, J. Atkinson, S. Purcell

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Journal Information

The Association

The primary objective of AOAC International is to obtain, improve, develop, test, and adopt precise, accurate, and sensitive methods for analysis of foods, vitamins, food additives, pesticides, drugs, cosmetics, plants, feeds, fertilizers, hazardous substances, air, water, and any other products, substances, or phenomena affecting the public health and safety, the economic protection of the consumer, or the protection of the quality of the environment; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in the analytical sciences related to foods, drugs, agriculture, the environment, and regulatory control of commodities in these fields; and to afford opportunity for the discussion of matters of interest to scientists engaged in relevant pursuits.

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

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Instructions to Authors

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

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

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

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(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**

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SPECIAL REPORTS

Methods of Analysis for Toxic Elements in Foods. Part IV. General Method of Ashing for the Determination of Toxic Elements

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As a result of the request of the Codex Alimentarius Committee on Methods of Analysis and Sampling, a general method for ashing of foods for the determination of toxic elements was assembled. This method consolidates the results of more than a decade of interlaboratory work of numerous USSR food laboratories and specifies the details of wet and dry ashing procedures.

An important component of determining the safety of food is the toxic element content. A number of methods of analysis are available for this purpose, such as atomic absorption spectrometry (AAS), polarography, and colorimetry. All of these instrumental measurement techniques require a preliminary treatment of the test sample by ashing to destroy interfering organic combinations and to bring the inorganic elements into a soluble state.

Many variants of "dry" and "wet" mineralization exist; they differ in temperature, volume, nature of reagents, and the quantities of test portion used. The equipment conditions used may vary from open to closed beakers and from high pressure vessels heated directly or heated by microwaves. Although the magnitude of the test portion is important for mineralization, vague recommendations are given in the majority of cases. For example, the method for the determination of copper in food products (1) gives the general recommendation of 5–100 g test portion. The method promulgated by the Nordic Committee on Food Analysis for the determination of metals in foods by AAS (2) gives the general recommendation of 10–20 g test portion. The International Organization for Standardization standard for the dry ashing method of fruits and vegetables and their products requires a test portion of 5–20 g (3); for the wet decomposition method, the test portion required is 5–10 g (4). The Council for Mutual Economic Assistance (CMEA) standard recommends a test portion of 5–20 g for dry ashing and 5–100 g for wet decomposition, irrespective of the type of product (5). Until recently, few official recommendations for the quantity of mineralization test portions for different product types were made by international organizations, except for the AOAC International commodity-oriented mineralization methods.

In this discussion, the elements As, Cd, Hg, and Pb are considered as toxic elements; Cu, Fe, and Zn are essential at low concentrations but toxic at high concentrations. Maximum limits (MLs) are prescribed for all 7 elements and for Sn in many national and Codex standards, together with official methods of measurement, usually by AAS, polarography, or colorimetry. The problem of dissolution of the test portions by mineralization is more complicated.

The wide variety of food products such as meat, milk, beverages, liver. grain, and fats, requires a number of different test portions and a variety of mineralization procedures. The mineralization process for high-fat products differs from that for high-sugar products, and the requirements for liquids are different than those for solids. Details also depend on the type of toxicant; the determination of arsenic requires a slightly different mineralization procedure than that for lead or tin. Unfortunately, no official, detailed recommendations for mineralization were validated by interlaboratory experiments. Therefore, individual researchers work out their own recommendations for their particular food. In many cases, the application of different methods of mineralization results in poor interlaboratory reproducibility. The necessity for the standardization of the mineralization process has become evident, but such standardization must consider the peculiarities of food composition and the chemical form of the element in the food before and after mineralization. Because the form of the toxic element present after mineralization is not usually known, the method of additions, commonly used to measure trace quantities, is often inapplicable for the determination of toxic elements.

The standardization work with respect to specificity of the product and the nature of the toxic element was undertaken in the USSR during 1981–1985. The state standard (6) was developed by interlaboratory research conducted by 22 laboratories. The main ideas of this standard were presented in a slightly different form in a previous paper (7). This description was recommended at the 17th Session (1991) of the Codex Alimentarius Committee on Methods of Analysis and Sampling as the basis for the creation of a general Codex Method. Comments on the method were received from the International Dairy Federation, the Nordic Committee on Food Analysis, and from the governments of Canada, Cuba, Czechoslovakia,

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Denmark, Finland, Sweden, Switzerland, and the United States. All of the comments were of a positive nature and suggested certain modifications in the application of AAS. The attached mineralization procedure takes these comments into account.

Some additional comments concerned the following: In the description of the method, only the simplest equipment is mentioned. The modern techniques using high pressure, ultrasound, microwaves, etc., are omitted to permit use of the method in all of the laboratories of the world that recognize the Codex Alimentarius. For routine work within a country, the modern methods of mineralization may be used. But in a case of interlaboratory discrepancy, it is necessary to use the Codex approved harmonized method of mineralization.

Nitric acid is recommended for use at the final stage of dry ashing. This additive not only accelerates ashing, but in many cases (especially in the presence of chlorides) it prevents the loss of some elements by volatilization. For the same purpose, the addition of magnesium oxide and magnesium nitrate is recommended for the determination of arsenic by dry ashing.

A slightly diluted (57%) perchloric acid solution is recommended for wet ashing. Although this acid is considered safer than the concentrated form, hydrogen peroxide may be substituted.

The method of acid extraction is recommended for fats, oils, and similar products. As compared to other methods of dry and wet mineralization, acid extraction results in a smaller loss, not exceeding about 5%, which is permissible for the purposes of food safety.

The test portions required for AAS are considerably less than those required for polarography and spectrophotometry. In the majority of cases, test portions are close to those recommended by the Nordic Committee on Food Analysis (2), but they are given in more detail with respect to food type.

General Method of Ashing for the Determination of Toxic Elements

This method is for the determination of As, Cd, Cu, Fe, Pb, Sn, and Zn in foods by wet and dry mineralization and by acid extraction.

Dry Mineralization

Principle

This method involves complete decomposition of organic substances by oxidation of the organic matter in an electric furnace at a controlled temperature. It is applicable to all raw materials and to foods containing <60% fat.

Apparatus

(a) *Balance.*—Capacity, 200 g.

(b) *Drying oven.*—Capable of maintaining a specific temperature within the operating range of 40–150° ± 5°C.

(c) *Electric oven.*—Or programmable or muffle furnace capable of maintaining a specific temperature within 150–500° ± 25°C.

(d) *Infrared lamp.*—250–500 W.

(e) *Shaker.*

(f) *Crucibles.*—Quartz, platinum, or porcelain.

(g) *Electric hot plate.*

(h) *Water bath.*

(i) *Flask with bulbs.*—To remove CO_2 under vacuum in *Dry Mineralization, Preparation for Mineralization.*

(j) *Pipets.*

(k) *Cylinders.*

(l) *Ashless filters.*

Reagents

All reagents must be at least analytical grade for trace element analyses.

(a) *Water.*—Double distilled or deionized. Use throughout, except where tap water is specified.

(b) *Nitric acid (1 + 1) and (1 + 9).*

(c) *Sulfuric acid (1 + 9).*

(d) *Hydrochloric acid (1 + 1).*

(e) *Magnesium oxide.*

(f) *Magnesium nitrate.*—Ethanolic solution 50 g/L.

(g) *Sodium hydroxide.*

(h) *Ethanol.*—Not denatured.

(i) *Sodium hydroxide.*—Ethanolic solution, for cleaning: 130 g NaOH + 130 mL water + 880 mL ethanol.

Preparation for Mineralization

Wash crucibles with water and detergent, and rinse with tap water. Wash with NaOH ethanolic solution, and rinse with reagent water, HNO_3 (1 + 9), and then 4–5 times with reagent water.

Remove CO_2 from beer, mineral water, soft drinks, and carbonated and sparkling wines by pouring 250–300 mL beer or aqueous products into a 1 L Erlenmeyer flask, warm to room temperature, close flask with 1-hole rubber stopper with glass tube inserted, and shake flask in automatic shaker 20–30 min. Remove CO_2 from wine by bubbling air through the test sample for 3–5 min or by evacuating 1–2 min in flask with bulbs until foaming ceases and large bubbles form on the surface.

Weigh, to nearest 0.01 g, the test portion specified in Table 1 into a crucible or dish. Use pipet for liquids.

Mineralization for Determination of Cd, Cu, Pb, Zn, and Fe—AAS Method

With foods containing <20% moisture, add 40 mL ethanol to test portion, mix, close with glass stopper, and leave overnight at room temperature. Place container with test portion on electric hot plate, and cautiously char until smoking stops. To hasten charring, infrared lamp may also be used. Place container with charred residue in electric furnace at ca 250°C.

With foods containing 20–80% moisture, place container with test portion in air or vacuum oven at 105°C, or any other device that will dehydrate the food, and hold about 3 h. Transfer to an electric hot plate, and char until smoking stops. To hasten charring, infrared lamp may also be used. Place container with charred residue in electric furnace at ca 250°C.

Table 1. Recommended test portion size (g or mL) to be used for mineralization of food for determination of trace elements

Food	AAS for Cd, Cu, Fe, Pb, and Zn	Polarography[a]				Colorimetry			
		Pb	Cd	Zn	Cu	Cu	Sn	Fe	As
Fruit, vegetables	10–20	25	25	10–25	10–25	10–25	—	5–10	30
Processed fruit and vegetable products	10–20	25	25	10–25	10–25	10–25	2–5	5–10	30
Red and poultry meat	10–20	25	25	5–10	5–10	5–25	—	5–25	30
Liver, kidney, and other organs	10	15	15	5	5	5–10	2	1–5	25
Canned meat and meat-vegetables	10–20	25	25	5–10	5–10	5–25	2–5	5–25	30
Gelatin	15	10	20	10	10	10	—	3	20
Frozen whole eggs	15	25	50	5	10	25	—	10	30
Egg powder	5	10	10	2	5	10	—	3	10
Oils, fats, and their products	20	35	35	35	40	40	—	35	40
Fish, seafood, algae, and their products	10–20	25	25	10	10–25	10–25	1–2	5–10	30
Grain and grain products	5	10–25	10–25	2–10	2–10	5–10	—	10–20	30
Bread and bakery products	10	10–25	10–25	5–10	5–10	5–10	—	10–20	30
Confectionery products	10	10–25	25	1–10	1–10	5–40	—	1–10	30
Milk and milk products									
Liquid	50	50–100	100	25	10–50	50	—	50	100
Dry	5–10	10	10	5	1–10	1–10	—	5	25
Curds	10–20	25	25	5–10	5–10	10–20	—	10–20	50
Canned	20–40	25–50	50	10–15	5–10	10–20	1–2	5–10	50
Butter	10–20	50	50	25	25	50	—	20–30	50
Beverages									
Wine and wine products	20	25	25	10	10	20	—	2–20	50
Cognac	20	25	25	10	10	20	—	50	50
Beer	50	50	50	50	50	50	20	20	50
Mineral water	50	100	100	100	100	100	—	100	50
Soft drinks	50	50	50	50	50	50	—	50	50

[a] Test portion stated is for the determination of a single element. For simultaneous analysis of all elements, use maximum value mentioned in the table.

With liquid foods containing >80% moisture, proceed as follows:

(**a**) *Wine products.*—Evaporate in water bath to dryness and place in cold electric furnace.

(**b**) *Beer, canned fruit, and vegetables.*—Evaporate to dryness on hot plate, char until smoking stops, and place in electric furnace at ca 150°C.

(**c**) *Milk, fermented dairy products, and canned milk products.*—Add HNO_3 (1 + 1), 1 mL/50 g test portion, mix, place on hot plate, and char until smoking stops. To hasten charring, infrared lamp may also be used. Place container with charred residue in electric furnace at ca 250°C.

(**d**) *Products containing >20% sugar.*—Stewed fruit and jam: Add 5 mL H_2SO_4 (1 + 9)/1 g dry substance, mix, let stand 48 h, evaporate on electric hot plate to dryness, and char and ash as in (**c**).

(**e**) *Fatty foods containing 20–60% fat.*—Such as cheese and oil seeds and their products: Add HNO_3 (1 + 1), 1.0–1.5 mL/10 g test portion, mix, place on electric hot plate, and char and ash as in (**c**).

Heat residue gradually, increasing temperature 50°C at 30 min intervals to 450°C, until ash is grey. Remove container from furnace, cool to room temperature, and moisten with 0.5–1.0 mL HNO_3 (1 + 1).

Slowly evaporate acid to dryness on electric hot plate; place container in an electric furnace at ca 250°C, increase temperature gradually to 450°C, and hold 1 h. Mineralization is considered complete when ash is white or slightly colored, with no

charred particles. If carbon is present, repeat treatment with HNO_3 (1 + 1) or water.

Mineralization for Colorimetric Determination of Fe

Mineralize food other than cheese, raw fruit, vegetables, and their products as in *Dry Mineralization, Preparation for Mineralization.*

Dehydrate test portions of cheese and fruit and vegetable products to dryness in an air or vacuum oven at ca 105°C. Wet entire residue with 1–2 mL HNO_3 (1 + 1), and heat in a water bath until vapors cease. Repeat acid treatment twice. Char residue on a hot plate. To hasten charring, infrared lamp may also be used. Place container in an electric furnace at ca 250°C. Increase temperature by 50°C at 30 min intervals to 450°C until ash is grey.

Mineralization for Determination of As

To container with test portion of product containing <80% moisture, add 10% by weight of dry substance MgO (1 + 1 suspension) and the same amount (calculated as the dry salt) of $Mg(NO_3)_2$ ethanolic solution, followed by some water to form a slurry, and let stand overnight. With products containing >80% moisture, use 5% instead of 10% MgO.

Evaporate slurry to dryness on water bath or in air or vacuum oven at 80–100°C. Transfer to hot plate, and char gently until smoking stops. To hasten charring, an infrared lamp may also be used. Place container in an electric furnace at ca 250°C, and gradually increase temperature 50°C/h to 450°C until ash is grey.

Remove container from furnace, cool to room temperature, and moisten with 0.5–1.0 mL water. Slowly evaporate to dryness on hot plate and replace in furnace at ca 250°C. Gradually increase temperature to 450°C, and hold 1 h. Mineralization is considered complete when ash is white or slightly colored, with no charred particles. If carbon is present, repeat treatment with water.

Wet Mineralization

This method involves complete destruction of organic substances by heating with concentrated H_2SO_4, HNO_3, and 57% $HClO_4$ or 30% H_2O_2, or by heating in the presence of H_2O_2 only. The method is applicable to all raw materials and foods other than butter and animal fats.

Apparatus

(a) *Balance.*—Capacity, 200 g.
(b) *Electric hot plate or gas burner.*
(c) *Kjeldahl or flat-bottom flask.*
(d) *Glass cylinders.*
(e) *Funnel.*
(f) *Pipets.*
(g) *Glass beads.*—To facilitate boiling.
(h) *Ashless filters.*

Reagents

All reagents must be at least analytical grade for trace element analyses.

(a) *Water.*—Double distilled or deionized. Use throughout, except where tap water is specified.
(b) *Nitric acid, concentrated and (1 + 5).*
(c) *Sulfuric acid, concentrated.*
(d) *Hydrochloric acid, concentrated and (1 + 36).*
(e) *Perchloric acid, 57% solution.*
(f) *Hydrogen peroxide, 30% solution.*
(g) *Hydrazine sulfate.*

Preparation for Mineralization

Wash glassware and rinse with HNO_3 (1 + 5), tap water, and reagent water.

Weigh test portion of liquid or puree-type product (Table 1) into beaker, and transfer to Kjeldahl or flat-bottom flask without depositing product on walls of flasks.

Weigh test portion of solid or paste-like product (Table 1) onto ashless filter paper, wrap product, and with aid of a glass rod, place it into digestion flask.

Prepare beer, mineral water, or soft drinks as in *Dry Mineralization, Preparation for Mineralization.* Measure quantity indicated in Table 1 with pipet, transfer to Kjeldahl flask, and evaporate on hot plate to 10–15 mL.

Filter wine-making products, and pipet quantity indicated in Table 1 into 50 mL Kjeldahl flask.

Place test portion of solids, including gelatin and egg products indicated in Table 1, into Kjeldahl flask, add 15 mL water, and mix. Allow gelatin to swell 1 h.

Acid Mineralization for the Determination of Cu, Fe, and Sn

For raw materials and food products other than wine-making products, vegetable oils, margarine, and edible fats proceed as follows: To flask containing test portion, add HNO_3 (10 mL/5 g product or 20 mL beer, mineral water, or soft drinks), and let stand at least 15 min. Add 2–3 glass beads, close loosely with pear-shaped glass stopper, and heat gently on hot plate. Increase heating rate, and heat until liquid is reduced to 3–5 mL. Cool flask.

Add 10 mL HNO_3, 5 mL H_2SO_4, and 4 mL $HClO_4$ or 4 mL H_2O_2/5 g test portion of food products or 20 mL beer, mineral water, or soft drinks. When analyzing dairy products for Cu and Fe, omit H_2SO_4. Do not change sequence of adding acids; $HClO_4$ is always added last. Evaporate contents to ca 5 mL while avoiding browning of liquid. If solution darkens, stop heating, cool flask to room temperature, add 5 mL HNO_3, and 2 mL $HClO_4$ or 2 mL H_2O_2, and heat until white vapors appear. If solution is colored, repeat procedure. Mineralization is considered complete if the cooled solution remains colorless.

To eliminate residual acid, add 10 mL water to cooled flask, boil 10 min while white vapors are being released, cool, add 10 mL water, and repeat twice. If precipitate forms, add 20 mL water, 2 mL H_2SO_4, and 5 mL HCl, and boil until precipitate dissolves, replenishing water occasionally. Cool, and either use

digest directly or transfer it quantitatively to a volumetric flask, dilute to mark with water, and mix.

Acid Mineralization for the Determination of Cu and Fe in Fats and Oils

For vegetable oils, margarine, and edible fats, use following procedure: Place test portion in 100 mL Kjeldahl flask, and heat 7–8 h until viscous mass is formed. Cool, carefully add 25 mL HNO_3 and 12 mL $HClO_4$, and heat until a colorless liquid is obtained. If liquid darkens during digestion, add 5 mL portions HNO_3 as necessary, and continue heating until mineralization is complete, as indicated by cooled solution remaining colorless. Continue with removal of acids as in *Acid Mineralization for the Determination of Cu, Fe, and Sn.*

Acid Mineralization for Determination of As

For raw materials and food products other than wine-making products, beer, vegetable oils, margarine, and cheese, use following procedure: To Kjeldahl flask containing test portion (Table 1), add 2–3 mL HNO_3/g dry solids (acid should cover product completely), and let stand at least 12 h. Add 2–3 glass beads, and heat gently on hot plate 1.5–2.0 h, avoiding violent reaction. Cool, carefully add mixture of H_2SO_4 and HNO_3 (1 + 1), and heat 1.5–2.0 h. Stop heating when white fumes are evolved.

If solution in flask does not become discolored, cool and add quantity of HNO_3 or mixture of HNO_3 and H_2O_2 (2 + 1) equal to initial quantity. Boil 1.5–2.0 h longer, until liquid is discolored. If it is not discolored, add HNO_3 again, and boil until release of brown vapors of nitric oxides ceases, maintaining at least 5 mL liquid in the flask.

To eliminate residual acid, add twice as much water as initial quantity of HNO_3 to cooled flask, and boil until white vapors start to be released. Cool, add 20 mL water and 0.1–0.2 g hydrazine sulfate, and boil 1.5–2.0 h. Final solution should be colorless or straw yellow.

Acid Mineralization for the Determination of Cu in Wine

For wine and wine-making products, use the following procedure: To the Kjeldahl flask containing the test portion (Table 1), add 5 mL H_2O_2 for products containing <10 g sugar/L and cognacs or 10 mL H_2O_2 for products containing >10 g sugar/L. Heat to boiling. For products containing up to 100 g sugar/L, boil to 5–6 mL, place flask in a boiling water bath, and evaporate contents to 2–3 mL. For wines containing >100 g sugar/L, boil to 7–10 mL, cool, add additional 5 mL H_2O_2, boil to 7–10 mL, cool, add additional 5 mL H_2O_2, boil to 5–6 mL, and evaporate in the water bath to 2–3 mL.

If liquid darkens during evaporation in water bath, cool, add additional 5 mL H_2O_2, boil to 5–6 mL, and evaporate again to 2–3 mL. After mineralization, add 1 mL HCl (1 + 36), and evaporate in water bath to 2–3 mL. Use entire solution without dilution for measurement.

Acid Extraction (Incomplete Mineralization)

Toxic elements are extracted from vegetable oils, butter, margarine, edible fats, and cheese by boiling with dilute HCl or HNO_3.

Apparatus and Reagents

In addition to the following apparatus, see *Apparatus and Reagents* for *Dry Mineralization* and *Wet Mineralization.*

(**a**) *Flask with reflux condenser.*

(**b**) *Separating funnel.*

Preparation for Mineralization

Wash glassware, and rinse with HNO_3 (1 + 99), tap water, and reagent water.

Extraction

Add 40 mL HCl (1 + 1) or HNO_3 (1 + 2) to flask containing test portion (Table 1). Add several glass beads, insert reflux condenser, place on hot plate covered with a mat, and boil 1.5 h from beginning of boiling. Slowly cool contents of flask (extraction mixture) to room temperature without removing condenser.

With solid animal fats, place flask with extraction mixture in cold water bath to harden fat. Pierce layer with glass rod, and filter aqueous layer through filter wetted with the acid used for the extraction into a reaction flask, Kjeldahl flask, or quartz or porcelain dish, depending on the element to be determined. Melt fat remaining in flask in water bath, add 10 mL acid solution, shake, cool, pierce cold fat, and transfer wash liquid to same container through the same filter. Wash filter with 5–7 mL water.

With vegetable oils, transfer extraction mixture to separating funnel. Rinse flask with 10 mL acid solution, and transfer rinse into the same funnel. Shake, let separate, and transfer the lower aqueous layer through a filter wetted with the same acid solution used for the extraction into a receiving flask or dish, as for fats. Rinse flask with 10 mL acid solution, filter through same filter, and wash filter with 5–7 mL water.

Preparation of Test Solution for As

If HCl was used for extraction, filter extraction mixture directly into distillation flask.

If HNO_3 was used for the extraction, filter extraction mixture into conical flask, add 0.2 g hydrazine sulfate, and boil 1.5–2 h. Transfer solution quantitatively with water to the distillation flask.

Preparation of Test Solution for Colorimetric Determination of Cu and Fe

For colorimetric measurement, use following procedure: Filter extraction mixture into Kjeldahl flask. If HNO_3 was used for the extraction, evaporate contents of flask to 5–7 mL on the hot plate, cool, and add 1 mL $HClO_4$. Boil to colorless or faintly colored solution. If HCl was used for the extraction, add 5 mL HNO_3, evaporate to 5–7 mL on hot plate, cool, and add 4 mL HNO_3 and 1 mL $HClO_4$. Boil to colorless or faintly colored solution.

To remove acid residues, add 10 mL water to cooled flask, boil 10 min, and cool. Repeat the addition of water and heating procedure twice. Transfer solution quantitatively to volumetric flask, dilute to the mark with water, and mix.

Acknowledgment

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SPECIAL REPORTS

New Metrological Characteristics of Analytical Methods of Analysis Used for Safety Control of Food and Environmental Materials

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MQS and MQS_m are new metrological characteristics of the limit of determination of methods used for safety control of foods and environmental objects. MQS is the minimum quantity of an analyte that can be determined by any applicable method in an analytical sample. MQS_m is the minimum quantity of analyte that can be measured in the test solution in an interlaboratory, method-performance study of a specific method, under the condition that $MQS_m \leq MQS$. Examples are given of their calculation and use.

Analytical control of contaminants in food and the environment is a significant problem. New devices, methods, and methodology appear that enable us to measure many contaminants at extremely low concentrations. In addition to the generally accepted metrological characteristics of methods that include intralaboratory repeatability (s_r) and interlaboratory reproducibility (s_R), the problem of the limit of analytical determination (LAD) of methods exists. We will discuss the complexity of the problem with examples from methods used for the control of food safety.

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For methods characterizing nutritional value, the LAD in most cases is not needed, as long as the true concentrations are above LAD. The maximum quantity of contaminants in foods is characterized by an officially established, maximum permitted concentration (ML). For safety control of foods, we should use only those methods that enable us to reliably determine analytes whose concentrations are equal to or less than ML under practical working conditions. This is the primary condition for the acceptability of methods considered in one laboratory or in disputed cases in several laboratories; the standards of the International Organization for Standardization (ISO) (1) are used in interlaboratory studies. When comparing methods, with all other characteristics being equal, preference should be given to such methods that enable the reliable determination of the analyte at the ML level. This characteristic can only be established under interlaboratory conditions.

Of all analytical chemistry problems, the one connected with LAD is the most complicated and intricate. Numerous suggestions have been made by individuals and international organizations concerning terminology and definitions. As mentioned by Currie (2), many researchers use the same terms for different concepts and different terms for the same concept. Unfortunately, the majority of published proposals on the problem of LAD concern only the single laboratory aspect of the problem. For safety control, metrological characteristics re-

quire the broader, interlaboratory concept. This aspect of LAD is mentioned by Currie (3), who observed that the systematic uncertainty of individual laboratories are transformed into a random uncertainty in the interlaboratory environment.

From our experience in the development of the USSR set of standards for methods for the determination of toxic elements, we proposed the introduction of 2 new metrological characteristics: (*1*) *MQS*.—A metrological characteristic used only for safety control defined as the minimum absolute quantity of analyte that can be determined by any applicable method in an analytical sample; it must be below the ML level. MQS will have a single meaning for the safety control of homogeneous materials such as air, water, and soil that have the same ML level. As different food products have different ML values, individual MQSs may be calculated for specific foods. In this case, a common MQS applicable to all types of food is calculated as the lowest individual MQS of the element. (*2*) MQS_m.—A metrological characteristic used only for safety control defined as the absolute minimum quantity of an analyte in the analytical sample that can be reliably measured with a stated degree of confidence by a specific method of analysis. The measurement is made in a certain quantity of the test solution required by the instrument, as determined in an interlaboratory study. The study is conducted under the condition that $MQS_m \leq MQS$, and RSD_R does not exceed the maximum permitted values that depend on analyte concentration.

These characteristics take into account the peculiarities of safety control, which require a reliable determination of the analyte at the ML level under interlaboratory conditions. These proposals were previously published (4).

Of the numerous classifications concerning LAD, we believe that the proposal of Currie (2) is the most appropriate. Currie suggests the establishment of the limit of detection (L_D), limit of identification (L_I), and limit of quantitative determination (L_Q). Although most authors discuss general analytical situations, many discussions of limits of measurements imply or use results obtained with pure solutions within one laboratory. But as was correctly mentioned by Horwitz in his comments on the International Union of Pure and Applied Chemistry (IUPAC) proposals for the "limit of determination" (5), different researchers, even if they apply the same method, obtain quite different results for the values of L_D, L_I, and L_Q. This variability is explained by a number of reasons, such as skillfulness of the researcher, purity of the reagents, and idiosyncracies of the equipment.

With regard to the LAD problem, one of the main idiosyncracies of a method is the practically determined, fixed working volume, V_{opt}, of the prepared analyte solution, which is used completely or partially for the quantitative measurement. The majority of methods used for the control of food contaminants deal only with the final stage of the studied food solution and the accompanying reference solutions. The term "working volume" applied to the analysis of trace elements in food analysis means the fixed volume into which the mineralized test portion of food is converted for use in the analytical measuring instrument. "Test portion" is used as defined by ISO and IUPAC as the measured quantity of food taken for the analysis.

Working Volume Concept

The equipment that is used in some polarographic and colorimetric methods requires a minimum volume. In the polarography modifications developed in USSR, the working volume depends on the volume of the polarographic cell and is usually about 5–10 mL. In most spectrophotometers and photometers, the working volume varies from 1 to 25 mL according to the cuvet design. The problem is more complicated when dealing with atomic absorption spectrophotometers that do not have cells with a fixed volume. Nevertheless, a test portion of food after mineralization is transformed to a solution with a fixed volume, which is usually within the 5–20 mL range. However, because sensitivities of atomic absorption spectrophotometers vary greatly, a wide range of working volumes can be used, although the practical lower limit is about 1 mL. In special cases, a volume of 0.1 mL can be attained by complex formation, extraction of the mineralized solution with an organic solvent, and concentration of the solvent to a small volume. Even if an insignificantly small aliquot of the test solution is required for the analysis, the test solution must be converted before the measurement to some fixed volume that is convenient for the work, which is called the "working volume."

When analyzing foods for trace elements with low MLs such as cadmium and mercury, the limiting factor often is the quantity of test portion that can be practically handled. When using the recommended test portions (6), the entire working volume must be used for the measurement to obtain a concentration sufficient for reliable final results.

Some laboratories, depending on experience, purity of reagents, and instrument differences, are able to obtain values for the LAD that greatly differ from those obtained by other laboratories. Very low values are obtained when working with pure (model) solutions. Such limits may be called, in Currie's classification, the "ideal intralaboratory limit of quantitative determination," L^{ii}_Q. In the practical analysis of foods, the test portion undergoes a number of chemical operations, including mineralization, that require addition of reagents to test, control, and blank materials. As a result, the LAD values increase noticeably. These values of the LAD may be called the "real intralaboratory limit of quantitative determination," L^{ir}_Q, where $L^{ir}_Q \geq L^{ii}_Q$.

Even following the ISO instructions for interlaboratory studies (1), some laboratories with reduced sensitivity are not able to achieve the LAD of other laboratories. In such cases, a series of interlaboratory studies should be organized, with analyte concentrations equal to or greater than L^{ir}_Q, to find a concentration at which the maximum RSD_R values do not exceed those recommended by IUPAC (11). This concentration is designated as L_QR, which is greater than or equal to L^{ir}_Q. In the final interlaboratory study, L_QR should correspond to the analyte concentration in the test solution prepared for the analysis. For some modern sensitive methods of analysis such as flameless atomic absorption spectrophotometry and liquid chromatography, higher concentrations of the analyte can be several times more than L_QR. This concentration is marked L_QRR; with $L_QRR > L_QR$. Actually, L_QR in pure analytical

chemistry and for safety control should be understood as "the limit of quantitative interlaboratory determination of methods." L_QRR is used practically for the determination of the analyte in safety control. The interlaboratory determination of L_QR and L_QRR is important for determining the reliability of the results. For determining reliability, we may use the so-called "Horwitz curve," which relates the relative standard deviation, RSD_R, to the analyte concentration. As a result of the generalization of a large amount of experimental material, Horwitz showed that RSD_R is largely dependent on the concentration, C, rather than on the nature of the analyte or on the method of analysis. This dependence is logarithmic and may be expressed by the formula, Typical RSD_R (%) = $2\ C^{(-0.1505)}$, where C is expressed as a decimal fraction. This equation applies to the ideal case. The maximum permitted values for an acceptable method correspond to the equation, Maximum RSD_R (%) = $4C^{(-0.1505)}$. The proposals made by Horwitz are published by IUPAC (11) for the evaluation of method reliability. We also consider these recommendations to be sufficient for the evaluation of the parameters L_QR. If the results at the tested concentrations, L_QR, are above the values indicated by the "Horwitz curve," then a new interlaboratory study should be organized with higher concentrations of the analyte that provide acceptable parameters, or the method must be "improved."

For reliable analyses, the test solution introduced into the measuring instrument must have a definite concentration (L^R_Q) of the analyte in a definite volume (V_{opt}). Thus, in the final analysis it is not the relative quantity of the analyte (concentration) in the measured solution that is important but the absolute quantity prepared for the analysis and presented to the instrument. This quantity, MQS_m, may be calculated according to the following equation:

$$MQS_m = L^R_Q \times V_{opt} \qquad (1)$$

Particular attention must be paid to compatibility of units; if L^R_Q is expressed in μg/mL (mg/L), then V_{opt} must also be in mL and MQS_m must be in μg.

Many scientists express the value of the limit of determination not for analytical concentrations used for the introduction of the working solution into the instrument (i.e., L^R_Q), but for the analyte concentrations that are directly in the tested product, such as mg/kg or μg/mL product. This concentration may be designated as L^{RP}_Q. To get to the recommended concentration of the analyte, L^R_Q, it is necessary to make additional recalculations from the recommended test portions and V_{opt} to the concentration level of the working solution, L^R_Q. For example, if the optimal value of the test portion, M_{opt}, is 25 g, the limit of determination of the studied analyte in a specific product in the interlaboratory study, L^{RP}_Q, is 0.001 mg/kg, and V_{opt} is 1 mL, then $L^R_Q = 0.025$ (kg) × 0.001 (mg/kg)/0.001 (L) = 0.025 mg/L or μg/mL. This is the value to be used in Equation 1. The reverse calculation is also possible; when we know L^R_Q, we can calculate L^{RP}_Q. Note that although L^R_Q should be expressed as μg/mL, ML may be expressed as μg/mL for liquids and μg/g for solid products.

In this connection, we should point out that Currie introduces, together with the related concepts L_D, L_I, and L_Q, a new value, L_R, the "regulatory limit," which is equivalent to our ML. We do not consider these identical terms, because ML refers to products (matrixes) and L^R_Q, L_D, L_I, and L_Q refer to analyte concentration in the solution to be measured. If L_R refers to the analyte concentration, then to compare them the additional calculations mentioned above must be made.

Use of MQS

The determination of MQS is still not a sufficient evaluation of the method for the purpose of safety control of food. It must be compared with the quantity of the contaminant actually contained in the examined product (test sample) to ensure that the analyte concentration is at the ML level. For practical reasons such as time required for mineralization and losses incurred in mineralization, optimum quantities of test portions of examined food products, M_{opt}, were defined in a previous paper (6). Considering the limitations imposed by M_{opt} and ML, the minimum absolute quantity of the analyte, *MQS*, is calculated as follows:

$$MQS = ML \times M_{opt} \qquad (2)$$

Again, compatibility of units must be achieved. If from toxicological considerations we know the absolute quantity of a toxic analyte that must be determined in a test portion of food, then methods must be used that will permit us to reliably determine this quantity MQS_m, where:

$$MQS_m \leq MQS \qquad (3)$$

This formula reiterates that acceptable methods must reliably measure absolute quantities that are limited by the quantity required by the legal limit in one test sample of the product. Because MQS_m will differ from method to method, the values obtained will suggest which method, if any, should be chosen for safety control of the food.

Examples of MQS_m and MQS Calculation and Use

The initial calculations of MQS_m were obtained from the interlaboratory study that developed the methods for the toxic elements As, Cd, Cu, Fe, Hg, Pb, Sn, and Zn for incorporation into the state standard. A total of 22 USSR independent laboratories participated in the work from 1981 to 1985. Ten laboratories participated in the elaboration of each method, and one served as a coordinator. This laboratory prepared the exact description (protocol) of the methods and conducted the preliminary intralaboratory research on model solutions. This research was done to determine the real intralaboratory limit of quantitative determination, L^{π}_Q, and the parameters of the calibration curve from 5 points or more. By definition, the first (lowest) point of the curve corresponds to L^{π}_Q. Homogeneous food products with the specific trace element added at levels of 0.5, 1.0, and 1.5 × the ML were examined by all members of the group. Results were statistically analyzed in conformity with the ISO standard (1) and were discussed at meetings of the experts representing all participating laboratories. Reasons for

Table 1. Common MQS (μg) for any food (Table 2) and MQS_m (limit of determination of a specific method) of various methods for determination of toxicologically significant elements

Element	Common MQS (less than)	MQS_m		
		Polarography[a]	Colorimetry	Method
Pb	4	0.6	8[b]	Dithizone, **25.095**[c]
Cd	0.5	0.2	5[b]	Dithizone, **25.021**[c]
Zn	100	2	10[b]	Dithizone, **25.143**[c]
Cu	20	—	5[a]	Zinc dibenzyldithiocarbamate
	Electrolyte D	1		
	A	2	5[a]	Sodium diethyldithiocarbamate
	B	20		
	C	20		
Sn	200	—	10[a]	Quercetin
Fe	50	—	10[a]	*o*-Phenanthroline
As	3	—		Ag diethyldithio-carbamate with:
			2.5[a]	ethanolamine
			5[a]	methenamine
Hg[d]	0.2	—	0.15[a]	Tetraiodomercurate
			5[b]	Dithizone, **25.117**[c]

[a] According to USSR standards (Part 2) [7].
[b] According to our data.
[c] *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA.
[d] By AAS method, $MQS_m = 0.01^a$.

the occurrence of outliers were explored, and the protocol was revised, if necessary, to avoid their reoccurrence. If L^{π}_Q turned out to be too low, i.e, some of the laboratories did not find the element at this concentration, additional research was conducted with other concentrations of L^{π}_Q. Finally, as a result of the interlaboratory research, L^R_Q was determined, and MQS_m was calculated (Table 1) for every method studied (8). All RSD_R values at the ML concentration should be no larger than the maximum RSD_R values.

As a typical example, consider the calculation of MQS_m for the determination of copper in foods by the sodium diethyldithiocarbamate colorimetric method using a photoelectrocolorimeter with a working volume, V_{opt}, of 25 mL. As a result of intralaboratory research, the coordinating laboratory recommended the interlaboratory value of L^R_Q and an intralaboratory value for L^{π}_Q, the first point of the calibration curve, of 0.2 mg/L (0.2 μg/mL). Interlaboratory research showed that the relative intralaboratory repeatability, RSD_r, calculated by ISO 5725 (1) was 15%, and the interlaboratory reproducibility, RSD_R, was 30% at a Cu concentration of 0.2 mg/L. At the meeting of experts, this value was considered to be satisfactory. Because RSD_R is less than the maximum permissible recommendations of IUPAC (11), maximum RSD_R of 40% at 0.2 mg/L (or 0.2 μg/mL), no additional research was required. In this particular case, $L^R_Q = L^{\pi}_Q$. For the majority of food products with high ML for copper, it was more convenient to work with higher L^{RR}_Q values (0.4–1.0 mg/L), which came from the average values of 7% for RSD_r and 14% for RSD_R in the interlaboratory studies. However, to calculate MQS, we should take minimum values of L^R_Q according to Equation 1:

$$MQS_m = 0.2 \times 25 = 5\ \mu g$$

Similarly, using the recommended protocols, specified instruments, and preset working volumes, MQS_m values were calculated for all the studied methods and are presented in Table 1. For the As and Hg colorimetric methods at the concentrations corresponding to L^R_Q, the RSD_Rs were about 40%. The RSD_R could not be improved (lowered) by increasing the size of the test portion to increase the concentration corresponding to L^R_Q without changing the methods.

In the USSR, all foods were divided into 20 groups according to ML values (10). Optimum values for the analytical test portions, M_{opt}, were defined for these groups. Because different groups of products have different ML and M_{opt} values, they will have their own MQS values according to calculations by Equation 1. We named these values "individual MQS" to differentiate them from "common MQS," which is the same for any product analyzed according to the given method. For the analysis of water, air, and soil that have a homogeneous composition and single ML values, an individual MQS is not always needed. The results of the calculation of common and individual MQS values according to Equation 2 are given in Table 2. The numerical value of the common MQS was determined by the lowest value of individual MQS values. For example, the common MQS for Pb was determined by the individual MQS for oil (4 μg).

To determine the acceptability of the investigated methods for food safety control, the MQS_m of each method is compared with the common and individual values for MQS given in Table 1.

The MQS values may also be used to characterize a number of currently recommended methods for safety control of food. Table 1 compares MQS_m values for official dithizone methods for the determination of certain trace elements from *Official*

Table 2. Minimum quantity (μg) of analyte that can be determined in test portions of different products for toxicologically important elements (MQS-values)

Product	Element							
	Pb	Cd	Zn	Cu	Sn	Fe	As	Hg
Fruit, vegetables	12	0.9	250	125	—	50	6	0.8
Fruit and vegetable products, processed	12	0.9	250	125	1000	50	6	0.8
Red and poultry meat	12.5	1.25	700	125	—	—	3	1.2
Liver, kidney, and other internal organs	15	2.5	350	50	200	—	25	4
Meats and meat and vegetables, canned	12.5	1.25	700	125	500	—	3	1.2
Gelatin	20	0.6	1000	150	—	—	10	2
Eggs, whole frozen	7.5	0.5	250	75	—	—	3	0.8
Egg, powdered	30	1	200	150	—	—	5	1
Vegetable oils and their products, animal fats	4	1.2	200	20	—	200	4	1.2
Fish, sea mammals and invertebrates, algae, and their products	30	6	400	250	200	—	25	0.9
Grain and grain products	7.5	0.75	500	100	—	—	6	0.6
Bread and bakery products	7.5	1.25	250	50	—	—	3	0.4
Flour and confectionery products	4.5	0.75	500	400	—	—	6	0.4
Milk and dairy products—liquid, dry (reconstituted)	5	2	500	100	—	—	5	0.2
Milk curds	15	10	500	80	—	—	10	0.8
Dairy products, canned	15	5	150	30	200	—	7.5	0.3
Butter	5	1.5	125	22.5	—	150	4.5	1.2
Cheese	7.5	5	250	48	—	—	8	0.8
Wine and cognac	7.5	0.75	100	50	—	300	10	0.25
Beer	15	1.5	500	250	—	300	10	0.25

Methods Analysis (9) and polarographic methods in the USSR state standard (7). The dithizone methods for Cd, Hg, and Pb cannot be used for the safety control of food at the currently existing USSR ML levels (10) because $MQS_m > MQS$ for all 3 elements. However, the colorimetric methods for the determination of Cu, Fe, and Sn, the atomic absorption methods for the determination of Hg, and the polarographic methods for the determination of Cd, Pb, and Zn given in the USSR state standards may be used.

In the case of the polarographic method for Cu (Table 1), the MQS_m values are 20 μg, with electrolytes B and C at the limit of the common MQS. The corresponding standard (7) indicates that these electrolytes can be used only to analyze grain, bread, bakery and confectionery products, and beverages, for which individual MQS values are 100, 500, 400, and 50 μg, respectively (Table 2). These MQS values are considerably higher than the MQS_m values for the polarographic methods. For the Cu determination in vegetable oils and milk, which have low individual MQS values, the colorimetric method with an MQS_m of 5 μg is recommended.

In the case of As determination in most foods, the monoethanolamine method is recommended (Table 1). The methenamine method is useful only with foods of high MQS (fish, beverages, and internal organs of animals).

Therefore, Table 1 is useful for comparing the MQS_m of the different methods and evaluating their applicability to the control of toxic elements in specific foods. The MQS_m values may also be used for the opposite task of determining the minimum value of the test portion of a test sample (food), M_{min}, through the equation $M_{min} = MQS_m/ML$, where all the values are expressed in compatible units: M_{min} in g, MQS_m in μg, and ML in μg/g (or mL).

In many cases, such calculations enable us to reduce the quantity of test portion, without reducing the accuracy of the method, and to hasten the analysis. For example, in the analysis of lead in canned meat by the polarographic method (MQS_m = 0.6 μg) at ML = 1 mg/kg = 1 μg/g, we obtain: $M_{min} = 0.6/1 = 0.6$ g ≈ 1 g. For the determination of lead only in canned meat at the ML level, the quantity of test portion should be about 1 g.

The same principles can be used for the evaluation of methods for environmental safety control in air, water, soil, or any matrix where an ML has been established and the size of the test portion must be limited for practical reasons.

We tried to show that contrary to pure chemistry, where the characterization of the limit of determination of a method usually uses the results of intralaboratory research (L^n_Q and L^n_Q) for safety control purposes compared with ML, the limit of determination must be established through interlaboratory research (L^R_Q). However, for the objective comparison of different control methods, it became necessary to establish 2 additional metrological characteristics, MQS and MQS_m, because they address factors that are not significant in pure chemistry: the need for the ML determination and the importance of using an optimum test portion. Therefore, we suggested (4) in the requirements for Codex Methods that the term "limit of determination for the method responsible for safety" be used,

or in short, the "limit of determination in place of Codex Methods" for the classical term "limits of determination." The special functions of the Codex methods require the complex criteria involving MQS and MQS_m; for pure chemistry, the old criteria are sufficient.

Relationship to Accuracy

The concept of "accuracy" is one of the most important characteristics of analytical chemistry. It refers to the relationship of the results of analysis to the true composition of the test sample. As a rule, accuracy is not essential for safety control for several reasons: Only a limited number of officially sanctioned methods are used for the safety control of food. These methods were accepted as alternatives on the basis of their comparability. If bias exists, it will influence the results from all the participating laboratories equally. Furthermore, ML values were set on the basis of toxicological work with animals. Such limits necessarily have a large uncertainty that is considerably greater than the acceptable bias of the chemical methods for toxic elements, which usually are less than 10%.

Discussion and Recommendations

The majority of authors who discussed the problem of LAD and LQ focussed primarily on the aspects of terminology and concepts at the intralaboratory level. The practical limitations of analytical chemistry necessitated the development of a new sphere of analytical metrology, the LAD from interlaboratory studies, which we have attempted to handle through the introduction of the new metrological characteristics of MQS and MQS_m. But several problems still remain. The nomenclature of terms and concepts used in interlaboratory studies need to be harmonized. This task is underway in IUPAC by Currie (2) and Horwitz (5). Indixes similar to the "ideal intralaboratory limit of quantitative determination" of Currie are needed for "real" food products studied in individual laboratories and for the interlaboratory environment.

Stated criteria for the calculation of these indixes and for critical values of RSD would also be appropriate. Currie mentions an RSD value for intralaboratory studies of 10% (2). However, Horwitz commented (5) that one value cannot be universal, because RSD is a function of concentration. Before final proposals appear, critical RSD_R values derived from maximum values of the "Horwitz curve" should probably be established, depending on the concentration at the ML level: only RSD_R values that are below the maximum RSD_R values should be considered admissible.

The practical values of RSD_r in the determination of $L^{\tilde{n}}_Q$ and L^{n}_Q cannot exceed 50%, or the analysis is not considered quantitative.

In addition, the harmonized protocols developed by IUPAC (11) should be expanded to include the peculiarities required for determining LAD and possibly even including the preliminary determination of $L^{\tilde{n}}_Q$, L^{n}_Q, L^{R}_Q, and L^{RR}_Q, as well as criteria for the calculation of their reliability and the determination of the maximum values of RSD_r and RSD_R. We plan on stating our proposals on these two items in our next paper.

Acknowledgment

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ARTIFICIAL SWEETENERS

Determination of Acesulfam-K in Foods

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A liquid chromatographic method was evaluated for the determination of the intense sweetener acesulfam-K in tabletop sweetener, candy, soft drink, fruit juice, fruit nectar, yogurt, cream, custard, chocolate, and biscuit commercial preparations. Samples are extracted or simply diluted with water and filtered. Complex matrixes need a clarification step with Carrez solutions. An aliquot of the extract is analyzed on a reversed-phase μBondapak C_{18} column using 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (90 + 10) as mobile phase. Detection is performed by UV absorbance at 220 nm. Recoveries ranged from 95.2 to 106.8%. With one exception, all analyzed values were within ±15% of the declared levels. The repeatabilities and the repeatability coefficients of variation were, respectively, 0.37 mg/100 g and 0.98% for products containing less than 40 mg/100 g acesulfam-K and 2.43 mg/100 g and 1.29% for other products. The same procedure also allowed detection of many food additives or natural constituents, such as other intense sweeteners, organic acids, and alkaloids, in a single run without interfering with acesulfam-K. The method is simple, rapid, precise, and sensitive; therefore, it is suitable for routine analyses.

Acesulfam-K is the potassium salt of 6-methyl-1,2,3-oxathiazine-4(3*H*)-one-2,2-dioxide. This intense sweetener was first prepared by Clauss and Jensen in 1967 and commercialized by Hoechst Ltd under the brand name Sunett™.

Acesulfam-K is about 200 times sweeter than a 3% sucrose solution. It is used in a wide range of products, either by itself or in conjunction with other sweeteners. Under normal food processing and storage conditions, acesulfam-K is extremely stable.

The human body rapidly absorbs acesulfam-K but excretes it completely unmetabolized (1). Furthermore, it is not fermented by oral bacteria (2). Therefore, acesulfam-K is a nonnutritive and noncariogenic sweetening agent.

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Toxicological studies showed that acesulfam-K did not exhibit any mutagenicity or carcinogenicity (1). For that reason, the joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives approved the use of acesulfam-K, to which an acceptable daily intake of 0–15 mg/kg body weight was allocated (3).

Acesulfam-K is authorized in about 30 countries and should be declared on the label. Recently, the European Community issued a proposal for a Directive (4) in which precise maximum permitted levels are given for acesulfam-K in many different foods. Rapid and robust analytical methods are needed to check food labeling or to monitor the quality of finished products.

Although thin-layer chromatographic (5, 6), photometric (7), or isotachophoretic (8, 9) methods were developed, liquid chromatography (LC) is the most frequently applied technique for the determination of acesulfam-K. LC procedures using amino (10), ion-exchange (11), or reversed-phase columns (9, 12–19) were reported. Among the reversed-phase procedures, elution with a mobile phase consisting of a mixture of KH_2PO_4 and acetonitrile is the most powerful system. Under these conditions, acesulfam-K separates well from aspartame, saccharin, and other food additives (18, 19). Interestingly, the same technique can be applied for the determination of aspartame and its breakdown products (20). Although several recovery experiments were performed, analyses of food samples and comparison of the results with the acesulfam-K contents declared on the label has never been published.

In this study, we performed a thorough evaluation of the above LC technique for the separation of acesulfam-K from other sweeteners, food additives, and natural constituents (organic acids, alkaloids) that may interfere. A wide range of commercial products were analyzed, and the figures were compared with the acesulfam-K levels declared on the label.

Experimental

Materials

The 20 samples analyzed in this study were all commercial products purchased in Switzerland and England.

Reagents

(a) *Solvents and chemicals.*—All chemicals were of analytical reagent grade. LC quality acetonitrile was purchased from Machler. Demineralized water was obtained from a Milli-Q system (Millipore).

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Table 1. Influence of the amount of acetonitrile in the mobile phase on capacity factor (k′)[a]

	0.0125M KH_2PO_4 (pH 3.5)–acetonitrile				
Standard	(98 + 2)	(95 + 5)	(90 + 10)	(85 + 15)	(80 + 20)
Phenylalanine	3.14	1.68	0.79	0.47	0.37
Acesulfam-K	2.49	1.63	0.94	0.61	0.45
Theobromine	13.7	4.57	1.32	0.63	0.45
Hydroxymethylfurfural	5.01	2.64	1.37	0.84	0.69
Saccharin	6.72	3.50	1.54	0.91	0.52
Aspartylphenylalanine	8.08	4.12	1.76	0.85	0.50
Theophylline	>17.5	7.94	2.22	0.96	0.56
DKP	>17.5	11.0	3.85	1.74	0.91
Caffeine	>17.5	>17.5	4.72	1.89	1.01
Aspartame	>17.5	>17.5	8.72	3.69	1.65
Vanillin	>17.5	>17.5	10.2	5.42	3.25
Dulcin	>17.5	>17.5	13.8	6.88	3.78
Alitame	>17.5	>17.5	15.1	6.09	2.68
Benzoic acid	>17.5	>17.5	15.2	8.79	5.25
Sorbic acid	>17.5	>17.5	15.6	9.05	5.33

[a] $k' = (t_R - t_o)/t_o$; t_o values: 3.29 min (98 + 2), 3.26 min (95 + 5), 3.23 min (90 + 10), 3.13 min (85 + 15), and 3.21 min (80 + 20).

(b) *Carrez I solution.*—3.6 g potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) was dissolved in water and diluted to 100 mL.

(c) *Carrez II solution.*—7.2 g zinc sulfate heptahydrate ($ZnSO_4\cdot 7H_2O$) was dissolved in water and diluted to 100 mL.

(d) *Mobile phase.*—0.0125M KH_2PO_4 buffer (pH 3.50)–acetonitrile (90 + 10). The phosphate buffer was made up of 0.17% KH_2PO_4 solution precisely adjusted to pH 3.50 with 5% phosphoric acid. The buffer and acetonitrile were separately filtered through 0.45 µm filter (Millipore), precisely mixed in the desired proportions (with pipets), and degassed 5 min in an ultrasonic bath. The mobile phase was maintained under a slight overpressure of helium (avoid bubbling) during the analyses and was prepared daily.

(e) *Standard solutions.*—1 mg/mL aqueous stock solutions were made up for acesulfam-K (Hoechst); diketopiperazine (Bachem Feinchemikalien); aspartame, saccharin, sodium cyclamate, phenylalanine, theobromine, and theophylline (Fluka); alitame (Pfizer); dulcin (ICN Pharmaceuticals); sucralose and thaumatin (Tate & Lyle); neohesperidin dihydrochalcone (Jafora); stevioside (Sato Stevia); aspartylphenylalanine (Sigma); vanillin (Givaudan); and caffeine, citric acid, benzoic acid, sorbic acid, lactic acid, L-malic acid, and hydroxymethylfurfural (Merck). Stock solutions were further diluted as required for LC analysis.

Apparatus

(a) *Liquid chromatograph.*—Model 420 pump (Kontron), manual injector (Rheodyne) fitted with a 10 µL loop, Model 480 column oven and Model 480 oven controller (Kontron), Model ERC-7215 absorbance detector (Erma) set at 220 nm, and Model 450 computing integrator (Kontron).

(b) *LC column.*—µBondapak-C_{18} (10 µm, 300 × 3.9 mm) reversed-phase analytical column (Waters); Guard-Pak µBondapak-C_{18} (10 µm) precolumn (Waters).

(c) *Chromatographic conditions.*—Column temperature, 25°C; flow rate, 0.8 mL/min; detector attenuation, 0.2 AUFS.

Sample Preparation

(a) *Tabletop sweeteners and candies.*—Samples were dissolved in water to yield an acesulfam-K concentration of 10–30 µg/mL. All solutions were filtered through 0.2 µm filters (Sartorius) before 10 µL injection into the chromatograph.

(b) *Liquid beverages.*—Soft drinks were first degassed. An aliquot of the sample was diluted to yield an acesulfam-K concentration of 10–30 µg/mL. Undissolved matter, if any, was filtered through folded filter paper (Schleicher & Schuell 597 1/2). Highly colored solutions were decolorized by passing through a C_{18} disposable cartridge (Supelclean LC-18, Supelco). All diluted solutions were filtered through 0.2 µm filters before injection.

(c) *Other products.*—A quantity of homogenized sample was accurately weighed into a 100 mL volumetric flask to yield an acesulfam-K concentration of 10–30 µg/mL. About 70 mL water was added, and the sweetener was extracted at 60°C for 15 min. The solution was cooled to room temperature, and 5 mL Carrez I solution and 5 mL Carrez II solution were successively added. The flask was shaken vigorously and diluted to 100 mL with water. The solution was filtered through folded filter paper. The filtrate was passed through 0.2 µm filter before injection.

Procedure

Two 10 µL volumes of standard and sample solutions were injected. Results were calculated according to the following formula:

$$100 \times (Co/Cs) \times (As/Ao)$$

where Co = concentration of acesulfam-K in standard solution (mg/100 mL), Cs = concentration of product in sample solution

Table 2. Influence of phosphate buffer pH on capacity factor (k')[a]

	pH[b]			
Standard	3.0	3.5	4.0	4.5
Phenylalanine	0.89	0.79	0.74	0.86
Acesulfam-K	1.01	0.94	0.97	1.07
Theobromine	1.36	1.32	1.37	1.69
Hydroxymethylfurfural	1.37	1.37	1.34	1.55
Saccharin	1.69	1.54	1.44	1.75
Aspartylphenylalanine	2.61	1.76	1.11	0.96
Theophylline	2.25	2.22	2.30	2.86
DKP	4.47	3.85	2.92	2.62
Caffeine	4.76	4.72	4.80	6.13
Aspartame	9.40	8.72	8.65	10.3
Vanillin	10.2	10.2	10.1	12.1
Dulcin	13.8	13.8	13.8	16.7
Alitame	15.5	15.1	>17.6	>16.8
Benzoic acid	16.9	15.2	11.6	9.21
Sorbic acid	16.2	15.6	14.2	12.8

[a] t_o values: 3.22 min (pH 3.0 + 4.0), 3.23 min (pH 3.5), and 3.27 min (pH 4.5).
[b] Mobile phase: 0.0125M KH_2PO_4–acetonitrile (90 + 10).

(mL or g/100 mL), *As* = peak area of product in sample solution, and *Ao* = peak area of acesulfam-K in standard solution.

Results and Discussion

Method Evaluation

The basic conditions for the evaluation of the LC method were those described by Tsang et al. (20) for the determination of aspartame and its major breakdown products: aspartylphenylalanine, diketopiperazine (DKP), and phenylalanine. We tested the influence of the amount of acetonitrile in the mobile phase and the pH of the phosphate buffer to find the best conditions for the separation of acesulfam-K from different food additives and natural constituents. We also determined the detection wavelength for optimum sensitivity.

Mobile phase.—The amount of acetonitrile in the mobile phase was varied (Table 1). As the proportion of acetonitrile increased, capacity factors and retention times of all standards decreased rapidly. The effect was much less pronounced for acesulfam-K. The sweetener was well-separated from most of the tested food additives and possible interfering products for all mobile phases studied.

However, acesulfam-K was poorly separated from phenylalanine when using 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile ([95 + 5] and [80 + 20]), and acesulfam-K coeluted with theobromine, a natural cocoa alkaloid, in the (85 + 15) and (80 + 20) media. The 2 compounds would obviously interfere in the determination of acesulfam-K in aspartame-containing or chocolate-containing foods under these conditions. Furthermore, retention times of most standards were too long (>60 min) with the (98 + 2) and (95 + 5) mixtures, whereas their resolution was insufficient with the (80 + 20) proportions. Finally, a mobile phase consisting of 90% phosphate buffer and 10% acetonitrile was selected. In addition to a rapid determination of acesulfam-K, it allows the separation of all standards in less than 60 min.

pH.—Using the phosphate buffer–acetonitrile ratio chosen above, the pH of the phosphate buffer was varied (Table 2). The resolution of acesulfam-K from phenylalanine and theobromine at pH 3.0 was not as good as that found at different pHs. Furthermore, the use of mobile phases in the pH range of 3.5–6.5 is recommended by the manufacturer for maximum column life. For most standards, capacity factors and retention times at pH 4.5 differed greatly from those measured at other pHs. Under these conditions, distortions in the DKP and benzoic acid peaks were observed. More importantly, the separation of acesulfam-K from aspartylphenylalanine was poor. This separation was also poor at pH 4.0.

These results would have precluded a precise determination of the sweetener in aspartame-containing foods at these pHs. Consequently, the pH of the phosphate buffer in the mobile phase was set at 3.5.

Because the capacity factors of all standards are sensitive to the composition and pH of the mobile phase, great care and precision are required in mobile phase preparation. Following these recommendations, reproducible retention times were obtained.

Wavelength.—Table 3 shows the influence of the detection wavelength on sensitivity. Optimum sensitivity of acesulfam-K was obtained at 230 nm.

In conclusion, a mobile phase consisting of 0.0125M KH_2PO_4 buffer (pH 3.5)–acetonitrile (90 + 10) and a detection wavelength of 230 nm are the optimized chromatographic conditions for the determination of acesulfam-K. However, 220 nm was also selected in this study to detect the other compo-

Table 3. Influence of the detection wavelength on sensitivity[a]

	Sensitivity, mV/min[b]				
Standard	205 nm	214 nm	220 nm	230 nm	240 nm
Phenylalanine	27.6	20.1	12.9	0.89	0.11
Acesulfam-K	6.11	12.8	17.7	24.4	21.2
Theobromine	33.4	29.7	20.4	9.08	5.66
Hydroxymethylfurfural	14.2	5.95	5.59	8.71	7.90
Saccharin	56.5	44.4	35.5	23.2	10.8
Aspartylphenylalanine	30.9	17.1	11.8	2.40	0.31
Theophylline	55.2	45.9	29.9	12.9	8.36
DKP	33.9	19.1	12.4	2.31	0.26
Caffeine	46.3	50.0	35.1	14.7	9.59
Aspartame	24.9	14.3	9.49	1.60	0.22
Vanillin	35.9	32.3	24.6	34.3	31.2
Dulcin	67.1	24.0	14.7	22.1	30.9
Alitame	18.0	12.9	7.80	1.69	0.26
Benzoic acid	12.5	55.7	43.3	39.9	32.9
Sorbic acid	10.2	8.86	8.47	12.6	25.8

[a] Mobile phase: 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (90 + 10).
[b] Peak area corresponding to 1 µg of standard on the column (10 µL injection of a 100 µg/mL solution).

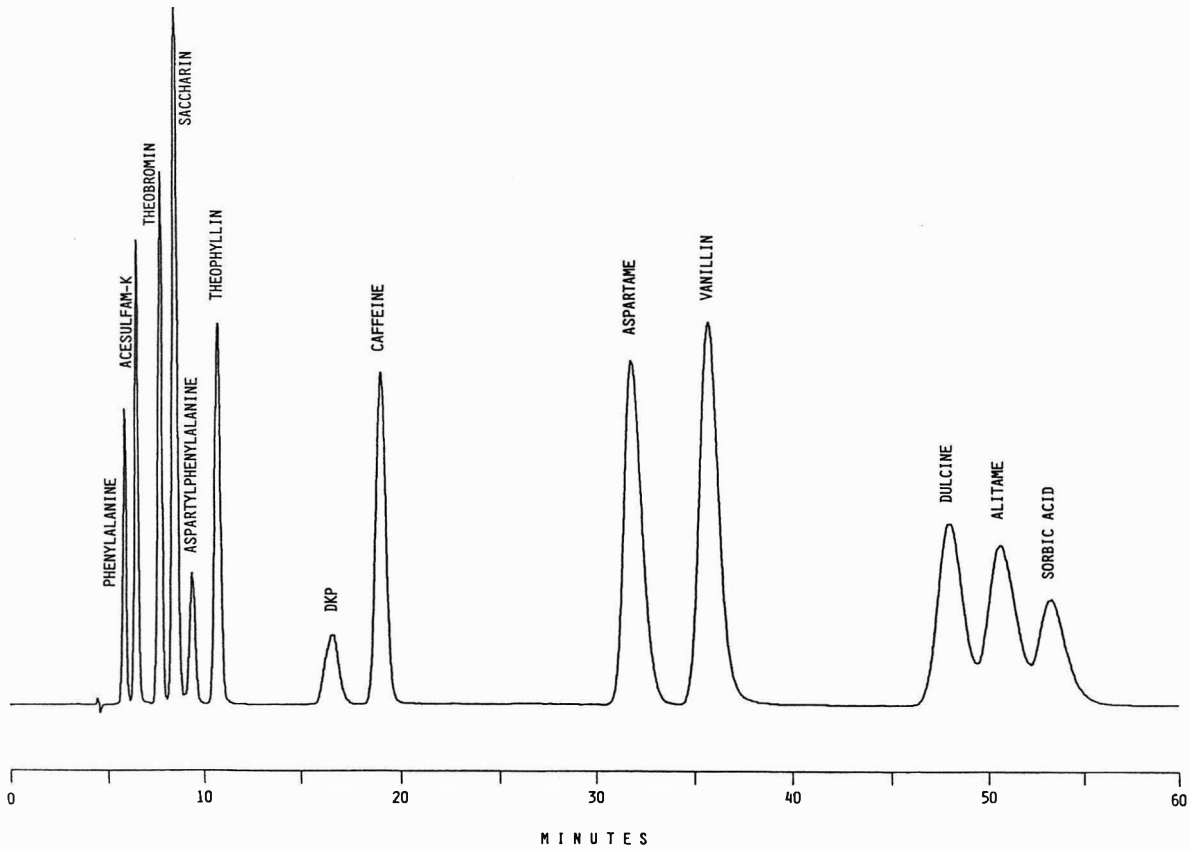

Figure 1. Chromatogram of mixed standard solution.

nents that are, for the most part, much more sensitive at this wavelength than at 230 nm. The proposed chromatographic conditions differ slightly from those applied by Hagenauer-Hener et al. (19), who used a more concentrated phosphate buffer (0.02M) at a higher pH (4.2–4.4). Figure 1 shows a typical chromatogram of a mixed standard solution containing acesulfam-K and other food additives or natural constituents. Under these conditions, retention times of citric, malic, and lactic acids were very close to t_o, whereas the intense sweeteners cyclamate, sucralose, neohesperidin dihydrochalcone, stevioside, and thaumatin were not detected.

Linearity and Detection Limit

Under the above-mentioned conditions, a linear detector response (correlation coefficient, r = 0.9996) was obtained over acesulfam-K concentrations ranging from 0.5 to 300 µg/mL. The detection limit, measured on a standard solution and defined arbitrarily as the amount of product on the column that produces a signal-to-noise ratio of 5, was about 100 pg sweetener.

Recovery Experiments

Table 4 presents recoveries of various amounts of acesulfam-K added to isotonic drink, black currant juice drink, apricot yogurt, caramel cream, pralined chocolate biscuit, and rice pudding samples. Percent recoveries ranged from 95.2 to 106.8% and were not influenced by the food matrix or the added level of sweetener. Recoveries determined in the highly colored black currant juice drink indicated that no loss of acesulfam-K was induced by the decolorizing step (C_{18} cartridge). Results obtained for complex foods also showed that acesulfam-K is completely recovered after clarification with Carrez solutions.

Analysis of Foods

Table 5 shows results obtained for the analysis of 20 different commercial diet foods with the proposed method. Except for the biscuits, all results were within ±15% of the declared levels, when available. This tends to show that the addition of acesulfam-K to the product is under control and confirms the stability of the sweetener during manufacturing and storage of food products. The repeatabilities and the repeatability coefficients of variation of the measurements, calculated from the differences between the duplicates, were, respectively, 0.37 mg/100 g and 0.98% for products containing less than 40 mg/100 g acesulfam-K and 2.43 mg/100 g and 1.29% for other products.

No particular problems in sample preparation or in LC separation were encountered. Tabletop sweeteners and candies were simply dissolved in water and injected into the chromatograph. Figure 2 shows a chromatogram of a fruit candy sample containing aspartame along with acesulfam-K. Both sweeteners were easily detected. Furthermore, the presence of DKP and phenylalanine indicated some degree of aspartame decomposition.

Table 4. Recovery of acesulfam-K added to various products

Product	Added level, mg/100 g	Rec., %
Isotonic drink	5	98.2
	10	100.7
	15	99.5
	20	96.4
Black currant juice drink	10	102.0
	15	102.6
	20	104.9
Apricot yogurt	5	99.8
	10	104.5
	15	99.9
	20	100.8
Caramel cream	3	95.2
	5	96.3
Pralined chocolate biscuits	5	98.7
	10	99.0
	15	104.0
	20	98.8
Rice pudding	5	106.8
	10	104.3
	15	105.4
	20	104.1

Table 5. Analysis of acesulfam-K in commercial products[a]

Product	Declared	Found
Tabletop sweetener	10 mg/tablet	10.6 mg/tablet
Spiced candy	50 mg/100 g	43.8 mg/100 g
Cooked sugar	46 mg/100 g	43.0 mg/100 g
Fruit candy	20 mg/100 g	16.8 mg/100 g
Marzipan	130 mg/100 g	127.0 mg/100 g
Cola soft drink	—	11.6 mg/100 mL
Soft drink	—	3.1 mg/100 mL
Isotonic drink	13 mg/100 mL	12.8 mg/100 mL
Black currant juice drink	—	18.4 mg/100 mL
Orange juice	—	78.4 mg/100 mL
Lemon juice	—	75.1 mg/100 mL
Pineapple nectar	5.0 mg/100 mL	5.0 mg/100 mL
Orange nectar	5.5 mg/100 mL	5.3 mg/100 mL
Apricot yogurt	40 mg/180 g	45.5 mg/180 g
Caramel cream	15 mg/100 g	13.2 mg/100 g
Custard	—	12.8 mg/100 g
Bitter chocolate	68 mg/100 g	64.8 mg/100 g
Pralined chocolate biscuits	20 mg/100 g	25.3 mg/100 g
Rice pudding	—	8.9 mg/100 g
Creamed pudding	—	13.8 mg/100 g

[a] All results are average of duplicate determinations.

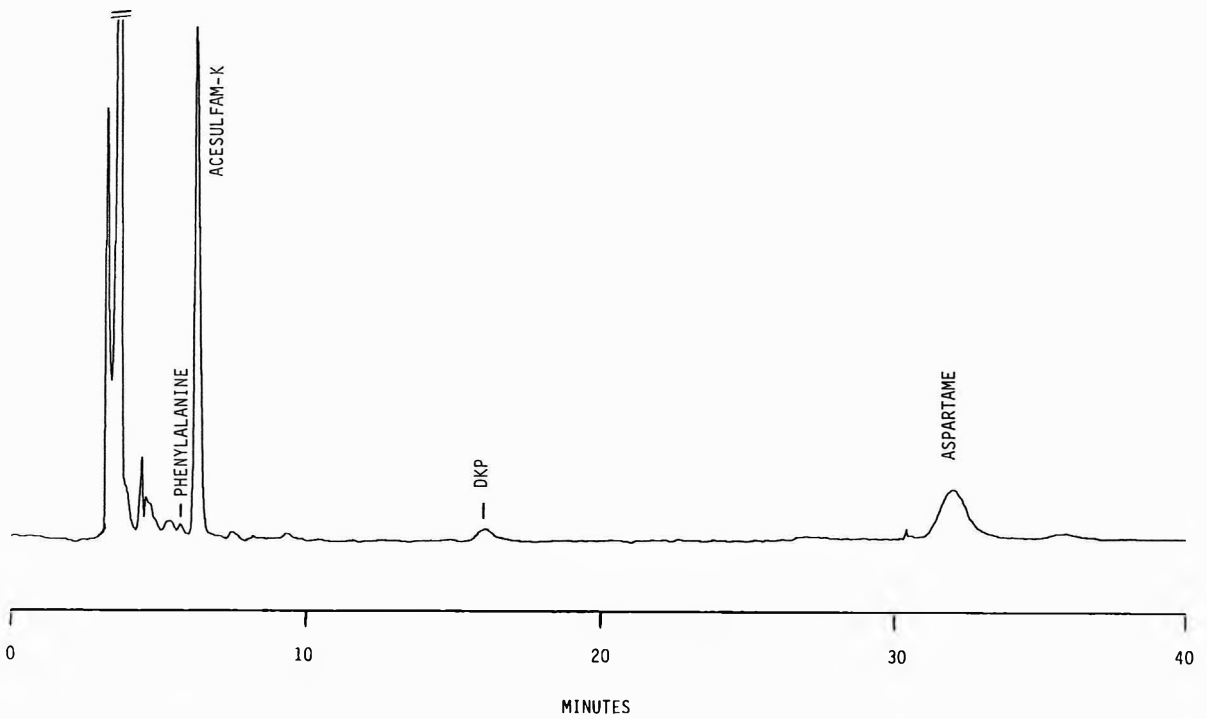

Figure 2. Chromatogram of fruit candy.

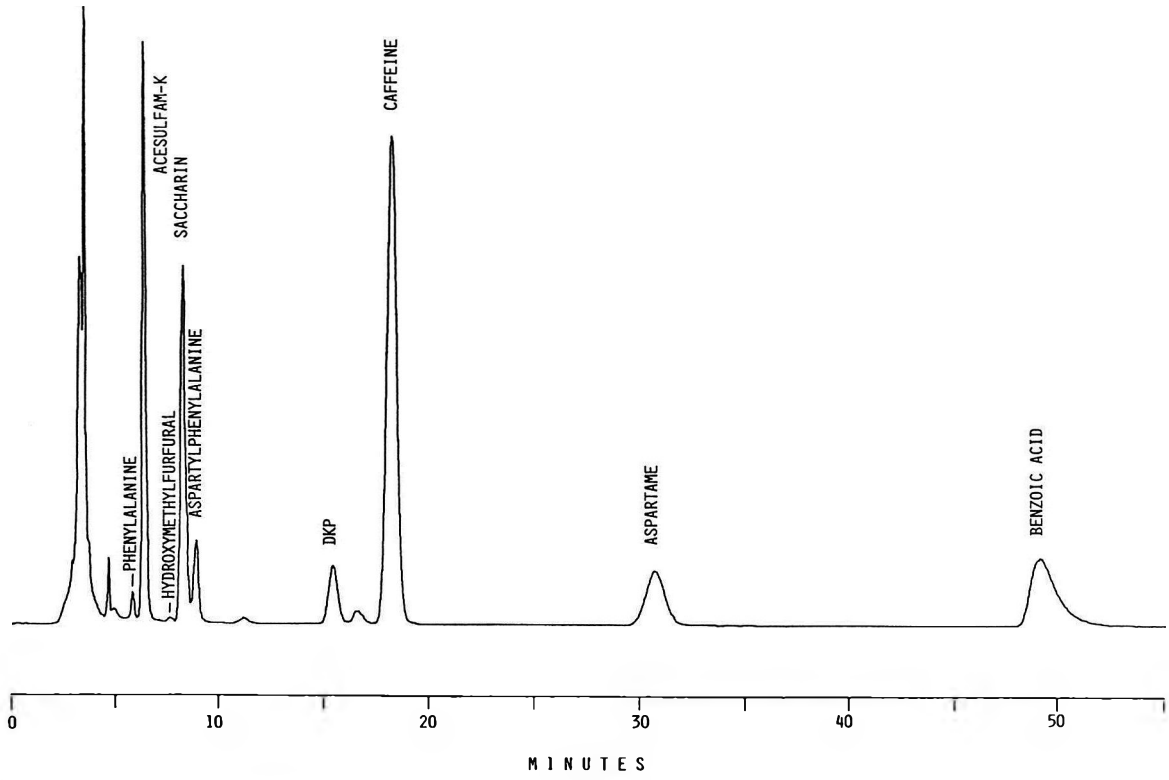

Figure 3. Chromatogram of diet cola.

Clear liquid beverages such as soft drinks were degassed and diluted before analysis. Cloudy products were first filtered to eliminate insoluble matter. An LC separation of a diet cola sample containing acesulfam-K, aspartame, and saccharin is shown in Figure 3. The 3 artificial sweeteners were well-separated from hydroxymethylfurfural, caffeine, and benzoic acid, as well as from the 3 major aspartame breakdown products.

More complex foods needed a clarification step with Carrez solutions before injection. Figure 4 shows a chromatogram of pralined chocolate biscuits containing vanillin. Good resolu-

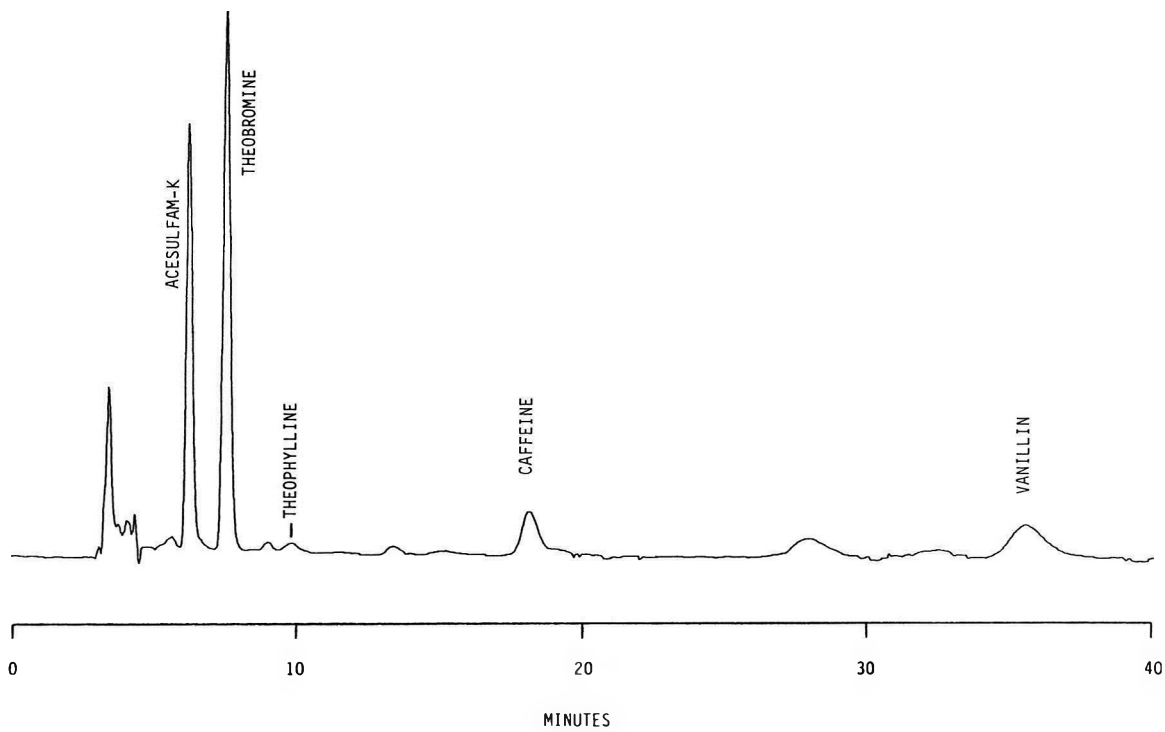

Figure 4. Chromatogram of pralined chocolate biscuit.

tion of acesulfam-K from natural cocoa alkaloids (theobromine, theophylline, and caffeine) and added vanillin was obtained.

Conclusions

The optimized LC method described in this work is applicable to the determination of acesulfam-K in all types of foods. The technique uses simple, aqueous extraction of the sweetener and isocratic elution on a reversed-phase column. Good recoveries were obtained. The acesulfam-K levels found in commercial products were close to the declared values. The proposed method is rapid, precise, and sensitive. Consequently, it is a very powerful tool for routine analyses.

The same technique also allows the detection of many other food additives or natural constituents in a single run, without interfering with acesulfam-K. The applicability of the method to the determination of aspartame and its major decomposition products (21) and saccharin in commercial foods will also be studied.

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ARTIFICIAL SWEETENERS

Determination of Aspartame and Its Major Decomposition Products in Foods

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A liquid chromatographic procedure already evaluated in a preceding study for the analysis of acesulfam-K is also suitable for the determination of the intense sweetener aspartame in tabletop sweetener, candy, fruit beverage, fruit pulp, soft drink, yogurt, cream, cheese, and chocolate preparations. The method also allows the determination of aspartame's major decomposition products: diketopiperazine, aspartylphenylalanine, and phenylalanine. Samples are extracted or diluted with water and filtered. Complex matrixes are centrifuged or clarified with Carrez solutions. An aliquot of the extract is analyzed on a reversed-phase μBondapak C_{18} column using 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile ([85 + 15] or [98 + 2]) as mobile phase. Detection is performed by UV absorbance at 214 nm. Recoveries ranged from 96.1 to 105.0%. Decomposition of the sweetener was observed in most food samples. However, the total aspartame values (measured aspartame + breakdown products) were within –10% and +5% of the declared levels. The repeatabilities and the repeatability coefficients of variation were, respectively, 1.00 mg/100 g and 1.34% for products containing less than 45 mg/100 g aspartame and 4.11 mg/100 g and 0.91% for other products. The technique is precise and sensitive. It enables the detection of many food additives or natural constituents, such as other intense sweeteners, organic acids, and alkaloids, in the same run without interfering with aspartame or its decomposition products. The method is consequently suitable for quality control or monitoring.

Aspartame, *N*-L-α-aspartyl-L-phenylalanine-1-methyl ester, is an intense sweetener first discovered in 1965 by J. Schlatter and commercialized by Searle & Co. under the brand name Nutrasweet™.

Aspartame has about 200 times the sweetness of sucrose and exhibits a sugarlike taste without bitter or metallic aftertaste. It extends and intensifies flavors, particularly fruit flavors. It is used in a variety of foods, sometimes in combination with other sweeteners.

The stability of aspartame in dry-product applications is relatively good. However, decomposition occurs in liquid products under certain conditions of temperature and pH (Figure 1). The decomposition products have no sweet taste or aftertaste.

Metabolic studies showed that aspartame is rapidly digested and metabolized as the corresponding dipeptide (1–5). Consequently, it has the same caloric value as proteins (17 kJ/g). However, because of its intense sweetness, the amounts of aspartame used in foods are small enough for the sweetener to be considered virtually nonnutritive and noncariogenic (6).

Numerous studies showed that aspartame was nontoxic and safe for the general population (1). Therefore, the joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives approved the use of aspartame, to which an acceptable daily intake of 0–40 mg/kg body weight was allocated (7).

Aspartame received regulatory clearance in more than 60 countries and should be labeled properly. The European Community recently published a proposal for a Directive (8) that set the maximum permitted level of aspartame in various foods.

Many analytical procedures were proposed to check food labeling or to measure the degree of aspartame decomposition during manufacturing and storage of food products. Spectrophotometric (9, 10), enzymatic (11, 12), titrimetric (9), capillary isotachophoretic (13, 14), thin-layer chromatographic (15), and gas chromatographic (16) methods were described, but liquid chromatography (LC) is by far the most frequently used technique. Elution is usually performed on a reversed-phase column with a mobile phase of phosphate buffer–acetonitrile at low pH (17–31). Variations in the pH and/or the acetonitrile content of the mobile phase greatly influence the retention times of aspartame and its decomposition products (19, 20). In the preceding study to this article, we confirmed these observations and found that similar LC conditions could be used for the determination of the sweetener acesulfam-K (31).

Most reported studies were limited to beverages (3, 20, 22, 25, 27, 32) and yogurts (21, 23, 30). Surprisingly, comparison of the results with the aspartame content declared on the label has rarely been published. In the present work, we determined aspartame and its major decomposition products in a large variety of commercial products. Whenever possible, the figures

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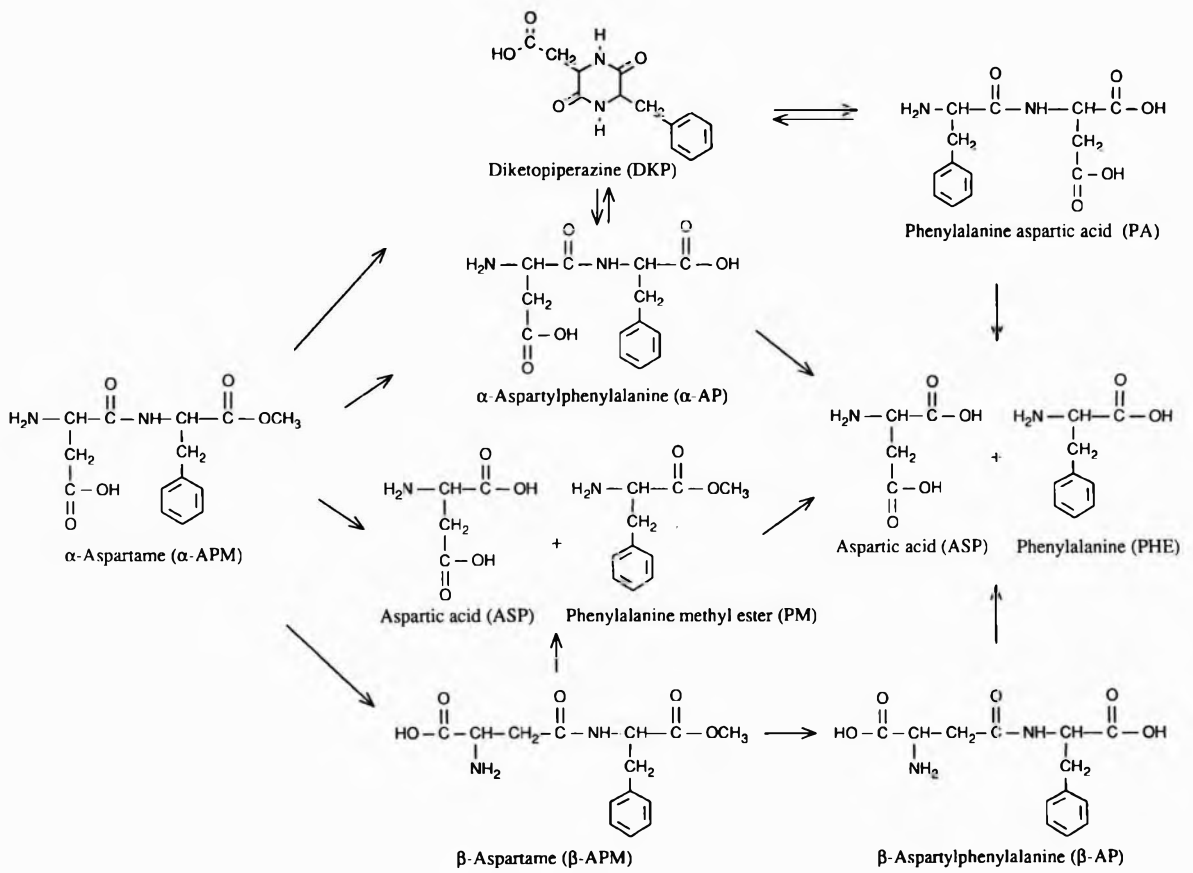

Figure 1. Decomposition products of aspartame (1–5).

were compared with the declared values. The applied LC conditions were derived from those previously established (31) for the separation of acesulfam-K from other sweeteners (including aspartame), food additives, and natural constituents (organic acids and alkaloids).

Experimental

Materials

The 24 samples analyzed in this study were all commercial products purchased in Switzerland and France.

Reagents

(a) *Solvents and chemicals.*—All chemicals were of analytical reagent grade. LC quality acetonitrile was purchased from Machler. Demineralized water was obtained from a Milli-Q system (Millipore).

(b) *Carrez I solution.*—3.6 g potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) was dissolved in water and diluted to 100 mL.

(c) *Carrez II solution.*—7.2 g zinc sulfate heptahydrate ($ZnSO_4\cdot 7H_2O$) was dissolved in water and diluted to 100 mL.

Table 1. Influence of acetonitrile concentration in the mobile phase on the capacity factors (k')[a]

	0.0125M KH_2PO_4 (pH 3.5)–acetonitrile				
Standard	(98 + 2)	(95 + 5)	(90 + 10)	(85 + 15)	(80 + 20)
Methanol	ND[b]	ND	ND	ND	ND
Aspartic acid	0	0	0	0	0
Phenylalanine	3.14	1.68	0.79	0.47	0.37
Phenylalanine aspartic acid	3.62	1.74	0.72	0.44	0.36
α-Aspartylphenylalanine	8.08	4.12	1.76	0.85	0.50
β-Aspartylphenylalanine	8.12	4.09	1.80	0.90	0.53
DKP	>17.5	11.0	3.85	1.74	0.91
Phenylalanine methyl ester	>17.5	10.1	3.85	1.83	1.04
β-Aspartame	>17.5	>17.5	8.62	3.67	1.64
α-Aspartame	>17.5	>17.5	8.72	3.69	1.65

[a] $k' = (t_R - t_o)/t_o$; t_o values: 3.29 min (98 + 2), 3.26 min (95 + 5), 3.23 min (90 + 10), 3.13 min (85 + 15), and 3.21 min (80 + 20).
[b] ND, not detected at 214 nm.

(d) *Mobile phase.*—0.0125M KH_2PO_4 buffer (pH 3.50)–acetonitrile. Phosphate buffer was made up of 0.17% KH_2PO_4 solution precisely adjusted to pH 3.50 with 5% phosphoric acid. The buffer and acetonitrile were filtered separately through 0.45 μm filter (Millipore), precisely mixed in the desired proportions (with pipets), and degassed 5 min in an ultrasonic bath. The mobile phase was maintained under a slight overpressure of helium (avoid bubbling) during analyses and was prepared daily.

(e) *Standard solutions.*—1 mg/mL aqueous stock solutions were made up for *N*-L-α-aspartyl-L-phenylalanine-1-methyl ester, L-phenylalanine, L-phenylalanine-1-methyl ester hydrochloride (Fluka); 3,6-dioxo-5-(phenylmethyl)-2-piperazine-acetic acid (diketopiperazine, DKP), *N*-L-βaspartyl-L-phenylalanine-1-methyl ester, *N*-L-phenylalanine-L-aspartic acid (Bachem Feinchemikalien); *N*-L-α-aspartyl-L-phenylalanine (Sigma); L-aspartic acid (Serva); and methanol (Merck). Stock solutions were further diluted as required for LC analysis.

(f) *β-Aspartylphenylalanine solution.*—100 mg β-aspartame was hydrolyzed in 100 mL 0.1N hydrochloric acid at 80°C for 2.5 h. Complete hydrolysis was not required, because the solution was only used to determine the capacity factor and the retention time of β-aspartylphenylalanine.

Apparatus

(a) *Liquid chromatograph.*—Model 420 pump (Kontron); manual injector (Rheodyne) fitted with a 10 μL loop; Model 480 column oven (Kontron); Model 480 oven controller (Kontron); Model ERC-7215 absorbance detector (Erma) set at 214 nm; Model 450 computing integrator (Kontron).

(b) *LC column.*—μBondapak-C_{18} (10 μm, 300 × 3.9 mm) reversed-phase column (Waters); Guard-Pak μBondapak-C_{18} (10 μm) precolumn (Waters).

(c) *Chromatographic conditions.*—Column temperature, 25°C; flow rate, 0.8 mL/min; and detector attenuation, 0.2 AUFS.

Sample Preparation

(a) *Tabletop sweeteners.*—Samples were dissolved in water to yield an aspartame concentration of 20–100 μg/mL. All solutions were filtered through 0.2 μm filters (Sartorius) before 10 μL injection into the chromatograph.

(b) *Liquid beverages.*—Soft drinks were first degassed. An aliquot of the sample was diluted to yield an aspartame concentration of 20–100 μg/mL. Undissolved matter, if any, was eliminated by filtration through folded filter paper (Schleicher & Schuell 597 1/2). Highly colored solutions were decolorized by passing through a C_{18} disposable cartridge (Supelclean LC-18, Supelco). All diluted solutions were filtered through 0.2 μm filters before injection.

(c) *Creams, yogurts, and fruit pulps.*—A quantity of homogenized sample was accurately weighed into a 100 mL volumetric flask to yield an aspartame concentration of 20–100 μg/mL. About 60 mL water was added. The flask was shaken vigorously and placed in an ultrasonic bath for 10 min. After dilution to 100 mL with water, the solution was centrifuged 5 min at 1600 *g*. The supernatant was filtered through 0.2 μm filter before injection.

(d) *Candies and powdered beverages.*—A quantity of homogenized sample was accurately weighed into a 100 mL volumetric flask to yield an aspartame concentration of 20–100 μg/mL. About 70 mL water was added, and the sweetener was extracted 30 min at 40°C. The solution was cooled to room temperature, diluted to 100 mL with water, and filtered through folded filter paper. The filtrate was passed through 0.2 μm filter before injection.

(e) *Other products.*—A quantity of homogenized sample was accurately weighed into a 100 mL volumetric flask to yield an aspartame concentration of 20–100 μg/mL. About 70 mL water was added, and the sweetener was extracted 30 min at 40°C. The flask was then placed in an ultrasonic bath for 10 min. The solution was cooled to room temperature, and 5 mL Carrez I solution and 5 mL Carrez II solution were successively added. The flask was shaken vigorously and diluted to 100 mL with water. The solution was filtered through folded filter paper. The filtrate was passed through 0.2 μm filter before injection.

Procedure

Two 10 μL volumes of standard and sample solutions were injected. Results were calculated according to the following formula:

$$100 \times (Co/Cs) \times (As/Ao)$$

where Co = concentration of aspartame or decomposition product in standard solution (mg/100 mL); Cs = concentration

Table 2. Recovery of aspartame added to various products[a]

Product	Added level, mg/100 g	Rec., %
Fruit candy	10	99.9
	20	102.0
	40	105.0
Diet cola	10	104.0
	15	100.4
	20	104.2
Vanilla cream powder	100	97.6
	200	98.3
	400	96.1
	600	98.0
Strawberry yogurt	10	96.3
	20	100.2
	30	99.5
	40	96.5
Milk chocolate	20	101.4
	30	101.6
	40	102.1
	50	103.2

[a] Mobile phase: 0.0125M KH_2PO_4 (pH 3.50)–acetonitrile (85 + 15).

Table 3. Analysis of aspartame and its major decomposition products in commercial foods[a]

	Aspartame			DKP		Aspartylphenylalanine		Phenylalanine	
Food	Declared	Found	Total	Found	α-APM equiv.	Found	α-APM equiv.	Found	α-APM equiv.
Tabletop sweetener	18 mg/tablet	18.1	18.2	0.1	0.1	—	—	—	—
Peppermint	30.2 mg/100 g	28.0	28.0	—	—	—	—	—	—
Fruit candy	45.3 mg/100 g	42.6	42.6	—	—	—	—	—	—
Spiced candy	3.6 mg/candy	3.8	3.8	—	—	—	—	—	—
Diet cola No. 1	29.7 mg/100 mL	28.6	28.9	0.3	0.3	—	—	—	—
Diet cola No. 2	ND[b]	15.3	23.6	4.4	4.9	2.6	2.7	0.4	0.7
Diet tonic	ND[b]	14.1	16.6	0.6	0.7	1.7	1.8	—	—
Ice tea	27 mg/100 mL	24.4	24.4	—	—	—	—	—	—
Ice coffee	20 mg/100 mL	19.4	19.4	—	—	—	—	—	—
Orange juice	ND[b]	18.3	18.9	0.2	0.2	—	—	0.2	0.4
Fruit beverage	24 mg/100 mL	23.6	24.0	0.2	0.2	—	—	0.1	0.2
Chocolate beverage	140 mg/100 g	122.5	127.7	0.3	0.3	3.6	3.8	0.6	1.1
Malted beverage	150 mg/100 g	117.0	151.6	5.0	5.6	—	—	16.3	29.0
Strawberry pulp	290 mg/100 g	269.6	284.6	6.1	6.8	6.5	6.8	0.8	1.4
Exotic fruit pulp	260 mg/100 g	241.6	253.3	4.8	5.4	4.0	4.2	1.2	2.1
Prune pulp	290 mg/100 g	259.8	273.3	6.9	7.7	4.0	4.2	0.9	1.6
Strawberry yogurt	39 mg/100 g	31.4	35.8	1.1	1.2	0.5	0.5	1.5	2.7
Exotic fruit yogurt	35 mg/100 g	31.5	35.7	0.6	0.7	0.8	0.8	1.5	2.7
Prune yogurt	35 mg/100 g	29.6	34.6	1.0	1.1	1.1	1.2	1.5	2.7
Vanilla cream	300 mg/23 g	274.2	296.1	19.5	21.9	—	—	—	—
Fruit cream	90 mg/100 g	53.7	84.8	9.7	10.9	18.1	19.0	0.7	1.2
Fresh cheese	50 mg/100 g	48.8	51.2	2.1	2.4	—	—	—	—
Muesli	38 mg/100 g	38.1	38.1	—	—	—	—	—	—
Milk chocolate	45 mg/100 g	33.5	42.1	2.8	3.1	2.2	2.3	1.8	3.2

[a] All results are average of duplicate determinations and are expressed in the same units as the declared values.
[b] ND, not declared; units are mg/100 mL.

of product in sample solution (mL or g/100 mL), *As* = peak area of product in sample solution, and *Ao* = peak area of aspartame or decomposition product in standard solution.

Results and Discussion

Choice of Mobile Phase

Previously, we evaluated an LC procedure for the determination of acesulfam-K in foods (31). Chromatographic conditions were found for the optimum separation of the sweetener from different food additives and natural constituents. Under these conditions, aspartame and its major decomposition products were also well-resolved from each other and from the compounds considered in the study. We have extended the evaluation of the method to all aspartame breakdown products so far identified (Figure 1). The pH of the phosphate buffer was kept at 3.5, and the detection wavelength was changed to 214 nm for better sensitivity (see Table 3, ref. 31). However, the proportion of acetonitrile in the mobile phase was varied to find the optimum separation conditions. Results are shown in Table 1.

Except aspartic acid (not retained by the column) and methanol (not detected at 214 nm), the capacity factors and retention times of all standards decreased rapidly as the amount of acetonitrile increased. Four different pairs of products could be distinguished that separated very well from each other, regardless of mobile phase composition. However, separation within the pairs phenylalanine (PHE)/phenylalanine aspartic acid, α-aspartylphenylalanine (α-AP)/β-aspartylphenylalanine, diketopiperazine (DKP)/phenylalanine methyl ester, and α-aspartame (α-APM)/β-aspartame was never achieved. This means that aspartame and its 3 detectable major decomposition products (α-AP, DKP, and PHE) are each coeluting with a minor decomposition product. In this study, the corresponding peaks in the chromatogram were attributed to the former compounds only.

To select the proper working conditions, the capacity factors in Table 1 were also compared with those given for other food additives and constituents (see Table 1, ref. 31).

In all cases, aspartame was well-separated from the possible interfering substances studied. A mobile phase consisting of 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (80 + 20) was suitable for a very rapid analysis of the sweetener alone but would have precluded the determination of its decomposition products in acesulfam-K-, saccharin-, caffeine-, or cocoa-containing foods. Quantitation of both α-APM and DKP could be achieved in reasonable time using the (85 + 15) and (90 + 10) media. Nevertheless, several compounds still interfered with α-aspartylphenylalanine and phenylalanine, particularly in

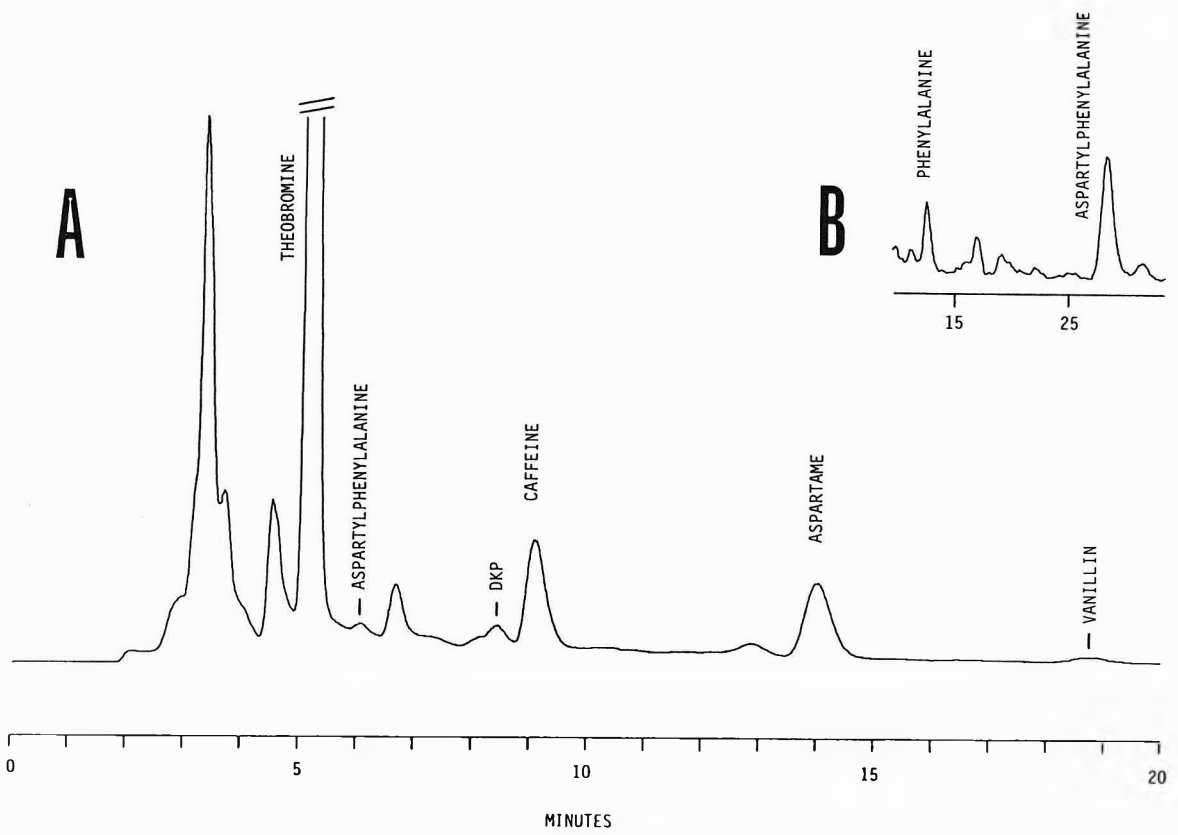

Figure 2. Chromatogram of a powdered chocolate beverage with mobile phase (A) 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (85 + 15) and (B) 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (98 + 2).

complex foods. These 2 products were, however, very well-resolved from all standards with the (98 + 2) mixture.

Therefore, the proper mobile phase depends mainly on the analytes and on the complexity of the food matrix. In this study, we choose the medium (85 + 15) as the starting chromatographic conditions for the analysis of aspartame and DKP and the medium (98 + 2) for the determination of α-AP and PHE. Because the capacity factors of all analytes are sensitive to the composition of the mobile phase, great care and precision are required in its preparation. Following these recommendations, reproducible retention times were obtained.

Linearity and Detection Limit

The linearity and the detection limit were assessed for aspartame only. With a mobile phase consisting of 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (85 + 15), a linear detector response (correlation coefficient, r = 0.9999) was obtained over aspartame concentrations ranging from 0.5 to 500 μg/mL. The detection limit, measured on a standard solution and defined arbitrarily as the amount of product on the column that produces a signal-to-noise ratio of 5, was about 1 ng sweetener.

Recovery Experiments

Table 2 presents recoveries of various amounts of aspartame added to fruit candy, diet cola, vanilla cream powder, strawberry yogurt, and milk chocolate samples. Percent recoveries ranged from 96.1 to 105.0% and were not influenced by the food matrix or the amount of sweetener added. Results obtained for complex foods showed that no loss of aspartame was induced by the extraction and the clarification steps.

Analysis of Foods

Table 3 shows results obtained for the analysis of aspartame in 24 different commercial diet foods. In 66.7% of the samples, the level of sweetener found accounted for more than 90% of

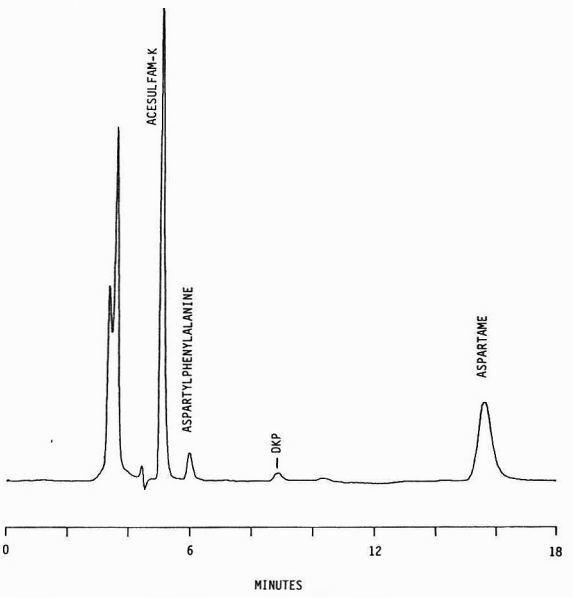

Figure 3. Chromatogram of a diet tonic.

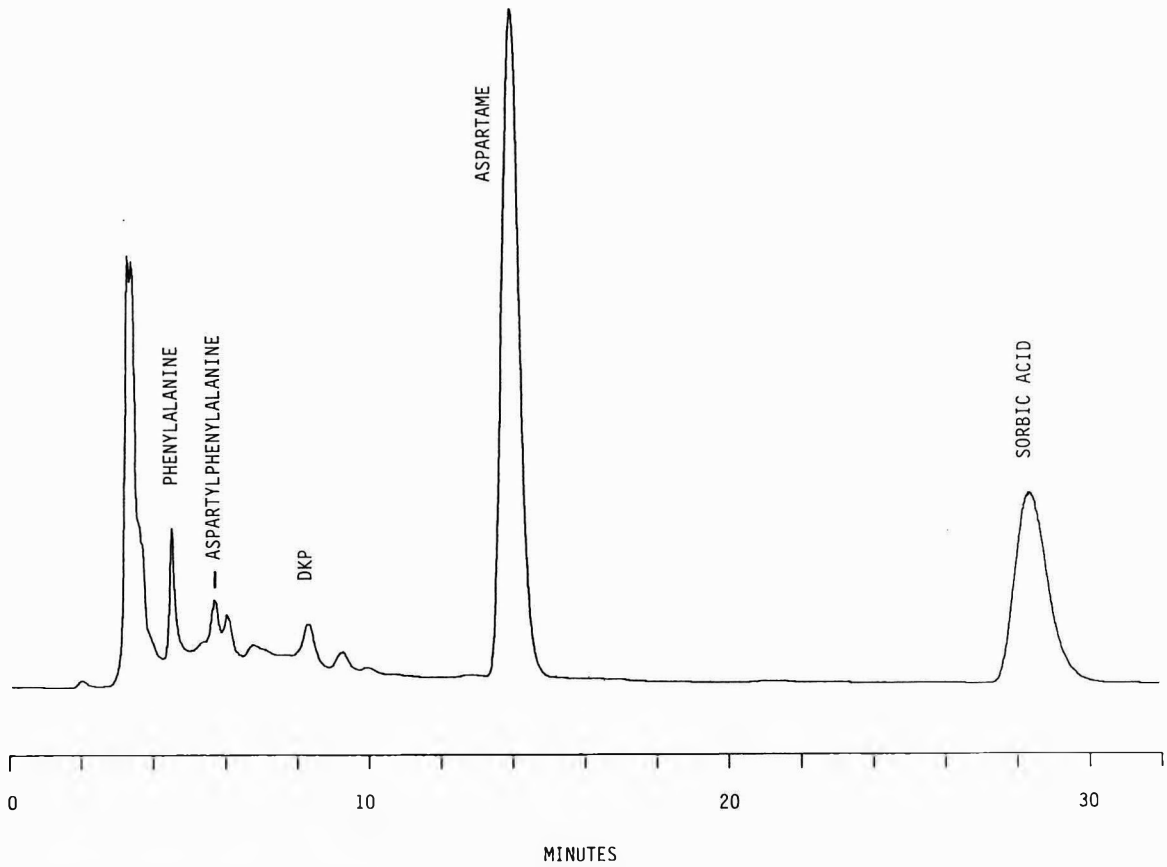

Figure 4. Chromatogram of a strawberry pulp.

the claimed value. However, DKP, aspartylphenylalanine, and phenylalanine were observed in most foods. The formation of these compounds was attributed to aspartame degradation only, assuming that their natural content in food was negligible. When the measured amounts of α-APM and decomposition products, expressed on a molar basis in aspartame (α-APM) equivalents, were summed up, the resulting total aspartame values were within −10% and +5% of those declared on the labels.

α-APM decomposition was particularly high for fruit cream (40%), milk chocolate (26%), malted beverage (22%), and fruit yogurt (15%). Fruit yogurt samples were prepared by the addition of 13% aspartame-containing fruit pulp after fermentation of milk. Interestingly, the amounts of DKP and α-AP found in the yogurts approximately correspond to those expected from the presence of 13% of the corresponding fruit preparations. This suggests that no further thermal or chemical decomposition of aspartame occurred in the finished product. However, levels of phenylalanine measured in yogurts were much higher than expected if they originated only from the fruit preparations. This could be explained by a postfermentation, biological degradation of aspartame by the yogurt cultures that had not reached a stationary phase (21).

The repeatabilities (r) and the repeatability coefficients of variation (CV_r) for the determination of aspartame, calculated from the differences between the duplicates, were, respectively, 1.00 mg/100 g and 1.34% for products containing less than 45 mg/100 g sweetener and 4.11 mg/100 g and 0.91% for the other products.

Different sample preparations were applied, depending on the complexity of the food matrix. Tabletop sweeteners were simply dissolved in water and injected into the chromatograph. An extraction step was included for the analysis of candies and powdered beverages. Figure 2 shows chromatograms of a powdered chocolate beverage containing vanillin. With 15% acetonitrile in the mobile phase, aspartame separated from natural cocoa alkaloids (theobromine and caffeine) and added vanillin (Figure 2A). The presence of DKP and aspartylphenylalanine indicated some degree of aspartame decomposition. A precise quantitation of α-AP and phenylalanine was possible only by reducing the amount of acetonitrile to 2% (Figure 2B).

When needed, liquid beverages were degassed, filtered, and diluted before analysis. A chromatogram of a diet tonic containing acesulfam-K and aspartame is given in Figure 3. The 2 artificial sweeteners were well-resolved from DKP and aspartylphenylalanine.

Creams, yogurts, and fruit pulps required an extraction step and a separation of the extract from the solid matter by centrifugation. Figures 4 and 5 show chromatograms of a strawberry pulp sample and a prune yogurt, respectively. In each case, sorbic acid, which was added as a preservative, and the 3 major aspartame breakdown products could be detected in the same run. As above, quantitation of α-AP and PHE had to be performed with a mobile phase containing only 2% acetonitrile (Figure 5B).

Finally, the other products needed a clarification step with Carrez solutions. We found that unknown compounds eluted

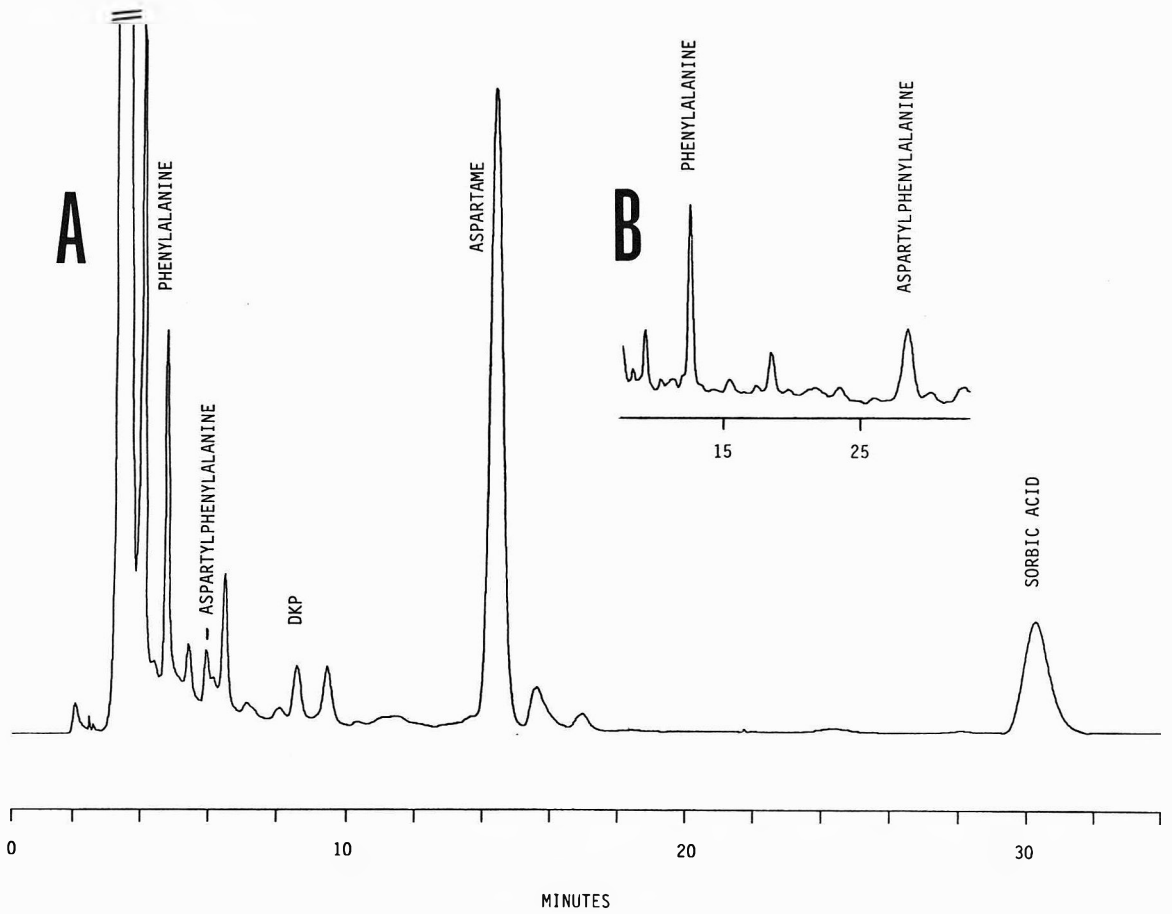

Figure 5. Chromatogram of a prune yogurt with mobile phase (A) 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (85 + 15) and (B) 0.0125M KH_2PO_4 (pH 3.5)–acetonitrile (98 + 2).

very close to aspartame and DKP in some chocolate samples when phosphate buffer–acetonitrile (85 + 15) was used. These peaks were separated when reducing the proportion of acetonitrile to 10%.

Conclusions

The LC procedure evaluated in a preceding study for the determination of acesulfam-K is also applicable to the analysis of aspartame and its major decomposition products in all types of foods. Sample preparation is straightforward, and elution is performed isocratically on a reversed-phase column. The choice of the amount of acetonitrile in the mobile phase depends on the analytes and on the complexity of the food matrix. Good recoveries of aspartame were obtained, and this result indicated no loss of the sweetener during sample preparation. Various degrees of aspartame decomposition were found in most of the commercial food samples analyzed in this study. However, the total aspartame values (measured aspartame + breakdown products) were close to the declared levels.

The technique also allows the detection of other food additives or natural constituents in the same run without interfering with aspartame or its decomposition products. Furthermore, the method is precise and sensitive. It is well-adapted for quality control or monitoring.

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CHEMICAL CONTAMINANTS MONITORING

Organochlorine Pesticide Residues in Dairy Milk in and around Delhi

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A survey of dairy milk and milk products in and around Delhi, India, was undertaken to monitor the levels of DDT and HCH. The survey revealed that pesticide residue levels in milk often exceeded the limits recommended by the Food and Agriculture Organization/World Health Organization Expert Committee. Maximum residue levels of total HCH in milk (fat/whole milk basis) has not yet been prescribed. The 15 samples of dairy milk and 4 samples of milk products analyzed had residue levels ranging from 0.022 to 0.166 μg/g for HCH and from 0.042 to 0.382 μg/g for DDT.

The problem of environmental contamination by persistent chlorinated hydrocarbons evokes major concern due to the presence of their residues in the environment and human tissues. Pesticide residues in food and animal feed are of interest because pesticides enter the human system through direct consumption of contaminated food or through milk, meat, and other products obtained from animals that feed on contaminated feed and fodder.

In developing countries, organochlorines such as DDT (1,1,1-trichloro-2, 2-bis-(4-chlorophenyl)ethane) are extensively used in malaria control programs. DDT was first introduced in India in 1948, and the current production is 8000 tons/year. It is manufactured solely by Hindustan Insecticides Ltd (HIL) (1). Reports (2, 3) indicate the buildup of DDT residues in soil and in earthworms in the vicinity of the HIL factory, which is located in a metropolitan area. This buildup is attributed to volatilization and consequent dispersal of DDT. However, DDT residues decreased with increasing distances from the factory (2).

Another organochlorine, HCH (1,2,3,4,5,6-hexachlorocyclohexane), was first introduced in India in 1949 for the control of agricultural pests. The present production is 36 000 tons/year (4). About 30 000 tons of HCH is used in agriculture alone each year; the remaining 6000 tons is used in public health programs. Commercial HCH contains only 13% lindane, the active ingredient (HCH gamma isomer).

Prolonged and uncontrolled use of organochlorines coupled with poor enforcement of related regulations have contributed to the widespread presence of pesticide residues in food commodities.

Typical contaminants of milk are persistent fat soluble organochlorine pesticides such as DDT, hexachlorobenzene (HCB), HCH isomers, and, to a lesser extent, the cyclodiene compounds.

Attempts have been made to ensure that the residues are kept at a minimal level so that the health risk due to their ingestion is minimized, if not eliminated. This requires permissible levels in food. Also, food must be monitored to ensure that the legal limits prescribed for the pesticide on different commodities are not exceeded. A survey of dairy milk and milk products was undertaken to monitor the levels of DDT and HCH.

Experimental

Apparatus

(a) *Gas chromatographs.*—(*1*) Varian 3400 equipped with ^{63}Ni electron capture detector. Operating conditions: column temperature, programmed from 175° to 220°C at 5°C/min; injector port, 220°C; detector, 250°C; column was held at initial temperature (175°C) for 8 min and then at 220°C for 10 min; nitrogen flow rate, 30 mL/min. (2) Hewlett Packard 5890 Series II with electron capture detector. Operating conditions: column temperature, programmed from 155°C, held at this temperature for 5 min and then raised to 210°C at 10°C/min; injector port, 250°C; detector, 300°C; nitrogen flow rate, 20 mL/min.

(b) *Chromatographic columns.*—(*1*) Glass, 2 m×2 mm id, packed with 1.5% OV-17 and 1.95% OV-210 on 80–100 mesh Chromosorb W. (2) HP1 (U.S. patent No. 4,293415, methyl silicone gum) megabore column, 10 m × 0.53 mm id and 2.65 μm film thickness.

(c) *Centrifuge.*—Model R 8 C, with 15 mL centrifuge tube (Remi Laboratory).

(d) *Separatory funnel.*—Pear-shaped, BOROSIL, 250 mL.

Reagents

(a) *Solvents.*—Hexane (Qualigens) and acetone (Merck). Distilled before use.

(b) *Sulfuric acid.*—Density 1.835 g/mL at 20°C (BDH India).

(c) *Water.*—Distilled-in-glass.

(d) *Anhydrous sodium sulfate.*—BDH India.

(e) *Groundnut oil.*—Commercial sample (Postman).

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Table 1. HCH and DDT residues in whole milk samples from different sources in Delhi

No.	Sample source	Frequency	HCH residues, μg/g				
			alpha	beta	gamma	delta	Total residues
1.	Dairy milk (Zone 1)	4	0.032 (0.009–0.084)	0.021 (0.008–0.040)	0.003 (0.002–0.005)	0.002 (0.001–0.003)	0.058
2.	Dairy milk (Zone 2)	3	0.069 (0.002–0.104)	0.007 (tr–0.012)	0.003 (tr–0.005)	0.001 (tr–0.002)	0.080
3.	Dairy milk (Zone 3)	3	0.042 (0.034–0.051)	0.016 (tr–0.024)	0.011 (0.003–0.026)	tr[a]	0.069
4.	Dairy milk (Zone 4)	3	0.069 (0.003–0.128)	0.012 (0.001–0.024)	0.002 (tr–0.003)	0.003 (tr–0.009)	0.086
5.	Buffalo milk	2	0.112 (0.022–0.202)	0.024 (0.013–0.034)	0.018 (tr–0.036)	0.012 (tr–0.023)	0.166
6.	Condensed milk	1	0.045 (0.030–0.075)	0.031 (0.016–0.056)	0.004 (0.003–0.005)	0.004 (0.001–0.009)	0.084
7.	Cheese	1	0.065 (0.061–0.070)	0.011 (0.008–0.017)	0.013 (0.009–0.019)	0.021 (0.019–0.023)	0.110
8.	Cream	1	0.017 (0.017–0.018)	ND	0.038 (0.036–0.040)	0.077 (0.071–0.083)	0.132
9.	Curd	1	0.018 (0.017–0.018)	ND	0.004 (tr–0.007)	tr	0.022
			DDT residues, μg/g				
			p,p'-DDE	*p,p'*-DDD	*p,p'*-DDT		
1.	Dairy milk (Zone 1)	4	0.018 (0.008–0.036)	0.045 (0.019–0.089)	0.134 (0.075–0.231)		0.197
2.	Dairy milk (Zone 2)	3	0.007 (tr–0.013)	0.044 (0.025–0.056)	0.053 (0.031–0.070)		0.104
3.	Dairy milk (Zone 3)	3	0.042 (0.038–0.046)	0.054 (0.032–0.075)	0.097 (0.032–0.139)		0.193
4.	Dairy milk (Zone 4)	3	0.023 (tr–0.044)	0.027 (0.006–0.058)	0.058 (0.016–0.098)		0.108
5.	Buffalo milk	2	0.050 (0.027–0.073)	0.037 (0.034–0.039)	0.133 (0.113–0.153)		0.220
6.	Condensed milk	1	0.052 (0.041–0.065)	0.009 (0.007–0.011)	0.127 (0.120–0.147)		0.188
7.	Cheese	1	0.040 (0.036–0.043)	0.072 (0.067–0.077)	0.233 (0.231–0.237)		0.345
8.	Cream	1	tr	0.149 (0.149–0.151)	0.233 (0.229–0.239)		0.382
9.	Curd	1	tr	0.010 (0.007–0.015)	0.032 (0.032–0.033)		0.042

[a] tr = <0.001, ND = nondetectable.

Sample Collection

Dairy milk was collected from different sources in and around Delhi, and samples were classified on the basis of 4 different zones from where they were obtained. Four samples were collected from Zone 1, 3 samples from Zone 2, 3 samples from Zone 3, and 3 samples from Zone 4 as indicated under the frequency column of Table 1. Buffalo milk samples from 2 different zones were also analyzed.

Milk products (cheese, cream, and curd) were prepared from a dairy milk sample. Condensed milk was obtained from the market.

Extraction and Cleanup

Each experiment was performed in triplicate. The density of each milk sample was found by weighing an aliquot of the liquid. Milk (20 mL) was transferred to a clean glass-stoppered cylinder, 80 mL hexane-acetone (1 + 1, v/v) was added, and the mixture was shaken vigorously. A colloidal mass separated out. The supernatant organic solvent was decanted off and stored. The colloidal mass was shaken thoroughly 3 times with 30 mL hexane, and the solvent decanted off and combined. The precipitate was centrifuged 2 min at 2000 rpm, and the remaining solvent was also combined. The total hexane extract was transferred to a separatory funnel, and the solution was cleaned up by the dropwise addition of 35 mL concentrated sulfuric acid. The hexane layer was washed 3 times with 50 mL distilled water, dried over anhydrous sodium sulfate, concentrated to 10 mL, and analyzed. Milk products were weighed and processed similarly (as given above) for analysis.

Analysis of Organochlorine Pesticides

Determination of HCH and DDT residues was carried out by gas chromatography on the Varian 3400. The retention times for *p,p′*-DDE, DDT, and DDD were 14.28, 16.55, and 16.60 min, respectively. The alpha, gamma, beta, and delta isomers of HCH eluted at 4.68, 5.99, 7.32, and 8.15 min, respectively.

The average recovery of the procedure conducted by overspiking non-blank milk samples was 80% for *p,p′* isomers of DDT, DDE, and DDD at 0.5 μg/g fortification level. The average recovery of the alpha, beta, gamma, and delta isomers of HCH from spiked samples of milk at 0.5 μg/g levels was 90%. In the absence of a control sample of milk without HCH or DDT residues, groundnut oil was spiked at 0.1 and 0.5 μg/g levels with *p,p′*-DDT and then extracted and cleaned up to find the efficiency of the procedure. The average recovery of the method was 83.6 ± 0.08%. In short, recoveries were more than 80% for DDT and its metabolites from milk and groundnut oil and more than 89% for HCH and its stereoisomers from milk.

The limit of detection was 0.005 μg/g for *p,p′*-DDT, DDE, and DDD and 0.002 μg/g for alpha HCH, 0.004 μg/g for beta HCH, 0.0008 μg/g for gamma HCH, and 0.0008 μg/g for delta HCH.

The identity of the pesticides was confirmed by carrying out the analysis on the Hewlett Packard 5890 Series II using electron capture detector and alternate column packing. The retention time of the alpha, beta, gamma, and delta isomers of HCH were 2.87, 3.08, 3.43, and 3.65 min, respectively. The *p,p′*-DDE, *p,p′*-DDD, and *p,p′*-DDT eluted at 10.54, 12.09, and 15.20 min, respectively, under the above temperature conditions.

Results and Discussion

All samples of dairy milk analyzed contained HCH and DDT. Total HCH ranged from 0.058 to 0.166 μg/g, and DDT residues ranged from 0.104 to 0.220 μg/g. Average, minimum, and maximum values of different isomers recorded from each zone are given in Table 1. A dairy milk was processed, and cheese, cream, and curd were also estimated for HCH and DDT content. Curd contained only 0.042 μg/g of total DDT compared to cheese and cream, which recorded a total of 0.345 and 0.382 μg/g of DDT, respectively. Total HCH was significantly higher in processed milk, cream and cheese, than in original dairy milk. Cream recorded the highest HCH levels of 0.132 μg/g. DDT residues exceeded the extraneous residue limit of 0.05 μg/g (whole milk basis) in all the dairy milk samples.

Most dairy milk samples contained HCH residues (alpha, beta, gamma. and delta). Of all HCH isomers, the residue limit was established only for lindane, which is 0.004 μg/g (whole milk basis). Among the dairy milks, only 4 samples contained gamma HCH above this limit. Alpha HCH was found to be invariably higher than gamma HCH, which is found in the original HCH formulation used commercially in India. Total HCH residues were found to be highest in buffalo milk (0.166 μg/g). Gamma HCH content was highest in cream (0.038 μg/g) in contrast to cheese (0.013 μg/g) and curd (0.004 μg/g). The maximum residue level of total HCH in milk (whole milk/fat basis) is not yet established.

Total HCH and DDT residues had coefficients of variation of 28.4 and 28.9%, respectively, in 5 types of milk samples and 41.8 and 45.3%, respectively, in all 19 samples. The respective values for Zones 1, 2, 3, and 4 were 55.8, 84.5, 14.1, and 87.0 for HCH and 45.6, 25.5, 41.2, and 71.1 for DDT.

DDT, HCH, and their metabolites show consistent biomagnification in the upper trophic zone in food (5). There are reports of DDT and its metabolites in human blood samples (6, 7). Because milk is one of the major routes through which DDT and HCH enter the human system, it is essential to monitor the presence of these insecticides in milk at regular intervals.

Acknowledgment

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COLOR ADDITIVES

Liquid Chromatographic Determination of 2-Chloro-4-nitroaniline, 2-Naphthol, and 2,4-Dinitroaniline in D&C Red No. 36

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A method is described for the determination of the intermediates and a related impurity in D&C Red No. 36 by reversed-phase liquid chromatography. This method may be used to ensure that limits set forth in the *Code of Federal Regulations* on the amounts of these 3 impurities in the color are not exceeded. The pigment is dissolved in boiling dioxane and then precipitated. The filtrate is chromatographed by isocratic elution, and then the column is washed and reequilibrated. Impurities were identified as 2-chloro-4-nitroaniline (2-Cl-4-NA), 2-naphthol, and 2,4-dinitroaniline (2,4-DNA) by comparison of their retention times and spectra with those of standards. Peak area calibrations were linear to at least 0.375% 2-Cl-4-NA, 1.25% 2-naphthol, and 0.025% 2,4-DNA, all with zero intercepts. At the specification levels, 99% confidence limits were 0.30 ± 0.006% for 2-Cl-4-NA, 1.0 ± 0.03% for 2-naphthol, and 0.020 ± 0.0004% for 2,4-DNA. The limits of determination calculated from calibration data were 0.019% for 2-Cl-4-NA, 0.10% for 2-naphthol, and 0.0014% for 2,4-DNA at the 99% confidence level. Recoveries were 100–104% for 2-Cl-4-NA added to purified D&C Red No. 36, 100% for 2-naphthol, and 100–110% for 2,4-DNA; relative standard deviations were 0.8–3.4%. A survey of certified D&C Red No. 36 samples showed that the batches contained higher levels of intermediates than were determined previously by a cellulose column method in which the pigment was not dissolved.

D&C Red No. 36 (Figure 1, Colour Index No. 12085, CAS No. 2814-77-9, 1-[(2-chloro-4-nitrophenyl)azo]-2-naphthalenol), is an azo pigment that is manufactured by diazotization of 2-chloro-4-nitroaniline (2-chloro-4-nitrobenzenamine, 2-Cl-4-NA) and coupling with 2-naphthol (2-naphthalenol) (1). The color may be used in the United States in drugs and cosmetics (except for those used in the area of the eye) after the U.S. Food and Drug Administration (FDA) certifies that each batch of the color additive meets specifications published in the *Code of Federal Regulations*, including limits of 0.30% for 2-Cl-4-NA, 1.0% for 2-naphthol, and 0.020% for 2,4-dinitroaniline (2,4-dinitrobenzenamine; 2,4-DNA) (2).

The intermediates, 2-Cl-4-NA and 2-naphthol, were previously quantitated in D&C Red No. 36 by FDA with a method that is a modification[1] of a general procedure (3) for the determination of intermediates in FD&C colors. The pigment is mixed with 95% ethanol, water, and cellulose, and the mixture is transferred to a cellulose column. The intermediates are eluted from the column with ammonium sulfate in aqueous ethanol and quantitated from the absorption spectra of fractions of the eluate. The cellulose column method was also used for determination of intermediates in a related pigment, the former D&C Orange No. 17; however, reversed-phase liquid chromatographic (LC) analysis of the color showed that extraction of the intermediates from the color in the cellulose column method was incomplete (4).

Consequently, an LC method for the determination of the intermediates in D&C Red No. 36 was investigated. In addition to the 2 intermediates, 2,4-DNA was identified as an impurity in certain batches of the color by comparison of the retention times and spectra of the impurities with those of standards. The 2,4-DNA may be an impurity in 2-Cl-4-NA, or it may be produced by substitution of the chlorine in 2-Cl-4-NA with nitrite during diazotization, or both. These identifications were confirmed by gas chromatographic/mass spectrometric analysis of a 95% ethanol extract of certified D&C Red No. 36. The LC method that was previously used to determine 2-naphthol and 2,4-DNA in D&C Orange No. 17 (4) could not be used to analyze D&C Red No. 36, because the chromatographic peaks for 2-Cl-4-NA and 2-naphthol overlapped. Therefore, a modification of this LC method was developed and is reported here.

The test extract is prepared by boiling a mixture of 50 mg color (or laked color containing 50 mg pigment) and 8 mL dioxane in a 15 mL beaker to dissolve the color. The solution is cooled in ice, and the resulting mixture is warmed to room temperature. This procedure dissolves the impurities and removes most of the color. The mixture is transferred to a 10 mL volu-

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[1] A copy of the modified method can be obtained from the U.S. Food and Drug Administration, Office of Cosmetics and Colors, HFS-126, Washington, DC 20204.

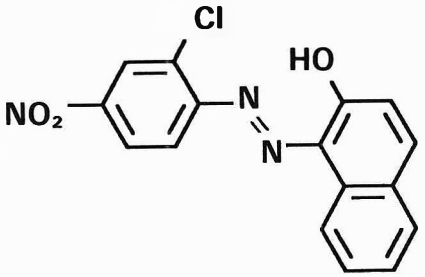

Figure 1. D&C Red No. 36.

metric flask and diluted to volume with dioxane. The mixture is filtered through a membrane filter. The filtrates of the color, dioxane blanks, and standard dioxane solutions of the intermediates and 2,4-DNA are chromatographed with detection at 254 nm for 2-naphthol and 2-Cl-4-NA and at 340 nm for 2,4-DNA (near its λ_{max}, 344 nm). Detection at 340 nm for 2-Cl-4-NA is also feasible. Calibration lines (area = slope × equivalent % analyte) are calculated by least squares linear regression from the peak areas in chromatograms of the blank and standard solutions. The concentrations of the intermediates and 2,4-DNA in the color are calculated from the calibration lines and the peak areas in chromatograms of the filtrates of D&C Red No. 36.

Experimental

Apparatus

(**a**) *Syringe.*—10 mL poly(ethylene) and poly(propylene); Aldrich "all-poly" (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(**b**) *Syringe filter unit.*—25 mm diameter, 0.5 μm pore, poly(tetrafluoroethylene) membrane filter with poly(ethylene) housing; Millipore Millex-SR (Millipore Corp., Bedford, MA 01730).

(**c**) *Liquid chromatograph.*—With continuous gradient capability. A system that can switch between 2 eluants may be used, but making minor changes in eluant strength will be difficult. (*1*) *Pump and autoinjector controller, and data collector and analyzer.*—Waters Model 860 with LAC/E interface and VAX 3300 computer equipped with version 2.2 software. (*2*) *Pump.*—Waters 600E at 2 mL/min. (*3*) *Injector.*—Waters Model 712 WISP autoinjector set at 25 μL. (*4*) *Detector.*—Waters Model 490E UV-vis detector set at 254 and 340 nm and 1 AU/V (Waters Chromatography Division, Millipore Corp., Milford, MA 01757).

(**d**) *LC column.*—Waters RCM-C18, 10 μm particle size, 100 × 8 mm id, in radial compression unit; used at ambient temperature.

(**e**) *Guard column.*—Waters 2.4 × 25 mm guard cartridge containing Waters Bondapak C18 Corasil, 37–50 μm particle size packing.

Table 1. Preparation of working solutions for LC determination of intermediates and 2,4-DNA in D&C Red No. 36

	Volume of standard solution, mL		
Flask	Stock solution of 2-Cl-4-NA	Stock solution of 2-naphthol	Intermediate solution of 2,4-DNA
1	1	2	4
2	4	5	2
3	3	4	1
4	5	1	5
5	2	3	3

Reagents

(**a**) *Water for LC eluants.*—LC grade (Burdick & Jackson Laboratories Inc. [B&J], Muskegon, MI 49442).

(**b**) *Methanol.*—LC grade (B&J or J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(**c**) *Ammonium acetate.*—LC grade (Fisher Scientific Co., Pittsburgh, PA 15219).

(**d**) *2-Cl-4-NA.*—Recrystallized from aqueous alcohol, (79 g in 1800 mL ca 95% ethanol–water [1 + 2.5]). Analytically pure (±0.4% for C, H, and N).

(**e**) *2-Naphthol.*—Fisher certified recrystallized reagent or Fluka puriss (Fluka Chemical Corp., Ronkonkoma, NY 11779–7238).

(**f**) *2,4-DNA.*—Recrystallized from aqueous alcohol (5). Analytically pure (±0.4% for C, H, and N).

(**g**) *1,4-Dioxane.*—Distilled-in-glass (B&J). Use only in hood; this compound is a suspected carcinogen. Test for peroxides with peroxide test strips (Cat. No. 10011-1, EM Science, Gibbstown, NJ 08027).

Solutions

(**a**) *LC eluants.*—(*1*) *Eluant A.*—Aqueous 0.2M ammonium acetate. Dissolve 15.417 g ammonium acetate in water and dilute to 1 L. (*2*) *Eluant B.*—Methanol.

(**b**) *Stock and intermediate standard solutions.*—(*1*) *2-Cl-4-NA stock solution.*—Accurately weigh ca 4.688 mg 2-Cl-4-NA, and transfer to 50 mL volumetric flask. (*2*) *2-Naphthol stock solution.*—Accurately weigh ca 15.625 mg 2-naphthol and transfer to 50 mL volumetric flask. (*3*) *2,4-DNA stock solution.*—Accurately weigh ca 15.625 mg 2,4-DNA, and transfer to 50 mL volumetric flask. Dissolve each compound in dioxane, and dilute each solution to 50 mL. (*4*) *2,4-DNA intermediate solution.*—Transfer 1 mL 2,4-DNA stock solution, (*3*), to 50 mL volumetric flask, and dilute to 50 mL with dioxane.

(**c**) *Working solutions.*—Transfer random combinations of 1, 2, 3, 4, or 5 mL aliquots of the 2-Cl-4-NA and 2-naphthol stock solutions and the 2,4-DNA intermediate solution, (**b**)(*1*), (**b**)(*2*), and (**b**)(*4*), to five 25 mL volumetric flasks. Dilute to 25 mL with dioxane. All 5 volumes of each analyte solution must be used (e.g., see Table 1). The working solutions contain the equivalent of 0.075–0.375% 2-Cl-4-NA, 0.25–1.25% 2-naphthol, and 0.005–0.025% 2,4-DNA in D&C Red No. 36 (referred to as "equivalent % analyte").

Table 2. Conditions for LC determination of intermediates and 2,4-DNA in D&C Red No. 36[a]

Time, min	Eluant A, %	Eluant B, %	Action
0	50	50	Inject, inhibit integration
2.9	—	—	Begin integration
10.8	50	50	Begin wash
10.9	0	100	—
11.8	—	—	End integration
13.0	0	100	End wash
13.1	50	50	Initial conditions
18.0	50	50	End run

[a] Flow rate, 2 mL/min.

Preparation of Sample Extracts

Accurately weigh ca 50 mg color or laked color containing ca 50 mg pigment into 15 mL beaker. Add small Teflon-coated stirring magnet (0.5 × 5/16 in.) and 8 mL dioxane, and cover beaker with watch glass. Boil gently with stirring for 5–7 min on preadjusted stirrer-hot plate. Cool 5–10 min in ice water with stirring as color and dioxane precipitate. Stir for 5 min in room-temperature bath; transfer mixture, by using Pasteur pipet, into 10 mL volumetric flask. Wash watch glass, magnet, beaker, and pipet with four 0.5 mL portions of dioxane. Transfer washings to volumetric flask, and dilute to volume with dioxane. Attach barrel of syringe to filter unit, and place outlet in autosampler vial. Pour mixture from volumetric flask into filter unit, insert plunger, and apply moderate pressure to filter.

Liquid Chromatography

Sparge eluants with helium, and set detector to 254 and 340 nm with LC conditions, as shown in Table 2. The effective analysis time is 19.2 min, because automatic injection takes 1.2 min. Prime and purge pumps and injection system. Wash column at 2 mL/min with 100% eluant B for 15 min and then with 50% eluant B for 6 min. Chromatograph 2 preliminary dioxane blanks.

For calibration, chromatograph the 5 working solutions and a dioxane blank. Plot peak area vs equivalent percentage for each analyte from chromatograms of standards and blank, and calculate slopes and intercepts of calibration lines (area = slope × equiv. % analyte + intercept) by least-squares linear regression. The intercepts should be indistinguishable from zero. Calculate the slopes of new calibration lines that are forced through the origin (area = slope × equiv. % analyte).

For analysis, chromatograph color filtrate (as shown in Figure 2) and dioxane blank. If analysis is not concurrent with calibration, prepare and chromatograph a working standard solution containing the 3 analytes at the specification levels or other level(s) of interest. The data from the chromatography of the confirmatory standard solution should be within the confidence limits of the calibration lines for an individual value. From the slopes of calibration lines forced through the origin and the peak areas of color filtrate, calculate the percent of each intermediate in the sample.

If this method was not used previously, prepare a test mixture containing 1 mL each of the 2-Cl-4-NA and 2-naphthol stock solutions and 2,4-DNA intermediate solution, (**b**)(*1*), (**b**)(*2*), and (b)(*4*), diluted to 50 mL with dioxane. Prepare 3 solutions containing individual analytes by separately diluting 1 mL each of the 2-Cl-4-NA and 2-naphthol stock solutions and 2,4-DNA intermediate solution, (**b**)(*1*), (**b**)(*2*), and (**b**)(*4*), to 10 mL with dioxane. Chromatograph the 4 test solutions followed by a dioxane blank. Identify the peaks, and confirm that they are well-separated and that 2-naphthol elutes before 10 min. Confirm that carryover is negligible and that the transient gradient induced by dioxane does not interfere with the peaks. If necessary, increase or decrease % eluant B to decrease or increase retention times of intermediates. (An increase of 2% eluant B decreased the retention time of 2-naphthol by 1.2 min.) Adjust detector attenuation for proper display at the level of interest, and adjust integration parameters for proper quantitation at the lowest level.

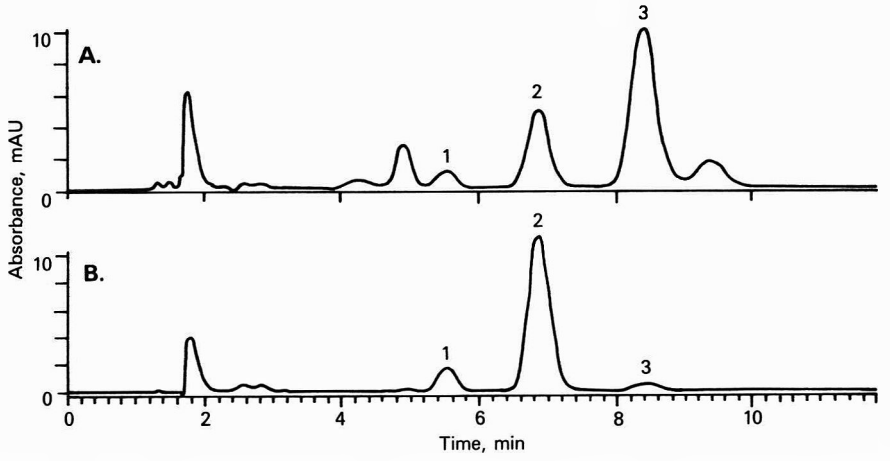

Figure 2. Chromatograms of filtrate of D&C Red No. 36 (sample 18, Table 4): A = 254 nm, B = 340 nm; 1 = 2,4-DNA, 2 = 2-Cl-4-NA, 3 = 2-naphthol.

Table 3. Recovery data for LC determination of intermediates in D&C Red No. 36[a]

Added, %	Found ± SD, %	Rec., %[b]	RSD, %
	2-Cl-4-NA		
0	0.0020 ± 0.0019	—	—
0.0377	0.0415 ± 0.0016	104	3.8
0.1509	0.1552 ± 0.0020	102	1.3
0.3019	0.3019 ± 0.0038	100	1.2
	2-Naphthol		
0	0 ± 0	—	—
0.1310	0.1307 ± 0.0012	100	0.9
0.5240	0.5230 ± 0.0045	100	0.8
1.048	1.050 ± 0.014	100	1.3
	2,4-DNA		
0	0.00016 ± 0.00022	—	—
0.00248	0.00288 ± 0.00010	110	3.4
0.00994	0.01049 ± 0.00036	104	3.4
0.01988	0.02007 ± 0.00029	100	1.4

[a] Quintuplicate analyses.
[b] Corrected for blank.

Recovery Studies

D&C Red No. 36 that contained 0.23% 2-Cl-4-NA, 0.68% 2-naphthol, and 0.12% 2,4-DNA was recrystallized once from dioxane (12.5 g/1600 mL). Five unfortified and 15 fortified recovery mixtures were prepared by adding 2-Cl-4-NA, 2-naphthol, and 2,4-DNA to recrystallized D&C Red No. 36. These 20 mixtures were extracted and filtered by following the procedure for the samples, and the filtrates were chromatographed in random order.

Spiking solutions.—The stock solutions of 2-Cl-4-NA and 2-naphthol and the intermediate solution of 2,4-DNA, (**b**)(*1*), (**b**)(*2*), and (**b**)(*4*), were used.

Recovery mixtures.—For each of 20 recovery mixtures, ca 50 mg recrystallized D&C Red No. 36 was accurately weighed into a 15 mL beaker, and a stirring magnet was added. Aliquots of 0.2, 0.8, or 1.6 mL of all 3 spiking solution were added by using adjustable pipettors and reversed-mode pipetting (overfill pipet tip and then expel set volume of liquid [to first stop]) to 15 of the beakers in random combination. Fortification levels are shown in Table 3. Volumes were adjusted to 8 mL with dioxane.

Results and Discussion

Calibration

The calibration solutions and blanks were chromatographed in random order. The peak areas were plotted vs the equivalent percentages of analytes, and the data were analyzed by least-squares linear regression (area = slope × equiv. % analyte + intercept). Plots of the data were linear with all correlation coefficients (R) ≥ 0.9995, and the intercepts were indistinguishable from zero at the 99% confidence level. Therefore, the data were reanalyzed by using least-squares linear regression with a zero intercept (area = slope × equiv. % analyte), and values of R ≥ 0.9997 were calculated. Limits of determination were 0.019% for 2-Cl-4-NA, 0.10% for 2-naphthol, and 0.0014% for 2,4-DNA at the 99% confidence level (6). At the specification levels, 99% confidence limits were 0.30 ± 0.006% for 2-Cl-4-NA, 1.0 ± 0.03% for 2-naphthol, and 0.020 ± 0.0004% for 2,4-DNA.

Recovery Studies

Recoveries (Table 3) from the analyses of solutions fortified at the specification levels were 100% each for 2-Cl-4-NA added at the 0.30% level, 2-naphthol added at the 1.0% level, and 2,4-DNA added at the 0.020% level. Recoveries at lower levels were 100–110%. If the amounts of 2-Cl-4-NA and 2,4-DNA in the unfortified color had been 0.0040 and 0.00042% instead of 0.0020 ± 0.0019% and 0.00016 ± 0.00022%, respectively, then all recoveries would have been 100%.

From the standard deviations (SDs) of the quintuplicate determinations at the lowest fortification levels shown in Table 3, the limits of determination were 0.0060% for 2-Cl-4-NA, 0.0046% for 2-naphthol, and 0.00037% for 2,4-DNA. These limits were calculated from the following equation:

$$c_L = 3.75 \times SD_{blank}$$

in which a 99% one-sided *t*-value was used instead of $k = 3$ (7). These limits are lower than the corresponding limits of determination of 0.019, 0.10, and 0.0014% that were calculated from calibration data. In other calibration experiments in which more data points were used, the limit of determination was 0.02% for 2-naphthol.

Survey

Thirty-two samples of certified D&C Red No. 36, consisting of colors and laked colors that included 3 samples from lots used for pharmacological testing, were analyzed by the LC method. Results of these analyses and those obtained during certification analysis by the cellulose column method are summarized in Table 4. All lots of D&C Red No. 36 were found to contain higher levels of intermediates by the LC method than by the cellulose column method. Because analyses of D&C Red No. 36 fortified with solutions of intermediates demonstrated satisfactory recoveries by the cellulose column method, the lower survey results for the cellulose column method are attributed to incomplete extraction of the intermediates from the solid color.

Conclusions

This LC method for the determination of intermediates and a related impurity in D&C Red No. 36 may be used to certify that the color additive meets published specifications for these analytes. Dissolution of the color additive in dioxane and analysis by reversed-phase LC is a significant improvement over the previously used cellulose column method in which the

Table 4. Survey of certified D&C Red No. 36 for intermediates and 2,4-DNA

Sample	Color, %	2-Cl-4-NA, %[a,b]	2-Naphthol, %[a]		2,4-DNA, %[a,c]
			LC	Cellulose column	
1	19[d]	0.03	0.06	0.03	0.000
2	22[d]	0.03	0.10	0.02	0.000
3[e]	97	0.04	0.21	0.18	0.000
4	99	0.13	0.25	0.11	0.000
5	97	0.09	0.26	0.14	0.000
6	97	0.13	0.28	0.19	0.002
7	99	0.03	0.29	0.08	0.000
8	97	0.01	0.29	0.04	0.001
9[e]	98	0.15	0.29	0.20	0.000
10	98	0.04	0.35	0.10	0.000
11	98	0.10	0.35	0.17	0.000
12[e]	97	0.20	0.39	0.15	0.000
13	97	0.04[b]	0.39	0.10	0.001
14	97	0.02	0.40	0.06	0.002
15	93[d]	0.06	0.41	0.10	0.000
16	100	0.03	0.42	0.15	0.000
17	97	0.13	0.42	0.12	0.001
18	97	0.14	0.42	0.10	0.011
19	98	0.13	0.44	0.14	0.000
20	96	0.07	0.44	0.18	0.000
21	97	0.02	0.46	0.12	0.000
22	98	0.15	0.48	0.14	0.000
23	98	0.05	0.48	0.12	0.000
24	98	0.08	0.52	0.16	0.000
25	97	0.19	0.66	0.20	0.016
26	97	0.16	0.68	0.20	0.033[f]
27	98	0.23	0.68	0.11	0.122[f]
28	96	0.07	0.69	0.20	0.000
29	97	0.21	0.72	0.16	0.021[f]
30	98	0.19	0.73	0.14	0.046[f]
31	99	0.07	0.75	0.20	0.000
32	97	0.03	0.82	0.15	0.000

[a] As % of sample.
[b] Results shown are for LC method; results for cellulose column method were 0%, except for sample 13 for which trace 2-Cl-4-NA was reported.
[c] Results shown are for LC method; 2,4-DNA was not determined by the cellulose column method.
[d] $BaSO_4$ lake.
[e] Pharmacology sample.
[f] Certified before the specification of 0.020% for 2,4-DNA was established.

color additive is not dissolved. The discrepancy between the flawed cellulose column method and this LC method illustrates that recovery studies may not be valid if the fortified samples are heterogeneous. To ensure validity, proof is needed that no analyte remains in the undissolved portion. The validity of analysis of D&C Red No. 36 lakes by this LC method needs to be confirmed; proof is needed that the laked analytes are quantitatively extracted from the laked color and substratum with boiling dioxane.

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COSMETICS

Specific Determination of Ethylene Oxide and Ethylene Chlorohydrin in Cosmetics and Polyoxyethylated Surfactants by Gas Chromatography with Electron Capture Detection

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A simple specific determination method was developed for ethylene oxide (EO) and ethylene chlorohydrin (ECH) in cosmetics and surfactants. EO is desorbed from samples by using a nitrogen stream and absorbed into acidic potassium iodide solution, where it is converted to ethylene iodohydrin (EIH). Any remaining ECH in the samples is converted to EO by the addition of sodium hydroxide, and the desorption procedure is repeated with a fresh acidic potassium iodide absorbing solution. EIH is extracted with benzene and determined by gas chromatography with electron capture detection. EO and ECH contents in the samples are calculated from EIH results. Recoveries from water and shampoo samples were 70.3 ± 5.4 and 58.9 ± 1.2%, respectively, for EO and 66.3 ± 4.0 and 64.5 ± 4.6%, respectively, for ECH. Detection limits in 0.2–2.0 g samples were in the 0.005–0.03 μg/g range for EO and 0.01–0.07 μg/g for ECH. High levels of EO (30–394 μg/g as ECH) were found in 5 of 18 polyoxyethylated surfactant samples, but only small amounts (0.07–4.0 μg/g) of ECH were detected in the samples. EO was not detected in cosmetic samples tested, but ECH was present in small quantities (≤1.11 μg/g).

Ethylene oxide (EO) has been used for the sterilization of medical devices, foods, and powdered ingredients of cosmetics. Whenever the aeration procedure fails to remove EO from sterilized products, significant amounts of EO may remain in the products. EO is also used to prepare a number of chemical products. Many nonionic surfactants used in cosmetics contain a polyoxyethylene chain that is formed by the polymerization of EO, and EO starting material can be retained in these surfactants. Ethylene chlorohydrin (ECH) is formed by the reaction of EO with chloride ion in foodstuffs and medical devices (1, 2). EO was shown to be toxic, mutagenic, and carcinogenic in test animals (3), and ECH was shown to be toxic and mutagenic (4, 5).

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EO or ECH or both were determined by gas chromatography (GC) in rubber catheters (2); intraocular lenses (6); honey, beeswax, and pollen (7); cocoa and chocolate (8); food products (9); and surfactants (10). The determination of EO in cosmetics by GC/flame ionization detection was reported by Helms (11). The method of Jensen (9), based on the conversion of EO and ECH to ethylene iodohydrin (EIH) followed by GC determination of EIH, is simple and sensitive for determining total amounts of EO and ECH. However, individual concentrations of EO and ECH cannot be obtained by the method.

This report describes a method for specific determination of EO and ECH in cosmetics and surfactants by GC with electron capture detection (ECD).

METHOD

Reagents and Apparatus

All chemicals and organic solvents were analytical reagent grade. Distilled water was used throughout.

(a) *Standards.*—10 μg/mL EO in acetone (Kanto Chemical Co., Inc., Japan), EIH (Tokyo Chemical Industry Co., Ltd, Japan), and >99.0% pure ECH (Wako Pure Chemical Industries, Ltd, Japan). ECH was found to contain 0.2% EIH by GC/ECD. A series of standard solutions of EIH and ECH in hexane were prepared for GC/ECD analysis. Aqueous solutions of EO and ECH were used for the recovery tests. (*Caution*: EO is volatile and a potential mutagen and carcinogen. EO solution should be prepared in well-ventilated hood.)

(b) *Desorption apparatus for EO.*—See Figure 1. All-glass, connected tightly with clamps. N_2 is fed to sample container (A) through 4 mm id inlet tube placed 1.5 cm above surface of sample solution; 4 mm id inlet tube in absorbing tube (C) is placed 0.5 cm above bottom.

(c) *Gas chromatograph.*—Model GC-9AM (Shimadzu Co., Japan) equipped with electron capture detector and glass column, 1.5 m × 3 mm id glass column, packed with 5% PEG-20M on Uniport HP (60–80 mesh). Operating conditions: injection port, 250°C; column oven, 120°C; detector, 300°C; carrier gas, nitrogen; flow rate, 50 mL/min; sample injection volume, 2–3 μL.

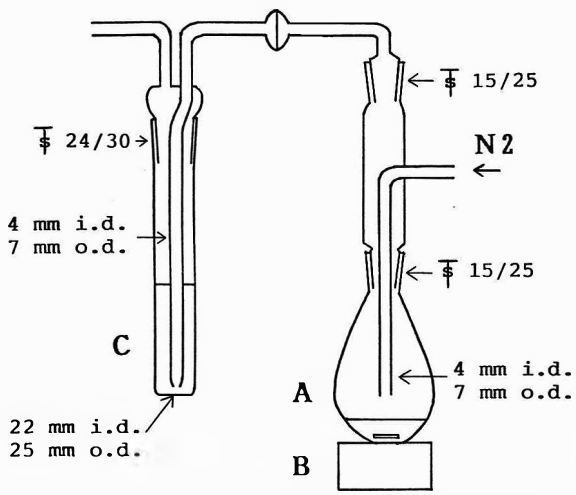

Figure 1. Apparatus for EO desorption: A, 100 mL round-bottom flask; B, magnetic stirrer; and C, 50 mL absorbing tube (17 × 2.2 cm id).

Samples

Cosmetics were purchased from a market in Tokyo during 1990 and 1991. Surfactants were chemical reagents purchased from Wako and Tokyo Chemical Industry Co.

Determination

(**a**) *Method 1 (determination of total EO and ECH).*— Weigh 0.2–2 g sample into 100 mL round-bottom flask, and add Teflon-coated stir bar 3 cm long. Weigh 5.2 g potassium iodide into 50 mL absorbing tube, and dissolve it with 10 mL 0.02N H_2SO_4. Add 30 g glass beads (2 mm id) to absorbing tube. Set up apparatus as shown in Figure 1. Add 10 mL 2N NaOH to sample through gas inlet. Introduce nitrogen stream (300 mL/min) into apparatus. Take care that no droplets splash on sides of flask. Start magnetic stirrer. Allow nitrogen stream to sweep sample surface for 30 min at room temperature.

Transfer absorbing solution to 100 mL separatory funnel, wash tube and glass beads with 15 mL water, and add washing to funnel. Add 0.1 g anhydrous Na_2SO_3, and shake funnel until solution is decolored. Add 6 g anhydrous Na_2SO_4, and extract 3 times with 10, 5, and 5 mL benzene (*Caution*: benzene is a known carcinogen). Dry extract over anhydrous Na_2SO_4, transfer to 25 mL volumetric flask, and adjust volume to mark with benzene; use as test solution for GC/ECD analysis. Obtain EIH concentration in test solution from calibration curve based on peak height method.

(**b**) *Method 2 (specific determination of EO and ECH).*— Set up apparatus as for Method 1. Add 5 mL dimethyl sulfoxide (DMSO) or water to sample, and allow nitrogen stream to sweep sample surface at 300 mL/min for 30 min. Replace absorbing solution with fresh solution. Add 10 mL 2N NaOH to sample through gas inlet, and allow nitrogen to flow again for 30 min. Treat absorbing solutions as in Method 1. Use first absorbing solution for EO determination and second for ECH determination.

Calculations

Calculate EO and ECH contents by using the following equations.

$$\text{EO } (\mu g/g) = \frac{C \times 25.0 \times 44.1}{W \times 172.0}$$

$$\text{ECH } (\mu g/g) = \frac{C \times 25.0 \times 80.5}{W \times 172.0}$$

where C = EIH concentration in test solution (μg/mL), W = weight of sample (0.2–2 g), 25.0 = mL test solution, 44.1 = molecular weight of EO, 80.5 = molecular weight of ECH, and 172.0 = molecular weight of EIH.

Results and Discussion

The desorption procedure for EO is similar to a method described by Jensen (9). However, Jensen used sodium iodide to convert EO to EIH and ethyl acetate to extract EIH. Consequently, significant amounts of sodium iodide are used, and iodine can be formed during the desorption procedure and subsequently extracted with ethyl acetate together with EIH. Elec-

Table 1. Recoveries of EO and ECH from water and shampoo and EIH from absorbing solution

Treatment	Sample	Chemicals spiked[a]	NaOH[b]	Rec., %[c]
Method 1	Water	ECH	+	64.1 ± 2.3
Method 2	Water	ECH	–	0
			+	66.3 ± 4.0
		EO	–	70.3 ± 5.4
			+	0
	Shampoo	ECH	–	0
			+	64.5 ± 4.6
		EO	–	58.9 ± 1.2
			+	0
Extraction	Absorbing solution	EIH		65.1 ± 0.6

[a] Water was fortified with ECH (1.26 μg) or EO (0.4 μg), and shampoo (No. 2 in Table 3) was fortified with ECH (10 μg) or EO (5 μg). Absorbing solution was fortified with 0.55 μg EIH.
[b] (+) and (–) means the desorption with and without NaOH, respectively.
[c] Values are mean ± SD of 3–5 determinations.

Table 2. EO and ECH in polyoxyethylated surfactants and polyethylene glycols[a]

	EO and ECH, μg/g[b]			
		Method 2[c]		
Chemicals	Method 1, EO + ECH	EO – NaOH	ECH + NaOH	ECH, μg/g[d]
POE (10) mono-*p*-nonylphenyl ether[e]	62	66	0.07	<10
POE (15) mono-*p*-nonylphenyl ether	373	394	0.16	<10
POE (18) mono-*p*-nonylphenyl ether	1.5	1.4	0.61	—[f]
POE (20) mono-*p*-nonylphenyl ether	285	339	—	<10
POE (10) monooleyl ether	ND[g]	—	—	—
POE (20) monooleyl ether	40	30	4.0	—
POE (50) monooleyl ether	3.3	—	—	—
POE (10) mono-*p*-octylphenyl ether	1.3	1.4	—	—
POE monocetyl ether	244	216	1.6	—[h]
POE (10) monostearate	4.1	—	—	—
POE (45) monostearate	7.2	—	—	—
POE (20) sorbitan monooleate (Tween 80) 1	ND	—	—	—
POE (20) sorbitan monooleate (Tween 80) 2	ND	—	—	—
POE (20) sorbitan monolaurate (Tween 20) 1	ND	—	—	—
POE (20) sorbitan monolaurate (Tween 20) 2	ND	—	—	—
POE (20) sorbitan trioleate (Tween 85)	0.07	—	—	—
POE (23) lauryl ether (Brij 35)	0.09	—	—	—
POE (10) oleyl ether (Brij 96)	ND	—	—	—
Poly(ethylene glycol) 600	4.0	—	—	—
Poly(ethylene glycol) 1000	0.27	—	—	—
Poly(ethylene glycol) 4000	0.23	—	—	—
Poly(ethylene glycol) 20000	ND	—	—	—

[a] Surfactants and polyethylene glycols tested were chemical reagents. Each value is the result of single determination.
[b] EO was calculated as ECH, μg/g.
[c] After EO was distilled without NaOH for EO determination, NaOH was added to the sample to convert ECH to EO, which was then distilled again for ECH determination.
[d] ECH was determined by direct injection of sample solution to gas chromatograph.
[e] POE, polyoxyethylene.
[f] —, not determined.
[g] ND, not detected.
[h] A peak appeared near the retention time of the ECH peak, but its shape was different from that expected for ECH.

tron capture detectors can be damaged by the presence of high concentrations of iodine if the extract is injected into the gas chromatograph without removing the iodine. Although iodine can be reduced to iodide by $Na_2S_2O_3$ treatment (9), air oxidation can cause iodide to gradually reoxidize in the test solution during storage. Therefore, we sought alternatives to sodium iodide and ethyl acetate. Potassium iodide reacts with EO as easily as sodium iodide and is less susceptible to air oxidation than sodium iodide. Hexane does not extract EIH from aqueous solutions. Although benzene extracts EIH with only a 65.1% recovery from absorbing solution (Table 1), it has the advantage of not extracting any sodium iodide or potassium iodide. From these results, we decided to use potassium iodide instead of sodium iodide and benzene instead of ethyl acetate. A small amount of iodine is still formed in the absorbing solution in the modified method, but it is easily reduced by the addition of Na_2SO_3 before benzene extraction. We used Na_2SO_3 for the reduction of iodine. These modifications result in a lower recovery than Jensen (9) experienced; however, damage of the electron capture detectors by iodine is avoided.

In the analyses of surfactant and shampoo samples, the addition of antifoam was not effective in preventing foaming during desorption. Therefore, we introduced the nitrogen stream to the apparatus by merely blowing on the surface (Figure 1) rather than bubbling in the sample solution. This modification did not affect the recovery.

In Method 1 (for the determination of total EO and ECH), varying the time of desorption between 20 and 60 min resulted in only small differences in the recoveries of EO and ECH from water and shampoo. The recovery of ECH at 30 min desorption from water samples fortified with ECH was 64.1 ± 2.3% (n = 3, Table 1). Reproducibility of the analysis was satisfactory if the sample was thoroughly dissolved in NaOH solution. However, if the sample would not readily dissolve in the NaOH solution, it had to be dissolved in a small amount of acetone, and then the NaOH solution could be added.

In Method 2 (for the specific determination of EO and ECH), EO could be recovered by the desorption without NaOH, whereas ECH could not. Recoveries of EO from water and shampoo samples were 70.3 ± 5.4 % (n = 5) and 58.9 ± 1.2 %

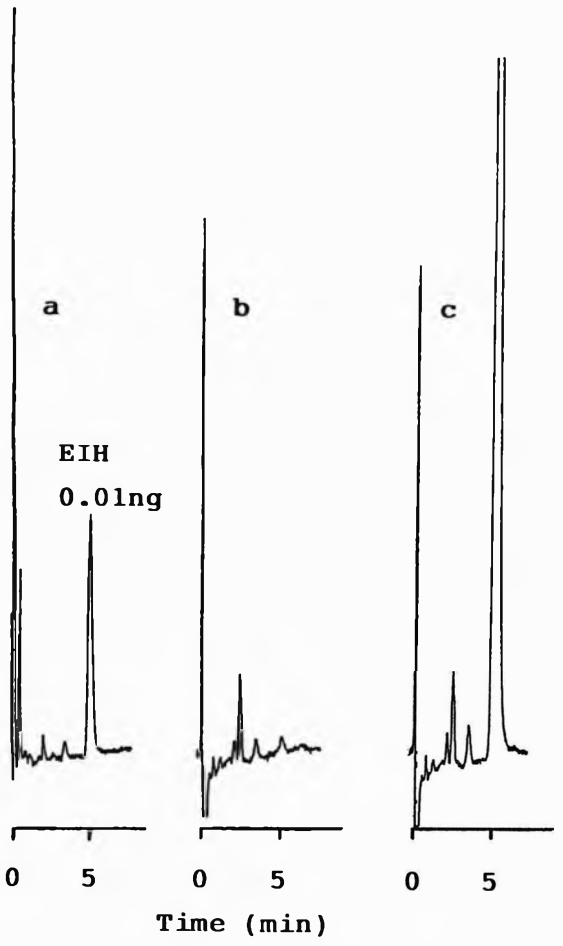

Figure 2. Gas chromatograms of EIH standard solution (a), sample solution from shampoo desorbed without NaOH (b), and with NaOH (c).

Table 3. EO and ECH in cosmetics

Sample	No.	EO and ECH, μg/g[a]		
			Method 2[b]	
		Method 1, EO + ECH	EO – NaOH	ECH + NaOH
Baby powder	1	0.08	ND[c]	0.08
	2	0.05	0.002	0.01
	3	ND	—[d]	—
Shampoo	1	1.32	ND	1.11
	2	0.51	ND	0.46
	3	0.44	ND	0.55
	4	1.11	ND	1.09
	5	0.36	—	—
	6	0.12	—	—
	7	0.11	—	—
	8	0.04	—	—
	9	ND	—	—
Hair cream	1	ND	—	—
Hair lotion	1	0.11	—	—
	2	0.09	ND	0.10
	3	—[e]	—	—
Milky lotion	1	ND	—	—
Face pack	1	0.04	ND	0.07
Skin lotion	1–6	ND	—	—

[a] EO was calculated as ECH, μg/g. Each value is the result of single determination.
[b] After EO was distilled without NaOH for EO determination, NaOH was added to the sample to convert ECH to EO, which was then distilled again for ECH determination.
[c] ND, not detected.
[d] —, not determined.
[e] EIH content could not be determined, because an interfering peak overlapped over the EIH peak.

(n = 3), respectively (Table 1). ECH could be quantitatively converted to EO by the reaction with NaOH. Therefore, ECH could be recovered by desorption after the addition of NaOH. Recoveries of ECH from water and shampoo samples were 66.3 ± 4.0% and 64.5 ± 4.6%, respectively. The standard deviations of the recoveries of spiked EO and ECH were between 1.2 and 5.4%. The precision of the method was satisfactory. When DMSO was used to dissolve water-insoluble samples, a small amount of DMSO was sometimes volatilized into absorbing solution. Although DMSO gave a broad peak on the GC chromatogram, it did not interfere with the EIH determination.

The gas chromatograms of EIH standard solution and sample solution prepared from shampoo (No. 2 in Table 2) by Method 2 are shown in Figure 2. The detection limits using 0.2–2.0 g samples were in the 0.005–0.03 μg/g range for EO and 0.01–0.07 μg/g for ECH (signal-to-noise ratio = 3).

Table 2 shows the results of EO and ECH in surfactants. Five of 18 surfactants were found to contain considerable amounts of EO (30–394 μg/g as ECH), but only small amounts of ECH (0.07–4.0 μg/g) were detected in surfactant samples. Results obtained by the 2 methods exhibited reasonable agreement with each other. ECH was also determined by direct injection of sample solution in benzene to GC/ECD under the same GC conditions used for EIH (detection limit, 10 μg/g in the sample). ECH was not detected in the surfactants that contained high levels of EO.

Table 3 shows the results of EO and ECH in cosmetics. EO was not found in commercially available cosmetics, but small amounts of ECH were detected (≤1.11 μg/g) in these samples. An interfering peak for the determination of EIH was observed in a hair lotion. Results shown in Table 3 indicate that shampoos consisting mainly of polyoxyethylated surfactants contained more ECH than other cosmetics and suggest that the ECH found in cosmetics might be derived from EO in the surfactants.

To answer the question of what happened to the EO in surfactants, milky lotions were prepared by the reversed emulsification method at 75°C on a laboratory scale from polyoxyethylene mono-p-nonylphenyl ethers containing 66 and 394 μg/g EO (as ECH), sorbitan monostearate, cetyl alcohol, liquid paraffin, and water. Only ≤4% of initial EO contents remained in the products. Most of the EO seems to be lost by evaporation during the emulsification process. In addition, EO

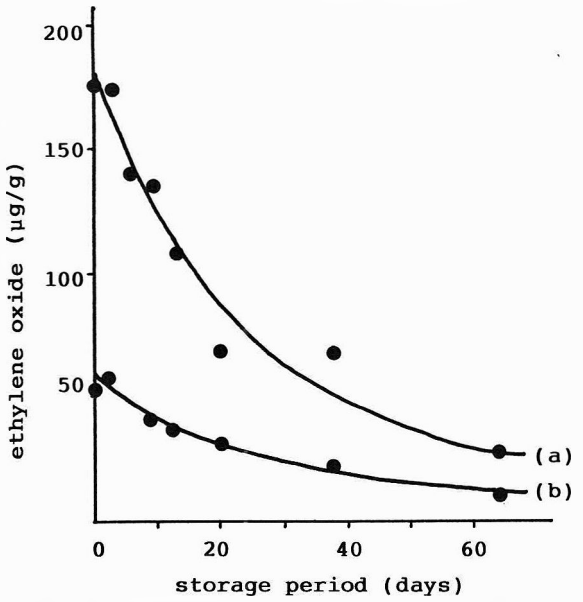

Figure 3. Decrease of EO in surfactants during storage: 2% polyoxyethylene(15) mono-*p*-nonylphenyl ether (a) and polyoxyethylene monocetyl ether (b) were dissolved in water and stored in the dark at room temperature.

in the surfactants dissolved in distilled water gradually decreased during storage at room temperature in the dark (Figure 3). These results confirmed the observations that surfactants contained EO but cosmetics did not.

The proposed method is useful for the rapid specific determination of EO and ECH in cosmetics and surfactants.

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DAIRY PRODUCTS

Automated Technique for Sampling Milk from Farm Bulk Tanks: Collaborative Study

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An automated, in-line, mechanical technique for sampling milk from farm bulk tanks was evaluated in a collaborative study. The automated sampling device, which is mounted on the milk intake line, contains an electronically controlled peristaltic pump. The device takes a representative sample of the entire volume pumped through the system. Samples taken can be analyzed for both composition and microbiological quality. The study was performed in 3 phases. In the first 2 phases, samples taken by manual and automated methods were compared in analyses for somatic cell count, antibiotics, fat, protein, lactose, and solids-not-fat. The third phase, using a modified procedure, was designed to compare sampling methods in analyses for total bacteria count (standard plate count), psychrotrophic bacteria count, and coliform count. Evaluation of the data by a nested ANOVA indicated no difference between results for samples taken by the automated and manual methods ($P = 0.05$) in Phases 1 and 2, irrespective of whether the bulk milk was agitated before sampling. By introducing a sanitizing step between farms in Phase 3, the automated method also provided samples comparable with those taken manually for microbial analyses. The automated method has been adopted first action by AOAC International.

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The recommendation was approved by the General Referee and the Committee on Foods I and was adopted by the Official Methods Board of AOAC. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, 251–255.

Milk in bulk tanks is usually sampled manually by the milk hauler after agitation of the milk for a prescribed length of time. The time required to blend the milk before sampling varies by size of bulk tank and/or manufacturer's recommendations, usually ranging between 5 and 10 min (1). Even at the longer interval, some questions exist as to whether milk supplies in some bulk tanks, particularly those of very large capacity, are indeed properly blended for sampling purposes (2, 3). Certainly, sufficient basis exists for exploring mechanical methods of sampling milk from a bulk tank.

Preliminary studies (2, 3) indicated the potential viability of an automated milk-sampling device. The present collaborative study was done (*1*) to evaluate the automated sampling method under relatively diverse weather and operating conditions and under recommended bulk milk-blending procedures (agitation times) and (*2*) to evaluate automated sampling without prior blending of the milk supply. The latter study was undertaken to determine if satisfactory samples could be obtained without prior agitation, thus providing for more efficient pickup of milk at the dairy farm.

The automated method was presumed to be capable of sampling milk for both composition/chemical and microbiological analyses. However, the study demonstrated the necessity of a between-farm sanitizing step when the automated device is used to sample milk for microbial analyses.

Collaborative Study

Three bulk milk pickup trucks were fitted with Foss type 20500 Autosampler (AS) devices (A/S Foss Electric, Hillerod, Denmark, manufacturer; Foss Food Technology Corp., Eden Prairie, MN 55344, U.S. agent). The unit is a proportionate sampling device that composites small aliquots of all milk withdrawn from the tank by taking and adding aliquots throughout the time milk is being pumped from the bulk tank to the tank truck. Two trucks were provided with one AS unit

each; the third truck was fitted with 2 AS units, to enable a direct comparison of 2 different samplers taking samples from the same bulk milk supplies.

One tank truck operated out of a Land O'Lakes, Inc., dairy plant in Browerville, MN; the second out of a Land O'Lakes, Inc., dairy plant in Volga, SD; and the third from the Associated Milk Producers, Inc., dairy plant in Arlington, TX. The different locations were purposely selected to provide a reasonably wide range of ambient temperatures and other operating conditions. In particular, we thought it desirable to evaluate the AS unit in situations where milk supplies were large and, hence, long sampling times were required.

Milk haulers were given an opportunity to become familiar with operation of the unit before the study started. All milk haulers were licensed by their respective states for bulk milk sampling and pickup. However, to standardize pickup procedures, all haulers were required to follow manual sampling requirements prescribed by Wisconsin Administrative Code, AG 107.03 (4). This code requires that bulk milk be agitated for at least 5 min before taking a sample from tanks of 1500 gal. capacity or less. Milk in tanks of over 1500 gal. capacity must be agitated for at least 10 min before sampling. Other agitation times must be adhered to as prescribed by 3-A Standards (1).

In this study, no bulk tanks under 500 gal. capacity were sampled. The minimum sample volume was 70 mL. All samples were taken in rigid plastic vials with hinged, over-the-lip closures. All samples were maintained at 0–4.4°C, transported to certified laboratories within 30 h from time of collection, and handled, in general, as prescribed by Wisconsin Administrative Code, AG 107.03, Subsection (3)(ref. 4).

For Phases 1 and 2 of the study, each milk hauler sampled milk from bulk tanks of 4 different producers on 3 separate days. Three samples were taken manually from each tank, one directly below the port in the location officially approved for this purpose, the second to the left of the port and deeper than the first sample, and the third to the right of the port and deeper than the second sample. A fourth sample was then obtained using the automated sampler. On the truck fitted with 2 AS units, 2 samples were taken simultaneously, one from each AS unit.

In Phase 1, all AS samples were taken following the required agitation time, i.e., as normally prescribed for sampling milk from a bulk tank. In Phase 2, field representatives (licensed milk samplers) were asked to precede the tank truck to the dairy farm and to take a manual sample from each bulk tank after proper (prescribed) agitation of the milk. Not less than 90 min later, an AS sample was taken, this one by the hauler, but without prior agitation of the milk. Two AS samples were again obtained from the truck on which 2 AS units were installed.

Initially, the AS samples were taken without any between-farm sanitization of the device. This procedure ultimately proved unsuccessful for samples subjected to standard plate count (SPC), psychrotrophic bacteria count (PBC), and coliform count (CC) enumeration. In particular, contamination of AS samples occurred fairly frequently and somewhat more extensively on milk picked up at the first stop of the day. Contamination of the unit was considered to be the cause, with multiplication of bacteria resulting in greater contamination of loads the longer the interval between use. Further evaluation suggested that the unit was simply not being cleaned and sanitized properly. For this reason, a second trial was implemented and became Phase 3 of the study. In this trial, procedures were varied on alternate pickups. On 2 consecutive days of the week, the unit was used without sanitization between farm pickups of milk. At the next 2 pickup days of the same week, the entrance port to the unit was sanitized using a pipe cleaner dipped in chlorine sanitizer (200–300 ppm hypochlorite). For this purpose, we used a hypochlorite solution and not a sanitizers with the potential to maintain microbial killing power in the presence of organic solids. In this manner, the device was sanitized before each pickup (between each farm). We thought that this approach would both test the capability of the unit for sampling milk for microbial analyses and, if successful, also pinpoint the specific source(s) of contamination that had been encountered in earlier work.

Because Phase 3 was undertaken later in the fall, it was done only in Texas, where it was still reasonably warm. However, to provide and maintain additional and consistent heat during overnight storage of the unit, an electric light bulb was placed inside the cabinet of the tank truck where the unit was housed.

In Phases 1 and 2, all samples were picked up on a Monday and Wednesday or Tuesday and Thursday of each week over 3 weeks during July and August 1990. Phase 3 was undertaken in late October, early November 1990 and involved 2 samplings of each farm milk supply each week over a 4-week period.

In Phase 1, a total of 156 milk samples was obtained: 3 milk haulers × 4 farms × 4 samples/farm × 3 sampling days = 144 + 12 additional AS samples from the truck carrying 2 AS units = 156. In Phase 2, a total of 84 samples was obtained: 3 milk haulers × 4 farms × 2 samples/farm × 3 sampling days = 72 + 12 additional AS samples from the truck carrying 2 AS units = 84. In Phase 3, a total of 96 samples was obtained: 1 milk hauler × 3 farms × 2 samples/farm × 4 sampling days/ week × 4 weeks = 96.

Each milk hauler was provided with a master reporting form. On this form, randomized sample-identification numbers were recorded along with farm identification, bulk milk temperature, ambient temperature, length of agitation of the bulk milk before sampling, temperature in the rear cabinet of the truck at the location where the AS sample was being taken, distance to the farm from the dairy plant or from the previous farm, and time required to reach the farm from either the dairy plant or the previous pickup.

To evaluate as critically as possible any possible carryover effect of one sample to the next, we tried to select dairy farms with milk supplies that ranged widely in composition and microbiological quality. The pickup routes were then planned such that supplies of widely varying composition and/or quality were picked up consecutively.

Operation of Automated Sampler

The automated milk sampler is mounted on intake lines of tank trucks, preferably on the suction side of the milk pump. It operates on either 12 or 24 V DC supply. The sampler contains an electronically controlled peristaltic pump. By presetting the

desired sample volume, volume of liquid (set at individual farms), and pump speed, the sampler operates throughout the pumping cycle, giving a representative sample of the entire volume being pumped through the system.

During operation of the sampler, milk passes through a short length of silicon tubing. This tubing can be used for 1 day of milk pickup (i.e., several consecutive bulk tank samplings) or can be replaced at each farm before sampling. Initially, however, the silicon tubes are sterile. They are in fact available in quantity in sterile condition in aseptically packaged containers so that it is possible to replace the "used" tube with a fresh, sterile tube after each pickup. In our study, the AS sampling was done in this manner. In addition, just before sampling, a 2 s delay was invoked during which a small amount of milk passed through the AS unit without being collected. The unit was also preset to perform a 5 s air purge at the completion of the sampling cycle to clear remaining milk from the unit.

In Phases 1 and 2, milk samples were analyzed in duplicate for milk fat, protein, lactose, and solids-not-fat (SNF) by the infrared spectroscopic method, **972.16** (5). Somatic cell counts were determined by the automated fluorescent dye procedure described in *Standard Methods for the Examination of Dairy Products* (6). Analyses for beta-lactam residues were conducted by the *Bacillus stearothermophilus* qualitative disk method II, **982.17**, and freezing point of milk, as a measure of water adulteration, was determined by the thermistor cryoscope method, **961.07**.

As mentioned, data from Phases 1 and 2 indicated unsatisfactory results for total aerobic bacteria count, psychrotrophic bacteria count, and coliform bacteria count. Therefore, results are not reported. However, a separate study was implemented as Phase 3, in which manually taken samples and AS samples were analyzed singly, and for total aerobic bacteria, psychrotrophic bacteria, and coliform bacteria counts only. Total aerobic bacteria count was determined by the standard plate count described in *Standard Methods for the Examination of Dairy Products* (6). Methods from the same reference (6) were used to determine the 10-day psychrotrophic bacteria count and the violet red bile presumptive test for coliforms.

All analyses were performed by certified laboratories. Samples from the Upper Midwest were analyzed by Dairy Quality Control Institute, Inc., St. Paul, MN, and those from Texas were analyzed by Associated Milk Producers, Inc., Arlington, TX.

After analysis of samples, all data were sent directly to the Associate Referee. Randomized sample numbers were decoded, and data were evaluated statistically.

970.26 Sampling of Milk from Bulk Tanks and Other Storage Equipment— Alternative II–Automated Method

First Action 1992

(Applicable to automated sampling of milk from bulk storage tanks)

Method performance:

A nested ANOVA indicated no difference (at $P = 0.05$) between milk samples obtained by automated device and manually collected when analyzed for composition (fat, protein, lactose, SNF, and total solids), for microbiological quality (total aerobic count, psychrotrophic count, and coliform count), and for somatic cell count, drug residues, and added water.

A. Principle

Automated sampling device, mounted on milk intake line and containing electronically controlled peristaltic pump, takes representative sample of entire volume pumped through system.

B. Apparatus and Reagent

(a) *Automated sampling device.*—Consists of electronically controlled peristaltic pump, roller wheel, inlet orifice, sample-container holder, sterile silicon tubes, and tool for installing tubes (Foss 20500 AutoSampler, Foss Electric, or equivalent). Install on milk intake line of milk truck or other tank, preferably on suction side of pump.

(b) *Hypochlorite solution.*—200–300 ppm hypochlorite in H_2O.

(c) *Sample container.*—Aseptic plastic vial with snap-on cap, 50–100 mL; or whirl-pak plastic sample bag; or other aseptic sample container that sample-container holder will adjust to fit.

C. Sampling Procedure

(a) *Installation or replacement of sterile silicon tube.*—Open upper cover of device. Sanitize installation tool with 200–300 ppm hypochlorite solution by immersion for >15 s. Using sanitized tool, remove new tube from sterile packaging. Place tube on sample intake port of device and press in place. Still using sanitized tool, pull tube down over roller wheel and force outlet end into clip holder.

(b) *Operation of sampling device.*—Insert sample container into holder and slide holder upward to lock in place. Set device scale to estimated volume of milk to be pumped. Turn on device and then turn on pump. When pumping is finished, turn off device, remove sample container, and properly store for analysis. (*Note*: Just prior to taking samples for microbiological analysis, sanitize inlet orifice of device by working pipe cleaner dipped in hypochlorite solution back and forth several times within inlet orifice. Change to fresh tube between producers.)

Ref.: JAOAC **76**, March/April issue (1993)

Results and Discussion

The raw data for Phases 1, 2, and 3 are on file at AOAC Technical Services as Appendices A, B, and C, respectively. Aside from the general agreement between results for AS and manually taken samples, which may be observed without benefit of statistical analysis, it should be noted that the milk supply from farm 292 sampled on July 30 was found positive for beta-lactam residues. All samples were found positive irrespective of sampling method; however, no sample was found positive in the milk supply sampled at the very next farm (No. 266). This fact is mentioned solely to suggest that no carryover oc-

Table 1. Summary of averages and ranges of raw data for Phases 1, 2, and 3

	Phase 1		Phase 2		Phase 3	
Condition/factor	Range	Av.	Range	Av.	Range	Av.
Temp. of milk in bulk tank, °C	2.0–6.7	4.0	1.7–9.0	4.0	1.0–4.0	3.7
Ambient temp., °C[a]	14.4–33.3	22.6	14.4–36.1	23.3	2.2–24.4	12.9
Temp. inside truck cabinet where unit was housed, °C	14.4–27.2	20.4	12.2–28.3	32.2	4.0–23.9	13.7
Overnight temp. in cabinet where unit was housed, °C	ND[b]	ND	ND	ND	17.8–35.5	31.3
Distance between farms, km	1.6–103	23.5	1.6–72.4	18.4	1.6–75.6	34.9
Travel time between farms, min	3–180	36.6	5–180	40.3	10–190	71.2
Somatic cell count/mL, (× 1000)	65–1100		60–910			
Cryoscope reading, °C	–0.537 to –0.497		–0.539 to –0.504			
Fat, % w/w	3.04–4.35		3.14–4.40			
Protein, % w/w	2.86–3.49		2.88–3.43			
Lactose, % w/w	4.36–5.01		4.56–4.96			
Solids-not-fat, % w/w	8.02–9.00		8.00–8.91			
Standard plate count, cfu/mL[c]					400–250000	
Coliform count, cfu/mL					0–1500	
Psychrotrophic bacteria count, cfu/mL					<10–11000	

[a] A temperature reading was taken at each farm at time of milk pickup.
[b] ND, Not determined.
[c] cfu, colony-forming units.

curred between the 2 milk supplies, at least within the limits of detectability of the test for beta-lactam residues.

Raw data from Phase 3 show general agreement between sampling methods in analyses for total aerobic bacteria, psychrotrophic bacteria, and coliform bacteria counts.

Table 1 summarizes the weather, other operational considerations, and the ranges in test values for the analytical measurements undertaken in this study. Ambient temperature ranged from 2 to 36°C (36 to 97°F). Temperature inside the cabinet where the AS unit was housed varied from 4 to 28.3°C (39 to 83°F). The distance between farms (milk pickups) ranged from 1.6 to 103 km; travel between farms required from 3 to 190 min. In addition, the Appendixes show that some milk supplies were agitated for as long as 15 min before manual samples were taken. Hence, the conditions under which milk sampling and pickup were made varied considerably.

Similarly, analytical data (Table 1) show wide variations. Somatic cell counts ranged from 60 000 to 1 100 000/mL; cryoscope readings from –0.497 to –0.539°C; and bacteria, coliform and psychrotrophic counts to as high as 250 000, 1500, and 11 000/mL, respectively. Percent fat ranged from 3.04 to 4.40, with equivalent variations in protein, lactose, and SNF. Again, such values reflect significant farm-to-farm variations with attendant demands on representative sampling of milk in bulk tanks.

Statistical analysis of data from the 3 phases of this study focused on 2 areas: (*1*) estimating components of variation and (*2*) testing for differences between results from the AS and manual (standard) methods.

The results reported in Phases 1 and 2 for determinations of somatic cells; antibiotics; freezing point; and percentages of fat, protein, lactose, and SNF were obtained by analyzing each of 2 aliquots taken from each of the coded samples. The statistical analysis encompassed these data, except for the presence of antibiotics (beta-lactam residues), which is obvious (either detected or not detected) and, therefore, not an appropriate variable for the analysis of variance used in this evaluation.

Estimation of Components of Variation

In Phases 1 and 2, one tank truck was equipped with dual AS units able to withdraw independent samples from the same bulk milk supply. This experimental format was designed to allow estimation of the pure AS sampling error encountered when sampling milk from a single farm supply. In this analysis, there were 2 (phases) × 3 (days) × 4 farm pickups/day = 24 individual bulk tank unloadings with 2 (AS duplicates) × 2 (laboratory duplicates) = 4 determinations/bulk tank milk supply. The components of variance for differences among bulk tanks (σ_T^2), differences among AS samples from the same bulk tank (σ_F^2), and differences among laboratory determinations on the same sample vial of milk (σ_L^2) were estimated using the nested analysis of variance in Table 2.

It should be noted that the laboratory "duplicates" are not blind in the sense needed to test laboratory reliability, because aliquots were taken from a single vial and then run side by side. Hence, the laboratory component of variance in this analysis may be regarded as being conservatively small to very small. By performing appropriate algebra on the mean squares in the ANOVA table, estimates of σ_T^2, σ_F^2, and σ_L^2 can be isolated. The square roots of these estimated variances are shown in Table 3 as standard deviations of the sampling error distributions. The AS standard deviation should be compared with the laboratory

Table 2. Analysis of variance for duplicate sample of one truck

Source of variation	Degrees of freedom	Mean squares	Expectation of the mean squares
Tank pickups	23	MS (T)	$\sigma_L^2 + 2\sigma_F^2 + 4\sigma_T^2$
AS samples on same tank pickup	24	MS (F)	$\sigma_L^2 + 2\sigma_F^2$
Lab. detns on same sample	48	MS (L)	σ_L^2

standard deviation and the tank standard deviation. One would expect the standard deviation of the tank distribution to be very much larger, and this is indeed the case for the response variables shown.

No data are given for SNF specifically. Because protein and lactose are the major components of SNF and because both of these components show standard deviations considerably lower than the tank values, it is assumed that AS samples are equally valid for SNF analysis.

Data in Table 3 provide preliminary indications that the AS unit can sample with minimal variance for the components indicated. However, the main intent of this study was to compare results obtained by automated and traditional (manual) methods.

Statistical Tests for Differences between Sampling Methods

Phases 1 and 2.—The statistical approach used in this study was a "repeated measure" (or split plot) design, with experimental units (whole plots) being bulk tanks arranged in 9 blocks of size 4 tanks each. On each of 3 days, each of 3 milk haulers picked up milk from 4 bulk tanks. The whole plot treatment factor was the order of sequence of picking up milk from the first to the fourth farm during any 1 day. We were interested in testing for a "sequence" effect, and the appropriate error term in mean square values shown in Table 4 is "tank variation."

The repeated measures (sample location) on each tank were the primary issue in this study, which was designed to have a great deal of power to detect small differences. The main effect for sample location contains the comparison of the AS sample with the 3 in-the-tank samples. In Table 4, the correct mean square for testing for sample location differences is labeled "sampling error." The laboratory error term is included to complete the data in the table but was not used to test any effects. The laboratory error term shows the mean square for split determinations from the same vial of milk and, as mentioned above, is likely as small as it possibly could be and may in fact overstate laboratory reliability.

From data in Table 4, the only effect showing any significance is for electronic somatic cell counting, with an interaction of pickups by sample location at the 5% level of confidence. The AS sample results are very much in line with those for the tank samples, and this "significant" F-ratio could easily be an artifact of calculating many F-ratios. Over all other parameters, the automated method showed no differences from the traditional (manual) method.

Table 5 shows the mean squares obtained on analyses of samples from Phase 2. In this phase, the question is whether the AS unit can take representative samples from a bulk tank without prior agitation of the milk. The data indicate no differences from those obtained manually after normal agitation procedures ($P \leq 0.01$). The AS samples in this case were taken not less than 90 min after the manually obtained samples and, as indicated, without prior agitation.

Phase 3.—In Phase 3, only SPC, PBC and CC assays were performed. Data consider the differences in results of these tests on samples taken manually compared with results for those taken by the automated method. On some days, the inlet orifice to the AS unit was sanitized between farm pickups; on other days, no sanitizing step was used.

Scatter plots showing the relationship between the data pairs for AS and manual samples are shown in Figure 1. Separate plots are shown for samples taken from the AS unit when it was sanitized and when it was not sanitized between farm milk pickups. In all 3 plots of data from the nonsanitized AS sampling, evidence of contamination may be seen. It is noteworthy that contamination was not limited to the first pickup of the day, i.e., after overnight storage of the sampling device when the ambient temperature was high. These latter events may be seen as data points far to the lower right of the plots of 23 pairings. (A laboratory accident eliminated one AS/tank pair; hence, there are only 23 data points.)

The 3 plots of data from tank samples compared with those for AS sampling when the entrance port to the AS unit was sanitized between farms exhibit excellent agreement for SPC, PBC, and CC. In Figure 1F, the one point isolated in the lower right was the result of a procedural error, which is described below.

The raw data generated by this study were transformed as follows to normalize the error distributions associated with sampling and laboratory variation:

Table 3. Standard deviations of sampling error distributions among bulk tanks, AS samples, and laboratory analyses for Phases 1 and 2

Source of variation	Cryoscope	ESCC[a]	Protein	Fat	Lactose
Tanks, σ_T	0.123	212	0.150	0.361	0.101
AS samples from same tank, σ_F	0.001	19	0.004	0.033	0.011
Lab. analyses on same vial, σ_L	0.002	18	0.010	0.014	0.005

[a] Electronic somatic cell count.

Table 4. Mean squares obtained on data from Phase 1

Source of variation	df[a]	Mean square				
		Cryoscope (× 10^6)	ESCC[b] (× 10^4)	Fat (× 10^4)	Protein (× 10^4)	Lactose (× 10^5)
Days of pickup	8	75.0	1134.0	8019.0	1544.0	11330.0
Daily sequence of pickups	3	1420.0	2645.0	6769.0	1858.0	13370.0
Tank variation	24	560.0	3198.0	3399.0	1424.0	10720.0
Sample location	3	7.0	3.7	4.4	0.04	16.7
Pickups × location	9	5.4	16.1[c]	3.9	2.1	23.4
Sampling error	96	4.2	7.1	4.9	2.3	30.2
Lab. error	141	1.8	6.1	1.8	0.7	8.2

[a] Degrees of freedom.
[b] Electronic somatic cell count.
[c] Statistically significant at 5% level.

SPC transformed to log (SPC + 1000) = ls
PBC transformed to log (PBC + 100) = lp
CC transformed to log (CC + 10) = lc

Tests for statistical significance of differences between results for samples from the sanitized AS unit and those taken manually were undertaken. In this analysis, d_i is defined as the paired difference between the transformed AS unit (l_{AS}) and the transformed manual tank sample results (l_{TANK}):

$$d_i = (l_{AS} - l_{TANK})_i$$

where i indexes the pickups. These data are shown in Table 6.

In a statistical sense, data in Table 6 indicate that samples taken by the AS unit yield slightly higher PBC counts. This difference does not appear to be of biological significance. Moreover, this finding is only possible if laboratory results are very precise, which they appear to be.

Use of data from the first and second pickups of the day make possible, at least, an evaluation of the question of carryover effect, i.e., the transfer of microbial contaminants from one sample to the next via contamination of the AS unit. Only data obtained when the unit was sanitized are useful in this regard.

In addition, only data from 7, not 8, days can serve this evaluation because of obvious contamination of the AS sample on one day of pickup (Table 6). Although instructed to sanitize the entrance port to the unit before each pickup on days when the sanitization treatment was rendered, the milk hauler, at the first stop on October 31, had no chlorine sanitizer. The unit was simply rinsed with water before the milk sample was taken. As a result, microbial contamination occurred. By the second stop of the day, sanitizer was available, and the unit was appropriately sanitized throughout this and remaining pickups.

Data in Table 7 provide the basis for establishing a model for testing the carryover effect (shown in Table 8). In this model, l_{A1}, l_{A2}, l_{T1}, and l_{T2} represent the transformed data for the AS and manually obtained samples of the first and second pickups of a day on which the entrance port to the unit was sanitized between farm pickups. Then, the best estimate of the first pickup value would be $(l_{A1} + l_{T1})/2 = \bar{l}_1$ the average of the first pickup samples. If a carryover effect occurred, then one would expect l_{A2} to be contaminated by a contribution from $\bar{l}_1$. This consideration can be modeled as a proportion, k, of $\bar{l}_1$. The difference between the second farm AS sample and the second farm "manual" tank sample will be inflated by $k\bar{l}_1$ if carryover is occurring. By defining Diff2 = $l_{A2} - l_{T2}$, the following statistical effects are expected:

$$\text{expected value of } (l_{A1} + l_{T1})/2 = \bar{l}_1$$

$$\text{expected value of } (l_{A2} - l_{T2})/2 = k\bar{l}_1$$

A plot of Diff2 vs $\bar{l}$ should show an increasing relation with slope k. For a carryover effect to make sense, the estimated slope in the regression of the observed differences vs the observed first stop readings should be positive. These data are shown in Table 8. The rank correlation and estimated k, respectively, were –0.35 and –0.05 for SPC, –0.78 and –0.15 for PBC, and –0.17 and –0.02 for CC.

Table 5. Mean squares obtained on data from Phase 2

Source of variation	df[a]	Mean square				
		Cryoscope (× 10^6)	ESCC[b] (× 10^4)	Fat (× 10^4)	Protein (× 10^4)	Lactose (× 10^5)
Days of pickup	8	65.0	2158.0	1651.0	980.0	4242.0
Daily sequence of pickups	3	314.0	2082.0	2581.0	1205.0	3194.0
Tank variation	24	283.0	1067.0	1359.0	503.0	4056.0
Sample location	1	0.4	1.4	107.0	92.0	1604.0
Pickups × location	3	8.1	7.0	38.0	136.0	440.0
Sampling error	32	7.6	7.3	60.0	174.0	569.0
Lab. error	72	4.8	2.3	1.4	152.0	7.7

[a] Degrees of freedom.
[b] Electronic somatic cell count.

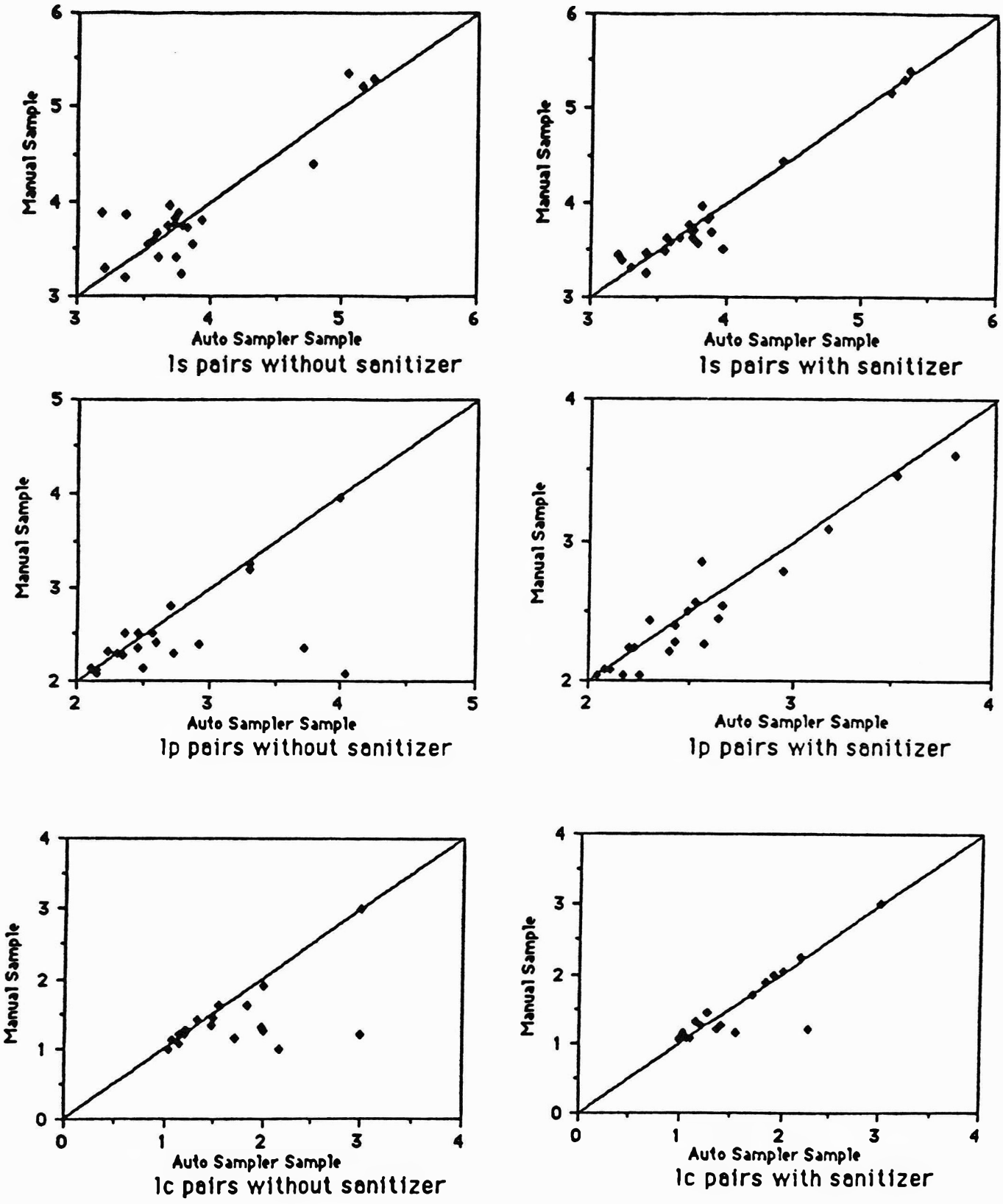

Figure 1. Scatter plots showing relationship between data pairs for bacterial counts on samples in collaborative study on automated sampling of milk from farm bulk tanks. Data for manual and automated sampling are log transformed. ls = standard plate counts; lp = psychrotrophic bacteria counts; lc = coliform counts. Data are shown for sampling by sanitized and nonsanitized units. See text for further explanation.

The negative sign on the k estimates is not consistent with a carryover effect. The data show no indication of carryover of microbial contaminants from one AS sample to the next.

Again, it is noteworthy to mention that the sampling conditions were conducive to microbial growth if contamination occurred. Results of microbial analyses made on AS samples collected during Phases 1 and 2 indicated that contamination was generally more frequent and more extensive at the first pickup of the day. To evaluate this specific concern, the temperature inside the truck cabinet that housed the AS unit was kept very high throughout the night (Table 1). Atmospheric temperatures during this phase of the study were not high but were certainly in a range in which bacterial contaminants would readily grow and multiply. Finally, distances and times between pickups were, in some instances, very long, with ample time for growth of contaminating organisms. Hence, the AS unit appears to have served the sampling purpose for both chemical and component analyses as well as microbial analyses.

Recommendation

We recommend that use of an automated device for sampling milk from bulk storage tanks for microbial, chemical, and/or component analyses be adopted first action.

Acknowledgments

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Table 6. Tests for statistically significant differences between results[a] from samples obtained by sanitized AS and samples obtained manually in Phase 3

Statistic[b]	SPC	PBC	CC
$\bar{d}$	0.023	0.117	0.011
SE	0.056	0.059	0.060
t	0.40	1.97	0.18
P-value	NS[c]	5% < P <10%	NS

[a] SPC = standard plate count; PBC = psychrotrophic bacteria count; CC = coliform count.
[b] $\bar{d}$ = average difference; SE = standard error; t = t-value.
[c] NS = not significant.

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Table 7. Bacteria counts (cfu/mL)[a] of samples from first and second pickups of days on which AS unit was sanitized before use

		SPC		PBC		CC	
Date	Order of pickup	AS	Manual	AS	Manual	AS	Manual
10/24	1	24000	26000	800	510	145	167
10/24	2	6000	5900	350	240	72	85
10/25	1	4900	4100	20	20	25	4
10/25	2	600	1000	80	<10	13	6
10/30	1	160000	140000	3200	2800	>1000	>1000
10/30	2	700	1500	10	<10	15	8
10/31[b]	1	5300	2200	280	80	179	6
10/31	2	6700	3800	150	60	3	2
11/7	1	200000	200000	1400	1100	>1000	>1000
11/7	2	1000	1100	30	20	5	4
11/8	1	4600	3200	260	600	7	8
11/8	2	2500	2100	240	270	60	65
11/13	1	220000	250000	6200	4200	>1000	>1000
11/13	2	6300	5600	210	210	1	4
11/14	1	4800	4300	70	70	1	3
11/14	2	1000	400	170	50	19	16

[a] cfu = colony-forming unit; SPC = standard plate count; PBC = psychrotrophic bacteria count; CC = coliform count.
[b] Automatic sampling unit not sanitized before use.

Table 8. Regression values[a] for estimated slope of Diff2 plotted against $\bar{I}_1$

SPC		PBC		CC	
Diff2	$\bar{I}_1$	Diff2	$\bar{I}_1$	Diff2	$\bar{I}_1$
0.042	4.41	0.122	2.87	–0.064	2.22
0.097	3.74	0.234	2.11	0.157	1.35
–0.167	5.18	0.020	3.49	0.143	3.00
–0.021	5.30	0.035	3.13	0.030	3.00
0.053	3.69	–0.037	2.70	–0.030	1.24
0.044	5.37	0.00	3.72	–0.105	3.00
0.155	3.74	0.255	2.23	0.047	1.08

[a] SPC = standard plate count; PBC = psychrotrophic bacteria count; CC = coliform count; Diff2 = $I_{A2} - I_{T2}$; $\bar{I} = (I_{A1} + I_{T2})/2$.

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DRUGS

Analysis of Testosterone Esters by Tandem Mass Spectrometry

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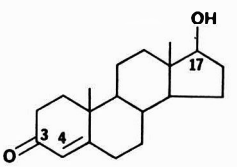
The electron ionization (EI) and chemical ionization (CI) mass spectra of 12 representative testosterone esters were examined to explore the various analytical options available for identification and confirmation of the esters. Using EI, a number of fragment ions indicated the identification of the testosterone moiety, but structural confirmation of the individual esters often required the observance of the molecular ion at very low relative abundance ratios. The acceptable analytical method involved CI/tandem mass spectrometry based on the production of the 2 generic product ions derived from the protonated molecule ion.

Anabolic steroids are defined as synthetic derivatives of the male hormone, testosterone, which are used clinically to promote growth and to repair body tissue damaged by senility, debilitating illness, or convalescence.

Unfortunately, anabolic steroids are abused for nontherapeutic purposes. The primary source of the anabolic steroids abused by body-builders and others seeking muscle bulk, such as football players, is the black market. The demand by athletes for anabolic steroids has created a lucrative marketplace, which has resulted in a large number of preparations that are offered for illegal distribution and sale.

With this dramatic increase of counterfeit preparations of domestic and imported anabolic steroids, the need to devise a concerted analytical protocol involving structural identification and confirmation has become a high regulatory priority. The constituents of these preparations vary from complex mixtures of various testosterone esters to vegetable oils (i.e. placebos). In the majority of cases, the declared contents on the counterfeit label have no bearing on the actual chemical contents manufactured under clandestine conditions. To bring legal action against such preparations, the exact contents must be identified before regulatory action can be initiated.

The lack of analytical standards for many of the unusual testosterone esters used in these counterfeit preparations precluded the use of gas chromatography (GC) or liquid chromatography (1, 2) as a primary method of analysis. Reliance on GC/mass spectrometry (MS) became the method of choice (3, 4). Previously, analysis of oil-injectable preparations and tablets (5) uses MS/MS as a confirmatory technique to provide the required number of structurally related ions for confirmation (6).



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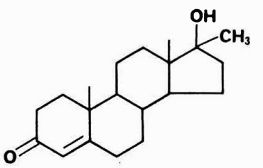

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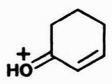

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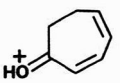

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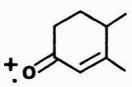
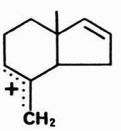
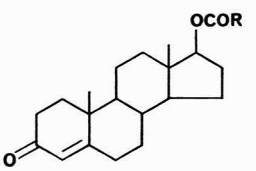


m/z 124



m/z 147

Ion structures.



Structures. III, R + CH_3; IV, R = CH_2CH_3; V, R = $CH(CH_3)_2$; VI, R = $CH_2(CH_2)_2CH_3$; VII, R = $CH_2(CH_2)_3CH_3$; VII, R = $CH_2(CH_2)_4CH_3$; IX, R = Ph; X, R = $CH_2CH_2(CH_2)_5$; XI, R = $CH_2(CH_2)_7CH_3$; XII, R = CH_2CH_2Ph.

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The presence of ions at $[M + 29]^+$ and $[M + 41]^+$ in the methane chemical ionization (CI) spectrum of a testosterone ester (5) cannot be considered sufficient for confirmatory purposes. Those adduct ions can only support the designation of the molecular weight of the compound under investigation and do not address structural confirmation. In the examination of counterfeit preparations of various testosterone esters, an analytical approach was required to screen for all possible compounds and, concurrently, to provide the necessary structure confirmation for legal action.

This paper reports the application of GC/MS/MS in the development of a rapid analytical protocol. Reaction monitoring is used for 2 generic product ions. The ions can be used to screen for testosterone (I), methyl testosterone (II), and various testosterone esters (III–XIII) in all forms of counterfeit preparations (tablets, injectables, etc.) as well as bona-fide domestic preparations of anabolic steroids.

Experimental

Reference standards of the various steroids were obtained from Research Plus, Bayonne, N.J. (I, III–VII, IX, X, and XIII) and K & K Laboratories, Plainview, NY (II). Testosterone caproate (VIII), testosterone decanoate (XI), and testosterone phenylpropionate (XII) were synthesized by modifications of the procedure originally developed by Junkmann et al. (7)

Testosterone (500 mg) was dissolved in a minimum amount of methylene chloride (ca 0.5 mL). The esterifying agent was then added (caproic anhydride [0.5 mL], decanoyl chloride [430 μL], or phenylpropionyl chloride [310 μL]). The solution was allowed to stand 30 min (for the acid chloride) or heated on a steam bath 2 h (for anhydride addition).

The solution was diluted with 5 mL water to stop the esterification process, and 50 mL 5% NaOH was added. A reaction mixture (50 mL) was then extracted with 100 mL hexane, and the organic layer was washed 5 times with 25 mL 5% NaOH, then five times with 25 mL 5% H_2SO_4, and finally 5 times with 25 mL water. The extract was then taken to near-dryness.

The extract was cleaned up on an acetonitrile–celite column (10 g Celite 545 with 10 mL acetonitrile). The column was initially washed with hexane (50 mL) before the transfer of the reaction extract dissolved in 10 mL hexane. The column was then washed with six 5 mL portions of hexane.

Each eluate was examined by GC for the presence and purity of the appropriate testosterone ester. Testosterone is retained on the column. Eluates were combined to provide a pure reference standard that was taken to dryness.

Mass spectra were recorded on a Finnigan Model 45A triple stage quadrupole mass spectrometer equipped with an Incos data system.

Operating conditions for the various steroids were as follows: 10 m DB-5 megabore column programmed from 250 to 290°C at 15°C/min with a flow rate of 4 mL/min He; electron energies, 70 eV (electron ionization [EI]) and 100 eV (CI); quadrupole entrance, −5 V; collision energy, −10 V (CAD); source pressure for methane CI, 400 mT; electron multiplier, 1100 V (EI) and 1500 V (CI/CAD); source, 170°C; injection port, 260°C; separator oven, 260°C; MS scan rates, 45–500 daltons in 2 s.

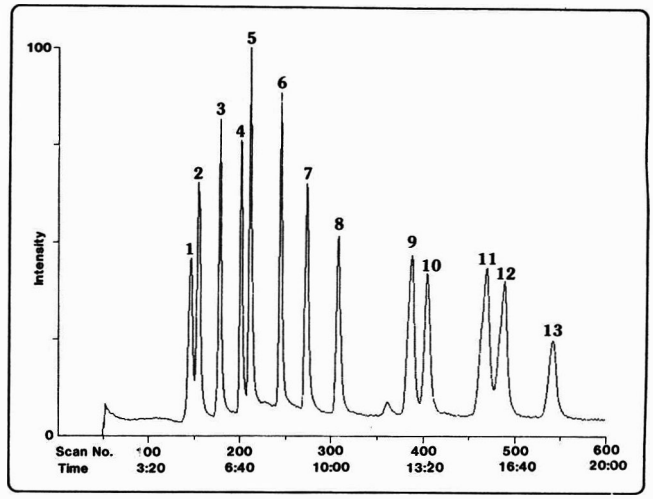


Figure 1. Reference standard mixture of testosterone esters under capillary GC/MS/EI: 1, testosterone (I); 2, methyl testosterone (II); 3, testosterone acetate (III); 4, testosterone propionate (IV); 5, testosterone isobutyrate (V); 6, testosterone valerate (VI); 7, testosterone caproate (VII); 8, testosterone enanthate (VIII); 9, testosterone benzoate (IX); 10, testosterone cypionate (X); 11, testosterone decanoate (XI); 12, testosterone phenylpropionate (XII); and 13, testosterone undecanoate.

Results and Discussion

Gas Chromatography

Separation of the various esters of testosterone was accomplished by using a 10 m DB-5 capillary column operated under a temperature program (Figure 1). These recording conditions were chosen to optimize the separation of a homologous series of testosterone esters that increased in molecular weight by 14 amu. Such an approach would then allow examination of various anabolic steroid products to give an indication of the potential molecular weight composition via retention time. Under the conditions selected, no thermal degradation of these steroids was noticed. Previously, LC was assumed to be the only analysis suitable for such steroids, because they suffered from thermal instability and were sufficiently polar to render GC difficult. From the present work, such conclusions appear to be conservative.

If thermal lability were operative, a concentration dependence of representative spectra throughout the elution profile would have been observed similar to that previously detected with dexamethazone (8). No such variance of the protonated molecule ion in the spectra taken throughout the elution profile was detected. This preliminary screening protocol permitted a survey of the various constituents of the counterfeit products before MS confirmation.

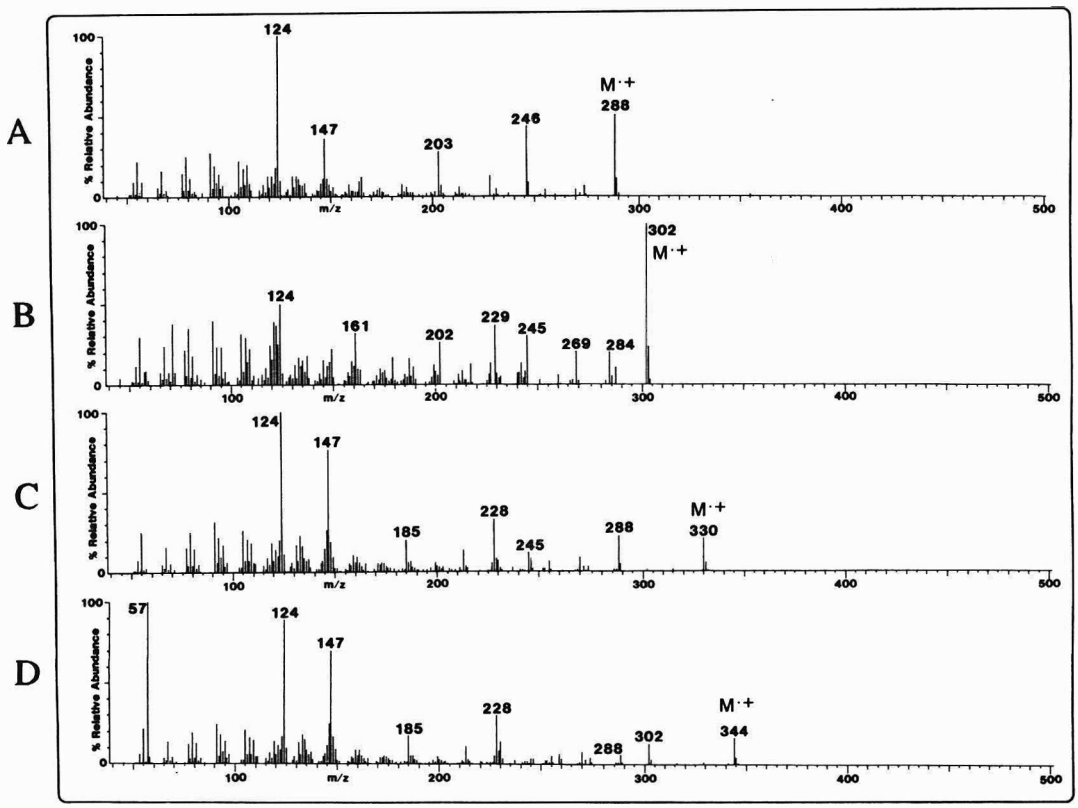


Figure 2. EI mass spectra: A, testosterone (I); B, methyl testosterone (II); C, testosterone acetate (III); and D, testosterone propionate (IV).

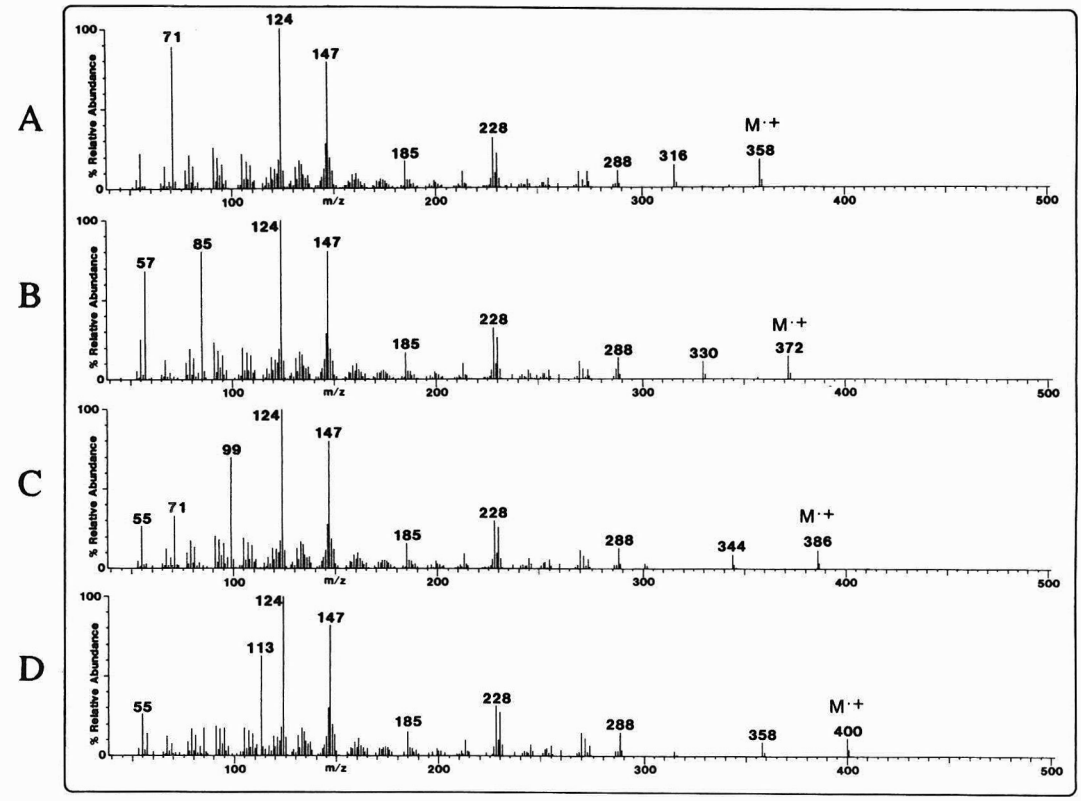


Figure 3. EI mass spectra: A, testosterone isobutyrate (V); B, testosterone valerate (VI); C, testosterone caproate (VII); and D, testosterone enanthate (VIII).

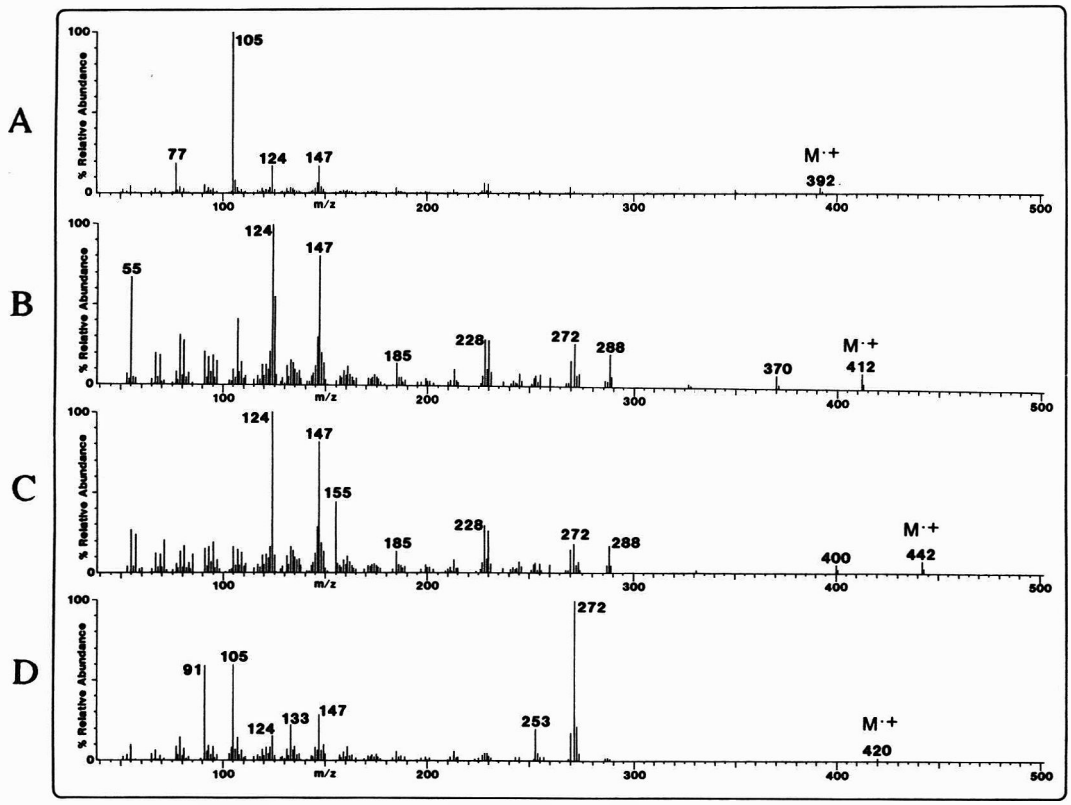
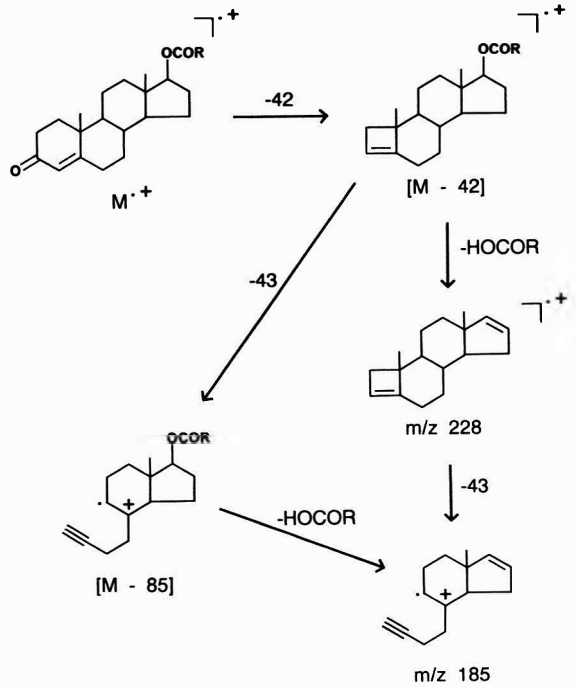


Figure 4. EI mass spectra: A, testosterone benzoate (IX); B, testosterone cypionate (X); C, testosterone decanoate (XI); and D, testosterone phenylpropionate (XII).



Scheme 1. Fragmentation pathway and ion structures for generic fragment ions detected in testosterone esters under EI.

Electron Ionization

The EI spectra of testosterone (I), methyl testosterone (II), and the various testosterone esters (III–XII) are shown in Figures 2–4. A number of general observations can be made. The relative abundance of the molecular ion progressively decreases as the molecular weight of the steroid increases. The overall fragmentation pattern for all compounds examined exhibited a high degree of similarity below *m/z* 245. Two prominent fragment ions seemed to be generic to all the compounds examined, namely *m/z* 124 and 147. From the original study of androst-4-en-3-ones by Shapiro and Djerassi (9), the base peak in the EI spectra at *m/z* 124 was confirmed to result from the fission of the 6–7 and 9–10 allylic bonds with double hydrogen transfer to the charged fragment ion. The appearance, however, of a second prominent fragment ion at *m/z* 147 was not observed in this previous study of various androstenones, perhaps because of different source conditions. However, in the original report on the EI spectrum of testosterone by Spiteller et al. (10), the structure of the ion at *m/z* 147 was thought to be a loss of water from a ring C/D moiety with a ring D exocyclic double bond, i.e., the ion at *m/z* 165.

In this present extended study of testosterone esters, product ion chemistry studies determined the precursor ion of the ions at *m/z* 124 and 147 to be the ion at *m/z* 272, i.e., the ion representing the complete loss of the C-17 substituent ester grouping. Therefore, the fission of the 6–7 and 9–10 bonds can result in the production of 2 prominent fragment ions, *m/z* 124 and 147, depending on the site for charge transfer. Previous conclu-

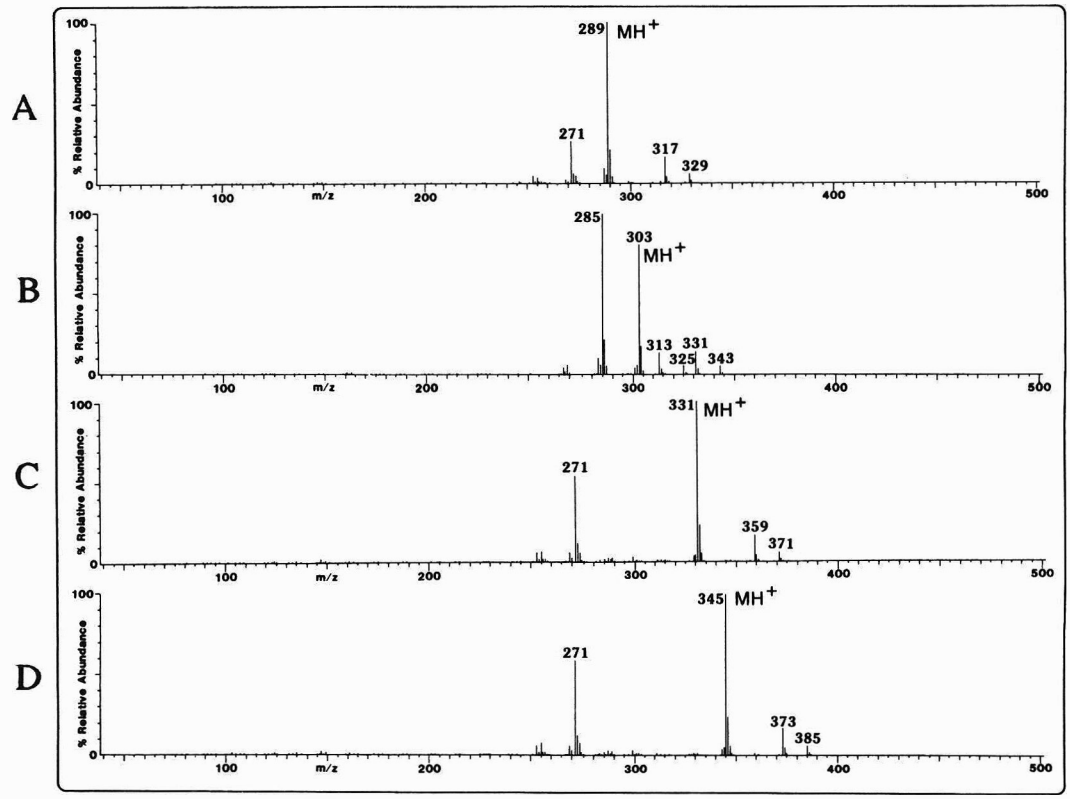


Figure 5. CI mass spectra: A, testosterone (I); B, methyl testosterone (II); C, testosterone acetate (III); and D, testosterone propionate (IV).

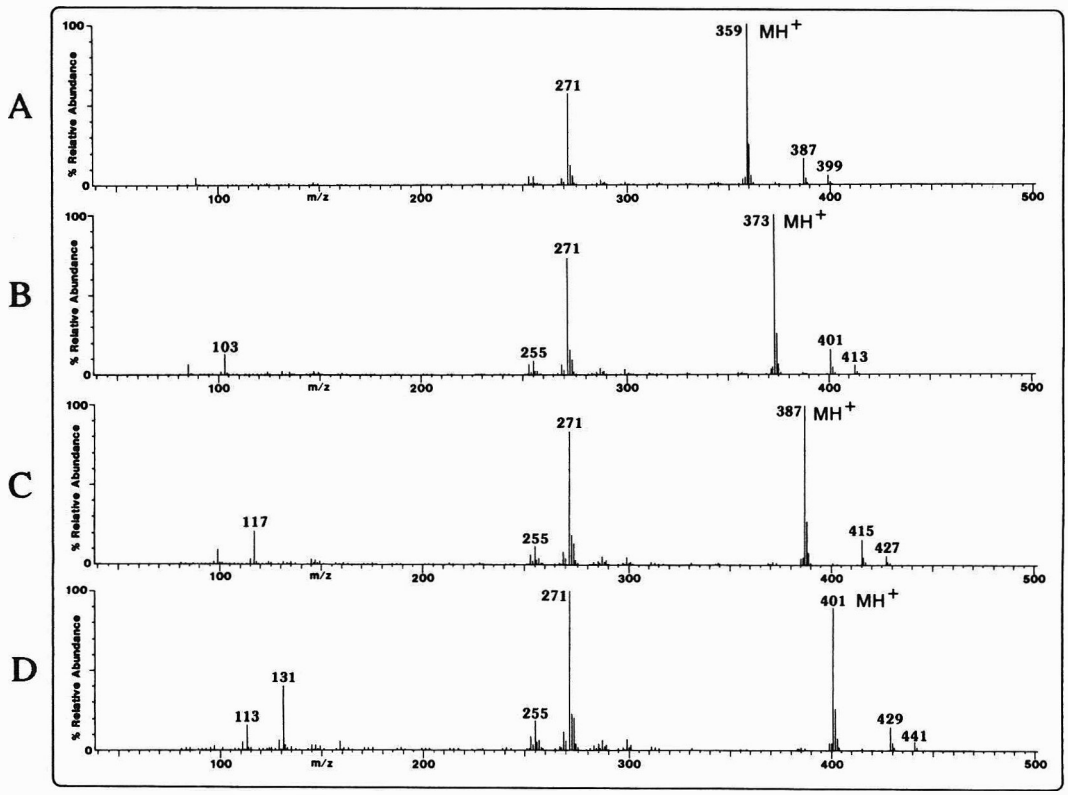


Figure 6. CI mass spectra: A, testosterone isobutyrate (V); B, testosterone valerate (VI); C, testosterone caproate (VII); and D, testosterone enanthate (VIII).

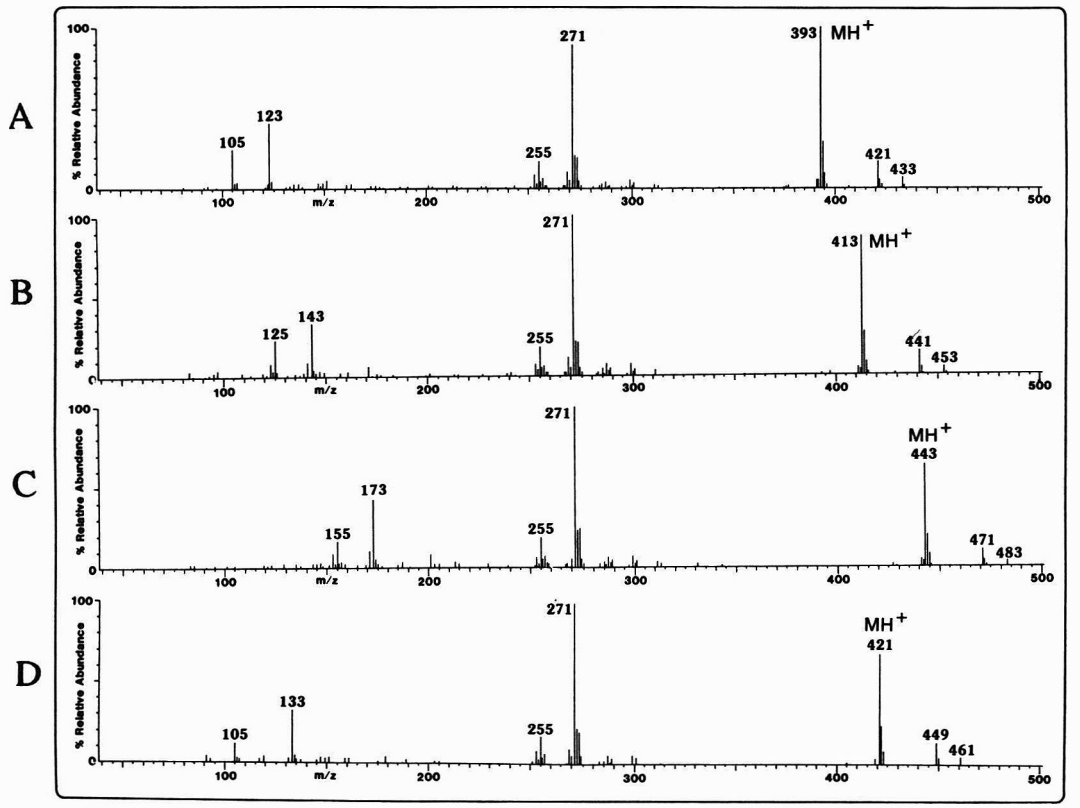


Figure 7. CI mass spectra: A, testosterone benzoate (IX); B, testosterone cypionate (X); C, testosterone decanoate (XI); and D, testosterone phenylpropionate (XII).

sions (10) about the origin of these 2 fragment ions have been modified to reflect the true parentage as the dehydrated testosterone moiety itself, regardless of the substituent group at C-17. The presence of these 2 generic fragment ions can be used to predict confidently the presence of a testosterone ester.

The origin of 2 less abundant generic fragment ions, *m/z* 228 and 185, in the EI spectra of these testosterone esters were investigated. From collision studies, we concluded that *m/z* 185 could be derived via 2 competing pathways. The first route of fragmentation was via ketene loss from ring A, followed by a loss of 43 amu to give an ion corresponding to [M – 85] (Scheme 1), where the ester grouping at C-17 remained intact. This loss of 85 amu was observed in all esters examined. Finally, loss of the ester side chain gave the ion at *m/z* 185. The alternate route to the ion at *m/z* 185 was experimentally established via MS/MS as occurring via a primary ketene loss from ring A, followed by loss of the C-17 ester side chain and fragmentation of ring A-B.

Chemical Ionization Mass Spectrometry

Under methane CI conditions, the various testosterone esters displayed the base peak as the protonated molecule ion together with adduct ions at $[M + 29]^+$ and $[M + 41]^+$ (Figures 5–7). The only fragment ion of sufficient relative abundance for comment was at *m/z* 271, and this ion represented the complete loss of the C-17 ester side chain. Therefore, the CI spectra were relatively featureless for further structural diagnosis. A preliminary study on the product ion spectra of a number of steroids containing the 4-en-3-one system in ring A, together with an angular methyl group at C-10, namely testosterone, testosterone propionate, progesterone, and methyl testosterone, was already reported (11). In that report, 2 principal product ions, *m/z* 97 and 109, were declared to be of important diagnostic value in detecting the presence of such structural systems. We concluded that the structure of both these product ions were derived from ring A via fission of the 6–7 and 9–10 bonds with concomitant transfer of 2 hydrogen atoms originally bound to C–8 and C–11, followed by loss of methyl groups. These preliminary findings suggested that observation of these 2 product ions, *m/z* 109 and 97, could be regarded as highly characteristic for the stereochemistry of ring A.

The present study extended that finding to the 13 testosterone esters under investigation (I–XIII) and verified that such product ions were highly characteristic of the testosterone moiety whatever the substituent side chain at C-17.

Reaction Monitoring Protocol

To make use of these generic product ions observed for all testosterone esters, a reaction monitoring protocol was developed to screen samples for the presence of anabolic steroids while effectively ignoring all other constituents. This experimental approach adopted the logic that, in a triple quadrupole instrument, the collision experiment should be able to pinpoint anabolic steroid components in a complex mixture by permitting only the detection of the responsible precursor ions of the 2 selected generic products ions, *m/z* 109 and 97. Results for

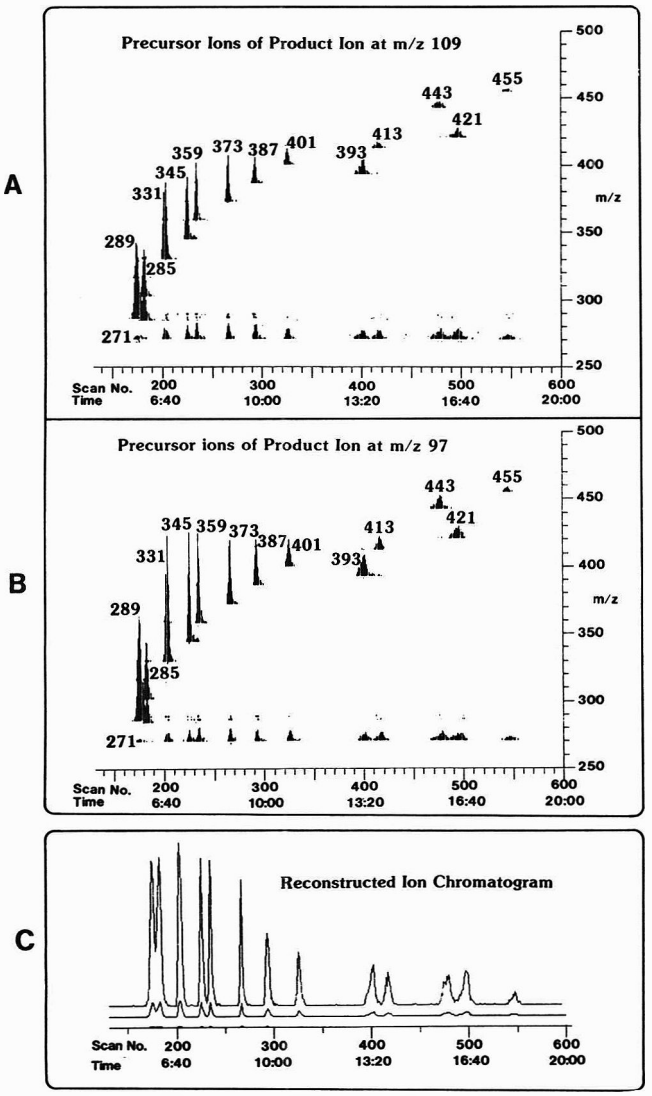


Figure 8. Reaction monitoring profiles under MS/MS: A, precursor ions detected as parents of product ion at *m/z* 109; B, precursor ions detected as parents of product ion at *m/z* 97; and C, reconstructed ion chromatogram.

such an experiment performed on the reference mixture (Figure 1) are illustrated in Figure 8. Clearly, the precursor ions responsible for production of the product ions *m/z* 109 and 97 are the protonated molecule ions representing the various testosterone esters. A minor contributing precursor ion to these product ions was at *m/z* 271, representing the steroid structure after the loss of the C-17 side chain. The lower detection limit established for this method of monitoring for anabolic steroids was experimentally established at 1 ng injected on-column (signal-to-noise ratio, 10:1; with RSD = 5%).

Conclusion

These studies provided a number of optional approaches to the detection and confirmation of anabolic steroids in pharmaceutical preparations derived from both bonafide and clandestine sources. Under EI conditions, full mass spectral scans can easily indicate the presence of a testosterone ester by observing 2 principal generic fragment ions at *m/z* 124 and 147, as well as a number of lesser abundant fragment ions at *m/z* 185, 228, and 288. As the molecular weight of the steroid increases, the relative abundance of the molecular ion decreases; also, its presence in the spectrum may be difficult to verify when the ester side chain contains more than 4 carbons. Because these fragment ions are common to all testosterone esters studied, the presence of the molecular ion and the appropriate retention time are necessary before a confirmation could be considered acceptable for legal purposes. Under CI conditions, the presence of the protonated molecule ion is the dominating feature. An increased presence of the ion at *m/z* 271 represents the testosterone structure after loss of the ester side chain.

Clearly, such structural evidence derived from 2 ions, together with the appropriate retention time, could be considered strong evidence for proof of presence. Therefore, 2 generic product ions (*m/z* 97 and 109) derived from the protonated molecule ion are necessary for confirmation of presence. The use of this combination of protonated molecule ion and product ions can be streamlined into a reaction monitoring protocol whereby counterfeit preparations of testosterone esters can be screened for anabolic steroids while providing all the necessary data for confirmation.

This approach may provide a potential experimental approach for biomedical matrixes such as blood and urine where sample cleanup can be minimized or even ignored before analysis.

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DRUGS

Determination of Residual Ethylene Oxide in Spectinomycin Hydrochloride Bulk Drug by Dynamic Headspace Gas Chromatography

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A dynamic headspace gas chromatographic method was developed for the determination of residual ethylene oxide (EtO) in a pharmaceutical bulk drug, spectinomycin hydrochloride. The recommended column is PoraPLOT Q; PoraPLOT U was demonstrated to be equivalent. A detection limit of 2 ppb was achieved, and linearity was established to the highest value tested (5 ppm). The detection limit was 2–3 orders of magnitude lower than that achieved in previously published methods for EtO in pharmaceutical bulk drugs. Precision studies yielded relative standard deviations ranging from 2 to 10% over the 10–575 ppb concentration range. The method was applied to support EtO sterilization studies conducted at The Upjohn Company and was implemented for routine use. The sterilization studies demonstrated that residual levels of EtO <10 ppb are achievable.

Historically, ethylene oxide (EtO) was widely used in the sterilization of pharmaceutical bulk drugs. Because of concerns over the toxicity and potential carcinogenicity of EtO, however, its use as a sterilizing agent is being viewed with increasing disfavor by European regulatory agencies. Several countries stipulated maximum allowable residual levels for EtO in bulk drugs. These concerns have culminated in regulatory actions by the Federal Health Office in Germany banning the use of EtO for the sterilization of pharmaceutical starting materials. Further, work is also underway on a European Economic Community Regulatory Guideline that would implement similar regulations throughout the European Community. As a result of this heightened regulatory climate, pharmaceutical companies are exploring alternative sterilization measures for bulk drugs. Foremost among these are the application of terminal heat, gamma irradiation, and aseptic processing.

Because of the time required to develop and validate alternative sterilization procedures, a rapid switchover for drugs that are unstable to heat or radiation is unrealistic. Nevertheless, for EtO to gain continued acceptance until alternative sterilization procedures can be implemented, it is imperative that residual EtO be reduced to the lowest practical levels. Accordingly, The Upjohn Company embarked on studies to minimize residual EtO levels in all products for which alternative sterilization procedures are not currently available.

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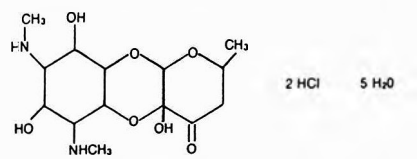
For these efforts to succeed, suitable analytical methodology must be available for measuring low concentrations of EtO. At present, suitable analytical methodology does not exist for measuring EtO in bulk drug at concentrations below about 1 ppm. In this report, we describe a method for trace determination of residual EtO in the antibiotic spectinomycin hydrochloride (Sp·2HCl). Because of improved sterilization technology for this drug achieved at Upjohn, a procedure capable of detecting EtO to the low parts per billion (ppb) level was needed.

The structure of Sp·2HCl is shown in structure 1. Sp·2HCl is produced by fermentation by the soil microorganism *Streptomyces spectabilis* and is the active ingredient in the Upjohn product Trobicin®. The primary indication for Trobicin is gonorrhea.



Structure 1.

Most modern methods for the determination of EtO are based on gas chromatography (GC), usually combined with solvent extraction, headspace sampling, vacuum extraction and distillation, steam distillation, or chemical derivatization. Various procedures were developed for determining EtO jointly with its 2 reaction products, ethylene chlorohydrin and ethylene glycol (1–4). Most of these procedures rely on extraction followed by direct injection. Derivatization permits indirect, sensitive detection of EtO by electron capture GC, as was demonstrated for intraocular lenses (5), foods (6), and air (7). Packed-column GC procedures were developed by Hartman and Bowman (8) for the determination of EtO in both water-soluble and insoluble pharmaceutical bulk drugs.

Most newer methods for EtO use headspace sampling. Kaye and Nevell (9) presented 2 methods based on static headspace GC for the determination of EtO in surgical materials. Margeson et al. (10) devised a procedure in which Carbopack B is used to quantitate EtO in emissions from commercial sterilizers (10). A definitive method for the determination of monomeric EtO in pharmaceutical raw materials produced totally or partly from EtO was developed by the TEGEWA Association in Germany (11). This method, using capillary GC, is applicable over the 0.5–2.5 µg/g concentration range. To our knowledge, no methods have been published for the determination of EtO at low ppb levels in pharmaceutical bulk drugs. We describe such a method in this paper.

Experimental

Dynamic headspace GC (or purge/trap GC) was used in this work. The GC was an HP 5890A with flame ionization detection. A Tekmar LSC 2000/2016 (fully automated) with 16 vessel capability served as the purge/trap instrument. A companion capillary cryofocusing unit was used to focus thermally desorbed volatiles onto the GC column. A block diagram of the experimental arrangement used is shown in Figure 1. The developed method specifies either of 2 porous layer open tubular (PLOT) columns, 27.5 m × 0.32 mm id, each with an appended 2.5 m particle trap. Initial development work was conducted on an $R_t x - 5$ column (Restek, Bellefonte, PA), 30 m × 0.32 mm, with d_f = 3.0 µm. Later work was conducted on a PLOT column, PoraPLOT Q (Chrompack, Raritan, NJ), containing a 10 µm thick coating of particles. Equivalent performance was demonstrated on a PoraPLOT U column (Chrompack).

Chemicals

Lecture bottles each equipped with a control valve and containing 0.5 lb (0.227 kg) of 99.7% pure liquid EtO were purchased from either Matheson Gas Products or AGA Specialty Gas. Bottles were stored in a freezer to inhibit polymerization. All water was high-purity (Milli-Q or NANOpure), additionally boiled to remove a potential interferant for EtO.

Standards Preparation

Standard stock solutions were prepared either by (*1*) bubbling EtO at a moderate rate for 2–3 min into a chilled, tared 25 mL volumetric flask containing ca 20 mL water, then bubbling in 0.25–0.5 g gas, or (*2*) transferring liquid EtO into a vial with a Teflon-lined septum cap and then removing 0.25–0.5 g liquid EtO with a gas-sampling syringe and transferring it to a 25 mL volumetric flask as in (*1*). The concentration of EtO in the stock solution was calculated by weight difference. Successive serial dilutions were made with high-purity water. Concentrations of the diluted standards ranged from ca 0.1 to 1000 ng/µL. These operations were performed as carefully and rapidly as possible because of the toxicity and volatility of EtO. The resulting standard solutions were transferred to 1 dram vials containing Teflon-lined septum caps. Vials were filled to overflowing to avoid headspace and stored in a refrigerator. To maintain 90% potency of these preparations, new standards were prepared every 3 weeks. The accuracy and reproducibility of the standards preparation are attested to by the equivalent results obtained in an interlaboratory comparison in which standards were prepared independently (*vide infra*).

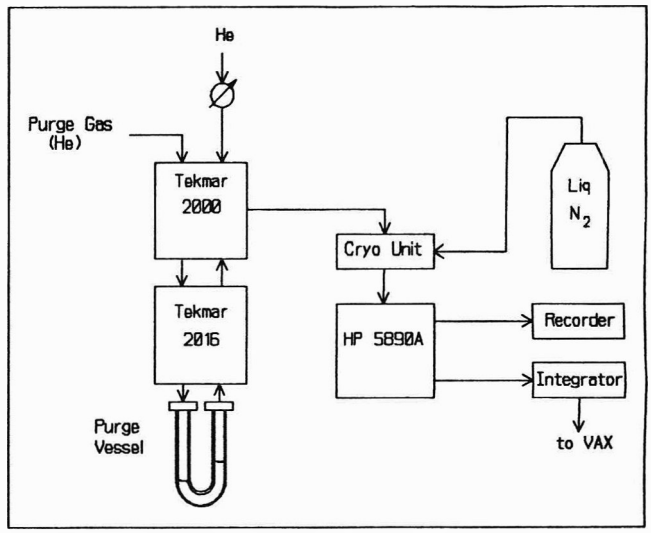


Figure 1. Block diagram of the experimental arrangement.

METHOD

Blank

Because this method is designed to detect EtO at the low ppb level, a clean blank must be obtained. Further, blanks should be interspersed with samples and standards to ensure the integrity of the chromatography. As noted above, purified water was additionally boiled to eliminate a potential unidentified interferant for EtO. This boiled water served as the blank.

Sample Preparation

Bulk drug samples were delivered to the laboratory in bags (usually) or jars as quickly as was feasible to avoid loss of EtO. If samples cannot be analyzed upon delivery, they should be stored in a freezer. Sample preparation consists of dissolving Sp·2HCl bulk drug in boiled water in a volumetric flask at a concentration of 0.1 g/5 mL. Jars or bags containing bulk drug were agitated, and the powder was then weighed to the nearest 0.1 mg; these operations should be performed as quickly as possible. Solutions of bulk drug do not lose measurable EtO, either through volatilization or reaction, within 24 h (not tested for a longer time).

Samples were introduced into the purge vessel via a 3-port valve using a 5 mL hypodermic syringe (preferably gas-tight). The volumetric flask was first inverted to recapture any EtO that had partitioned into the neck of the flask. The solution was poured into the syringe to overflowing, the plunger was inserted, and excess solution was expelled until 5 mL remained. For standards, the same procedure was followed using water in

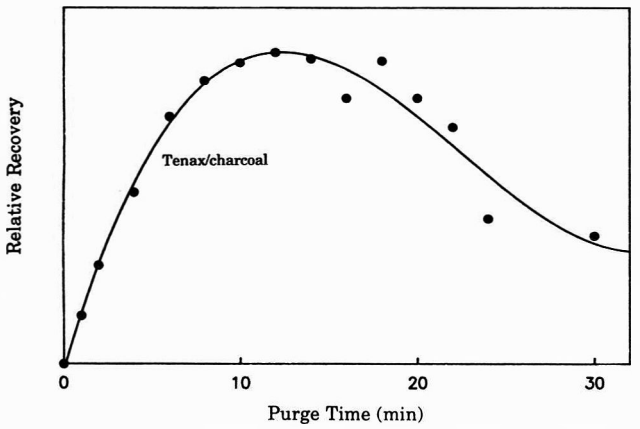


Figure 2. Relative recovery vs purge time for EtO on Tenax/charcoal.

lieu of the sample, and 1–10 µL of an appropriate standard was added from a 10 µL syringe through the Luer tip of the 5 mL syringe. Conventional, nonfritted, 5 mL purge vessels ("needle spargers," Tekmar) were used throughout.

All concentrations stated in this report are referenced to the bulk drug; this also applies to standards run without bulk drug. The procedure given above specifies that 100 mg bulk drug be dissolved in 5 mL water, then purged. Hence, the weight/volume (w/v) concentration of EtO in solution is 1/50 of the w/w concentration calculated relative to bulk drug.

Purge/Trap and GC Conditions

A series of optimization studies was conducted to determine the purge/trap conditions. The sample was stripped by using high-purity (99.999%) helium gas that had been further purified by passage through copper coils immersed in liquid nitrogen. Purges were conducted at room temperature for 12 min at a flow rate of 40 cm^3/min. The stripped compounds were trapped on Tenax/charcoal (Tekmar trap No. 4). Thermal desorption was conducted at 190°C for 3.5 min onto PoraPLOT Q (or U) cooled to –75°C. The cryofocused volatiles were subsequently injected (flash-heated) into the GC at 200°C (duration, 1 min). A trap bake at 225°C for 15 min was also included in each cycle to maintain a clean trap. Valve, transfer line, and capillary union temperatures were maintained at 130, 130, and 110°C, respectively. The PoraPLOT columns, 27.5 m × 0.32 mm, with a 2 m particle trap appended, were operated at a flow rate of 1.9 cm^3/min. A typical GC temperature cycle (for PoraPLOT Q) consisted of an initial 6 min isothermal period at 100°C, followed by a rapid ramp (10°C/min) to 180°C, and a subsequent hold to remove other volatiles before the next run. A beta version of Dry Lab GC, a GC optimization program developed by LC Resources (Lafayette, CA), was found to be helpful in arriving at GC conditions.

Results and Discussion

A trap containing an admixture of Tenax and charcoal (trap No. 4 supplied by Tekmar) proved effective in trapping EtO. Tenax GC, commonly used in purge/trap analysis, is unsuited for EtO because of premature breakthrough. A recovery-time curve for EtO on Tenax/charcoal is shown in Figure 2. There is some scatter to the data starting at 16 min, where breakthrough is first noted. The scatter is a consequence of a broad breakthrough profile that is not precisely repeatable. Although absolute recovery was not determined (by comparison with direct injection), the plateau region of the curve in Figure 2 is taken to signify complete recovery. Supporting evidence for this interpretation is provided by the observation that recovery was enhanced 30% by a 4 min purge conducted at 50°C compared to ambient temperature, whereas a 12 min purge at 50°C gave no enhancement. In practice, recovery is of little consequence; repeatability, regardless of absolute recovery, is the critical figure of merit. On the basis of the curve of Figure 2, a 12 min purge time was selected.

The sample was purged at room temperature. We noted in our studies that the purgeability of EtO from water (no bulk drug present) is only slightly enhanced by temperature. This is consistent with the modest enhancements reported by Ramstad and Nestrick (12) for other water-soluble compounds. In the present work, however, far greater *apparent* enhancements were observed for EtO by purging at elevated temperature (55°C) from solution containing bulk drug. Further investigation led to the conclusion that EtO was formed in situ at the higher temperature from the dehydrochlorination of ethylene chlorohydrin (ECH, 2-chloroethanol); ECH itself formed from the reaction of EtO with inorganic chloride (HCl in this case). Although the dehydrochlorination of ECH in alkaline media is well known (13), our observation indicates that this reaction also proceeds to a slight extent at lower pH (pH 5) at elevated temperature, although not at ambient temperature. These experimental results suggest that in situ generation of EtO from ECH followed by purge/trap analysis could provide an indirect means of determining ECH at low levels.

Method Evaluation

Linearity

The method is linear from the limit of detection (2 ppb) to >5 ppm, which is the highest concentration tested (r = 0.99995). The high sensitivity afforded by the purge/trap technique is not required at concentrations above about 1 ppm. The slope of the response-concentration curve was the same for EtO purged from water or from an aqueous solution of bulk drug, demonstrating that purgeability is neither attenuated nor enhanced by bulk drug. Percent recovery for bulk drug fortified with EtO over the 2–1150 ppb concentration range is given in Table 1. Recoveries were determined relative to a 23 ppb standard, which is the concentration of standard adopted for this assay. A calibration curve for these data over the most relevant concentration range, 2–115 ppb, is shown in Figure 3. A correlation coefficient of 0.999 was obtained for this plot. The slight positive intercept stems from a significant positive bias at 2.3 ppb and a smaller positive deviation at 11.5 ppb. The intercept does not differ statistically from zero at the 95% confi-

Table 1. Recovery of EtO added to bulk drug

EtO added, ppb	EtO rec., ppb	Rec., %
2.30	3.27	142
5.75	5.94	103
11.5	12.9	112
23.0	23.0[a]	100[a]
57.5	59.7	104
115	116	101
230	230	100
575	588	102
1150	1200	104

[a] Recoveries measured relative to 23.0 ppb.

dence interval. Similarly, the slope is not significantly different from 1.

Precision

Precision studies were conducted both with and without the benefit of the ALS 2016 autosampler. In manually conducted experiments, relative standard deviations (RSDs) of 9.0 and 4.0% were obtained by area for bulk drug containing 30 and 360 ppb EtO, respectively. Eight replicate runs were made at the lower concentration and 10 at the higher. Corresponding values obtained by using fully automated equipment (ALS 2016) are given in Table 2. Either peak height or peak area may be used for quantitation, although area gave slightly better precision. The precision is comparable at the 2 higher concentrations (115 and 575 ppb). Although for this brief study the precision was best at the lowest concentration (2.5% at 12 ppb), we do not claim enhanced precision at the lowest levels.

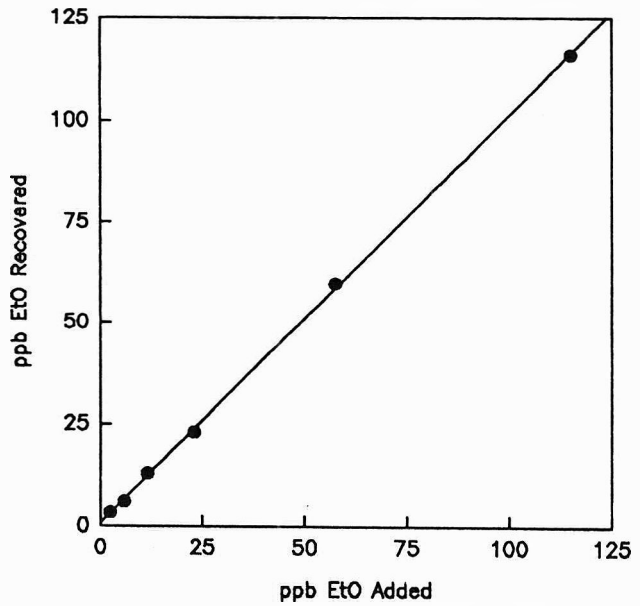


Figure 3. Calibration/recovery curve for EtO from fortified bulk drug solution.

Table 2. Precision of EtO assay

EtO concn, ppb	Area	Ht	No. runs	RSD, %
12	X		6	2.5
12		X	6	2.5
115	X		6	4.2
115		X	6	6.6
575	X		7	3.7
575		X	7	4.0

In a separate, automated, 2-day precision study, EtO-free bulk drug was fortified with 23 and 115 ppb EtO and each was run 6 times on each day. An RSD of 4.9% was obtained at 23 ppb on day 1 and 3.7% on day 2. The 2-day interday precision was 4.4% (interday RSD defined according to reference 14). At 115 ppb the RSD was 2.0% on day 1 and 2.3% on day 2, for a 2-day precision of 2.1%. At concentrations of 10 ppb and below, the RSD may approach 10% (no data shown). Hence, over the entire applicable range, precision is 10% or better; above approximately 10 ppb, a precision of 5% or better may be expected. In an interlaboratory comparison, application of F- and Student's *t*-tests revealed no statistical difference between results (95% confidence interval) at residual EtO concentrations of 10 and 98 ppb.

A brief intertrap precision study was conducted as part of method ruggedness testing. Six traps were evaluated, 1 on each of 6 consecutive days. Four of the traps had not been used previously. Six analyses were conducted for each trap for bulk drug containing 40 ppb EtO. RSDs of 5.4, 1.7, 4.8, 3.6, 8.0, and 7.8% were obtained for an intertrap RSD of 5.7%. Whether the last 2 traps would have yielded better precision after a "settling in" time is unknown.

Chromatography

Illustrative chromatograms on PoraPLOT Q are shown in Figures 4–6. A blank, with the position of EtO marked, is seen in Figure 4a. The blank was high-purity water that was additionally boiled. The peak preceding EtO is due to acetaldehyde. The source of acetaldehyde is believed to be 3A alcohol, which is used as a rinse solvent in the laboratory, and which contains acetaldehyde as an impurity. A trace amount is presumed to leak into the system during the purge step. Acetaldehyde serves as a convenient marker for EtO and also was designed into a system suitability test. The identity of other peaks is unknown. In assaying for EtO in Sp·2HCl bulk drug, an approximately 23 ppb standard is used (external standard procedure). A chromatogram of a standard is shown in Figure 4b. A sample of spectinomycin sterilized with EtO by the "old process" is shown in Figure 4c; the peak corresponds to 98 ppb residual EtO. Figure 5 shows (a) a blank and (b) a sample sterilized by the new process; in this case, the peak corresponds to 6 ppb residual EtO. Detailed analysis revealed a routinely obtainable detection limit of 2 ppb for this procedure; the detection limit is defined as the total signal, S_t, minus the blank contribution, S_b, equal to 3 times the standard deviation of the blank (15). A

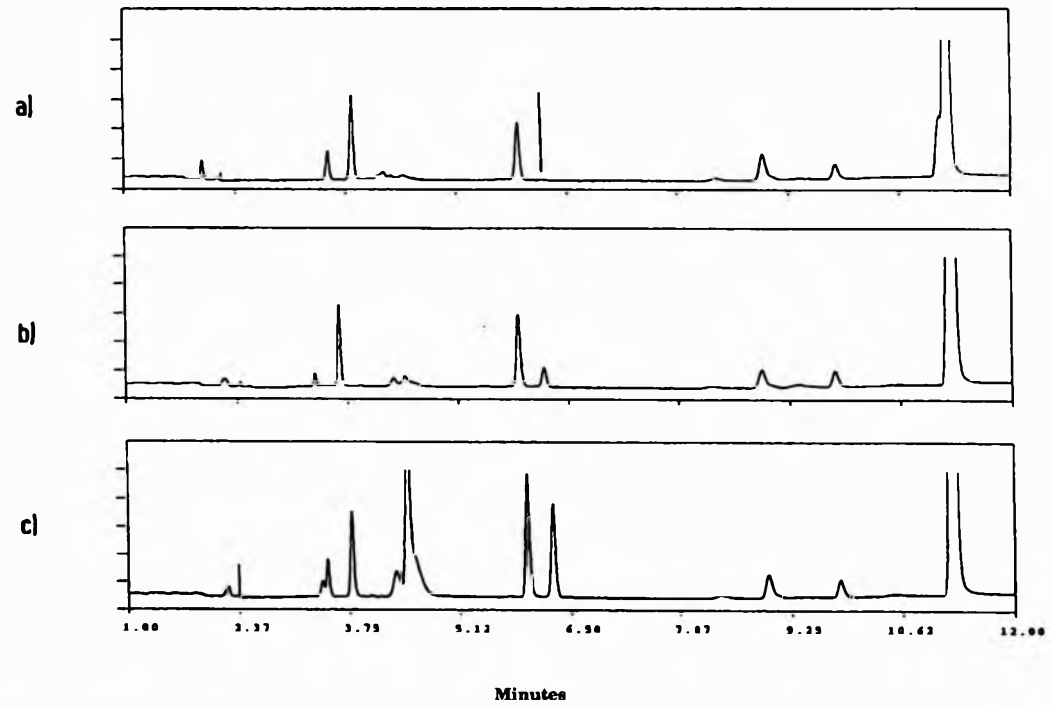


Figure 4. Chromatograms obtained on PoraPLOT Q for (a) blank, (b) 24 ppb standard, and (c) sample containing 98 ppb EtO. The position of EtO is marked by the arrow.

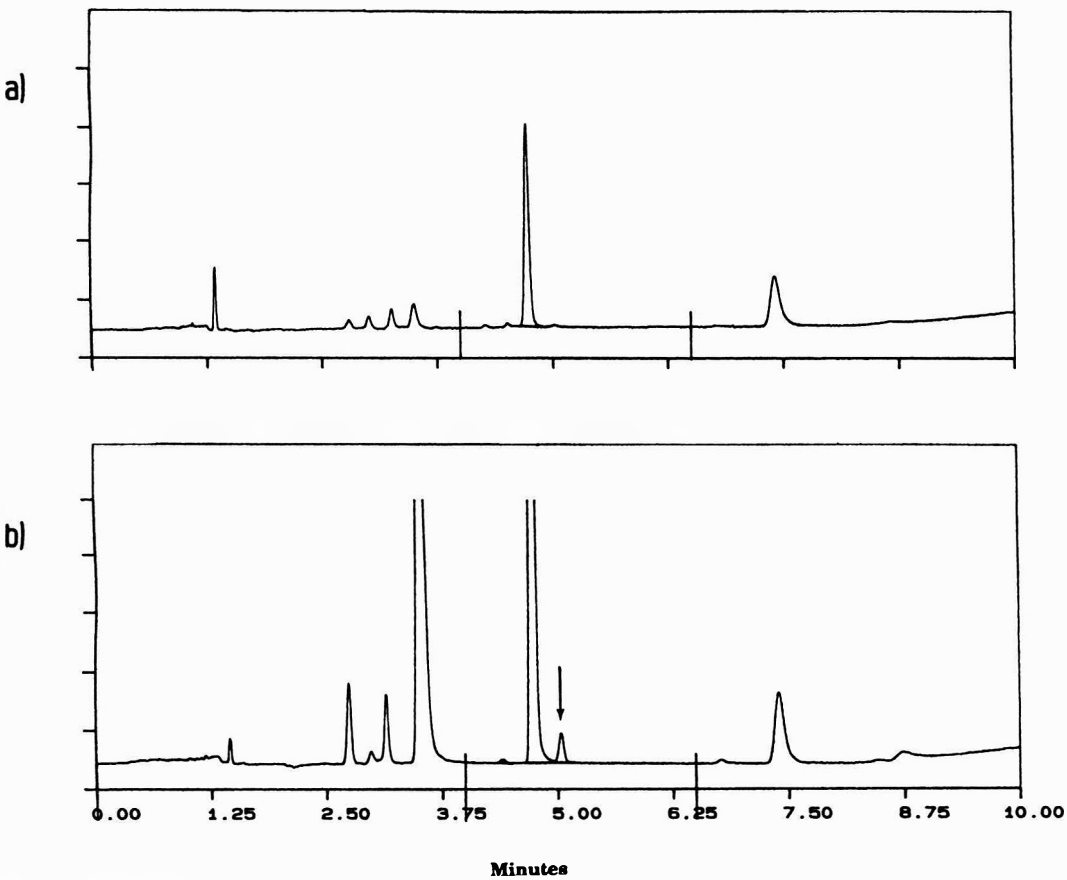


Figure 5. Chromatograms obtained on PoraPLOT Q for (a) blank and (b) sample containing 6 ppb EtO that was sterilized by the new process.

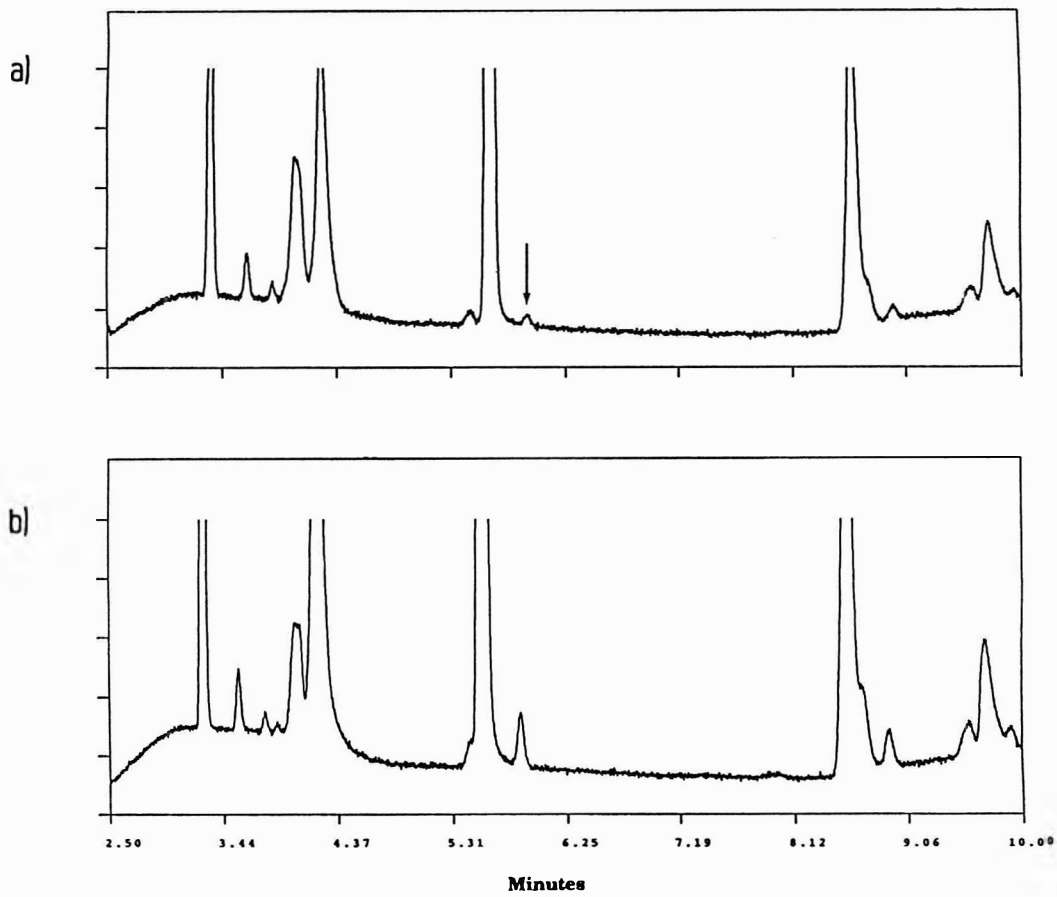


Figure 6. Chromatograms obtained on PoraPLOT Q (a) for blank and (b) at the detection limit (2 ppb).

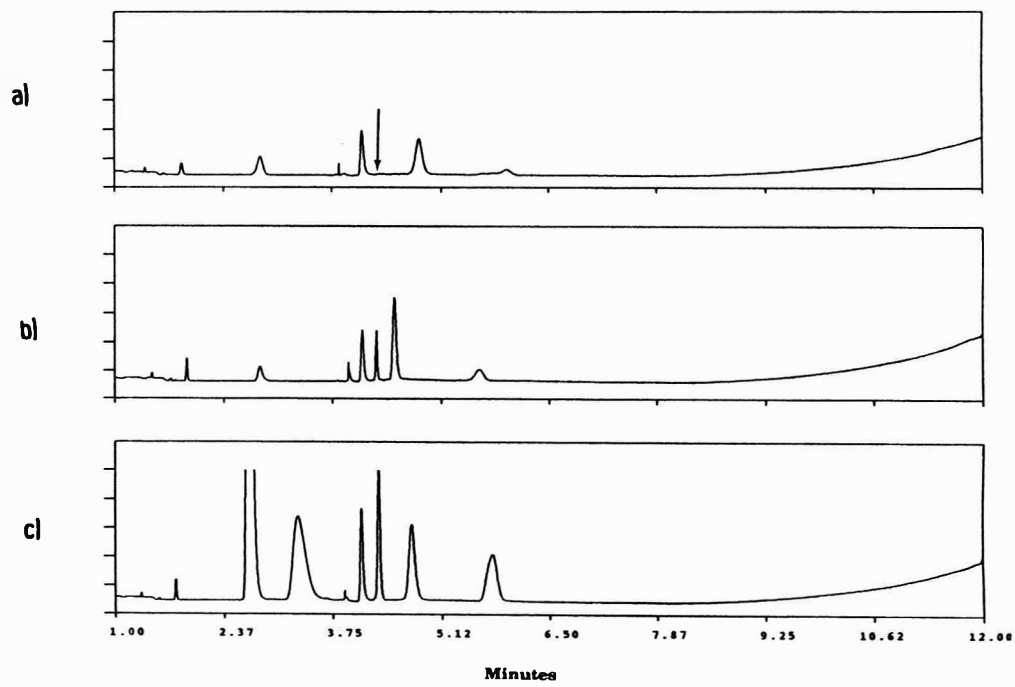


Figure 7. Chromatograms obtained on PoraPLOT U for (a) blank, (b) 24 ppb standard, and (c) sample containing 90 ppb EtO.

Table 3. Residual EtO in EtO-sterilized spectinomycin hydrochloride

Sample[a]		EtO concn, ppb
1	a	7
	b	7
	c	5
	d	6
	e	1
	f	1
	g	1
	h	1
2	a	6
	b	2
	c	2
	d	3
	e	2
3	a	2
	b	3
	c	2
	d	3
	e	2
4	a	5
	b	4
	c	2.5
	d	5
	e	4

[a] The letter designations refer to sampling sites within a sterilized bag.

chromatogram at the detection limit, along with the corresponding blank, is shown in Figure 6.

In the event that an interference could preclude low-level quantitation of EtO on PoraPLOT Q, an alternative (contingency) column was sought. Figure 7 shows (a) blank, (b) standard (24 ppb), and (c) sample (90 ppb) chromatograms for EtO obtained on a PoraPLOT U column. The difference in selectivity compared to PoraPLOT Q (Figure 4) is apparent. In comparative testing, PoraPLOT U proved equivalent to PoraPLOT Q for the assay of EtO. A third PLOT column manufactured by Chrompack, PoraPLOT S, was also evaluated; its selectivity is similar to that of PoraPLOT U. However, this column is not currently available in a 0.32 mm id format.

Quantitation of EtO in Bulk Drug

Table 3 gives results obtained for residual EtO in spectinomycin bulk drug sterilized under various conditions in a sterilization optimization study (new process optimization). To our knowledge, no previously published method allows the determination of EtO in a pharmaceutical bulk drug at the levels shown in Table 3.

Conclusion

A dynamic headspace GC method was developed for the determination of residual EtO in Sp·2HCl bulk drug. Extensive validation studies were conducted on R_tx – 5 and PoraPLOT Q columns, with the latter the recommended column. Assay equivalence was further demonstrated for PoraPLOT Q and PoraPLOT U. Relative precision ranged from about 2 to 10% over the 10–575 ppb concentration range. Linearity was demonstrated from the limit of detection (2 ppb) to the highest value tested (>5 ppm). This new methodology provides a detection limit for EtO that is 2–3 orders of magnitude lower than that previously obtained for a water-soluble pharmaceutical bulk drug.

Acknowledgment

We thank K. Mills for his contributions to this work.

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DRUGS IN FEEDS

Liquid Chromatographic Method for the Simultaneous Analysis of Sulfadimethoxine and Ormetoprim in Animal Feeds

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An analytical method was developed to simultaneously determine sulfadimethoxine (SDM) and ormetoprim (OMP) in medicated animal feeds. SDM and OMP are extracted from the feed into methylene chloride at pH 10 following the addition of tetrabutylammonium hydroxide to form an ion pair with SDM. After the methylene chloride extract is dried over sodium sulfate, an aliquot is purified by solid-phase extraction using a PrepSep silica extraction column. Both compounds are separated by liquid chromatography on an Ultrasphere ion-pair column at 30°C with UV detection at 275 nm. The average recoveries for OMP and SDM in feeds fortified between 0.01 and 0.5% total combined drug ranged from 96.4 to 101.4%, respectively. Replicate assays of typical feeds yielded coefficients of variation of 1.7–3.1%.

The antimicrobial sulfadimethoxine (SDM) and the potentiator ormetoprim (OMP) (Figure 1) are an approved drug combination for use as an antibacterial and coccidiostat in poultry (in a 5:3 ratio of SDM:OMP called Rofenaid^{R}) and catfish, trout, and salmon (in a 5:1 ratio of SDM:OMP called Romet^{R}).

The presently accepted methods for the determination of SDM and OMP in animal feeds are based on colorimetric (1) and spectrofluorometric (2) assays, respectively. These methods lack specificity, require each drug to be assayed individually, and include extensive cleanup and derivatization steps.

Our objective was to develop a more convenient means to assay feed samples for SDM and OMP at use levels (0.01–0.08% for Rofenaid and 0.25 to 0.5% for Romet). Previously, we reported a liquid chromatographic (LC) method for the simultaneous determination of SDM and OMP in blood and tissues of various species in which both compounds are simultaneously extracted with methylene chloride at pH 10 (3). OMP is extracted because it is basic and is in the nonionized state at pH 10. To extract SDM as well, which is amphoteric and is ionized at pH 10, tetrabutylammonium hydroxide is added to form an ion pair that preferentially partitions into the organic phase. Both compounds are then simultaneously quantitated by normal-phase LC using a silica gel column and a mobile phase consisting of chloroform–methanol–water–ammonium hydroxide in a 1000 + 28 + 2 + 0.6 ratio.

This method probably could have been adapted to the assay of feeds; however, our aim was to use as little potentially hazardous solvents as possible by eliminating the use of methylene chloride in the extraction step and chloroform in the LC analysis (preferably by going to an aqueous, reversed-phase system). We became aware of a recently published (4) method describing the use of a Beckman Ultrasphere C_{18}-Ion Pair column with a mobile phase consisting of acetonitrile–methanol–0.1M phosphate buffer, pH 4.0, for the analysis of SDM and OMP residues in salmon muscle tissue. We found that a modification of this mobile phase (the addition of the ion-pair reagent heptanesulfonic acid sodium salt and a different proportion of organic solvents to buffer) gave good chromatography of SDM and OMP. For the extraction step, we tried several solvent systems (e.g., aqueous methanol, acetonitrile, acetonitrile–trichloroacetic acid), but none was satisfactory. Either the extraction efficiencies were not adequate or the extract could not be cleaned up adequately for LC. To obtain a satisfactory assay for both compounds, we went back to the use of an extraction procedure similar to the one we used previously for the assay of SDM and OMP (3), and we added a solid-phase cleanup step before LC analysis. A procedure for the simultaneous quantitation of SDM and OMP in feed medicated at use levels is described.

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METHOD

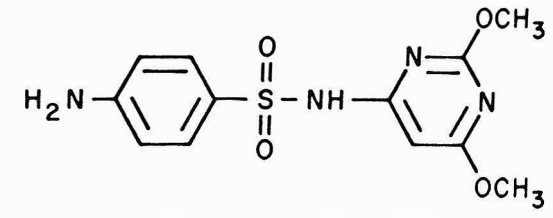
Apparatus

(a) *Liquid chromatograph.*—Pump, Model 510 (Waters); autosampler, WISP Model 712 (Waters); UV detector, Model 785A set at 275 nm (Applied Biosystems); chromatographic data system, Maxima 820, version 3.3 (Dynamic Solutions, Waters).

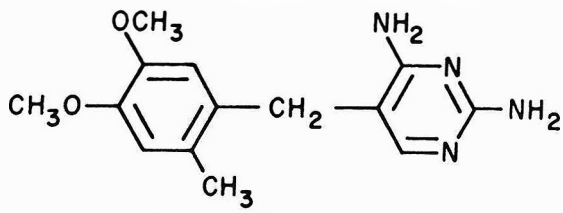
(b) *LC column.*—Ultrasphere C_{18}-IP, 5 μm, 15 cm × 4.6 mm (Beckman Instruments, Inc.) with LC precolumn filter, 2 μm (No. 84560, Waters).

(c) *Column heater.*—Temperature Control System, set at 30°C (Waters).

(d) *Centrifuge tube.*—100 mL, round-bottom, Pyrex (No. 8240, Corning).



SULFADIMETHOXINE



ORMETOPRIM

Figure 1. Structures of sulfadimethoxine and ormetoprim.

(e) *Homogenizer.*—Polytron Model PT10/35 with PTA 10S generator (10 mm diam., Brinkman Instruments, Inc.).

(f) *Solid-phase extraction vacuum manifold.*—Spe-ed Mate-30 (Applied Separations, Inc.).

(g) *Solid-phase extraction column.*—PrepSep Silica, 300 mg (Fisher Scientific).

(h) *pH meter.*—Accumet Model 610A with pH electrode (Fisher Scientific).

Reagents

Reagent grade chemicals are used throughout method, unless stated otherwise.

(a) *Rofenaid-40 (15% OMP and 25% SDM) and Romet-30 (5% OMP and 25% SDM) premixes.*—For sample fortification (Hoffmann-La Roche, Nutley, NJ).

(b) *Buffer solution, pH 10.0.*—0.05M Potassium carbonate–potassium borate–potassium hydroxide buffer (Fisher Scientific).

(c) *Tetrabutylammonium hydroxide (TBAH), 0.4M.*—LC grade (Eastman Kodak Co.).

(d) *Solvents.*—LC grade methylene chloride, methanol, and acetonitrile (Fisher Scientific).

(e) *Water.*— Distilled and deionized (Hydro-Service, Paramus, NJ).

(f) *Phosphoric acid, 85%.*—LC grade (Fisher Scientific) with a specific gravity of 1.7.

(g) *1-Heptane sulfonic acid sodium salt.*—Premixed, concentrate (20 mL), LC grade (Eastman Kodak).

(h) *0.1M Phosphate buffer, pH 4.0.*—Combine 0.1M sodium phosphate dibasic heptahydrate with 0.1M phosphoric acid (1:1), and adjust to pH 4.0, if necessary.

(i) *Mobile phase.*—(Acetonitrile–methanol [5 + 3])–0.1M phosphate buffer, pH 4.0–1-heptanesulfonic acid sodium salt concentrate (250 + 750 + 20). This produces a 5mM ion-pair working solution.

Standard Solutions

All standard solutions are stable for up to 3 months when refrigerated.

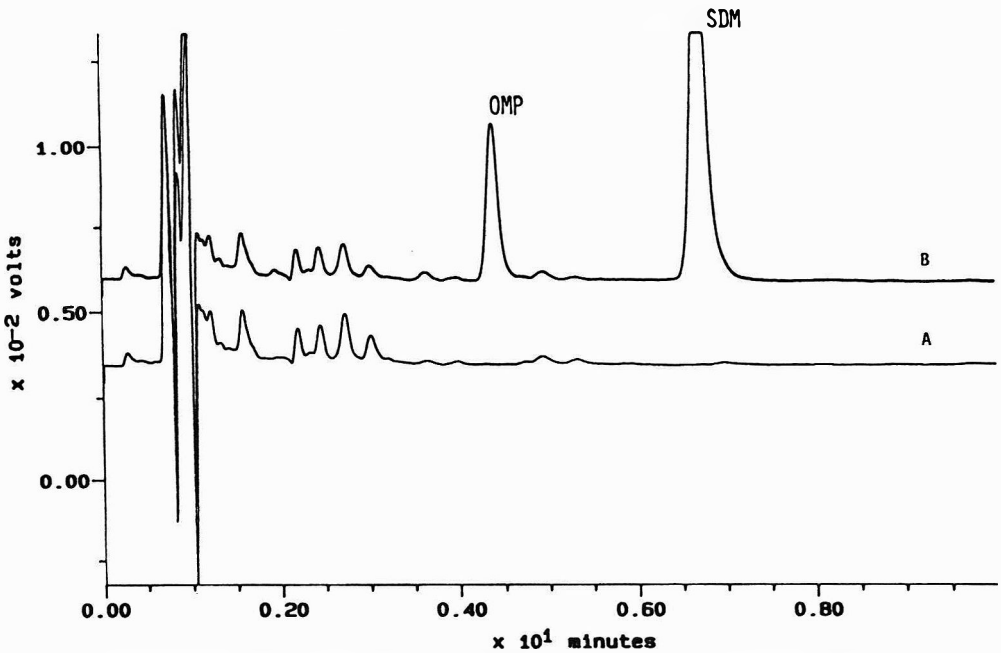


Figure 2. Typical chromatograms obtained for (A) control feed sample and (B) medicated Rofenaid (0.01% drug) feed sample.

Table 1. Recovery of OMP and SDM from fortified animal feeds

	Added, ppm[a]		Recovered, ppm		Recovery, %[b]		Rec. SD, %		CV, %	
Drug level, %	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM
					Rofenaid					
0.01	36.7	60.8	35.4	60.3	96.4	99.1	5.9	8.9	6.2	9.0
0.02	76.6	127.0	74.6	127.2	97.4	100.3	4.6	6.3	4.8	6.3
0.04	153.0	253.6	151.3	256.5	98.9	101.2	4.6	7.2	4.7	7.1
0.08	316.4	524.5	314.2	529.9	99.3	100.9	2.8	2.7	2.8	2.7
Mean					98.0	100.4	4.4	6.2	4.5	6.2
					Romet					
0.25	408.9	2095.3	403.0	2070.0	98.5	98.8	1.7	1.0	1.8	1.0
0.50	817.1	4186.3	827.6	4246.0	101.3	101.4	1.0	0.9	1.0	0.9
Mean					99.9	100.1	2.0	1.6	2.0	1.6

[a] Premix added to control feed and assayed.
[b] All levels based on average of 5 replicates.

(**a**) *Standard solutions for Rofenaid samples.—(1) Stock solution, 600 µg/mL OMP + 1 mg/mL SDM.*—Accurately weigh 60 mg OMP and 100 mg SDM (reference standards, Hoffmann-La Roche, Nutley, NJ) into a 100 mL volumetric flask. Add methanol to dissolve, and dilute to volume with methanol. *(2) 60 µg/mL OMP + 100 µg/mL SDM.*—Dilute 10.0 mL stock solution (*1*) to 100 mL with methanol. *(3) Solutions for standard curve.*—Prepare at least 3 appropriate concentrations from solutions (*1*) and (2) in methanol to bracket the expected concentrations of the samples.

(**b**) *Standard solutions for Romet samples.—(1) Stock solution, 200 µg/mL OMP + 1 mg/mL SDM.*—Weigh accurately 20 mg OMP and 100 mg SDM (reference standards) into a 100 mL volumetric flask. Add methanol to dissolve and dilute to volume with methanol. *(2) Solutions for standard curve.*—Prepare at least 3 subsequent dilutions of stock solution (*1*) in methanol to bracket the expected concentrations of the samples.

Extraction and Sample Preparation

Mix ground feed sample well, and weigh 5.0 g into a 100 mL centrifuge tube. For recovery studies, add an appropriate quantity of premix or OMP and SDM reference standards to give 0.01, 0.02, 0.04, and 0.08% drug in unmedicated Rofenaid feed (1.25, 2.5, 5.0, and 10.0 mg premix) and 0.25 and 0.50% drug in unmedicated Romet feed (41.25 and 82.5 mg premix). Add 2.75 mL pH 10 buffer solution, 1.0 mL 3N sodium hydroxide, 1.25 mL TBAH, and 50 mL methylene chloride. Homogenize contents of centrifuge tube for 1 min with a Polytron set at 4–4.5. Wipe generator thoroughly after each sample. In addition, rinse generator with methylene chloride between samples containing different drug levels, and discard rinse. Decant ca 10–20 mL extract through ca 10 g anhydrous sodium sulfate contained in a funnel plugged with cotton, and collect filtrate in a glass vial.

Solid-Phase Extraction

Transfer 3.0 mL filtered extract to a PrepSep silica extraction column fitted into vacuum manifold. Let sample flow through column by gravity. If gravity percolation is slow (>15 min), apply minimum vacuum (<5 in. Hg) to assist flow. After last portion of solvent exits the PrepSep, thoroughly dry column by applying maximum vacuum for at least 5 min. Elute OMP and SDM from column with 5 mL methanol into a 10 mL volumetric flask by using gravity flow. If elution takes longer than 5 min, apply minimum vacuum (<5 in. Hg) to assist elution. Apply maximum vacuum for 30–60 s to elute all the methanol. Remove volumetric flask from the manifold, and dilute to volume with methanol. Transfer an aliquot of the methanol to an LC autosampler vial.

LC Determination

Set column temperature at 30°C, flow rate at 2 mL/min, detector at 275 nm, and injector volume at 10 µL. Equilibrate system with mobile phase until retention times and peak areas are reproducible. The retention times are ca 4.5 min for OMP and 6.7 min for SDM. First, inject set of standards (at least 3 concentrations), followed by samples, with an external standard interspersed among the samples (ca every 10 samples) to check chromatographic consistency. Using peak areas, set the data system to calculate a weighted (1/c) linear-regression curve from the initial set of standards. From these standard curve parameters, calculate the OMP and SDM concentration in the samples (µg/mL) and the original quantity of each in the feed (ppm; dilution factor, 33.333) by using the data system. At the end of each day, rinse the system with a minimum of 10 column volumes of methanol–water (1 + 1).

If an equivalent data system is not available, use weighted linear regression analysis to obtain the linear standard curves for OMP and SDM, respectively, and calculate the concentration (µg/mL) of OMP and SDM in the injected samples. Cal-

Table 2. Method reproducibility[a]

	Rofenaid found, ppm		Romet found, ppm	
Replicate	OMP	SDM	OMP	SDM
1	41.812	64.216	853.396	4156.979
2	42.535	64.373	849.781	4136.715
3	42.154	64.064	831.993	4071.203
4	40.931	61.927	794.261	3939.025
5	41.026	62.198	807.555	4000.296
Mean	41.692	63.356	827.397	4060.844
SD	0.70	1.19	25.93	91.67
CV, %	1.68	1.88	3.13	2.26

[a] Medicated feed sample; 5 replicate assays/sample.

culate the concentration (ppm) of each in the original feed as follows:

$$\text{ppm} = C \times DF$$

where C = concentration (μg/mL) in the injected sample and DF = volume (mL) of extraction solvent divided by volume (mL) of feed extract placed on the PrepSep multiplied by the final volume (mL) of feed extract after solid-phase extraction column, all divided by weight (g) of feed taken for analysis. For this method:

$$DF = [(50/3) \times 10]/5 = 33.333$$

Results and Discussion

To determine system suitability, 5 replicate injections of a standard solution were made. The coefficients of variation (CVs) for OMP and SDM, respectively, were 0.51 and 0.36% for the peak areas and 0.14 and 0.09% for the retention times. A plot of response versus concentration for each compound was linear ($r^2 \geq 0.999$) over the range of standards for Rofenaid (OMP, 0.6–12 μg/mL; SDM, 1–20 μg/mL) and Romet (OMP, 8–40 μg/mL; SDM, 40–200 μg/mL). Representative chromatograms of a control feed sample (A) and OMP and SDM in medicated feed sample (B) are shown in Figure 2.

The accuracy of this method over the use levels for OMP and SDM (50-fold total drug concentration range) in animal feeds is shown in Table 1. For these analyses, a weighed quantity of the appropriate premix was added to 5 replicate control feed samples per level and analyzed by this method. The average recoveries for OMP and SDM at the use levels in Rofenaid and Romet ranged from 96.4 to 101.4%.

To establish the reproducibility of the method, representative feed samples at a use-level for Rofenaid (0.01% drug) and Romet (0.5% drug) were assayed. Five replicates of each sample were carried through the complete analytical procedure. Results are shown in Table 2. Good reproducibility was obtained, with average CVs for OMP and SDM of 1.7 and 1.9%, respectively, in the Rofenaid sample and 3.1 and 2.3%, respectively, in the Romet sample.

To compare the proposed LC method with the accepted methods, 4 different levels of medicated Rofenaid and 2 levels of medicated Romet feeds were assayed for OMP and SDM by both methods. Results are shown in Table 3. Assays for OMP averaged 102.7% of claim by the current methods and 102.6% of claim by the LC method; for SDM, they averaged 105.6 and 98.3% of the claims, respectively. In addition, the average ratios for OMP and SDM obtained using the 2 methods (LC/current methods) were 1.00 and 0.93. These results indicate good agreement between the 2 methods.

To test the method in the hands of a second analyst, 4 levels of medicated Rofenaid feeds were assayed by 2 analysts on separate days. The number of samples assayed per level is indicated in Table 4. Data show good correlation between the

Table 3. Comparison of assay results for current and LC methods for OMP and SDM in medicated feeds

				% of Claim					
		Claim, ppm		Current methods[a]		LC assay[b]		Ratio[c]	
Feed type	Drug level, %	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM
Rofenaid	0.01	37.5	62.5	99.7	102.4	111.2	101.4	1.12	0.99
Rofenaid	0.02	75	125	104.7	106.8	94.3	88.2	0.90	0.83
Rofenaid	0.04	150	250	103.7	102.4	106.3	101.4	1.03	0.99
Rofenaid	0.08	300	500	93.7	96.4	101.8	101.4	1.09	1.05
Romet	0.25	412.5	2062.5	115.4	112.9	101.7	99.1	0.88	0.88
Romet	0.5	825	4125	98.9	112.9	100.3	98.4	1.01	0.87
Mean				102.7	105.6	102.6	98.3	1.00	0.93
SD				7.4	6.5	5.7	5.1	0.10	0.09
CV, %				7.2	6.2	5.6	5.2	9.5	9.4

[a] Two replicates/level.
[b] Five replicates/level.
[c] Ratio of LC assay/current methods.

Table 4. Results of interassay variation of method for OMP and SDM in feeds

		% Label claim (mean)							
		0.01%		0.02%		0.04%		0.08%	
Analyst	No. samples/level	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM
1	5	111.2	101.4	94.3	88.2	106.3	101.4	101.8	101.4
2	3[a]	101.2	89.3	101.3	88.3	97.0	92.3	97.4	96.4
Mean		106.2	95.4	97.8	88.3	101.7	96.9	99.6	98.9

[a] Only 2 samples for the 0.04% level.

analysts. Therefore, the method can be reproduced and is not analyst-dependent.

To test for possible interferences, 33 approved feed additives for use in poultry, cattle, and aquaculture were chromatographed in the LC system. Only oxolinic acid eluted close to either OMP or SDM, eluting just before SDM. Oxolinic acid was added to feed at its use level (0.1%) with OMP and SDM and processed through the SDM/OMP feed method. The oxolinic acid did not interfere with the SDM analysis.

The LC method described here allows for the simultaneous assay of OMP and SDM in animal feeds. It is more specific and much faster than the currently accepted methods. Good recoveries and reproducibilities are obtained by this LC method for both compounds from multiple matrixes (poultry and aquaculture feeds). This method is presently being used on a routine basis, and approximately 200 samples have been assayed.

Acknowledgment

We thank C. Flores for her assay assistance with the currently accepted methods.

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DRUG RESIDUES IN ANIMAL TISSUES

Assay of Oxytetracycline Residues in Salmon Muscle by Liquid Chromatography with Ultraviolet Detection

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A novel method for the assay of oxytetracycline residues in salmon muscle is described. Tissue is homogenized with 4 volumes of 1% metaphosphoric acid in the presence of a small amount of dichloromethane (0.4 volume), shaken, and centrifuged. The residue is washed twice (4 and 2 volumes, respectively) with 1% metaphosphoric acid, and the combined aqueous supernatants are concentrated to about 2 mL by flash evaporation and then diluted to 5 mL. Filtered aliquots of the extract are subjected to liquid chromatography using a C_{18} column with a mobile phase of acetonitrile–tetrahydrofuran–0.025M aqueous oxalic acid (9 + 1 + 30) containing octanesulfonic acid at a final concentration of 10mM. Eluted peaks are monitored at 355 nm. Calibration and standard curves were linear from 0.01 to 0.5 μg on-column, with a limit of quantitation better than 0.05 μg/g. Recoveries from spiked blanks varied from 85.8 to 90.3% (relative standard deviation, 13.1–2.4%) in the 0.05–1.00 μg/g concentration range and were 21.1% at 0.02 μg/g.

Aquaculture is under rapid development in North America and especially in Canada. As in land farming, aqua farming uses a wide variety of drugs for prevention and treatment of disease in cultured fish. Among these drugs, oxytetracycline (OTC) is commonly used to treat bacterial infections such as vibriosis and furunculosis. A tolerance limit of 0.1 μg/g was established in the United States for uncooked edible tissues of salmonids, catfish, and lobsters (1). In most other countries, a zero tolerance is implied, and exact withholding periods were established.

In Canada, OTC is approved for use in aquaculture. In salmon farming, withholding times of 40 days above 10°C and 80 days below 10°C are required before slaughter of the fish destined for human consumption. Whether such long withholding periods are justified depends on the exact elimination characteristics of the drug, but pharmacokinetic studies are hampered by a lack of proper assay methods.

Traditionally, antibiotic residues were estimated by microbiological assays. Despite their high sensitivity, they lack the specificity required for regulatory purposes. Recently, liquid chromatographic (LC) methods were reported for the determination of OTC in animal tissues and fluids (2–8) and, more specifically, in fish (9–11). The methods for fish use a solid-phase extraction (SPE) technique to purify the extract before the LC determination.

Recently, we found that recoveries of OTC varied drastically depending on the supplier of the solid-phase cartridge and even from batch to batch from the same supplier. To alleviate this problem, a new extraction that avoids SPE was developed with an LC assay for the determination of OTC in salmon muscle.

Materials and Methods

Fish Samples

Wild fish, Pacific Pink Salmon, purchased at a local supermarket.

Reagents

Use analytical grade reagents and deionized water (Four Bowl System, Millipore Corp., Bedford, MA 01730) unless otherwise specified.

(a) *Solvents.*—Acetonitrile, methanol, and dichloromethane (LC grade, J.T. Baker Inc., Phillipsburg, NJ 08665); tetrahydrofuran stabilized with 250 ppm butylated hydroxytoluene (Accusolv, Anachemia, Mississauga, ON, L5C 4A7, Canada).

(b) *Metaphosphoric acid.*—1% (w/v, extracting solution) and 10% (w/v), both in water.

(c) *Oxalic acid.*—0.025M in water.

(d) *Mobile phase.*—Mix 750 mL 0.025M oxalic acid solution with 225 mL acetonitrile and 25 mL tetrahydrofuran. Add 2.17 g octanesulfonic acid sodium salt (Sigma Chemical Co., St. Louis, MO 63178), and mix until completely dissolved. Filter through 0.2 μm membrane (Ultipor $N_{66}R$, Pall Trinity Micro Corp., Cortland, NY).

(e) *OTC stock solution.*—1000 μg/mL. Accurately weigh 100 mg OTC hydrochloride (OTC free base: 895 μg/mg, Sigma) and dissolve in 100 mL methanol. May be kept at –8°C

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Table 1. Recovery of OTC from spiked salmon muscle

Concn added, μg/g[a]	N[b]	Av. concn found, μg/g	Rec., %	RSD_r[c], %	RSD_R[d], %
0	10	0.004[e]	—	—	—
0.02	8	0.019	94.5	15.0	21.1
0.05	10	0.045	90.3	12.3	13.1
0.10	10	0.088	88.4	7.5	7.3
0.20	10	0.173	86.3	7.0	6.2
0.50	10	0.429	85.8	1.8	8.1
1.00	10	0.887	88.7	1.7	2.4
Average (0.05–1.0 μg/g)			87.9		
RSD, %			2.1		

[a] Actual concentration, as the hydrochloride salt equivalent to 89.5% OTC, free base (label claimed).
[b] No. of determinations.
[c] Within-day relative standard deviation, by analysis of variance (repeatability).
[d] Between-day relative standard deviation, by analysis of variance (reproducibility).
[e] Five out of the 10 control fish tissue extracts contained an interference with retention time similar to OTC (range of apparent concentration, 0.002–0.01 μg/g). Recoveries were corrected for the corresponding blank value calculated as OTC.

for no more than 2 weeks. (**Note:** All OTC concentrations refer to the salt as supplied by Sigma.)

(**f**) *Fortification solutions.*—50 and 5 μg/mL. Before use, dilute 5 mL stock solution to 100 mL with water (fortification). Further dilute 10 mL fortification solution to 100 mL with water (1/10 fortification).

(**g**) *Solutions for standard curve.*—Dilute fortification or 1/10 fortification solution to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μg/mL with 10% metaphosphoric acid.

Apparatus

(**a**) *LC pump.*—Spectroflow 400 (Kratos Analytical, Ramsey, NJ 07446). Set flow rate at 1.0 mL/min.

(**b**) *Autosampler.*—Model SP8780XR (Spectra-Physics, San Jose, CA 95134) equipped with 500 μL sample loop.

(**c**) *Detector.*—Variable UV detector (Spectroflow 783, Kratos Analytical) set at 355 nm.

(**d**) *Chromatographic data system.*—Model 727 (Axxiom Chromatography Inc., Calabasas, CA 91302).

(**e**) *Column.*—Ultrasphere ODS, 5 μm, 150 × 4.6 mm (Beckman Instruments Inc., Berkeley, CA 94710).

(**f**) *Guard column.*—7 μm, 15 × 3.2 mm (New Guard RP-18, Brownlee Labs Inc., Santa Clara, CA 95050).

(**g**) *Filter unit.*—Millex-GS, 0.22 μm pore size (Millipore).

(**h**) *Homogenizer.*—Polytron Model PT 10/35 with 11.0 diameter PT 10ST Probe (Brinkmann Instruments Division, Rexdale, ON, M9W 4Y5, Canada).

(**i**) *Centrifuge.*—Centrifuge, Hermle Model Z320, with No. 22072V01 rotor (Mandel Scientific Co. Ltd., Guelph, ON, N1H 6J3, Canada), operated at 4100 rpm (2500 × *g*).

Sample Preparation

Transfer ca 5 ± 0.1 g fish muscle, accurately weighed, to 50 mL screw-capped poly(propylene) centrifuge tube. For recovery studies, spike at this point with 20, 50, 100, or 200 μL of the 1/10 fortification solution (0.02, 0.05, 0.1, or 0.2 μg/g), or 50 or 100 μL of the fortification solution (0.5 or 1.0 μg/g). Add 2 mL dichloromethane and 20 mL extracting solution. Homogenize tube contents 45–60 s with Polytron at medium speed. Rinse probe in second centrifuge tube containing 20 mL extracting solution, and reserve rinsing for second extraction of the sample. Rinse probe with distilled water, discard water, and wipe probe. Shake homogenized sample 10 min on horizontal shaker. Centrifuge 10 min at 4100 rpm (2500 × *g*), and decant clear supernatant into 250 mL round-bottom flask. Repeat extraction twice, first with 20 mL rinse from probe, and then with 10 mL extracting solution. Combine all supernatants in round-bottom flask, and discard final tissue sediment. Evaporate supernatants to ca 2 mL on rotary evaporator at 35°C, and quantitatively transfer residue to 5 mL volumetric flask. Rinse round-bottom flask with ca 2 mL water, making sure to dissolve all residue adhering to glass, and add rinsings to 5 mL volumetric flask. Dilute to volume with water and mix. Filter through filter unit, (**g**), attached to 10 mL syringe, collecting filtrate in screw-capped glass tube. Transfer ca 1.5 mL to autosampler vial.

Liquid Chromatography

Inject 500 μL aliquots onto chromatographic column. Chromatograph one set of standard solutions at beginning and one at end of every sample set, and construct calibration curves, using peak areas. Obtain parameters of linear regression (Y = mX + b), using least-squares fit. Calculate concentration in sample by using the following formula:

$$X = \frac{Y - b}{m}$$

where Y = μabsorbance units/min, b = Y-intercept or constant, m = slope or X-coefficient, and X is concentration in μg/g equivalent.

Results and Discussion

We found that recoveries of the method of Oka et al. (6) were variable, apparently because of a lack of SPE cartridge consistency. To avoid this problem, we set out to develop an

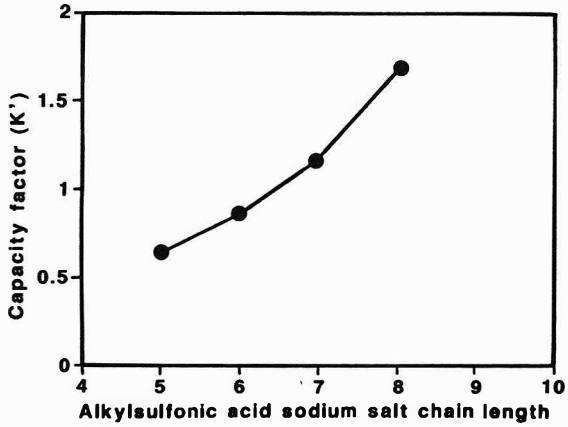

Figure 1. Effect of alkylsulfonic acid sodium salt alkyl chain length on capacity factor (k') of OTC.

alternative sample preparation that would omit the use of SPE. Tetracyclines are not easily extracted into organic solvents, but they do pass easily into aqueous acidic solutions. However, the large volumes required for deproteination of the tissue create a need to reconcentrate the extract to make it suitable for LC.

Ueno et al. (9) explored 3 common protein-precipitating agents for extracting OTC from rainbow trout muscle: perchloric, trichloroacetic, and metaphosphoric acids. The precipitation step was followed by a complex extraction procedure involving SPE. They reported a recovery of 64% with 0.5% aqueous metaphosphoric acid. However, we found that the extraction could be simplified and better recoveries could be obtained by the addition of 2 mL dichloromethane, as recommended by Mulders and Van de Lagemaat (8). The best recoveries (85.8–94.5%) were obtained by using 3 extractions with 1% metaphosphoric acid (20, 20, and 10 mL) followed by flash evaporation, redissolution, and filtration (Table 1). The average recovery from the 0.05–1.00 μg/g concentration range was 87.9 ± 2.1%.

The LC conditions of Oka et al. (6) needed to be modified, because a small interfering peak eluted with a retention time close to that of OTC. To obtain better separation between OTC and the interference, the effect of ion-pairing reagents was evaluated. Addition of quaternary alkyl ammonium salts, although improving the chromatography of OTC, could not provide adequate separation. The addition of alkylsulfonic acid sodium salts improved peak symmetry, increased the capacity factor (k'), and separated the interfering peak. Figures 1 and 2 show the effects of chain length and concentration, respectively, on k' of OTC. The k' was directly proportional to chain length and to the concentration of the alkylsulfonic acid sodium salts. When methanol was replaced with tetrahydrofuran, polar materials from tissue extract eluted faster. These combined effects of the alkylsulfonic acid chain length and concentration, together with organic modifiers, allowed adjustment of the retention time of OTC, which eluted in a window between 2 peaks (retention times, 8.7 and 11.4 min) endogenous to salmon muscle (Figure 3). A third peak (not identified yet) having the same retention time as OTC was found in fish from aquaculture but was absent from wild fish. Several attempts to resolve it from OTC were unsuccessful. Although its concentration did not exceed 0.01 μg/g when calculated as OTC, recoveries reported in Table 1 were corrected according to the amount found in corresponding unspiked sample.

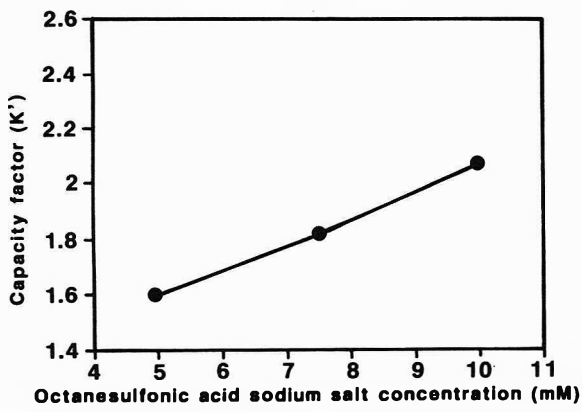

Figure 2. Effect of octanesulfonic acid sodium salt concentration on k' of OTC.

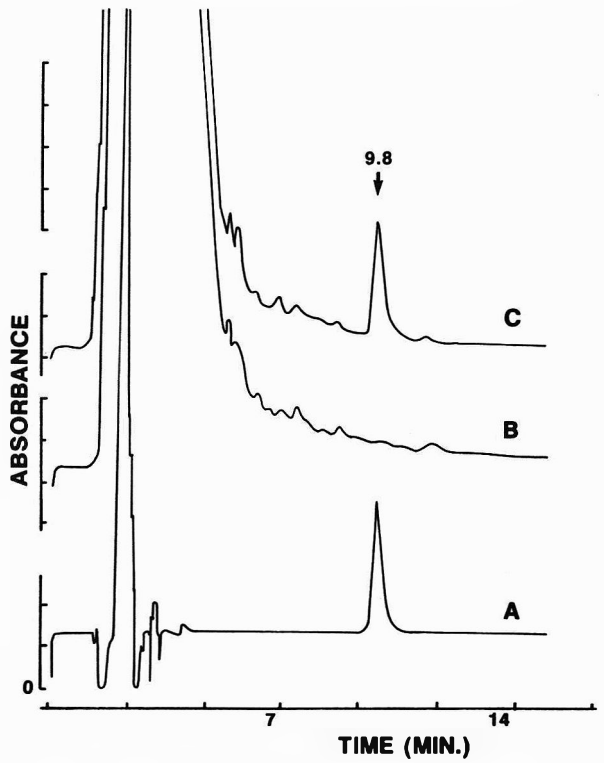

Figure 3. Typical chromatograms of A, 0.1 μg/mL standard solution; B, blank salmon muscle extract; and C, extract of salmon muscle spiked with 0.1 μg/g OTC. Retention time of OTC was 9.8 min (arrow).

Although a relatively large volume (500 μL) of the concentrated extract was used, it was well tolerated by the LC column. A small shift in retention time was experienced from the beginning to the end of each run, and the peak symmetry and the peak height were also affected. The column could be easily regenerated by washing it weekly or after every 50 sample injections by passing, in reverse flow, 75 mL of each of the fol-

Table 2. Comparison of the slopes of the calibration curves obtained by peak height and peak area from standard solutions of OTC[a]

	Slope by peak height		Slope by peak area	
Day	Before	After	Before	After
1	31740	35946	12247	12189
2	37127	35394	12384	12386
3	36140	33046	12170	12280
4	39812	35860	12107	12161
5	26805	26368	12316	12227
Within-day variation RSD_r, %[b]	6.35		0.42	
Day-to-day variation RSD_R, %[b]	13.57		0.81	

[a] Two calibration curves were done with each sample run, one before and the second after. For each regression, $N = 8$. See Table 1 for the concentrations used.

[b] RSD (relative standard deviation) obtained by analysis of variance.

lowing solvents: water, methanol, chloroform, tetrahydrofuran, *n*-hexane, chloroform, methanol, water, and mobile phase. The guard column was replaced as needed. This simple maintenance procedure made it possible to use the same column throughout this project (6 months; >1500 injections) without any apparent deterioration.

All calibration curves were linear over the concentration range studied (0.005–0.5 µg on-column), had a Y-intercept not significantly different from zero, and a correlation coefficient (r) better than 0.999 ($n = 10$, over 5 days). When the slopes of the calibration curves (before and after) from 5 analytical runs were compared, peak height measurement had a within-day variation of 6.35% and a day-to-day variation of 13.57%, whereas peak area showed only 0.42 and 0.81% for within-day and day-to-day variations, respectively (Table 2). The great variability in peak height resulted from changes in peak shape that were probably caused by accumulation of impurities on the column. This had little effect on the peak area, however. Therefore, peak area was chosen for measuring the OTC concentration in spiked samples.

OTC could be detected at 0.5 ng on-column for standard solution and at 0.002 µg/g for salmon extract; the limit of quantitation was found to be better than 0.05 µg/g (see Table 1). At this concentration, the day-to-day relative standard deviation (RSD) was 13.1%, which was well below the 20% limit recommended for residues at 0.1 µg/g (12). At 0.02 µg/g, the RSD was 21.1%, which was just above the recommended maximum.

The method described is fast, simple, and sensitive. It has a limit of quantitation that is less than half of the U.S. tolerance for OTC in salmonid tissues, which would make the method suitable for regulatory purposes.

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Simultaneous Determination of Multiple Tetracycline Residues in Milk Using Metal Chelate Affinity Chromatography

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A method was developed for the determination of 7 tetracyclines in milk. Raw milk samples are defatted, acidified, and centrifuged to remove proteins, and the tetracyclines are specifically absorbed from the milk by chelation with metal ions bound to small Chelating Sepharose Fast Flow columns. The tetracyclines are removed from these columns with EDTA-containing buffer, and the extracts are further cleaned up by centrifugal ultrafiltration. Finally, the extracts are concentrated and analyzed simultaneously by on-line concentration. This method has limits of detection for individual tetracyclines of <5 ng/mL and was validated with fortified milk samples at 15, 30, and 60 ng/mL. Recoveries exceeded 60% for all tetracyclines at all levels, with good precision. The method was also tested on milk from cows dosed with each of the tetracyclines. Advantages of this method over existing methods include its sensitivity, minimal use of organic solvents, and speed; with an autosampler, at least 14 samples can be processed and analyzed in 1 day.

Tetracyclines are a class of antibiotics that chelate divalent metal ions and have a fused, partially aromatic, 4-ring structure. Veterinary uses in food-producing animals are approved for oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC). The U.S. Food and Drug Administration (FDA), Center for Veterinary Medicine (CVM) set "safe levels" for residues in milk of 30, 80, and 30 ppb of these drugs, respectively. In addition to these 3 major drugs, doxycycline (doxy), minocycline (mino), demeclocycline (DMCTC), and methacycline (metha) are human drugs that may also be available for veterinary use. CVM needed to have a multiresidue regulatory method available that would detect the presence of these residues in milk.

Several liquid chromatographic (LC) methods were published for the determination of TC residues (TCs) in tissues or milk (1–9). Most of these methods did not have the desired sensitivity. The sensitivities of the Thomas procedure (3) and the matrix solid-phase dispersion technique (8) were clearly limited by the presence of endogenous milk peaks. The use of metal chelate affinity chromatography (MCAC) as a class-specific extraction step sounded quite promising, although the procedure developed by Farrington's group was somewhat cumbersome and time-consuming (1, 2).

This paper describes an LC method for determining 7 TCs in milk using MCAC as a precolumn cleanup and concentration step (1, 2), on-line sample concentration (3, 4, 10), and UV detection. The sample is defatted, acidified, and centrifuged, and the clear supernatant solution is applied to a small chelating agarose column that was previously charged with copper ions. This column is washed, and the TCs are specifically eluted with EDTA-containing buffer. The eluate is ultrafiltered to remove proteins and then injected on the LC. It is further concentrated by using 100% aqueous mobile phase as the starting condition. The TCs are adsorbed onto the column and then eluted by changing mobile phase conditions to include 22% acetonitrile and 8% methanol.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Perkin-Elmer Series 4 pump, LC-95 UV detector set at 355 nm, LCI-100 integrator, PE 7700 computer with Chromatographics 3 software, ISS-100 autosampler equipped with 2 mL loop, or equivalent LC system.

(b) *LC column.*— PLRP-S, 5 μm, 100 Å, 150 × 4.6 mm column, equipped with guard column containing same packing material (No. 1111-3500, Polymer Labs, Amherst, MA 01002).

(c) *Refrigerated centrifuge.*—Sorvall RC 3, or equivalent, with fixed-angle rotor holding 18 mm diameter tubes and rotor holding disposable 15 mL centrifuge tubes.

(d) *Minicolumns.*—Bio-Rad disposable poly(propylene) columns (No. 731–1550, Hercules, CA 94547), or equivalent.

(e) *Rack to hold minicolumns.*—Optional; can be rigged from wire test tube racks, but rack manufactured expressly for this purpose (Bio-Rad, No. 731–7005) simplifies procedure.

(f) *Centrifugal ultrafilters.*—Amicon Centricon 30 (No. 4208 or 4209, Beverly, MA 01915).

(g) *Filters.*—LC solvent filtration apparatus with 0.2 μm nylon filters to fit.

(h) *Volumetric flasks.*—Class A, 1 L, 100 and 5 mL.

Reagents

(a) *LC grade water.*—Use wherever water is called for. Distilled water is deionized then further treated with UV irradiation to remove trace organic impurities (Nanopure II/Organicpure system, Sybron Barnstead, Boston, MA 02132).

(b) *Solvents.*—LC grade methanol and acetonitrile.

(c) *Tetracycline analytical standards.*—OTC, and hydrochloride salts of TC, CTC, and metha (USP Reference Standards, U.S. Pharmacopeial Convention, Rockville, MD 20857). Hydrochloride salts of mino, DMCTC, and doxy (Sigma Chemical Company, St. Louis, MO). All TCs used for oral dosing of cows were purchased from Sigma, except metha, which was a gift from Wallace Laboratories.

(d) *Metal chelate resin.*—Chelating Sepharose Fast Flow (No. 17-0575-01, Pharmacia, Piscataway, NJ 08854). Store refrigerated.

(e) *Sodium succinate.*—0.1M, pH 4.0. Dissolve 11.8 g succinic acid (Sigma reagent grade, or equivalent) in ≤1 L water. Titrate to pH 4.0 with 10N NaOH, and dilute to 1 L. Store under refrigeration. Discard if any particulate matter appears.

(f) *Copper sulfate.*—10mM. Dissolve 0.5 g $CuSO_4 \cdot 5H_2O$ (Sigma reagent grade, or equivalent) to 200 mL with water. Store at room temperature.

(g) *Ethanol.*—20% (v/v).

(h) *McIlvaine–EDTA–NaCl buffer.*—Prepare McIlvaine buffer by dissolving 12.9 g citric acid monohydrate (Sigma reagent grade) and 10.9 g Na_2HPO_4 (Fisher reagent grade) in 1 L water. Store under refrigeration. Prepare McIlvaine buffer 0.1M in EDTA and 0.5M in NaCl by adding 37.2 g $Na_2EDTA \cdot 2H_2O$ (Fisher reagent grade, or equivalent) and 29.2 g NaCl (Mallinkrodt reagent grade, or equivalent) to flask and diluting to 1 L with buffer. Filter through 0.2 μm nylon filter. Store at room temperature.

(i) *LC mobile phase.*—To 1 L volumetric flask, add 0.90 g oxalic acid (Aldrich Chemical Co., Milwaukee, WI), dissolve, and dilute to volume with LC grade water. Filter through 0.2 μm Nylon 66 membrane, and place in one pump reservoir. Separately fill remaining reservoirs with LC grade methanol, acetonitrile, and water. Sparge with helium (3).

Standard Solutions

(a) *Tetracycline stock solutions.*—100 μg/mL. Place 10 mg (corrected for potency) of each tetracycline in individual amber or foil-wrapped 100 mL volumetric flask. Dilute to volume with methanol. Shake well to dissolve. Store at −10°C. Stable for 2 months.

(b) *Combined working tetracycline stock solution.*—1 μg/mL. Pipet 0.5 mL of each stock solution into 50 mL amber or foil-wrapped flask. Dilute to volume with methanol. Store at −10°C. Stable for 5 days.

(c) *Chromatographic standards.*—Dilute 0.750 mL working stock to 5.0 mL with McIlvaine–EDTA–NaCl buffer in a volumetric flask to make 150 ng/mL standard solution. Dilute this solution 2 + 1 (4 mL 150 ng/mL standard + 2 mL buffer) to make 100 ng/mL. Dilute 100 ng/mL standard 1 + 1 (2 mL + 2 mL buffer) to make 50 ng/mL, and 1 + 4 (1 mL diluted to 5 mL with buffer in volumetric flask) to make 20 ng/mL. The 20, 50, 100, and 150 ng/mL chromatographic standards are equivalent to milk concentrations of 10, 25, 50, and 75 ng/mL. Not stable; prepare daily.

Milk Fortification

Fortify milk to 15, 30, and 60 ng/mL by adding 75, 150, and 300 μL TC working stock, respectively, to 5 mL whole raw milk. Milk may have been stored frozen, but should show no signs of souring or curdling.

Animal Treatment

Cows were given a single oral bolus containing either 5 mg/lb body weight (CTC, mino, DMCTC, or doxy) or 2.5 mg/lb body weight (OTC or TC). Milk was collected for analysis twice daily postdosing. Samples were analyzed within 18 h while still fresh. Aliquots of each milking were also immediately frozen and stored for future use. Frozen milk containing residues at the levels of interest was then used in the method validation studies. To produce milk containing more than 1 residue at the desired concentration, in some instances 2 milk samples containing individual incurred residues were blended before analysis.

MCAC Column Preparation

Swirl Chelating Sepharose Fast Flow resin in its bottle to obtain even suspension. Transfer two 0.7 mL portions of suspension to minicolumn, using P1000 pipetman (or equivalent) equipped with blue tip from which bottom 2–3 mm was removed with sharp razor to create larger bore pipet tip.

Open bottom outlet of minicolumn, and allow shipping buffer to drain out. Add or remove resin if necessary, so that bed volume is between 1.0 and 1.2 mL. Wash resin with three 2 mL portions of water; then, "charge" with 2 mL 10mM $CuSO_4$. Wash column with two 2 mL portions of water. It is now ready for use.

All MCAC column operations are accomplished by using gravity feed. Many columns can be prepared simultaneously. Column appearance at this point should be bed volume between 1.0 and 1.2 mL, with approximately the top 0.7 mL blue from Cu^{2+} adsorption. Bottom 1/3 of column should remain white.

Sample Preparation

Place 5.0 ± 0.1 mL milk in 15 mL disposable centrifuge tube, and centrifuge 15 min at 3000 rpm (1500 × *g*) and 10°C to separate cream.

Remove lower "skim" layer, using 9 in. Pasteur pipet, and transfer to clean 15 mL centrifuge tube. Alternatively, puncture solid fat layer with Pasteur pipet on opposite sides while still cold, and decant skim milk through holes. Discard fat. Add 10 mL 0.1M sodium succinate (pH 4) buffer to milk, cap tube, invert several times to mix, and centrifuge 30 min at 3000 rpm to pellet resulting precipitate.

Apply clear supernatant solutions directly to MCAC columns. (If using Bio-Rad columns, apply in 2 batches, because reservoir is not large enough to hold entire supernate.) Filter

through by gravity. Avoid disturbing column bed excessively. Proceed with subsequent step only after no liquid is visible above resin. Do not let columns dry out.

Wash columns sequentially with 2 mL 0.1M sodium succinate (pH 4), 2 mL water, 2 mL methanol, and then 2 mL water.

Next two steps are critical for good recoveries.—(*1*) Carefully apply 0.70 ± 0.05 mL McIlvaine–EDTA–NaCl buffer to columns. Drip buffer along sides of column so as not to disturb column bed. Discard clear flowthrough. Use gravity feed only on this and next step. (*2*) Elute TCs from column with additional 2.5 ± 0.05 mL McIlvaine–EDTA–NaCl buffer, collecting eluate. MCAC columns should appear white at this point; eluted solution is blue.

Clean columns with additional 2–3 mL McIlvaine–EDTA–NaCl buffer, and wash with three 2 mL portions of water and then 5–10 mL 20% ethanol in water. Cap columns with excess of 20% ethanol and store in refrigerator. Before next use, use vortex mixer or invert several times to resuspend resin thoroughly. Same column may be used at most 6 times. On subsequent uses, open top of column and start at "Open bottom outlet of..." in *MCAC Column Preparation*. Avoid reusing columns that were exposed to sour milk or excessive amounts (>5 μg) of TCs. Columns are good for at least 2 months when stored properly.

Ultrafiltration

Caution: Blue MCAC eluates are not stable. Eluates develop precipitate that can clog and effectively destroy LC column. For this reason, I strongly recommend that samples be deproteinized further before analysis. Ultrafiltration through Centricon 30 effectively removes proteins of MW 30 000 daltons and higher without significantly reducing TC concentration.

Wash filters before use by centrifuging (15 min at 1500 × *g*) 2 mL water through them. Shake both retentate and filtrate chambers to remove all water. Wash filters while MCAC columns are being loaded and washed.

Collect MCAC eluates directly in upper (retentate) chamber of Centricon 30 centrifugal ultrafilters. Cap and invert several times to ensure sample homogeneity. Centrifuge MCAC samples 30–90 min at 6500 rpm (5000 × *g*) in fixed-angle rotor. Centrifugation may be stopped when there is ≥1 mL filtrate in bottom chamber.

Liquid Chromatography

Mobile phase composition.—Use gradient reported by Thomas (3). Briefly, inject sample with mobile phase at 100% 0.01M oxalic acid with 1 mL/min flow rate. After 1 min, linearly change mobile phase over 5 min to 0.01M oxalic acid–methanol–acetonitrile (70 + 8 + 22). Maintain this composition for 11 min at 1 mL/min then return to initial conditions. Monitor UV absorbance at 355 nm. (Substitution of polymeric reversed-phase column significantly improves resolution and column longevity.) Store column in water–acetonitrile (50 + 50). Flush column and LC system with pure water before and after storage to prevent precipitation of oxalic acid by high concentrations of organic solvent.

Sample injection.—Use 2.0 mL sample loop. Sample injection size may vary; 600 μL is convenient amount for PE ISS 100 autoinjector to handle. To quantitate accurately, inject identical volumes each time. Use water as flushing solvent in autosampler to prevent salt precipitation.

Peak identification.—TCs elute in following order: mino, OTC, TC, DMCTC, CTC, metha, and doxy. (Retention times tend to shift slightly both as column ages and as number of runs in experiment increases. It is necessary to define very tight windows for peak identification, because TCs cluster closely together. For this reason, a standard must be included every 5–10 injections. All putative residue peaks should have retention times within 0.05 min of retention times observed in bracketing standards.)

Extract stability.—TCs are not stable at room temperature under acidic conditions (i.e., McIlvaine–EDTA–NaCl buffer). TC and CTC, in particular, degrade significantly (≥50%) within 24 h. Degradation products tend to elute earlier than parent compound and usually migrate with OTC. To avoid this problem, all centrifugation steps should be performed at 10°C, and sample extracts either should be refrigerated or should be analyzed within 4 h of preparation. MCAC samples may be stored refrigerated up to 2 days or frozen up to 1 week with only slight changes in concentration.

Calculations

Prepare standard curve for each of 7 TCs from standard chromatograms. Inject equal volumes of standards and samples on LC system. Determine sample concentrations by linear regression, using formula $y = mx + b$, where x = peak area or height (both were used successfully in this laboratory) and y = concentration of extract injected in ng/mL. Correlation coefficients for each of TC standard curves are routinely ≥0.995. Because MCAC reduces sample volume from 5 mL to 2.5 mL, determine TC concentration in original milk sample by dividing concentration determined for injected sample by 2.

Care should be taken with integration. Baseline determined by automated data systems should be individually inspected for each chromatogram. See chromatograms in Figure 1 for appropriate baseline construction.

Results

Three separate experiments were conducted on different days in which TCs were determined in duplicate samples of control milk and control milk fortified with mino, OTC, TC, DMCTC, CTC, metha, and doxy at 15, 30, or 60 ng/mL each. Figure 1 shows chromatograms of control milk and control milk fortified at 30 ng/mL.

Noise levels in the 6 control milk samples were determined at the retention times for each of the TCs and used to calculate the limit of detection (LOD) and limit of quantitation (LOQ) (11) for each residue for a 600 μL sample injection (Table 1). The LOD and LOQ determinations are consistent with observed ability to detect and quantitate low levels of TCs in the analysis of several-day postdose incurred milk samples.

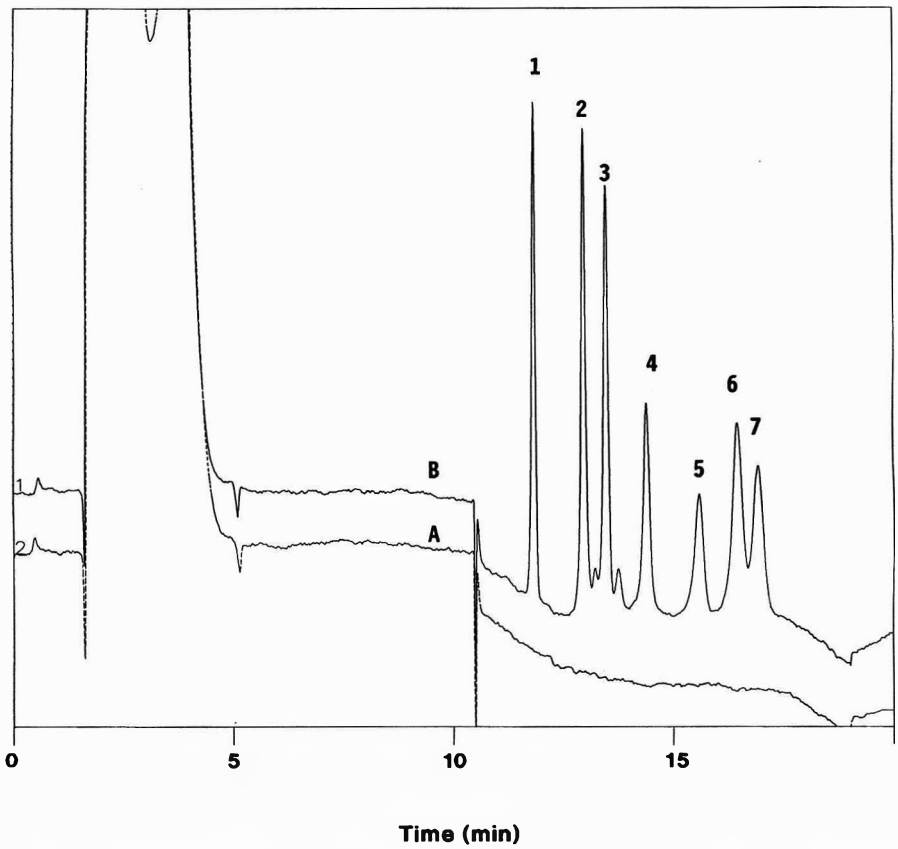

Figure 1. Chromatograms of MCAC extracts: (A) control milk; (B) control milk fortified at 30 ng of each TC/mL, (1) Minocycline, (2) oxytetracycline, (3) tetracycline, (4) demeclocycline, (5) chlortetracycline, (6) methacycline, and (7) doxycycline.

Table 2 shows the recovery data for the fortified milk samples. Approximate retention times, average percent recoveries, and percent coefficients of variation are presented. Recoveries varied from a low of 63% for mino to a high of 91% for OTC; they tended to be higher for the samples fortified at lower concentrations. The reasons for this are not clear; however, this phenomenon was observed before in TC analysis (1, 5). In this method, the capacity of the MCAC columns may be a factor in reduced recoveries at higher residue levels, although samples containing >500 ng/mL were successfully analyzed.

Five replicates of milk containing incurred residues were analyzed. The results, with coefficients of variation, are reported in Table 3. In a separate set of exhaustive extraction experiments, dosed milk samples containing relatively high levels of TCs were analyzed. The discarded fat layers from these samples were extracted with water, which was added to the discarded pellets from the sodium succinate precipitate. An additional 10 mL of sodium succinate was added to these solutions, followed by vortexing and centrifugation. The supernatant solutions were applied to separate MCAC columns, which were then treated as described in the *METHOD* section. Table 4 shows the levels determined in the primary extract or method, the levels in the secondary extract, and the percentages of the total extract in the primary and secondary extracts.

This method was developed by using milk from cows housed at Division of Veterinary Medical Research (DVMR). These cows had the advantage of having a known treatment history. None of the control milk produced here contained any background peaks that would interfere with the analysis. To confirm that this method works with milk from other sources, we acquired milk from cows or herds with known treatment histories (typically from agricultural schools) from other regions of the United States. Control milk from North Dakota was found to contain an endogenous peak with a retention time

Table 1. Approximate retention time (RT), LOD, and LOQ of method for TCs residues in milk: Calculations assume a 600 μL injection volume

Parameter	Mino	OTC	TC	DMCTC	CTC	Metha	Doxy
Approx. RT, min	11.7	12.8	13.3	14.2	15.3	16.1	16.6
LOD, ng/mL	0.50	0.42	0.52	1.02	1.27	0.93	1.15
LOQ, ng/mL	1.03	0.83	1.01	2.18	2.35	1.90	2.22

Table 2. Recovery and coefficient of variation (CV) of TCs from fortified milk samples

Fortified sample concn, No. of samples	Rec., % (CV, %)													
	Mino		OTC		TC		DMCTC		CTC		Metha		Doxy	
15 ng/mL, $n = 5$	66.6	(8.9)	91.0	(9.5)	79.4	(10.7)	81.0	(12.0)	80.3	(21.3)	74.6	(16.7)	79.3	(13.0)
30 ng/mL, $n = 6$	65.1	(12.2)	85.6	(8.0)	73.6	(4.5)	75.2	(2.8)	68.3	(9.5)	67.1	(6.7)	72.4	(6.0)
60 ng/mL, $n = 6$	63.4	(13.5)	82.0	(10.1)	69.8	(5.5)	68.6	(4.7)	65.5	(6.5)	65.3	(6.1)	71.6	(6.4)

Table 3. Residue concentration (ng/mL) in milk from cows dosed with TCs

Parameter	Mino	OTC	TC	DMCTC	CTC	Metha	Doxy
	27.73	28.07	33.50	22.89	28.00	16.19	32.78
	26.76	28.21	32.99	21.06	27.50	15.19	36.42
	29.19	29.78	34.53	22.04	27.39	16.72	31.43
	23.18	27.74	32.81	20.27	28.22	15.83	33.12
	26.49	28.76	32.06	19.27	25.95	14.26	30.48
Av. concn	26.67	28.52	33.18	21.11	27.41	15.64	32.85
CV, %	8.32	2.80	2.76	6.75	3.24	6.07	6.89

similar but not identical to that of TC (Figure 2). Analysis of the North Dakota milk using a minor variation of the procedure published by Thomas (3) revealed that the MCAC procedure removes most (>90%) but not all of this endogenous peak. The MCAC procedure was modified to include more stringent wash steps (approximately double the volume described), which effectively removed the interfering peak. However, the increased washes also reduced the recoveries of some of the TCs, particularly mino; therefore, they were not incorporated into the standard method. Recovery of TC itself is only mildly affected by the increased washes; if the identity of a peak is uncertain, the milk sample could be re-analyzed and the more stringent wash procedure used.

This method was tested for interferences resulting from other veterinary drugs. Solutions containing approximately 1 μg/mL of the following drugs were chromatographed by the LC system described above: chloramphenicol, gentian violet, ivermectin, spectinomycin, hygromycin B, and a mixture of 11 different sulfa drugs. Of these, chloramphenicol, spectinomycin, hygromycin B, and the sulfa drugs resulted in any peaks detectable at 355 nm. (It was strongly suspected that the spectinomycin/hygromycin B resulted from a contaminant in the stock solution.) Milk samples were prepared containing 1 μg/mL of the above drugs. Samples were also prepared containing 1 μg/mL of the above drugs and 30 ng/mL of each of the 7 TCs. These samples were extracted and analyzed by this method. The first set of samples resulted in chromatograms indistinguishable from control milk chromatograms. Recoveries of TCs in the second set of samples were similar to recoveries from control milk fortified with TCs only. No detectable interference from these other drugs was found with the TC analysis.

During its development, this method was evaluated for ruggedness according to Youden's system (12). Except where indicated, minor changes in volumes and buffer compositions in the MCAC column preparation and extraction steps did not significantly affect results. The performance of this method was also tested by having a second analyst in the laboratory use the method to analyze fortified samples. Results were similar to those reported here.

MCAC is a convenient, TC class-specific extraction method. Because milk samples prepared by this method are free of chromatographic interferences, detection limits are low and peaks are readily identified. This method has the additional advantages that numerous samples may be processed at one time, and almost no hazardous waste is generated by the extraction procedure.

Table 4. Exhaustive extraction of milk from cows dosed with OTC, TC, or CTC

Extraction	Parameter	OTC	TC	CTC
First extraction	Concn, ng/mL	51.21	70.94	52.70
	CV %, $n = 3$	1.66	3.94	11.39
	% of total extracted	87.70	92.38	90.21
Second extraction	Concn, ng/mL	7.18	5.85	5.72
	CV %, $n = 3$	9.18	4.42	36.75
	% of total extracted	12.30	7.62	9.79

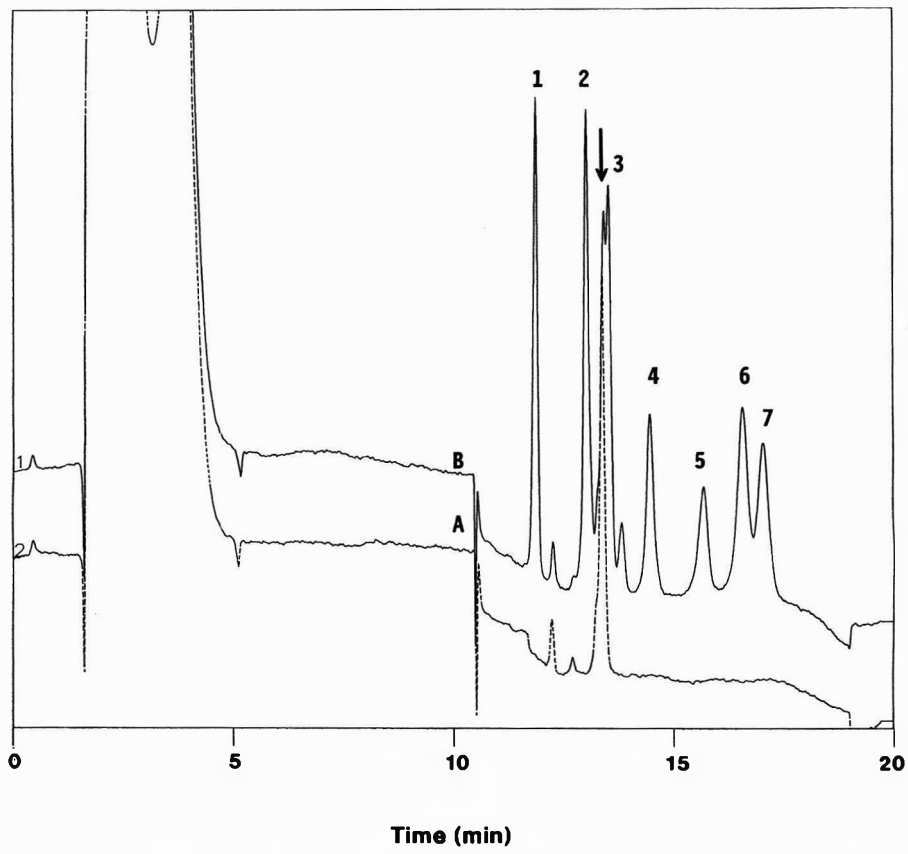

Figure 2. Chromatograms of MCAC extracts of milk from North Dakota: (A) control milk and (B) ND control milk fortified at 30 ng/mL with each TC. Peaks are labeled as in Figure 1. Arrow indicates endogenous milk peak.

Acknowledgments

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DRUG RESIDUES IN ANIMAL TISSUES

Quantitation of Sulfamethazine in Pork Tissue by Thin-Layer Chromatography

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Our earlier method to detect and quantitate sulfamethazine (SMZ) in milk at the 10 ppb level was modified to quantitate SMZ in pork tissue. Sulfabromomethazine (SBZ) is added to the tissue as an internal standard. SMZ and SBZ are extracted from the tissue into water as the supernatant of a centrifuged, aqueous homogenate and are cleaned up and concentrated by a series of solid-phase extractions. The sulfonamide-containing eluate is then separated on a silica gel thin-layer chromatographic plate. SBZ and SMZ are derivatized with fluorescamine, and their fluorescence is quantitated with a scanning densitometer. The limit of detection was estimated at 0.25 ppb (signal-to-noise ratio, 3:1). The average accuracy over the analysis range (0.54–21.8 ppb [μg/kg]) was 95.6% (standard deviation = 29.4%, $n = 54$).

Sulfamethazine (SMZ) is a commonly used sulfonamide that is effective for treating various bacterial infections in food-producing animals. The United States has a current tolerance of 0.1 ppm for SMZ residues in edible swine tissues (21 CFR 556.670) (1), but recent reports from the National Center for Toxicological Research on the possible carcinogenicity of SMZ (2, 3) prompted the U.S. Food and Drug Administration (FDA) to consider lower tolerance limits, possibly into the low ppb levels (4, 5).

Current AOAC official methods for quantitating SMZ in swine tissue (**982.40, 982.41,** and **982.31**) (6) are applicable only down to the 50 ppb level. Moreover, these methods are time-consuming and produce an appreciable amount of waste solvents. Goals of new methodology are to decrease the analysis time and reduce the amount of solvents used.

Solid-phase extraction (SPE) is one way to reduce high solvent consumption. A recently reported multiresidue method for sulfonamides in swine tissue uses a type of SPE (7) that requires very small amounts of halogenated and nonhalogenated solvents per sample (8 mL each) in the analyte isolation phase. However, the method uses liquid chromatography (LC) for analyte separations, which produces a substantial volume of waste solvent; the method also uses a photodiode array detection system for quantitation, which results in a minimal detectable limit between 31 and 62 ppb (7).

Aerts et al. reported a sensitive multiresidue LC method (reported limit of detection, 5 ppb) for sulfonamides in meat tissue (8, 9). A continuous flow system involving column switching is used to isolate and concentrate the analytes from an aqueous tissue extract before LC analysis. Postcolumn derivatization with dimethylaminobenzaldehyde enhances both the sensitivity and selectivity of this method. The use of aqueous saline to quantitatively extract sulfonamides and chloramphenicol from tissues is successful at trace levels (10).

We recently reported a very sensitive method (quantitative range of analysis, 0.5–15 ppb) for detecting SMZ in milk (11). The method uses a series of solid supports to extract, isolate, and concentrate the analyte. After thin-layer chromatographic (TLC) separation, the analyte is quantitated by fluorescence densitometry. The method uses a total of about 20 mL nonhalogenated organic solvents per sample. Small modifications to the method for SMZ in milk permitted the use of an aqueous tissue extract (8–12) in conjunction with the series of solid supports. These modifications resulted in the method we now report. Results obtained from assays of swine tissue fortified with SMZ in the 0.5–20 ppb range are presented.

Experimental

Reagents

(a) *Solvents.*—LC grade.

(b) *Water.*—LC grade, from Modulab Polisher I system (Continental Water Systems, San Antonio, TX).

(c) *Reagents.*—Baker analyzed (J.T. Baker, Phillipsburg, NJ) except for fluorescamine, SMZ, *N*-acetylsulfanilyl chloride, and 2-amino-4,5-dimethylpyrimidine, which were obtained from Sigma Chemical Co. (St. Louis, MO).

(d) *Solutions.*—Prepare stock solutions of SMZ (Sigma Chemical Co., S-6256) and SBZ (11) at 1 mg/mL in acetone, and store at −80°C. Prepare working solutions in water of 1.0 μg SBZ/mL and 2.0, 1.5, 1.0, 0.8, 0.75, 0.50, 0.40, 0.25, 0.20, 0.10, and 0.05 μg SMZ/mL by diluting stock solutions. Prepare fresh working solutions monthly, and store at 0–5°C.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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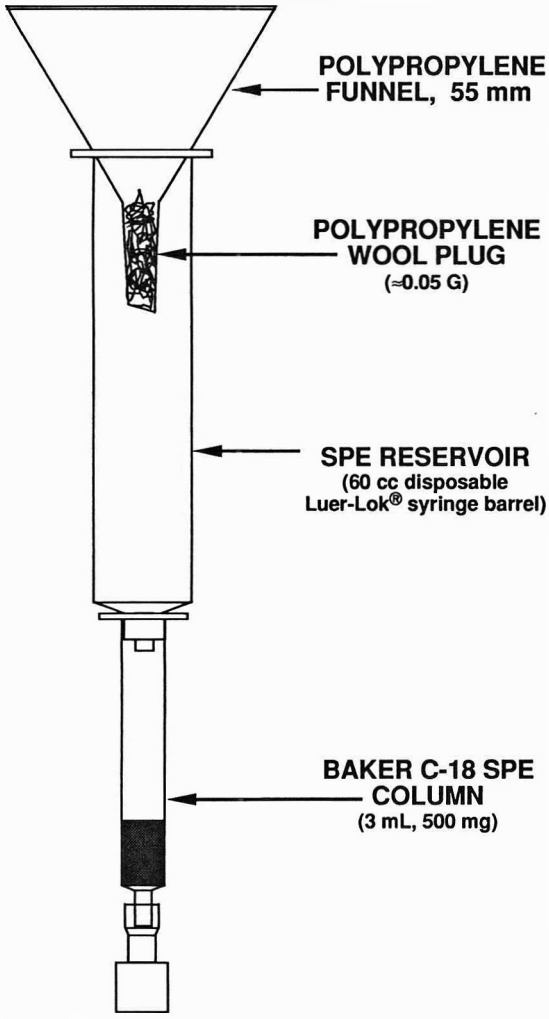

Figure 1. Filtration and extraction column setup. The Luer-Lok fits tightly into the 500 mg C_{18} SPE column without the need for an adapter. The poly(propylene) wool plug is loosely packed to fill the stem of the funnel and is only intended to retain the fat that floats on the supernatant.

(**e**) *Acidic alumina.*—Purchase as activated and use as received, 95+%, –60 mesh (Alfa Products, Danvers, MA).

(**f**) *Cation-exchange resin.*—AG MP-1, 100–200 mesh, chloride form (Bio-Rad Labs, Richmond, CA).

Apparatus

(**a**) *Homogenizer.*—Polytron Model PT 10/35 (Brinkmann Instruments, Inc., Westbury, NY) equipped with Model PTA-20S generator, and operated at a setting of ca 4½ for 20 s.

(**b**) *Centrifuge.*—Refrigerated centrifuge (IEC Centra-7R, International Equipment Co., Needham Heights, MA) with 12 × 50 mL tube capacity rotor (Model 822A). Low-density poly(ethylene) centrifuge tubes with closures (Nalge No. 3112–0050, Fisher Scientific, Pittsburgh, PA). Mark tubes at 25 mL level before analysis. Centrifuge samples 15 min at 15°C and 4800 rpm (ca 4000 × *g*).

(**c**) *SPE manifold.*—Visiprep™, equipped with vacuum gauge and optional Teflon solvent guide needles (Supelco, Inc., Bellefonte, PA). Fit 1/4 in. piece of 3/8 in. rubber tubing around sample control valves of manifold to increase fingertip control of sample flow. Collect eluates in 10 × 75 mm disposable culture tubes (VWR, San Francisco, CA).

(**d**) *SPE setup.*—A reservoir, ≥50 mL, attached to extraction column (Bakerbond C_{18}, 3 mL, Baker) is required [we used 60 mL Luer-Lok poly(propylene) syringe barrel (Becton Dickinson & Co., Rutherford, NJ) with end cut off at 60 mL graduation mark; presence of Luer-Lok permits attachment without need of connecting adaptor]. Place 55 mm disposable poly(propylene) funnel (Fisher Scientific) in top of reservoir. Pack funnel stem loosely with ca 0.05 g teased poly(propylene) wool (Aldrich Chemical Co., Inc., Milwaukee, WI) to prefilter sample (see Figure 1). Attach C_{18} columns to the vacuum manifold and wash with two 3 mL volumes each of methanol then water; place an additional volume of 1.5 mL water above the bed.

(**e**) *Preparation of anion-exchange resin.*—Shake 10 g AG MP-1 (ca 1 min) with 300 mL 10% acetic acid in acetone, let settle 15 min, and decant. Shake resin with 300 mL water, let settle 15 min, and decant. Shake resin with 300 mL 2M HCl, let settle 5 min, and decant. After rinsing with water in a course-fritted funnel until water is neutral, shake resin 1 h, using a mechanical shaker, with 300 mL 0.2M K_2HPO_2 buffer, pH 7.9. Filter resin through a course-fritted funnel, wash with water until water is neutral, and dry in the funnel (vacuum 5 min). Store the 10 g of resin, refrigerated, in 200 mL ethanol–water (1 + 1), and use 0.5 mL suspension for column B.

(**f**) *Concentration column (column B).*—Use the end of a 1 mL disposable pipet tip with a 70 µm porous poly(propylene) disc (2.5 mm disc punched from 1.59 mm sheet 70 µm Fritware® [BEL-ART, Pequannock, NJ]), and add 0.5 mL anion-exchange resin suspension and let drain to waste.

(**g**) *Clean-up column (column A).*—Fill a Quik-Snap column (Isolab, Akron, OH) to the reservoir with methanol, and slowly pour 0.50 ± 0.02 g acidic alumina into the column. Place a bed of course sand (ca 5 mm) on top of the alumina after it has settled. Snap the bottom closure off, and place the column above the concentration column (column B, Figure 2), letting methanol drain through column B to waste.

(**h**) *TLC development and application.*—Use ascending one-dimensional development in a twin trough chamber, 10 × 10 cm (Camag, Muttenz, Switzerland) with chamber saturation for 10 min. TLC plates (10 × 10 cm) precoated with Silica Gel 60 were obtained from Merck (Darmstadt, Germany). Wash plates by immersing in methanol 5 min and then dry at 80°C for 30 min. Apply samples to TLC plate with Camag Linomat IV (Camag, Wrightsville Beach, NC). Use N_2 to spray samples onto TLC plate 10 mm from bottom edge at rate of 6 s/µL. Starting 10 mm from plate edge, apply samples in 6 mm bands separated by 4 mm. This arrangement permits 8 lanes per plate, 3 of which will always be standards.

(**i**) *TLC detection and quantitation.*—Use ethyl acetate–toluene (1 + 1) as solvent; split 10 mL evenly between troughs. Running time and distance are 11 min and 63.0 ± 0.4 mm from plate bottom, respectively. To allow detection, mechanically dip the dried chromatogram (5 min under flow of nitrogen at

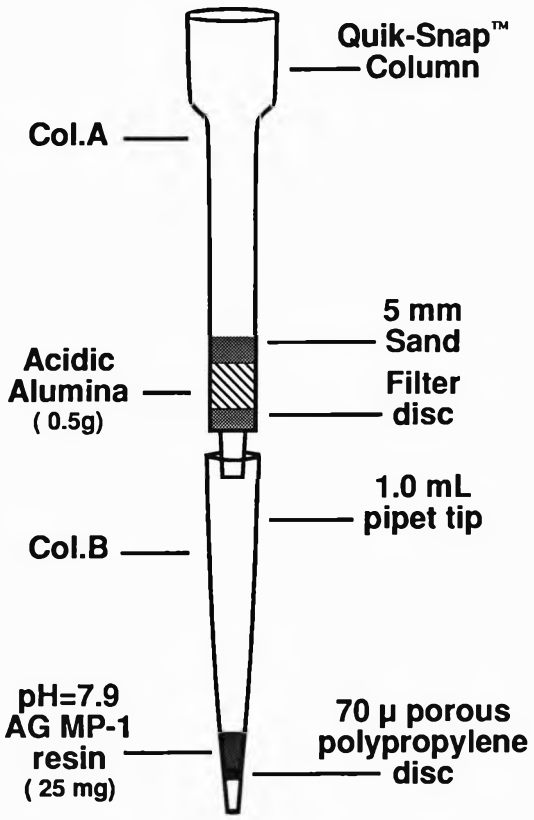

Figure 2. Cleanup and isolation column setup.

room temperature) (Camag Immersion Device II) at low speed for 2 s in 100 mL fluorescamine solution (25 mg in 10 mL acetone, to which 90 mL hexane is added). Dry the dipped plate 5 min with nitrogen then spray with 0.2M H_3BO_3 (adjust pH to 8.0 with 1M NaOH). After spraying with borate buffer, immediately place plate in –20°C freezer for ≥5 min before densitometry. Place positioning plate in –20°C freezer for ≥15 min before scanning. Place TLC plate prepared for chilling on positioning plate and chill parts together. For densitometry, scan lanes with a 0.025 × 5 mm band at 0.5 mm/s in the fluorescence mode using a Camag TLC Scanner II. After placing positioning plate (with respective TLC plate) back in densitometer, place similarly chilled Ace flexible cold compress (purchased at local drug store) on positioning plate to help maintain low-temperature environment during densitometry. Measure excitation at 366 nm (Hg lamp), and measure emission after a 400 nm cut-off filter. Record the densitogram, and measure peak heights on a Camag SP4290 integrator.

Sample Preparation

Intact pork loin roast purchased from local market was used for all work reported here. Pork tissue was manually deboned, and all grossly exposed connective tissue and fat were manually removed. Muscle tissue was cut into ca 1 in. cubes and ground twice through 2 mm hole plate (household model meat grinder; Sears Roebuck & Co., Chicago, IL). Ground meat was divided into ca 140 g lots and stored at –80°C in Whirl-Pak bags until analyzed.

Determination

Place frozen tissue (still in closed bag) under running cold water until meat is thawed. Weigh 5.00 g samples into aluminum weighing dishes, and fortify each analytical sample with 50 µL SBZ internal standard working solution. Up to 12 samples can be analyzed concurrently on equipment described (centrifuge and vacuum manifold). Designate 3 samples as calibration standards, and fortify 1 each at 0, 7.5, and 15.0 ppb by adding 50 µL water or respective working solution (0.75 or 1.50 µg SMZ/mL). For recovery studies, fortify each of the other 9 samples with 50 µL SMZ working solutions containing 2.0, 1.0, 0.8, 0.5, 0.4, 0.25, 0.2, 0.1, and 0.05 µg SMZ/mL. Let tissue sample stand at room temperature for 15 min to allow incorporation of drug into tissue. Transfer tissue sample into 50 mL centrifuge tube, dilute to mark (25 mL) with water (use wash bottle), and then homogenize sample. After homogenization, carefully use water wash bottle to rinse generator probe (direct rinse into centrifuge tube). A total of 40–45 mL homogenate should now be in centrifuge tube. After all samples are homogenized, cap tubes, shake briefly (5 s), and centrifuge. While samples are centrifuging, prepare C_{18} SPE setup as described in *Apparatus*, (**d**). After centrifuging, decant supernatants into their respective reservoirs through poly(propylene) wool plug in funnels. After all samples are filtered into their reservoirs, open each sample valve on vacuum manifold one full turn. Vacuum should be off at this point. Samples will begin to flow slowly by gravity through columns. Carefully apply vacuum to manifold until pressure is 20–30 kPa (ca 7 in. Hg). This should result in flow of 6–8 mL sample solution/min through column. Close sample valve of each tube when sample solution level reaches top of C_{18} column bed. Wait until all samples reach this point. Remove reservoirs, and wash all columns with 3 mL water at full vacuum (ca 90 kPa, 27 in. Hg), followed by 3 mL hexane (use wash bottles to apply wash water and hexane, and fill column barrel to top). Air-dry columns 10 min by applying full vacuum. While C_{18} columns are drying, prepare cleanup and isolation setup (Figure 2) as described in *Appartus*, (**f**) and (**g**). After columns are dry, attach Teflon solvent guide needles inside manifold chamber and setup to collect column eluants. Discard previous column eluants from samples and washings to waste.

Elute C_{18} columns with three 1 mL portions of methanol (use 1000 µL automatic pipet for this and subsequent solvent applications). Apply vacuum at 20–30 kPa (ca 7 in. Hg) to assist this elution only after first milliliter has completely wetted column by gravity percolation. Remove culture tubes from manifold, and pour eluates into Col. A of tandem setups (Figure 2). Flow from this point on in method is controlled by gravity. Rinse culture tubes with two 1 mL portions of methanol, and apply rinses to tandem setup only after previously applied methanol has completely passed through both columns. Rinse walls of Col. A with 1 mL methanol. After methanol has passed through, discard Col. A. Rinse walls of Col. B with 1 mL methanol. Discard methanol eluates from tandem column setups to waste.

Pass 250 µL acetone–acetic acid–methanol (94 + 5 + 1) through Col. B, and collect in 5 cc Reacti-Vials (Pierce Chem-

Table 1. Effect of scanner temperature[a]

	Chilled		Unchilled	
SMZ added, ng/g	SMZ found, ng/g ± SD[b]	Av. % accuracy[c]	SMZ found, ng/g ± SD[b]	Av. % accuracy[c]
0.51	1.12 ± 1.2	218.21	0.27 ± 2.4	52.54
1.02	2.67 ± 1.5	260.88	2.13 ± 0.56	208.19
5.11	4.58 ± 0.9	89.49	4.38 ± 1.8	85.77
7.67	7.35 ± 1.4	95.80	7.25 ± 2.5	94.50
10.22	9.21 ± 1.2	90.06	10.01 ± 2.4	97.97
15.34	14.99 ± 2.1	97.77	16.59 ± 0.8	108.15

[a] Results obtained during method development, not using the final procedure presented.
[b] $n = 3$; one analysis at each concentration on each of 3 days.
[c] Relative % accuracy based on SMZ found by using the internal standard calculation and the SMZ added.

ical Co., Rockford, IL), or similar conical vials. SMZ and SBZ are contained in this eluant. Cap vials and mix by vortexing 10 s. Apply 50 µL sample to TLC plate as described in *Apparatus*, (**h**). If 12 tissue samples (vacuum manifold and centrifuge capacity) are analyzed, 2 TLC plates are needed for analysis, and 3 calibration standards each must be applied to both plates. Calibration standards are routinely applied to tracks 2, 4, and 6 to space standards over plate. When sample application is completed, develop each plate for 11 min in toluene–ethyl acetate (1 + 1). Dry plate 5 min with N_2 before fluorescamine dipping, and dry after dipping ca 2 min with N_2 before spraying with borate buffer. Do not dry TLC plate after spraying with buffer, but immediately place on positioning plate of TLC scanner, already in –20°C freezer, for 5 min before scanning. Scan plate and record data as described above in *Apparatus and Reagents*, (**g**). Calculate linear calibration equations as follows for each plate from standards by using least-squares fit option in Cricket Graph software:

$$[\text{ ng SMZ/g} = m\frac{\text{SMZ height}}{\text{SBZ height}} + b\ ] \qquad (1)$$

Calculate SMZ found for each fortified sample by substituting observed SMZ/SBZ height ratios into calibration equation (Eq. 1) obtained for plate on which sample was developed.

Results and Discussion

Three parameters of the milk method were changed to permit tissue analysis: (*1*) the sample size was reduced to 5 g, (*2*) the fluorescence densitogram was obtained at a reduced temperature, and (*3*) the AG MP-1 resin elution volume was reduced to 250 µL.

The sample size was reduced, because we could not reliably homogenize 10 g tissue with 15 mL water. Mixing 5 g tissue with 20 mL water changed the liquid/solid ratio enough to allow complete and reliable homogenization and extraction, and the homogenate volume was kept within the capacity of a 50 mL centrifuge tube.

The internal temperature of the TLC scanner often reached 34°C after the Hg lamp was turned on and permitted to warm up. Surface temperature measurements of the positioning plate in the scanner yielded similar temperatures. Because fluorescamine derivatives are not heat-stable, increased temperature (>10°C above room temperature) probably is adversely influencing the results. To assess this effect, 6 fortification levels were evaluated each day for 3 days, with cooling and then without cooling (Table 1). The standard error of the estimate was calculated for each condition according to the following formula:

$$S_{xy} = \sqrt{\sum (X - \hat{Y})^2 / (n - 2)} \qquad (2)$$

where Y is the amount of SMZ found and $\hat{Y}$ is the amount of SMZ added. $S_{xy} = 1.41$ when the plate is scanned chilled, and $S_{xy} = 1.83$ when the plate is scanned at the densitometer's ambient operating temperature (about 30–34°C). In addition to the reduced standard error, an increase in the signal-to-noise ratio was also observed when scanning was done at the reduced temperatures. Therefore, with a reduction in the S_{xy} and an increase in the signal-to-noise ratio, reduced temperature scanning was incorporated into the method. The effect of reduced temperature scanning is being investigated further, and we devised an alternative positioning plate that conveniently maintains the reduced temperature for enough time (13) without the need of the Ace cold pack.

The precision and accuracy values obtained at the 0.5 and 1.0 ppb levels (Table 1) were not as good as those we obtained with the method for SMZ in milk (11). We reduced the sample size from 10 mL (about 10 g) to 5 g tissue and applied a 100 µL sample to the TLC plate to compensate for this reduction. However, when 100 µL was applied to the plate, the chromatographed bands were not as narrow as when 50 µL was applied. We believe that this is because of an excess of acetic acid in the sample band that interferes with the chromatography. To correct the problem, the elution volume of the AG MP-1 column was reduced to 250 µL from 500 µL. This change permitted a smaller volume (50 µL) to be applied to the plate. The 50 µL volume did not affect the chromatography, and the reduction to 250 µL did not affect the recoveries.

Once the described procedure was developed, SMZ was successfully extracted with water and then cleaned up from pork tissues fortified with SMZ at 1.1 and 2.2 ppb (Figure 3). A good signal-to-noise ratio was obtained even at the 1.1 ppb

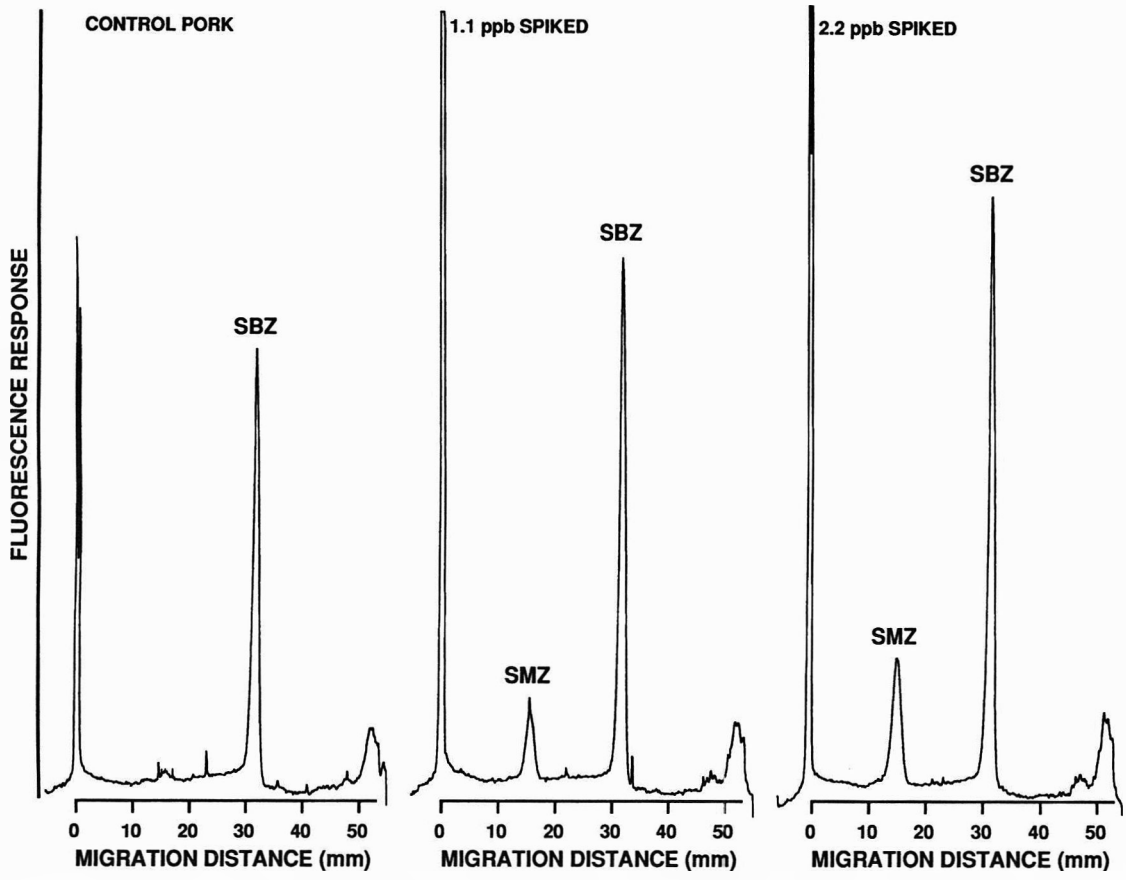

Figure 3. Sample densitograms: Tissue samples fortified with 10 ppb SBZ and either 0, 1.1, or 2.2 ppb SMZ are illustrated. Origin is at 0 mm migration, and solvent front is at 53 mm migration.

level (about 10:1). All samples were fortified with 10 ppb SBZ. The different response heights for the SBZ peak are evidence of the variability of the absolute percent recovery in the method and demonstrate the need for an internal standard correction. Calibration lines were calculated from samples fortified at 0, 7.5, and 15 ppb SMZ (as described above), with SBZ as an internal standard. The average squared correlation coefficient for the calibration curves was 0.995 ± 0.006 ($r^2 \pm$ SD, $n = 12$). The high correlation for the curves over the 15 ppb range illustrates the suitability of SBZ for use as an internal standard. Table 2 shows the results obtained for pork tissue fortified at 9 levels and analyzed daily for 6 days. The average accuracy over the analysis range of 0.5–21 ppb was 95.65% (SD = 29.45%, $n = 54$).

Close examination of the control sample (Figure 3) reveals a small interference at 16 mm migration distance ($R_f = 0.30$). This coincides with the SMZ migration in the fortified samples. The level was undetectable by the integrator and was not subtracted from the results. Because pork used in these studies was obtained from a local meat market, samples may not have been completely sulfonamide-free. To determine whether the peak might be SMZ, three 5 g samples of unfortified pork (neither SMZ nor SBZ was added) were extracted. The methanol eluates from the 3 C_{18} SPE columns were combined and passed through the same aluminum oxide and AG MP-1 tandem setup; then, 50 µL of the acidic acetone eluate of the AG MP-1 column was applied to a TLC plate and developed as described. The baseline interference at the 16 mm migration distance increased in height approximately 3 times, and the rest of the densitogram remained unchanged. This result indicates 2 conclusions. First, SBZ is a good choice as an internal standard, because no evidence was found of interferences at SBZ's migration distance of 31 mm ($R_f = 0.58$). Second, the pork tissue used for this study may have been contaminated with an SMZ residue of approximately 0.1 ppb. No attempt was made to confirm the identity of the interference by other means. Other sulfonamides could be the cause of the interference; sulfamerazine and sulfadiazine were both isolated by the method and both migrate with SMZ in the TLC system presented. SMZ could be confirmed by using a different TLC system (15) or one of several TLC (16, 17) or LC systems (8, 11, 18). Sulfaethoxypyridazine and sulfadimethoxine are 2 other sulfonamides isolated by the method, but they are both resolved from SMZ. The only other sulfonamide to which the method was applied was sulfapyridine (SPD), which is not isolated. SPD's pKa is 8.43 (19), and thus, it is not retained by the 7.9 AG-MP1 resin. Altering the pH of the AG-MP1 resin to 9 or above should isolate SPD. SPD is chromatographically resolved from SMZ, as are many other sulfonamides (15). The potential for a multisulfonamide method is present but has not been evaluated.

The identity of the interference, although important, does not greatly influence the determination of SMZ at the 5–10 ppb

Table 2. Accuracy results for sulfamethazine-fortified pork tissue

SMZ added, ng/g	SMZ found, ng/g ± SD[a]	Av. % accuracy[b]	CV, %
0.54	0.56 ± 0.39	102.19	71.51
1.09	1.16 ± 0.51	106.08	43.98
2.18	1.84 ± 0.42	84.51	22.50
2.73	2.31 ± 0.27	84.64	11.93
4.36	3.72 ± 0.33	85.16	8.97
5.45	4.75 ± 0.27	87.18	5.61
8.72	8.19 ± 0.29	93.88	3.62
10.91	11.06 ± 0.63	101.40	5.72
21.81	24.93 ± 3.73	114.31	14.95

[a] $n = 6$; one analysis at each concentration on each of 6 days.
[b] Relative % accuracy, based on SMZ found using the internal standard calculation and the SMZ added.

range, but it should affect the method's detection limit. Without correcting for the interfering residue and using the signal-to-noise ratio of 3:1, we estimate the method's limit of detection at approximately 0.25 ppb; however, this noise level is not suitable for regulatory purposes. The FDA's general guidelines for methodology for residue analyses below 100 ppb require the background noise at the regulatory level to be less than 10% of the residue's signal and the coefficient of variation to be less than 20%. According to these guidelines, the presented method could be suitable for regulatory purposes at and above the 2 ppb level (see Table 2 and Figure 3).

The upper limit of the method must also be considered. Figure 4 shows 2 plots of the residuals (SMZ found – SMZ added) for the values used in Table 2. The average residual and the upper and lower control limits (+3SD and –3SD, respectively) are indicated as lines across each plot. Plot A includes the values found for the 21.81 ppb fortification; Plot B does not. There is a larger control limit span in Plot A, and 2 values in the 21.81 ppb zone exceed the upper control limit. This indicates a problem with the method at this level of analysis. The standard error of analysis calculated as defined above (Eq. 2) with and without the 21.81 ppb data yields values of 1.66 and 0.58, respectively. An F-test on the variances indicates that the 2 sets of data are not of the same group. A possible cause is the saturation of the photomultiplier tube with the 21.8 ppb samples. Therefore, we suggest setting the upper quantitative limit of the method to approximately 15 ppb, which, from our experience with SMZ in milk (11), is within the linear range of the photomultiplier tube. If quantitation at a level higher than 15 ppb is desired, we suggest decreasing the sample size, lowering the sensitivity of the photomultipler, or fortifying with SBZ at the target level desired and then either decreasing the volume applied to the TLC plate or increasing the elution volume of the AG MP-1 column,.

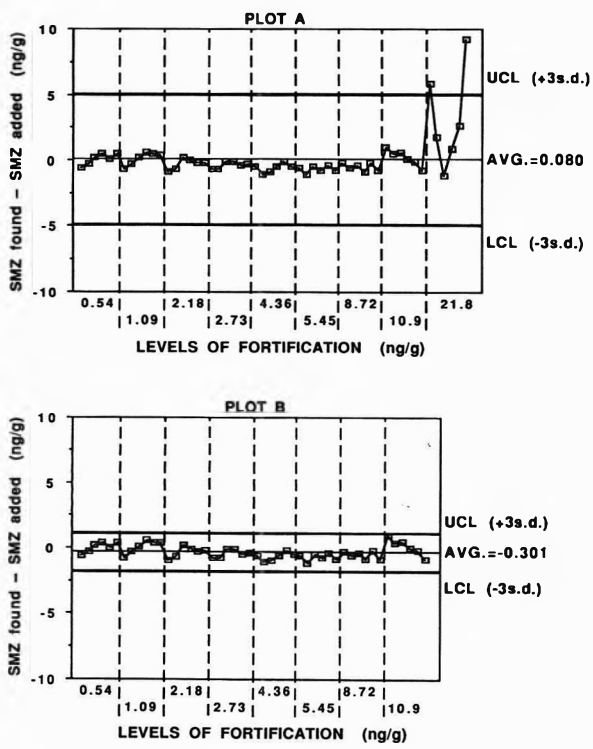

Figure 4. Quality control plots: Values for the difference between the SMZ found and SMZ added are plotted for each fortification level. The average difference, 99% upper control limit (UCL = +3SD) and 99% lower control limit (LCL = –3SD) were calculated and are illustrated.

Conclusions

The method is rapid (one analyst can manually complete 12 samples in 8 h), uses little solvent (about 20 mL solvent per sample for sample preparation and chromatography), and is sensitive (detection limit is approximately 0.25 ppb, with a linear range of quantitation of approximately 2–15 ppb).

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FOOD ADULTERATION

Liquid Chromatographic Detection of a Variety of Inexpensive Sweeteners Added to Pure Orange Juice

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Liquid chromatography with pulsed amperometric detection was used to analyze pure orange juice adulterated with a variety of inexpensive sweeteners. This method can be used to detect low levels (5–10%) of high fructose corn syrup (42 and 55), cane sugar hydrolysates (50 and 80%), and beet sugar hydrolysates (chemically or enzymatically prepared).

The adulteration of orange juice has progressed from simple addition of water and sugar to the addition of adulterants designed to mask detection by regulatory agencies. The simplest form of adulteration is overdilution of concentrate. This type of adulteration can be detected by determining the °Brix value of the orange juice (1). If this value is lower than the minimum of 11.8 °Brix set by industry, the juice has been diluted with water.

Other inexpensive fruit juices have also been used to adulterate orange juice. Tillmans and Kiesgen (2) developed the formol index, which quantitates free amino acids to detect this type of adulteration. An excellent review on the expansion of this method to the detection of individual amino acids was published (3). Analysis of flavanone glycosides by liquid chromatography (LC) was also successfully used (4) to detect the presence of grapefruit juice in orange juice.

Another significant adulterant in the orange juice industry is the addition of pulpwash. To detect this type of adulteration, Petrus and Attaway (5) developed methodology based on the difference in the visible and ultraviolet absorption and fluorescence excitation and emission characteristics of orange juice and orange pulpwash.

Attempts were also made to establish orange juice adulteration by determining trace components. These components include phenolics (6), chloramine-T (7), lipids (8), minerals (9), organic acids (10), and vitamins, sugars, and nicotinic acid (11). Vandercook et al. (12) introduced a microbiological assay to determine the fruit content of orange juice products; however, this method achieved limited success in the detection of adulterants in orange juice.

The major soluble solid present in orange juice is carbohydrate (13); therefore, sophisticated adulteration of orange juice requires addition of carbohydrate. Isotope ratio ($^{13}C/^{12}C$) was used extensively for the detection of sugar cane and corn-derived syrups in orange juice (14, 15). However, low-level addition of these inexpensive sweeteners (<25%) to orange juice can go undetected (13). Because of identical CO_2 fixation mechanisms, this isotope ratio measurement cannot be used to detect beet sugar/beet sugar hydrolysate. Progress was made in determining $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios (16, 17) for beet sugar addition to orange juice concentrate. However, climatic variation in the $^{18}O/^{16}O$ ratio and difficulty in routinely determining the $^{2}H/^{1}H$ isotope ratio limit the widespread use of these methods. These isotope methods for beet sugar determination cannot be used with single strength orange juice because of the addition of water.

Recently, our research group (18) developed a method to detect the adulteration of orange juice with the beet sugar hydrolysate, beet medium invert sugar (BMIS). This material is an ideal adulterant for orange juice, as it contains the same major carbohydrate ratio as orange juice (1:1:2, glucose:fructose:sucrose) and is inexpensive (<$0.30/lb). The addition of this material to orange juice can be readily detected by LC analysis of "fingerprint" oligosaccharides. This method was widely accepted by regulatory agencies and testing laboratories for the routine analysis of orange juice suspected of being adulterated with BMIS.

In this paper, we present an extension of this LC method to the detection of a variety of inexpensive sweeteners in a single chromatographic run. These sweeteners include high fructose corn syrup (HFCS 42 and 55), cane medium invert syrup (CMIS, 50% hydrolyzed cane sucrose), cane invert syrup (CIS, 79–80% hydrolyzed cane sucrose), beet invert syrup (BIS, >90% hydrolyzed beet sucrose) prepared both chemically and enzymatically, and BMIS (50% hydrolyzed beet sucrose) produced chemically and enzymatically. We also introduce a much simpler sample preparation scheme and a more rapid elution program, which shortens the analysis time to 96 min.

Experimental

Samples

A reconstituted orange juice concentrate (Florida Department of Citrus pilot plant) consisting of 11.71 °Brix and 0.78% acid was used in this study.

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Commercial BMIS (Michigan Sugar Co., MI), HFCS (42 and 55; CASCO Inc., ON, Canada), and CIS (50%, 79–80%; Tate and Lyle, London, UK) were used as adulterants in this study.

BIS and BHIS (chemically produced) were prepared from 90 g liquid beet sucrose (65.5 °Brix, Alberta Sugar Co.). To this solution, 100 µL concentrated hydrochloric acid solution (37%) was added. The solution was heated to 65°C, and aliquots were analyzed by LC until 96–98% of the sucrose was hydrolyzed. The total reaction time was ca 90 min (60 min for medium invert sugar). The solution was neutralized by the dropwise addition of 25% sodium hydroxide solution.

BIS and BMIS (enzymatically produced) were prepared from 205 g liquid beet sucrose (65.5 °Brix, Alberta Sugar Co.) with 30.2 mg invertase (Sigma Chemical Co., I–4504 Grade VII, 852 units/mg). The enzyme was solubilized in water (minimum amount) before it was added. The reaction mixture was heated at 55°C, and aliquots were analyzed until the desired glucose:fructose:sucrose ratio was obtained (ca 40 min for 1:1:2; 80 min for glucose:fructose ratio of 1:1). The solution was then heated for 5 min at 80°C to denature the enzyme, filtered, and added to orange juice.

Analytical samples (additions based on sweetener solutions of 11.0 °Brix) were as follows:

(a) *Reconstituted orange juice concentrate.*—Sample A, 11.71 °Brix, 0.78% acid.

(b) *Sample A (80%) + 10% HFCS 42 + 10% beet sucrose.*

(c) *Sample A (80%) + 10% BIS (chemically produced) + 10% beet sucrose.*

(d) *Sample A (80%) + 10% BIS (enzymatically produced) + 10% beet sucrose.*

(e) *Sample A (80%) + 12% CIS + 8% beet sucrose.*

(f) *Sample A (80%) + 20% CMIS.*

(g) *Sample A (80%) + 20% BMIS (chemically produced).*

(h) *Sample A (80%) + 20% BMIS (enzymatically produced).*

Sample Preparation

Samples were diluted with LC grade water to 5.5 °Brix. The resulting solution was centrifuged (Beckman Instruments Inc.) 15 min at 4°C (2000 × *g*). The supernatant was passed through 5 cm^3 100–200 mesh AG 50W-X8 cation exchange resin and 5 cm^3 100–200 mesh AG1-X4 anion exchange resin (formate form) (Bio-Rad Laboratories). Passage of the sample through these resins removes proteins, amino acids, organic acids, most cations, and most anions. The sample was then passed through a C_{18} Sep-Pak cartridge to remove flavanones and phenolics. Finally, the sample was passed through a 0.22 µm syringe filter (Waters Associates). Ninhydrin and Bradford tests were performed on selected samples to ensure the absence of amino acids and proteins. All samples were either analyzed immediately or stored at –20°C until analyzed.

Liquid Chromatography

The resulting samples were analyzed on a Waters 625 metal-free gradient liquid chromatograph. Carbohydrates were separated on 2 Carbo Pac PA1 (Dionex) pellicular anion exchange columns (250 × 4 mm) connected in series. A 100 µL sample was injected by a Waters 712 Wisp autosampler for analysis of the orange juice samples. The flow rate was 0.70 mL/min. The carbohydrates were detected by a pulsed amperometric detector (PAD; Waters Model 464) with a gold electrode and triple-pulsed amperometry at a sensitivity of 50 µA. The electrode was maintained at the following potentials and durations: $E_1 = 0.05$ V ($T_1 = 0.299$ s), $E_2 = 0.60$ V ($T_2 = 0.299$ s), and $E_3 = -0.80$ V ($T_3 = 0.499$ s). A postcolumn delivery system (Waters) of 300mM NaOH at 0.80 mL/min was used to minimize baseline drift. The linear gradient elution shown in Table 1 was used to achieve separation of the oligosaccharides.

Table 1. Linear gradient elution for separation of oligosaccharides

	Composition, %		
Time, min	A	B	C
0.00	100	0	0
6.59	100	0	0
7.00	95	5	0
23.00	89	11	0
30.00	60	40	0
35.00	60	40	0
45.00	10	90	0
50.00	10	90	0
53.00	0	0	100
68.00	0	0	100
68.10	100	0	0
96.00	100	0	0

[a] Where A is 100mM NaOH, B is 100mM NaOH/100mM NaOAc, and C is 300mM NaOH.

Carbohydrates eluting from the columns were plotted by either a Maxima 825 Chromatography Workstation (Millipore) or a Spectra-Physics Model 4290 integrator.

Glucose, Fructose, and Sucrose Determination

Samples were prepared for analysis by dissolving 200 mg sample in a 250 mL volumetric flask and diluting to volume with LC grade water. Samples were filtered through a 0.22 µm syringe filter (Waters Associates).

Filtered samples were analyzed by using the aforementioned equipment with the following modifications: a single Carbo Pac PA1 column was used, and solutions were eluted with an isocratic mobile phase of 60mM NaOH at 1.0 mL/min with no postcolumn addition of NaOH.

Sugar standards were prepared for each carbohydrate both individually and as a mixture. Sample concentrations for each ranged from 0.01 to 0.10 mg/mL. LC analysis of these standards gave *r* values of 0.993, 0.994, and 0.983 for glucose, fructose, and sucrose, respectively. The standard carbohydrate mixture had a correlation coefficient of 0.992.

Titratable Acidity

Samples were analyzed in triplicate by **942.15** (*Official Methods of Analysis*, 15th ed.).

Table 2. Chemical composition of pure and adulterated orange juice samples

Sample	pH	°Brix	%Acid	Glucose:fructose:sucrose
Pure Florida orange juice (PFOJ)	3.77	11.7	0.79	1: 1.2: 2.4
PFOJ + 10% HFCS (42) + 10% beet sucrose (BeS)	3.73	11.5	0.63	1: 1.2: 2.1
PFOJ + 10% HFCS (55) + 10% BeS	3.73	11.5	0.62	1: 1.2: 2.3
PFOJ + 10% BIS chemically produced + 10% BeS	3.71	11.5	0.64	1: 1.1: 2.2
PFOJ + 10% BIS enzymatically produced + 10% BeS	3.75	11.5	0.63	1: 1.2: 2.1
PFOJ + 12% CIS + 8% BeS	3.73	11.5	0.62	1: 1.1: 2.2
PFOJ + 20% CMIS	3.71	11.5	0.65	1: 1.1: 2.2
PFOJ + 20% BMIS chemically produced	3.72	11.5	0.63	1: 1.2: 2.4
PJOF + 20% BMIS enzymatically produced	3.73	11.5	0.63	1: 1.1: 2.3
Representative literature values[a]	3.4–4.3	10.8[b]	0.65–1.52	1: 1.1: 2.1[c]

[a] From reference 29.
[b] Minimum.
[c] Average/Florida.

°Brix

Brix value for each sample was obtained on a refractometer (Canlab, Edmonton, AB) maintained at 22°C.

pH

A Fisher Accumet (Model 620) pH meter was used to determine sample pH values.

Results and Discussion

The major carbohydrates present in pure orange juice are glucose, fructose, and sucrose in an approximate ratio of 1:1:2 (19, 20). More than 95% of the carbohydrate present in sugar beets and sugar cane is the disaccharide sucrose (21). The simple addition of either of these materials to orange juice would alter the major carbohydrate profile, which would indicate adulteration. Hydrolysates such as BMIS or CMIS have the same major carbohydrate profile as authentic orange juice. Detecting the addition of either of these materials to orange juice proved to be a difficult analytical problem. Other inexpensive sweeteners that maintain the correct major carbohydrate ratio, such as HFCS (42 or 55, basically a 1:1 ratio of glucose:fructose) + beet sucrose and CIS or BIS (1:1 ratio of glucose:fructose) + beet sucrose, could also be used as adulterants for orange juice.

Table 2 contains standard chemical information on the samples analyzed in this study. In each case, the °Brix, pH, % acid, and glucose:fructose:sucrose ratio indicate that these samples would be considered authentic based on these tests.

Isotope ratio methods were extensively used to detect cane- and corn-derived syrups in orange juice. Although this method is useful, it cannot detect beet sugar/beet sugar hydrolysates because of identical CO_2 fixation mechanisms. On the basis of the natural range in isotopes of oranges, corn, and cane sugar, a sample could contain up to 25% of these adulterants and still have an acceptable isotope value (22).

Our original paper was based on the hypothesis that all natural products (oranges, sugar beets, etc.) contain trace levels of naturally occurring oligosaccharides (18). These trace oligosaccharides would be specific for each food product, and their presence could be used as fingerprints to detect the addition of one product to another. Our current research shows that the majority of the oligosaccharides found in the sweeteners used in this study are formed during their production, as no significant oligosaccharide levels are evident in the original materials (beet or cane sucrose).

Figure 1 shows the carbohydrate pattern for an authentic orange juice from Florida. The large, off-scale peaks having retention times of approximately 11.5–27 min are glucose, fructose, and sucrose. The carbohydrates of interest (fingerprint carbohydrates) are those that have retention times between 35 and 65 min. Both the quantity and number of oligosaccharides are low. Only 2 fingerprint oligosaccharides in this region have retention times between 35 and 65 min, one at 39.28 and another at 40.38 min. We have analyzed approximately 300 authentic orange juice samples from growing regions all over the world, and the natural oligosaccharide patterns were all very similar.

Because of an identical CO_2 fixation mechanism and a wide natural $^{18}O/^{16}O$ isotope ratio, the addition of beet sugar hydrolysates to orange juice is extremely difficult to detect. Because this material is inexpensive and readily available, it has become the adulterant of choice for this type of fraud. Figures 2–5 show the carbohydrate pattern of BMIS and BIS produced in our laboratory under both acidic and enzymatic conditions. The chromatogram of BMIS chemically produced (Figure 2) is identical to commercial BMIS with respect to oligosaccharide pattern (18). In addition, the chromatogram of BIS has an oligosaccharide pattern very similar to that of commercial BIS. The chromatogram of chemically produced BMIS has 4 major fingerprint oligosaccharides eluting in the 42–53 min region. We have analyzed BMIS samples from 6 countries (United States, Canada, UK, Finland, Denmark, and Holland). In each case, the oligosaccharide patterns were chemically identical to our laboratory-produced BMIS.

Enzymatically produced BMIS (Figure 3) also has a number of fingerprint oligosaccharides with retention times in the 40.7–49.4 min range. Addition of either of these materials to pure orange juice would clearly show the presence of these

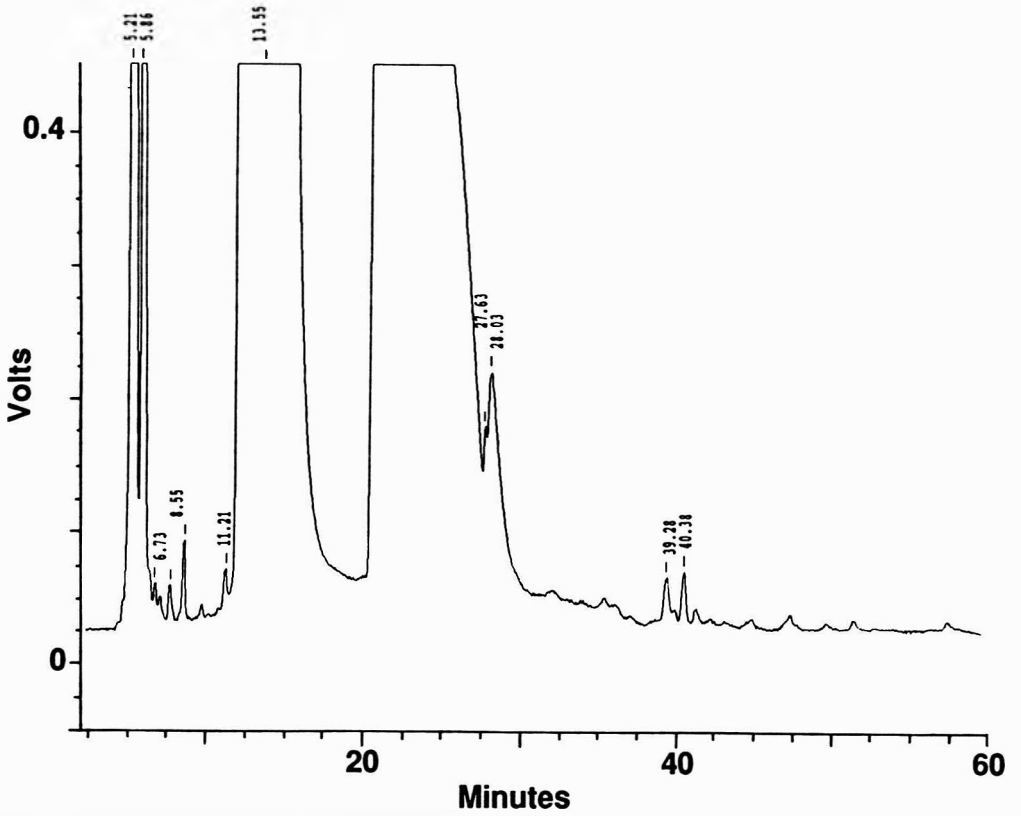

Figure 1. Liquid chromatogram of pure Florida orange juice.

fingerprint compounds and would reveal their origin. Figure 4 is the chromatogram of BIS produced chemically. This material also has a unique fingerprint oligosaccharide profile, with 3 major peaks having retention times in the range of 42.3–49 min. Although the concentrations of these oligosaccharides are not as large as those observed for BMIS, they occur in a unique region (when compared to pure orange juice) and are readily detected. Enzymatically produced BIS (Figure 5) has a much more diverse oligosaccharide pattern in both number and concentration. These unique oligosaccharides could be readily used to detect the addition of this material to pure orange juice.

The formation of oligosaccharides under acid hydrolysis conditions is referred to as "reversion" (23, 24). A number of researchers observed the formation of oligosaccharides during the enzymatic hydrolysis of sucrose via transferase activity (25–27). Therefore, regardless of the method used to produce beet sugar hydrolysates, a fingerprint pattern will be produced that is different from that observed in the authentic material. This pattern can then be used to prove conclusively the adulteration of orange juice with these materials.

Figures 6–9 are chromatograms of the authentic orange juice adulterated to levels of 20% (total adulteration) with beet sugar/beet sugar hydrolysates. When BIS was used as the adulterant, 10% beet sugar was added to maintain the correct glucose:fructose:sucrose ratio. As can be seen from the carbohydrate profile of each of the adulterated samples, there is a dramatic increase in the oligosaccharide pattern. New peaks are observed; that is, oligosaccharides are present that are not present (or are present at extremely low levels) in authentic orange juice. The power of this method can be demonstrated by close examination of Figure 8, which shows pure orange juice adulterated with only 10% BIS. In this chromatogram, a significant peak (area of 1.7 million) still exists with a retention time of 42.35 min. The addition of 20% BMIS (chemically produced) to orange juice results in the appearance of 4 major peaks (Figure 6). The peak at approximately 53 min can be used as the fingerprint for BMIS addition. At 20% adulteration, this peak has an area >4 million. Therefore, 5% adulteration would result in a peak area of >1 million. It is clear from these chromatograms that the detection of 20% adulteration is simple, and that levels of adulteration as low as 5% could be readily detected.

The presence of fingerprint oligosaccharides can also be seen in each of the other inexpensive sweeteners examined in this study. Figures 10–13 are chromatograms of commercial cane and corn hydrolysates.

The oligosaccharide pattern for commercial CMIS (Figure 10) shows a large peak at approximately 42 min, with 6 other fairly large peaks in the 46.5–53.2 min range. Commercial CIS (Figure 11) shows 2 major peaks at approximately 42 and 48 min. As was the case with beet sugar hydrolysates, the natural abundance of the fingerprint oligosaccharides in these materials would reveal their addition to orange juice.

The production of HFCS involves enzymatic hydrolysis of corn starch, which results in the formation of trace levels of maltose, maltotriose, and other dextrose polymers (28). Figures 12 and 13 are chromatograms of commercial HFCS 55 and 42, respectively. The chromatograms conclusively show

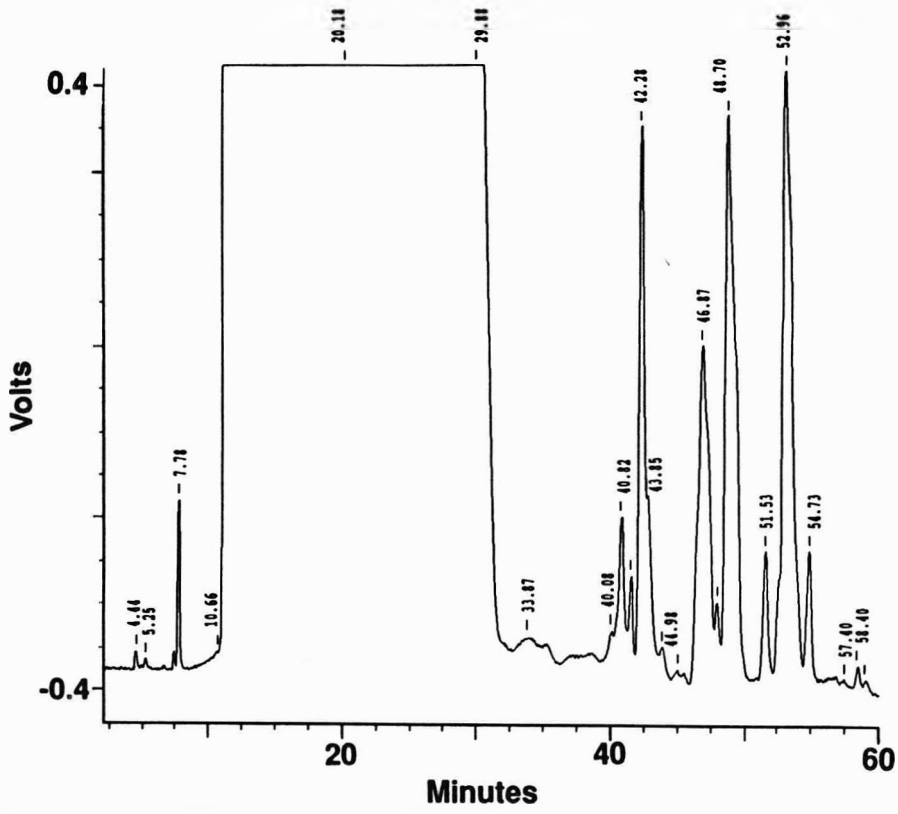

Figure 2. Liquid chromatogram of chemically produced BMIS.

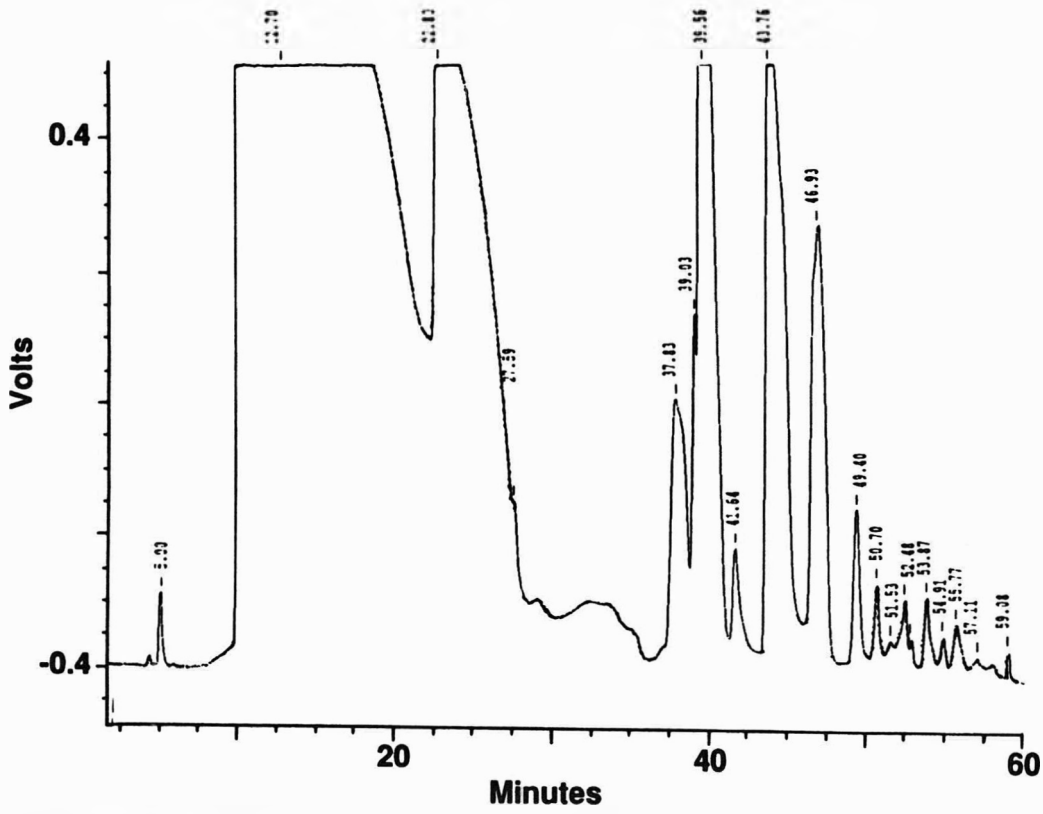

Figure 3. Liquid chromatogram of enzymatically produced BMIS.

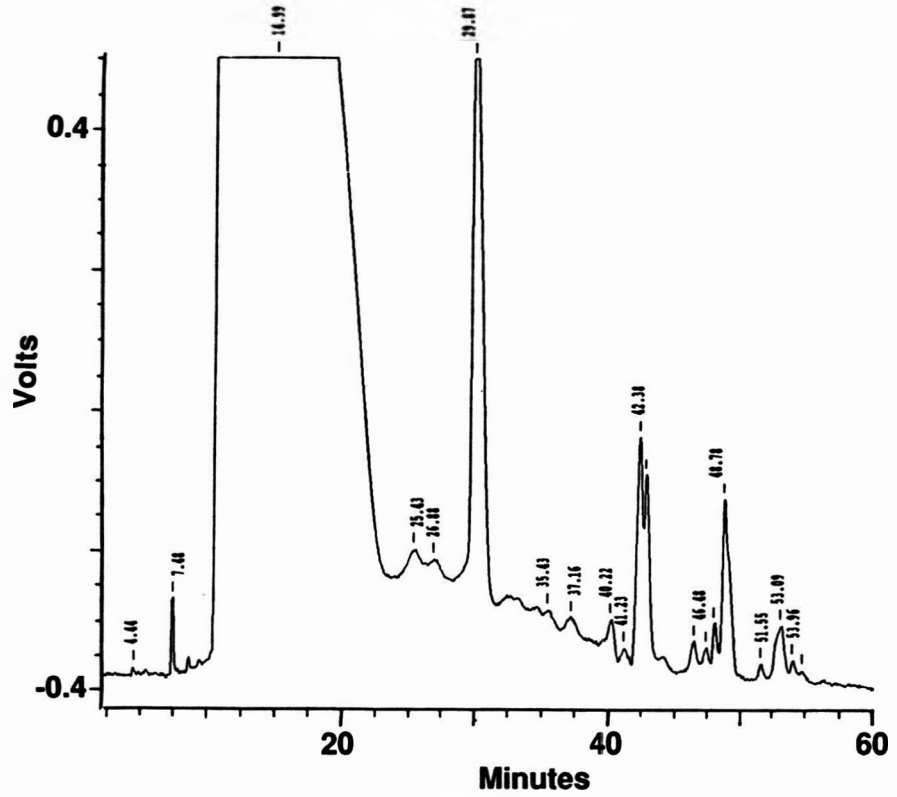

Figure 4. Liquid chromatogram of chemically produced BIS.

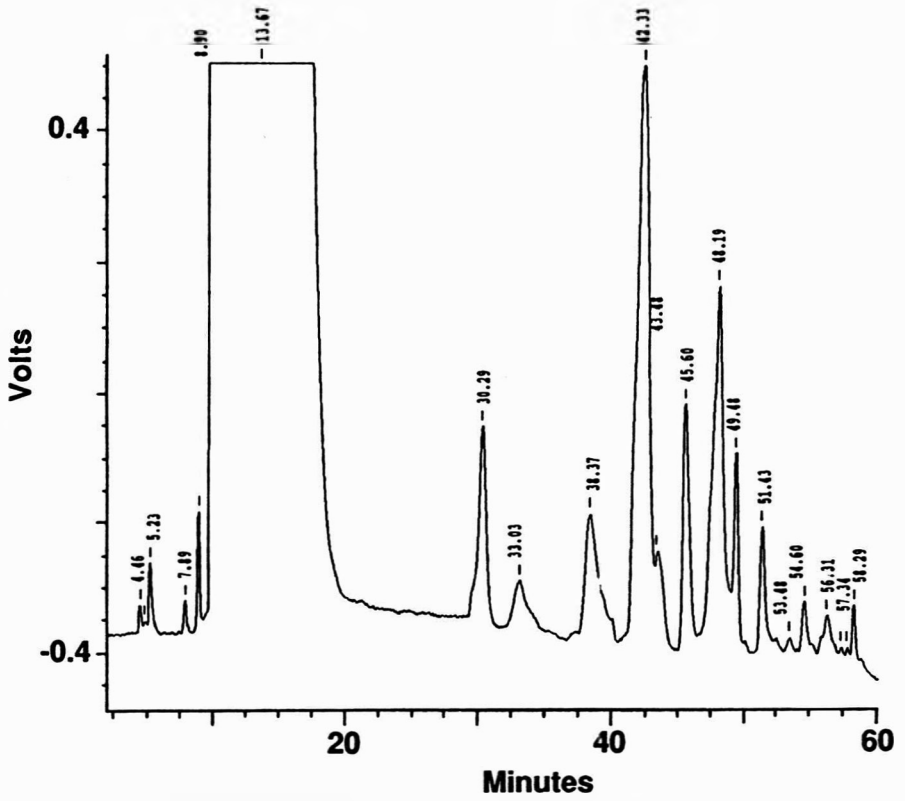

Figure 5. Liquid chromatogram of enzymatically produced BIS.

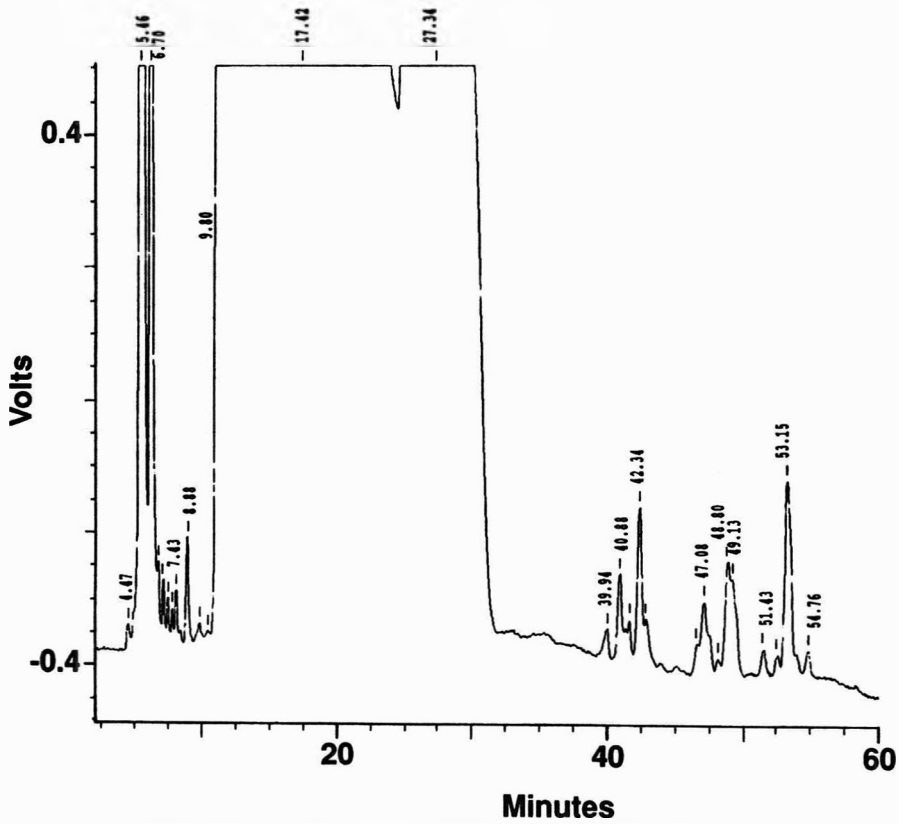

Figure 6. Liquid chromatogram of pure Florida orange juice + 20% chemical BMIS.

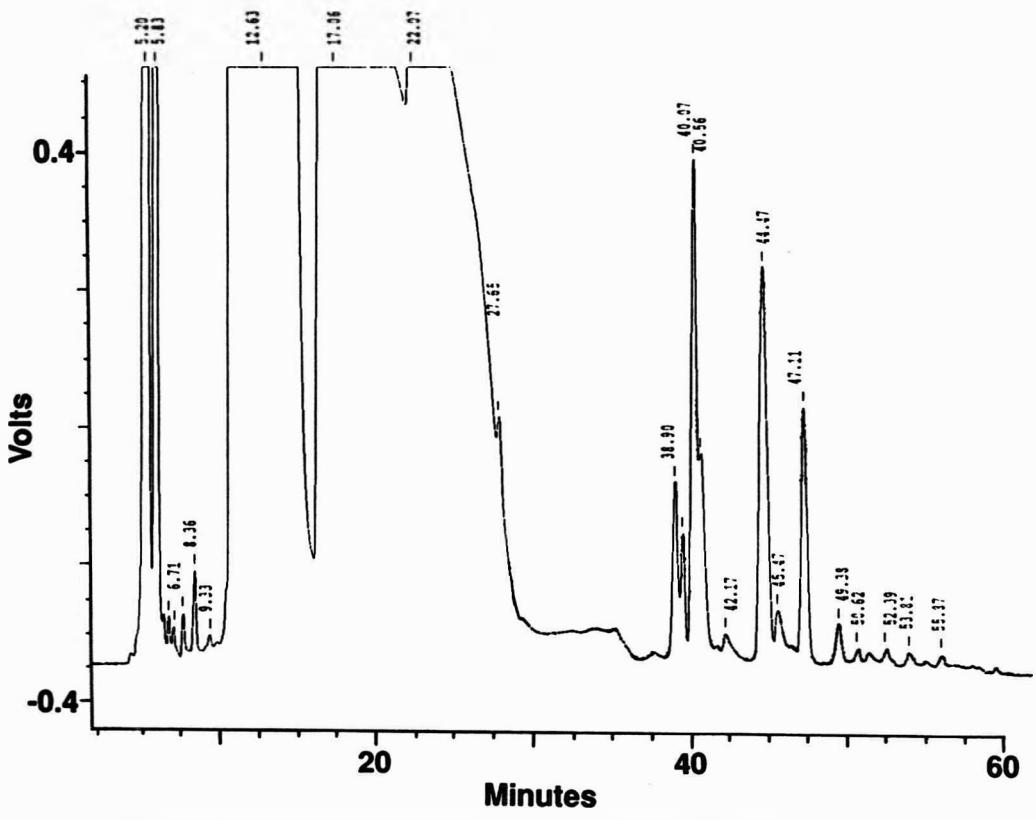

Figure 7. Liquid chromatogram of pure Florida orange juice + 20% enzymatic BMIS.

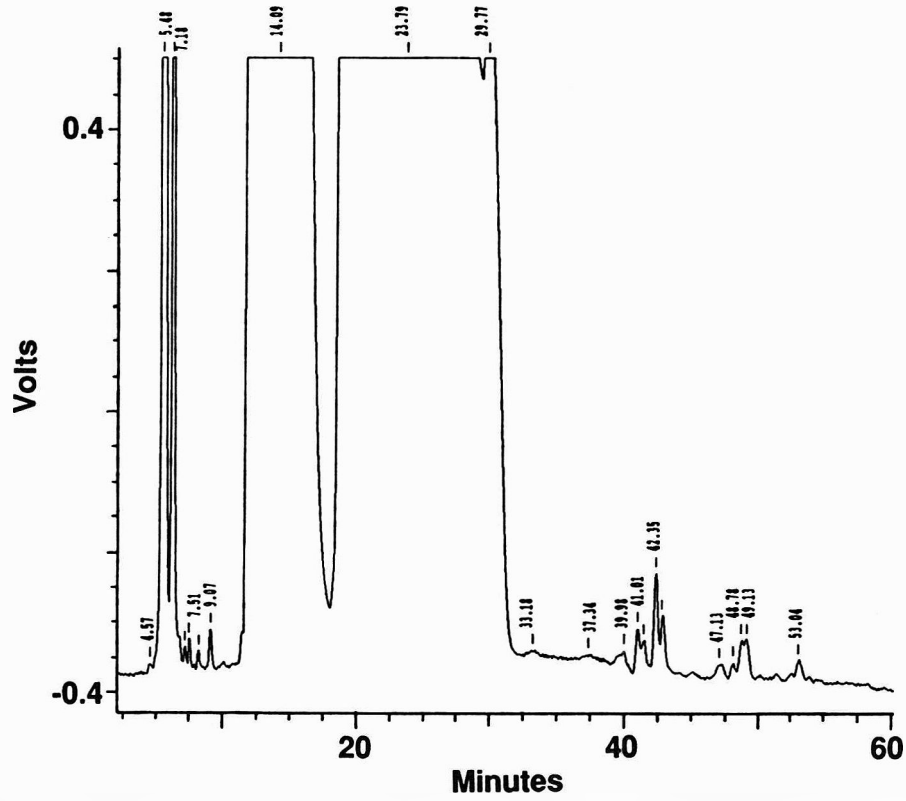

Figure 8. Liquid chromatogram of pure Florida orange juice + 10% chemical BIS + 10% beet sucrose.

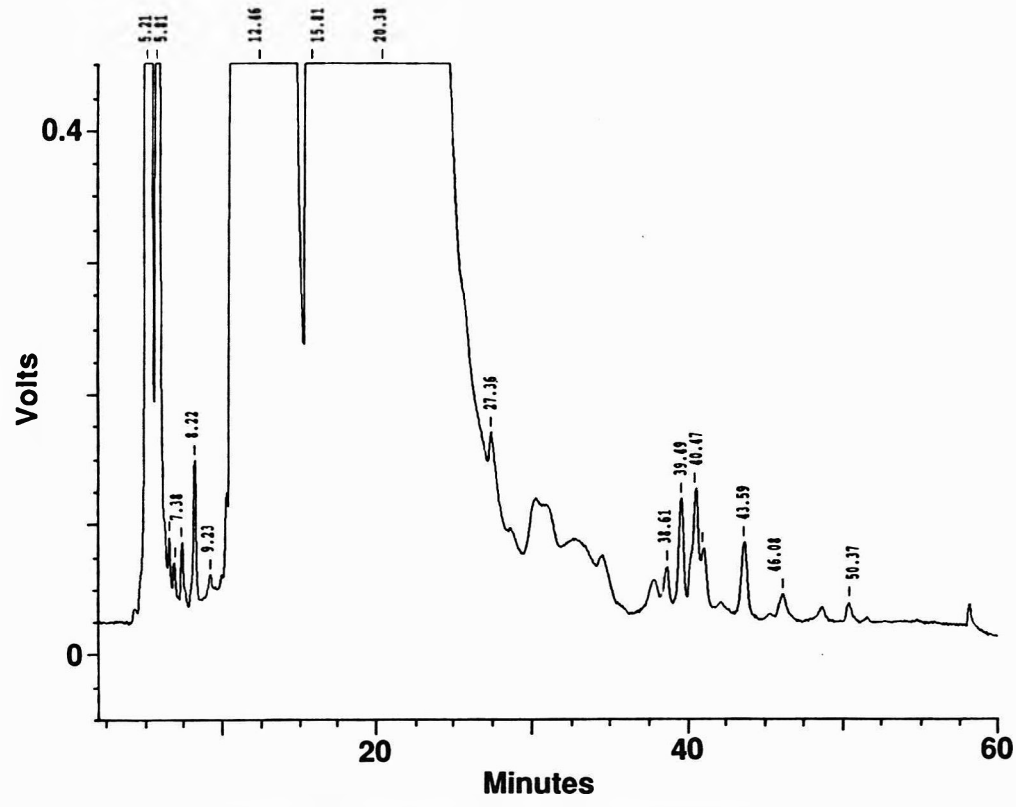

Figure 9. Liquid chromatogram of pure Florida orange juice + 10% enzymatic BIS + 10% beet sucrose.

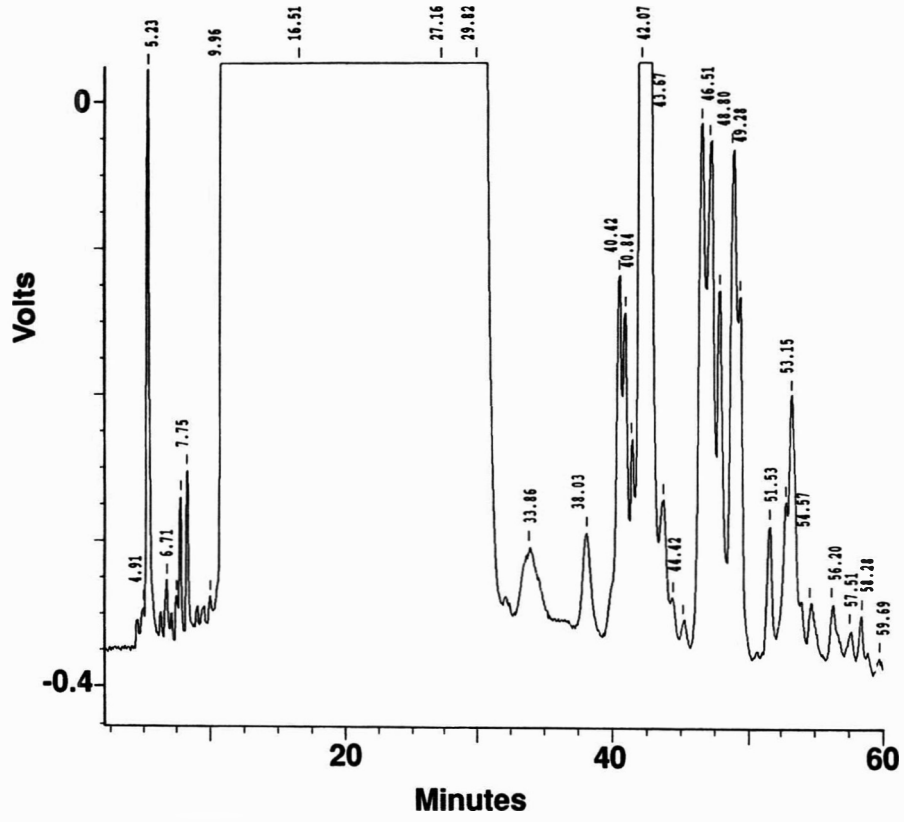

Figure 10. Liquid chromatogram of CMIS.

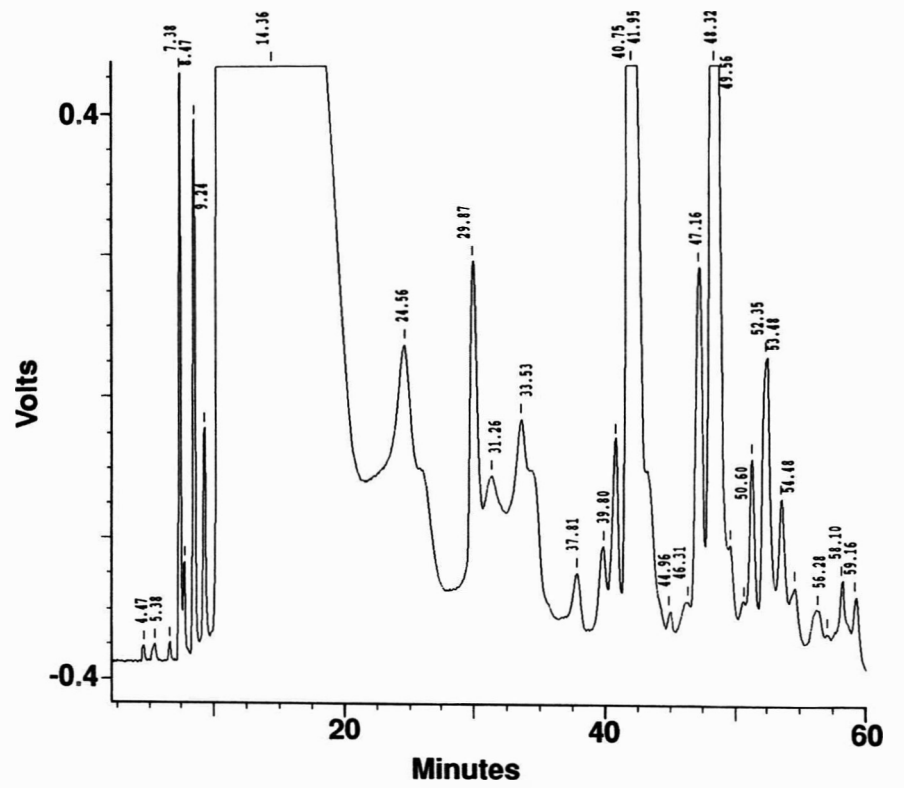

Figure 11. Liquid chromatogram of CIS.

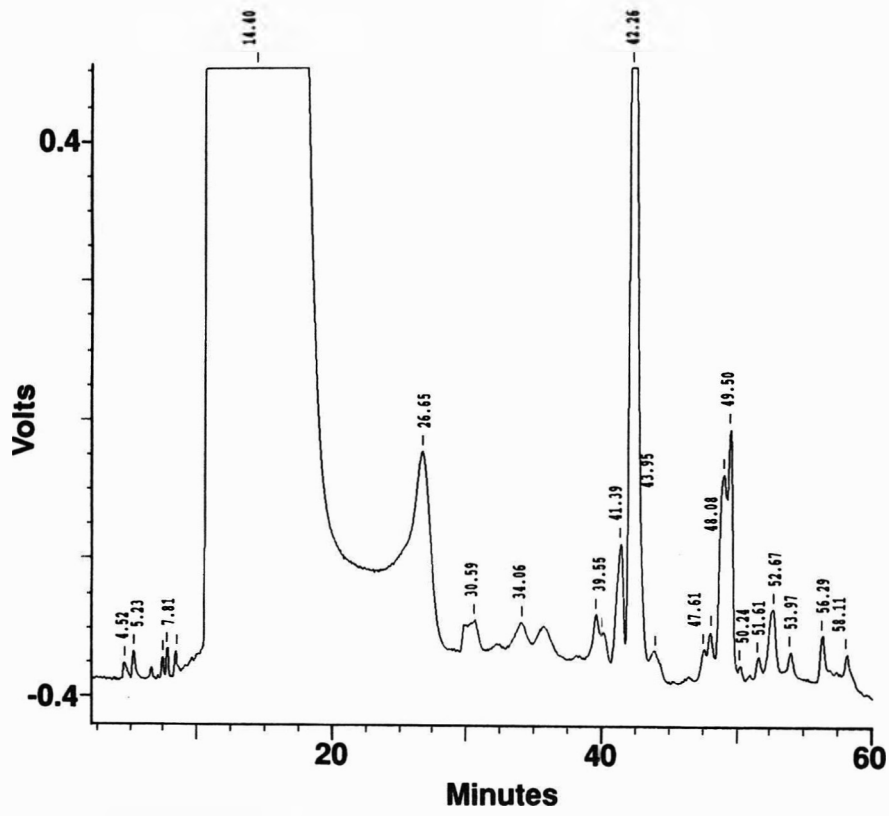

Figure 12. Liquid chromatogram of HFCS 55.

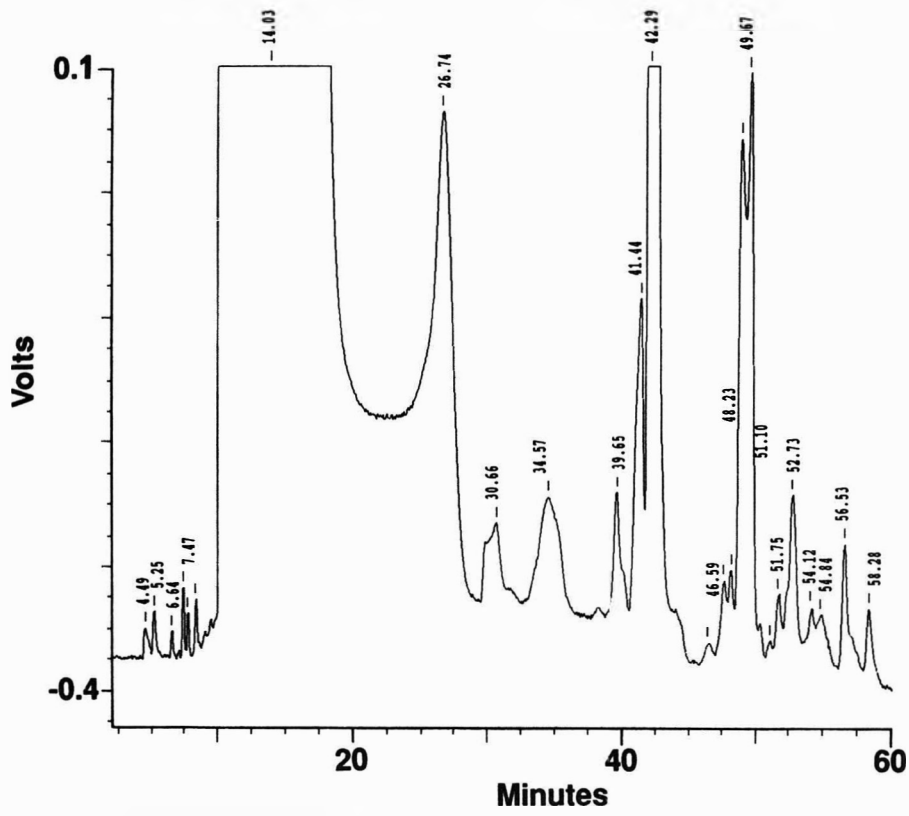

Figure 13. Liquid chromatogram of HFCS 42.

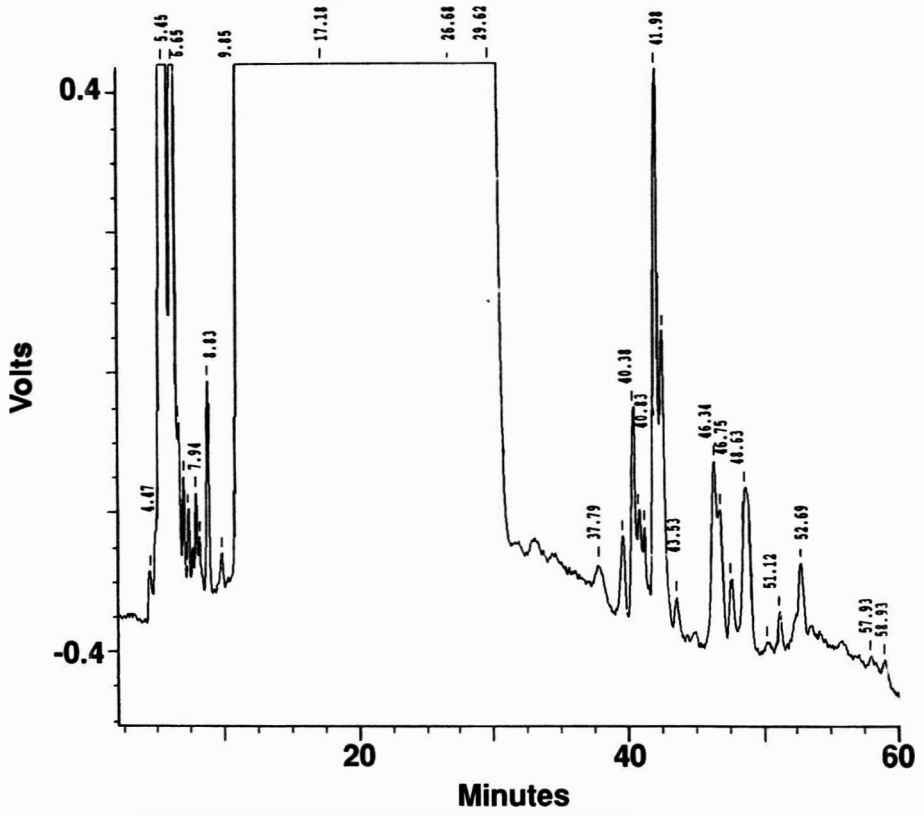

Figure 14. Liquid chromatogram of pure Florida orange juice + 20% CMIS.

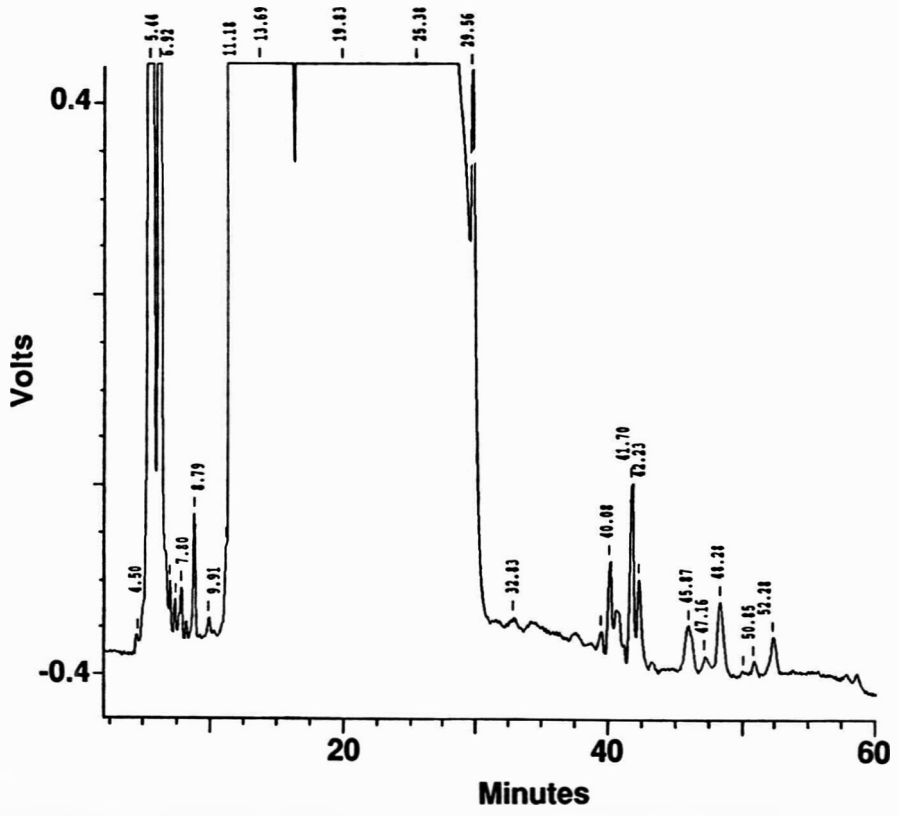

Figure 15. Liquid chromatogram of pure Florida orange juice + 12% CIS + 8% beet sucrose.

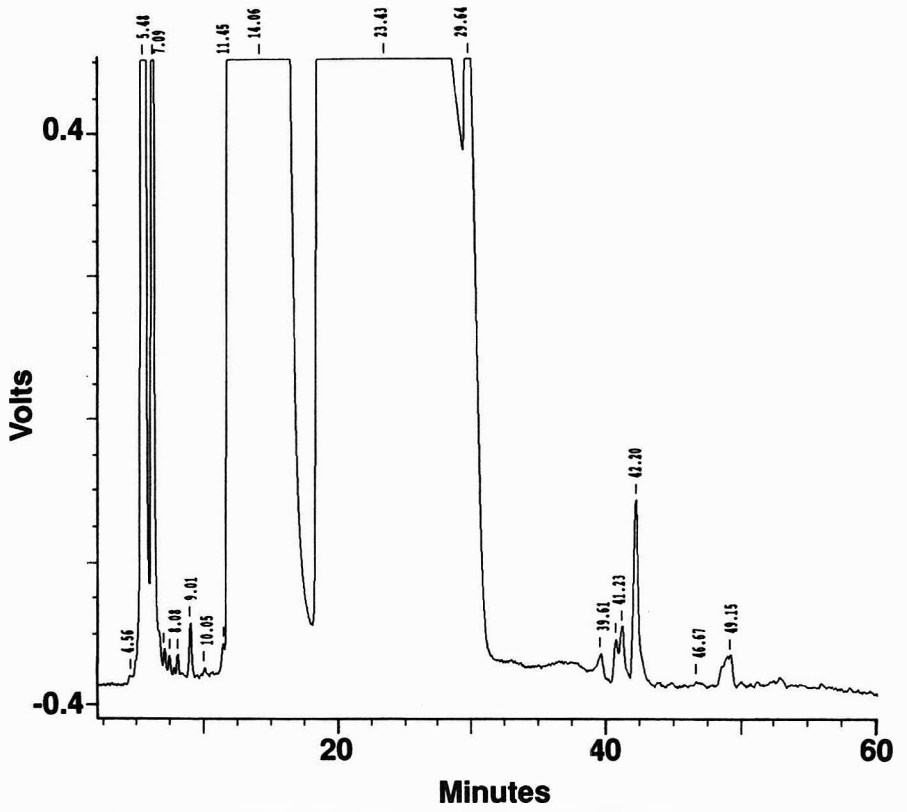

Figure 16. Liquid chromatogram of pure Florida orange juice + 10% HFCS 42.

that these inexpensive sweeteners contain appreciable levels of maltose (retention time of approximately 42.3 min) and maltotriose (retention time of approximately 49.5 min).

Research has shown that pure orange juice contains no maltose (29, 30). Therefore, our LC method would conclusively show the addition of HFCS to orange juice by the presence of maltose in an adulterated sample. This method could be used to detect low-level addition (<5%) of HFCS to orange juice.

Figures 14–16 show pure orange juice adulterated with low levels of cane/corn hydrolysates. As was the case with BIS, when CIS or HFCS was used as the adulterant, beet sucrose was added to maintain the correct glucose:fructose:sucrose ratio. It is quite obvious from these chromatograms that adulteration with these inexpensive sweeteners results in the appearance of fingerprint oligosaccharides. Although the inexpensive sweetener levels shown here are 20% (total adulteration), detection levels below this are easily attainable. We have identified adulteration with these materials to 5% levels.

In our laboratory, we use the presence of specific peaks (see above) to determine if an orange juice was adulterated and which inexpensive sweetener was used. We also use the areas of these peaks to approximate the level of adulteration (see above for BMIS and BIS). Because of differences in columns, specific batches of inexpensive sweetener, and detectors, we have not attempted to show an absolute correlation between peak area and adulteration level. Laboratories interested in using this technique to detect adulteration would be advised to analyze a number of pure juices and pure adulterants to determine fingerprint patterns and response factors for their system.

As we have shown in this research, oligosaccharides can be formed via the action of acid on sucrose. Therefore, we investigated the possibility of fingerprint oligosaccharide formation via the heating of single-strength/fresh-squeezed orange juice (SSOJ) during pasteurization. Experiments conducted in our laboratory show that oligosaccharides were not formed during this process. We also temperature-abused SSOJ (212°F for 5 and 10 min); analysis of these samples showed no oligosaccharide formation. The possibility does exist that temperature abuse of orange juice concentrate may result in oligosaccharide formation. However, as previously mentioned, we have analyzed pure orange juice concentrate from all growing and processing regions of the world, and these samples show very low concentrations of oligosaccharides.

In our previous work (18), the program used for carbohydrate elution and column re-equilibration was lengthy (3 h). In this research, we shortened the total analysis and column re-equilibration time to 96 min. This modification resulted in some loss of resolution of the oligosaccharides. However, the loss in resolution is not significant, and the shorter analysis time is more conducive to routine testing.

We also introduced a simpler sample cleanup that omits the use of charcoal/Celite. Initially, this cleanup was necessary to avoid column overload with glucose, fructose, and sucrose. By using a more dilute solution, we avoided this problem while maintaining a sufficient oligosaccharide concentration.

We extended this LC method to detect adulteration of grapefruit juice (31) with a variety of inexpensive sweeteners. In addition, we recently structurally identified 2 of the fingerprint oligosaccharides present in commercial BMIS (32).

Conclusion

A significant problem facing the orange juice industry today is the addition of inexpensive sweeteners to pure juices that maintain the correct major carbohydrate ratio. Analysis of trace carbohydrates in these sweeteners by LC with pulsed amperometric detection reveals the presence of several oligosaccharides unique to or in much greater concentration in these sweeteners than in pure orange juice. Research conclusively proves that the addition of any of these sweeteners to orange juice, even at low levels (<10%), can be detected.

This method is especially useful for the detection of HFCS in orange juice, because orange juice contains no maltose. The normal detection limits of this method (based on a 5.5 °Brix sample) can be greatly increased by monosaccharide removal and sample concentration before analysis (33). In the case of HFCS addition to orange juice, detection limits to 1% are possible.

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MICROBIOLOGICAL METHODS

Evaluation of Microbial Loads of *Bacillus subtilis* Spores on Penicylinders

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Three types of penicylinders were compared for their retention of spores of *Bacillus subtilis* testing for sporicidal activity of disinfectants. Glass, porcelain, and stainless steel penicylinders are inoculated with a water suspension of *B. subtilis* var. *niger* (ATCC 9372) spores and dried. One set of each type of penicylinder is submerged 1 h in 0.9% saline. One set of porcelain penicylinders is submerged 15 h in a neutralized chemical germicide, and one set is also inoculated with a culture filtrate of *B. subtilis* (ATCC 19659), dried according to the AOAC method, and submerged 1 h in 0.9% saline. Microbial loads simulate those held on carriers used to test sporicidal activity of disinfectants. Carriers are immersed in chemical germicide, transferred to a neutralizer, and placed in a culture medium. Average percentages of *B. subtilis* var. *niger* spores retained on 10 carriers after 1 h submersion in saline and in water were as follows: glass, 93.6%; porcelain, 99.9%; and stainless steel, 99.5%. Retention of spores after 15 h submersion in a neutralized chemical germicide and in water was 98.9%. Porcelain penicylinders inoculated from a culture filtrate of *B. subtilis* (ATCC 19659) retained 26% of the spores after being submerged 1 h in saline and placed in water. Glass penicylinders, which retained the lowest and most variable number of spores, were the least suitable for sporicidal activity testing. *B. subtilis* (ATCC 19659) spores tested on porcelain penicylinders met only the minimum HCl resistance requirements of ≥2 min. On porcelain penicylinders, the resistance of *B. subtilis* var. *niger* spores to 2.5N HCl was relative to the number of spores inoculated.

The AOAC method for testing sporicidal activity of disinfectants, **966.04** (1), calls for inoculating porcelain penicylinders in 72 h culture filtrates of *Bacillus subtilis* and *Clostridium sporogenes*. The method does not specify the number of spores to be inoculated on the penicylinders. Ascenci et al. (2) found that after *B. subtilis* spores were inoculated, dried on porcelain penicylinders, and placed in saline solution for 10 and 60 min, 71 and 72% of the spores, respectively, were washed off. In testing stainless steel penicylinders inoculated with different types of bacteria, Alfano et al. (3) found that the following percentages of cells were retained after carriers were exposed to phosphate-buffered dilution water: *Salmonella choleraesuis*, 10.1; *Pseudomonas aeruginosa*, 51.2; and *Staphylococcus* spp., 38.8.

To determine surface hydrophobicity, Ronner et al. (4) measured the contact angle of a drop of water on the surface and found it to be <5% (hydrophilic) for untreated glass and 25% (hydrophobic) for untreated stainless steel. They also found that spores of some species were strongly hydrophobic, as shown by their adherence to octylsepharose beads, and that this property corresponded to their adhesiveness to stainless steel (hydrophobic-low energy surfaces) and untreated glass (hydrophilic-high energy surfaces). Husmark and Ronner (5) reported that, as ionic concentration increased, the number of *B. subtilis* spores that adhered to hydrophilic glass also increased and that adhesion of spores was greater with different concentrations of $MgSO_4$ than with NaCl. Ascenci et al. (2) and Cole et al. (6) examined penicylinders by scanning electron microscopy and reported grooves and/or pits in porcelain and stainless steel penicylinders.

This study examined the retention of *B. subtilis* spores on 3 types of penicylinders and compared a water suspension of *B. subtilis* var. *niger* (ATCC 9372) spores with a culture filtrate of *B. subtilis* (ATCC 19659) spores for inoculating porcelain penicylinders in the AOAC sporicidal test for disinfectants.

Experimental

Media and Reagents

(a) *Soil extract nutrient broth.*—Prepared as directed in AOAC method **966.04A** (1), 10 mL in 20 × 150 mm tubes; Anatone (American Laboratories, Inc., Omaha, NE 68127), beef extract (Difco Laboratories, Detroit, MI 48232), an the author's garden soil extract.

(b) *Nutrient agar.*—Difco.

(c) *Manganese sulfate.*—Aldrich Chemical Co., Milwaukee, WI 53233.

(d) *Pour plate agar.*—Bacto tryptic soy agar (TSA) (Difco).

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(e) *Phosphate-buffered dilution water.*—Prepare as described in AOAC **960.09A**.

(f) *Distilled, deionized water.*—University of Minnesota, Minneapolis, MN 55455.

(g) *Sterile water.*—For irrigation, United States Pharmacopeial (USP) grade (Travenol Laboratories, Inc., Deerfield, IL 60015).

(h) *Sterile saline.*—0.9% sodium chloride, for injection; USP (American McGaw, Irvine, CA 92714).

(i) *Sodium hydroxide.*—Mallinckrodt Chemical Works, St. Louis, MO 63160.

(j) *Hydrochloric acid.*—Mallinckrodt.

(k) *Acid potassium phthalate 84j.*—National Institute of Standards and Technology, Gaithersburg, MD 20899.

(l) *Alcide expor.*—Alcide Corp., Norwalk, CT 06851.

(m) *Sodium bicarbonate.*—Mallinckrodt.

Organisms

(a) *Bacillus subtilis var. niger.*—ATCC 9372 (American Type Culture Collection, Rockville, MD 20852).

(b) *Bacillus subtilis.*—ATCC 19659 (American Type Culture Collection).

Apparatus

(a) *Borosilicate glass (Pyrex) penicylinders.*—8 × 10 mm (University Research Glassware, Carrboro, NC 27510).

(b) *Porcelain (steatite ceramic) penicylinders.*—8 × 10 mm (Fisher Scientific, Eden Prairie, MN 55344).

(c) *Stainless steel penicylinders.*—8 × 10 mm, polished (S&L Metal Products, Corp., Maspeth, NY 11378).

(d) *Scanning electron microscope (Jeol 840).*—Jeol USA, Inc., Peabody, MA 01960.

(e) *Sonicator.*—Healthsonics Corp., San Ramon, CA 94583.

(f) *Vortex mixer.*—Vortex Genie (Scientific Industries, Inc., Springfield, MA 01101).

(g) *Colony counter.*—Parkfield Quebec (American Optical Corp., Buffalo, NY 14215).

(h) *Medication tubes.*—Borosilicate glass, 25 × 150 mm (VWR Scientific, Chicago, IL 60660).

(i) *Morton culture tube closures.*—25 mm (Scientific Products, Minneapolis, MN 55441).

(j) *Petri dishes.*—Glass (Pyrex), 20 × 100 mm, and poly(styrene), 15 × 100 mm (Fisher).

(k) *Filter paper.*—Whatman No. 2, 9 cm diameter (Whatman Ltd, Maidstone, UK).

Preparation of Clean Spore Suspension

A lyophilized culture of a *B. subtilis* var. *niger* strain (ATCC 9372) was reconstituted into soil extract nutrient broth (1). A total of 3 daily transfers were made into the broth, which was incubated at 35°C. Nutrient agar with 5 ppm manganese sulfate (7) was inoculated with 0.5 mL inoculum, which was spread with a sterile, bent glass rod. Agar plates (150 × 20 mm) were incubated 10–14 days at 35°C until ca 95% sporulation occurred. Spores were washed from the agar by using a bent glass rod and sterile water and then cleaned by repeatedly centrifuging at 10 000 rpm and pouring off the supernatant liquid. Sterile water for irrigation, USP, was used for the spore suspension for inoculating the 3 types of penicylinders. Spores were suspended in sterile, filtered, deionized water for inoculation of porcelain penicylinders with a 15 h exposure time. The suspension was heated 20 min at 80°C. Pour plates were prepared with TSA, and the number of spores/mL of suspension was determined by aerobic plate counts. The spore suspension was stored in the refrigerator.

Inoculation of Penicylinders

New penicylinders were boiled 10 min in distilled water to remove any oils. The penicylinders were washed in distilled water containing sodium lauryl sulfate, rinsed repeatedly with distilled water, and then soaked in 1N NaOH overnight. The carriers were rinsed and tested with phenolphthalein according to AOAC **955.14C** (1). Penicylinders were placed in 25 × 150 mm tubes with metal caps and autoclaved 30 min at 121°C.

The 3 types of penicylinders were each inoculated with a suspension of 6.0×10^7 *B. subtilis* var. *niger* spores. The spore suspension was sonicated for 10 s to break up any small clumps. Ten mL of the spore suspension was placed in a 25 × 150 mm tube with a metal cap, and 10 penicylinders were placed in the suspension, which was then mixed in a vortex mixer. A nichrome wire hook was used to place the penicylinders upright on the filter in a petri dish. The penicylinders were then placed in a vacuum desiccator at 26–28 lb Hg and dried for at least 72 h.

B. subtilis (ATCC 19659) was cultured in soil extract nutrient broth, inoculated, and dried on new porcelain penicylinders, according to AOAC **966.04** (1).

Micrographs of Spores on Penicylinders

The 3 types of penicylinders were inoculated with a suspension of 6.0×10^7 *B. subtilis* var. *niger* spores. They were dried for several days in a low-temperature oven and then coated with carbon–gold in a vacuum evaporator. Micrographs were taken with a Jeol 840 scanning electron microscope at the Center for Electron Microscopy at the University of Minnesota.

Retention of Spores on Penicylinders

Four sets of penicylinders were inoculated with a water suspension of *B. subtilis* var. *niger* (ATCC 9372) spores. Inoculated penicylinders were individually immersed in 0.9% saline (10 mL in 20 × 150 mm tubes) to simulate carriers placed in germicide and then transferred to 10 mL sterile water instead of a neutralizer; they were then transferred to 10 mL buffered water instead of a culture medium. A flamed nichrome wire hook was used to transfer the penicylinders. Glass, porcelain, and stainless steel penicylinders were tested in sets of 10 for each type of carrier. The contact time in 0.9% saline was 1 h. A chemical germicide was mixed with filtered, deionized water according to label directions. Chlorine dioxide was produced when sodium chloride and organic acid were mixed (8).

One set of 10 porcelain penicylinders was immersed for 15 h in tubes containing 10 mL neutralized chemical germicide (9 mL chlorine dioxide + 1 mL 1M $NaHCO_3$). The set was then

Table 1. Quantities of *B. subtilis* var. *niger* (ATCC 9372) spores retained on glass (Pyrex) penicylinders

Carrier	No. of spores[a] In 0.9 % saline ($\times 10^4$)[b]	In water ($\times 10^4$)	Retained on penicylinders ($\times 10^5$)[c]	Retention, %
1	1.9	1.7	12	97.1
2	1.1	3.5	7.6	94.3
3	1.8	1.9	9.4	97.1
4	2.1	1.4	4.0	91.3
5	1.4	4.2	5.4	90.6
6	2.8	4.3	5.4	92.5
7	1.6	3.3	9.2	95.0
8	1.2	2.2	5.7	97.0
9	1.4	3.3	6.4	93.1
10	0.054	3.1	2.7	88.1
$\bar{X}$			6.7	93.6
SD			2.8	—
RSD, %			42	—

[a] Inoculated from water suspension of 6.0×10^7 spores/mL.
[b] Submerged for 1 h.
[c] Retained after being submerged in the 2 solutions.

Table 2. Quantities of *B. subtilis* var. *niger* (ATCC 9372) spores retained on porcelain penicylinders

Carrier	No. of spores[a] In 0.9 % saline ($\times 10^2$)[b]	In water ($\times 10^2$)	Retained on penicylinders ($\times 10^6$)[c]	Retention, %
1	1.5	4.0	1.7	99.97
2	1.5	18	2.0	99.90
3	2.0	7.0	1.3	99.93
4	3.0	8.5	1.6	99.93
5	2.0	23	2.1	99.88
6	4.0	14	1.9	99.91
7	1.5	7.0	1.3	99.94
8	1.5	3.0	1.0	99.96
9	3.5	8.0	1.4	99.92
10	2.0	5.0	1.7	99.96
$\bar{X}$			1.6	99.90
SD			0.33	—
RSD, %			21	—

[a] Inoculated from water suspension of 6.0×10^7 spores/mL.
[b] Estimates (counts at 10^{-2} dilution); submerged for 1 h.
[c] Retained after being submerged in the 2 solutions.

transferred first to 10 mL sterile water and then to 10 mL buffered water.

One set of 10 porcelain penicylinders inoculated with *B. subtilis* (ATCC 19659) from a culture filtrate of soil extract nutrient broth was immersed 1 h in 0.9% saline and transferred first to sterile water and then to buffered water. These 3 sets of tubes were then heated 20 min at 80°C to destroy vegetative cells and permit quantitation of spores.

Each penicylinder in buffered water was sonicated 5 min and then mixed in a vortex mixer 30 s to remove spores from the surface. Phosphate-buffered water was used for dilution blanks. Numbers of spores washed off in the 0.9% saline, in the neutralized chemical germicide, or in water (Tables 1–3), and the number of spores retained on the penicylinder after transfer to buffered water (Table 4) were determined by aerobic plate counts of pour plates prepared with TSA (incubated 48 h at 35°C).

The total number of spores inoculated on each penicylinder was determined by adding the number of spores on the carrier in the buffered water to that of spores washed off into the saline/chemical germicide and the water/neutralizer. The percent retention of spores on a penicylinder was calculated by dividing the total number of spores from all 3 solutions into the number of spores from the penicylinder in the buffered water.

HCl Resistance

Four 10-fold serial dilutions were made from a water suspension of *B. subtilis* var. *niger* (ATCC 9372) spores. The number of spores was determined by aerobic plate counts of pour plates prepared with TSA. Spores were tested for resistance to 2.5N HCl according to AOAC **966.04**, except that a water suspension rather than the soil extract nutrient broth filtrate was used to inoculate the porcelain penicylinders. Triplicate HCl resistance tests were performed. Three HCl resistance tests were also performed on spores of *B. subtilis* (ATCC 19659) on porcelain penicylinders that were inoculated from 72 h culture filtrates of soil extract nutrient broth, according to AOAC **966.04** (1).

Statistical Analysis

The percent retentions of spores on 3 types of penicylinders (Tables 1–3) were compared by the Kruskal-Wallis rank test (9). The sum of the spores in saline and water and on the penicylinders was assumed to represent the number of spores on the dried penicylinder before use.

Results and Discussion

Figure 1 shows micrographs of *B. subtilis* var. *niger* inoculated from a clean water suspension of spores on stainless steel, porcelain (steatite ceramic consisting of a hydrous magnesium silicate [AlSiMag Technical Ceramics, Inc., Laurens, SC]), and glass (Pyrex) penicylinders.

Tables 1–3 show the number of *B. subtilis* var. *niger* spores retained on the 3 types of penicylinders inoculated with 6.0×10^7 spores/mL and immersed 1 h in 0.9% saline. Quantities of spores determined simulated those expected to be retained on carriers in tests of the sporicidal activity of disinfectants.

Carriers were placed in a chemical germicide for the time specified on the label and transferred first to a neutralizer and then to a growth medium. Stainless steel penicylinders retained the most spores, and the relative standard deviation was only slightly higher on these carriers (23%) than on porcelain peni-

Table 3. Quantities of *B. subtilis* var. *niger* (ATCC 9372) spores retained on stainless steel penicylinders

Carrier	No. of spores[a] In 0.9 % saline ($\times 10^3$)[b]	In water ($\times 10^3$)	Retained on penicylinders ($\times 10^6$)[c]	Retention, %
1	1.2	2.1	2.6	99.9
2	4.6	8.8	1.9	99.3
3	3.4	2.8	2.4	99.7
4	8.8	6.8	2.6	99.4
5	10	15	3.0	99.9
6	3.9	4.9	2.3	99.6
7	18	22	2.8	98.6
8	3.1	7.9	1.4	99.2
9	2.3	4.4	1.7	99.6
10	2.2	1.1	2.1	99.8
$\bar{X}$			2.3	99.5
SD			0.52	—
RSD, %			23	—

[a] Inoculated from water suspension of 6.0×10^7 spores/mL.
[b] Submerged for 1 h.
[c] Retained after being submerged in the 2 solutions.

cylinders (21%). We observed that stainless steel penicylinders corrode when exposed to 2.5N HCl.

The number of spores retained on glass penicylinders was less than one-half of those held on either the porcelain or the stainless steel penicylinders. Ronner et al. (4) found that the hydrophobic property of some spores corresponds with their adhesiveness, e.g., stainless steel (hydrophobic) and treated

Table 4. Quantities of *B. subtilis* var. *niger* (ATCC 9372) spores retained on porcelain penicylinders

Carrier	No. of spores[a] In neutralized germicide ($\times 10^3$)[b]	In water ($\times 10^4$)	Retained on penicylinders ($\times 10^6$)[c]	Retention, %
1	6.5	0.81	1.1	98.8
2	3.8	1.9	2.0	98.9
3	1.1	0.71	1.3	99.4
4	1.7	1.2	1.4	99.0
5	2.7	1.2	1.3	99.0
6	2.6	1.4	1.9	99.1
7	2.3	2.2	1.8	98.8
8	0.45	1.0	1.4	99.3
9	2.8	2.3	1.0	97.6
10	6.9	1.7	2.3	99.0
$\bar{X}$			1.5	98.9
SD			0.42	—
RSD, %			28	—

[a] Inoculated from water suspension of 6.9×10^7 spores/mL.
[b] Submerged for 15 h.
[c] Retained after being submerged in the 2 solutions.

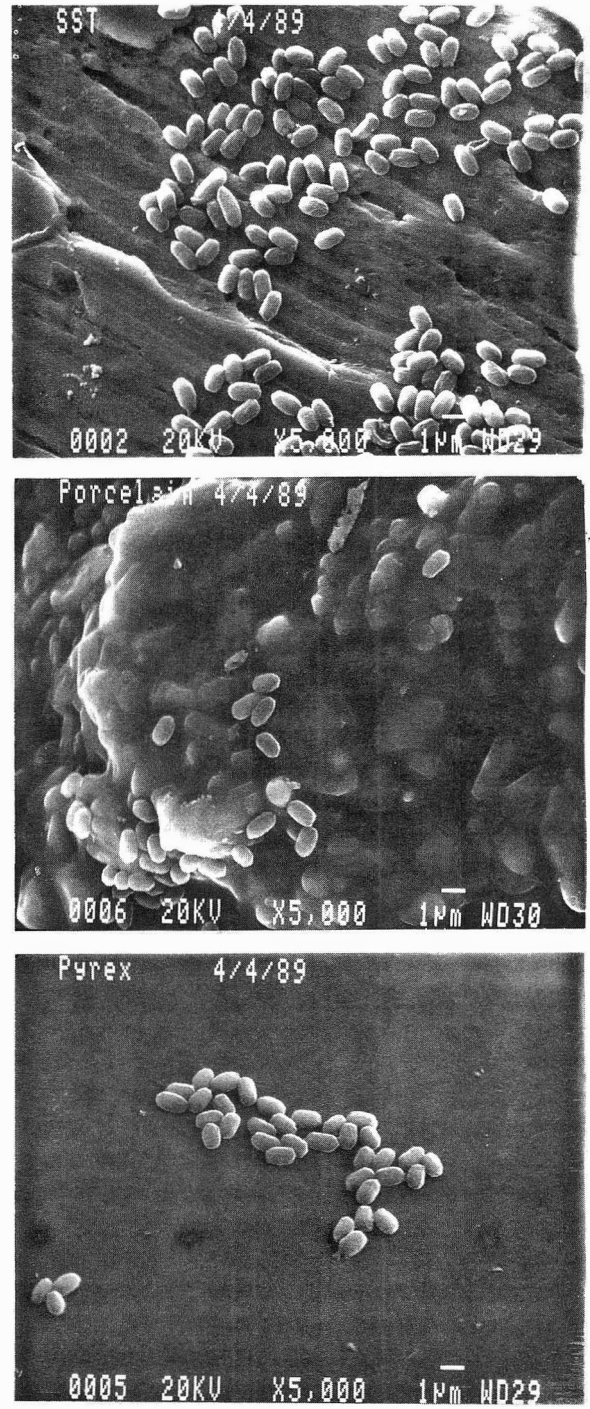

Figure 1. Scanning electron micrographs of *B. subtilis* var. *niger* spores attached to (a) stainless steel, (b) porcelain, and (c) glass (Pyrex) penicylinders.

glass (hydrophilic). The adhesiveness of *B. subtilis* var. *niger* spores indicated that the order of hydrophobicity of the 3 types of penicylinders is stainless steel > porcelain > glass. The percent retention of *B. subtilis* var. *niger* spores was significantly lower ($\alpha = 0.05$) for glass penicylinders than for the other 2 types of penicylinders.

Table 4 shows the quantities of *B. subtilis* var. *niger* spores retained on porcelain penicylinders that were submerged in a neutralized chemical germicide (chlorine dioxide neutralized

Table 5. Quantities of *B. subtilis* (ATCC 19659) spores retained on porcelain penicylinders

Carrier	No. of spores: In 0.9 % saline ($\times 10^4$)[a]	In water ($\times 10^4$)	Retained on penicylinders ($\times 10^4$)[b]	Retention, %
1	6.5	1.4	7.1	48
2	2.1	8.6	1.1	9
3	3.9	1.6	1.2	18
4	9.4	7.1	2.3	12
5	1.7	1.3	3.1	14
6	16	16	8.7	21
7	8.6	12	12	37
8	11	6.9	4.5	20
9	1.0	4.0	4.3	42
10	1.3	1.5	1.7	38
$\bar{X}$			4.6	26
SD			3.7	—
RSD, %			80	—

[a] Submerged for 1 h.
[b] Inoculated from soil extract nutrient broth culture filtrate according to AOAC sporicidal method; retained after being submerged in the 2 solutions.

with sodium bicarbonate), transferred first to water instead of a neutralizer, and then to buffered water. Carriers were exposed 15 h to the germicide, because label directions often specify 10 h or more of exposure time, and spore retention is affected differently by various surfactants in chemical sterilants.

Table 5 shows the quantities of *B. subtilis* (ATCC 19659) spores retained on porcelain penicylinders that were inoculated from a culture filtrate of soil extract nutrient broth and dried according to AOAC **966.04**. The average percent retention from 10 penicylinders was 26% (average washoff, 74%). Ascenci et al. (2) reported a similar washoff (72%) of *B. subtilis* (ATCC 19659) spores. Apparently, components of the dried culture medium are easily dissolved from the penicylinders, and most *B. subtilis* spores are dispersed into the disinfectant being tested.

Table 6 shows that the resistance of *B. subtilis* var. *niger* to 2.5N HCl correlates with the number of spores inoculated on porcelain penicylinders. The corresponding numbers of spores/mL in the water suspension used to inoculate the penicylinders are listed (Table 6). Resistance of ≤5 min to 2.5M HCl was obtained after porcelain penicylinders were inoculated with a concentration of 1.2×10^6 of *B. subtilis* var. *niger* spores. The 2.5N HCl resistance test results are also shown for *B. subtilis* (ATCC 19659), which was cultured in soil extract nutrient broth and then inoculated and dried on porcelain penicylinders according to AOAC **966.04**. These spores, tested according to the AOAC sporicidal method, met only the minimum HCl resistance requirements of ≥2 min. This low HCl resistance may result from a combination of a low retention of spores and this particular strain of *B. subtilis* cultured in soil extract nutrient broth. More resistance to 2.5N HCl can be obtained by increasing the concentration of the water suspension of *B. subtilis* var. *niger* spores used to inoculate the penicylinders.

Table 6. Triplicate HCl resistance tests of *B. subtilis* var. *niger* (ATCC 9372)[a] and *B. subtilis* (ATCC 19659)

Resistance to 2.5N HCl, min[b]: 2	5	10	20	Spore count from 5 porcelain penicylinders: Av.	SD	Water suspension concn of spores/mL in inoculum
B. subtilis var. *niger* (ATCC 9372)						
–	–	–	–			
–	–	–	–			
–	–	–	–	26	4.9	1400
+	–	–	–			
–	–	–	–			
–	–	–	–	224	95	14000
+	–	–	–			
+	–	–	–			
–	–	–	–	1100	273	150000
+	+	–	–			
+	+	–	–			
+	+	–	–	8900	3300	1200000
+	+	+	–			
+	+	–	–			
+	+	–	–	130000	66000	15000000
B. subtilis (ATCC 19659)[c, d]						
+	–	–	–			
+	–	–	–			
+	–	–	–			

[a] Inoculated from 10-fold dilutions of a water suspension.
[b] +, Growth in modified thioglycollate; –, no growth in modified thioglycollate.
[c] Penicylinders inoculated from filtrate of soil extract nutrient broth showed only 2 min resistance to 2.5N HCl.
[d] Tested according to AOAC method.

Conclusion

The retention of spores in both porcelain and stainless steel penicylinders was high after submersion in saline and transfer to water. However, stainless steel carriers were corroded by the chloride ion in the HCl resistance test. The glass penicylinders had the lowest and most variable spore retention and would be the least suitable carrier. *B. subtilis* (ATCC 19659) spores inoculated from a culture filtrate of soil extract nutrient broth on porcelain penicylinders met only the minimum HCl resistance requirements of ≥2 min. With a water suspension of *B. subtilis* var. *niger* spores for inoculating penicylinders, adequate resistance to 2.5N HCl can be obtained by using a known concen-

tration of spores. More uniform retention of microbial loads on porcelain penicylinders can be obtained by using the inoculum from a clean spore suspension of *B. subtilis* var. *niger* instead of *B. subtilis* (ATCC 19659) in a culture filtrate of soil extract nutrient broth.

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MYCOTOXINS

Study of the Reproducibility Characteristics of a Liquid Chromatographic Method for the Determination of Fumonisins B_1 and B_2 in Corn: IUPAC Collaborative Study

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An interlaboratory study of the reproducibility characteristics of a liquid chromatographic method for the determination of fumonisins B_1 and B_2 in corn was conducted in 11 laboratories in the United States, South Africa, Italy, Japan, United Kingdom, and The Netherlands. Each laboratory was supplied with 12 coded, blind duplicates of 6 samples of naturally contaminated corn containing different amounts of fumonisins B_1 and B_2. Samples are extracted with methanol–water (3 + 1), extracts are centrifuged, and supernatants are cleaned up on strong-anion-exchange cartridges, which were supplied to participants. Solutions are derivatized with *o*-phthaldialdehyde, and individual fumonisins are determined by reversed-phase liquid chromatography with fluorescence detection. Quantitation is by comparison with the supplied fumonisin standards. The within-laboratory repeatability was determined by statistical analysis of data after exclusion of outliers. Relative standard deviations for within-laboratory repeatability varied from 7.7 to 25.5% for fumonisin B_1 at concentrations between 200 and 2000 ng/g and from 12.5 to 36.8% for fumonisin B_2 at concentrations between 70 and 740 ng/g. Relative standard deviations for between-laboratory reproducibility varied from 18.0 to 26.7% for fumonisin B_1 and from 28.0 to 45.6% for fumonisin B_2 at the concentrations mentioned above. These measures of variability indicate that the method is suitable for adoption as an official method provided that the accuracy characteristics are verified collaboratively.

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Fumonisins are structurally related toxins originally isolated from cultures of *Fusarium moniliforme* (1). They are diesters of propane-1,2,3-tricarboxylic acid (tricarballylic acid) and 2-amino-12,16-dimethylpolyhydroxyicosanes in which the C_{14} and C_{15} hydroxyl groups are esterified with the terminal carboxyl group of tricarballylic acid (2). So far, 6 fumonisins have been isolated and characterized (3, 4). Three of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), are the major fumonisins produced in nature; fumonisin B_4 (FB_4) and fumonisins A_1 and A_2 (FA_1 and FA_2) are produced in relatively minor quantities (3, 4).

FB_1 causes leukoencephalomalacia (LEM) in horses (5, 6) and pulmonary edema in pigs (7) and is hepatotoxic and hepatocarcinogenic to rats (8). Fumonisin levels in food are statistically associated with an increased risk of human esophageal cancer in the Transkei, Republic of South Africa (9, 10). Furthermore, fumonisins occur worldwide on corn and in commercial corn-based human foods (11, 12). Exceptionally high levels of FB_1 and FB_2 were found in feed samples associated with confirmed outbreaks of LEM in horses (12–14). Therefore, an accurate, sensitive, and reproducible analytical method that can assess potential animal and human exposure to these toxic and carcinogenic compounds is essential.

Initial attempts to develop suitable analytical methods were recently discussed (12), as well as the rationale for studying the reproducibility of a modification of the method of Shephard et al. (15) in a collaborative study. This method uses precolumn derivatization of extracts with *o*-phthaldialdehyde (OPA) and liquid chromatographic (LC) separation of the derivatized fumonisins on reversed-phase media followed by fluorescence detection. The method of Shephard et al. (15) was used successfully for the determination of FB_1 and FB_2 in fungal cultures (16), corn (12), feed from field cases of LEM (12, 14, 15), and commercial corn-based food (11, 12). Because of the performance of this method when applied to a variety of commodities, it was selected for collaborative study under the sponsorship of the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry.

Collaborative Study

The study was executed according to the "Guidelines for Collaborative Study Procedure To Validate Characteristics of a Method of Analysis" of the AOAC (17). The method was optimized in our laboratory and shown to be reproducible with good recoveries for both FB_1 and FB_2. In a pilot study performed by 4 laboratories from 4 countries, blind duplicates of 3 naturally contaminated corn meals were analyzed by each laboratory. Statistical analyses of the results of the pilot study justified commencement of the full collaborative study, and suggestions by participants in the pilot study were incorporated.

Eleven laboratories from 6 countries were each supplied with 12 coded, blind duplicates of 6 naturally contaminated corn meals. Of these 6, one was a blank (containing <50 ng/g FB_1); the remaining 5 were blended to give a range of FB_1 values between 200 and 2000 ng/g. These levels covered the range previously found in naturally contaminated corn-based food (11, 12). The FB_2 levels were approximately one-third of the corresponding FB_1 level. Each participant was also furnished with method directions, 2 practice test samples, reference standards, 15 Bond-Elut strong-anion-exchange (SAX) cartridges, and an appendix containing information on suggested alternative reversed-phase columns and mobile phases, as well as chromatograms obtained under these conditions. Participants were requested to prepare an extract from each corn test sample, perform the cleanup using a Bond-Elut SAX cartridge, and perform an LC analysis of the cleaned-up extract.

The following description of apparatus and instructions for performing the analyses were supplied to each participant.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Pump (Waters M-45, Milford, MA 01757, with Waters U6K injector, or equivalent).

(b) *LC column.*—Stainless steel, 25 cm × 4.6 mm id, packed with Ultracarb 7 ODS 30 reversed-phase material (Phenomenex, Torrance, CA 90501, or equivalent) and in-line reversed-phase C_{18} guard column.

(c) *Fluorescence detector.*—Perkin-Elmer 650S fitted with 18 µL flow cell and set at wavelengths of 335 nm (excitation) and 440 nm (emission) and slit widths of 12 nm, or equivalent.

(d) *Data system.*—Waters Model 745, or equivalent.

(e) *Blender.*—Sorvall Omnimixer, or equivalent.

(f) *Solid-phase extraction (SPE) columns.*—Bond-Elut SAX cartridges, 3 cc capacity containing 500 mg sorbent (Varian, Harbor City, CA 90710).

(g) *SPE tube manifold.*—Supelco, Bellefonte, PA 16823, or equivalent.

(h) *Solvent evaporator.*—Silli-therm heating module (Pierce, Rockford, IL 61105, or equivalent).

Reagents

(a) *Solvents and reagents.*—Acetonitrile, methanol, *o*-phosphoric acid (>85%), glacial acetic acid, OPA, 2-mercaptoethanol, sodium dihydrogen phosphate, and disodium tetraborate. All solvents and reagents should be analytical grade.

(b) *LC mobile phase.*—Methanol–0.1M sodium dihydrogen phosphate (4 + 1). Dissolve 15.6 g $NaH_2PO_4 \cdot 2H_2O$ in 1 L distilled water, and adjust pH to 3.35 with *o*-phosphoric acid. Filter mobile phase through 0.45 µm Waters HV membrane. Pump mobile phase at a flow wate of 1 mL/min.

(c) *OPA reagent.*—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. Store reagent solution for no more than 1 week at room temperature in dark, in capped amber or aluminum foil-covered vial.

(d) *Fumonisin standard solution.*—Dissolve residue in vial provided (marked "Fumonisin Standards") in 1 mL acetonitrile–water (1 + 1), to give 50 and 25 µg/mL concentrations of FB_1 and FB_2, respectively. Store solution at 4°C.

Extraction and Cleanup of Corn

Caution! Do not open individual test sample containers until immediately before weighing ground corn for analysis (this will reduce absorption of atmospheric moisture by test portion).

Homogenize 25 g ground corn with 50 mL methanol–water (3 + 1) in blender for 5 min (at speed setting equivalent to 60% full speed).

Transfer mixture to 250 mL plastic centrifuge bottle, and centrifuge 10 min at 500 × *g* at 4°C. Filter supernatant through Whatman No. 4 fluted filter paper.

Fit SAX cartridge to SPE tube manifold. Condition cartridge by washing successively with 5 mL methanol followed by 5 mL methanol–water (3 + 1). Do not allow cartridge to run dry.

Apply 10 mL filtered extract to cartridge. Wash cartridge with 8 mL methanol–water (3 + 1) followed by 3 mL methanol. Maintain flow rate of 2 mL/min through cartridge. Do not allow cartridge to run dry.

Elute and collect FB_1 and FB_2 with 14 mL 0.5% acetic acid in methanol, in suitable collection vial.

Evaporate eluate to dryness in 4 mL capacity vial under stream of dry nitrogen at 60°C. Rinse collection vial with 1 mL methanol, and add washings to 4 mL vial, washing sides of 4 mL vial with methanol to concentrate total residue at base of vial. Evaporate additional methanol under nitrogen, and ensure that all acetic acid has evaporated. Retain dried residue at 4°C before LC analyses.

Derivatization and LC Analyses

(a) *Preparation of standard derivatives.*—Transfer 50 µL fumonisin standard solution to base of small test tube. Add 200 µL OPA reagent, mix, and inject 10 µL reacted mixture within 1 min. Time between addition of reagent and injection is critical because of fluorescence derivative decay.

(b) *Detector and recorder response.*—Adjust sensitivity settings of fluorescence detector so that FB_1–OPA derivative standard, prepared according to method, will give ≥80% recorder response.

(c) *Corn extracts.*—Redissolve purified test residue in 200 µL methanol. Transfer 50 µL of this extract (1.25 g test

portion equivalent) to base of small test tube, and add 200 µL OPA reagent. Mix, and inject 10 µL derivative within 1 min of adding OPA reagent.

(d) *Quantitation.*—Calculate fumonisin content of corn from chromatographic peak areas using the following equation:

$$A\ (\text{ng}) = \frac{G}{H} \times S$$

where A = ng fumonisin present in test extract injected into LC, G = fumonisin peak area in test sample, H = fumonisin peak area in standard, and S = amount of fumonisin standard injected into LC (100 ng for FB_1 and 50 ng for FB_2, based on supplied standard). Calculate C, concentration of fumonisin present in test sample in ng/g, by using the following equation:

$$C\ (\text{ng/g}) = \frac{A \times T \times D}{I \times W}$$

where A is calculated above, T = total volume of derivatized test solution (250 µL), D = dilution factor used, I = injection volume (10 µL), and W = test portion equivalent weight derivatized (1.25 g). The following equation is derived by using the parameter values listed above:

$$C\ (\text{ng/g}) = A \times 20 \times D$$

Results

All 11 collaborators completed the interlaboratory study. Laboratory 9 experienced technical problems while attempting to analyze the test samples and completed the study on a second batch of test samples. Data received from the participating laboratories are presented in Table 1. All values reported by Laboratory 3 were excluded from the statistical evaluation of the data, because this laboratory made changes to the derivatization procedure and reported that the fluorescence of fumonisin derivatives increased between 1.5 and 20 min after derivatization, which is completely contrary to all experience with OPA derivatives of fumonisins. (See also *Participants' Comments.*) None of the other results had to be rejected on the basis of a Grubbs test for the removal of extreme values (18). Results of the statistical analyses are reported in Table 2, including within-laboratory repeatability values (r), corresponding standard deviations (s_r), and relative standard deviations (RSD_r) and between-laboratory reproducibility values (R), corresponding standard deviations (s_R), and relative standard deviations (RSD_R).

Table 2 also contains HORRAT ratios calculated for both FB_1 and FB_2 at the 5 concentration levels tested. HORRAT is the ratio of the RSD_R found in an actual assay conducted in an interlaboratory study to that predicted from the determined or known concentration through the following equation:

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

where C = concentration expressed as a decimal fraction (19).

Discussion

The results reported in Table 1 and the chromatograms returned by the participants demonstrated that all the laboratories except Laboratory 3 experienced no problems in following the instructions for the method and had no difficulty in executing the individual steps in the procedure. All the participants except Laboratory 5 reported values below 50 ng/g for test sample 1. The reason for the extremely high values reported by Laboratory 5 for this test sample was clear from the chromatograms they returned. These chromatograms showed very broad peaks eluting in the regions of the fumonisins that could possibly have been caused by contaminants eluting from earlier injections that were erroneously identified as fumonisins by Laboratory 5. Statistical analyses were not performed on the values reported for test sample 1, because the levels were below what is conservatively considered to be the detection limit (50 ng/g) of the method.

Comparison of the blind duplicate values reported by each participant for samples 2–6 indicates that all participants except Laboratory 3 had no difficulty in repeating their results. This acceptable degree of within-laboratory precision is reflected in the relative RSD_r values reported in Table 2, which varied from 7.7 to 25.5% for FB_1 and from 12.5 to 36.8% for FB_2. The mean values ranged from 226 to 1983 ng/g for FB_1 and from 74 to 741 ng/g for FB_2. The satisfactory repeatability of the method is further demonstrated because any lack of homogeneity in the blind replicates distributed to the participants would also have been reflected in the values reported for the RSD_r. In this regard, test sample 3 seems to have been the least homogeneous and test sample 4 the most homogeneous, because the highest and lowest relative standard deviations (RSD_r), respectively, were found for both FB_1 and FB_2 in these samples.

As can be expected, the between-laboratory variability of the data was somewhat higher than the within-laboratory variability (Table 2). The RSD_R values varied from 18.0 to 26.7% for FB_1 and from 28.0 to 45.6% for FB_2 at the different levels of FB_1 and FB_2 included in this study (Table 2). These values are of the same order as those obtained in the collaborative study of an LC method for the determination of aflatoxins in corn, which showed good between-laboratory variability (20). The 2 studies are comparable, because they both describe the determination of mycotoxins in corn, involve extraction and solid-phase cleanup of extracts, and use precolumn derivatization before chromatographic separation and fluorescence detection. However, the collaborative study on aflatoxins (20) involved corn containing levels of total aflatoxins varying from 0 to 130 ng/g, whereas the present study on fumonisins was performed on corn containing considerably higher levels of toxins (200–2000 ng/g FB_1 and 70–740 ng/g FB_2). The acceptable repeatabilities and reproducibilities obtained in the present study enhance the confidence in the performance of this method, especially because none of the collaborators had previous experience with the method.

The acceptability of the method is also reflected in the HORRAT ratios reported in Table 2. Horwitz and Albert (19) "found this ratio to be the best single index of acceptability of method performance" and stated that "in general values above 2 are considered unacceptable." All the HORRAT ratios calculated for FB_1 were well below 2, and only 1 value for FB_2 was above 2. On the basis of this index, the method produced ac-

Table 1. Results for determination of fumonisins B_1 and B_2 (ng/g) in blind duplicate samples of naturally contaminated corn

Sample No.	Code No.	Laboratory No. 1	2	3	4	5	6	7	8	9	10	11
		Fumonisin B_1										
1	2	SL[a]	0	0	0	152[b]	0	7	0	16	29	20
	10	0	0	9	15	1903[b]	0	18	19	15	27	13
2	6	234	185	2	242	268	171	214	299	174	267	254
	11	219	168	51	228	240	181	258	290	155	346	221
3	1	SL	276	28	462	451	274	375	308	233	394	418
	9	281	298	29	342	144	353	369	227	264	453	371
4	3	514	500	29	628	699	534	631	514	424	744	631
	7	493	510	30	617	538	490	597	555	350	733	602
5	5	1034	902	73	1421	1233	1078	1165	1430	417	1502	1452
	12	1032	865	247	1168	1145	960	1468	1771	819	1406	1116
6	4	1739	1489	77	1966	2019	1842	2181	2073	1529	2425	1993
	8	1715	1543	198	1930	2277	2058	2097	2846	944	2904	2080
		Fumonisin B_2										
1	2	SL	0	0	0	93[b]	0	0	0	15	0	0
	10	0	0	0	0	0	0	0	0	15	0	0
2	6	69	67	0	79	80	65	93	52	47	121	77
	11	32	56	14	77	75	66	84	54	57	158	66
3	1	SL	72	8	19	159	105	125	79	68	172	145
	9	67	94	0	91	31	118	122	55	89	195	113
4	3	182	222	8	242	296	250	293	161	179	405	292
	7	219	233	0	316	227	236	274	237	165	425	250
5	5	348	267	20	479	439	434	435	393	142	733	510
	12	327	289	89	365	401	369	566	535	278	695	406
6	4	568	533	25	692	686	790	840	524	507	1202	826
	8	572	533	65	607	794	790	800	959	313	1507	778

[a] SL = sample lost.
[b] Invalid data, see text.

ceptable reproducibilities at all concentrations of FB_1 tested and at 4 of the 5 concentrations of FB_2 tested.

This study was primarily aimed at determining the reproducibility characteristics of the method; therefore, the products distributed to participants were all naturally contaminated rather than "spiked" samples. This was considered to be a better measure of the reproducibility of the method, because extraction of the fumonisins from the corn matrix is an essential aspect of the method. Therefore, the accuracy (recovery) of the method was not tested collaboratively but was investigated extensively in our laboratory. Recoveries of FB_1 and FB_2 were found to be 99.5 and 85.9%, respectively (15). These recoveries were not determined by spiking corn but by spiking corn extracts at levels corresponding to 2000–4000 ng/g FB_1 and 4000–8000 ng/g FB_2.

Participants' Comments

All participants confirmed that the description of the method was easy to follow and that they had no difficulty during the analysis. One participant found the instructions for calculation unnecessarily complicated; another found the chromatographic rnn time too long. Two participants complained about the large solvent volumes used for washing and elution of the ion-exchange cleanup cartridges, which also led to a long evaporation time for the 14 mL eluate from these cartridges. All participants returned chromatograms that showed good resolution of the individual toxins and other components. The only exceptions were the broad peaks obtained by Laboratory 5 in the positions of the fumonisins in the "blank" samples.

Table 2. Statistical analysis of data for LC determination of fumonisins B_1 and B_2 in corn

Sample No.	Mean, ng/g	s_r, ng/g	RSD_r, %	s_R, ng/g	RSD_R, %	Outliers No. of laboratories	HORRAT ratio
			Fumonisin B_1				
1[a]	<50	—	—	—	—		
2	226	33.2	14.7	55.7	24.6	1	1.2
3[a]	337	84.5	25.5	86.2	26.0	1	1.4
4	565	43.5	7.7	101.5	18.0	1	1.0
5	1169	170.1	14.6	311.8	26.7	1	1.7
6	1983	255.1	12.9	457.3	23.1	1	1.6
			Fumonisin B_2				
1[a]	<50	—	—	—	—		
2	74	12.6	17.1	27.8	37.7	1	1.6
3[a]	101	37.1	36.8	46.1	45.6	1	2.0
4	255	32.0	12.5	71.3	28.0	1	1.4
5	421	66.2	15.7	144.3	34.3	1	1.9
6	741	130.9	17.6	270.0	36.4	1	2.2

[a] One sample lost by Laboratory 1.

Laboratory 3 commented that "when the standard (after derivatization) was left past 1.5 min the peak areas went up rather dramatically" and that "after 20 min the peak areas were 20–50% higher than at 1.5 min." This observation is completely contrary to all experience with OPA derivatives, including fumonisin derivatives. Laboratory 3 reported that a significant change was made to the method "in order to get results on our system" by using a "0.1M borate buffer in the derivatization step instead of the straight 0.1M borate." Results of Laboratory 3 (Table 1) were clearly not in agreement with those from the other 10 participants and were excluded from the statistical calculations on the basis of their deviation from the prescribed method.

One participant (Laboratory 7) noted that an independent comparison of this method was done with a procedure that used a C_{18} reversed-phase cleanup. On the basis of the analysis of a single sample analyzed by both methods, results indicated that the SAX cleanup procedure gave recoveries that were approximately 50% lower than those obtained from the alternative C_{18} cleanup procedure. Results of this participant in the collaborative study were, however, in excellent agreement with those from the other 9 laboratories used for statistical evaluation of the method. Lower recoveries may be obtained from SAX cartridges if the prescribed elution flow rate of 2 mL/min is exceeded or the pH of the sample extract (before the sample is applied to the cartridge) is <5.8. The effect of pH and other parameters that can influence fumonisin recoveries from SAX cartridges were addressed by Sydenham et al. (21).

Conclusion

This study indicated that the reproducibility characteristics of the basic method are suitable for its adoption as an official method provided that the accuracy characteristics are studied collaboratively. Since this collaborative study was performed, an updated version of the basic method was published (21) in which the codetermination of FB_3 with FB_1 and FB_2 is described and the cleanup procedure on the ion-exchange cartridges is simplified. The reproducibility characteristics of this updated version will be similar or superior to those determined in the present study. Depending on the availability of toxin standards, both methods should be suitable for reliable determinations of the major naturally occurring fumonisins. The accuracy characteristics of the method for the codetermination of FB_1, FB_2, and FB_3 should be investigated collaboratively.

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MYCOTOXINS

Liquid Chromatographic Determination of Aflatoxins in Animal Feeds and Feed Components

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A method is described for the determination of aflatoxins B_1, B_2, G_1, and G_2 in animal feeds and feed components by liquid chromatography (LC) with fluorescence detection. In this modified procedure, the aflatoxins are extracted from the samples with methanol–water (75 + 25), and the solution is vacuum-filtered through Whatman No. 541 filter paper. An aliquot of the extract is first defatted with petroleum ether, and then the aflatoxins are partitioned into chloroform. The chloroform extract is purified on a silica gel chromatographic column, aflatoxins B_1 and G_1 are derivatized by trifluoroacetic acid to their hemiacetals, and the aflatoxins are determined by reversed-phase liquid chromatography. Recoveries from 11 samples ranged from 90 to 98% for aflatoxins B_1 and G_1 (spiking range 2–25 ng/g) and B_2 and G_2 (spiking range 0.2–2.5 ng/g).

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Local veterinarians have often suspected that mycotoxins were involved in disease outbreaks, especially in the poultry industry. Although these suspicions were based on clinical symptoms and autopsy data, the presence of myco-

toxins was not proved. The levels of aflatoxins in local foods and feeds are regulated by law; however, the variety of analytical techniques required for different sample types has posed problems for regulatory agencies. Recent developments in immunoassay (1) and affinity chromatographic procedures (2) appear to be capable of handling a wide range of sample types. However, the cost of such systems and their uncertain shelf lives under variable storage conditions are factors discouraging their use in developing countries at present.

With the persistent shortage of trained laboratory personnel and with large numbers of incoming feed components and premixes, there is need for reasonably rapid and reliable methods capable of determining aflatoxins in a wide range of sample types. The methods of Romer (3) and Shannon et al. (4) were not suitable for local samples because of interferences in the final extracts when determination by liquid chromatography (LC) was attempted. On the other hand, the AOAC methods (5) tend to be too specific with respect to sample types.

Of all the other methods tested for the determination of aflatoxins B_1, B_2, G_1, and G_2 in a wide range of animal feeds and feed components, we found the method of Trucksess et al. (6), which was developed for corn and peanut butter, to be most readily adapted to animal feeds and their components. Unfortunately, with the exception of corn and wheat middling, the original method was inadequate; recoveries from spiked samples were low, and sensitivity of LC detection was poor because of the presence of interferents. This communication describes extensive modifications made to the method of Trucksess et al. (6) to provide a reliable method for the determination of aflatoxins B_1, B_2, G_1, and G_2 in a variety of animal feeds and feed components.

METHOD

Reagents and Materials

(a) *Solvents.*—(British Drug House, or equivalent.) Analar grade acetonitrile, isopropyl alcohol, methanol, and petroleum ether (80–100°C). Extract reagent-grade chloroform and dichloromethane with concentrated H_2SO_4, 2M NaOH, and distilled, deionized water; then distill in all-glass apparatus. Extract reagent grade diethyl ether with water to remove ethanol, distill in all-glass apparatus, and store over acid-washed copper turnings.

(b) *Silica gel.*—70–230 mesh (No. 7734, E. Merck, or equivalent).

(c) *Aflatoxin.*—(Sigma Chemical Co., St. Louis, MO.) Prepare 40 and 4 μg/mL working solutions of aflatoxins B_1 and G_1 and aflatoxins B_2 and G_2, respectively, in $CHCl_3$. Confirm concentration and purity before use by measuring absorbance at 365 nm and by thin-layer chromatography (TLC), respectively. Wrap standards in amber-colored bottles with foil and keep at 4°C when not in use.

(d) *Sodium sulfate.*—Anhydrous, analytical grade.

(e) *Sodium chloride.*—Saturated aqueous solution.

(f) *Trifluoroacetic acid (TFA).*—99% purity (Aldrich Chemical Co., Milwaukee, WI). Store in desiccator when not in use.

Apparatus

(a) *Wrist action shaker.*—Model SGL-705-010X (Gallenkamp, or equivalent).

(b) *Rotary evaporator.*—Model SB (Buchi, or equivalent).

(c) *Evaporator.*—Sillivap® with Reactivials® and Teflon-lined screw caps (Pierce Chemical Co., Rockford, IL).

(d) *Fluorescence spectrophotometer.*—Model MPF-44B, fitted with LC flow cell (Perkin-Elmer, Norwalk, CT).

(e) *Liquid chromatograph.*—Consta Metric III pump (Milton Roy, FL); injection valve with 20 μL loop, Model 7125 (Rheodyne); reversed-phase column, RP18 (10 μm); 25 cm × 0.4 cm id with 2.5 cm × 4 mm id RP18 guard columns (Brownlee).

(f) *Glass chromatographic columns.*—25 cm × 0.8 cm id, each plugged with glass wool/washed cotton wool at lower end.

(g) *Glassware for storage of aflatoxin standards and sample extracts.*—Silanize clean, dry glassware by filling with 5% solution of trimethylsilyl chloride in petroleum ether, and letting stand 2 h in fume hood. Wash all glassware with distilled water and acetone before use.

Sample Preparation

Each laboratory sample of animal feeds and feed components was ground and sieved to <1 mm particle size. Test portions were obtained by appropriate subsampling techniques (7) to maintain homogeneity of samples.

Sample Extraction

Add 250 mL methanol–water (75 + 25) to 500 mL extraction flask containing 50 g test portion. Stopper and shake 30 min on wrist-action shaker. Filter through Whatman No. 541 filter paper and collect filtrate. Immediately transfer 40 mL to a 250 mL separatory funnel and defat by shaking with 2 successive volumes of petroleum ether for 2 min. For broiler starter, broiler grower, broiler finisher, corn, dairy ration, duck feed, wheat middling, and soybean meal, extract with two 50 mL portions of petroleum ether. For fish meal and pig grower, use two 100 mL portions, and for coconut meal two 150 mL portions of petroleum ether.

Collect lower methanolic layer into another 250 mL separatory funnel and add 10 mL saturated sodium chloride solution. Extract aflatoxins with three 25 mL portions of chloroform, and pool extracts. Add 5 g anhydrous sodium sulfate to combined chloroform extract and swirl to remove water. Decant into 100 mL round-bottom flask. Rinse sodium sulfate with two 5 mL portions of chloroform and add rinsings to flask. Evaporate contents of flask to dryness on rotary evaporator at 50°C water bath temperature.

Purification of Extract

Redissolve dry extract in 2 mL dichloromethane and transfer with Pasteur pipet to chromatographic column packed with silica gel in petroleum ether. For fish meal, pig grower, and coconut meal, use 2.0 g silica gel. For all other sample types,

Table 1. Slopes and y-axis intercepts of aflatoxin calibration curves in different matrixes

Aflatoxins		Sample matrixes									
		LC solvent	Broiler starter	Broiler finisher	Corn	Wheat middling	Dairy ration	Soybean meal	Pig grower	Fish meal	Coconut meal
B_{2a}	Slope[a]	24.8	24.4	23.8	24.6	24.0	25.6	24.2	25.2	25.2	24.0
	C[b]	7.1	7.2	8.0	7.2	7.3	6.5	7.0	6.8	6.6	7.3
G_{2a}	Slope[a]	20.5	20.2	20.3	20.2	20.8	19.2	19.0	20.5	22.1	20.8
	C	7.5	7.1	7.2	7.2	7.4	7.0	7.4	7.1	7.2	7.4
B_2	Slope[c]	522.6	510.4	508.6	514.2	529.4	535.6	520.4	525.8	523.6	529.4
	C	9.4	10.4	10.8	10.0	9.5	8.0	9.2	9.4	9.3	9.5
G_2	Slope[c]	327.5	320.4	330.6	331.4	320.4	328.5	330.8	326.8	322.4	320.4
	C	7.6	7.8	7.2	7.0	8.2	7.6	7.3	7.8	8.0	8.2

[a] Calibration range, 0.5–16 ng.
[b] Y-axis intercept.
[c] Calibration range, 0.03–1.5 ng.

use 1.5 g silica gel. Rinse flask with two 1 mL portions of dichloromethane and add rinses to column.

Elute 1.5 g silica gel chromatographic column in turn with 15 mL each of petroleum ether, diethyl ether, and diethyl ether–chloroform (3 + 1) at flow rate of ca 2 mL/min, and discard eluates. Elute aflatoxins with 20 mL chloroform–acetone (9 + 1) in 50 mL round-bottom flask. For 2.0 g silica gel chromatographic column, elute in turn with 20 mL each of petroleum ether, diethyl ether, and diethyl ether–chloroform (3 + 1), and discard eluates. Elute aflatoxins with 30 mL chloroform–acetone (9 + 1) in 100 mL round-bottom flask and evaporate to dryness on rotary evaporator.

Derivatization

Redissolve residue of chloroform–acetone (9 + 1) in 1 mL chloroform and transfer with Pasteur pipet to 5 mL Reactivial wrapped with aluminum foil. Rinse flask with two 1 mL portions of chloroform and add to vial. Evaporate chloroform with gentle stream of warm nitrogen, using Sillivap evaporator. Place capped vial with residue in water bath at 3–5°C and equilibrate 15 min. Add 200 µL TFA and let stand 15 min more. Remove most of unreacted TFA with warm nitrogen. Near dryness, add 0.5 mL chloroform and evaporate to remove all excess TFA.

Chromatographic Analysis

Redissolve contents of vial with 1 mL degassed LC mobile phase (acetonitrile–isopropyl alcohol–methanol–water, 9.5 + 5 + 15 + 100) just before analysis.

Set fluorescence spectrophotometer at λ_{max} excitation 365 nm and λ_{max} emission 425 nm, and LC mobile phase flow rate of 1.75 mL/min. Inject various concentrations of mixed authentic B_{2a}, G_{2a}, B_2, and G_2 (20–2000 ng B_{2a} or G_{2a} mL and 2–200 ng B_2 or G_2 mL) into LC system and construct calibration curve.

Because 20 µL injection of final sample solution is 1/50 of 1 mL, which represents 8 g sample, calculate aflatoxin concentration in samples, using the following formula: aflatoxin, ng/g = (Concentration interpolated from calibration curve × 50)/8.

Influence of Sample Matrix on Accuracy of LC Determination

Spike chloroform–acetone (9 + 1) column eluates of each sample type, previously checked by liquid chromatography and found to contain no detectable aflatoxins, with 20–200 ng B_1 and G_1, and 2–20 ng B_2 and G_2. Extract to dryness on rotary evaporator and derivatize B_1 and G_1 according to TFA method. Remove unreacted TFA and redissolve residue in 1 mL LC mobile phase. Analyze by LC, and compare slopes and y-axis intercepts with calibration curves of standards prepared in LC mobile phase only (Table 1).

Recovery Studies on Aflatoxins

Add aflatoxins B_1 and G_1 (100–1250 ng) and B_2 and G_2 (20–125 ng) in chloroform to 500 mL extraction flask. Evaporate solvent and add 50 g uncontaminated sample to aflatoxins, followed by extraction solvent. Shake on wrist action shaker, filter, and collect 40 mL filtrate. Defat and purify on silica gel chromatographic column as described previously. Derivatize by TFA method and analyze by liquid chromatography. Determine percent recovery by interpolation on calibration curve generated from standards in LC mobile phase (Table 2).

Results and Discussion

Aqueous methanol is an inexpensive and efficient extractant for aflatoxins from agricultural commodities (8–10). However, care must be taken not to let the aflatoxins remain more than 1 h in this solvent, because they are unstable when exposed to methanol for long periods (2). Aflatoxins were, thus, defatted and partitioned immediately after sample extraction. Of the various ratios of methanol–water tested for extraction of the aflatoxins from the different sample types, methanol–water (75 + 25) was found to be best suited for animal feeds and feed components. This ratio, in addition to efficiently extracting the aflatoxins from the samples, also reduced the tendency for emulsion formation during the partitioning with chloroform to remove the aflatoxins from aqueous extracts.

Table 2. Recoveries of aflatoxins added to various sample types

	Aflatoxins			
	B_1	B_2	G_1	G_2
Samples	2–25 ng/g[a]	0.2–2.5 ng/g	2–25 ng/g	0.2–2.5 ng/g
Broiler starter	93.2 ± 2.2[b] (91.0–95.6)	95.3 ± 2.2 (91.5–96.8)	96.3 ± 1.2 (94.2–98.0)	95.3 ± 1.8 (92.5–98.0)
Broiler grower	92.7 ± 3.2 (90.2–98.0)	95.2 ± 1.7 (94.1–96.8)	91.8 ± 1.7 (90.2–94.6)	93.3 ± 2.5 (90.6–96.4)
Broiler finisher	93.0 ± 2.4 (91.5–95.2)	94.0 ± 2.0 (91.6–96.4)	95.4 ± 0.9 (94.2–96.6)	91.5 ± 0.8 (90.6–92.4)
Soybean meal	94.2 ± 3.1 (95.0–98.3)	96.6 ± 1.1 (95.0–98.0)	96.2 ± 1.5 (94.3–98.6)	97.5 ± 1.0 (96.4–98.8)
Wheat middling	96.6 ± 1.4 (95.0–98.3)	95.3 ± 1.1 (94.0–96.8)	93.4 ± 2.5 (90.4–96.4)	96.8 ± 1.1 (95.5–98.3)
Corn	90.9 ± 0.8 (90.2–92.3)	95.4 ± 1.4 (92.4–96.8)	90.9 ± 0.7 (90.0–92.0)	95.5 ± 1.3 (94.0–97.5)
Dairy ration	95.7 ± 4.5 (86.4–98.6)	97.0 ± 1.1 (96.0–98.2)	96.2 ± 1.9 (94.3–98.6)	96.8 ± 1.3 (95.4–98.4)
Duck feed	96.5 ± 1.3 (95.4–98.6)	96.9 ± 1.4 (95.0–98.6)	97.2 ± 2.1 (94.2–100.2)	96.5 ± 1.1 (95.4–98.4)
Pig grower	91.0 ± 0.9 (90.2–92.6)	91.1 ± 0.4 (90.6–91.6)	91.7 ± 0.9 (90.4–92.8)	90.8 ± 0.5 (90.4–91.8)
Fish meal	95.6 ± 2.2 (91.8–98.2)	95.1 ± 3.0 (90.4–98.3)	93.5 ± 2.7 (90.3–96.4)	96.5 ± 2.0 (93.2–98.4)
Coconut meal	90.7 ± 0.6 (90.0–91.8)	94.3 ± 2.3 (91.0–96.8)	91.3 ± 1.2 (90.0–92.8)	91.9 ± 1.8 (90.4–95.2)
Mean of all samples	92.6 ± 2.3	95.1 ± 1.7	94.0 ± 2.3	94.8 ± 2.4

[a] Spiking range, consisting of 6 spiking levels.
[b] Overall mean of 12 determinations ± standard deviation; range of values in parentheses.

Some samples are higher in lipid content than others and require increased quantities of petroleum ether to remove the chromatographic interferent. Coconut meal contained the highest proportion of lipids, followed by pig grower, fish meal, soybean meal, poultry feeds, corn, and wheat middling. Petroleum ether was used instead of hexane, as described by Trucksess et al. (6) because it is cheaper. Also, the sodium chloride solution was not added to the defatting stage; salting out may cause some of the aflatoxins to partition into the organic phase and become lost. Instead, sodium chloride was added just before partitioning into chloroform.

Different masses of residues (0.5–0.8 g) were obtained when the chloroform extracts were evaporated to dryness, and different masses of silica gel were needed for efficient column purification. For corn and wheat middling, residue masses varied from 0.5 to 0.55 g. For broiler starter, broiler grower, broiler finisher, soybean meal, duck feed, and dairy ration, residue masses ranged from 0.55 to 0.65 g and for fish meal, pig grower, and coconut meal, from 0.7 to 0.8 g. Columns loaded with 2.0 g silica gel for fish meal and coconut meal and 1.5 g for all others provided efficient cleanup of sample extracts. The volumes of eluting solvents were optimized by collecting 1 mL aliquots of each eluate, concentrating, and analyzing by thin-layer chromatography. In the case of petroleum ether, diethyl ether, and diethyl ether–chloroform (3 + 1), the volumes of eluting solvents were optimized by maximizing the removal of co-extractives in these fractions. On the other hand, the optimum eluting volumes of chloroform–acetone (9 + 1) were established for the different samples by determining the minimum volume needed to collect all detectable aflatoxins. These volumes of eluting solvents in the column purification step were larger than those used by Trucksess et al. (6) for corn and peanut butter, but they were necessary to remove interferents present in feeds and feed components.

Aflatoxins B_1 and G_1 can be derivatized to their respective hemiacetals, B_{2a} and G_{2a}, by treatment with TFA at room temperature (5, 11, 12). However, we experienced formation of fluorescent side reaction products that could interfere in afla-

Table 3. Detection limits (ng/g) for aflatoxins in 50 g samples

Aflatoxins	Pig grower[a]	Fish meal	Coconut meal	All other sample types[b]
B_1[c]	0.3	0.4	0.4	0.2
B_2	0.04	0.04	0.12	0.02
G_1[c]	0.8	1.0	1.5	0.5
G_2	0.06	0.06	0.09	0.03

[a] Composed of corn, wheat middling, fish meal, soybean meal, and beef tallow.
[b] Other samples refer to corn, wheat middling, soybean meal, dairy ration, broiler starter, and broiler finisher.
[c] Determined as hemiacetals B_{2a} and G_{2a}.

toxin determination by liquid chromatography. Consequently, our investigations showed that TFA at 3–5°C effectively converts 90–93% of aflatoxins B_1 and G_1 to their hemiacetals with minimal side reaction (13) before their determination by liquid chromatography. Established LC mobile phases (14–17) were tried but did not efficiently resolve B_{2a} and G_{2a}; overall retention times were >20 min, and sensitivity of detection was >50 ng/g. The use of our LC mobile phase acetonitrile–isopropyl alcohol–methanol–water (9.5 + 5 + 15 + 100) at a flow rate of 1.75 mL/min was successful in resolving B_{2a} and G_{2a} (>90%) with much improved sensitivity and retention times (12 min total) for the 4 aflatoxins. Table 3 lists the detection limits for 50 g samples of the different sample types.

It should be noted that samples with the highest lipid contents also gave higher detection limits, indicating the importance of the defatting process. Our detection limits are superior to those obtained by Trucksess et al. (6), Jain and Hatch (18), Pons et al. (19), and Manabe et al. (20) but inferior to that obtained by Beebe (17) using 500 µL injection volumes compared to our 20 µL injections.

The relative sensitivities for aflatoxins in different sample matrixes (Table 1) deviated only slightly from the calibration curves generated by authentic aflatoxins in the LC mobile phase. Except for G_{2a} in fish meal, there were no significant differences ($P < 0.05$) in the quantities of B_{2a}, G_{2a}, B_2, and G_2 obtained by interpolation from calibration curves generated by using standards in LC solvent, compared to those in sample matrixes. It is evident that calibration curves for aflatoxins obtained with authentic aflatoxins in the LC mobile phase can be used for quantitative analyses.

The recoveries for aflatoxins B_1, B_2, G_1, and G_2 from all samples were greater than 90% for spiking range 2–25 ng/g for B_1 and G_1 and 0.2–2.5 ng/g for B_2 and G_2 (Table 2). Analysis of variance revealed no significant differences ($P < 0.05$) between the quantities of individual aflatoxins recovered from different sample types or between the quantities of each aflatoxin recovered from a given sample. These results are expected for animal feeds, which are composed mainly of fish meal, soybean meal, wheat middling, and corn and vary only in the percentage composition of individual feed components.

Recoveries of aflatoxins B_1, B_2, G_1, and G_2 from corn were comparable to those obtained by Trucksess et al. (6), Beebe (17), and Hutchins and Hagler (16), and greater than those obtained by Francis et al. (21). For poultry and other animal feeds, the recoveries were comparable to those obtained by Shannon et al. (4), Jain and Hatch (18), and Pennington (22).

Sample analysis by the present method takes about 2 h/sample. However, the extraction, defatting, and partitioning can be done in batches, followed by simultaneous chromatographic purification with multiple column units. With the present method, 8 samples in duplicate can be analyzed daily by a single operator.

The present method provides us with the accuracy and sensitivity required for aflatoxin determination in animal feeds and feed components. We are confident that it can be applied equally well to other sample types.

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OILS AND FATS

Gas Chromatographic Determination of Hydrocarbons in Crude Palm Kernel Oil

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A method for the determination of hydrocarbons (HCs) in crude palm kernel oil (CPKO) is described. HCs are extracted from CPKO by passing through a silica gel column by using *n*-hexane as the eluting agent. The eluate is collected for analysis. Identification and confirmation of the HCs in the eluate are performed with the aid of a gas chromatograph equipped with a mass selective detector, and the HCs in CPKO are quantitated by comparison of peak areas with reference to an external standard, eicosane ($C_{20}H_{42}$). Recoveries and coefficients of variation for 5 samples of oil spiked with 1–300 μg/g eicosane were 70.0–86.6% and 3.5–13.2%, respectively. The method was used for the analysis of CPKO suspected of being contaminated with mineral oil.

Hydrocarbons (HCs) constitute one of the components found in the unsaponifiable fraction of vegetable oils. However, contamination with mineral HCs from the atmosphere or industrial processing equipment is a possibility. In the case of crude palm kernel oil (CPKO), a number of possible sources contribute to the final level of HCs in the oil. Naturally occurring or endogenous HCs could arise in the course of natural deterioration of components in the oil such as hydroperoxides and free fatty acids. The steam conditioning or cooking of palm kernels in readiness for oil extraction could also hasten this deterioration process.

Recently, HCs in oil have received a lot of attention because of possible contamination from ships' tanks carrying previous cargoes such as mineral oil or diesel fuel. Many methods were published for the determination of various HCs in vegetable oils (1–6), but none gave a satisfactory analysis that could differentiate between endogenous HCs of the oil and HCs resulting from contamination. Because HCs are nonsaponifiable, many of these methods attempted to estimate the HCs content of the unsaponifiable fraction of oils. However, not all HCs are recovered by isolation of the unsaponifiable fraction. This is especially so for the lighter HCs (C_{14} and below). Losses can occur during the saponification process itself and also during the evaporation of the solvent. In addition, methods involving saponification are time-consuming and tedious.

In this paper, we propose a combination of silica gel column chromatography and gas chromatography (GC) for the quantitation of HCs in CPKO. This technique is rapid and sensitive enough for HCs in CPKO, although on-line coupled liquid chromatography/GC (7) was reported to give much higher sensitivities.

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METHOD

Reagents

Use analytical grade chemicals and solvents.

(a) *Solvents.*—*n*-Hexane, methanol, and chloroform.

(b) *Silica gel.*—Kieselgel 60, 70–230 Mesh ASTM (Merck). Wash with chloroform–methanol (3 + 1, v/v), and activate 2 h at 120°C.

(c) *Anhydrous sodium sulfate.*

(d) *Eicosane standard solutions.*—Eicosane, 99% purity (Sigma Chemical Co.). *Stock solution.*—1 mg/mL. Prepare by dissolving ca 50 mg eicosane, accurately weighed, in 50 mL *n*-hexane in a 50 mL volumetric flask. *Working standard solutions.*—Dilute stock solution in *n*-hexane to give working standards of 1, 5, and 10 μg/ml in *n*-hexane.

Apparatus

(a) *Glass chromatographic column.*—With sintered glass disc (250 × 20 mm) fitted with Teflon stopcock.

(b) *Gas chromatograph.*—Hewlett Packard Model 5890 Series II linked to Vectra QS/165 Workstation, with flame ionization detector and on-column injector. Operating conditions: fused silica capillary column, 25 m × 0.32 mm id; cross-linked methyl silicone gum; detector at 310°C; oven temperature programmed from 70 to 280°C at 5°C/min and held until all peaks have eluted.

(c) *Gas chromatograph/mass selective detection (MSD) system.*—Hewlett Packard 5890 and 5970 MSD with 59970 MS Chemstation. Operating conditions: Carbowax fused silica capillary column, 25 m × 0.33 mm id; on-column injector mode, 70 eV; injector, 280°C; transfer line, 280°C; MSD system, 172°C; mass analyzer, 180°C; hold oven at 30°C for first 2 min, then program at 7.5°C/min from 30 to 280°C and hold

Table 1. Data on calibration curve

Amt injected, ng	Mean peak area, arbitrary units[a]	SD	CV, %
1	7.521	0.696	9.25
5	35.692	2.032	5.69
20	144.470	8.959	6.20
50	330.368	34.422	10.42
100	718.319	46.950	6.54
200	1452.318	110.040	7.58
400	3486.191	190.184	5.46
N	7		
R^2	0.9911		
Slope	8.3426 ± (0.1237)		

[a] For 6 injections.

Table 2. Absolute response factor, Y, for eicosane

Eicosane concentration, ng	Absolute response factor, Y[a]
1	0.1330
5	0.1401
20	0.1384
50	0.1513
100	0.1392
200	0.1377
400	0.1147

[a] $Y = \frac{\text{Mass of eicosane}}{\text{Peak area}}$.

until all peaks have eluted. This hold time was determined by a trial run of a sample in the GC system until no more peaks were seen eluting through the column.

Preparation of Silica Gel Column

Thoroughly mix 30 g silica gel with *n*-hexane, and transfer the slurry into the glass chromatograph column. Wash column with *n*-hexane. Let *n*-hexane level fall to ca 20 mm above the top of the silica gel column before adding a 5 mm layer of anhydrous sodium sulfate. The top of the column bed must not dry out, or the effectiveness of the stationary phase will be impaired. Likewise, channeling of the column will affect separation. Elute the column with 50 mL *n*-hexane, and collect this fraction for GC injection. This is to ensure that the column is hydrocarbon-free before use in the extraction of HCs from CPKO.

Recovery Studies

Calibration curve.—Dilute 10 and 1 µg/mL working standards accordingly in 150 mL *n*-hexane in a 250 mL pear-shaped flask to obtain solutions containing 1, 5, 20, 50, 100, 200, and 400 g eicosane. Evaporate solvent from these 7 solutions in a gentle stream of nitrogen with the pear-shaped flask standing on a warm water bath at ca 50°C. Redissolve residue in an accurately measured volume of 1 mL *n*-hexane to give solutions representing 1, 5, 20, 50, 100, 200, and 400 µg/mL. Inject 1 µL of each solution into the GC system. Measure peak area, and calculate linear regression (r^2) against concentration.

Spiked oil standard curve.—Refined palm olein (RPOo) is used as the external standard for spiking, because it does not contain eicosane. This oil has gone through the complete process of physical refining, which includes bleaching, filtration, deacidification, and vacuum steam deodorization. Accurately weigh 10 g RPOo into each of five 100 mL volumetric flasks. Add 2, 15, and 50 mL working standard solution (5 µg/mL) to 3 of the RPOo samples, and dilute to 100 mL with *n*-hexane. This will give a spiked solution containing 1, 7.5, and 25 µg/g eicosane in oil. Add 2 and 3 mL 1 mg/mL stock solution to the remaining 2 RPOo samples, and make up solution with *n*-hexane to give a spiked solution containing 200 and 300 µg/g eicosane in oil, respectively. Pipet 20 mL sample containing 1 µg/g eicosane in oil into the prepared silica gel column. Elute the spiked oil sample from the column with an additional 150 mL *n*-hexane, and collect eluate in a 250 mL round-bottom flask. Evaporate the solvent in a gentle stream of nitrogen with the flask standing in a warm water bath (50°C). Redissolve the residue with an accurately measured volume of 1 mL *n*-hexane, and inject 1 µL into the GC system. Extract remaining spiked samples as described, using a freshly prepared column each time. Triplicate extractions are performed for each concentration of the spiked oil. Obtain the peak area of the eicosane peak, and calculate recoveries by comparison with corresponding concentration of calibration curve.

Extraction and Analysis of HCs from CPKO

Dissolve an accurately weighed sample of 5 g CPKO in *n*-hexane, and dilute to the 50 mL mark in a volumetric flask. Pipet 20 mL of this CPKO solution onto a prepared silica gel

Table 3. Recovery of eicosane in oil

Amt added, µg/g	Peak area, arbitrary units[a]	Amount found, µg/g	SD	CV, %	Rec., %
1.0	11.109	0.7	0.023	3.48	70.0
7.5	92.037	5.5	0.666	12.09	73.3
25.1	343.097	20.6	1.550	7.54	82.1
200.8	2901.052	173.8	22.971	13.22	86.6
301.2	4115.034	246.6	11.829	4.80	81.9

[a] Mean of triplicates with at least 2 injections for each sample.

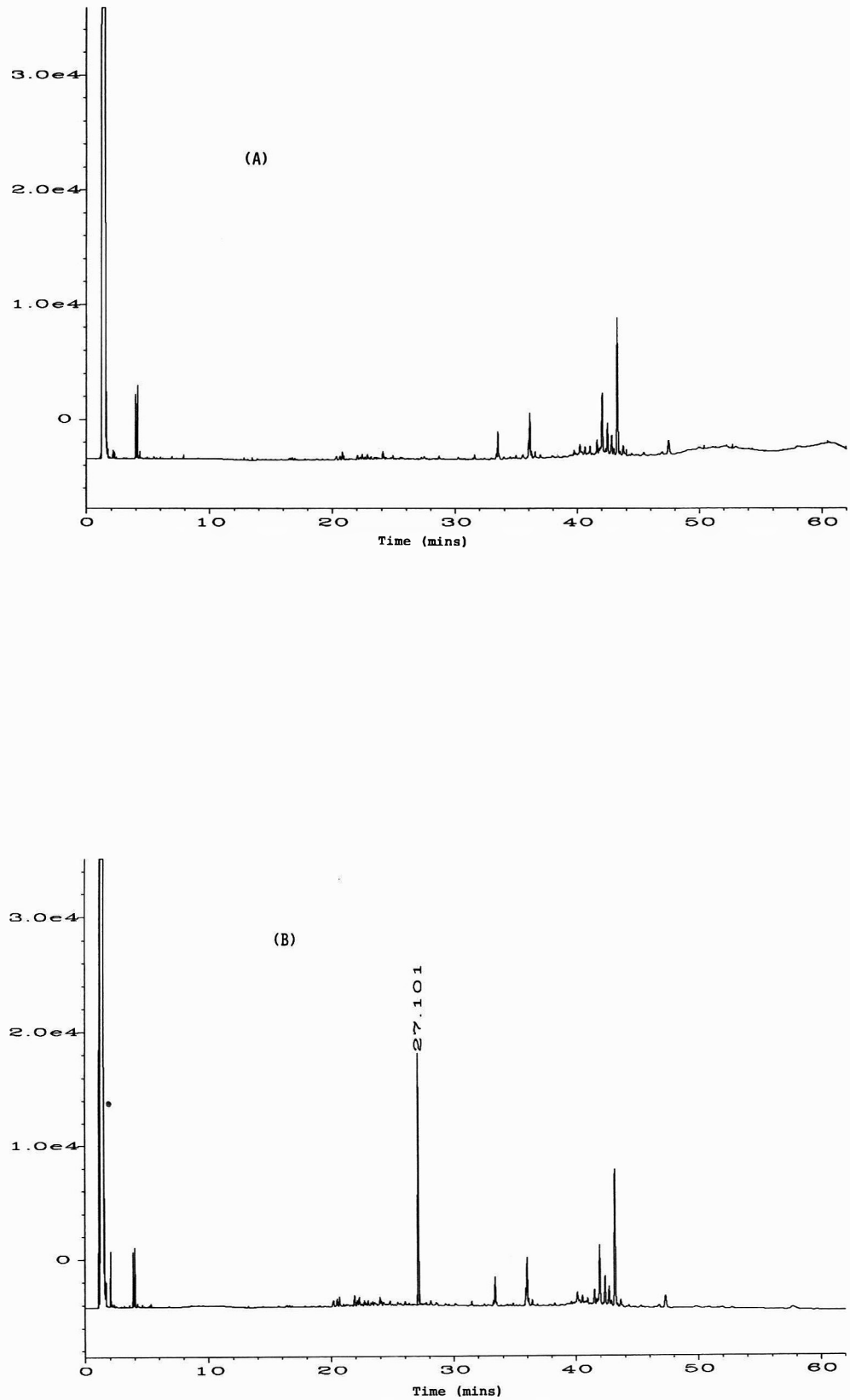
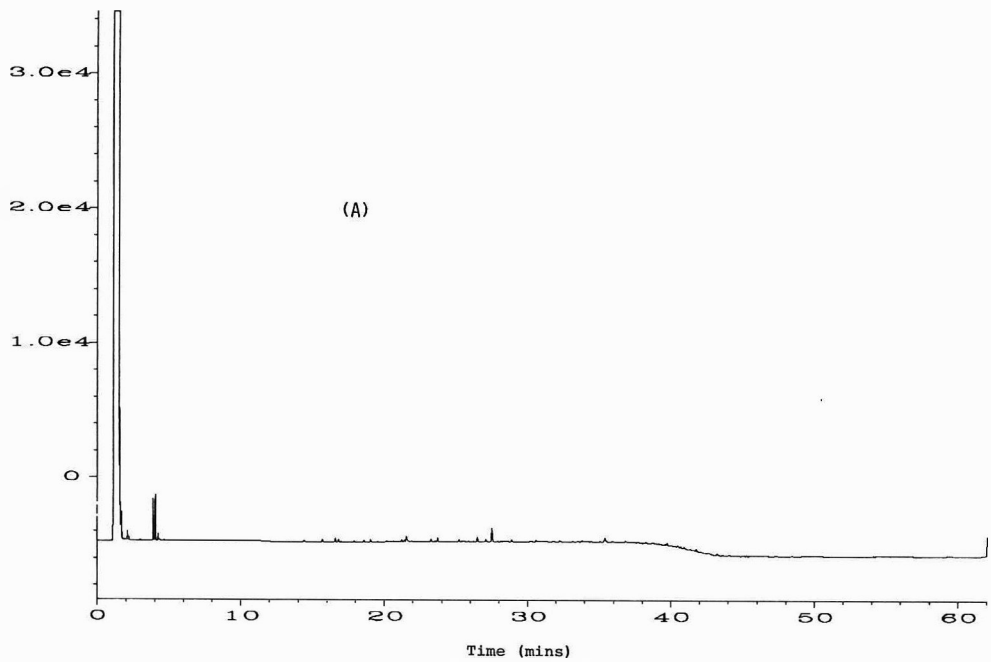


Figure 1. Chromatograms of RPOo extract (external standard): (A) Blank RPOo and (B) RPOo spiked at 7.5 μg/g.



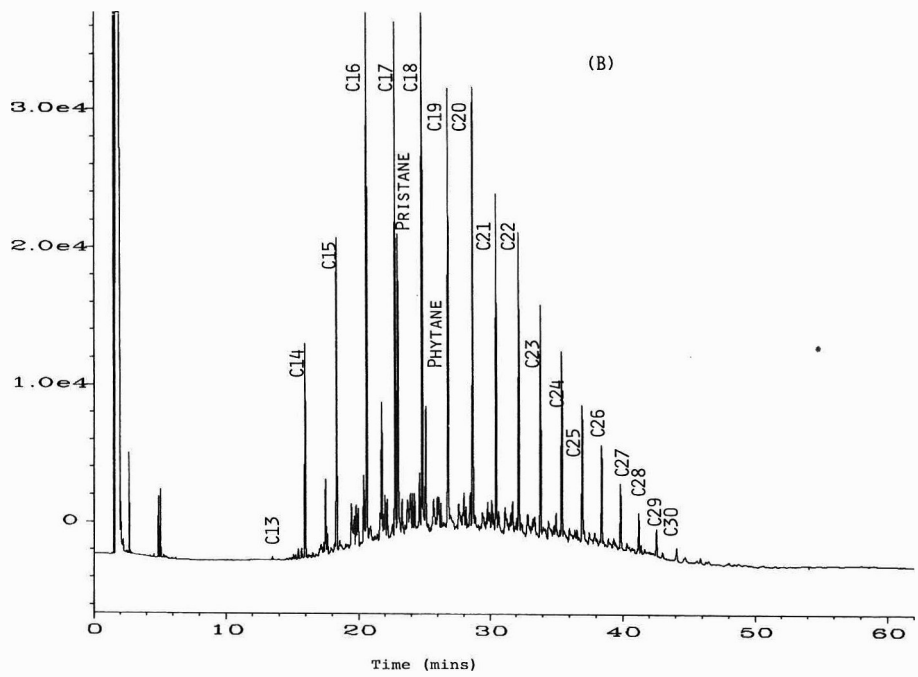


Figure 2. Chromatograms of CPKO extract: (A) Commercial CPKO sample and (B) CPKO sample suspected to be contaminated with mineral oil.

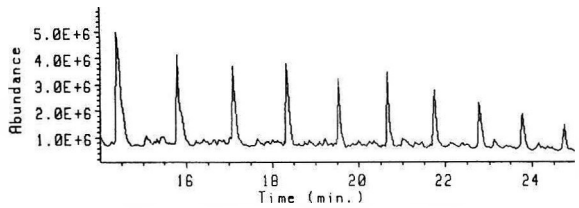


Figure 3. Ion chromatogram of part of the hydrocarbon fraction of CPKO.

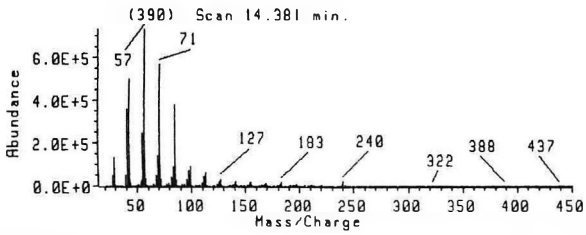


Figure 4. Mass spectrum of C_{17} alkane of CPKO.

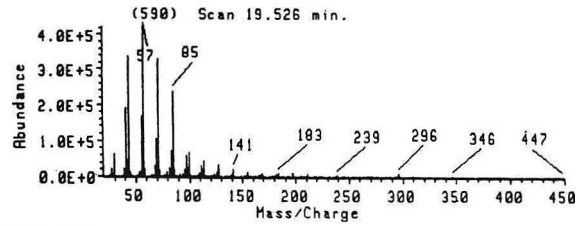


Figure 5. Mass spectrum of C_{21} alkane of CPKO.

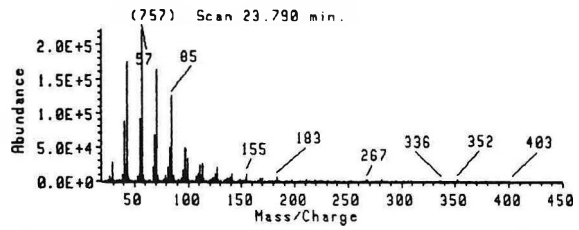


Figure 6. Mass spectrum of C_{25} alkane of CPKO.

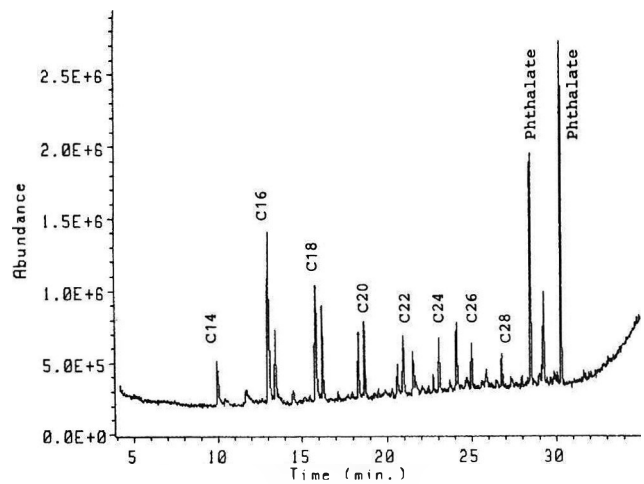


Figure 7. Total ion chromatogram of hydrocarbon fraction of CPKO kept in a plastic bottle.

column. In this way, an effective weight of ca 2 g CPKO is loaded onto the silica gel column. Elute HCs from the CPKO with 150 mL *n*-hexane, and collect this eluate in a 250 mL round-bottom flask. Evaporate *n*-hexane in a gentle stream of nitrogen as described. Redissolve the remaining HC residue in an accurately measured volume of 1 mL *n*-hexane, and inject 1 μL into the GC system and 1 μL into the GC/MSD system.

Results and Discussion

A number of factors may affect the efficiency of HC extraction from the silica gel column. The purity of the silica gel and of the solvent, *n*-hexane, are the 2 most important considerations. Silica gel with adsorbed HCs will result in extra peaks and an increased HC peak area recorded in the gas chromatograms. Prior washing of the silica gel with chloroform–methanol (3 + 1, v/v) followed by activation for 2 h at 120°C and elution with about 100 mL analytical grade *n*-hexane ensures that a clean column is used for the HC extraction.

Before loading the CPKO samples onto the silica gel column, about 50 mL *n*-hexane was collected from the column and evaporated to dryness. The residue was redissolved in 1 mL *n*-hexane, and 1 μL of this solution was injected into the GC system. This confirms that the silica gel column and the *n*-hexane used in the elution are free from other HCs. Additional factors that were considered before starting the extraction were the cleanliness of the glass apparatus and the presence of other possible HC contaminants, such as plastic caps, bottles, and stoppers that are known to contain the plasticizer phthalates.

To determine the reproducibility of the injection technique and the linearity of the flame ionization response, repeated injections of 1–400 ng eicosane in *n*-hexane were made into the capillary GC column. Table 1 shows the calibration curve data determined from measurement of eicosane peak area. Use of an automated injector would probably increase the reproducibility of the response. Linearity is acceptable, as indicated by the constancy of the absolute response factor (Table 2).

Sensitivity and recovery of the method were determined by analysis of RPOo samples spiked with eicosane. Figure 1 shows typical chromatograms of blank RPOo and RPOo spiked at 7.5 μg/g eicosane. Results of triplicate analysis, each solution injected twice, are summarized in Table 3. The recovery can be improved if a Kuderna-Danish evaporator is used to concentrate the sample down to 1 mL. In this case, the solution need not be taken down to dryness by blowing with nitrogen. Sensitivity for the eicosane was demonstrated to be at least 1 μg/g, which is adequate for the monitoring of HC level in CPKO. Recoveries and coefficients of variation ranged from 70.0 to 86.6% and from 3.5 to 13.2%, respectively.

The method was applied to studies of HC levels in commercial CPKO samples. Because certain samples of CPKO contained closely spaced long-chain HCs, an internal standard could not be used to quantify the HCs. Eicosane was chosen as the external standard, because it is of intermediate chain length and is found in CPKO and mineral oil. HCs in CPKO samples were calculated by comparing the sum of the areas of all the

HC peaks in the sample against the calibration curve. CPKO samples contaminated with mineral oil gave a chromatogram as shown in Figure 2 and are compared to the uncontaminated sample shown in the same figure. Figure 3 shows part of the ion chromatogram of a CPKO sample, and Figures 4–6 show the mass spectra of some of the hydrocarbons identified in that CPKO sample. The presence of a contiguous envelope of evenly distributed HC peaks ranging from C_{12} to C_{30} indicates the presence of mineral oil contamination. Phthalates were also detected in some samples of CPKO and, together with the identity of the HCs found in CPKO, were confirmed by GC/MSD (Figure 7). Pristane and phytane found in the contaminated sample (Figure 2B) were absent in Figure 7.

The procedure described in this paper is sensitive enough for the determination of HCs in CPKO. It can also be used for the analysis of samples suspected of mineral oil contamination.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Residual Imazethapyr in Soybeans by Gas Chromatography/Nitrogen–Phosphorus Detection

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Imazethapyr, a recently introduced imidazolinone herbicide, is registered for use on soybeans and other legumes in the United States and Canada. Its maximum allowed residue level in soybeans is 0.1 ppm. A method was developed to allow the measurement of ≥0.01 ppm imazethapyr on soybeans. The method exploits the ready cyclization of the nonvolatile acid to the volatile imidazoisoindol-3,5-dione by using an excess of easily removed, water-soluble carbodiimide and the selectivity of gas chromatography with nitrogen–phosphorus detection. A simple cleanup on a diol-bonded silica minicolumn reduces the complexity of the soybean extract. The derivatization works equally well for other imidazolinone herbicide acids. Because imidazoisoindolediones have characteristic fragmentation patterns, they are useful for mass spectroscopic confirmation of suspected residues.

Imidazolinone herbicides (Figure 1) were recently introduced for control of broadleaf weeds in a variety of legume and cereal crops in North America. Imazamethabenz and imazethapyr were registered for use in Canada: imazamethabenz on wheat and barley and imazethapyr on soybeans. Both pesticides have a maximum allowable residue limit of 0.1 ppm. Until Newsome and Collins (1) developed an enzyme-linked immunosorbent assay for imazamethabenz in cereal grain, no methods were published in the scientific literature for determining these herbicide residues on food products. Renner et al. (2) and Loux et al. (3) measured residual levels of imazaquin and imazapyr in soil by gas chromatography with nitrogen–phosphorus detection (GC/NPD) of the corresponding methyl esters. Wells and Michael (4) used liquid chromatography with ultraviolet detection at 195 nm for determining imazapyr in aqueous solutions.

With the exception of imazamethabenz, which is a methyl ester, the imidazolinone herbicides and the imazamethabenz metabolites are nonvolatile carboxylic acids. This paper introduces a novel approach to preparing a volatile derivative for GC analysis that is potentially applicable to all the imidazolinone herbicides. This approach is demonstrated by the determination of imazethapyr in soybeans.

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Experimental

Reagents

(a) *Solvents.*—Glass-distilled grade (Caledon Laboratories, Ltd, Georgetown, ON, L7G 4R9, Canada).

(b) *Chemicals.*—Imazapyr, imazethapyr, imazaquin, and imazamethabenz acid were donated by American Cyanamid, Princeton, NJ, as analytically pure standards (Figure 1). Dimethylaminopropyl ethylcarbodiimide hydrochloride was used as received (Aldrich Chemical Co., Milwaukee, WI 53233). A stock solution of imazethapyr (0.094 mg/mL) was prepared in ethyl acetate for spiking soybeans (10 µL for 10 g = 0.1 ppm).

Apparatus

(a) *Gas chromatograph.*—Model 3400 with NPD system and on-column injector (Varian Instrument Group, Sunnyvale, CA 94034). DB-5 capillary column, 30 m × 0.25 mm id, 0.24 µm film thickness (J&W Scientific, Folsom, CA 95630) with a 1 m deactivated, fused-silica retention gap, 0.53 mm id (Chromatographic Specialties, Brockville, ON, K6V 5W1, Canada). Temperature parameters: column, initially held 1 min at 100°C then raised to 215°C at 30°C/min, held 19 min, raised to 275°C at 30°C/min, and held 5 min to clean column; injector, initially held 0.25 min at 100°C then raised to 275°C at 300°C/min and held 29 min; detector, 300°C. Carrier gas, He at 1.7 mL/min. Volume injected, 1.5 µL.

(b) *Integrating recorder.*—Model 3390A (Hewlett-Packard, Avondale, PA 19311).

(c) *Solid-phase extraction cartridge.*—BondElut Diol (Analytichem, Harbor City, CA 90710) or Bakerbond Diol (J.T. Baker, Toronto, ON, M5W 1C5, Canada) or Supelclean Diol (Supelco, Oakville, ON, L6K 3V1, Canada); 3 mL.

(d) *Homogenizer.*—Kinematika PT 10-35 (Brinkmann Instruments, Rexdale, ON, M9W 4Y5, Canada).

(e) *Gas chromatograph/mass spectrometer.*—VG Analytical Model 7070EQ hybrid mass spectrometric (MS) system (with configuration EBQQ) coupled to a Varian 6000 GC system with on-column injector. DB-5 capillary column, 30 m × 0.25 mm id, held initially at 80°C for 1 min then ramped to

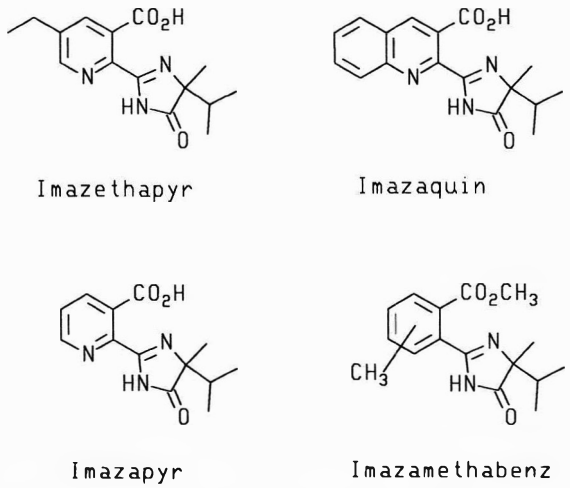

Figure 1. Structures of imidazolinone herbicides.

220°C at 30°C/min, held 18 min, and then heated to 280°C at 30°C/min. Only the conventional section (configuration EB) of the mass spectrometer was used. Conditions: resolution, 1 K; electron energy, 70 eV; trap current, 200 μA; source, re-entrants, and GC transfer line temperatures, 200°C. Full scanning mass range, *m/z* 40–450, exponential down at scanning rate of 0.6 s/decade and interscan delay of 0.2 s. For selected-ion monitoring (SIM), 80 ms dwell time for each ion.

(f) *Nuclear magnetic resonance (NMR) spectroscopy.*— Proton NMR spectra were run in deuterochloroform with tetramethysilane as internal reference on a Bruker AM200 spectrometer (Bruker Spectrospin Ltd., Milton, ON, Canada). The aromatic protons of each isomer were observed as 2 narrow doublets: isomer I at 8.16 and 8.93 ppm and isomer II at 8.07 and 8.89 ppm.

Extraction of Soybean Meal

A 10 g portion of soybean meal from ground, dry beans (40-mesh, Wiley mill) was homogenized 3 min in methanol (50 mL) at moderate speed. The homogenate was filtered through Whatman No. 1 filter paper. The rotor and sample vessel were rinsed with 15 mL methanol, and the rinsate was passed through the filter cake. The filtrate was evaporated to a yellow-green oil under reduced pressure at 38°C. The oil was partitioned between 25 mL 1N NaOH and 25 mL hexane–EtOAc (1 + 1). The aqueous layer was removed, and the organic layer was extracted with an additional 10 mL 1N NaOH. The combined aqueous layers were first back-extracted with 15 mL hexane–EtOAc (1 + 1), acidified to pH 3 with 10N H_2SO_4, and extracted with two 20 mL portions of CH_2Cl_2. The combined CH_2Cl_2 layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to a small volume. This concentrate was transferred to a 3 mL vial and evaporated to dryness under a stream of N_2 on a warm hot plate (the bulk of the coextracted acetic acid was removed).

Cyclization Reaction and Cleanup

A 2 mL aliquot of dimethylaminopropyl ethylcarbodiimide hydrochloride solution (10 mg/mL in methylene dichloride) was added to the vial containing the soya extract or an aliquot of imazethapyr standard solution and sealed with a Teflon-lined screw cap. The mixture was shaken briefly and left at room temperature for 1 h, and then a 1.0 mL portion was applied to a 3 mL diol-bonded minicolumn that was prewashed with 3% acetone in CH_2Cl_2. Collection of the eluant was begun as soon as sample was applied to the minicolumn. Once the sample descended to the top of the packing, a small volume (ca 0.1 mL) of 3% acetone in methylene chloride was used to rinse the interior wall of the tube, and then an additional 2 mL of the same solvent was used to elute the product. The combined eluant was evaporated just to dryness under a stream of N_2 on a warm hot plate (prolonged heating will reduce yield!), and the residue was taken up in 1.0 mL toluene for subsequent GC analysis.

Reaction Rate

Aliquots (10 μL) of imazethapyr stock solution were cyclized as above for various times at room temperature (1 min to 17 h). After the appropriate reaction time, solvent was evaporated under nitrogen without heating. The resulting residue was partitioned between 2 mL toluene and 2 mL water. Toluene solutions were analyzed by GC.

Calibration Curves

Various volumes (1–50 μL) of imazethapyr stock were cyclized for 60 min at room temperature, cleaned up on diol-bonded columns, and analyzed by GC, as detailed above. Linear regressions were performed with SlideWrite 4.0 (Advanced Graphics Software, Carlsbad, CA).

Results and Discussion

The proximity of the carboxylic acid group and the secondary amino group in the imidazolinone ring of the herbicides suggests that they might be readily cyclized. Indeed, Wepplo noted in his review (5) of the chemistry of the imidazolinone herbicides that imazapyr is converted to a mixture of 2 isomeric imidazoiso indolediones by dicyclohexylcarbodiimide in a 90:10 ratio. The patent literature claims that a 0.4M solution of imazapyr is cyclized quantitatively at room temperature within 2.5 h (6). We found that at trace levels of imazethapyr (1 μg/mL) an excess of dicyclohexylcarbodiimide generated only 1% of the cyclized product after 1 h at room temperature. Diisopropylcarbodiimide was only slightly better, producing 6% of the product. In contrast, dimethylaminopropyl ethylcarbodiimide hydrochloride gave quantitative cyclization under the same conditions, yielding 2 isomers in a ratio of 84:16 (Figure 2). The smaller steric requirement of the latter reagent is presumably responsible for the greater rate of reaction. The kinetics experiment already showed that 1 h at room temperature was sufficient to complete cyclization with the polar carbodiimide. Although heating the sample could shorten the reaction time, all cyclizations were performed at room temperature for convenience. The ratio of the 2 isomers is not a result of the high temperature of the GC conditions, because

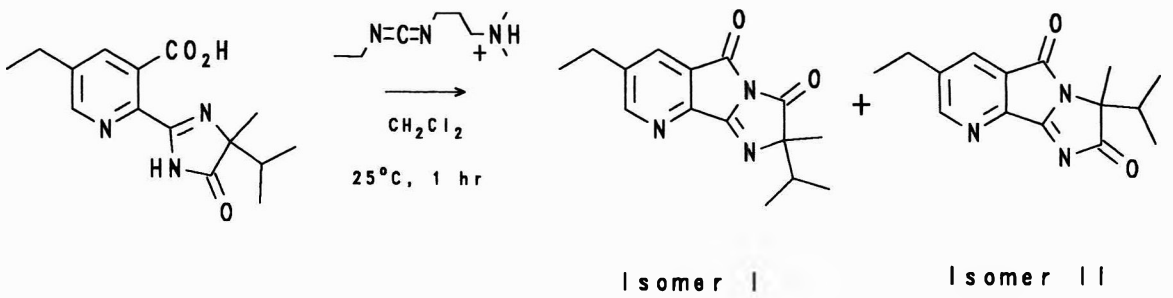

Figure 2. Derivatization reaction of imazethapyr.

proton NMR spectroscopy of a larger sample showed the same ratio at room temperature.

The presence of 2 isomers for analysis of a single component could be construed as disadvantageous with respect to quantitation. Isomer I is nearly 6 times as intense as isomer II; however, the disadvantage is negligible. In fact, the second isomer is very advantageous when MS confirmation is required, because 2 peaks will be seen by SIM (discussed later).

Calibration curves were prepared in the practical working range of 0.01–0.50 ppm and were good linear plots through the zero point for both isomers; the R values were 0.9983 and 0.9984 for the major and minor isomers, respectively. The smallest sample injected (75 pg) gave an isomer I peak with a signal-to-noise ratio of 25. This suggested that the limit of detection (signal-to-noise ratio = 3) was 0.002 ppm, but a more practical limit based on the noisier background of the soya extracts was approximately 0.01 ppm. The cyclized products were reasonably stable. After 3 days at room temperature under fluorescent lights or after 3 weeks in the dark at −20°C, a 5–10% decline in peak area was noted.

Initially, extracts of soybean meal were cyclized and worked up by evaporating the reaction mixture to dryness and then simply partitioning the residue between water and toluene to remove the excess of the water-soluble carbodiimide before GC analysis (Figure 3). The number and quantity of co-extracted interferences registered by NPD, however, necessitated a more substantial cleanup. A variety of chromatographic adsorbents were tried without success. Cleanup on Florisil, silica, or alumina gave poor recoveries as a result of irreversible binding of the cyclized products, even when the sorbent was deactivated in various ways. The ratio of the 2 isomers was also distorted. Cyanopropyl-bonded silica introduced many new interfering peaks in the chromatogram, and octadecyl-bonded silica was ineffective. Diol-bonded silica, however, worked very well, and solvent conditions were established so that cleanup was, in effect, a simple filtration of the reaction mixture through the column. Barron, in a study of strawberry aroma extractives (7), also observed irreversible binding of polar constituents on silica and found diol-bonded silica to be an effective substitute. The 2 isomers were recovered quantitatively, and their ratio did not change.

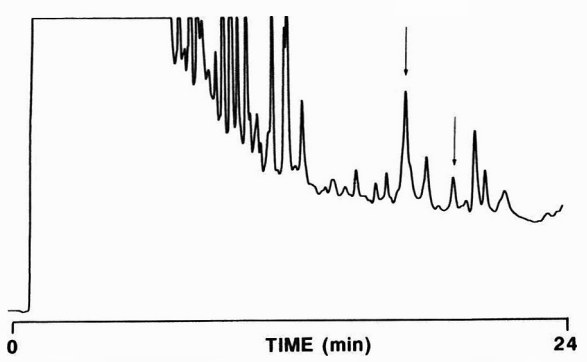

Figure 3. GC/NPD chromatogram of a derivatized soybean meal extract spiked with 0.1 ppm imazethapyr before cleanup. Arrows indicate imazethapyr derivatives.

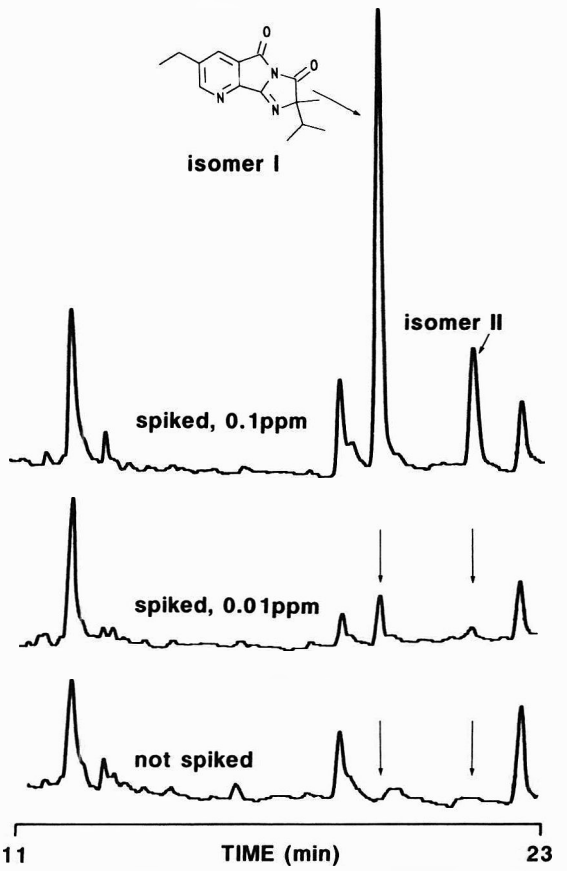

Figure 4. Sections of GC/NPD chromatograms of derivatized extracts of soybean meal spiked at various levels with imazethapyr after cleanup on the diol-bonded columns.

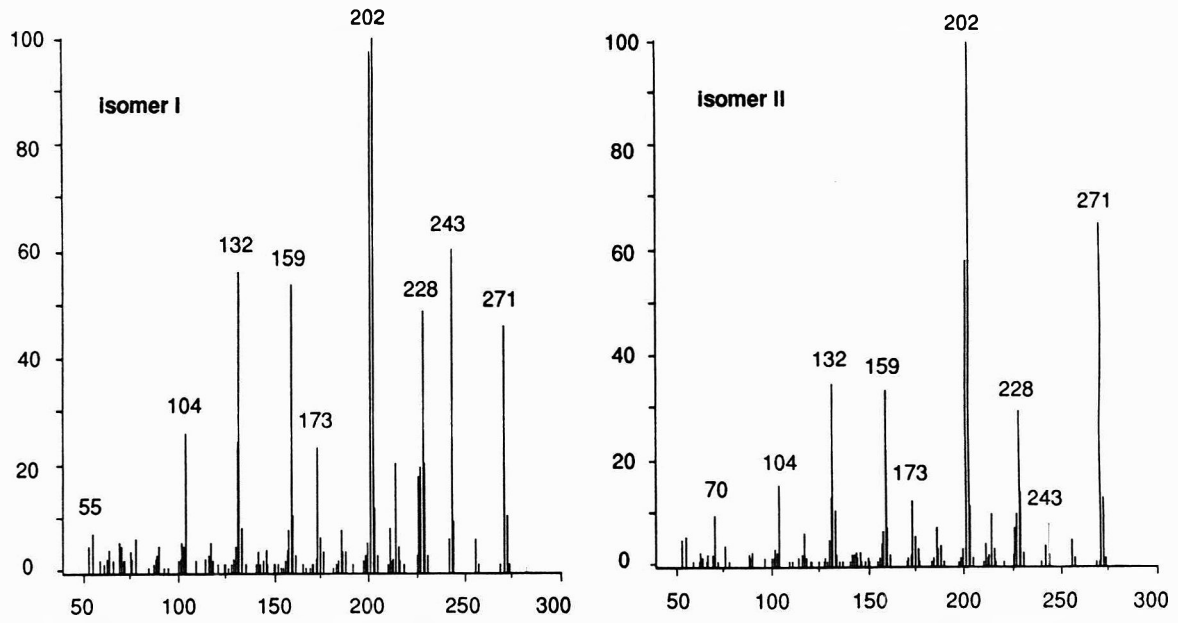

Figure 5. Mass spectra (EI) of the 2 isomeric imidazoisoindolediones from cyclization of imazethapyr (GC inlet).

GC conditions were then established to separate the principal isomer I from the remaining coextractives with little concern for the minor isomer II, because isomer II is a fixed percent of isomer I. Figure 4 compares sections of typical GC chromatograms from cleaned-up soya extracts that were spiked with 0, 0.01, or 0.1 ppm imazethapyr. Isomer I is clearly discernible at 0.01 ppm when compared with the control soya sample. Comparison of Figure 4, the extract after cleanup, with Figure 3, the extract before cleanup, demonstrates the improvement obtained by using the diol-bonded column.

Recovery of imazethapyr from spiked soybean meal was good; duplicate extracts each analyzed twice by GC gave recoveries of 80 ± 4% at 0.01 ppm and 97 ± 14% at 0.1 ppm. Extraction of soybean meal with acetone was not as effective as with methanol; recoveries were typically 30–40% lower with acetone.

The mass spectra of the 2 imazethaisoindolediones are illustrated in Figure 5. A number of ions of good intensity are common to both isomers. In fact, the imidazoisoindolediones of all 4 herbicide acids (prepared in the same way as that described for imazethapyr) have intense molecular ions and intense M^+-69 fragments: imazethapyr, 271 (70%) and 202 (95%); imazapyr, 243 (60%) and 174 (100%); imazaquin, 293 (80%) and 224 (100%); and imazamethabenz, 256 (95%) and 187 (100%). In addition, they have a number of common fragments of variable intensities (5–60%), such as the M^+-28 and M^+-43 ions. For SIM of imazethapyr diones, ions *m/z* 271 (molecular ion) and *m/z* 243 (loss of CO) were chosen rather than the more intense fragment at *m/z* 202. Not only are higher masses more selective for confirmation, but the drop in accelerating voltage is minimized by keeping the 2 monitored masses as close as possible.

Although the soybean extracts were too complex to be analyzed by GC/NPD without cleanup (Figure 3), GC/MS monitoring at *m/z* 243 and 271 was able to specifically find the 2 imazethapyr derivatives in the multitude of soybean coextractives (Figure 6). Note that in Figure 6, the control traces are amplified relative to the spiked traces, because the output is normalized to the largest peak. In addition, the ratio of the 2 derivatives appears different at *m/z* 243 and at *m/z* 271. In part, this is because of the different relative intensities of

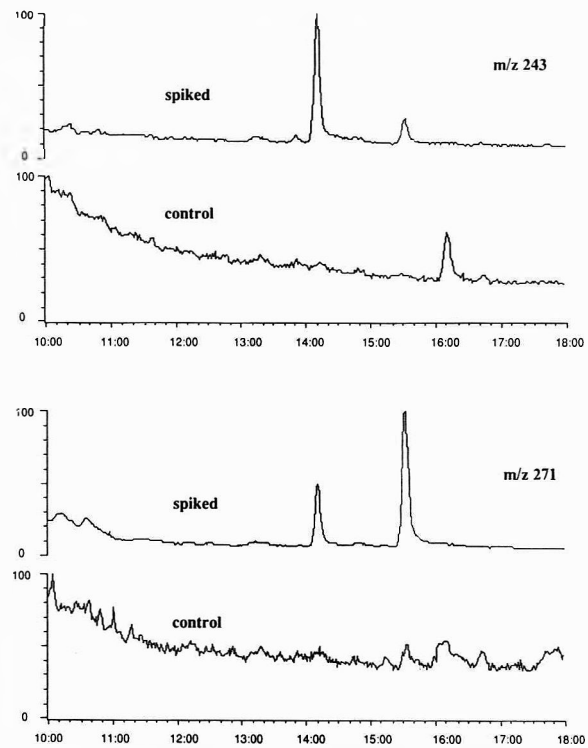

Figure 6. SIM traces (*m/z* 243 and 271) of 2 derivatized soya extracts before cleanup; one is spiked with 0.1 ppm imazethapyr, and one is not.

these 2 ions in the spectra of the 2 isomeric diones, but the difference may also be exacerbated by "junk" with a different mass that elutes at the same retention time and competes with the imazethapyr diones for ionization. Nevertheless, the ratio of ions at *m/z* 243 and 271 was consistent from sample to sample whether the sample was a pure standard, a cleaned-up soya extract, or an original soya extract. The selectivity of NPD obviates this problem.

In summary, trace levels of imazethapyr can be conveniently cyclized to the corresponding volatile and stable imidazolinedione that lends itself to GC analysis. We applied this reaction to the analysis of residual imazethapyr in soybeans. After a simple cleanup, NPD has the selectivity and sensitivity to measure this residue at ≥0.01 ppm in the derivatized extract. In addition, we pointed out that the cyclization reaction is successful with imidazolinone herbicide acids, such as imazapyr, imazaquin, and imazamethabenz acid, and that the derivatives have characteristic mass spectra that are useful for confirmation by SIM.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Development of Enzyme Immunoassay for Captan and Its Degradation Product Tetrahydrophthalimide in Foods

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A simple, sensitive, and precise enzyme-linked immunosorbent assay (ELISA) is described for the quantitation of captan as its degradation product tetrahydrophthalimide (THPI) in foods using polyclonal antibodies. Three hapten analogues of THPI with different alkyl spacer arm lengths were synthesized. Immunogens and coating proteins were prepared by coupling these haptens to human serum albumin and ovalbumin, respectively. A 5-carbon spacer arm appeared to be optimum for the production of antibodies. Heterologous coating proteins did not improve the sensitivity, but reduction of homologous coating protein concentration did improve the sensitivity, resulting in a concentration of test compound required to inhibit binding by 50% of 15.5 ng/mL. The antiserum is specific for captan, captafol, and THPI, but not other structurally related compounds. The minimum detection limit was 1 ng/mL; the linearity was 1–200 ng/mL. The overall recoveries of captan and THPI from 11 commodities spiked at 4 levels were 92 and 100%, respectively. The intra-assay and interassay coefficients of variation were 9.1 and 16.8% for apple blanks and 5.9 and 4.2% for apple spiked with 3 ppm THPI, respectively. The ELISA described is suitable for measuring captan and THPI at levels comparable to those typically found in fruit.

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Captan [*N*-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide] was introduced in the early 1950s and is still widely used for the protection of many fruit crops against a broad range of fungal pathogens. In Canada, about 300 000 kg captan is used annually, with a use pattern of 82% on leaves and fruits, 10% for seed treatment, 5% for home and garden uses, and 3% for nonagricultural applications (1). The maximum residue limit is 5.0 ppm in apples, apricots, blueberries, cranberries, cherries, grapes, peaches, pears, plums, raspberries, strawberries, and tomatoes (2).

Gas chromatography (GC) is the most widely used technique for the determination of captan. Electron capture (3–6), flame photometric (7), electrolytic conductivity (8), and mass spectrometric (9) detection methods were reported. Other methods of analysis include liquid chromatography (10, 11) and thin-layer chromatography (TLC) (12, 13). GC methods were also developed for THPI, a degradation product formed

by hydrolysis (14, 15) or as a metabolite (16, 17). Onley (5) described a procedure involving derivatization with pentafluorobenzyl bromide and electron capture detection that was capable of determining 0.02–0.04 ppm in milk or meat, but involved extensive cleanup. Winterlin et al. (7) developed a gas–liquid chromatographic method for THPI in urine using nitrogen–phosphorus detection, but the method also required multiple extractions and column chromatographic cleanup.

Immunochemical methods for the determination of pesticides have gained popularity over the last decade. The development of simple, sensitive, and rapid immunoassay methods for a range of synthetic pesticides (18) suggests that this is a viable alternative route for the determination of captan or THPI. Small molecules such as pesticides usually do not stimulate an immunogenic response. A hapten is usually linked with a spacer arm to a large molecule such as protein to become immunogenic. The present paper describes the production of antibodies and their use in an enzyme-linked immunosorbent assay (ELISA) for the determination of captan and THPI in foods.

Materials and Methods

Pesticide standards were obtained from the pesticide repository of the Food Research Division and were stated to be at least 99% pure by the respective manufacturers. *cis*-Δ4-tetrahydrophthalimide (THPI), tetrahydrophthalic anhydride, phthalimide, phthalic acid, sodium iodoacetate, 3-iodopropionic acid, 5-bromovaleric acid, tri-*n*-butylamine, and *iso*-butyl chloroformate were purchased from Aldrich Chemical Co., Milwaukee, WI. Captan, captafol, and folpet were dissolved in acetonitrile; the other structurally related compounds were dissolved in methanol to give standard solutions for standard curves and fortifications.

Bovine serum albumin (RIA grade), ovalbumin, human serum albumin, goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20, *o*-phenylenediamine dihydrochloride, and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chemical Co., St. Louis, MO. Freund's complete and incomplete adjuvants were obtained from Gibco, Grand Island, NY. Dialysis tubing (10 mm in diameter with a 12 000–14 000 molecular weight cutoff) was purchased from Spectrum Medical Industries Inc., Los Angeles, CA. Flat-bottom polystyrene microtiter plates, 96-well, were obtained from Dynatech Laboratories, Inc., Chantilly, VA.

Phosphate-buffered saline (PBS), pH 7.2, contained 20 mmol NaH_2PO_4 and 140 mmol NaCl/L of deionized water. Coating buffer (pH 9.6) contained 13 mmol Na_2CO_3 and 11 mmol $NaHCO_3$; citric buffer (pH 5.0) consisted of 0.6 mol Na_2HPO_4 and 0.1 mol citric acid/L deionized water. The substrate consisted of 35 mg *o*-phenylenediamine dihydrochloride and 20 μL 30% H_2O_2 in 50 mL citric buffer (19).

TLC was conducted on 25 cm glass plates coated with 0.25 mm Silica Gel G containing a fluorescent indicator. Plates were developed in solvent systems consisting of either toluene–ethyl acetate–acetic acid (50 + 50 + 1), solvent A; acetonitrile–water–diethylamine (88 + 12 + 1), solvent B; or dichloromethane–methanol–acetic acid (80 + 19 + 1), solvent C. Components were visualized by UV illumination.

Instrumentation

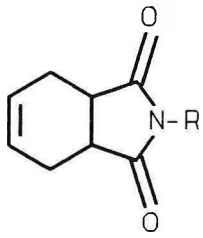
Microtiter plates were washed first with 1% Tween 20 in PBS (PBS-T) in a Model 120 Titertek Microplate washer (Flow Laboratories, Mississauga, ON, Canada) with 5 wash cycles and 3 soak periods of 30 s each and then 3 wash cycles of deionized water. Absorbances of microtiter wells were measured on a dual-beam Titertek Multiscan MCC with a 492 nm sample filter and 620 nm reference filters. A 12-channel Titertek pipet (25–250 μL) from Flow Laboratories was used for dispensing liquids. Data were transmitted to an Olivetti M280 microcomputer for analysis with a Lotus spreadsheet. The instrument was checked for reading bias by using *p*-nitrophenol and the reversed plate protocol described by Harrison and Hammock (20).

Mass spectra were obtained at a resolution of 1000 on a VG ZAB 2F instrument using a direct probe inlet and 70 eV electron impact ionization. An HP 5890 GC system equipped with an HP 5970 mass selective detection (MSD) system and a Supelco 25 m DB-5 column were used for quantitation, with minor modification of the method of Schoen and Winterlin (17).

Syntheses

To provide a functional group with which to couple THPI to immunizing or coating protein and to study the effect of the length of the spacer, derivatives were prepared by alkylating the imide nitrogen with either iodoacetic, 3-iodopropionic, or 5-bromovaleric acid. The potassium salt of THPI was prepared by dissolving 3.0 g THPI (20 mmol) in 15 mL ethanol containing 1.1 g KOH (20 mmol). The solvent was removed on a rotary evaporator, and 4.1 g sodium iodoacetate (20 mmol) was added. An exothermic reaction occurred on mixing of the solids. After the reaction subsided, 4 mL dimethyl formamide was added and the solution was refluxed 16 h. The reaction mixture was then cooled, transferred to a separatory funnel with 45 mL water, acidified to litmus with dilute HCl, and extracted with 60 mL dichloromethane. The aqueous phase was discarded, and the dichloromethane was extracted with 30 mL 5% $NaHCO_3$. The dichloromethane was discarded, and the solution was carefully acidified with dilute HCl; then, the aqueous layer was extracted with 30 mL fresh dichloromethane. This acid-base cycle was repeated, and the dichloromethane was dried with anhydrous Na_2SO_4. Removal of the solvent yielded 2.29 g (55%) of pale yellow crystals, mp 98–101°C: R_f, 0.21 in solvent A and 0.43 in solvent B; MSD, *m/z* (relative intensity) 209 ($[M]^+$, 14.9), 191 ($[M]^+$-H_2O, 18.2), and 163 ($[M]^+$-H_2O-CO, 55.3).

The *N*-propionic acid and *N*-valeric acid derivatives were prepared in a similar fashion by using 2 equivalents of KOH to prepare the potassium salts of THPI and the respective acids. The yield of propionate was 2.12 g (48%, yellow wax, mp 45–49°C). In solvent A, the R_f was 0.36, and in solvent B it showed



a. R = CH_2CO-NH-HSA
b. R = $(CH_2)_2CO$-NH-HSA
c. R = $(CH_2)_4CO$-NH-HSA
HSA: human serum albumin

Figure 1. Structure of THPI immunogens.

a major component at 0.43 and a minor one at 0.61: MSD, *m/z* (relative intensity) 223 ($[M]^+$, 2.2), 205 ($[M]^+$-H_2O, 100), and 177 ($[M]^+$-H_2O-CO, 5.8). The yield of valerate was 1.35 g (27%, yellow syrup). TLC revealed 3 components in solvent A, a major component at R_f 0.44 and minor components at 0.34 and 0.26. Chromatography in solvent B showed a major component at R_f 0.43 and a minor component at 0.75: MSD, *m/z* (relative intensity) 251 ($[M]^+$, 0.7), 233 ($[M]^+$-H_2O, 73.3), and 205 ($[M]^+$-H_2O-CO, 39). The material was used without further purification.

Tetrahydrophthalic anhydride (10 mg in 10 mL H_2O) or THPI (10 mg in 10 mL 0.1N NaOH) was hydrolyzed at 95°C for 30 min. The pH of the hydrolysate was adjusted to 6.7 with 1N HCl to yield tetrahydrophthalic acid and tetrahydrophthalamic acid, respectively. TLC in solvent C showed 1 spot at R_f 0.50 for tetrahydrophthalic acid and 2 spots at R_f 0.50 and 0.55 for tetrahydrophthalamic acid. The composition of the spots was determined by GC/MSD to be 87:13 tetrahydrophthalamic acid:tetrahydrophthalic acid, assuming both compounds had the same detector response. These compounds were used in the cross-reactivity study without further purification.

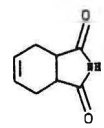
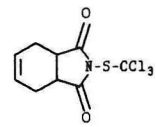
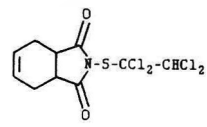
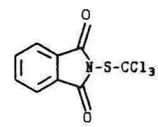
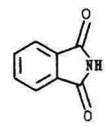
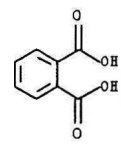
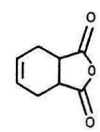
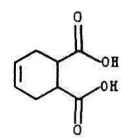
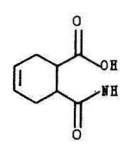
Immunogen

The immunogen was coupled to human serum albumin as previously described (21). Briefly, 40 µmol THPI derivative was dissolved in 1.0 mL 0.05M sodium phosphate, pH 7.0, and 100 mg EDC was added. Then 28 mg human serum albumin was added in 1.5 mL of the same buffer, and the solution was mixed and placed in a 4°C refrigerator for 18 h. The resulting conjugate was dialyzed against distilled water and stored frozen in 1 mL aliquots.

Coating Protein

Coating proteins were prepared by a mixed anhydride coupling reaction to ovalbumin to avoid recognition of EDC reaction by-products as previously described (21). The respective derivative of 40 µmol THPI was dissolved in 0.5 mL 1,4-dioxane and 15 µL tri-*n*-butylamine, and then 8 µL isobutyl chloroformate was added. After 30 min reaction, the solution was added with stirring to 44 mg ovalbumin in 3 mL 0.2M $NaHCO_3$, pH 9.3. After 22 h at 4°C, the solution was dialyzed against deionized water and diluted to 10 mL, and 200 µL aliquots were stored at −20°C.

Table 1. Specificity of antisera toward tetrahydrophthalimide and other structurally related compounds

Compound	Structure	IC_{50}, ng/mL[a]
Tetrahydrophthalimide		16.3
Captan		57.0
Captafol		71.9
Folpet		>1000
Phthalimide		>1000
Phthalic acid		>1000
Tetrahydrophthalic anhydride		>1000
Tetrahydrophthalic acid		>1000
Tetrahydrophthalamic acid		>1000

[a] IC_{50} = concentration of test compound required to inhibit binding by 50%.

Table 2. Percent recovery of captan as THPI from various commodities[a]

	Captan added, ppm				
	1.25	2.5	5.0	10.0	Mean ± SD
Strawberry	97.3	83.9	81.4	84.1	86.7 ± 6.2
Blueberry	95.6	85.1	96.7	84.8	90.6 ± 5.6
Raspberry	122.5	92.4	106.0	83.8	101.2 ± 14.6
Cherry	100.2	82.8	94.9	82.7	93.1 ± 7.6
Nectarine	88.5	99.9	110.1	109.9	102.1 ± 8.9
Peach	94.6	99.6	83.1	81.8	89.8 ± 7.5
Apricot	100.7	94.8	97.7	83.9	94.3 ± 6.3
Plum	89.9	85.2	111.8	83.3	92.6 ± 11.4
Apple	91.5	92.4	84.0	97.9	91.4 ± 5.0
Grape	86.8	95.3	115.5	104.6	100.5 ± 10.7
Tomato	62.2	66.3	73.2	79.0	70.2 ± 6.4

[a] Values are corrected for blank. All data are the means of duplicate samples.

Microtiter Plate Coating

Microtiter plates were rinsed with deionized water, and the wells were filled with 200 µL of a solution of 1.1 µg coating protein/mL plus 10 µg ovalbumin/mL in coating buffer. After 16 h at 4°C, the wells were washed with PBS-T using a Titertek Microplate washer with 5 wash cycles and 3 soak periods of 30 s each, and then 3 wash cycles of deionized water. The sensitized plates were stored in plastic bags at –20°C. These plates can be stored up to 6 months without any problem.

Immunization

Male White New Zealand rabbits, 3 groups of 4 animals each, were used to obtain antibodies against the THPI-human serum albumin conjugates. The immunogen was diluted in PBS and emulsified in Freund's complete or incomplete adjuvant to give 1 mg/mL. Rabbits were injected subcutaneously with 0.5 mL of the emulsion at 5 sites. Booster injections, in which incomplete adjuvant was substituted for complete adjuvant, were given at monthly intervals. Blood was collected from the marginal ear vein 1 week after each boost.

The serum from each animal was tested for titer by adding serial dilutions to a microtiter plate coated with 4.4 µg homologous coating protein/mL and incubating at 4°C for 30 min. Similar dilutions of serum from nonimmunized rabbits served as controls. After the excess antibody was removed by washing, the amount of adsorbed antibody was determined with goat anti-rabbit IgG peroxidase conjugate under the same conditions as for the immunoassay below. Serum dilutions that gave an absorbance of ca 1.0 were selected for competitive inhibition of binding by various concentrations of captan or THPI. Competitive inhibition was tested by preincubation of the appropriate dilution of antiserum with ≤500 ng captan or THPI/mL, followed by addition to a coated plate and measurement of the degree of binding as described below. Animals having the highest titers and most sensitive inhibition curves were exsanguinated under anesthesia 6–12 months after the initial immunization. The serum was stored frozen at –20°C in

Table 3. Percent recovery of THPI from various commodities[a]

	THPI added, ppm				
	0.67	1.35	2.7	5.4	Mean ± SD
Strawberry	114.2	114.7	85.0	84.2	99.5 ± 14.9
Blueberry	91.7	114.3	114.9	98.9	105.0 ± 10.0
Raspberry	117.9	118.6	96.6	80.2	103.1 ± 16.1
Cherry	117.2	112.2	98.9	86.8	103.8 ± 11.8
Nectarine	99.2	119.5	90.4	96.9	101.5 ± 10.9
Peach	89.7	84.7	81.0	73.4	82.2 ± 6.0
Apricot	84.17	112.6	113.5	86.9	99.3 ± 13.8
Plum	102.4	117.1	101.6	86.3	102.4 ± 10.2
Apple	99.3	96.8	103.2	108.8	102.0 ± 4.5
Grape	95.7	108.3	82.7	80.4	91.8 ± 11.2
Tomato	116.3	109.7	116.4	121.5	116.0 ± 4.2

[a] Values are corrected for blank. All data are the means of duplicate samples.

Table 4. Blank determinations of THPI in various commodities[a]

	ELISA	GC/MSD
Raspberry	0.027 ± 0.007	0.042 ± 0.001
Nectarine	0.019 ± 0.014	0.010 ± 0.002
Apricot	0.081 ± 0.015	0.071 ± 0.005
Apple	0.175 ± 0.016	0.135 ± 0.010
Grape	0.034 ± 0.010	0.033 ± 0.002

[a] Values are the mean of 6 replicates expressed in ppm ± standard deviation.

200 µL aliquots. To standardize the assay, serum collected from a single rabbit 12 months after the initial injection was used in all subsequent studies on cross-reactivity and in all applications to food extracts.

Sample Preparation

A 10 g subsample of previously homogenized commodity was blended 10 s with 60 mL methanol in a 125 mL flat-bottom boiling flask by a Polytron homogenizer. The solution was then refluxed 20 min. After cooling, the solids were removed by filtration through Whatman No. 1 paper, and the filtrate was diluted to 100 mL with methanol. Then, 5 mL extract was shaken twice with an equal volume of 10% ether in hexane for 5 min and centrifuged at 1000 rpm for 5 min. The ether–hexane layer was discarded. A 100 µL aliquot of the extract was left in a cupboard overnight to dry, and the residue was taken up in 25 µL PBS for ELISA. In the recovery studies, the homogenized samples were spiked with either captan or THPI at 4 levels. These fortified samples were incubated 30 min at room temperature before polytron extraction.

Immunoassay

A 1 mL aliquot of antiserum diluted 1:6000 with 0.1% BSA in PBS diluent was added to 25 µL sample or standard in a 12 × 75 mm culture tube. The solution was mixed and incubated at 4°C for 30 min, and 200 µL was added to triplicate wells of a cold coated plate. After further incubation at 4°C for 30 min, the wells were emptied and washed with PBS-T. A 200 µL aliquot of a 1:5000 dilution of second antibody goat anti-rabbit IgG peroxidase conjugate in 0.1% BSA in PBS diluent was added to each well, and after 30 min incubation at room temperature the plate was again emptied and washed. An equal volume of substrate consisting of 35 mg *o*-phenylenediamine dihydrochloride and 20 µL H_2O_2 in 50 mL citrate buffer, pH 5.0 (19), was added. The enzymatic reaction was allowed to proceed at room temperature in darkness for 30 min. The reaction was stopped by the addition of 50 µL 4M H_2SO_4. The absorbances were read at 492 nm, and the amount of THPI was determined by a least-squares plot of the logit of the absorbance against the log of the concentration of the standards. The standard curve consisted of 8 concentrations of THPI: 0, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, and 100.0 ng/mL. The concentration of test compound required to inhibit binding by 50% (IC_{50}), which is useful for comparing the degree of inhibition exhibited by various related compounds, is defined as the concentration of inhibitor necessary to produce a 50% decrease in absorbance. This value was calculated from the least-squares plot of the log of the concentration of inhibitor against the percentage decrease in absorbance relative to that obtained in the absence of inhibitor.

Results and Discussion

Animals immunized with a conjugate of the *N*-carboxymethyl derivative (Figure 1a) did not produce significant antibody titers within 3 months, and they produced only low titers at 4 months after initial injection. The *N*-carboxyethyl (Figure 1b) and *N*-carboxybutyl homologues (Figure 1c) were much more effective immunogens, eliciting high titers as early as 1 month. However, antiserum from the *N*-carboxyethyl–THPI immunized animals produced poor inhibition curves when tested with THPI and required approximately 500 ng/mL for 50% inhibition. Antiserum to the *N*-carboxybutyl hapten showed 50% inhibition of binding with THPI at 150 ng/mL and was selected for further optimization. These results demonstrated the importance of spacer arm length of hapten for a successful antibody production. The use of heterologous coating protein (*N*-carboxymethyl or *N*-carboxyethyl haptens) did not improve the sensitivity, but reduction of the coating protein concentration to 1.1 µg/mL was effective in increasing the sensitivity and resulted in an IC_{50} of approximately 16 ng/mL.

The specificity of the antiserum was evaluated by comparing the IC_{50} value of some structurally related compounds. The cross-reactivity of the antibodies to captan and captafol on a nanomole basis was 57 and 52%, respectively, relative to THPI. Other structurally related compounds were essentially unrecognized. Data are summarized in Table 1. Results demonstrated the high specificity of the antibodies against the THPI moiety.

Table 2 summarizes the recoveries from several captan-registered commodities that were spiked with captan at 1.25, 2.5, 5, and 10 ppm. Recoveries were calculated in terms of THPI, because the method converts all captan to THPI. Table 3 summarizes the recoveries of THPI spiked at 0.67, 1.35, 2.7, and 5.4 ppm levels. Good recovery was achieved for both captan and THPI (92 and 100%, respectively). The conversion of captan to THPI was essentially completed by refluxing 20 min in methanol. All data were corrected for blanks, which consisted of the sample matrix that was subjected to the entire extraction and ELISA procedure. Table 4 depicts the reproducibility of the blanks analyzed by ELISA and confirmed by GC/MSD method (17). The correlation coefficient of the ELISA and GC/MSD methods was 0.96 when 6 samples were analyzed.

The intra-assay and interassay coefficients of variation in this method were 9.1 and 16.8% for apple and 5.9 and 4.2% for apple spiked with 3 ppm THPI, respectively (n = 6), as indicated in Table 5. These latter values are similar to those observed for paraquat (22) or quinine (23) that was not subjected to extraction and cleanup steps. Table 6 shows that the average IC_{50} for THPI was 15.5 ng/mL. The ruggedness of the assay

Table 5. Intra-assay and interassay coefficients of variation[a]

	Intra-assay		Interassay	
	Mean ± SD	CV, %	Mean ± SD	CV, %
Apple	0.18 ± 0.02	9.1	0.20 ± 0.03	16.8
Apple + 3 ppm THPI	3.16 ± 0.18	5.9	2.85 ± 0.12	4.2

[a] Values are expressed in ppm of THPI. All data are the means of 6 separate samples.

Table 6. Interassay variability of IC_{50}

	THPI, ng/mL, Mean ± SD[a]	CV, %	*N*
May	14.21 ± 4.79	33.75	22
June	16.15 ± 4.40	27.22	17
July	16.11 ± 5.36	33.24	26
Overall	15.48 ± 5.01	32.39	65

[a] Values are expressed in ng/mL of THPI derived from 8 points standard curve from each plate.

was demonstrated by the variability of the IC_{50} derived from standard curves obtained over a 3-month period. The coefficient of variation was 32% ($n = 65$). The minimum detection limit was 1 ng/mL, with at least 10% binding inhibition; the linearity of the assay was 1–200 ng/mL. Taken together, the validation data for this ELISA procedure appear suitable for routine surveillance or compliance monitoring of captan and THPI in fruits. Because captafol yields THPI, it would be determined as well. The precursor of THPI can be identified by GC/MS, if required. Although this study was restricted to fruit commodities, the method should be adaptable to other matrixes, such as those encountered in human exposure monitoring.

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PESTICIDE FORMULATIONS

Liquid Chromatographic Assay of Bentazon Formulations

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A reversed-phase liquid chromatographic method is presented for determining the active agent in technical bentazon and in aqueous formulations of the sodium salt of bentazon. The procedure is an isocratic, external standard method specifying an octadecylsilyl column, an acetate-buffered mobile phase of methanol–water, and UV detection. The 340 nm detection wavelength eliminates interference from formulation impurities.

Bentazon, 3-(1-methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide, CAS 25057-89-0, is the active agent of certain commercial herbicides. Bentazon is used worldwide for the selective postemergence control of broad-leaf weeds in many crops, especially soybeans and peanuts.

Because bentazon is an organic acid with a phenyl ring, reversed-phase liquid chromatography (LC) with UV detection is the most common method of determination. Bentazon can be determined in the presence of other herbicides by a variety of reversed-phase columns and mobile phases that are often buffered to mildly acidic pH (1–7). Bentazon may be concentrated from water by extraction of the acid into ethyl acetate (1, 2) or by solid-phase extraction (SPE) (4–8). Reversed-phase LC with methanol–water, pH 2.70, was used for determination of residues of cyanazine, bentazon, and bentazon metabolites (9). Schuessler reported that a similar system, with acetone–1.25% acetic acid (20 + 80) as mobile phase, was effective for determining low levels of bentazon in the presence of phenoxy herbicides (10). He showed that UV absorbance, fluorescence, or electrochemical detection could be used; electrochemical detection gave the highest sensitivity.

Bentazon can also be determined by gas chromatography (GC), if a volatile derivative is first prepared. GC is preferred for residue work because of the sensitivity that can be attained with a nitrogen- or sulfur-selective detector (11–14). The derivative may be prepared with diazomethane, pentafluorobenzyl bromide, or *m*-(trifluoromethyl)benzyl chloride (13). Formation of the pentafluorobenzyl derivative gives the greatest sensitivity with electron capture detection.

An enzyme-linked immunosorbent assay (ELISA) was developed for determination of the *N*-methyl derivative of bentazon. For trace analysis, the bentazon is concentrated by SPE, followed by formation of the methyl derivative and determination by ELISA (8).

Unfortunately, none of the procedures listed above is directly applicable to analysis of the concentrated formulation but instead measure bentazon residue levels in water and crops. Bentazon absorbs strongly in the UV region (Figure 1). The absorption obeys the Beer-Lambert law, but UV absorbance is not suitable for analysis of commercial formulations because of the presence of impurities with similar absorbance spectra. During synthesis, bentazon formulations are contaminated with salts of organic acids, which also have aromatic character.

Bentazon can be converted to the acid form by treatment with a cation exchange resin in the H^+ form. It may then be quantitatively determined by nonaqueous potentiometric titration with, for example, tetrabutylammonium hydroxide. However, ion-exchange treatment not only converts bentazon congeners to titratable acids but also converts inorganic salts such as sodium chloride to the corresponding acids. A superior method of forming the acid is direct acidification and extraction into methylene chloride or chloroform. However, certain impurities are coextracted and give high results.

The most useful method for bentazon assay was found to be LC (15). Although most published procedures were designed to separate bentazon from many other herbicides, an assay method must be optimized to separate bentazon from its by-products and decomposition products. The following method is designed for the analysis of herbicide formulations containing 20–100% bentazon.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Precision pumps, gradient elution controller, autosampler, and variable-wavelength UV absorbance detector (Waters Chromatography Division, Millipore Corp., Milford, MA) and chromatographic data system (Hewlett-Packard, Palo Alto, CA, and Perkin-Elmer/Nelson, Cupertino, CA).

(b) *LC column.*—Steel, μBondapak C_{18}, 300 × 3.9 mm (Waters) and Radial-Pak (Waters).

(c) *Eluant.*—0.075M sodium acetate–methanol (60 + 40), prepared by dissolving 10 g reagent grade or LC grade sodium acetate trihydrate in 1 L reagent water, adjusting pH to 6.0 by dropwise addition of reagent grade glacial acetic acid, and add-

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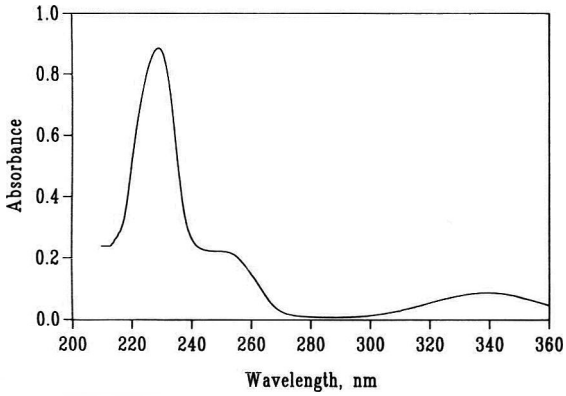

Figure 1. UV absorbance spectrum of aqueous bentazon, sodium salt.

ing reagent grade methanol. LC eluant was filtered and degassed before use.

(**d**) *Bentazon standard solutions.*—Prepared by weighing 60–70 mg bentazon standard (BASF Corporation) into 100 mL volumetric flask, dissolving in 4 mL methanol, adding 6 mL sodium acetate buffer solution, and diluting to volume with LC eluant.

Sample Preparation

Solid 60–70 mg samples of water-insoluble bentazon, acid form, were weighed into 100 mL volumetric flasks and dissolved in 4 mL methanol; 6 mL sodium acetate buffer solution was added to each flask, and the samples were diluted to volume with LC eluant. Liquid samples of aqueous formulations of bentazon sodium salt (60–70 mg bentazon) were weighed into 100 mL volumetric flasks and diluted to volume with LC eluant.

Determination

Various LC conditions were evaluated to optimize separation. Final conditions for assay were as follows: ambient temperature; flow rate, 1.0 mL/min; UV detector, 340 nm, 1 AUFS; chart speed, 1 cm/min; bentazon retention time, ca 5 min. Typical chromatograms are shown in Figures 2 and 3.

Results and Discussion

The method was developed by first working out a gradient elution procedure to resolve bentazon from impurities in the formulation. A reversed-phase method was used in which the methanol concentration was increased from about 10% to about 90%, with the balance being aqueous sodium acetate buffer solution, pH 6. When conditions were established under which bentazon was resolved from all impurities, trial and error was used to find an isocratic system that separated bentazon from the impurities. Accuracy was confirmed when the same assay value was returned by the isocratic and the gradient elution methods. This result showed that unresolved impurities do not contribute to a high result for bentazon.

Experiments in BASF laboratories showed excellent linearity for solution concentrations in the 100–1000 ppm range,

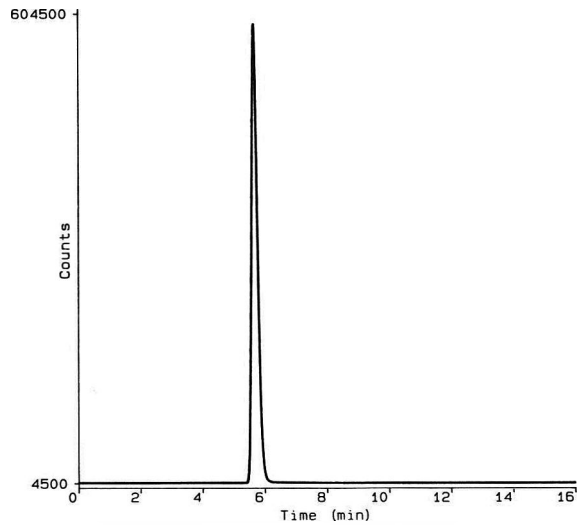

Figure 2. Chromatogram of bentazon standard.

with a correlation coefficient of 0.9998. The quantities specified in the recommended procedure will give solution concentrations in the 600–700 ppm range. However, the linear range is dependent, to some extent, on the particular detector. During the collaborative study reported in the companion paper, several laboratories noted that they obtained better linearity at lower concentrations, i.e., in the 200–300 ppm range (16).

Figure 1 shows the UV absorbance curve for the salt of bentazon in aqueous solution. We chose absorbance at 340 nm for detection, because the impurities associated with technical bentazon have little absorbance in this region, and simple fixed-wavelength detectors can generally operate at 340 nm if fitted with the proper accessories.

In the past, we used sodium saccharin as an internal standard for the determination of bentazon. It is inexpensive, chemically similar to the analyte, available in excellent purity, and elutes near to bentazon but is well-resolved. Because the assay

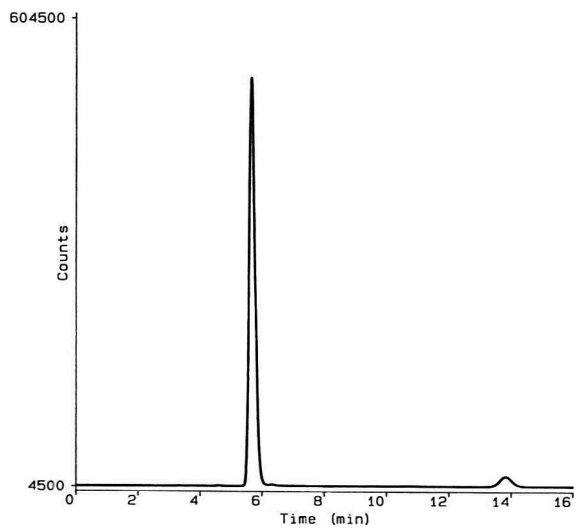

Figure 3. Chromatogram of formulated bentazon containing impurities.

method involves no separation of bentazon from the matrix, the only advantage of using an internal standard is that injections of sample and standard solutions need not be precise (17). We found that modern autosamplers and fixed-loop manual injectors provided very good precision for these aqueous solutions. The use of an internal standard actually had a deleterious effect on the precision of the method because of the uncertainties in determining the area of 2 peaks instead of a single peak. Therefore, we based the assay method on external standardization rather than internal standardization.

We investigated whether a method could be developed that would allow determination of bentazon in the presence of other herbicides that might be present in bentazon formulations. Worldwide, the compounds most frequently encountered in mixtures with bentazon are acifluorfen, atrazine, and phenoxycarboxylic acids such as 2,4-D. The presence of such compounds usually does not interfere with the evaluation of the bentazon peak in a single chromatographic run but produces late-eluting peaks that interfere in subsequent runs unless impractically long intervals are set between injections. Changes in solvent composition and lowering of the mobile phase pH are required to shorten analysis time and permit quantitation of all components of the mixture. No single set of conditions is suitable for analysis of all commercial mixtures. Therefore, we limited the scope of this method to the analysis of the most common formulations, which contain only bentazon as active agent.

No special safety considerations apply to this method. Bentazon itself has low toxicity, and the reagents and apparatus are common in industrial and government laboratories. The ruggedness of the method was demonstrated over a period of 12 years in BASF laboratories. The validation data developed in the AOAC collaborative study are the most relevant and are described in another paper (16).

The LC methodology is applicable to determination of part-per-million levels of bentazon if the matrix is very clean. We found that positive interference is a severe problem if either isocratic or gradient elution LC is applied to detection of bentazon in environmental samples. Other methods are preferred, as discussed at the beginning of this paper.

Acknowledgments

This paper could not have been written without the contributions over a period of 15 years by my colleagues at BASF, particularly P. Schmidt, E. Winkler, M. Liesner, G. Whitney, and R. Ziegler (now with Millipore/Waters).

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VITAMINS AND OTHER NUTRIENTS

Liquid Chromatographic Analysis of Niacin in Fortified Food Products

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An ion exchange liquid chromatographic (LC) method using an anion exchange resin column was developed for the determination of niacin in fortified foods. Samples were extracted by autoclaving with H_2SO_4 (1 + 1). Florisil open column chromatography was used to remove interferences from the sample extracts. Niacin levels were quantitated by an LC system using a 250 × 4.1 mm Hamilton PRP-X100 column, a mobile phase of 2% glacial acetic acid in water, and UV detection at 254 nm. The limit of detection was 0.11 μg niacin/mL, and the standard curve was linear from 0.24 to 0.80 μg niacin/mL. The system reproducibility was evaluated by completing 10 repetitive analyses on an infant formula and a macaroni product, which gave an average CV of 2.7%. Mean recovery (± standard deviation) was 99.8 ± 7.7 (n = 15). The results compared favorably with those by the AOAC microbiological method.

Current AOAC methods for the determination of niacin (nicotinic acid) use chemical and microbiological procedures (1). The AOAC chemical method uses cyanogen bromide to yield a colored pyridinium derivative that can be quantitated spectrophotometrically. This reaction is nonspecific and occurs with all substituted pyridines, including bound niacin (2). Furthermore, cyanogen bromide is similar in human toxicity to hydrogen cyanide, tending to make the assay unfavorable among analysts. Organisms used in the microbiological assay, such as *Lactobacillus plantarum*, may give erroneous results because of growth-stimulating or growth-depressing materials (3). As a result, both chemical and microbiological assays may yield higher niacin values than methods providing greater specificity.

Over the past several years, much work has been done in developing liquid chromatographic (LC) methods for the determination of niacin in foods. Poor resolution due to interferences has been a major difficulty, leading to development of extensive sample cleanup and complex chromatographic systems. VanNiekerk et al. (4) developed a method for foods which used a column-switching technique. The extract was initially injected onto a Nucleosil 5 C_{18} reversed-phase column. At the projected time when niacin elutes, the effluent was diverted via a 6-port valve to a Nucleosil 5 SB anion exchange column. Although no chromatograms of acid extracts were illustrated, the niacin peak from a basic extract was resolved in 40 min.

Hirayama and Maruyama (5) used a basic extraction for vinegars and jams. The extracts were subjected to an anion exchange column, followed by a cation cartridge column before being injected onto a Asahipak NH2P-50 column for analysis. The extracts needed to be evaporated with a rotary evaporator before each column, making the method fairly cumbersome. The niacin peak was well resolved and eluted in about 16 min; however, the baseline shifted to about 60% of the highest peak at 5 min and still did not returned to its initial point at 25 min.

Dawson et al. (6) developed an LC method to assay riboflavin, niacin, and thiamine in meats. Autoclaved acid extracts were digested with 5% takadiastase and 10% papain before LC analysis. A phosphate mobile phase was used in conjunction with an Alltech C_{10} 10 μm column. Although the niacin peak eluted in less than 10 min, the peak was an unresolved shoulder on a larger interference peak.

Vidal-Valverde and Reche (2) used a Dowex 1-X8 ion exchange resin to clean up the acid-digested niacin extracts from legume and meat samples before injecting them on a reversed-phase LC system. The LC system used a mobile phase of tetrabutylammonium bromide, methanol, and a sodium acetate buffer with a Waters μBondapak C_{18} column. Although good separation of niacin from the food extracts was achieved, the column life was very short. Attempts to use the system in our laboratory confirmed the short column life.

Earlier work by Chase and Soliman (7) resulted in the simultaneous analysis of thiamine, riboflavin, pyridoxine, and niacin in multivitamin premixes and supplements. However, niacin could be assayed only in samples in which ascorbic acid was not present, because vitamin C and niacin coeluted.

Recently, Chase et al. (8) developed a procedure to simultaneously assay thiamine, riboflavin, and pyridoxine in infant formulas. Niacin, however, could not be determined by this method, because the cold perchloric acid digestion failed to

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extract the vitamin. The objective of this study was to develop a fast, reliable LC method with minimum interferences for niacin and suitable for the analysis of a variety of food products.

METHOD

Reagents

(a) *Florisil.*—Pesticide grade (Floridin, Co., Pittsburgh, PA 15235), activated 12 h at 130°C.

(b) *Methanol.*—Suitable for LC (Baxter Healthcare Corp., Muskegon, MI 49442).

(c) *Stock standard solution.*—Niacin solution, 20.0 μg/mL, was prepared by dissolving USP Reference Standard Nicotinic Acid (U.S. Pharmacopeial Convention, Rockville, MD 20852) in 0.5N H_2SO_4. The stock standard was stable for several months under refrigeration.

(d) *Mobile phase.*—Glacial acetic acid, 20 mL, was diluted to 1.0 L with water and filtered through a 0.45 μm nylon filter (Universal Scientific, Inc., Atlanta, GA 30360).

Apparatus

(a) *Liquid chromatograph.*—Model 625 LC solvent delivery system, Model 440 detector, and Model 712 WISP auto injector or U6K injector for manual injection (Waters Associates, Milford, MA 01757).

(b) *Integrator.*—Model 3392A, or equivalent (Hewlett-Packard, Atlanta, GA 30339).

(c) *LC column.*—PRP-X100 stainless steel, 250 × 4.1 mm, No. 79433 (Hamilton Co., Reno, NV 89520) with in-line precolumn filter, No. 84560 (Waters Associates).

(d) *Glass columns.*—10.5 × 300 mm, 30 mL capacity, No. 420550–0213 (Kontes, Vineland, NJ 08360), to prepare Florisil open columns.

Chromatographic Conditions

(a) *Preparation of Florisil column.*—Add 4 g Florisil to column and top with small pledget of glass wool. Prewash column with 30 mL methanol, followed by two 15 mL portions of 0.5N H_2SO_4. Do not allow liquid level to fall below glass wool pledget at any point.

(b) *Instrument parameters.*—Injection volume, 100 μL WISP auto injector, or equivalent (Waters Associates); flow rate, 1.5 mL/min; wavelength, 254 nm.

Sample Description and Preparation

Several products representing a variety of food matrixes were evaluated. Infant formula samples consisted of 2 powders and 3 concentrates. The formulas represented formulation bases of soy, whey/milk, and milk/whey. In addition, bread; beef soup; tuna; egg noodles; pasta; and fortified wheat, corn, oat, and rice cereals were included in the study.

The infant formula composites were prepared under subdued light according to AOAC (9) instructions for proper warming, opening, mixing, and storage under nitrogen atmosphere and refrigeration. The bread composite was prepared after slices were air-dried, ground, and passed through a 1 mm sieve. The egg noodles, pasta, and cereal composites were ground and passed through a 1 mm sieve. The beef soup and tuna composites were blended and homogenized in a food processor.

Sample Extraction

Accurately weigh sample analytical portion to contain 100 μg niacin, based on the declared amount, into a 500 mL Philips beaker. Add ca 50 mL water to each sample, followed by 6.0 mL H_2SO_4 (1 + 1), and mix well to break any clumps. Autoclave sample at 121–123°C for 45 min and let extract come to room temperature.

Using magnetic stirrer, adjust extract to pH 6.0–6.5 with 7.5N NaOH; then immediately adjust to pH 4.5 with H_2SO_4 (1 + 1). Dilute extract to 100.0 mL with water, and filter through Whatman No. 40 paper (Whatman, Ltd., U.K.). Adjust 20.0 mL aliquot of filtrate to pH 0.5–1.0 with H_2SO_4 (1 + 1), and transfer to Florisil column. Wash column with two 15 mL portions of 0.5N H_2SO_4, and discard effluent. Elute niacin with 25 mL 0.5N NaOH into 50 mL volumetric flask containing 1.0 mL glacial acetic acid, and dilute to volume with water. Filter sample through 0.45 μm nylon filter (Rainin Instrument Co., Woburn, MA 01801) before injection.

Recovery Studies

Run sample recovery by spiking sample with standard at 200% of the declared niacin value. Use same sample weight for recovery as for sample analysis. From this point, treat recovery same as sample, beginning with "add ca 50 mL water..." except dilute extract to 200.0 mL with water and filter.

Standard Extraction

Prepare working niacin standard by combining 10.0 mL stock standard with 40 mL water and 6.0 mL H_2SO_4 (1 + 1). From this point, treat standard same as sample, beginning with "autoclave sample at..." except prepare standard curve by diluting 3.0, 6.0, 8.0, and 10.0 mL final filtered solution to 10.0 mL with mobile phase to give final concentrations of 0.24, 0.48, 0.64, and 0.80 μg niacin/mL, respectively.

AOAC Method

The AOAC microbiological method (10), based on growth of *Lactobacillus plantarum* ATCC 8014, was used. DIFCO niacin assay media (DIFCO, Detroit, MI 48232) were used throughout the assay.

Results and Discussion

The Florisil cleanup step is a modified version of a similar procedure using Lloyds reagent or hydrated aluminum silicate (11). This method requires several cumbersome centrifugation steps. It was proposed that an open chromatographic column could accomplish the same purpose; however, the particle size of the hydrated aluminum silicate was 0.1–4 μm, which made it virtually impossible to filter. For this reason, magnesium silicate or Florisil was used. The effect of the Florisil column was illustrated by injecting sample extracts into the LC column with

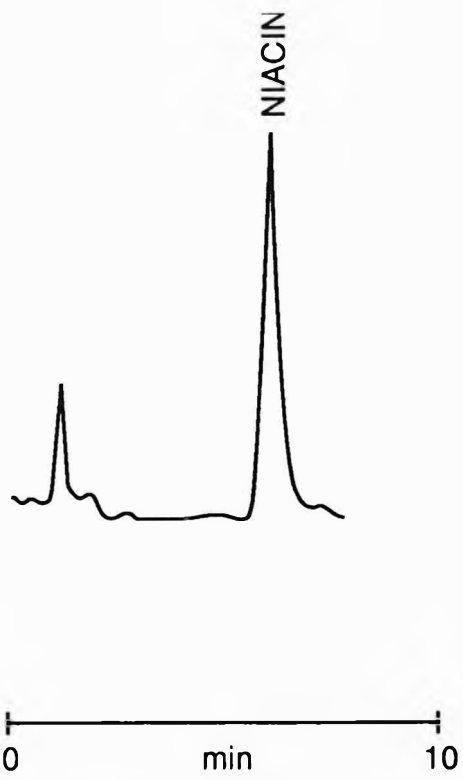

Figure 1. Chromatogram of a niacin standard solution (1.0 μg/mL) by UV detection (254 nm), using a Hamilton PRP-X100 column, 100 μL injection volume, and flow rate of 1.5 mL/min.

and without previous cleanup on the Florisil column. LC chromatograms of sample extracts that were subjected to the Florisil column yielded clean, interference-free niacin peaks, compared with the several interfering peaks from extracts without previous cleanup. To verify the purity of the niacin peak, a diode array detector was used. The UV spectrum of the standard niacin peak coincided with the UV spectrum of the niacin peak of a sample extract.

The efficiency of the Florisil column was studied by increasing the volume of the extract applied to the column and by increasing the concentration of niacin in the 20 mL aliquot of extract. Column retention capacity was evaluated by individually applying 10, 20, 30, 40, 50, and 60 mL portions of a whey/milk-based infant formula extract to each of 6 Florisil columns. In addition, the concentration of niacin in five 20 mL portions of extract was varied between 35 and 92 μg by individually spiking each portion. In both situations, as the niacin concentration on the Florisil column approached 60 μg, the deterioration of column efficiency resulted in appreciable losses of niacin. To avoid overloading the Florisil column, no more than 40 μg niacin should be applied. However, increasing Florisil quantities above 4 g will reduce flow rate and increase analysis time.

This method is capable of yielding interference-free, baseline-resolved chromatograms of niacin in <10 min for the standard and sample extracts, as illustrated in Figures 1 and 2, respectively. Each of the foods studied required at least 15 min to

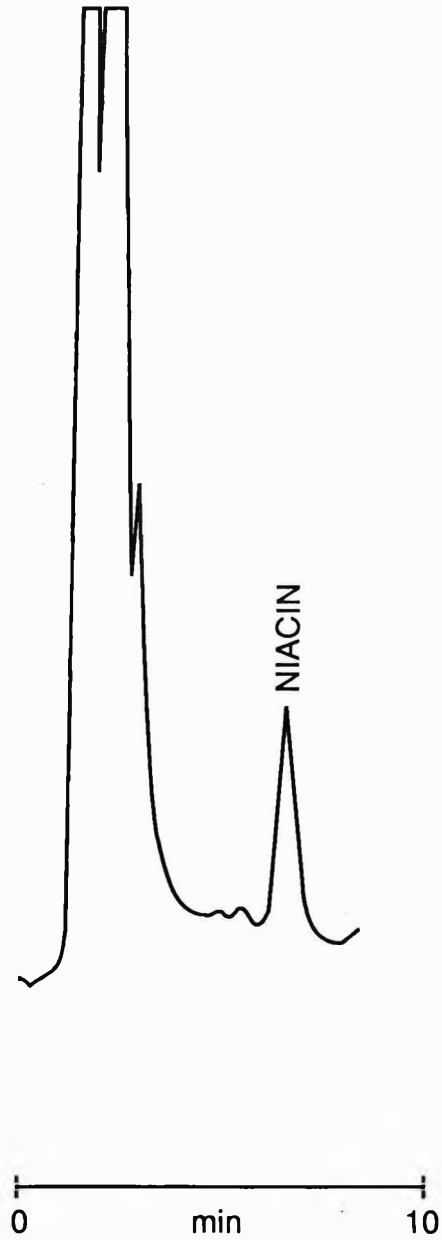

Figure 2. Chromatogram of niacin in a milk-based infant formula. (Conditions similar to those for Figure 1.)

elute all peaks and return to baseline before the next injection, except for the soy-based infant formulas and beef soup samples, which required 60 min to allow for late-eluting peaks. The time of 60 min was, however, reduced to 35 min by increasing the flow to 2.5 mL/min for 20 min immediately after the niacin peak eluted. The flow rate was then reset to 1.5 mL/min, and the system was allowed to equilibrate for 3 min before the next injection.

The LC standard curve response was linear from 0.24 to 0.80 μg niacin/mL, with a detection limit of 0.11 μg niacin/mL. The system reproducibility was evaluated by completing 10 repetitive analyses of a whey/milk-based infant formula and a macaroni product (labeled at 66.1 and 70.5 μg/g, respectively). Mean niacin values were 71.5 ± 1.7 μg/g (CV, 2.4%) for the formula and 126 ± 3.6 μg/g (CV, 2.9%) for the pasta.

Table 1. Comparison of results for niacin by LC and AOAC microbiological methods

Sample	Declared, µg/g	Niacin, µg/g	
		LC[a]	AOAC[b]
Bread	52.9	55.6 ± 2.1	61.2 ± 1.6
Spaghetti	70.5	126 ± 3.6	117 ± 0
Egg noodles	52.9	69.3 ± 0	75.5 ± 1.3
Macaroni	52.9	95.4 ± 3.7	111 ± 1.4
Corn cereal	176	48.6 ± 0.8	42.5 ± 1.3
Wheat cereal	176	229 ± 7.8	252 ± 0.7
Oat cereal	176	216 ± 3.5	234 ± 2.1
Rice cereal	176	203 ± 2.8	224 ± 2.8
Infant formula (soy concentrate)	17.3	21.3 ± 1.6	23.1 ± 1.1
Infant formula (whey/milk concentrate)	15.6	17.1 ± 0.9	18.6 ± 0.4
Infant formula (milk/whey concentrate)	9.64	11.5 ± 0.1	12.5 ± 0.2
Infant formula (soy powder)	35.4	38.4 ± 1.6	58.9 ± 2.8
Infant formula (whey/milk powder)	66.1	71.5 ± 1.7	82.4 ± 0.6
Beef soup	14.9	15.1 ± 2.3	15.1 ± 0.2
Tuna	123	146 ± 7.1	125 ± 0.7

[a] LC data are the mean ± SD of triplicate injections for duplicate extractions except for spaghetti and whey/milk formula samples, which were analyzed 10 times.
[b] AOAC data are the mean ± SD of duplicate determinations.

Table 1 presents the declared amounts of niacin and the values obtained by the LC and AOAC microbiological methods (10). The declared values are expressed as % USRDA/serving and, for infant formula concentrates, as µg niacin/5 fluid ounces of ready-to-feed preparation. For this study, the declared units were converted to µg niacin/g. Except for low levels of niacin found in corn cereal, all the other LC and AOAC assay values exceeded label claims. Overall, the microbiological assay results averaged 11% higher than the LC results. A linear regression analysis comparing the LC and AOAC methods, where $y = mx + b$, gave a correlation coefficient (r) of 0.990. Recovery studies were determined by spiking each sample at 200% of the declared value. To avoid overloading the Florisil column, however, the extract was diluted to 200.0 mL instead of 100.0 mL, as the method requires. The mean percent recovery for LC was 99.8 ± 7.7 ($n = 15$).

In addition to fortified products, several foods containing naturally occurring low levels of niacin were evaluated. Table 1 shows the results for 2 of these samples, namely, beef soup and tuna. Beef soup gave the same results by both LC and microbiological methods, whereas the LC result for tuna was 16.8% higher. Other foods containing naturally occurring niacin that were evaluated were canned corn and frozen lima beans. When these foods were extracted as described in this study, the niacin levels in the final dilution approached the detection limit of 0.11 µg niacin/mL. Because of sample matrix interferences at these low levels, niacin could not be accurately quantitated by the LC method.

Conclusion

This method provides an alternative to the AOAC chemical method based on cyanogen bromide and the AOAC microbiological assay for niacin in a variety of fortified food products. The method could not be used on some low-potency, unfortified products because of chromatographic interferences present in undiluted sample extracts. LC results showed very good agreement with those by the AOAC microbiological method.

Acknowledgments

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VITAMINS AND OTHER NUTRIENTS

Niacin I: Dissolution Profiles of Sustained-Release Niacin Products by Automated and Manual Procedures

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Automated and manual procedures were developed to obtain dissolution profiles of sustained-release niacin formulations. The procedures are based on the United States Pharmacopeia XXII apparatus 1 (basket) at 100 rpm with 900 mL 0.1N HCl as the dissolution medium. Filtered aliquots are read at 260 nm. No interference was found from excipients. The procedures are straightforward and will discriminate between sustained-release and regular niacin formulations.

Niacin is reported to be useful in lowering blood cholesterol levels (1) and reducing cardiovascular risk (2). Niacin is recommended as a first-choice drug for hypercholesterolemia (3). Frequent cutaneous and gastrointestinal side effects have restricted its use (3, 4). Niacin was formulated in sustained-release products in an attempt to lessen side effects (5–9). Bioavailability studies were done on these formulations, but some side effects were still reported (5, 7).

The U.S. Food and Drug Administration (FDA) became aware that sustained-release niacin formulations administered for cholesterol control to patients under a physician's care may cause liver problems (private communication). A sample of such niacin tablets was submitted to our laboratory for examination. Because no dissolution test is available for these products and United States Pharmacopeia (USP) XXII has only a disintegration test for niacin tablets, a test to examine the dissolution profiles of niacin products was sought. It was of some concern that niacin formulated as sustained-release medication may in fact be acting as regular niacin formulations because of inadequate controls.

The purpose of this work was to design an in-vitro dissolution test that would discriminate between regular and sustained-release formulations, that may correlate with bioavailability data, and that may help in pinpointing the problem with the sample of interest.

Limited dissolution data are available for regular niacin formulations (10, 11). No comparisons of dissolution data for different marketed products of either regular or sustained-release niacin formulations are available in the literature. A bioavailability-dissolution study was done on isonicotinic acid hydrazide tablets (12), with good correlation.

Several sustained-release and regular niacin products were examined. USP XXII apparatus 1 (basket) and apparatus 2 (paddle) were both evaluated with 2 dissolution media, water and 0.1N HCl. No work with buffers was done, because Ogata et al. (12) reported correlation of a related product in acid medium. In addition, we observed that the absorbance obtained for niacin at 260 nm in pH 6.8 buffer was only about half that obtained in acid. The final procedure presented here uses 900 mL 0.1N HCl with USP XXII apparatus 1 (basket) at 100 rpm. Filtered and/or diluted aliquots were read at 260 nm. Excipients did not interfere.

The dissolution profiles of the sustained-release formulations were all very similar, except for one product that apparently is no longer on the market. Sustained-release formulations were easily distinguished from regular niacin formulations. The method could be used as a regulatory procedure. Average dissolution values of sustained-release formulations were about 25% at 1 h and 60% at 4 h. Regular niacin formulations had values of about 80–100% at 1 h. The dissolution profile from the sample of clinical interest was similar to those of the other sustained-release formulations.

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Experimental

Automated System

Apparatus and Reagents

(a) *Automated dissolution system.*—Hewlett-Packard 8450A diode array UV-vis spectrophotometer, Hewlett-Packard 85 personal computer, dissolution analysis software for sampling at 15 min intervals, 0.1 cm flowcell, 8-port automatic switching valve (VICI, Velco Instrument Co., Houston, TX), and proportioning pump I (Technicon Corp., Tarrytown, NY); sample probe and filter previously described (13).

(b) *Dissolution unit.*—6-Spindle, with baskets rotated at 100 rpm (Hanson 381, Hanson Research Corp., Northridge, CA), 1 L round-bottom resin kettles, and 900 mL dissolution medium at 37°C. Apparatus complied with previously described guidelines (13).

(c) *Dissolution medium and diluting solvent.*—0.1N HCl.

Standard Preparation

For dissolution tests of 500 mg dosage units: 55 mg niacin, of known purity and accurately weighed, was dissolved with 0.1N HCl in a 100 mL volumetric flask. Concentration of standard was adjusted for lower or higher dosages.

Sample Preparation

A single 500 mg dosage unit was placed in each of the 6 baskets of the dissolution apparatus.

Determination

The dissolution apparatus was connected to the automated sampling system. Separate probes were placed in standard, blank (0.1N HCl), and 6 dissolution kettles. Software controlled the sampling sequence and the start of each basket at 1 min intervals. Sampling time was every 15 min for 4 h. Absorbances of the aliquots were measured at 260 nm and compared with the stored standard. Results were calculated, corrected for amounts of dissolution medium withdrawn, and printed.

Manual System

Apparatus and Reagents

(a) *Spectrophotometer.*—Hewlett-Packard 8450A diode array UV-vis with 1 cm cells.

(b) *Dissolution unit.*—6-Spindle, with baskets rotated at 100 rpm (Hanson 381), 1 L round-bottom resin kettles, and 900 mL dissolution medium at 37°C. Apparatus complied with previously described guidelines (13).

(c) *Syringes.*—5 mL glass with Leur-Lock fitting (Scientific Products, McGaw Park, IL). For aliquot removal, each syringe was fitted with either a long 16-gauge stainless steel cannula or a glass pipet tip.

(d) *Filter disks.*—0.45 μm membrane filter, 13 mm, held in a Swinnex adapter (Millipore Corp, Milford, CT).

(e) *Dissolution medium.*—0.1N HCl.

Standard Preparation

A standard solution of niacin (0.02 mg/mL in 0.1N HCl) was accurately prepared.

Sample Preparation

A single dosage unit was placed in each of the 6 baskets of the dissolution apparatus. The test was started at basket 1, and the other baskets were started at 1 min intervals.

Determination

At 1 and 2 h, a 2.5 mL aliquot was withdrawn from the appropriate kettle by using the syringe-cannula arrangement. At the end of the test (4 h), a 5 mL aliquot was withdrawn. Dissolution medium was not replaced. Each aliquot was filtered through a 0.45 μm membrane filter. The first few drops (ca 0.5 mL) were discarded, and the rest was collected. For the 1–2 h samples, 1.0 mL filtrate was diluted with 0.1N HCl to 10.0 mL in a volumetric flask. For the 4 h samples, 3.0 mL fil-

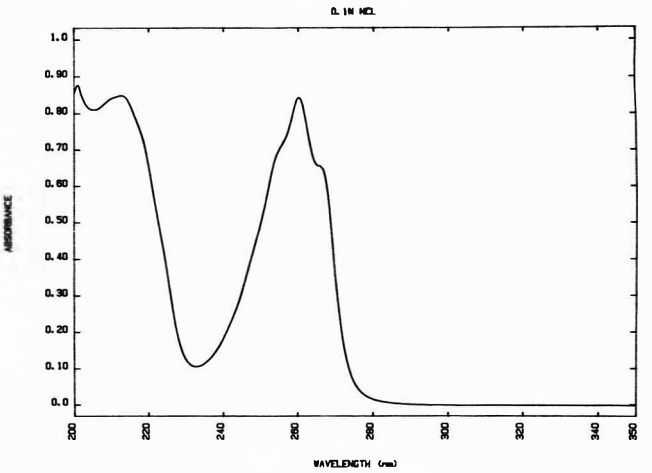

Figure 1. UV spectrum of 0.022 mg/mL niacin in 0.1N HCl.

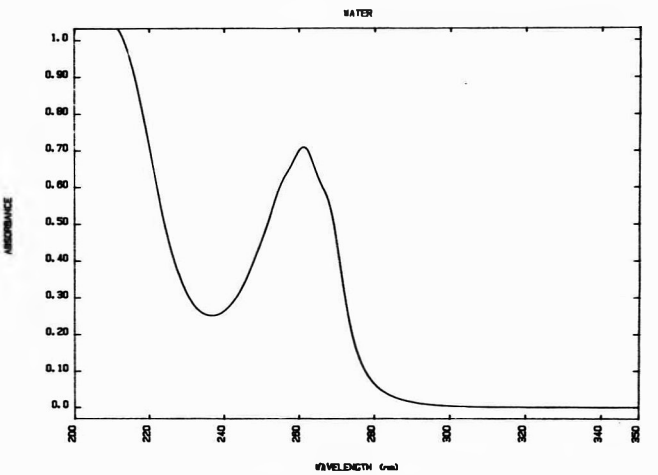

Figure 2. UV spectrum of 0.022 mg/mL niacin in water.

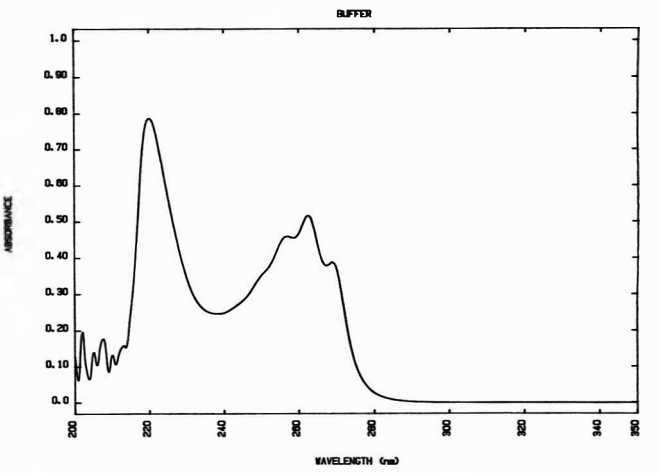

Figure 3. UV spectrum of 0.22 mg/mL niacin in pH 6.8 buffer.

Table 1. Comparison of dissolution results[a] (% of declared) for regular and sustained-release niacin tablets in 2 media and 2 apparatuses

	Regular 100 mg tablets[b]				Sustained-release 500 mg tablets[c]			
	Water		0.1N HCl		Water		0.1N HCl	
Time, min	App. 1	App. 2	App. 1	App. 2	App. 1	App. 2	App. 1	App. 2
15	23.1	24.7	32.2	35.6	5.3	6.3	10.0	9.8
30	39.0	40.0	50.1	55.5	8.3	10.0	15.4	15.5
45	52.9	53.8	64.6	70.2	10.8	13.0	19.8	20.0
60	65.8[d]	63.9[e]	76.6[f]	80.2[g]	13.1	15.8	23.8	24.0
75	77.8	71.8	85.2	87.2	15.3	18.3	27.2	27.7
90	85.8	78.8	90.9	92.0	17.3	20.8	30.5	31.0
105	89.9	84.2	94.2	96.7	19.2	23.1	33.6	34.1
120	92.5	88.3	96.4	99.7	21.0	25.4	36.5	37.0
135	94.3	91.4	97.7	100.3	22.8	27.5	39.2	39.9
150	95.6	93.6	98.8	100.6	24.5	29.7	41.9	42.5
165	96.7	95.7	99.6	100.6	26.2	31.8	44.9	45.1
180	97.5	97.7	100.2	100.8	27.9	33.8	46.9	47.5
195	98.2	99.5	100.7	100.9	29.5	35.7	49.3	49.8
210	98.9	100.1	101.0	101.1	31.1	37.6	51.6	52.2
225	99.5	100.4	101.0	101.2	32.6	39.5	53.8	54.3
240	100.0	100.5	101.3	101.4	34.1[h]	41.4[i]	55.9[j]	56.4[k]
360	—	—	—	—	—	—	66.1	—
480	—	—	—	—	—	—	77.4	—

[a] Average of 6 tablets.
[b] Walgreens brand.
[c] Slo-Niacin brand.
[d] Standard deviation is 15.9 at 1 h.
[e] Standard deviation is 18.3 at 1 h.
[f] Standard deviation is 9.4 at 1 h.
[g] Standard deviation is 17.8 at 1 h.
[h] Standard deviation is 0.3 at 4 h.
[i] Standard deviation is 0.3 at 4 h.
[j] Standard deviation is 0.9 at 4 h.
[k] Standard deviation is 0.4 at 4 h.

trate was diluted to 50.0 mL. Absorbances of the sample and standard solutions were concomitantly measured from 200 to 350 nm at a concentration of ca 0.02 mg/mL. The amount of niacin dissolved from each tablet was calculated from the maximum absorbance at 260 nm.

Results and Discussion

To set adequate limits of acceptance, the objectives of the dissolution test were to obtain a measurable concentration of niacin in the medium in a reasonable time and still be able to discriminate among formulations. Additionally, niacin must be stable and have a useable, interference-free UV spectrum in the medium selected. The procedure presented meets these criteria.

As part of the method development, we measured the UV absorbance spectrum of niacin in water, 0.1N HCl, and pH 6.8 phosphate buffer, because the dissolution test would probably use one of these media as a solvent. The wavelength of maximum absorbance of niacin did not differ much in the 3 solvents: 260, 261, and 262 nm in 0.1N HCl, water, and buffer, respectively. Absorbance at the maximum wavelength varied greatly: 0.489, 0.661, and 0.802 at a niacin concentration of 0.022 mg/mL in buffer, water, and acid, respectively. The shoulders observed at about 259 and 269 nm on either side of the maximum absorbance in the spectrum of niacin in acid and water became pronounced peaks in the buffer (Figures 1–3). Further testing showed niacin is stable for at least 1 month in 0.1N HCl, which historically has been one of the preferred dissolution media. Thus, 0.1N HCl seemed a good first choice. Because niacin is very soluble in water, comparisons were made between water and 0.1N HCl as the dissolution media.

To reduce experimental time, a quick examination of the effect of rotational speeds was undertaken before the final comparisons were made between the basket and paddle procedures. Three rotational speeds, 50, 100, and 125 rpm, were tried with the basket apparatus and 900 mL water. The basket apparatus was chosen, because results obtained by the basket procedure would normally be higher than those obtained by the paddle procedure. Individual tablets from one regular and one sustained-release niacin formulation were examined. At 50 rpm, the results for the sustained-release product were very low; at 125 rpm, the basket speed seemed excessive, and the ability of the test to discriminate between the regular and sustained-release products did not improve over that at 100 rpm. Therefore, the 100 rpm speed was selected.

A sample of 500 mg sustained-release niacin tablets and a sample of 100 mg regular niacin tablets were compared in

Table 2. Dissolution results[a] (% of declared) for regular and sustained-release niacin tablets by automated method

Dosage		Time, h			
Declared, mg	Type[b]	1	2	4	SD[c]
500	SR[d]	24.1	34.6	48.4	3.1
500	SR[e]	26.1	41.7	62.1	2.3
500	Reg[e]	100.4	102.4	103.1	3.8[f]
500	SR[g]	25.5	37.1	52.3	0.8
250	SR[d]	31.8	45.0	61.3	1.5
500	SR[h]	26.0	37.2	52.0	—
500	SR[i]	6.1	8.9	13.6	—

[a] Average of 6 tablets.
[b] SR = sustained release; Reg = regular.
[c] Standard deviation is for results at 4 h.
[d] Nature Made brand.
[e] Optimum brand.
[f] Standard deviation is for results at 1 h.
[g] Family Independent Pharmacy brand.
[h] Sample of clinical interest; average of 3 tablets (13 tablets in sample).
[i] Health for Life (Fibercin) brand; 1 tablet of 3 remaining tested; formed gel in basket.

water and acid with USP XXII apparatuses 1 and 2 at 100 rpm (Table 1). Approximately the same results were obtained using the paddle and basket procedures in 0.1N HCl. However, with apparatus 2, the tablets wobbled on the bottom of the dissolution vessel (buoyancy), which could lead to higher results when using the paddle procedure. This phenomenon was noticed with other formulations. For this reason, the USP XXII apparatus 1 (basket) procedure was chosen.

Dissolution results obtained from the sustained-release formulation in 0.1N HCl were easily measurable, higher than the results in water, and easily distinguished from the results obtained from the regular niacin formulation, which dissolved relatively quickly. The regular niacin formulations tested reached 80–100% dissolution in the first hour. Sustained-release formulations were below 32% in the first hour. Ogata et al. (12) reported correlation between bioavailability and dissolution for isonicotinic acid hydrazide tablets with hydrochloric acid at pH 1 and a rotational speed of 75 rpm.

Several formulations collected by FDA investigators were tested by the USP XXII apparatus 1 (basket) procedure described in this paper (Table 2). To check the procedure for interference, an aliquot of the dissolution medium was taken from each of the formulations after the last sampling time. Solutions were filtered and diluted, and absorbances were measured from 200 to 350 nm. The spectra were compared with that of a standard solution and examined for background excipient interference; none was found. Independent analysis of the formulations by in-house UV and liquid chromatographic procedures confirmed niacin was present within 5% of the labeled amount. In addition, all the sustained-release tablet formulations failed the USP XXII disintegration time requirement of 30 min for regular niacin tablets, as would be expected. Elsbaugh et al. (11) reported that the time required for 90% of the drug to dissolve was longer than the disintegration time. Our dissolution results for the regular niacin tablet formulation confirm this observation.

For the test to be useful in many laboratories and possibly adopted as an official method, a manual version was needed. Results of the comparison between the automated and manual procedures are listed in Table 3. In the manual method, concentrations were not corrected for removed medium, because these corrections were small.

Table 3. Comparison of dissolution results[a] (% of declared) for regular and sustained-release niacin tablets by automated and manual methods

Dosage		Automated				Manual			
		Time, h				Time, h			
Declared, mg	Type[b]	1	2	4	SD[c]	1	2[d]	4[d]	SD[c]
100	Reg[e]	76.6	96.4	101.3	9.4[f]	80.1	101.6	98.8	11.0[f]
250	SR[g]	29.8	43.0	60.0	0.7	30.0	41.4	57.1	1.0
500	SR[g]	28.0	39.9	55.2	0.8	26.9	38.3	63.8	2.2
250	SR[h]	30.0	46.2	55.9	0.4	27.7	41.5	64.2	1.5
500	SR[h]	23.8	36.5	55.9	0.9	22.8	35.5	52.4	0.6

[a] Average of 6 tablets.
[b] Reg = regular; SR = sustained release.
[c] Standard deviation is for results at 4 h.
[d] Uncorrected for removed medium.
[e] Walgreens brand.
[f] Standard deviation is for result at 1 h.
[g] Endur-acin brand.
[h] Slo-Niacin brand.

In conclusion, the dissolution method presented in this paper is simple and free from interference. Differences in sustained-release and regular niacin formulations could easily be determined within 60 min, and the method is suggested for routine testing. No statistical treatments were done to see what minimum dissolution time would be needed for differentiation. From literature indications, results from this procedure should correlate with in vivo results. One formulation swelled in the basket and formed a gel that would not allow migration of the media through the basket and gave low dissolution results. However, no more of this product could be found, and it is apparently no longer on the market.

Acknowledgments

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VITAMINS AND OTHER NUTRIENTS

Analysis of Milk-Based Infant Formula. Phase V. Vitamins A and E, Folic Acid, and Pantothenic Acid: Food and Drug Administration–Infant Formula Council: Collaborative Study

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In 1982, the U.S. Food and Drug Administration, the Infant Formula Council and its member companies, contract laboratories, and other government laboratories began a study of analytical methods for the nutrients listed in the Infant Formula Act of 1980 (P.L. 96–359). Four phases of the study have been completed and are discussed in earlier reports. The present report provides data on Phase V, in which 13 laboratories collaboratively studied individual methods for folic acid, pantothenic acid, and vitamin E, in addition to 2 methods for vitamin A. Vitamins A and E are determined by liquid chromatography. Folic acid and pantothenic acid are determined by microbiological methods using acidimetric and/or turbidimetric assays as the determinative step. In most cases, relative standard deviations for repeatability, RSD_r, and reproducibility, RSD_R, are as good as those that would be predicted from other collaborative studies. RSD_r and RSD_R values obtained for the 5 methods are 9.35 and 25.44% for folic acid, 4.59 and 10.23% for pantothenic acid, 8.46 and 11.69% for vitamin E, 3.62 and 9.72% for vitamin A (retinol isomers), and 4.9 and 10.5% for vitamin A (retinol). The 5 methods have been adopted first action by AOAC International.

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This report was presented at the 105th AOAC Annual International Meeting, Phoenix, AZ, August 12–15, 1991.
The recommendation was approved by the General Referee and the Committee on Foods II and was adopted by the Official Methods Board of AOAC. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, 251–255.

Infant formula is the most highly regulated consumer food product on the market today. In the past, deficient chloride content in one infant formula led to metabolic alkalosis in some children who received only formula for their total nutritional needs. Thus, on September 26, 1980, President Carter signed into law the Infant Formula Act of 1980 (P.L. 96–359) (Act), which placed on the manufacturers the responsibility of producing formula containing a specific minimum amount of nutrients and also charged them with maintaining records to support this action.

The Act specifies minimum and in some cases maximum amounts of listed nutrients, requires notification to the U.S. Department of Health and Human Services, U.S. Food and Drug Administration (HHS-FDA) when a manufacturer determines that an infant formula may be adulterated or misbranded, authorizes HHS-FDA to set requirements for voluntary recalls initiated by firms, and increases the inspection authority of HHS-FDA.

FDA has established a series of quality control procedures for the manufacture of infant formulas. The purpose of these quality control procedures is to ensure that infant formulas contain the necessary nutrients at levels required by the Act.

In April 1982, the FDA Commissioner met with executives of the infant formula industry on the subject of analytical variability in methodologies used by industry laboratories and FDA laboratories. In May and June 1982, members of the FDA Bureau of Foods (now the Center for Food Safety and Applied Nutrition) and members of the infant formula companies, the Infant Formula Council (IFC) staff, and representatives of AOAC and other interested laboratories met to develop a plan to conduct a collaborative study on methods of analysis for nutrients required in milk-based infant formula by the Act. Government laboratories in Canada and the United Kingdom also participated in the study under a tripartite agreement. The purposes of the collaborative study were (*1*) to undertake an

interlaboratory comparison of methods of analysis for nutrients in infant formula and (2) to identify reference methods of analysis for infant formula for inclusion in the AOAC compendium of methods.

Because of the large number of nutrient methodologies to be studied, the collaborative study was divided into different phases. Phase I of the study included determination of vitamin A, Vitamin B_6, calcium, magnesium, potassium, and sodium; Phase II included determination of vitamin C, riboflavin, niacin, manganese, copper, iron, and zinc; and Phase III included chloride, phosphorus, proximate analysis, thiamine, total pantothenates, and vitamins A, B_{12}, and E. Phase IV included iodide, essential fatty acids (linoleate), and vitamins D and K. The present report addresses Phase V, in which methods for folic acid, pantothenic acid, and vitamins A and E were studied.

Collaborative Study

FDA and Infant Formula Council members agreed that a milk-based formula with iron was the most commonly used formula and, therefore, would be the first product matrix studied. A ready-to-feed (RTF) formula was agreed on to minimize potential variability among samples. At the time, Bristol-Myers USPNG, Ross Laboratories, and Wyeth-Ayerst Laboratories manufactured essentially all the RTF milk-based formula in the United States. These companies agreed to produce typical formulas, similar to their usual product, for the collaborative study.

Robert Gelardi of the Infant Formula Council, representatives from the production departments of the 3 companies, and Ken Ewing of the FDA Detroit district office formed a subcommittee to produce and distribute the sample materials such that participating laboratories could not identify their manufacturer. All products were manufactured, and then packaged in unlabeled, identical 32 oz cans. Approximately 1000 cans were produced by each manufacturer. Shortly before Phase V began, sufficient formula from each company was sent to Ewing for coding and distribution to participating laboratories.

For Phase V, the FDA Detroit district office sent each laboratory 6 sets (12 cans per set) of materials. The six 12-can sets included 3 different products, produced by each of 3 manufacturers, plus a duplicate set of the 3 products (blind duplicates). A separate 12-can composite was prepared from each of the 6 sets and was analyzed for the specified nutrients according to the protocol.

Collaborators were instructed to conduct assays on the 3 sets of sample on day 1 and to repeat the assays on the blind duplicates on day 2, unless otherwise noted in the methodology, to obtain the within-laboratory variability. Immediately before compositing the test samples, laboratories were instructed to warm previously shaken individual cans for a sufficient time, not to exceed 2 h at 60°C, to achieve uniformity, while shaking occasionally. Each composite was prepared from 12 cans by transferring equal aliquots from each can under nitrogen and subdued light to a flask prepared as in **968.12B(b)** (1). Composites were thoroughly mixed under nitrogen by stirring gently to avoid foaming or excessive inclusion of air. Portions of the composites were poured into thoroughly cleaned or sterile containers (with air-tight caps), closed securely, identified, and refrigerated at 4°C until assayed.

Sources of Methods

Folic Acid

Folic acid (total folate activity) is determined in accordance with an IFC method (2), which was originally written with reference to a method described by Cooperman (3), and an AOAC method, **39.076(a)** (4). Folate activity is determined by acidimetric or turbidimetric measurement of the growth response of the organism *Lactobacillus casei*. Growth responses of the organism in standard solutions and unknowns are quantitatively compared.

The IFC method was studied and evaluated by Hazleton Laboratories before its selection for collaborative study. Precision as indicated by relative standard deviation, RSD_r, was 6.0% (n = 16); recovery averaged 96.7% (n = 3).

Pantothenic Acid

Pantothenic acid is determined by first subjecting samples to simultaneous attack of alkaline intestinal phosphatase and avian liver peptidase, as described by Zook et al. (5). The intestinal phosphatase removes the triphosphoadenine from coenzyme A, and the avian liver peptidase enzyme breaks the peptide linkage. Bound pantothenic acids released to the free form are then assayed microbiologically as in **945.74** (1) by means of turbidimetric microbial growth response. The method is performed as described except **945.74D(c)** is modified to use 1.0N HCl to adjust the mixture to pH 4.4–4.6.

Vitamin A (Retinol Isomers)

A liquid chromatographic (LC) method for vitamin A isomers, all-*trans*-retinol and 13-*cis*-retinol, was provided by Neville Thompson ("Determination of Vitamin A in Milk and Infant Formula by HPLC," Version 3.01). Tests were performed before the collaborative study to evaluate the method. The method was subsequently published by Thompson and Dural (6).

In the LC method, the sample is digested in ethanolic potassium hydroxide at room temperature, and a portion of the digest is extracted with a mixture of diethyl ether and hexane in a centrifuge tube. Hexadecane is added to the extract to prevent destruction of the vitamin A after evaporation of the extracting solvents. The residue is dissolved in heptane and chromatographed on a silica column to separate and quantitate vitamin A.

Thompson (6) reported that recovery of all-*trans*-retinol had been investigated. The addition of 0.308 μmol of the palmitate to five 100-mL portions of simulated formula resulted in mean recoveries of 0.282 μmol all-*trans*-retinol/100 mL. Thompson also states, "Three samples of infant formula were examined by the present method and by a colorimetric method using

trifluoroacetic acid; the values of the HPLC method were on average 7% higher."

Precision was also evaluated by Thompson. The analysis of 6 different cans of liquid infant formula from the same lot produced a relative standard deviation of 4.0% for determinations of the *trans*-isomer.

Vitamin A (Retinol)

Wyeth-Ayerst provided an unpublished method for vitamin A ("Determination of Retinol in Infant Formula Products by HPLC"). The method is applicable to infant formula products containing >500 IU vitamin A/qt (ready-to-feed basis).

The sample is saponified, partitioned with organic solvent, and separated from the matrix by LC using isopropanol in hexane as the mobile phase and a silica column. UV detection is performed at 336 or 340 nm.

Wyeth-Ayerst reported that the RSD_r of the method was 1.97%, and recovery of retinyl palmitate added to milk-based formula averaged 99% (n = 5, RSD_r = 1.24). A comparison of results of vitamin A determinations by saponification (retinol) and direct extraction (retinyl palmitate) produced highly comparable data.

Vitamin E

The method selected for the collaborative study was an unpublished method submitted by Wyeth-Ayerst ("Determination of all-rac-Tocopherol (Vitamin E Activity) in Infant Formula Products by HPLC").

Vitamin E is determined by LC. The sample is saponified with potassium hydroxide, partitioned with organic solvent, and chromatographed on a silica column using isopropyl alcohol in hexane. UV detection is performed at 280 nm.

Recovery of all-rac-tocopherol added to infant formula averaged 100.4% (RSD_r = 1.73%, n = 5).

992.03 Vitamin E Activity (All-rac-α-Tocopherol) in Milk-Based Infant Formula—Liquid Chromatographic Method

First Action 1992

(Applicable to determination of vitamin E activity in milk-based infant formula.)

Method Performance (milk-based liquid, ready-to-feed, 3 manufacturers):

Mean = 24.14 IU vitamin E/L infant formula

s_r = 2.04; s_R = 2.82; RSD_r = 8.46%; RSD_R = 11.69%

A. Principle

Vitamin E activity in samples of infant formula is determined by saponification of all-rac-α-tocopherol, partitioning with organic solvent, separation from matrix, and quantification by liquid chromatography.

B. Apparatus

(**a**) *Liquid chromatograph (LC).*—Capable of pressures up to 3000 psi with injector capable of 100 µL injections. Operating conditions: eluent flow rate, 2.0 ± 0.2 mL/min; temperature, ambient.

(**b**) *Detector.*—Capable of measuring absorbance at 280 nm, with sensitivity of 0.02 AUFS.

(**c**) *Precolumn.*—2 mm id × 2 cm stainless steel, packed with 40 µm pellicular reversed-phase C_{18} (Alltech 28551 is suitable).

(**d**) *Column.*—4.6 mm id × 25 cm stainless steel, packed with 5 µm silica (Hypersil Silica is suitable).

(**e**) *Shaking water bath.*—Capable of maintaining 70 ± 2°, with variable speed capable of 60 oscillations/min, ca 11 × 14 in. sample area (Precision Scientific model 25 is suitable).

(**f**) *Glassware.*—(*1*) 125 mL separatory funnels. (*2*) 5 mL volumetric flasks. (*3*) 100 mL low-actinic volumetric flasks.

C. Reagents

(**a**) *Mobile phase solution.*—Hexane–isopropyl alcohol (99.92 + 0.08, v/v), HPLC grade solvents. Degas 2–5 min under vacuum.

(**b**) *Wash solution.*—H_2O–absolute ethanol (3 + 2, v/v).

(**c**) *Extraction solution.*—Hexane–methylene chloride (3 + 1, v/v), HPLC grade solvents.

(**d**) *Saponification solution.*—10.5N potassium hydroxide (KOH). Dissolve 673 g KOH in 1 L H_2O.

(**e**) *Antioxidant solution.*—1% pyrogallol. Dissolve 5.0 g pyrogallol (1,3,5-trihydroxybenzene, 98%; Aldrich is suitable source) in 500 mL absolute ethanol.

(**f**) *Standard solutions.*—(*1*) *Stock standard solution.*—0.5 mg/mL all-rac-α-tocopheryl acetate in hexane. Accurately weigh ca 50 mg all-rac-α-tocopheryl acetate (USP Reference Standard) into 100 mL low-actinic volumetric flask and dilute to volume with hexane (HPLC grade). Shake well to dissolve. Make fresh every 3 weeks. Store at –20° in explosion-proof freezer when not in use.

(*2*) *Working standard solution.*—10 µg/mL all-rac-α-tocopheryl acetate. Pipet 2 mL stock standard solution, (*1*), into 100 mL low-actinic volumetric flask. Evaporate to dryness under nitrogen. Dissolve residue in antioxidant solution, **C(e)**, and dilute to volume. Prepare fresh daily.

(**g**) *Suitability test solution.*—Approximately 15 µg/mL all-rac-α-tocopherol (USP Reference Standard) and all-rac-α-tocopheryl acetate (USP Reference Standard) in hexane (HPLC grade).

D. Extraction of Standard and Samples

Pipet 10.0 mL working standard solution, **C(f)***(2)*, or sample volume containing ca 0.095 IU vitamin E activity (10 mL for ready-to-feed formulas) into 150 mL centrifuge tube. Bring sample volume to 10 mL with H_2O, if necessary. To standard tubes, add 10 mL H_2O, 20 mL antioxidant solution, **C(e)**, and 5 mL saponification solution, **C(d)**. To sample tubes, add 30 mL antioxidant solution and 5 mL saponification solution. Cap tubes and swirl briefly to mix. Place tubes in 70° shaking H_2O bath (ca 60 oscillations/min) for 25 min. Remove tubes and place in ice 5 min, or until contents cool to room temperature.

Quantitatively transfer contents to separate 125 mL separatory funnels. Wash remaining sample or standard from tube

into funnel with 30 mL H_2O. Pipet 30.0 mL extraction solvent, **C(c)**, into funnel and shake ca 2 min. When layers separate, discard aqueous (lower) layer. Add 30 mL wash solution, **C(b)**, to funnel and shake very gently 30 s, venting frequently. Let phases separate and discard aqueous layer. Repeat wash step 3×. Pipet 20.0 mL portion from funnel into 50 mL tube and evaporate to dryness under nitrogen. Transfer residues quantitatively to separate 5 mL volumetric flasks and dilute to volume with mobile phase solution, **C(a)**. Inject 100 µL standard or sample into LC.

E. System Suitability Test

Inject 100 µL test solution, **C(g)**, into LC. Typical peak retention times for tocopherol and tocopheryl acetate are 6.0 and 5.0 min, respectively. Calculate resolution (R) factor between tocopherol and tocopheryl acetate as follows:

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

where t_1 and t_2 = retention time measured from injection time to elution time of peak maximum of tocopherol and tocopheryl acetate, respectively, and W_1 and W_2 = widths of peaks measured by extrapolating relatively straight sides to baseline of alcohol and acetate, respectively.

If R factor is >1.0, proceed with sample analysis; if R factor is <1.0, decrease amount of isopropyl alcohol added per liter [mobile phase solution, **C(a)**] by ca 0.01%. Inject working standard solution, **C(f)***(2)*, 5×. Calculate reproducibility of replicate injections in terms of standard deviations (per USP), which should be ≤2%. Typical relative standard deviation values for peak height are ±1.5%.

F. Liquid Chromatography

Inject 100 µL standard or sample into LC.

G. Calculations

Measure peak heights or peak areas of all-rac-α-tocopherol in both sample and standard chromatograms. Calculate IU per reconstituted quart of vitamin E activity (A) as follows:

$$A = H_{sam}/H_{std} \times C_{std} \times 0.001 \text{ IU/µg} \times 946.33 \text{ mL/quart}$$

where H_{sam} = peak height of sample; H_{std} = peak height of standard; C_{std} = concentration of standard, µg/mL.

Ref.: JAOAC **76**, March/April issue (1993)

CAS-59-02-9 (α-tocopherol)

CAS-7695-91-2 (α-tocopheryl acetate)

992.04 Vitamin A (Retinol Isomers) in Milk and Milk-Based Infant Formula—Liquid Chromatographic Method

First Action 1992

(Applicable to determination of all-*trans*-retinol and 13-*cis*-retinol in milk and milk-based infant formula.)

(Work in subdued artificial light.)

(*Caution*: See *Appendix: Laboratory Safety* for precautions on "Distillation, Extraction, and Evaporations," "Sodium and Potassium Hydroxides," "Safe Handling of Organic Solvents," "Diethyl Ether," and "Peroxides.")

Method Performance:

Mean = 2730.3 IU vitamin A/L infant formula

s_r = 98.8; s_R = 265.4; RSD_r = 3.62%; RSD_R = 9.72%

A. Principle

Sample is digested with ethanolic potassium hydroxide (KOH), and vitamin A is extracted into diethyl ether–hexane. Hexadecane is added to prevent destruction of the vitamin after evaporation. Residue is dissolved in heptane, and vitamin A isomers, all-*trans*-retinol and 13-*cis*-retinol, are determined by liquid chromatography on silica column.

B. Apparatus

(**a**) *Alkaline digestion apparatus.*—100 mL volumetric flask with ***T*** stopper (select flask with graduation mark low in neck), magnetic stirrer, and 25 mm × 8 mm (volume less than 1.5 mL) stirring bar.

(**b**) *Extraction apparatus.*—15 mL stoppered centrifuge tubes, Pasteur pipets with rubber bulbs, and water bath with clamps for evaporation of solvents under nitrogen.

(**c**) *Liquid chromatographic system (LC).*—Injector capable of 100 µL injections, pump capable of 3000 psi, 15 cm × 4.5 mm column packed with 3 µm silica (Apex 3 micron silica, Jones Chromatography, Columbus, OH, is suitable), detector capable of measuring absorbance at 340 nm, and data recording system (chart recorder or integrator).

(**d**) *Spectrophotometer.*—Capable of measuring absorbance at 324.5 nm.

C. Reagents

(**a**) *Ethanolic pyrogallol solution.*—2% pyrogallol (1,3,5-trihydroxybenzene, 98%; Aldrich is suitable source) in 95% ethanol or SDA 3A.

(**b**) *Ethanolic KOH solution.*—10% (w/v) KOH in 90% ethanol (prepared from absolute ethanol or SDA 3A).

(**c**) *Extraction solution.*—Hexane–diethyl ether (85 + 15), HPLC grade hexane. Prepare fresh daily. Store ether in metal container under nitrogen.

(**d**) *Hexadecane solution.*—1 mL hexadecane in 100 mL hexane.

(**e**) *Mobile phase solution.*—Heptane–isopropanol, HPLC grade reagents; proportions are adjusted to obtain retention times specified in **D**, *System Suitability Test.*

(**f**) *Standard oil solution.*—*(1) All-trans-retinol.*—Dissolve 100 mg crystalline all-*trans*-retinol in 50 g cottonseed oil, stirring under nitrogen with magnetic stirrer. Store all-*trans*-retinol oil solution under nitrogen in low-actinic airtight container at 4°. Weigh (±0.1 mg) 3 drops of oil solution (ca 50 mg) into each of three 50 mL volumetric flasks; dissolve and dilute to volume with isopropanol. Read solution absorbance at least monthly at 324.5 nm, using 0.1% solution of cottonsed oil in isopropanol in reference cell. Calculate concentration of all-*trans*-retinol (ng/mL) in solutions by multiplying absorbance by 5460. Calculate mean content of oil (C_f) in ng/mg from weight of oil used.

(2) 13-cis-Retinol.—Prepare as in *(1)*, reading absorbance at 326 nm, and calculating concentration of 13-*cis*-retinol in solution by multiplying absorbance by 5930.

(**g**) *Standard working solutions.—(1) All-trans-retinol.*—Weigh (±0.1 mg) 3 drops of all-*trans*-retinol oil solution into each of three 50 mL volumetric flasks. Dissolve and dilute to volume with heptane containing 0.5% isopropanol. Prepare daily. Calculate concentration of all-*trans*-retinol (1–2 μg/mL) in standard working solution from weight of oil solution. Inject all-*trans*-retinol standard working solution to calibrate LC. Prepare standard solutions containing 50, 500, and 1000 ng/mL all-*trans*-retinol to verify linearity of standard curve by making appropriate dilutions of standard working solution in heptane containing 0.5% isopropanol. Verify by LC that ratio of all-*trans*-retinol to 13-*cis*-retinol in these solutions is >30.

(2) 13-cis-Retinol.—Prepare as in *(1)*.

D. System Suitability Test

Inject 100 μL standard working solutions into LC. Adjust amount of isopropanol (1–5%) in LC solution, **C(e)**, and flow rate (1–2 mL/min) until 13-*cis*-retinol and all-*trans*-retinol elute ca 4.5 and 5.5 min, respectively. LC measurements should be <2% relative standard deviation when 100 ng/mL all-*trans*-retinol standard working solution is injected.

E. Digestion and Extraction of Sample

(**a**) *Powdered infant formula, powdered milk.*—Weigh 140 g powder into 1 L volumetric flask, add H_2O (boiled and cooled) almost to mark, flush with nitrogen, stopper flask, and mix. When foam collapses, dilute to volume with H_2O and mix.

(**b**) *Liquid infant formula, milk.*—Pipet 20 mL concentrated formula plus 20 mL H_2O, or 40 mL ready-to-use formula, or 40 mL preparation obtained in (**a**), or 40 mL milk, into 100 mL digestion flask. Add 10 mL ethanolic pyrogallol solution, **C(a)**, and 40 mL ethanolic KOH solution, **C(b)**. Add ethanolic pyrogallol solution until liquid is ca 1 cm below graduation mark, stopper, shield contents from light by covering flask with Al foil, and stir 18 h. Dilute to volume with ethanolic pyrogallol solution, stopper, and stir to mix. Pipet 3 mL of digestate, using pipet filler safety bulb, into 15 mL centrifuge tube. Add 2 mL H_2O and 7 mL extraction solution, **C(c)**, and vortex 30 s, using gloves and safety glasses. Centrifuge 5 min at 2000 rpm. Transfer hexane (upper) layer, using Pasteur pipet with rubber bulb, to 25 mL volumetric flask. Transfer as much of hexane layer as possible with no more than trace of aqueous digest. Repeat extraction step 2× with 7 mL portions of extraction solvent, and pool hexane extracts in the 25 mL volumetric flask. Add 1 mL hexadecane solution, **C(d)**, and dilute to volume with hexane. Invert flask to mix, and then let stand ≥5 min to allow any aqueous digest to settle.

Pipet 15 mL of clear solvent from center of flask, avoiding contaminating aqueous digest, into 15 mL centrifuge tube, and evaporate under nitrogen. Dissolve residue immediately in 0.5 mL heptane, and transfer to sealable vials for LC determination.

F. Determination

Inject 100 μL standard working solutions into LC. Inject 100 μL sample extract, **E**. Measure peak areas for 13-*cis*- and all-*trans*-retinol.

G. Calculation

Calculate all-*trans*-retinol concentration, V_t, (ng/mL milk or diluted formula) as follows:

$$V_t = A_t/A_{st} \times W_t \times C_t \times 1/50 \times 25/15 \times 100/3 \times 1/2 \times 1/40$$
$$V_t = A_t/A_{st} \times W_t \times C_t \times 5/360$$

where A_t = peak area, all-*trans*-retinol in sample; A_{st} = peak area, all-*trans*-retinol in standard; W_t = weight, mg, oil solution used to prepare working standard solution; and C_t = concentration, ng/mg, all-*trans*-retinol in oil solution.

Calculate 13-*cis*-retinol concentration, V_c, (ng/mL milk or diluted formula) as follows:

$$V_c = A_c/A_{sc} \times W_c \times C_c \times 5/360$$

where A_c = peak area, 13-*cis*-retinol in sample; A_{sc} = peak area, 13-*cis*-retinol in standard; W_c = weight, mg, oil solution used to prepare working standard solution; and C_c = concentration, ng/mg, 13-*cis*-retinol in oil solution.

Ref.: JAOAC **76**, March/April issue (1993)

CAS-68-26-8 (vitamin A)

992.05 Folic Acid (Pteroylglutamic Acid) in Infant Formula —Microbiological Methods

First Action 1992

(Applicable only to free form of folic acid.)

Method Performance (milk-based liquid, ready-to-feed, 3 manufacturers):

Mean = 173.1 μg folic acid/L infant formula

s_r = 16.2; s_R = 44.0; RSD_r = 9.35%; RSD_R = 25.44%

A. Principle

Folic acid content of infant formula samples is estimated from acidimetric response of *Lactobacillus casei.*

B. Apparatus

(**a**) *Incubator.*—Forced-draft incubator or circulating H_2O bath, capable of maintaining within ±0.5° temperatures between 28 and 40°.

(**b**) *Centrifuge.*—Capable of operating at 3000 rpm for ≥3 min, with adapters to hold culture tubes.

(**c**) *Mechanical shaker.*—Capable of operating 72 h at 100–200 horizontal strokes per min; minimum capacity, 100 tubes; if shaker cannot be placed inside (**a**), shaker itself must maintain temperature specified in (**a**).

C. Basal Medium Preparation

(Store all solutions in dark at ca 10°. Store all solutions except those containing alcohol under toluene. Proportionate amounts may be prepared.)

(**a**) *Acid-hydrolyzed casein solution.*—Mix 400 g vitamin-free casein with 2 L HCl (1 + 1), and heat at reflux temperature 8 h. Remove HCl by distillation under reduced pressure until thick paste remains. Dissolve paste in H_2O and transfer to 4 L beaker using total of 1500 mL H_2O. Adjust to pH 3.5 ± 0.1 with ca 200 mL 40% NaOH (400 g NaOH/L H_2O). Transfer solution to 2 L mixing cylinder and dilute to volume with H_2O. Pour solution into 6 L Erlenmeyer flask. Add additional 2 L H_2O. Add 80 g activated charcoal (Norit A is suitable), stir 1 h, and filter through fluted paper. Repeat treatment with activated charcoal. Add 35 mL toluene, shake well, and store at 10°. If precipitate forms in storage, filter before use.

(**b**) *Adenine–guanine–uracil solution.*—Add 1.0 g each of adenine sulfate, guanine HCl, and uracil to 50 mL hot (90°) HCl (1 + 1) in 150 mL beaker. Heat at 90° until solids are dissolved. Cool and transfer solution quantitively to 1 L volumetric flask using H_2O. Dilute to volume with H_2O and mix thoroughly. Add 10 mL toluene, mix well, and store at 10°. Prepare fresh every 3 months.

(**c**) *Cystine solution.*—Weigh 1.25 g L-cystine; suspend in 40 mL H_2O. Add concentrated HCl dropwise with stirring (ca 2 mL required) until L-cystine dissolves. Transfer to 50 mL volumetric flask and dilute to volume with H_2O.

(**d**) *Salt solution II.*—Dissolve 10 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.5 g $FeSO_4 \cdot 7H_2O$, 1.5 g $MnSO_4 \cdot H_2O$, and 1 mL concentrated HCl in 250 mL H_2O. Add 2 mL toluene and store at 10°. Make fresh every 3 months.

(**e**) *Xanthine solution.*—Suspend 1.0 g xanthine in 200 mL H_2O in 400 mL beaker. Heat to 70°. Add 30 mL NH_4OH (2 + 3) and stir until dissolution is complete. Quantitatively transfer solution to 1 L volumetric flask, using H_2O. Dilute to volume with H_2O and mix thoroughly. Store under toluene at 10°. Prepare fresh every 3 months.

(**f**) *Basal medium stock solution.*—To 700 mL H_2O in 1 L beaker, add 127 mL acid-hydrolyzed casein solution, (**a**); 20 mL adenine–guanine–uracil solution, (**b**); 16 mL cystine solution, (**c**); 10 mL salt solution II, (**d**); and 10 mL xanthine solution, (**e**). Mix. Using pH meter, adjust to pH 6.4–6.5 by adding 1.0N NaOH and dilute to 1 L in glass-stoppered graduated cylinder. Dispense 200 mL portions into 16 oz polyethylene bottles, cap, and freeze (use within 2 months). (Difco Folic Acid Casein Medium is also available for basal medium.)

D. Other Solutions

(**a**) *Salt solution I.*—Dissolve 50 g K_2HPO_4 and 50 g KH_2PO_4 in 500 mL H_2O. Add 10 mL toluene and store at 10°. Make fresh every 3 months.

(**b**) *Polysorbate 80 solution.*—Dissolve 25 g Polysorbate 80 (polyoxyethylene sorbitan monooleate) in ethanol to make 250 mL solution.

(**c**) *Clarified skim milk.*—Add 10 g spray-dried skim milk powder to 80 mL H_2O. Mix thoroughly. Using pH meter, adjust pH to 4.2 with HCl (1 + 1). Centrifuge mixture 15 min at 3000 rpm to obtain clear supernate. Decant supernate into 150 mL beaker. Adjust pH to 7.0 by addition of NaOH solution. Centrifuge 15 min at 3000 rpm to obtain clear supernate. Decant supernate into 100 mL cylinder and dilute to 100 mL with H_2O.

(**d**) *Phosphate buffer solution.*—0.05M. Dissolve 5.85 g KH_2PO_4 and 1.22 g K_2HPO_4 in H_2O and dilute to 1 L. Prepare fresh daily.

(**e**) *Bromthymol blue indicator solution.*—Weigh 0.1 g bromthymol blue into small beaker. Dissolve in few mL ethanol, add 1.6 mL 0.1N NaOH, and dilute with H_2O to 250 mL.

(**f**) *Isotonic salt solution.*—Weigh 0.9 g NaCl and transfer to 100 mL volumetric flask. Dilute to volume with H_2O and shake until salt has dissolved. Transfer 10 mL portions to culture tubes, plug with cotton or cap with stainless steel caps and sterilize 20 min at 121°. Prepare fresh weekly.

(**g**) *Biotin solution.*—Weigh 50 mg biotin into 1 L volumetric flask and add 10 mL 0.2N NaOH. When biotin is dissolved, dilute to volume with H_2O and mix well. Store at 10°. Prepare fresh every 6 months.

(**h**) *Vitamin solution.*—Dissolve 50 mg niacin, 71.4 mg sodium riboflavin-5-phosphate (equivalent to 50 mg riboflavin), 50 mg thiamine hydrochloride, 50 mg calcium pantothenate, 10 mg *p*-aminobenzoic acid, and 10 mL biotin solution, **D(g)**, in 150 mL H_2O and dilute to 200 mL. Add 6 mL toluene, mix well, and store at 10°. Prepare fresh every 2 months.

(**i**) *Pyridoxamine solution.*—Dissolve 14.3 mg pyridoxamine dihydrochloride in H_2O and dilute to 100 mL. Add 3 mL toluene, mix well, and store at 10°.

(**j**) *Chick pancreas preparation.*—Weigh 100 mg desiccated chick pancreas (Difco Laboratories, Cat. No. 0459-12-2), add 20 mL H_2O, and stir 15 min. Centrifuge 10 min at 3000 rpm and use clear supernate. Prepare fresh daily.

(**k**) *Phosphate buffer (0.05M)–ascorbic acid solution.*—Dissolve 50 mg ascorbic acid in 100 mL 0.05M phosphate buffer solution, **D(d)**. Prepare just prior to use.

E. Culture and Maintenance Media

(**a**) *Maintenance medium.*—Add 10 g tryptone; 10 g anhydrous dextrose; 2 g K_2HPO_4; 3 g $CaCO_3$; 1 g Liver Concentrate NF (Pharmaceutical Division, Wilson & Co, Chicago, IL, is suitable); 5 mL salt solution I, **D(a)**; 5 mL salt solution II, **C(d)**; and 15 g agar to 990 mL H_2O. Bring mixture to boil and stir until agar is dissolved. Continue stirring while dispensing 10 mL portions into 18 × 150 mm hard glass culture tubes. Plug tubes with polyurethane or cotton plugs and sterilize 15 min at 121°. Cool to room temperature in upright position. Maintenance medium will keep indefinitely in closed container stored at 10°.

(**b**) *Culture medium.*—Add 9.6 g riboflavin assay medium (Bacto-Riboflavin Assay Medium, Difco Laboratories, is suitable) to 360 mL boiling H_2O and stir until dissolved. Cool to room temperature, add 40 mL clarified skim milk, **D(c)**, and mix well. Dispense 10 mL portions into 18 × 150 mm test tubes, cap with stainless steel caps, and sterilize 15 min at 121°. Cool and store at 10°. Make fresh after 3 months.

F. Stock Cultures of Test Organisms

Inoculate, by stab, 3 tubes of maintenance medium, **E(a)**, from pure culture of *Lactobacillus casei* (ATCC No. 7469).

Incubate 24 h at 37°, label as monthly cultures, and store at 10°. From 1 of monthly cultures, inoculate 4 tubes of maintenance medium, incubate 24 h at 37°, and label as weekly cultures. Each week, inoculate 4 or 5 tubes of maintenance medium from weekly culture, incubate 24 h at 37°, and label as daily cultures. Use daily culture to prepare inoculum. At beginning of next month, inoculate 3 new monthly cultures from unused monthly culture from fresh stock.

Titrimetric Method

G. Folic Acid Standard Solutions

(Do not shake standard solutions stored under toluene.)

(**a**) *Stock standard solution.*—500 μg/mL. Accurately weigh USP Folic Acid Reference Standard, equivalent to 55–56 mg folic acid, dried to constant weight and stored in dark over P_2O_5 in desiccator. Using 50 mL H_2O, quantitatively transfer to 100 mL volumetric flask. Add 2 mL NH_4OH (2 + 3). When completely dissolved, dilute to volume with H_2O, and add, by pipet, additional H_2O needed for final volume, calculated as follows, to bring stock standard solution to 500 μg/mL folic acid:

$$V_f = (W_s \times P_s \times 1000)/(100 \times 500) = (W_s \times P_s)/50$$

where V_f = final volume, mL, stock solution; W_s = weight, mg, folic acid standard; and P_s = purity, %, folic acid standard (from label).

Mix well. Store in red or amber bottle at 10°. Prepare fresh after 4 months.

(**b**) *Intermediate standard solution.*—50 μg/mL. Accurately pipet 10 mL stock standard solution, (**a**), into 100 mL amber or red volumetric flask, dilute to volume with H_2O, and mix thoroughly. Store at 10°. Make fresh after 1 month.

(**c**) *Working standard solution.*—0.1 ng/mL. Pipet 1 mL intermediate standard solution, (**b**), into amber or red 100 mL volumetric flask, dilute to volume with H_2O and mix. Pipet 1 mL this first solution into another 100 mL amber or red volumetric flask, dilute to volume, and mix. Pipet 5 mL this second solution into 250 mL amber or red volumetric flask, dilute to volume with phosphate buffer solution, **D(d)**, and mix. Label this as standard solution (0.0001 μg/mL or 0.1 ng/mL). Prepare fresh for each assay.

H. Inoculum

Inoculate tube of culture medium, **E(b)**, with cells from daily culture, **F**, and incubate 16–20 h at 37°. Under aseptic conditions, centrifuge culture 10 min at 2000 rpm to obtain clear supernate, discard supernate, and resuspend cells by shaking or swirling in 10 mL isotonic salt solution, **D(f)**. Repeat from "centrifuge culture 10 min ..." 2×. Add 1 mL of final suspension to 10 mL isotonic salt solution, **D(f)**, and swirl to form uniform suspension. Use as inoculum.

I. Assay Solution

For powders, accurately weigh sample containing ca 5 μg folic acid into 100 mL beaker. Reconstitute in 25–30 mL H_2O and quantitatively transfer to 100 mL volumetric flask. Dilute to volume with H_2O.

For liquids, pipet volume containing ca 5 μg folic acid into 100 mL volumetric flask. Dilute to volume with H_2O. (Sample solutions contain ca 0.05 μg [50 ng] folic acid/mL.)

Pipet 1 mL diluted sample and 1 mL chick pancreas preparation, **D(j)**, into 18 × 150 mm screw top culture tube, and mix well. Add 18 mL 0.05M phosphate buffer–ascorbic acid solution, **D(k)**, and 1 mL toluene. Mix.

For enzyme and reagent blank, pipet 1 mL H_2O and 1 mL chick pancreas preparation into 18 × 150 mm screw-top culture tube and mix. Add 18 mL 0.05M phosphate buffer–ascorbic acid solution and 1 mL toluene. Mix.

Incubate sample and blank tubes 16 h at 37°. Sterilize 10 min at 121°, and centrifuge 10 min at 2000 rpm to obtain clear supernate. Dilute supernates with 0.05M phosphate buffer–ascorbic acid solution to volume, V_f, **G(a)**. Label as assay solution.

J. Assay Tubes

Meticulously cleanse by suitable means (Na lauryl sulfate USP has been found satisfactory as detergent), hard glass tubes and other necessary glassware. (Test organisms are highly sensitive to minute amounts of growth factors and to many cleansing agents. Therefore, it may be preferred to follow cleansing by heating glassware 1–2 h at ca 250°.)

Prepare tubes containing standard solution, **G(c)**, as follows: To tubes (in duplicate or replicate) add: 0.0 (for uninoculated blanks), 0.0 (for inoculated blanks), 1.0, 2.0, 3.0, and 4.0 mL standard solution.

Prepare tubes containing assay solution, **I**, as follows: To similar tubes (in duplicate or replicate) add 1.0, 2.0, 3.0, and 4.0 mL assay solution.

To each tube, standard and assay solutions, add phosphate buffer solution, **D(d)**, to make 5.0 mL. To bottle containing 200 mL basal medium stock solution, **C(f)**, add 1.0 mL vitamin solution, **D(h)**, and 0.8 mL pyridoxamine solution, **D(i)**, and mix well. Add 5.0 mL this medium to each standard and sample tube. Cover tubes suitably to prevent bacterial contamination, and sterilize 10 min at 121°. Cool as rapidly as practicable to minimize color formation. Take precautions to keep sterilizing and cooling conditions uniform throughout assay. Too close packing of tubes in autoclave, or overloading autoclave, may cause variation in heating rate.

Aseptically inoculate each tube, except 1 set of duplicate or replicate tubes containing 0.0 mL standard solution (uninoculated blanks), with 1 drop inoculum. Incubate 60–72 h, at temperature between 28° and 40°, maintained within ±0.5°. Contamination of assay tubes with any foreign organism invalidates assay.

K. Determination

Prior to determination of response, visually inspect each set of tubes. Uninoculated blanks should be clear; standards and samples should be free of growth other than test organism. Titrate contents of each tube with 0.1N NaOH, using bromthymol blue indicator, **D(e)**, or using pH meter to pH 6.8. When deter-

mining pH, read pH values to nearest 0.01 pH unit after incubation. pH at 5.0 mL level of standard solution is normally 1.0–1.5 pH units below inoculated blank. Disregard results of assay if titer of inoculated blank is more than 1.5 mL greater than titer of uninoculated blank. Titer at 5.0 mL level of standard solution should be 8–12 mL.

Prepare standard concentration-response curve by plotting titration values, expressed in mL 0.1N NaOH for each level of standard solution used, against amount of reference standard contained in respective tubes.

Determine amount of folic acid for each level of assay solution by interpolation from standard curve. Discard results of any sample with titer of <0.5 mL or >4.5 mL.

L. Calculation

For each level of assay solution used, calculate folic acid content/mL assay solution. Calculate average values from tubes that do not vary by ≥15% from this average. If number of acceptable values remaining is <2/3 original number of tubes used in 4 levels of assay solution, data are insufficient. If number of acceptable values remaining is ≥2/3 original number of tubes, calculate folic acid concentration (μg/100 mL "as fed" formula) in sample from the average.

$$\text{Folic acid} = [(X \times D) - EB] \times [(100 \times R)/1000\ Q)]$$

where X = average value of folic acid per mL of assay solution, ng; D = dilution factor based on 1 mL sample dilution incubated with enzyme preparation, mL; EB = folic acid value found in enzyme blank, ng/mL enzyme preparation; Q = sample weight or volume, g or mL; and R = reconstitution value of sample required to make 100 mL of "as fed" formula, expressed as weight or volume.

Turbidimetric Method

(Not applicable in presence of extraneous turbidity or color in amount that interferes with turbidimetric measurements.)

M. Folic Acid Standard Solutions

Proceed as in **G**.

N. Inoculum

Proceed as in **H**.

O. Assay Solution

Proceed as in **I**.

P. Assay Tubes

Proceed as in **J**.

Q. Calibration of Photometer

See **960.46F**.

R. Determination

See **960.46G**.

S. Calculation

Proceed as in **L**.

Ref.: JAOAC **76**, March/April issue (1993)

CAS-59-30-3 (folic acid)

992.06 Vitamin A (Retinol) in Milk-Based Infant Formula—Liquid Chromatographic Method

First Action 1992

(Applicable to milk-based infant formulas containing >500 IU vitamin A per reconstituted quart.)

Method Performance (milk-based liquid, ready-to-feed, 3 manufacturers):

Mean = 2658 IU vitamin A/L infant formula

s_r = 129.6; s_R = 279.0; RSD_r = 4.9%; RSD_R = 10.5%

A. Principle

Vitamin A in infant formula is saponified, partitioned with organic solvent, separated from matrix, and quantified by liquid chromatography.

B. Apparatus

(**a**) *Liquid chromatograph (LC).*—Capable of pressures up to 3000 psi, with injector capable of 100 μL injections. Operating conditions: eluent flow rate, 1.5 ± 0.2 mL/min; temperature, ambient.

(**b**) *Detector.*—Capable of measuring absorbance at 336 nm, with sensitivity of 0.1 AUFS.

(**c**) *Column.*—4.6 mm id × 15 cm stainless steel, packed with 5 μm silica-based cyano group stationary phase (Sepralyte CN, Analytichem International, Harbor City, CA, is suitable).

(**d**) *Spectrophotometer.*—Capable of measuring absorbance at 325 nm.

(**e**) *Shaking water bath.*—Capable of maintaining 70 ± 2°, variable speed capable of 60 oscillations/min, with sample area ca 11 × 14 in. (Precision Scientific Model 25 is suitable).

(**f**) *Glassware.—(1)* 125 mL separatory funnels. *(2)* 5 mL volumetric flasks. *(3)* 100 mL low-actinic volumetric flasks.

C. Reagents

(**a**) *Mobile phase solution.*—Hexane–isopropyl alcohol (100 + 0.25, v/v), HPLC grade solvents. Degas 2–5 min under vacuum.

(**b**) *Wash solution.*—H_2O–absolute ethanol (3 + 2, v/v).

(**c**) *Extraction solution.*—Hexane–methylene chloride (3 + 1, v/v), HPLC grade solvents.

(**d**) *Saponification solution.*—10.5N potassium hydroxide (KOH). Dissolve 673 g KOH in 1 L H_2O.

(**e**) *Antioxidant solution.*—1% pyrogallol. Dissolve 5.0 g pyrogallol (1,3,5-trihydroxybenzene, 98%; Aldrich is suitable source) in 500 mL absolute ethanol.

(**f**) *Standard solutions.—(1) Stock standard solution.*—10 mg/mL retinyl palmitate in hexane. Quantitatively transfer contents of 1.0 g ampoule of retinyl palmitate (USP Reference

Standard) with hexane (HPLC grade) into 100 mL low-actinic volumetric flask, dilute to volume with hexane, and shake well to dissolve. Make fresh every 2 weeks. Store at −20° in explosion-proof freezer when not in use.

(2) Intermediate standard solution.—Pipet 2 mL of stock standard solution, *(1)*, into 250 mL volumetric flask and dilute to volume with hexane.

(3) Working standard solution.—Approximately 1.6 µg/mL retinyl palmitate. Pipet 2 mL intermediate standard solution, *(2)*, into 100 mL low-actinic volumetric flask. Evaporate to dryness under nitrogen. Dissolve residue in antioxidant solution, **C(e)**, and dilute to volume. Prepare fresh daily.

D. Extraction of Standards and Samples

Pipet 10.0 mL working standard solution, **C(f)***(3)*, or sample volume containing ca 20 IU vitamin A activity (10 mL for ready-to-feed formulas) into 150 mL centrifuge tube. Bring sample volume to 10 mL with H_2O, if necessary. To standard tubes, add 10 mL H_2O, 20 mL antioxidant solution, **C(e)**, and 5 mL saponification solution, **C(d)**. To sample tubes, add 30 mL antioxidant solution and 5 mL saponification solution. Cap tubes and swirl briefly to mix. Place tubes in 70° shaking H_2O bath (ca 60 oscillations/min) for 25 min. Remove tubes and place in ice 5 min, or until contents cool to room temperature.

Quantitatively transfer contents to separate 125 mL separatory funnels. Wash remaining sample or standard from tube into funnel with 30 mL H_2O. Pipet 30.0 mL extraction solvent, **C(c)**, into funnel and shake ca 2 min. When layers separate, discard aqueous (lower) layer. Add 30 mL wash solution, **C(b)**, to funnel and shake very gently 30 s, venting frequently. Let phases separate and discard aqueous layer. Repeat wash step 3×. Pipet 20.0 mL portion from funnel to 50 mL tube and evaporate to dryness under nitrogen. Transfer residues quantitatively to separate 5 mL volumetric flasks and dilute to volume with mobile phase solution, **C(a)**.

E. Determination of Standard Concentration

Pipet 2 mL of intermediate standard solution, **C(f)***(2)*, into 50 mL volumetric flask and dilute to volume with hexane. Transfer portion of this solution into 1 cm cell path length cuvet and measure absorbance at 325 nm. Calculate concentration of working standard solution, C_{std}, as follows:

$$C_{std} = [A_{325}/(2 \times \Sigma \times b)] \times 10^4$$

where A_{325} = absorbance of working standard solution at 325 nm; $\Sigma = 996$, extinction coefficient of retinyl palmitate in hexane at 325 nm; and $b = 1$ cm, cell path length.

F. System Suitability Test

Inject 100 µL of saponified working standard solution into LC. Typical retention times for 13-*cis*-retinol and *trans*-retinol are 7.5 and 9.0 min, respectively. Calculate R factor between 13-*cis*-retinol and *trans*-retinol as follows:

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

where t_1 and t_2 = retention time measured from injection time to elution time of peak maximum of 13-*cis*-retinol and *trans*-retinol, respectively, and W_1 and W_2 = widths of peaks measured by extrapolating relatively straight sides to baseline of 13-*cis*-retinol and *trans*-retinol, respectively.

If R factor is <1.3, increase amount of isopropyl alcohol added per liter [mobile phase solution, **C(a)**] by ca 0.05%. Inject saponified working standard solution 5×. Calculate reproducibility of replicate injections in terms of standard deviations (per USP), which should be ≤2%. Typical relative standard deviation values for peak height are ±1.3%.

G. Liquid Chromatography

Inject 100 µL standard or sample into LC.

H. Calculations

Since 13-*cis* vitamin A palmitate is not readily available, standard curve for all-*trans* vitamin A palmitate is used to determine biological potencies for both, correcting for 13-*cis* vitamin A palmitate, at 0.75 potency relative to all-*trans* vitamin A palmitate. This is based on assumption that relative molar absorptivities of both isomers are virtually equal at 336 nm.

Measure peak areas of *cis*- and *trans*-isomers of retinol in both sample and standard chromatograms. Multiply peak area under 13-*cis* vitamin A palmitate curve by 0.75. Sum the 2 areas to represent total peak area. Calculate IU per reconstituted quart of vitamin A activity (V) as follows:

$$V = (A_{sam}/A_{std}) \times C_{std} \times (1/0.55) \times 946.33$$

where A_{sam} = total peak area of sample; A_{std} = total peak area of standard; C_{std} = concentration of working standard solution, µg/mL; 1/0.55 = IU/µg retinyl palmitate; and 946.33 = mL/quart.

Ref.: JAOAC **76**, March/April issue (1993)

CAS-68-26-8 (vitamin A)

992.07 Pantothenic Acid in Milk-Based Infant Formula—Microbiological Turbidimetric Method

First Action 1992

(Applicable to determination of pantothenic acid in milk-based infant formula.)

Method Performance (milk-based liquid, ready-to-feed):

Mean = 4.80 mg pantothenic acid/L infant formula

$s_r = 0.22$; $s_R = 0.49$; $RSD_r = 4.59\%$; $RSD_R = 10.23\%$

A. Principle

Samples of infant formula are treated with alkaline intestinal phosphatase, which removes triphosphoadenine from coenzyme A, and avian liver peptidase, which breaks peptide linkage. Bound pantothenic acids are released to free form and assayed by turbidimetric microbial growth response.

B. Culture Media, Reagents, and Apparatus

(a) *Basal medium stock solution.*—Using ingredients in amounts prescribed for pantothenic acid, Table **960.46(d)**, proceed as below.

Using solutions prepared as in **960.46A**, add, with mixing, and in following order: acid-hydrolyzed casein solution, (**a**); cystine–tryptophan solution, (**e**); adenine–guanine–uracil solution, (**b**); vitamin solution IV, (**o**); vitamin solution VI, (**q**); salt solution A, (**i**); and salt solution B, (**j**). Add ca 100 mL H_2O and add, with mixing, anhydrous glucose and $NaOAc \cdot 3H_2O$. When solution is complete, adjust to pH 6.8 with NaOH solution, add, with mixing, polysorbate 80 solution, (**h**), and dilute with H_2O to 250 mL. [*Note*: Commercial equivalent, such as Difco pantothenate medium (AOAC USP 0816-15) is acceptable substitute for basal medium stock solution.]

(**b**) *Pantothenic acid stock standard solution.*—40 μg/mL. Accurately weigh, in closed system, 45–55 mg USP Calcium Pantothenate Reference Standard that has been dried to constant weight and stored in dark over P_2O_5 in desiccator. Dissolve in ca 500 mL H_2O, add 10 mL 0.2N HOAc and 100 mL 0.2N NaOAc, and dilute with additional H_2O to make Ca pantothenate concentration exactly 43.47 μg/mL (40 μg pantothenic acid/mL). Store under toluene in dark at ca 10°.

(**c**) *Pigeon liver acetone powder.*—Prepared to maintain NAD kinase activity of 0.5–1.5 units/mg solid (Sigma Chemical Co. is suitable supplier; 1 unit will phosphorylate 1.0 nmol β-NAD to β-NADP per min at pH 7.5 at 37° in presence of ATP).

(**d**) *Alkaline phosphatase.*—Type I (pfs), from bovine (calf) intestine; activity of 1–3 units/mg solid (Sigma Chemical Co. is suitable supplier; 1 unit will hydrolyze 1.0 μmol *p*-nitrophenyl phosphate per min at 37°).

(**e**) *Brewer's yeast.*—Dry powder.

(**f**) *Resin.*—200–400 mesh anion exchange resin (Dowex 1-X8, Bio-Rad Laboratories, Richmond, CA, is suitable).

(**g**) *Filters.*—Coarse particle retention, smooth surface, fast flow rate (No. 588, Schleicher & Schuell, Keene NH, is suitable).

(**h**) *Tris buffer solution.—(1) pH 8.3.*—Dissolve 24.2 g 2-amino-2-(hydroxymethyl)-1,3-propanediol and dilute to 200 mL with H_2O. Adjust pH to 8.3 with 30% HCl and filter, (**g**). *(2) pH 11.3.*—Dissolve 24.2 g 2-amino-2-(hydroxymethyl)-1,3-propanediol and dilute to 200 mL with H_2O; pH ca 11.3. Store at room temperature; discard after 2 weeks.

(**i**) *Sodium bicarbonate ($NaHCO_3$) solution.*—Dissolve 850 mg $NaHCO_3$ and dilute to 100 mL with H_2O. Prepare fresh on day of use and store refrigerated.

(**j**) *$KHCO_3$ solution.*—0.02M. Dissolve 1 g $KHCO_3$ and dilute to 500 mL with H_2O.

(**k**) *Other reagents.—(1)* 2-Octanol (*sec*-caprylic alcohol), purified grade (antifoam agent). (2) Toluene.

C. Enzyme Preparation

(**a**) *Activation of resin.*—Mechanically stir 100 g resin in 1 L 1.0N HCl 10 min, keeping resin completely suspended. Filter with suction through paper (Whatman No. 50 is suitable). Repeat stirring of resin in fresh 1 L 1.0N HCl and filter. Repeat stirring of resin in 1 L H_2O and filter. Repeat stirring of resin in 1 L H_2O and filtering another 10×. Adjust resin, adding enough H_2O to sufficiently mix well, to pH 8.0 by adding Tris buffer, pH 8.3, **B(h)***(1)*, dropwise. Store slurry in refrigerator. Discard after 2 days.

(**b**) *Equipment preparation.*—Chill overnight in freezer: mortar and pestle, 24 plastic centrifuge tubes (50 mL) with caps, 3 wide-mouth flasks (500 mL), 50 tubes (16 × 125 mm) with caps, 2 Mohr pipets (10 mL), and 2 glass rods. Chill overnight in refrigerator or 4° constant temperature room: $KHCO_3$ solution, **B(j)**; resin slurry, (**a**); and centrifuge head.

(**c**) *Pigeon liver extract solution.*—10%. Form suspension by rubbing 30 g pigeon liver acetone powder, **B(c)**, with ca 150 mL ice-cold 0.02M $KHCO_3$ solution, **B(j)**, in cold mortar held in ice–salt bath. Quantitatively transfer suspension, to 8 ice-cold centrifuge tubes, using ca 150 mL ice-cold 0.02M $KHCO_3$ solution (use total of 300 mL of 0.02M $KHCO_3$ solution). Shake capped tubes vigorously; chill 10 min in freezer.

Centrifuge tubes 5 min at ca 3000 rpm (to separate solids from liquid). Transfer supernate through cheesecloth to ice-cold 500 mL wide-mouth flask. Add half (150 g) activated resin and shake 5 min in ice–salt bath or shaker box in cold room. Transfer mixture to 8 ice-cold centrifuge tubes and centrifuge tubes 5 min at 3000 rpm. Transfer supernate through fresh cheesecloth to another ice-cold 500 mL wide-mouth flask and chill 10 min in freezer.

Add remaining half (150 g) activated resin and shake 5 min in ice–salt bath. Transfer mixture to 8 ice-cold centrifuge tubes and centrifuge tubes 5 min at 3000 rpm. Transfer supernate through previously used cheesecloth to another ice-cold 500 mL wide-mouth flask and chill 10 min in freezer.

Dispense supernate into ice-cold screw-cap tubes, and freeze until use. To use, thaw frozen enzyme suspension in ice bath.

(**d**) *Intestinal phosphatase solution.*—2%. Dissolve 20 mg/mL alkaline phosphatase, **B(d)**, in H_2O, making sufficient solution to add 0.4 mL to each tube in extraction, **D**. Prepare fresh for each assay. Keep refrigerated until use.

D. Assay Solution

(Throughout procedure, keep solution below pH 7.0 to prevent loss of pantothenic acid.)

Accurately measure amount of sample containing 50–100 μg total pantothenates and separately 5 mL (40 μg/mL) pantothenic acid stock standard solution, **B(b)**. With each assay, weigh 1.0 g Brewer's yeast (contains ca 100 μg total pantothenates) and assay with samples to test efficacy and consistency of enzyme hydrolysis procedure. Transfer each test sample, Brewer's yeast, and standard to separate 125 or 250 mL Erlenmeyer flasks containing 10 mL Tris buffer, pH 8.3, **B(h)***(1)* [or for very acid samples low in pantothenates requiring larger sample size, substitute Tris buffer, pH 11.3, **B(h)**(2)].

Add sufficient H_2O to obtain good suspension (typically ca 50 mL; keep total volume below 150 mL), 1.5 mL 2-octanol to prevent foaming and bumping, and autoclave 15 min at 121–123°. Cool and dilute flask contents with H_2O to concentration of 1 μg total pantothenates/mL (200 mL for standard, 100 mL for Brewer's yeast, according to known or assigned concentration for test samples).

For each test sample, carefully pipet (deliver to tube bottom, keep off tube walls) into separate 20 × 150 mm tubes: 1.0 mL test solution (ca 1 μg/mL); 0.1 mL $NaHCO_3$ solution, **B(i)**; 0.4 mL intestinal phosphatase solution, **C(d)**; 0.2 mL pigeon liver extract solution, **C(c)**; and 0.4 mL H_2O (cold). Prepare pantothenic acid standard tubes, using standard solution from previous paragraph, and treating with enzymes as for test samples (eliminates need for enzyme blank). Prepare additional pantothenic acid standard tubes, as secondary standards. (Total of 4 standard tubes needed per assay.) Prepare another tube using Brewer's yeast solution (ca 1 μg/mL) in place of test solution. As tubes are prepared, mix contents by individually rolling between palms (do not get mixture too far up tube walls), and set tubes immediately in rack in ice–salt bath. Add 0.1 mL toluene to all tubes. Cover tubes with wrapping film (Parafilm is suitable), and incubate tubes 4 h or overnight at 37°.

Adjust enzyme-treated samples, yeast, and standard (combine 4 tubes before pHing) to pH 4.5 using dilute HCl, dilute to volume calculated to contain 0.02 μg pantothenic acid/mL (typically 200 mL for standard, 50 mL for samples and yeast), and filter, **B(g)**.

To prepare primary standard (0.01 μg/mL), take 50 mL clear filtrate of standard from previous paragraph (at 0.02 μg/mL total pantothenate), adjust to pH 6.8 with dilute NaOH, and dilute to 100 mL with H_2O.

To prepare 2 secondary standards (0.008 and 0.14 μg/mL recommended), take portion of remaining clear standard (40 mL for 0.008 and 70 mL for 0.14 μg/mL), adjust pH to 6.8, and dilute to 100 mL with H_2O. Use secondary standards if sample value is either too high or too low for primary standard use.

To 25 mL clear filtrate (samples and yeast), add dilute HCl solution to check for complete precipitation. If no further precipitate forms, adjust mixture to pH 6.8 with NaOH and dilute with H_2O to volume calculated to contain ca 0.01 μg/mL total pantothenate (50 mL). If precipitate forms, adjust mixture to point of maximum precipitation (usually pH 4.5), dilute with H_2O to calculated volume, and filter. Take known aliquot of clear filtrate, adjust mixture to pH 6.8 with NaOH, and dilute with H_2O to volume calculated to contain ca 0.01 μg/mL. Designate this as assay solution.

E. Inoculum

(**a**) *Liquid culture medium.*—Dilute measured volume basal medium stock solution, **B(a)**, with equal volume aqueous solution containing 0.04 μg pantothenic acid/mL. Add 10 mL portions diluted medium to tubes, cover tubes to prevent contamination, sterilize 15 min in autoclave at 121–123°, and cool tubes as rapidly as practicable to avoid color formation from overheating. Store in dark at ca 10°.

(**b**) Make transfer of cells from stock culture of *Lactobacillus plantarum*, **960.46C(c)**, to sterile tube containing 10 mL liquid culture medium, (**a**). Incubate 6–24 h at any selected temperature between 30 and 40° held constant to ±0.5°. Under aseptic conditions, centrifuge culture and decant supernate. Wash cells with 3 ca 10 mL portions sterile 0.9% NaCl solution or sterile suspension medium, **960.46B(c)**. Resuspend cells in 10 mL sterile 0.9% NaCl solution or sterile suspension medium. Cell suspension so obtained is inoculum.

F. Assay

Using primary standard solution, **D**, and assay solution, **D**; basal medium stock solution, **B(a)**; and inoculum, **E**, proceed as in **960.46D, G**, and **H**. Value so obtained is potency of sample expressed as D-pantothenic acid. Multiply this value by 1.087 if potency is to be expressed as Ca D-pantothenate.

Ref.: JAOAC **76**, March/April issue (1993)

CAS-137-08-6 (calcium pantothenate)

CAS-79-83-4 (pantothenic acid)

Results and Discussion

Data and statistics for Phase V are provided in Tables 1–5. Statistical analysis used protocols in accordance with AOAC "Guidelines for Collaborative Study Procedure" (7). The data were screened for outliers by using the Dixon, Cochran, and Grubbs outlier tests. Subsequent estimation of repeatability and reproducibility parameters were performed with and without outliers.

For the nutrients studied, the ranges for reproducibility standard deviations (RSD_R) were as follows: folic acid, 22.5–25.5%; pantothenic acid, 7.6–10.8%; vitamin A (retinol isomers), 8.2–10.7; vitamin A (retinol), 8.8–13.3; and vitamin E, 9.0–12.8.

Figure 1 is a plot of the RSD_R values vs concentration of analyte on a log scale. A value of 7 for the negative log of the concentration corresponds to a concentration of 0.1 ppm; 6 corresponds to 1 ppm; 5 corresponds to 10 ppm, etc. The lower line in the graph, known as the Horwitz curve, represents the average RSD_R for a large number of collaborative studies at various concentrations; the upper line, which is twice the lower values, represents a guideline for the upper limit of acceptability of a method. A complete discussion of the Horwitz curve and its implication was presented by Horwitz et al. (8).

As shown in Figure 1, the precision of the Phase V data corresponds to the Horwitz curve with few exceptions. As noted in the AOAC "Guidelines for Collaborative Study" (7), "In some cases, there is no direct measure of bias, and consensus values derived from the collaborative study itself often must be used for the reference point."

Recommendations

Results of this collaborative study compare very favorably with the empirical norm developed via the Horwitz curve for each of the analytes studied.

On the basis of the precision of these data and the correspondence to the empirical norm, as well as the relative ease of use of methods and the fact that all the parties affected by the Infant Formula Act can perform the assays, it is recommended that the methods for folic acid, pantothenic acid, vitamin A by either determination, and vitamin E in milk-based infant formula be adopted first action.

Table 1. Collaborative results for determination of folic acid in milk-based formula (μg/L)[a]

Lab.	Sample 1		Sample 2		Sample 3	
A	120	120	210	210	240	230
C	59.8	81.7	110	156	139	203
D	96.8	100.2	184.7	187.6	246.5	184.9
E	116	134	216	244	301	312
H	110	0[b]	227	243	306	0[b]
I	125	127.4	244.9	254.9	272.4	277.4
J	76	80	180	180	200	220
O	0[c]	72	165	168	195	207
P	86.25	82.86	137.81	138.64	140.36	137.87

Sample	No. of labs.[d]	Average	RSD_r, %[e]	RSD_R, %[f]
1	9	98.3	7.9	23.9
2	9	192.1	7.02	22.5
3	9	228.8	10.2	25.5

[a] Samples were analyzed as blind duplicates.
[b] Out of standard range.
[c] Could not be calculated.
[d] No outliers were identified.
[e] RSD_r = repeatability relative standard deviation.
[f] RSD_R = reproducibility relative standard deviation.

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Table 2. Collaborative results for determination of pantothenic acid in milk-based infant formula (mg/L)[a]

Lab.	Sample 1		Sample 2		Sample 3	
A	4.0	4.0	5.4	5.2	4.9	4.9
C	4.33	4.26	5.09	5.61	5.00	5.44
D	4.18	4.08	5.08	5.19	5.24	4.72
E	3.80[b]	5.13[b]	5.10	4.03	4.82	4.78
H	4.8	4.6	6.1	5.8	5.6	6.0
J	4.0	4.0	5.6	5.1	4.5	4.7
O	4.1	4.1	5.3	5.3	5.1	5.0
P	3.60	3.80	4.33	4.32	4.22	4.33

Sample	No. of labs.	Average	RSD_r, %[c]	RSD_R, %[d]
1	8	4.2	8.2	9.4
	7[e]	4.1	2.0	7.6
2	8	5.2	6.5	10.8
3	8	5.0	4.2	9.5

[a] Samples were analyzed as blind duplicates.
[b] Outlier by Cochran test.
[c] RSD_r = repeatability relative standard deviation.
[d] RSD_R = reproducibility relative standard deviation.
[e] Outliers excluded.

Table 3. Collaborative results for LC determination of vitamin A (retinol isomers) in milk-based infant formula (IU/L)[a]

Lab.	Sample 1		Sample 2		Sample 3	
A	3070	2980	2480	2380	2660	2520
B	3198	3351	2596	2755	2782	2848
C	3100	3030	2510	2660	2720	2660
D	2418	2338	3099[b]	1914[b]	2184	2276
E	2270[b]	3160[b]	2140	2490	3020	3140
F	3153	3230	2548	2565	2780	2814
G	3059	3099	2425	2451	2640	2669
H	3533	3492	2688	2762	2753[b]	3215[b]
I	2641	2533	2352	1849	2314	2166
J	3400	3200	2600	2700	2900	2800
M	3028.9	3094.6	2493.8	2459.2	2638.0	2632.8
P	2967	2893	2480	2491	2680	2678

Sample	No. of labs.	Average	RSD_r, %[c]	RSD_R, %[d]
1	12	3010	6.5	11.6
	11[e]	3037	2.4	10.7
2	12	2495	11.1	11.1
	11[e]	2494	5.7	8.2
3	12	2687	4.1	10.0
	11[e]	2660	2.3	9.5

[a] Samples were analyzed as blind duplicates.
[b] Outlier by Cochran test.
[c] RSD_r = repeatability relative standard deviation.
[d] RSD_R = reproducibility relative standard deviation.
[e] Outliers excluded.

Table 4. Collaborative results for LC determination of vitamin A (retinol) in milk-based infant formula (IU/L)[a]

Lab.	Sample 1		Sample 2		Sample 3	
A	2920	2840	2380	2280	2550	2460
B	3255	3042	2572	2441	2795	2586
C	3380	3460	3170	2740	3310	2900
D	2812	2709	1758	1861	2647	2688
F	3010	3046	2491	2505	2523	2576
H	2551	3003	2278	2358	2342	2515
I	2543	2704	2144	2179	2207	2298
J	3000	3100	2300	2400	2600	2800
P	3042	3226	2407	2504	2613	2716

Sample	No. of labs.[b]	Average	RSD_r, %[c]	RSD_R, %[d]
1	9	2980	4.6	8.8
2	9	2376	5.0	13.3
3	9	2618	5.0	9.6

[a] Samples were analyzed as blind duplicates.
[b] No outliers were identified.
[c] RSD_r = repeatability relative standard deviation.
[d] RSD_R = reproducibility relative standard deviation.

Table 5. Collaborative results for LC determination of vitamin E in milk-based infant formula (IU/L)[a]

Lab.	Sample 1		Sample 2		Sample 3	
A	20.0	17.6	30.6	24.9	28.4	24.7
B	19.8	21.6	28.7	31.3	27.0	27.6
C	17.7	17.1	27.2	25.6	25.6	25.0
D	19.3	20.7	30.7	30.9	31.3	31.3
F	17.52	18.19	25.15	26.31	25.36	25.02
I	17.59	17.53	23.49	25.65	23.37	23.04
J	18.5[b]	31[b]	29	18.6	29	31
P	17	16	25	24	23	25

Sample	No. of labs.	Average	RSD_r, %[c]	RSD_R, %[d]
1	8	19.20	16.9	18.2
	7[e]	18.40	5.2	9.0
2	8	26.69	11.7	12.8
3	8	26.61	4.5	11.1

[a] Samples were analyzed as blind duplicates.
[b] Outlier by Cochran test.
[c] RSD_r = repeatability standard deviation.
[d] RSD_R = reproducibility standard deviation.
[e] Outlier excluded.

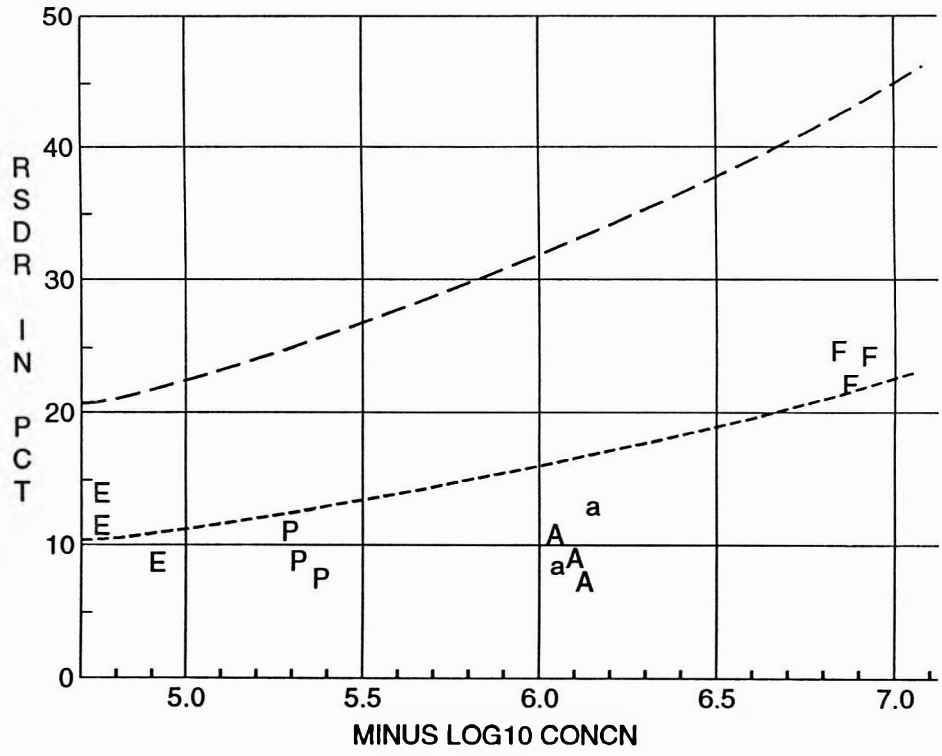

Figure 1. Plot of RSD_R values vs concentration of analyte (log_{10} scale) for determination of vitamin A-retinol (a), vitamin A-retinol isomers (A), vitamin E (E), folic acid (F), and pantothenic acid (P). Lower line is the Horwitz curve (8); upper line is twice the lower RSD_R values. On x-axis (minus log_{10} concentration), 7 corresponds to concentration of 0.1 ppm, 6 to 1 ppm, and 5 to 10 ppm.

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TECHNICAL COMMUNICATIONS

Comparison of Liquid Chromatographic Method to AOAC Microbiological Method for Determination of L-Tryptophan in Tablets and Capsules

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A reversed-phase liquid chromatographic (LC) method coupled with precolumn derivatization of L-tryptophan with phenylisothiocyanate was compared to the AOAC microbiological method for determining L-tryptophan in tablets and capsules. For the microbiological method, the concentrations of L-tryptophan were 4–8% lower in autoclaved test samples (hot method) than in test samples that were not autoclaved (cold method). When L-tryptophan values obtained by the LC method were compared to those obtained by the cold microbiological method, no significant differences were observed ($P > 0.05$). The mean relative standard deviations were 2.9% for the LC method and 1.6% for the cold microbiological method. The mean recoveries of standard L-tryptophan added before analysis were 99% for the LC method and 101% for the cold microbiological method. These results demonstrate that both methods are reliable for determining free L-tryptophan contained in tablets and capsules. However, the LC method has the advantages of using a smaller test portion and having a shorter analysis time.

In the summer and fall of 1989, an epidemic outbreak of eosinophilia-myalgia syndrome in the United States was associated with the consumption of products containing L-tryptophan (1, 2). Consequently, the U.S. Food and Drug Administration (FDA) recalled all products that contained L-tryptophan as the sole or major component, and this laboratory analyzed numerous L-tryptophan dietary supplement tablets and capsules for their L-tryptophan concentrations.

Because of the number of L-tryptophan-containing products that our laboratory received for analysis, both a liquid chromatographic (LC) method (3, 4) and the revised AOAC microbiological method (5, 6) were used to determine L-tryptophan concentrations. The purpose of this study was to assess the reliability of both methods by comparing the results of analyses of L-tryptophan-containing tablets and capsules.

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Experimental

Apparatus

(a) *Liquid chromatographic system.*—Pico-Tag Amino Analysis System (Waters Chromatography Div., Millipore, Milford, MA) consisting of reversed-phase C_{18} analytical column, 15 × 0.4 cm id; column temperature control module set at 38°C; 2 Model 510 pumps connected in series; Model U6K manual injector; Model 440 UV detector set at 254 nm; Model 840 data and chromatography control station; system interface module.

(b) *Pico-Tag workstation.*—Waters Chromatography Div., Millipore.

(c) *Reaction vial.*—Waters Chromatography Div., Millipore, Cat. No. 07363.

(d) *Filter disc.*—Gelman Sciences, Ann Arbor, MI, Cat. No. ACRO LC13.

(e) *Automatic pipetting machines.*—BBL Brewer (Becton Dickinson Microbiological Systems, Cockeysville, MD, Models 93112 and 60453), or equivalent.

(f) *Autoclave.*—American Sterilizer, Erie, PA.

(g) *Gilson Escargot fractionator.*—Model SC-30 (Gilson Medical Electronics, Inc., Middleton, WI) modified with air agitation system and connected to spectrophotometer.

Reagents

(a) *Mobile phase A.*—0.14M sodium acetate–acetonitrile (94 + 6), pH 6.40 ± 0.03, containing 0.05% triethylamine.

(b) *Mobile phase B.*—Acetonitrile–water (60 + 40).

(c) *Diluent.*—5mM disodium hydrogen phosphate–acetonitrile (95 + 5), pH 7.40.

(d) *L-Tryptophan standard.*—United States Pharmacopeial Convention, Inc., Rockville, MD.

(e) *Acetonitrile and methanol.*—Burdick & Jackson Laboratories, Inc., Muskegon, MI.

(f) *Sodium acetate trihydrate, disodium hydrogen phosphate, and barium hydroxide.*—Fisher Scientific Co., Pittsburgh, PA.

(g) *Triethylamine (TEA).*—Aldrich Chemical Co., Milwaukee, WI.

(h) *Phenylisothiocyanate (PITC).*—Pierce Chemical Co., Rockford, IL.

Table 1. Comparison of LC and AOAC microbiological (hot) methods for determination of L-tryptophan (TRP) in tablets

Tablet	Label claim, mg TRP	TRP concn, g/100 g	TRP, mg/tablet[a]		*P* value[b]
			LC	AOAC	
A	100	24.1	95.8 ± 1.0	93.3 ± 1.3	0.0195
B	100	26.1	103.8 ± 2.2	101.5 ± 1.3	0.1300
C	500	72.6	513.5 ± 4.4	487.8 ± 10.7	0.0043
D	500	68.1	489.3 ± 5.7	465.8 ± 5.0	0.0008
E	500	66.2	495.5 ± 4.4	465.0 ± 5.8	0.0002
F	500	41.4	486.5 ± 6.6	463.3 ± 4.3	0.0010
G	667	58.5	655.8 ± 13.5	613.0 ± 9.6	0.0021
Mean RSD, %[c]			1.4	1.4	

[a] Mean ± standard deviation, $n = 4$.
[b] *P* value > 0.05 indicates no significant difference (2-tailed *t*-test) between the 2 methods.
[c] RSD = relative standard deviation.

(i) *Hydrochloric acid.*—Ultrex grade (J.T. Baker Chemical Co., Phillipsburg, NJ).

(j) *Lactobacillus plantarum.*—ATCC 8014 (American Type Culture Collection, Rockville, MD).

Test Sample Preparation

L-Tryptophan tablets and capsules were collected by the FDA as a result of the tryptophan-associated eosinophilia-myalgia syndrome outbreak in the United States in 1989. These products were obtained from various geographic locations within the United States and represent several manufacturers.

Ten tablets or contents of 10 capsules from a single source were combined, weighed accurately, and ground to a fine powder to obtain a homogeneous test sample. The average weight per tablet or capsule was calculated from the combined weight of 10 tablets or 10 capsules.

For microbiological assay (hot method), 10 mL distilled water was added to a test portion equivalent in weight to that of 1 tablet or capsule and 15.4 g barium hydroxide in a 125 mL poly(propylene) Erlenmeyer flask, and the solution was autoclaved 30 min at 121–123°C, 15 psi. While the autoclaved solution was still hot, 50 mL distilled water and 15 mL 9N H_2SO_4 were added, and the pH was adjusted to 4.0–4.5 with either 9N H_2SO_4 or 1N NaOH. The solution was then quantitatively transferred to a 500 mL graduated cylinder, diluted to 500 mL with distilled water, filtered, and adjusted to a pH of 6.8 with 0.01N NaOH. The pH-adjusted solution was diluted with distilled water to a final L-tryptophan concentration of 1 μg/mL, based on the L-tryptophan content declared on the product label.

For cold microbiological assay, a test portion equivalent to that of 1 tablet or capsule was sonicated in 500 mL distilled water for 15–20 min and diluted with distilled water to a final L-tryptophan concentration of 1 μg/mL.

For LC analysis, a test portion of ca 100 mg ground tablets or capsules was accurately weighed into a 100 mL volumetric flask and diluted to volume with distilled, deionized water. The solution was sonicated 15–20 min.

Table 2. Comparison of LC and AOAC microbiological (hot) methods for determination of L-tryptophan (TRP) in capsules

Capsule	Label claim, mg TRP	TRP concn, g/100 g	TRP mg/capsule[a]		*P* value[b]
			LC	AOAC	
A	500	97.6	517.5 ± 9.0	488.3 ± 10.7	0.0058
B	500	91.4	515.5 ± 8.6	483.0 ± 7.4	0.0012
C	500	85.7	485.3 ± 7.2	455.3 ± 4.4	0.0004
D	500	86.9	476.3 ± 7.3	427.8 ± 3.8	0.0001
E	667	92.0	620.0 ± 3.6	577.3 ± 7.9	0.0001
F	1000	95.8	997.8 ± 8.7	935.3 ± 9.7	0.0001
G	1000	94.7	978.0 ± 16.1	918.5 ± 8.0	0.0006
Mean RSD, %[c]			1.4	1.3	

[a] Mean ± standard deviation, $n = 4$.
[b] *P* value > 0.05 indicates no significant difference (2-tailed *t*-test) between the 2 methods.
[c] RSD = relative standard deviation.

Table 3. Comparison of AOAC microbiological hot and cold methods for determination of L-tryptophan (TRP) in tablets and capsules

Tablet/capsule	Label claim, mg TRP	TRP concn, g/100 g	TRP, mg/tablet or capsule[a]		Loss by hot method, %
			Cold method	Hot method	
Tablet A	100	25.4	100.8 ± 1.0	96.3 ± 3.1	4.5
Tablet C	500	74.1	523.8 ± 7.9	480.5 ± 3.9	8.3
Tablet G	667	55.9	627.0 ± 23.2	587.0 ± 18.7	6.4
Capsule A	500	95.7	507.3 ± 6.1	483.8 ± 2.2	4.6
Capsule E	667	90.4	609.3 ± 8.9	582.0 ± 4.3	4.5
Capsule G	1000	96.8	1000.0 ± 7.4	942.0 ± 8.5	5.8
Mean					5.7
Mean RSD, %[b]			1.6	1.6	

[a] Mean ± standard deviation, $n = 4$.
[b] RSD = relative standard deviation.

Microbiological Assay

L-Tryptophan solutions were assayed according to the revised official AOAC method (5, 6), with *Lactobacillus plantarum* ATCC 8014 as the test organism.

LC Determination

(a) *Derivatization.*—A 20 µL portion of the sonicated solution was placed in a 6 × 50 mm test tube and dried under vacuum. The residue was mixed with 20 µL methanol–water–TEA (1 + 1 + 1), and the mixture was dried under vacuum. The redried residue was then derivatized 20–25 min with 20 µL methanol–water–TEA–PITC (7 + 1 + 1 + 1) at room temperature, and the derivatization mixture was dried 1 h under vacuum.

(b) *Chromatography.*—The residue was resuspended in 100 µL diluent, and 5 µL aliquots were injected into the LC system. Elution of L-tryptophan began with 100% mobile phase A at a flow rate of 1.0 mL/min. Mobile phase B was increased from 0 to 45% B in 10 min, increased from 46 to 100% B from 10 to 10.5 min, and maintained at 100% B until the end of analysis at 12 min. L-Tryptophan eluted at 9.4–9.6 min, and the chromatographic peak areas of the analytes were compared to standard L-tryptophan curves for quantitation.

Results and Discussion

In the revised AOAC microbiological assay for determination of L-tryptophan (6), infant formula test portions were hydrolyzed with barium hydroxide by autoclaving before the assay. For this study, L-tryptophan-containing tablets and capsules were similarly autoclaved (hot method) with barium hydroxide before the assay. Comparisons of L-tryptophan values determined by this hot microbiological assay to those values determined by the LC method for tablets and capsules are presented in Tables 1 and 2, respectively. Results show that for both tablets and capsules, the hot microbiological assay consistently yielded L-tryptophan values that were lower than those obtained by the LC method.

Consequently, because the tablets and capsules contained the free form of L-tryptophan, microbiological assays without autoclaving (cold method) were conducted. Results in Table 3 show that the concentrations of L-tryptophan were 4–8% lower

Table 4. Comparison of LC and AOAC microbiological (cold) methods for determination of L-tryptophan (TRP) in tablets and capsules

Tablet/capsule	Label claim, mg TRP	TRP concn, g/100 g	TRP, mg/tablet or capsule[a]		*P* value[b]
			LC	AOAC	
Tablet A	100	24.8	95.8 ± 1.5	100.8 ± 1.0	0.0004
Tablet C	500	73.0	507.8 ± 12.2	523.8 ± 7.9	0.0517
Tablet G	667	57.0	651.7 ± 22.3	627.0 ± 23.2	0.1295
Capsule A	500	95.6	506.0 ± 17.5	507.3 ± 6.1	0.8957
Capsule E	667	90.5	610.7 ± 16.4	609.3 ± 8.9	0.6754
Capsule G	1000	95.9	981.8 ± 35.6	1000.0 ± 7.4	0.3528
Mean RSD, %[c]			2.9	1.6	

[a] Mean ± standard deviation, $n = 4$.
[b] *P* value > 0.05 indicates no significant difference (2-tailed *t*-test) between the 2 methods.
[c] RSD = relative standard deviation.

Table 5. Recovery of L-tryptophan (TRP) standard added to tablets or capsules before analysis

	AOAC[a]		LC[a]	
Tablet/capsule	TRP added, mg	Rec., %	TRP added, mg	Rec., %
Tablet G	593.7	99.9	63.3	100.2
Tablet G	598.8	100.7	59.0	101.9
Capsule E	579.2	99.9	97.6	95.3
Capsule E	570.6	102.2	107.2	98.5
Mean ± SD[b]	100.7 ± 1.1		99.0 ± 2.8	

[a] Each value represents the average of duplicate analyses.
[b] SD = standard deviation.

in autoclaved tablets and capsules (hot method) than in those that were not autoclaved (cold method). These results suggest that the high temperature and pressure during autoclaving may degrade L-tryptophan; evidence of degradation can be detected as extra chromatographic peaks in LC analysis, if the degradation by-products react with the derivatizing reagent, PITC, for UV detection at 254 nm. Therefore, LC analyses of L-tryptophan-containing tablets and capsules after autoclaving to determine the presence of possible degradation peaks will be a topic for future study.

Comparisons of L-tryptophan values determined by the LC method and by the cold microbiological assay for tablets and capsules are shown in Table 4. No significant differences ($P > 0.05$, 2-tailed t-test) were observed between these 2 methods. The LC method and the cold microbiological assay demonstrated excellent repeatability based on mean relative standard deviations of 2.9 and 1.6%, respectively. Recoveries of standard L-tryptophan added to tablets or capsules before analysis averaged 101% for the cold microbiological assay and 99% for the LC method (Table 5).

Conclusions

This study demonstrated that the LC and the revised AOAC microbiological methods are reliable for determining L-tryptophan in tablets and capsules. On the basis of the statistical 2-tailed t-test, L-tryptophan values determined by either the cold microbiological method or the LC method showed no significant differences for identical test samples of tablets or capsules. Both methods demonstrated excellent repeatability and good recoveries of standard L-tryptophan added before the analysis. The LC method, however, has the advantages of using a smaller test portion and having a shorter analysis time.

This study also demonstrated that L-tryptophan values determined by the hot microbiological method were consistently 5–8% lower than L-tryptophan values determined by the cold microbiological method. Autoclaving of test samples by the hot microbiological method may have degraded some of the L-tryptophan in the tablets and capsules.

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TECHNICAL COMMUNICATIONS

Sample Preparation Bias in Carbon Stable Isotope Ratio Analysis of Fruit Juices and Sweeteners

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Two sample preparation methods are commonly used for carbon stable isotope ratio analysis (SIRA). One involves combustion of the sample with oxygen at 850°C; the other involves combustion of the sample with CuO in an evacuated glass tube at 550°C. I observed in our laboratory that these 2 methods yield different results for sugar-based products such as fruit juices, sweeteners, and vanillin. The CuO method yields results approximately 1‰ more positive than the oxygen combustion method. This bias is also observed in other laboratories, as shown in an analysis of the results of the AOAC collaborative studies of carbon SIRA of maple syrup, orange juice, honey, and honey protein. The oxygen combustion method is the AOAC method for honey, apple juice, and orange juice; both methods are incorporated into the AOAC method for maple syrup. I recommend that data generated by the CuO combustion method be appropriately corrected to yield results concordant with the official oxygen combustion method.

Carbon stable isotope ratio analysis (SIRA) has been very successful in detecting adulteration with undeclared corn syrup or cane sugar of maple syrup (1), honey (2), apple juice (3), orange juice (4), and other fruit products and sweeteners (5). It has also been useful in detecting the addition of artificial vanillin to vanilla extract (6).

The carbon SIRA method measures small variations in the ^{13}C content of the carbon of different plant types produced by different photosynthetic pathways. Most fruits and grains are Calvin cycle (C_3) pathway plants yielding ^{13}C values near −25‰; cane and corn are Hatch-Slack (C_4) pathway plants with ^{13}C values near −10‰; most succulents (including pineapple and vanilla) are crassulacean acid metabolism (CAM) plants that yield variable values.

Because the ability to detect added sweeteners by the carbon SIRA method is limited in part by the analytical reproducibility of the method, analytical biases due to variations in analytical technique must be understood and accounted for in data interpretation. There are 2 methods in widespread use in the United States for the sample preparation of fruit juices and sweeteners for carbon SIRA. The first method is the AOAC official method for sample combustion of honey, maple syrup, apple juice, and orange juice (7). In this procedure, the sample is combusted with oxygen at 850°C, and the combustion gases are recirculated over hot CuO. The second method is the alternative AOAC official method for sample combustion of maple syrup (7). In this procedure, the sample is combusted in vacuo over CuO at 550°C in sealed glass tubes.

In this paper, I report the existence of a significant and consistent sample preparation bias between these 2 methods of sample preparation.

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Experimental

Sample combustion with oxygen at 850°C.—The first method (**978.17C**) involves combustion of the sample in oxygen at 850°C, with recycling of the combustion gases over hot CuO. The CO_2 is then trapped and purified cryogenically in the usual manner.

Sample combustion with CuO in evacuated tubes at 550°C.—The second method (**984.23D**) involves combusting the sample with CuO at 550°C in sealed, evacuated Pyrex glass tubes. After combustion, tubes are cooled and opened under vacuum; the CO_2 is trapped and purified cryogenically in the usual manner.

Determination of $^{13}C/^{12}C$ ratio.—$^{13}C/^{12}C$ ratios were determined by AOAC method **978.17D**. ^{13}C values of all the CO_2 samples were measured blind by the same analyst on the same instrument using the same split of working reference gas to eliminate any effects of analyst and calibration bias.

Discussion

Table 1 lists the results for 17 samples of fruit juices and sweeteners analyzed by both sample preparation methods. Table 2 lists the results for 5 samples of natural vanillin analyzed by both sample preparation methods. Results from the sealed-tube combustion method were significantly more positive than those from the oxygen combustion method. The bias averages +0.6‰ for sugar products and +1.4‰ for vanillin.

These results are consistent with the results of 4 AOAC collaborative studies on orange juice (4), maple syrup (8), honey,

Table 1. Comparison of AOAC and sealed-tube methods of sample preparation of fruit juices and sweeteners (‰)

Sample	^{13}C (AOAC)	^{13}C (sealed tube)	Difference
Orange juice	–25.9	–25.5	+0.4
Orange juice	–25.9	–25.3	+0.6
Orange juice	–25.4	–25.1	+0.3
Orange juice[a]	–20.9	–20.5	+0.4
Apple juice	–25.0	–24.2	+0.8
Apple juice	–25.3	–24.5	+0.8
Apple juice	–25.5	–25.0	+0.5
Apple juice	–24.3	–24.0	+0.3
Concord grape juice	–25.3	–23.8	+1.5
Cranberry juice	–25.5	–24.8	+0.7
Maple syrup	–24.1	–23.5	+0.8
Maple syrup	–22.8	–22.5	+0.3
Maple syrup[a]	–20.8	–20.1	+0.7
Honey[a]	–21.7	–21.4	+0.3
Honey[a]	–22.5	–22.1	+0.4
Honey[a]	–23.5	–23.1	+0.4
Corn syrup	–11.7	–11.1	+0.6
Mean			+0.6

[a] Sample contains some added sugar.

Table 2. Comparison of AOAC and sealed-tube methods of sample preparation of vanillin (‰)

Sample	^{13}C (AOAC)	^{13}C (sealed tube)	Difference
Vanillin	–20.8	–19.3	+1.5
Vanillin	–21.0	–19.5	+1.5
Vanillin	–20.4	–19.4	+1.0
Vanillin	–21.8	–20.1	+1.7
Vanillin	–20.5	–19.3	+1.2
Mean			+1.4

Table 3. Sample preparation bias in the AOAC orange juice collaborative study (‰)

Sample	Coll. 2–7, Av.	Coll. 1	Difference
A	–21.2	–20.2	+1.0
B	–14.6	–13.2	+1.4
C	–25.7	–24.9	+0.8
D	–16.6	–15.5	+1.1
E	–19.0	–18.0	+1.0
Mean			+1.1

and honey protein (9). The collaborators for these studies that used the sealed-tube combustion procedure obtained the most positive values.

Table 4. Sample preparation bias in the AOAC maple syrup collaborative study (‰)

Sample	Coll. 3–5, Av.[a]	Coll. 1, 2; Av.	Difference
1	–21.8	–20.5	+1.3
2	–24.3	–23.4	+0.9
3	–22.7	–22.2	+0.5
4	–24.3	–23.6	+0.7
5	–22.7	–22.0	+0.7
6	–24.9	–24.0	+0.9
7	–24.7	–23.6	+1.1
Mean			+0.9

[a] Collaborator 6 deleted because of poor reproducibility.

Table 5. Sample preparation bias in the AOAC honey syrup collaborative study (‰)

Sample	Coll. 4, 7; Av.	Coll. 1–3, 5, 6, 8, 9; Av.	Difference
1	–23.3	–23.0	+0.3
2	–23.6	–23.1	+0.4
3	–23.0	–22.5	+0.5
4	–23.6	–23.1	+0.5
5	–23.3	–22.7	+0.6
6	–22.3	–21.9	+0.4
7	–22.7	–22.1	+0.6
8	–22.6	–22.1	+0.5
9	–22.5	–22.1	+0.4
10	–22.9	–22.7	+0.2
Mean			+0.4

In the AOAC orange juice collaborative study (Table 3), one laboratory indicated that sealed-tube combustions at 600°C were used. Results from that laboratory averaged 1.1‰ more positive than the average of the other 6 laboratories. This laboratory also had the most positive results of the 7 collaborators.

In the AOAC maple syrup collaborative study (Table 4), 2 laboratories indicated that they used sealed-tube combustions at 550°C. Results from those 2 laboratories averaged 0.9‰ more positive than the average of the other 3 laboratories. These 2 laboratories also had the 2 most positive results of the 5 collaborators providing reproducible results.

In the AOAC honey and honey protein collaborative studies (Tables 5 and 6), 7 laboratories indicated that they had used sealed-tube combustions. Results from these 7 laboratories were, on average, 0.4‰ more positive for honey and 0.8‰ more positive for honey protein than results from the other 2 laboratories that used oxygen combustion.

Determining which method yields results closer to the "true value" and which fractionations or contaminations are the ultimate cause of the sample preparation difference will require further study. I observed that combustion by the sealed-tube procedure often leaves a small amount of black residue deposited on the inside of the tube. I speculate that this material may

Table 6. Sample preparation bias in the AOAC honey protein collaborative study (‰)

Sample	Coll. 4, 7; Av.	Coll. 1–3, 5, 6, 8, 9; Av.	Difference
1	–23.9	–23.2	+0.7
2	–25.0	–23.3	+1.7
3	–24.0	–23.4	+0.6
4	–24.6	–23.8	+0.8
5	–24.3	–23.8	+0.5
6	–23.5	–22.8	+0.7
7	–24.2	–23.4	+0.8
8	–24.0	–23.7	+0.3
9	–24.6	–23.9	+0.7
10	–25.0	–24.3	+0.7
Mean			+0.8

contain a small amount of highly fractionated carbon and may account for the relatively positive values obtained by this combustion procedure.

Given the widespread use of ^{13}C data in evaluating flavor and sugar products, I recommend that data generated by the sealed-tube combustion method be appropriately corrected to yield results concordant with the oxygen combustion method.

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TECHNICAL COMMUNICATIONS

Liquid Chromatographic Determination and Liquid Chromatographic–Thermospray Mass Spectrometric Confirmation of Nicarbazin in Chicken Tissues: Interlaboratory Study

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The U.S. Food and Drug Administration sponsored an interlaboratory study of a liquid chromatographic determination with ultraviolet detection of nicarbazin in chicken liver and muscle tissues. The method determined the 4,4′-dinitrocarbanilide (DNC) portion of nicarbazin. The interlaboratory study of the determinative method was successful for nicarbazin at the 4 ppm level. Results showed good reproducibility for the fortified liver and muscle samples. Mean interlaboratory recoveries and percent coefficients of variation at about 4 ppm were 87.1 and 10.9%, respectively, for muscle and 87.4 and 7.5%, respectively, for liver. The interlaboratory analyses of the dosed liver and muscle tissues produced concentration levels similar to those obtained by the sponsor. The confirmatory procedure, which identified DNC in purified tissue extracts, used liquid chromatography–thermospray/mass spectrometry. The confirmatory procedure was successfully evaluated by one FDA laboratory.

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Nicarbazin is a 1 + 1 (mol/mol) complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyridine (HDP). The U.S. Food and Drug Administration (FDA) approved nicarbazin for the prevention of coc-

cidiosis in chickens (1), and a tolerance of 4 ppm was established in uncooked chicken muscle, liver, skin, and kidney (2). In the original official methods for tissue residues, DNC was measured polarographically (3–5). DNC was monitored, because it is excreted more slowly than HDP.

Eli Lilly and Co. developed a liquid chromatographic (LC) determination and an LC–thermospray/mass spectrometric (MS) confirmation for nicarbazin in chicken tissues in 1986 (6). The LC determination was successfully validated in 1987 in chicken liver and muscle tissues by 3 FDA laboratories and 1 Food Safety and Inspection Service (FSIS) laboratory. The confirmation was validated by 1 FDA laboratory. The methods used and the results of that interlaboratory trial are described in this report.

METHOD

Reagents

(a) *Solvents.*—Acetonitrile, dimethylformamide (DMF), ethyl acetate, hexane, and methanol: LC grade. Purify water with Millipore purification system, or equivalent.

(b) *Ammonium acetate.*—Reagent grade.

(c) *Nicarbazin analytical standard.*—Eli Lilly and Co.

(d) *Standard stock solution.*—1 mg/mL in DMF; dissolve by heating to 75–80°C. Solution is stable 1 month at room temperature.

(e) *Fortification solution.*—Dilute standard stock solution with ethyl acetate, usually to 20 μg nicarbazin/mL.

(f) *Standard curve solutions.*—Prepare series of standard solutions containing 2.0–8.0 μg nicarbazin/mL in methanol–water (75 + 25). These solutions are stable at least 1 month when stored cold. Because nicarbazin precipitated in the 8 μg/mL standard, some trial participants used 6 μg/mL as the most concentrated standard. Additionally, intermediate solutions used to prepare standards precipitated rapidly.

(g) *Determinative mobile phase.*—Methanol–water (75 + 25, v/v).

(h) *Confirmatory mobile phase.*—Dissolve 3.85 g ammonium acetate in 1 L of the determinative mobile phase.

Apparatus

(a) *Liquid chromatograph.*—Instrument capable of maintaining pulseless flow of mobile phase at 0.8–1.0 mL/min. Column: 25 cm × 4.6 mm id, stainless steel, packed with 3 μm C_{18}, Phenomenex IB-SIL, or equivalent. Operating conditions: flow rate, 0.8–1.0 mL/min; column temperature, ambient; injection volume, 50 μL; sensitivity, 0.2 AUFS. Effluent monitored by peak response measurements at 340 nm.

(b) *Mass spectrometer.*—Finnigan 4600, or equivalent, equipped with thermospray interface. Chromatograph samples from determinative method under LC conditions similar to determinative method. Selectively monitor ions at *m/z* 164, *m/z* 272, and *m/z* 302, using negative ion thermospray MS. Filament-on ionization was used, because the FDA thermospray was not equipped with discharge ionization as specified in the method.

(c) *Homogenizer.*—Sorvall Omni-Mixer. Do not substitute Tissumizer or high-speed blender, because homogenate filters slowly and recovery is reduced.

(d) *Evaporator.*—Rotary vacuum.

(e) *Cartridge chromatography column.*—Waters Sep-Pak Alumina B cartridge columns, No. 51820.

(f) *Filter paper.*—Schleicher & Schuell No. 588.

Sample Extraction

Homogenize 10 g ground control, fortified, or dosed chicken liver or muscle samples, at medium speed or ca 1/2 speed, 3 times with 25 mL ethyl acetate. Carefully decant each extract through filter paper, and evaporate combined extract to oily residue. Dissolve residue in 10 mL acetonitrile, and extract with 50 mL hexane. Save acetonitrile layer. Extract hexane layer twice with 10 mL acetonitrile. Combine acetonitrile layers; extract with 50 mL hexane. Discard all hexane layers. Evaporate combined acetonitrile extract to dryness. Connect 2 alumina B Sep-Paks in tandem, and condition with 4 mL DMF. Apply pressure gently with rubber bulb to initiate flow. Dissolve dry residue in 2 mL DMF and transfer to Sep-Paks. Wash with DMF (14 mL total), and eliminate as much DMF as possible from column. Add 10 mL methanol to Sep-Paks and elute DNC. Evaporate this extract to ca 2–3 mL, and dilute to 10 mL with methanol–water (75 + 25).

Data Analysis for Determinative Method

The concentration of nicarbazin in the final extract was determined from the peak response measurements for the standard nicarbazin solutions. A standard curve in the form of $y = mx + b$ was generated by linear regression analysis, where y = peak area, x = concentration in μg/mL, m = slope, and b = y intercept. In the equation for dosed tissue, the molar ratio of DNC to HDP is considered constant. The concentration of nicarbazin in dosed-tissue samples was calculated from the equation below, which does not adjust for recovery from fortified control tissue samples.

$$\text{Nicarbazin, } \mu g/mL = (Cs \times V \times AF \times Fdnc)/(W \times 0.7089)$$

where Cs = concentration (μg/mL) determined from the nicarbazin standard curve, V = final volume (usually 10 mL), AF = aliquot factor (usually 1.0), $Fdnc$ = actual fraction by weight of DNC in nicarbazin reference standard (0.7081), W = weight of tissue extracted (10 g), and 0.7089 = theoretical fraction by weight of DNC in nicarbazin reference standard.

Results and Discussion

Tables 1 and 2 summarize the data for fortified tissue from the 4 participating laboratories. All laboratories reported linear standard curves and no significant interferences. A trace of interference was reported in one control liver sample close to the retention time of DNC. Interferences of less than 2% of the tolerance were detected by another laboratory in control muscle samples at the retention time of DNC. The Center for Veterinary Medicine (FDA) guidelines for a method for determining an analyte at 0.1 ppm and above are an intralaboratory recovery of 80–110%, CV of 10% or less, and interferences of less than 10% of the response at the tolerance level. The liver and muscle recovery data meet these guidelines. Interlabora-

Table 1. Percent recovery of nicarbazin from fortified, control chicken liver tissues by LC method

	Laboratory			
Added, ppm	1	2	3	4
0	ND[a]	Trace	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
	ND	ND	ND	ND
2.00	78.3	95.5	89.5	93.5[b]
	87.6	99.0	90.0	96.4
	81.7	98.5	90.0	92.1
	76.8	99.5	85.0	94.2
	82.5	95.0	94.0	95.0
Mean, %	81.4	97.5	89.7	94.2
CV, %	5.2	2.1	3.6	1.7
4.00	81.1	86.8	93.3	—
	88.7	73.2	90.3	—
	81.3	82.0	80.8	—
	81.0	87.0	86.3	—
	79.1	90.0	94.8	—
Mean, %	82.2	83.8	88.5	—
CV, %	4.5	7.9	5.7	—
6.00	82.7	—	90.8	88.9[c]
	87.0	—	85.8	87.4
	77.5	—	85.3	101.0
	80.7	—	87.3	87.6
	76.0	—	91.7	89.8
Mean, %	80.8	—	88.2	90.9
CV, %	5.4	—	3.3	6.3
8.00	—	82.8	—	125.1[d]
	—	76.0	—	91.3
	—	74.8	—	89.9
	—	78.9	—	90.4
	—	79.6	—	88.2
Mean, %	—	78.4	—	97.0
CV, %	—	4.0	—	16.3

[a] ND = none detected.
[b] Fortified at 2.94 ppm.
[c] Fortified at 5.87 ppm.
[d] Fortified at 8.81 ppm.

Table 2. Percent recovery of nicarbazin from fortified, control chicken muscle tissues by the LC method

	Laboratory			
Added, ppm	1	2	3	4
0	0.06[a]	ND[b]	ND	ND
	0.08[a]	ND	ND	ND
	0.02[a]	ND	ND	ND
	0.11[a]	ND	ND	ND
	ND	ND	ND	ND
2.00	81.9	74.5	89.0	76.7[c]
	73.4	72.0	93.0	82.4
	74.9	70.0	87.0	80.2
	91.9	79.0	85.0	78.3
	91.5	82.0	86.0	80.7
Mean, %	82.7	75.5	88.0	79.7
CV, %	10.6	6.6	3.6	2.8
4.00	67.4	94.9	89.3	89.2[d]
	85.5	100.0	92.0	78.0
	88.5	89.8	91.3	74.3
	82.9	100.5	88.3	78.1
	76.7	107.0	92.0	83.3
	79.5			
Mean, %	80.1	98.4	90.6	80.6
CV, %	9.4	6.6	1.9	7.2
6.00	89.2	—	85.7	75.4[e]
	85.5	—	89.5	75.4
	83.7	—	93.7	73.5
	83.4	—	89.7	74.6
	82.5	—	91.0	76.4
Mean, %	84.9	—	89.9	75.1
CV, %	3.2	—	3.2	1.4
8.00	—	81.2	—	—
	—	85.8	—	—
	—	91.0	—	—
	—	95.5	—	—
	—	93.1	—	—
Mean, %	—	89.3	—	—
CV, %	—	6.5	—	—

[a] µg/mL.
[b] ND = none detected.
[c] Fortified at 2.08 ppm.
[d] Fortified at 4.17 ppm.
[e] Fortified at 6.25 ppm.

tory CV criteria are not provided in the guidelines. However, the interlaboratory percent recovery and CV data for control tissues fortified at about tolerance are close to the intralaboratory criteria. The respective values for these parameters are 87.1 and 10.9% (n = 21) for muscle tissue and 87.4 and 7.5% (n = 20) for liver tissue. This method performed well with fortified tissue samples.

Tables 3 and 4 summarize the data for dosed tissues (incurred residues). Because 2 laboratories incorrectly used the *Fdnc* factor in their calculations of the data for dosed tissue, we recalculated the data. One laboratory used DNC% for the *Fdnc* factor, and another did not use it at all. Concentrations determined for the dosed chicken tissues were more variable than those for the fortified samples. The sponsor determined the concentration of nicarbazin in the dosed-tissue samples before the samples were shipped to the participating laboratories. Be-

Table 3. Nicarbazin concentration (μg/g) in dosed chicken liver tissues determined by LC

	Laboratory			
Trial	1	2	3	4
1	4.44	5.51	5.34	4.48
2	3.95	4.77	5.73	4.48
3	3.98	4.96	4.55	4.95
4	4.17	5.29	4.68	5.02
5	4.25	4.90	4.87	6.50
Mean, %	4.16	5.09	5.03	5.09
CV, %	4.8	5.9	9.7	16.3

Table 4. Nicarbazin concentration (μg/g) in dosed chicken muscle tissues determined by LC

	Laboratory			
Trial	1	2	3	4
1	1.48	1.18	0.52	0.20
2	1.51	1.14	0.62	0.39
3	1.48	1.22	0.50	0.98
4	1.50	1.46	0.54	1.23
5	1.50	1.16	0.42	1.18
6	1.56			
Mean, %	1.51	1.23	0.52	0.80
CV, %	2.0	10.6	13.9	59.0

cause the dosed-tissue samples were stored frozen until analyzed, the sponsor again analyzed the dosed tissue after 10 months of storage. For the dosed liver, the values were 4.23 ppm and 2.6% CV initially and 4.81 ppm and 3.7% CV after 10 months. For the dosed lean (muscle), the values were 1.26 ppm and 8.6% CV initially, and 1.18 ppm and 2.6% CV after 10 months.

The interlaboratory CV of 12.9% for dosed liver is good, and the mean concentration of 4.84 ppm agrees with the sponsor's values. The interlaboratory dosed muscle mean concentration of 1.04 ppm is close to the sponsor's values; however, the interlaboratory CV of 43.8% is high. The increased variability probably occurred because the dosed muscle tissue was not uniformly mixed before subsamples were taken. Additionally, the concentration of DNC was below the lowest standard specified in the method. Because liver is the target tissue for regulatory purposes, the variability observed for the muscle tissue data was not considered critical to the performance of the method.

The data from the MS confirmatory procedure for dosed tissue and samples fortified at 4 ppm agreed with the data obtained for standards. No interferences were detected in the control tissue samples. For standards, the relative abundances of 2 ions monitored agreed within 10%. The variability observed for the third ion, *m/z* 302, was 14%. In the sample extracts, the data on the relative abundances of all 3 ions agreed to within the 10% limits.

In the familiarization stage of the trial, all participants reported low recovery of nicarbazin from fortified tissue samples. The problem was traced to low recovery of nicarbazin from the alumina cartridges. Thus, the solid-phase extraction was identified as a critical step. Three laboratories observed that residual DMF remaining on the alumina cartridges reduced the recovery of DNC. The precaution ensuring that residual DMF is removed from the alumina cartridges was added to the method. We substituted the 6 ppm standard and the fortified tissue samples for the 8 ppm standard in this trial because of the precipitation problems observed with the 8 ppm standard. Confusion about the use of the *Fdnc* factor caused problems. The concentrations of the standards and fortification solutions were calculated incorrectly; therefore, data for fortified samples were recalculated. As a result of the precipitation problems and the confusion about the *Fdnc* factor, the concentrations in samples of fortified tissue varied.

Once the participants became proficient in performing the solid-phase extraction, the method worked well. FDA approved this method for nicarbazin in chicken liver and muscle tissues at 4 ppm and above.

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TECHNICAL COMMUNICATIONS

Calculation of Juice Content in a Diluted Fruit Juice Beverage

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A 1990 amendment to the Food, Drug and Cosmetic Act states that if a food purports to be a beverage containing vegetable or fruit juice, it shall be deemed to be misbranded unless its label bears a statement of the total percentage of such fruit or vegetable juice content. The intent of the present paper is to assist beverage manufacturers in calculating the juice content of their products for the purpose of a label declaration.

In the juice industry, the percentage of each juice or concentrated juice ingredient in a beverage is calculated by using the known volume or weight of such ingredients, or by working backward, beverage manufacturers should be able to calculate the amount of juice ingredients required to support a previously determined juice content declaration. The fact that the amount of "juice" in the juice ingredients is measured in terms of weight units, whereas the percentage of juice in the beverage is reported by volume, complicates these calculations.

Because concentrated juices are widely used to make reconstituted 100% juices and diluted juice beverages, beverage manufacturers should also be able to calculate how much 100% juice can be made by diluting a concentrate of a known concentration with water. Juices, natural strength and concentrates, are described in part in terms of percent by weight of juice soluble solids. The term "soluble solids" includes all dissolved matter in the juice or beverage. The dissolved matter in juice is primarily sugar in the form of sucrose or invert sugar and natural citric, malic, or tartaric fruit acid. Corn sugar and syrup, additional acids, and other soluble ingredients may be added.

In the sugar industry, the concentration of sugar solutions is described in terms of "degrees Brix." Refractometers and hydrometers calibrated in degrees Brix and used on sucrose solutions such as those made from cane or beet sugar give readings equivalent to percent sugar by weight in the solution. The fruit juice industry commonly uses refractometers to measure degrees Brix and equates those readings to total soluble solids. However, in juices or beverages containing other soluble matter, e.g., citric acid, the reading from a refractometer is slightly less than true total solids. In the citrus industry, a correction is made for the deficiency on the basis of the amount of acid present, expressed as percent by weight of anhydrous citric acid. The U.S. Food and Drug Administration (FDA) Standard of Identity for Frozen Concentrate for Lemonade refers to "soluble solids taken as the sucrose value determined by refractometer and corrected for acidity." The standard for grapefruit juice uses similar terminology. The standards for orange juice products refer to "soluble solids" and to "Brix hydrometer readings," which imply the use of an acid correction when a refractometer is used. Table 1 shows the corrections used in the citrus industry (1, 2).

The standards for juice products usually include a statement of the minimum degrees Brix or the percent juice soluble solids to which a concentrate is to be diluted in preparation of a full strength, or "100%," juice. However, there are many fruit and vegetable juices for which FDA has not promulgated standards and for which no universally accepted definition of 100% juice previously existed. As part of a broad new regulation on food labeling, FDA published a list in the *Federal Register* of 46 fruits and 3 vegetables with a Brix level for each at 100% juice, and 2 fruits, lemon and lime, with an acidity level for 100% juice (3). The list of values (degrees Brix or percent citric acid) appears as Table 2. In the present paper, a Brix value from the FDA list is referred to as a "100% juice Brix." Calculations of juice content on the basis of Brix are applicable only to the fruits and vegetable juices for which Brix values are given on the FDA list. In the citrus industry, as previously noted, it is understood that when a Brix refractometer is used to obtain soluble solids, the readings are always corrected for acidity. However, the standard for pineapple juice states that the Brix of juice made from concentrate, determined by refractometer, is not less than 12.8° *un*corrected for acidity. Similarly, FDA has ruled that, in complying with the minimum Brix values in Table 2, refractometer readings for all fruits other than citrus are not corrected for acidity.

Table 2 shows that lemon and lime juices have values of 4.5% for anhydrous citric acid percent by weight. A lemon or lime juice is "100% juice" when the acidity is 4.5% by weight as citric acid. (A provision in the standard for lemon juice, including reconstituted lemon juice, establishes a minimum of 6° Brix by refractometer, uncorrected for acidity [21 CFR 146.114]). In the preparation of a diluted beverage such as lemonade, the calculation of juice content involves the density, which is influenced by the soluble solids content.

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Table 1. Refractometer correction table for anhydrous citric acid as used in the citrus industry[a, b]

Citric acid, %	+0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	0	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18
1.0	0.20	0.22	0.24	0.26	0.28	0.30	0.32	0.34	0.36	0.38
2.0	0.39	0.41	0.43	0.45	0.47	0.49	0.51	0.53	0.54	0.56
3.0	0.58	0.60	0.62	0.64	0.66	0.68	0.70	0.72	0.74	0.76
4.0	0.78	0.80	0.81	0.83	0.85	0.87	0.89	0.91	0.93	0.95
5.0	0.97	0.99	1.01	1.03	1.04	1.06	1.07	1.09	1.11	1.13
6.0	1.15	1.17	1.19	1.21	1.23	1.25	1.27	1.29	1.30	1.32
7.0	1.34	1.36	1.38	1.40	1.42	1.44	1.46	1.48	1.50	1.52
8.0	1.54	1.56	1.58	1.60	1.62	1.64	1.66	1.68	1.69	1.71
9.0	1.72	1.74	1.76	1.78	1.80	1.82	1.83	1.85	1.87	1.89
10.0	1.91	1.93	1.95	1.97	1.99	2.01	2.03	2.05	2.06	2.08
11.0	2.10	2.12	2.14	2.16	2.18	2.20	2.21	2.23	2.24	2.26
12.0	2.27	2.29	2.31	2.33	2.35	2.37	2.39	2.41	2.42	2.44
13.0	2.46	2.48	2.50	2.52	2.54	2.56	2.57	2.59	2.61	2.63
14.0	2.64	2.66	2.68	2.70	2.72	2.74	2.75	2.77	2.78	2.80
15.0	2.81	2.83	2.85	2.87	2.89	2.91	2.93	2.95	2.97	2.99
16.0	3.00	3.02	3.03	3.05	3.06	3.08	3.09	3.11	3.13	3.15
17.0	3.17	3.19	3.21	3.23	3.24	3.26	3.27	3.29	3.31	3.33
18.0	3.35	3.37	3.38	3.40	3.42	3.44	3.46	3.48	3.49	3.51
19.0	3.53	3.55	3.56	3.58	3.59	3.61	3.63	3.65	3.67	3.69
20.0	3.70	3.72	3.73	3.75	3.77	3.79	3.80	3.82	3.84	3.86
21.0	3.88	3.90	3.91	3.93	3.95	3.97	3.99	4.01	4.02	4.04
22.0	4.05	4.07	4.09	4.11	4.13	4.15	4.17	4.19	4.20	4.22
23.0	4.24	4.26	4.27	4.29	4.30	4.32	4.34	4.36	4.38	4.40
24.0	4.41	4.43	4.44	4.46	4.48	4.50	4.51	4.53	4.54	4.56
25.0	4.58	4.60	4.62	4.64	4.66	4.68	4.69	4.71	4.73	4.75
26.0	4.76	4.78	4.79	4.81	4.83	4.85	4.86	4.88	4.90	4.92
27.0	4.94	4.96	4.97	4.99	5.00	5.02	5.03	5.05	5.06	5.08
28.0	5.10	5.12	5.14	5.16	5.18	5.20	5.22	5.24	5.25	5.27
29.0	5.28	5.30	5.31	5.33	5.35	5.37	5.39	5.41	5.42	5.44
30.0	5.46	5.48	5.49	5.51	5.52	5.54	5.56	5.58	5.60	5.62
31.0	5.64	5.66	5.67	5.69	5.71	5.73	5.75	5.77	5.79	5.81
32.0	5.82	5.84	5.86	5.88	5.89	5.91	5.93	5.95	5.97	5.99
33.0	6.00	6.02	6.04	6.06	6.07	6.09	6.10	6.12	6.13	6.15
34.0	6.16	6.18	6.20	6.22	6.23	6.25	6.27	6.29	6.30	6.32
35.0	6.34	6.36	6.37	6.39	6.41	6.43	6.45	6.47	6.48	6.50
36.0	6.52	6.54	6.56	6.58	6.59	6.61	6.63	6.65	6.67	6.69
37.0	6.70	6.72	6.74	6.76	6.77	6.79	6.81	6.83	6.85	6.87
38.0	6.88	6.90	6.92	6.94	6.95	6.97	6.98	7.00	7.01	7.03
39.0	7.04	7.06	7.08	7.10	7.11	7.13	7.15	7.17	7.19	7.21
40.0	7.22	7.24	7.26	7.28	7.29	7.31	7.32	7.34	7.36	7.38
41.0	7.39	7.41	7.42	7.44	7.45	7.47	7.49	7.51	7.52	7.54

[a] Corrections to be added to readings of Brix refractometers (refractometric sucrose values) to obtain degrees Brix (1).

[b] Users of computers may find it practical to use the formula for corrections for citric acid content contained in the paper of Yeatman et al. (2); the correction to the refractometer reading is $0.012 + 0.193x - 0.0004\ x^2$, where x is the percent anhydrous citric acid content.

Calculations for Total Percent Juices in Beverages (Other Than Lemon and Lime Juices)

Beverage manufacturers use various procedures to measure the amount of juice ingredient going into a blend. Some plants may actually weigh the concentrate. More commonly, beverage manufacturers measure the volume of the juice ingredient, in a calibrated tank, by volumetric meter or by knowledge of the number of containers of known volume used.

If the *weight* of the concentrate is known, the equivalent weight of juice at the 100% juice Brix can be determined by using Equation 1:

$$\text{Weight of juice at 100\% juice Brix} = \text{Weight of concentrate} \times \frac{\text{Brix of concentrate}}{\text{100\% juice Brix}} \quad (1)$$

Table 2. Soluble solids content of 100% juices

Juice	100% juice Brix	Juice	100% juice Brix
Acerola	6.0	Guanabana (soursop)	16.0
Apple	11.5	Guava	7.7
Apricot	11.7	Honeydew melon	9.6
Banana	22.0	Kiwi	15.4
Blackberry	10.0	Loganberry	10.5
Blueberry	10.0	Mango	13.0
Boysenberry	10.0	Nectarine	11.8
Cantaloupe melon	9.6	Orange	11.8
Carambola	7.8	Papaya	11.5
Carrot	8.0	Passion fruit	14.0
Casaba melon	7.5	Peach	10.5
Cashew (Caju)	12.0	Pear	12.0
Celery	3.1	Pineapple	12.8
Cherry (red, sour)	14.0	Plum	14.3
Cherry (dark, sweet)	20.0	Pomegranate	16.0
Crabapple	15.4	Prune	18.5
Cranberry	7.5	Quince	13.3
Currant (black)	11.0	Raspberry (black)	11.1
Currant (red)	10.5	Raspberry (red)	9.2
Date	18.5	Rhubarb	5.7
Dewberry	10.0	Strawberry	8.0
Elderberry	11.0	Tangerine	11.8
Fig	18.2	Tomato	5.0
Gooseberry	8.3	Watermelon	7.8
Grape	16.0	Youngberry	10.0
Grapefruit	10.0		
		Lemon	4.5%[a]
		Lime	4.5%[a]

[a] Anhydrous citric acid, percent by weight, derived from the fruit as present in the diluted beverage.

The volume of the diluted juice at the 100% juice Brix can be calculated from the weight by using Equation 2 and available density tables for sucrose solutions. The tables are often called Brix tables or Specific Gravity Tables, although densities such as pounds per gallon or grams per liter are used in the present paper rather than specific gravity (4–7). (The densities of juices are essentially the same as the densities of sucrose solutions of comparable Brix values.)

$$\text{Volume} = \frac{\text{Weight}}{\text{Density}} \qquad (2)$$

The volume may be expressed in any units as long as the same units are used throughout. The available tables give densities in pounds per U.S. gallon at 20°C or in grams per liter at 20°C. Use Equation 3:

$$\text{Juice in beverage in percent} = \frac{\text{Volume of juice}}{\text{Volume of beverage}} \times 100 \qquad (3)$$

If the *volume* of the concentrated juice ingredient is known rather than the weight, the volume may be converted to the volume of the juice at the 100% juice Brix for that juice. First, equate the soluble solids in the concentrate to the soluble solids in the juice made from that concentrate by diluting to the 100% juice Brix: (volume of concentrate, Vc) × (pounds of sucrose per gallon in air at the Brix of the concentrate from the Brix tables) = (volume of juice, Vj) × (pounds of sucrose per gallon in air at the 100% juice Brix for that juice from the Brix tables).

Then, determine the volume of the juice at the 100% juice Brix by using Equation 4:

$$Vj = Vc \times \frac{\text{Pounds of sucrose per gallon for concentrate}}{\text{Pounds of sucrose per gallon for juice}} \qquad (4)$$

If grams of sucrose per liter from the Brix tables are used, the results are the same. Use Equation 3 to calculate the percent juice in the beverage. The sum of the juice ingredients from different sources is determined by adding the *volume* of each juice. The basis for the volume/volume calculation is a statement in the Code of Federal Regulations (21 CFR 102.5 [b] [1]), which reads, "The percentage of a characterizing ingredient or component shall be declared on the basis of its quantity in the finished product (i.e., volume/volume in the case of liquids)."

Note that the Brix of the finished beverage did not enter into these calculations. In a beverage with a total juice content of less than 100%, the Brix of the beverage may be different from

the "100% juice Brix" of the juice ingredients. The characteristic of the finished beverage involved in juice content calculations is its volume.

When juice used in a beverage is expressed juice not made from concentrate, the Brix of that juice as extracted or pressed may not be the same as the standard Brix given in the FDA list. FDA's rule states that undiluted "juices directly expressed from a fruit or vegetable (i.e., not concentrated and reconstituted) shall be considered to be 100 percent juice" (21 CFR 101.30 [i]). That is, regardless of the Brix, the juice content to be declared is the volume of the expressed juice divided by the volume of the beverage. If the beverage is offered to consumers in concentrated form, it is recommended that the label statement of juice content show the percentage of juice "When reconstituted as directed," or the equivalent (although FDA's recent rule is silent on this point).

In a beverage containing juice from more than one fruit or vegetable, the total juice content must be declared on the label. How the declarations must be made and whether the percentage of the individual juices must be declared is beyond the scope of this paper. In some cases, it may be necessary to declare in addition the content of one or more of the individual juices or to indicate that the beverage is "flavored" by such juices.

Calculations for Total Percent Lemon and Lime Juices in Beverages

An exception to the use of Brix values is made for lemon and lime juices. It has been a long standing practice in the citrus industry to base the sale of lemon and lime juices and concentrates on the acidity, inasmuch as these juices are most commonly used to add tartness to a food or beverage. That is, they are standardized to a specified acidity and priced accordingly. The acidity is usually expressed in grams of anhydrous citric acid per liter of concentrate, e.g., 400 GPL.

The FDA Standard of Identity for Lemon Juice, 21 CFR 146.114 (a)(1), states that lemon juice "has a titratable acidity content of not less than 4.5%, by weight, calculated as anhydrous citrus acid." The FDA list incorporates this value and applies it to lime juice, as well. Because a weight/volume measure (GPL) is used for concentrate and a weight/weight measure (%) is used for juice, the juice content calculations require knowledge of the density of the juice and, therefore, of the total soluble solids content. To obtain an accurate density from the Brix tables (4–7), the refractometric sucrose value for acidity must be corrected. Corrections are included in Table 1. To calculate the juice content of a lemon or lime beverage, follow Steps 1 through 10, or the *Alternative Procedure for Lemon and Lime Juices*:

(*1*) Determine the refractometer reading of the lemon or lime concentrate by using a Brix refractometer.

(*2*) Determine the acidity as citric acid in percent by *weight* of the concentrated juice by analysis, even though it was standardized during manufacture to a specified weight/volume acidity.

(*3*) Add the appropriate acidity correction to the refractometer reading to obtain total soluble solids (corrected Brix), and obtain the correction from Table 1.

(*4*) Calculate the actual acid concentration in GPL by multiplying the acidity determined in Step 2 by the density in grams per liter (from the Brix tables; 4–7) corresponding to the corrected Brix obtained in Step 3, and dividing by 100.

(*5*) Calculate the weight of the juice (obtained by diluting 1 lb of the concentrate to 4.5% acidity by weight) by using Equation 5:

$$\text{Pounds of juice per pound of concentrate} = \frac{\text{Acidity of concentrate in percent by weight (see Step 2)}}{4.5} \quad (5)$$

(*6*) Calculate the corrected Brix of the 100% juice (single-strength juice) by using Equation 6:

$$\text{Brix (corr.) of juice} = \frac{\text{Brix (corr.) of concentrate from Step 3}}{\text{Juice weight/pounds of concentrate from Step 5}} \quad (6)$$

(*7*) Use the Brix of the juice from Step 6 to obtain the density in grams per liter of the juice from the tables. Then, calculate the acidity in grams per liter of citric acid in juice that contains 4.5% citric acid by weight by using Equation 7:

$$\text{GPL of citric acid in juice} = \frac{4.5}{100} \times \text{density of juice} \quad (7)$$

(*8*) Calculate the volume concentration ratio (VCR) by dividing the GPL of the concentrate by the GPL of the single-strength juice (from Steps 4 and 7).

(*9*) Calculate the volume of the juice in the batch by multiplying the volume of concentrate by the volume concentration ratio.

(*10*) Use Equation 3 to calculate the percent juice in the beverage.

Juice Content Calculation of Punch Base Made From Three Concentrates

Table 3 shows the calculation of the total juice content in a punch base that contains concentrated orange juice (65° Brix, with concentrate in gallons), concentrated cherry juice (44° Brix, with concentrate in pounds), and concentrated lemon juice (400 GPL [grams of citric acid per liter]). Other ingredients include water, sugar, citric acid, and flavorings. The batch size for the base is 1000 gallons. The label of the punch base bears a statement that 1 volume of the base should be diluted with 3 volumes of water. Therefore, the final volume used in the juice content calculation on the basis of the single-strength beverage volume is 4000 gallons. Of course, the same calculation may be used for a ready-to-drink punch with a batch size of 4000 gallons.

The final result of the juice content calculation may be checked by using the procedures in the sections entitled *Calculations,* together with the data provided. The calculations for the orange juice concentrate in the punch base is as follows: 35 × 6.934 = 242.7, 242.7 × 100/4000 = 6.07%. For cherry juice,

Table 3. Juice content calculation of punch base made from 3 concentrates[a]

Data	Orange juice concentrate	Sour cherry juice concentrate	Lemon juice concentrate
Brix of concentrate	65°	44°	
Acidity of concentrate			400 GPL
Quantity of concentrate	35 gal	319 lb	5 gal
Brix of 100% juice, Table 2	11.8°	14.0°	
Acidity of 100% juice, Table 2			4.5% by wt
Equations to use	No. 4 & 3	No. 1–3	See steps 1–10
Data used in the calculations:			
Pounds of sucrose/gallon:			
Concentrate –65° Brix	7.135		
Juice –11.8° Brix	1.029		
Juice/concentrate ratio, volume	6.934		
Density of 14.0° Brix juice		8.795 lb/gal	
Density of 55.0° Brix concentrate			1256.5 g/L
Juice/concentrate ratio, weight		44.0/14.0 = 3.143	
Density of 7.8° Brix juice			1028.1 g/L
Acidity of 4.5% w/w juice (Step 7 or Figure 1)			46.3 GPL
Single-strength juice equivalent	242.7 gal	114.0 gal	43.2 gal
Juice in beverage	6.07%	2.85%	1.08%

[a] Total percent juice when diluted as directed = 10.0%.

it is 319 × 44.0/14.0 = 1003, 1003/8.795 = 114.0, 114.0 × 100/4000 = 2.85%. For lemon juice, the concentrated lemon juice used in the example has an acidity of 31.8% by weight, as determined in Step 2 of the procedure and an uncorrected refractometer reading of 49.2° from Step 1. In Step 3, the correction for acidity from Table 1 is 5.79, rounded to 5.8, which when added to the refractometer reading gives a Brix value of 55°. In Step 4, 31.8 × 1256.5 (density)/100 = 400 g acid/L, and confirms the supplier's specifications.

Step 5: [Equation 5] Weight of juice per pound of concentrate = 31.8/4.5 = 7.07 lb.

Step 6: [Equation 6] Corrected Brix of the single-strength juice = 55/7.07 = 7.78°.

Step 7: [Equation 7] Citric acid in juice = 4.5 × 1028.1/100 = 46.3 GPL.

Step 8: Volume concentration ratio = 400/46.3 = 8.64.

Step 9: Volume of juice = 5 × 8.64 = 43.2 gallons.

Step 10: Percent juice in beverage = 43.2 × 100/4000 = 1.08%.

Alternative Procedure for Lemon and Lime Juices

When the uncorrected refractometer reading of the concentrated juice has been determined (Step 1 in the above procedure), the acidity of the concentrate in terms of GPL is known with confidence and the concentrate into the batch has been measured by volume, Figure 1 may be used to obtain the number which results from Step 7. Then, the percentage of juice in the beverage may be calculated by using Equations 8 and 9. Steps 2 through 7 are eliminated:

$$\text{VCR} = \frac{V_j}{V_c} = \frac{\text{GPL of concentrate}}{\text{GPL of 4.5\% w/w juice from Figure 1}} \quad (8)$$

$$\text{\% juice in beverage} = \frac{\text{Volume of concentrate} \times \text{VCR}}{\text{Volume of beverage}} \times 100 \quad (9)$$

Calculation of Apparent Density from Degrees Brix

Specific gravity and density tables (4–7) are expressed in formulas that may be used in computers. Equations 10, 11, and 12 for apparent density in grams per milliliter and in pounds per U.S. gallon were published in a paper by Chen (8) (D is the apparent density of the sucrose solution and B is its degrees Brix.):

$$D = \sum_{n=0}^{5} b_n \times B^n, \text{ where } n = 0, 1, \ldots, 5 \quad (10)$$

in which $b_0 = 0.997174$, $b_1 = 3.857739 \times 10^{-3}$, $b_2 = 1.279276 \times 10^{-5}$, $b_3 = 6.191578 \times 10^{-8}$, $b_4 = -1.7774448 \times 10^{-10}$, and $b_5 = -4.199709 \times 10^{-13}$. The calculated values of D are in g/mL and the accuracy is estimated about $\pm 1 \times 10^{-5}$ g/mL.

The calculated apparent density from Equation 10 was then used to calculate the weight (pounds) of solution per U.S. gallon of solution and weight (pounds) of sucrose per U.S. gallon by using the following relations:

$$\text{lb of solution/gal} = 8.34536\ [\text{lb/g} \times \text{mL/gal}] \times D[\text{g/mL}] \quad (11)$$

and

$$\text{lb sucrose/gal} = B[^\circ\text{Brix}] \times D[\text{lb/gal}]/100 \quad (12)$$

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Table 4. Application of Equations 13 and 14

°Brix	Apparent specific gravity in air, 20°/20°C	Apparent density in air, g/mL, 20°C	Apparent density in air, lbs/gal, 20°C	Sucrose in air, lbs/gal, 20°C
0	1.00000	0.99718	8.322	0.0
20	1.08297	1.07991	9.012	1.802
40	1.17875	1.17542	9.809	3.924
60	1.28908	1.28544	10.727	6.436
80	1.41471	1.41072	11.773	9.418
100	1.55521	1.55082	12.942	12.942

Calculation of Apparent Specific Gravity and Density in Air from Degrees Brix

Computer-developed values for specific gravity of sucrose solutions in air and in vacuum, from which density in grams per liter and pounds per gallon may be easily calculated, were produced by James J. Hackbarth of the Stroh Brewery Co., Detroit, MI (9).

The apparent specific gravity (S) in dry air at 760 torrs 20°C/20°C was calculated from the specific gravity (S_T) in vacuum by using Equation 13 (10, 11):

$$S = S_T \times 1.00121 - 0.00121 \quad (13)$$

The relationship between apparent specific gravity and degrees Brix (B) or percent by weight sucrose was calculated by using Equation 14 (linear regression):

$$S = \sum_{n=0}^{8} a_n B^n \quad (14)$$

where $n = 0, 1, \ldots, 8$. $a_0 = 1.0$, $a_1 = 3.87217 \times 10^{-3}$, $a_2 = 1.20700 \times 10^{-5}$, $a_3 = 1.20485 \times 10^{-7}$, $a_4 = -2.37056 \times 10^{-9}$, $a_5 = 4.45367 \times 10^{-11}$, $a_6 = -5.15428 \times 10^{-13}$, $a_7 = 3.10578 \times 10^{-15}$, and $a_8 = -7.66564 \times 10^{-18}$.

The apparent density (D) of water in dry air at 760 torrs 20°C is 8.32162 lb/gal or 0.997177 g/mL; therefore, the density (D) of the solution is 0.997177 g/mL × S, or 8.32162 lb/gal × S, and the sucrose content is 8.32162 lb/gal × S × B/100.

Results that are computer generated by Equations 13 and 14 may be checked against the data in Table 4.

Notice that the Chen and Hackbarth formulas give densities in grams/mL, whereas the formulas used to calculate lemon juice volume specify densities in grams/L.

Other calculations commonly used in juice plants involving Brix, acidity, volume, formulation, etc., are not covered in the limited scope of this paper. J.B. Redd et al. (12) and D.A. Kimball (13) have written books that give more detail on such calculations. The *Cane Sugar Handbook* (14) has many useful tables on the physical characteristics of sugar solutions.

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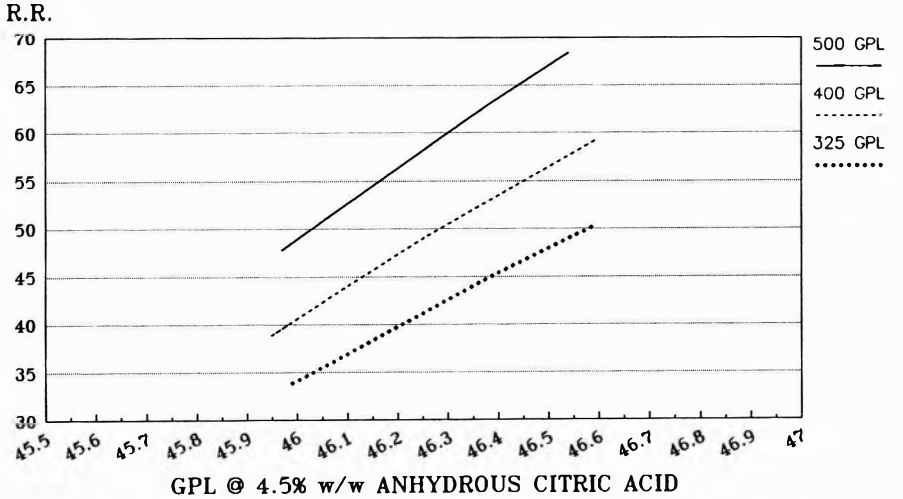

Figure 1. Refractometer reading, uncorrected, vs GPL at 4.5% w/w citric acid

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TECHNICAL COMMUNICATIONS

Incidence of Fungi in Shared-Use Cosmetics Available to the Public

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A survey was conducted to assess both the potential health risk from shared-use cosmetics caused by microorganisms and the microbial efficacy of preservatives in cosmetics. Samples of 3027 shared-use cosmetic products were collected from 171 retail establishments nationwide. Fungi were present in 10.4% of the products, and 3.9% contained fungal pathogens or opportunistic pathogens. The 423 fungal isolates identified represented 33 genera and at least 69 species. A disproportionately large share of the fungal isolates were from eye products; fewer were from lip products. Pathogenic or opportunistic pathogens made up 32.2% of the fungal isolates. A slightly lower percentage of samples that contained preservatives had fungi, a fact suggesting that preservatives reduce the incidence of fungi in cosmetics. Results of this survey indicate potential microbiological problems concerning the safety of shared-use cosmetics.

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[1] Deceased.

The U.S. Food and Drug Administration (FDA) is concerned with the microbiological safety of cosmetic products. Shared-use products available in retail establishments for use by many people are of particular interest. According to the Shared-Use Cosmetic Survey (FY 89) Assignment (1), many retail establishments that sell cosmetics also provide them at retail counters for in-store consumer experimentation and makeovers. The products may be offered for shared use on retail counters (often in a separate display case or drawer) and may be readily available to customers, or they may be held behind the counter and offered by the salesperson only upon request or by a cosmetician for makeup demonstration.

For this study, samples of shared-use cosmetics that were open and used for varying periods of time were collected at retail establishments and analyzed for the presence of viable bacteria and fungi. The bacterial findings will be summarized elsewhere.

Experimental

Sample Collection

FDA investigators visited 171 retail establishments nationwide that offered cosmetics for shared use and collected a total of 3027 samples, an average of 17.7 products per store. Products included liquid mascara, eye shadows, liquid eyeliners,

Table 1. Distribution of shared-use cosmetic samples tested, samples containing fungi, and fungal isolates by product type

Product type	Samples		
	Total No. tested (%)	Total No. containing fungi (%)	Total No. of fungal isolates (%)
Eye	1560 (51.5)	176 (55.7)	257 (60.8)
Face	640 (21.1)	80 (25.3)	97 (22.9)
Lip	778 (25.7)	55 (17.4)	63 (14.9)
Unknown	49 (1.6)	5 (1.6)	6 (1.4)
Total	3027 (100.0)	316 (100.0)	423 (100.0)

lipsticks, lip glosses, facial blushers (cream type), rouges, and foundations. The different products sampled at each retail establishment were unrelated except for being available at the same store. They included a variety of product categories (Table 1) and brands. Approximately 50% of the products were for the eyes, 20% were for the face, and 25% were for the lips.

Cosmetic Sampling

The opened cosmetic products were sampled by using the Transette III-R transport system (Spectrum Laboratories, Inc., Houston, TX), which consists of a sterile rayon swab on a plastic shaft and a modified Amies Transport Medium with charcoal. Approximately 1 sq. in. of the surface of each solid cosmetic was swabbed; for liquid or semisolid cosmetics, swabs were dipped into the containers.

Microbial Analyses

Inoculated swabs were transferred and cultured by analysts in the FDA District laboratories. Each swab was used to inoculate 2 agar plates for bacterial analysis and a third plate for fungal analysis. The swab surface was rotated approximately 120° between plates so that a different portion of the surface was used to inoculate each of the 3 plates. Swabs were streaked onto the entire surface of each agar plate.

For fungal detection, either malt extract agar or potato dextrose agar (each supplemented with 40 ppm chlortetracycline hydrochloride) was inoculated. Plates were incubated at 30 ± 2°C for 7 days, and colonies were counted. Suspected fungal isolates were transferred to malt extract or potato dextrose agar slants and sent to the FDA Division of Microbiology laboratory in Washington, DC, for identification.

Table 2. Distribution of number of fungal isolates found per sample of shared-use cosmetics

Isolates/sample	Samples (%)	Cumulative %
0	2711 (89.6)	89.6
1	239 (7.9)	97.5
2	55 (1.8)	99.3
3	16 (0.5)	99.8
4	4 (0.1)	99.9
5	2 (0.1)	100.0
Total	3027	100.0

Fungal Identification (Molds and Yeasts)

Mold cultures were transferred to Czapek, malt, and potato dextrose agar plates and incubated at room temperature until enough growth developed for identifications, which were based on the descriptions of Barnett (2), Gilman (3), Mislivec (4), Raper and Fennell (5), and Raper and Thom (6). Yeast cultures were transferred to potato dextrose agar plates and incubated as above. Identifications of yeasts were based on the descriptions of Lodder (7).

Results and Discussion

Occurrence of Fungi

The 423 fungal isolates identified, representing 33 genera, were classified as "nonpathogenic," "opportunistic pathogen," or "pathogenic" (2–9). Table 2 shows the distribution of the number of fungal isolates per test sample. Fungal isolates were identified in 316 (10.4%) test samples, and 2.5% of the test samples contained more than 1, but not more than 5, fungal species. The proportion of fungal isolates from eye products was higher and the proportion from lip products was lower than would be expected from the sample distribution (Table 1). Although only 51.5% of the samples were eye products, 60.8% of the fungal isolates were obtained from them. Only 14.9% of the fungal isolates were from lip products, which represented 25.7% of the samples.

The distribution by product type of the 316 samples containing fungi is similar to that of the samples overall (Table 1); however, proportionally fewer lip products contained fungi than would be expected from the overall sample distribution.

Sample Histories

Time in use.—Table 3 shows the distribution of the time in use of the products. Of the 2448 samples with known use times, 31 (1.3%) had been in use for 2 or more years. Time in use did not differ noticeably among various product types. The distribution of the time in use for the 316 samples that contained fungi was similar to that for samples overall (Table 3).

Preservatives.—On the basis of label declaration, preservatives were present in 69.2% of the eye products (1080/1560),

Table 3. Time in use for the shared-use cosmetic samples

	All samples			Samples containing fungi		
Time, months	No.	Percent	Cumulative %	No.	Percent	Cumulative %
Unknown	579	—	—	81	—	—
1	473	19.3	19.3	26	11.1	11.1
2	397	16.2	35.5	21	8.9	20.0
3	500	20.4	56.0	56	23.8	43.8
4	173	7.1	63.0	15	6.4	50.2
5	84	3.4	66.5	6	2.6	52.8
6	381	15.6	82.0	47	20.0	72.8
7	34	1.4	83.4	6	2.6	75.3
8	115	4.7	88.1	24	10.2	85.5
9	67	2.7	90.8	6	2.6	88.1
10	9	0.4	91.2	1	0.4	88.5
11	3	0.1	91.3	1	0.4	88.9
12	150	6.1	97.5	17	7.2	96.2
13	7	0.3	97.8	5	2.1	98.3
14	5	0.2	98.0	—	—	—
15	1	0.0	98.0	—	—	—
16	1	0.0	98.0	—	—	—
18	17	0.7	98.7	—	—	—
24	18	0.7	99.5	1	0.4	98.7
30	1	0.0	99.5	—	—	—
36	11	0.4	100.0	3	1.3	100.0
48	1	0.0	100.0	—	—	—
Total	2448	—	100.0	235	—	100.0

61.6% of the face products, and 51.5% of the lip products (Table 4). Of the samples containing fungi, 55.7% contained preservatives, compared with 62.5% of the samples overall (Table 4). Therefore, a slightly lower percentage of the samples that contained fungi had preservatives than did the samples overall, which suggested that preservatives may have helped reduce the incidence of fungi in the cosmetics. For products that contained fungi, 60.2% of the eye products, 62.5% of the face products, and only 36.4% of the lip products contained preservatives.

Fungal Organisms

Fungal genera and species.—Table 5 shows the occurrence of fungal genera among the isolates by product type. Table 6

Table 4. Presence of preservatives in all samples of shared-use cosmetics and in samples containing fungi

	Preservatives			
Product type	Present (%)	Absent (%)	Unknown (%)	Total (%)
	In all samples			
Eye	1080 (35.7)	321 (10.6)	159 (5.3)	1560 (51.5)
Face	394 (13.0)	207 (6.8)	39 (1.3)	640 (21.1)
Lip	401 (13.2)	278 (9.2)	99 (3.3)	778 (25.7)
Unknown	18 (0.6)	12 (0.4)	19 (0.6)	49 (1.6)
Total	1893 (62.5)	818 (27.0)	316 (10.4)	3027 (100.0)
	In samples containing fungi			
Eye	106 (33.5)	46 (14.6)	24 (7.6)	176 (55.7)
Face	50 (15.8)	28 (8.9)	2 (0.6)	80 (25.3)
Lip	20 (6.3)	30 (9.5)	5 (1.6)	55 (17.4)
Unknown	0 (0.0)	1 (0.3)	4 (1.3)	5 (1.6)
Total	176 (55.7)	105 (33.2)	35 (11.1)	316 (100.0)

Table 5. Occurrence of fungal genera (No. of isolates) in shared-use cosmetics by product type

Genus	Product type				
	Eye	Face	Lip	Unknown	Total
Actinomyces	1	1	0	0	2
Alternaria	13	8	7	0	28
Aspergillus	25	9	8	1	43
Brettanomyces	4	1	0	0	5
Candida	6	2	0	0	8
Candida-like	1	1	0	0	2
Chaetomium	2	1	1	0	4
Cladosporium	9	4	1	0	14
Cunninghamella	1	0	0	0	1
Epicoccum	4	1	1	0	6
Fusarium	5	1	2	0	8
Geotrichum	1	0	2	0	3
Helminthosporium	3	2	1	0	6
Kloeckera	1	0	0	0	1
Mold-nonviable	2	1	0	0	3
Mold-unidentified	3	0	0	0	3
Monilia	1	0	1	0	2
Mucor	1	0	0	0	1
Myrothecium	1	0	0	0	1
Neurospora	3	1	0	0	4
Paecilomyces	1	0	1	0	2
Penicillium	61	20	8	2	91
Phoma	1	0	0	0	1
Pullularia	28	8	8	0	44
Pyrenochaeta	0	0	1	0	1
Rhizoctonia	2	1	1	0	4
Rhizopus	8	10	4	0	22
Rhodosporidium	2	0	0	0	2
Rhodotorula	21	6	4	0	31
Saccharomyces	1	2	1	0	4
Saccharomyces-like	17	12	9	2	40
Schizosaccharomyces	1	1	0	0	2
Scopulariopsis	0	0	0	1	1
Stemphylium	4	1	0	0	5
Syncephalastrum	0	1	0	0	1
Trichoderma	6	0	0	0	6
Trichosporon	12	1	1	0	14
Yeast-nonviable	3	0	0	0	3
Yeast-unidentified	2	1	1	0	4
Total	257	97	63	6	423

shows the occurrence of fungal species and the classification of each according to hazard potential (nonpathogenic, opportunistic pathogen, or pathogenic). The 423 fungal isolates represent 33 genera and at least 69 species.

Three fungal species rarely found by our laboratory in consumer commodities occurred often: the molds *Penicillium notatum* and *Pullularia pullulans* and the yeast *Rhodotorula rubra*. Because these 3 species occur rarely and are not common constituents of human body microflora or of ambient air

Table 6. Occurrence of fungal species (No. of isolates) in shared-use cosmetics

Species	Frequency	Hazard potential[a]
Actinomyces spp.	2	N
Alternaria alternata	27	N
Alternaria spp.	1	N
Aspergillus aculeatus	1	O
Aspergillus amstelodami	5	N
Aspergillus chevalieri	2	N
Aspergillus flavus	1	O
Aspergillus fumigatus	5	P
Aspergillus niger	9	O
Aspergillus ochraceus	1	O
Aspergillus puniceus	1	N
Aspergillus sydowi	7	O
Aspergillus terreus	1	O
Aspergillus ustus	3	O
Aspergillus versicolor	6	O
Aspergillus wentii	1	O
Brettanomyces spp.	5	N
Candida albicans	5	P
Candida spp.	3	N
Candida-like	2	N
Chaetomium globosum	4	N
Cladosporium herbarum	14	N
Cunninghamella spp.	1	N
Epicoccum purpurascens	5	N
Epicoccum spp.	1	N
Fusarium spp.	8	N
Geotrichum candidum	3	P
Helminthosporium maydis	3	N
Helminthosporium spp.	3	N
Kloeckera spp.	1	N
Mold-nonviable	3	N
Mold-unidentified	3	N
Monilia spp.	2	N
Mucor spp.	1	O
Myrothecium spp.	1	N
Neurospora crassa	4	N
Paecilomyces varioti	2	P
Penicillium aurantio-candidum	1	N
Penicillium citrinum	17	O
Penicillium corylophilum	7	O
Penicillium cyclopium	3	N
Penicillium expansum	2	N
Penicillium fellutanum	1	N
Penicillium frequentans	2	N
Penicillium jensenii	1	N
Penicillium notatum	40	O
Penicillium oxalicum	2	O
Penicillium palitans	1	O
Penicillium purpurrescens	2	N
Penicillium restrictum	1	N
Penicillium roquefortii	2	N
Penicillium rubrum	1	N
Penicillium urticae	1	N
Penicillium variabile	6	N
Penicillium viridicatum	1	O

Table 6. (*Continued*)

Species	Frequency	Hazard potential[a]
Phoma spp.	1	N
Pullularia pullulans	44	N
Pyrenochaeta spp.	1	N
Rhizoctonia solani	3	N
Rhizoctonia spp.	1	N
Rhizopus nigricans	22	O
Rhodosporidium spp.	2	N
Rhodotorula rubra	31	N
Saccharomyces spp.	4	N
Saccharomyces-like	40	N
Schizosaccharomyces japonicus	2	N
Scopulariopsis brevicaulis	1	N
Stemphylium botryosum	2	N
Stemphylium verruculosum	3	N
Syncephalastrum spp.	1	N
Trichoderma glaucum	2	N
Trichoderma lignorum	4	N
Trichosporon pullulans	14	N
Yeast-nonviable	3	N
Yeast-unidentified	4	N
Total	423	

[a] N, nonpathogenic; O, opportunistic pathogen; P, pathogenic.

(personal observation), some of the cosmetics may have been contaminated before distribution.

Fungal pathogens.—Of the 423 fungal isolates found, 287 (67.8%) were nonpathogenic, 121 (28.6%) were opportunistic, and 15 (3.5%) were pathogenic. Opportunistic pathogens do not normally cause infections in healthy individuals, and therefore, they should not present a health problem for the general population. However, the elderly, the very young, and those with chronic disease (e.g., cancer, diabetes) or immunosuppression (e.g., transplant recipients, AIDS patients) are susceptible to infection from opportunistic pathogens. Most of the fungal species found during this survey can enter the body only by inhalation or by penetration of broken skin. In addition, many of the fungi found in this survey can elicit allergic responses that are usually of bronchopulmonary origin (8, 9).

Table 7. Distribution of number of pathogenic or opportunistic fungal isolates found per sample of shared-use cosmetics

Pathogenic or opportunistic isolates/sample	Samples (%)	Cumulative %
0	2908 (96.1)	96.1
1	104 (3.4)	99.5
2	13 (0.4)	99.9
3	2 (0.1)	100.0
Total	3027	100.0

Of the fungal isolates, 136 of 423 (32.2%) were either pathogenic or opportunistic pathogens. Table 7 shows the distribution of the number of pathogenic or opportunistic fungal isolates per sample. The 136 pathogenic or opportunistic fungal isolates came from 119 (3.9%) of the 3027 samples. Only one pathogenic or opportunistic fungal isolate per sample was found in 104 (87.4%) of the 119 samples containing pathogenic or opportunistic fungi. No sample yielded more than 3 pathogenic or opportunistic fungal isolates.

Table 8 shows the distribution of the 119 samples by product type that contained pathogenic or opportunistic fungi. This distribution is similar to that for samples containing any fungi (Table 1). However, the distribution of samples containing harmful fungi was proportionally higher for face products and lower for lip products than the distribution for samples overall (Tables 1 and 8). Of the 15 pathogenic fungal isolates, 9 (60.0%) were from eye products, 2 (13.3%) were from face products, and 3 (20.0%) were from lip products. When pathogens and opportunistic pathogens were combined, 78 of the 136 (57.4%) were from eye products, 35 (25.7%) were from face products, and 20 (14.7%) were from lip products. These proportions were similar to those for all fungal isolates (Tables 1 and 8).

Quantitation of Fungi

Table 9 shows the distribution of colony or plate counts (colonies per plate) obtained from plates prepared for the fungal plate count. Plate counts of 0 were found for 91.8% of the

Table 8. Hazard potential of fungal isolates from shared-use cosmetics

Product type	No. of samples with pathogenic or opportunistic isolates (%)	Hazard potential		
		Nonpathogenic isolates (%)	Opportunistic isolates (%)	Pathogenic isolates (%)
Eye	64 (53.8)	179 (42.3)	69 (16.3)	9 (2.1)
Face	33 (27.7)	62 (14.7)	33 (7.8)	2 (0.5)
Lip	19 (16.0)	43 (10.2)	17 (4.0)	3 (0.7)
Unknown	3 (2.5)	3 (0.7)	2 (0.5)	1 (0.2)
Total	119 (100.0)	287 (67.8)	121 (28.6)	15 (3.5)

Table 9. Fungal plate counts for shared-use cosmetics[a]

Plate counts	No. of samples	Percent	Cumulative %
Unknown	4	—	—
0	2775	91.8	91.8
1	93	3.1	94.9
2	35	1.2	96.0
3	15	0.5	96.5
4	11	0.4	96.9
5	6	0.2	97.1
6	10	0.3	97.4
7	3	0.1	97.5
8	3	0.1	97.6
9	2	0.1	97.7
10	5	0.2	97.8
11	3	0.1	97.9
12	2	0.1	98.0
13	2	0.1	98.1
14	3	0.1	98.2
15	1	0.0	98.2
18	2	0.1	98.3
19	2	0.1	98.3
20	1	0.0	98.4
21	2	0.1	98.4
22	1	0.0	98.5
23	1	0.0	98.5
24	2	0.1	98.6
26–50	13	0.4	99.0
51–75	5	0.2	99.2
76–100	6	0.2	99.4
101–200	6	0.2	99.6
237	1	0.0	99.6
437	1	0.0	99.6
TNTC[b]	11	0.4	100.0
Total	3023	—	100.0

[a] Colonies per plate.
[b] TNTC, too numerous to count.

samples. Plate counts were not converted to colonies per gram, because the amount of material was very small and accurate weighing of the cosmetic material picked up by the swabs was difficult.

Contamination Level per Product Type

Table 10 shows the percentage of samples of each product type that contained fungi of any type and pathogenic or opportunistic fungi. Fungi were present in 10.4% of the samples, and pathogenic or opportunistic fungi were identified in 3.9%. Proportionally fewer lip products contained fungi than would be expected from the overall sample distribution. Fungi were identified in 11.3% (176/1560) of the eye products, 12.5% of the face products, and 7.1% of the lip products. Pathogens were found in 4.1% of the eye products, 5.2% of the face products, and 2.4% of the lip products.

Follow-up Study

To determine if the fungi found in the shared-use cosmetics resulted from shared use or were present in the unopened cosmetics, FDA conducted a follow-up to this survey in 1990. A total of 407 unused and unopened retail samples equivalent to those shared-use cosmetic products that were found to be contaminated at high levels or with pathogens during the 1989 survey were collected and analyzed. One hundred percent of the samples had fungal plate counts of ≤100 colonies per gram (unpublished data).

Because high levels of microorganisms were found in the shared-use cosmetic testers analyzed in 1989, and the corresponding unopened retail products analyzed in 1990 were not contaminated, microbiological contamination appears to have occurred during consumer use.

Conclusions

Increased time in use did not correspond to higher fungal incidence. The presence of preservatives may have had some effect in reducing the incidence of fungi. Face products had the highest relative percentage of samples with fungi and pathogenic/opportunistic fungi. Lip products had the lowest relative percentage of contaminated samples. Fungal contamination in shared-use cosmetics was clearly identified, and even more important, significant levels of pathogens and opportunistic pathogens were found in 4–5% of the samples. This finding indicates potential problems with the safety of shared-use cosmetics.

Table 10. Contamination level for each product type for fungi and pathogenic or opportunistic fungi

	Samples		
Product type	Total No. tested (%)	No. containing fungi (%)	No. containing pathogenic or opportunistic fungi (%)
Eye	1560 (100.0)	176 (11.3)	64 (4.1)
Face	640 (100.0)	80 (12.5)	33 (5.2)
Lip	778 (100.0)	55 (7.1)	19 (2.4)
Unknown	49 (100.0)	5 (10.2)	3 (6.1)
Total	3027 (100.0)	316 (10.4)	119 (3.9)

Acknowledgments

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TECHNICAL COMMUNICATIONS

Small-Scale Survey of Organotin Compounds in Household Commodities

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Ninety-five commercially available items were analyzed for a small-scale survey of organotin compounds (OTCs) in household commodities such as textile products, shoe polish, adhesives, paint, and wax. Neither tributyltin nor triphenyltin species were found in any items; however, di- and trioctyltin and/or dibutyltin species (DOT, TOT, and DBT, respectively) were detected in 15 items; DOT was found in 14 items, TOT in 5 items, and DBT in 4 items. DOT was found in 2 diaper covers at about 0.1%. Detection frequencies of OTC were 6/10 for diaper covers, 3/10 for bibs, 2/10 for sanitary panties, 1/5 for outergarments, 1/5 for stockings, 1/5 for socks, and 1/10 for shoe polish.

Organotin compounds (OTCs) represented by tributyltin (TBT) and triphenyltin (TPT) compounds are generally formulated R_nSnX_{4-n} (R = aryl, alkyl, and alicyclic chains; X = inorganic or organic anionic substituents; and n = number of substituents). The concentration and/or accumulation of TBTs and TPTs in the aquatic environment and marine products have raised new pollution issues.

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TBTs and TPTs were used as biocides and fungicides for household commodities because of their strong biocidal activities. However, they were banned in 1979–1980 in Japan by the Law for the Control of Household Products Containing Harmful Substances (1) because of their strong skin-stimulating activities and intense toxicities. Household products designated under the Law are textile products, adhesives, paint, wax, and shoe polish. We have been inspecting both compounds in household commodities to protect consumer safety. Recently, we found 2 kinds of OTCs different from TBTs and TPTs in diaper covers by using a flame photometric detection/gas chromatography (FPD/GC) method after conversion of OTC to tetra-substituted derivatives with propyl magnesium bromide. Products were identified as di- and tri-*n*-octyltin (DOT and TOT, respectively) compounds by mass spectrometry (MS) (2). On the basis of these findings, public health agencies must survey the species of OTC contained in household commodities.

METHOD

Apparatus

(a) *FPD/GC system.*—GC-8A gas chromatograph (Shimadzu Co., Ltd, Kyoto, Japan) equipped with an FPD sys-

tem operated in the tin mode (filter for 610 nm) and Shimadzu C-R1B chromatopac using a megabore column DB-17, 15 m × 0.53 mm id (J & W Scientific, Inc., Folsom, CA 95630). Temperatures: column oven, programmed from 80°C to 250°C at 8°C/min; injection port and detector, 270°C. Gas flow rates; nitrogen (carrier gas), 20 mL/min; hydrogen, 0.5 kg/cm^2; air, 0.7 kg/cm^2.

(b) *GC/MS system.*—Hewlett Packard GC 5890 series II and JEOL JMS-AX505W (JEOL, Tokyo, Japan) operated in the electron impact mode at an ionization voltage of 70 eV and an ionization current of 300 μA. The column, programmed oven temperature, and injection port temperature were the same as for FPD/GC system. Separator and ion source temperatures: 250°C. Carrier gas: Helium, 20 mL/min.

Reagents

All chemicals were analytical grade unless otherwise stated. Organic solvents were pesticide grade. Deionized and distilled H_2O was used throughout.

(a) *OTCs.*—Tri-*n*-butyltin chloride (TBTC), triphenyltin chloride (TPTC), di-*n*-butyltin dichloride (DBTD), di-*n*-octyltin oxide (Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan). Diphenyltin dichloride (DPTD) (Aldrich Chemical Co., Milwaukee, WI 53201). Tri-*n*-octyltin chloride (TOTC) (Fluka Chemie AG, Buchs, Switzerland). Convert di-*n*-octyltin oxide to dichloride form (DOTD) according to the official method (1) before use. Purify DOTD on 10% hydrated alumina column by the method reported previously (2).

(b) *n-Propyl magnesium bromide (PMB).*—Approximately 2M PMB in tetrahydrofuran (Tokyo Kasei Kogyo Co., Ltd).

(c) *Phosphate–citrate buffer.*—Dissolve 1.43 g $Na_2SO_4 \cdot 12H_2O$, 17.3 g citric acid, and 5.0 g NaCl in 800 mL H_2O. Adjust pH to 2.0 with 1M HCl. Make up exactly 1000 mL with H_2O.

Samples

Ninety-five household commodities examined in this study were purchased, 2 items or less per manufacturer, at retail stores in Aichi Prefecture in 1990–1992. Each item was designated under the Law (1). Details of household commodities were as follows: 10 diapers, 10 diaper covers, 10 bibs, 10 sanitary panties, 10 pairs of underwear, 5 pairs of socks, 5 pairs of gloves, 5 outergarments, 5 stockings, 10 shoe polishes, 5 adhesives, 5 paints, and 5 waxes.

Extraction

A synopsis of sample preparation is provided here because of minute mention in the literature (1, 3, 4).

(a) *Textile products.*—Add 75 mL MeOH containing 0.05% HCl to 1 g sample, and reflux 30 min at 70°C. Add 50 mL phosphate–citrate buffer and 100 mL H_2O to the filtrate. Extract with 30 mL CH_2Cl_2 twice. Evaporate to dryness after drying over anhydrous Na_2SO_4.

(b) *Water soluble paint, adhesive, etc.*—Add 20 mL MeOH and 1 mL HCl to 1 g sample, successively, and shake

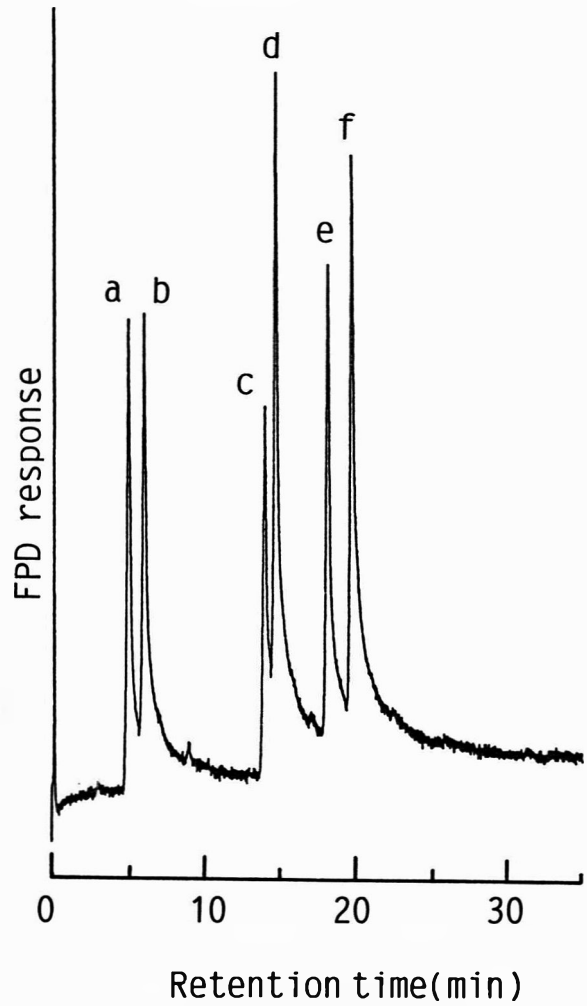

Figure 1. Typical FPD/gas chromatogram of standard propylated organotin compounds: a, 4.0 ng dipropyl-DBT; b, 5.0 ng propyl-TBT; c, 11.0 ng dipropyl-DOT; d, 15.0 ng dipropyl-DPT; e, 16.0 ng propyl-TOT; f, 18.0 ng propyl-TPT.

vigorously. Add 25 mL phosphate–citrate buffer and 50 mL H_2O to the filtrate. Extract with 30 mL CH_2Cl_2 twice, and evaporate to dryness.

(c) *Lipo-soluble shoe polish, wax, etc.*—Add 2 mL MeOH, 50 mL 3M HCl, and 10 g NaCl to 1 g sample, successively, and homogenize. Extract with 100 mL *n*-hexane–ethyl ether (4 + 6) twice. Wash combined organic phase with 50 mL H_2O. Evaporate to dryness.

Purification

Purify crude extract on a Florisil column (Florisil PR 3 g, 3 × 1.5 cm id) eluted with 40 mL ethyl ether and then 40 mL ethyl ether–acetic acid (99 + 1). Keep the later fraction and evaporate to dryness.

Propylation

Dissolve the later fraction in 1 mL *n*-hexane. Add 1 mL PMB carefully, and let solution stand 30 min at room temperature. Add 3 mL 0.5M H_2SO_4 dropwise to the solution to decompose extra PMB. Transfer the reaction mixture to separa-

Table 1. Recovery of OTCs from household commodities[a]

Item	Rec., %					
	DBT	TBT	DOT	TOT	DPT	TPT
Diaper cover	93.9 ± 3.5	91.8 ± 2.9	92.0 ± 3.2	92.3 ± 3.1	38.8 ± 5.5	91.9 ± 3.7
Socks	92.4 ± 3.8	90.5 ± 2.5	91.5 ± 2.5	92.8 ± 3.2	35.3 ± 5.1	93.1 ± 3.7
Adhesive	89.1 ± 3.1	88.5 ± 3.9	88.9 ± 3.0	85.0 ± 3.1	74.9 ± 4.5	91.1 ± 3.0
Shoe polish	87.1 ± 3.5	89.5 ± 3.5	90.3 ± 3.2	91.3 ± 2.9	89.6 ± 3.1	91.0 ± 3.5

[a] Each value is the mean of triplicate analyses ± standard deviation.

tory funnel, and rinse the flask with 10 mL EtOH. Add 70 mL H_2O, and extract with 20 mL *n*-hexane twice. Evaporate to dryness after drying over anhydrous Na_2SO_4. Redissolve the concentrate in *n*-hexane. Solution is ready for FPD/GC and GC/MS analysis.

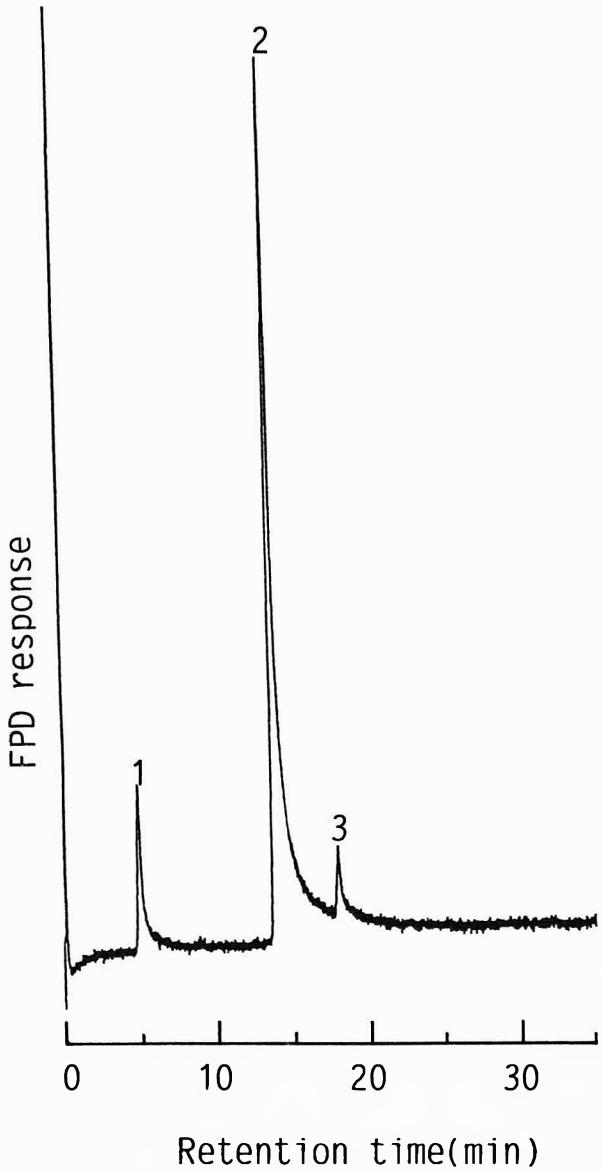

Figure 2. FPD/gas chromatogram obtained from diaper cover A: RTs of peaks 1, 2, and 3 were the same as those of dipropyl-DBT, dipropyl-DOT, and propyl-TOT, respectively.

Determination

Calculate the amount of OTC in the samples from the peak height of corresponding tetra-substituted organotin peak in FPD/gas chromatogram. Propylate standard OTC in the same manner as the above. Prepare working standard solutions for dipropyl-DBT and DOT and propyl-TOT by the dilution of the stock solution with *n*-hexane in the following ranges: 0.2–5.0 μg/mL (as DBTD), 0.2–10.0 μg/mL (as DOTD), and 0.5–15.0 μg/mL (as TOTC).

Recovery

Spike OTC at 3.0 ppm level in 4 items at sampling stage. Determine OTC contents according to above procedure, and calculate the overall recovery through whole analytical process.

Results and Discussion

Numerous FPD/GC methods with alkylation of OTC were published (4–7). Among these methods, we used propylation with PMB (4). A typical FPD/gas chromatogram for standard mixture of 6 kinds of propylated OTC is shown in Figure 1. Good resolution for propylated OTC was given by our GC conditions. Retention times (RTs) of dipropyl-DBT, propyl-TBT, dipropyl-DOT, dipropyl-DPT, propyl-TOT, and propyl-TPT were 5.12, 6.15, 14.11, 14.83, 18.23, and 19.83 min, respectively.

A plot of peak height of FPD/gas chromatogram vs amount of standard OTC injected resulted in linearity over the concentration range examined. As shown in Table 1, good recoveries were obtained; however, DPT recovery from textile products was poor. Ishizaka et al. (8) did not achieve sufficient recovery of DPT from fishes, in spite of their exhaustive efforts. When the survey is focused on DPT in textile products, the extraction procedure has to be modified efficiently to achieve satisfactory recovery of DPT from a Florisil column. In view of poor recovery of DPT from textile products, DPT was excluded from this study. Detection limits were 0.2 ppm for DBTD, TBTC, and DOTD and 0.5 ppm for TOTC and TPTC, respectively.

Peaks corresponding to TBTs and TPTs were not observed at all for 95 items of household commodities examined in this study; however, other OTCs were found in samples. FPD/gas chromatogram of diaper cover A is shown in Figure 2. Three

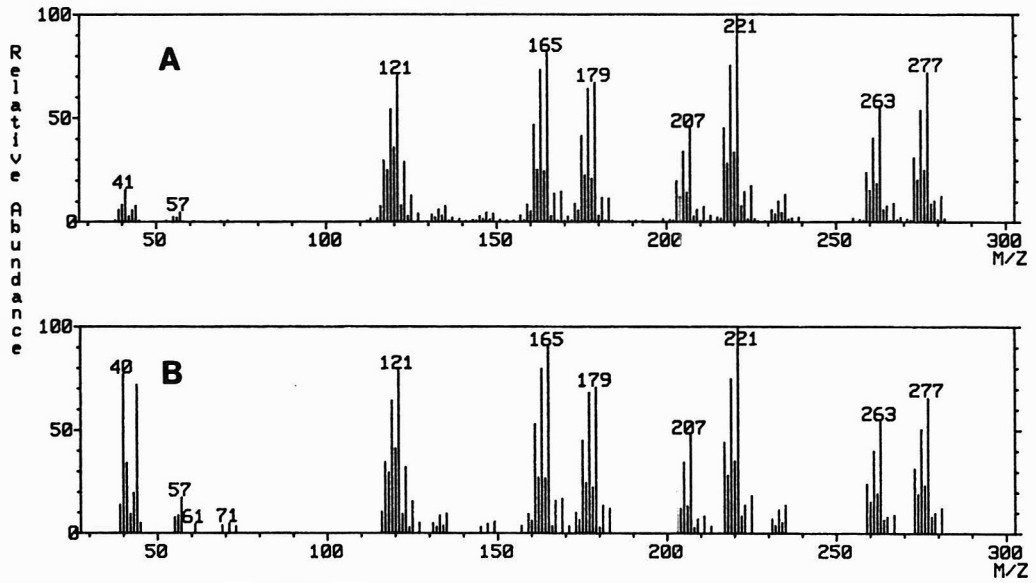

Figure 3. EI mass spectra of dipropyl-DBT: A, standard dipropyl-DBT; B, peak 1 in Figure 2.

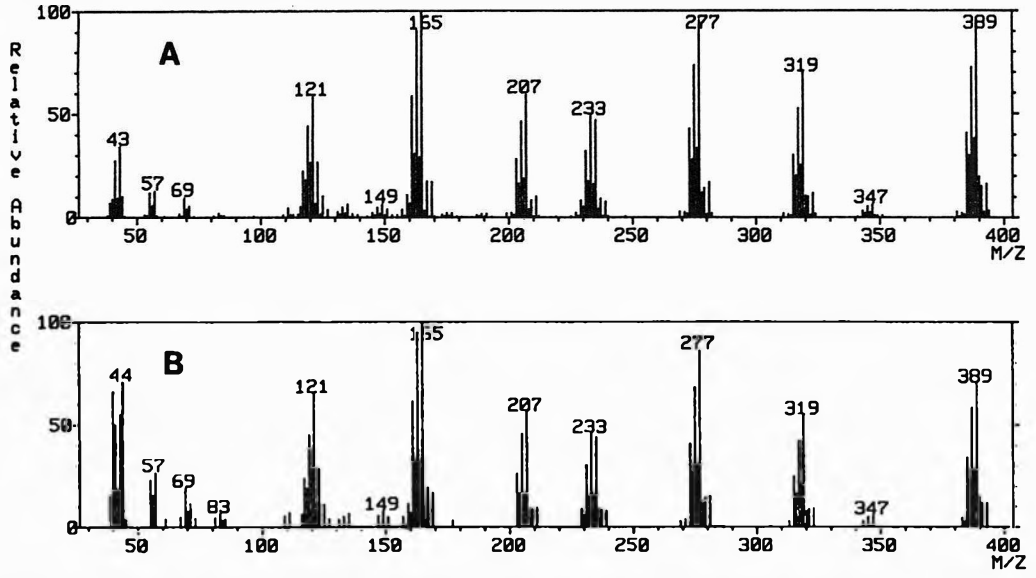

Figure 4. EI mass spectra of dipropyl-DOT: A, standard dipropyl-DOT; B, peak 2 in Figure 2.

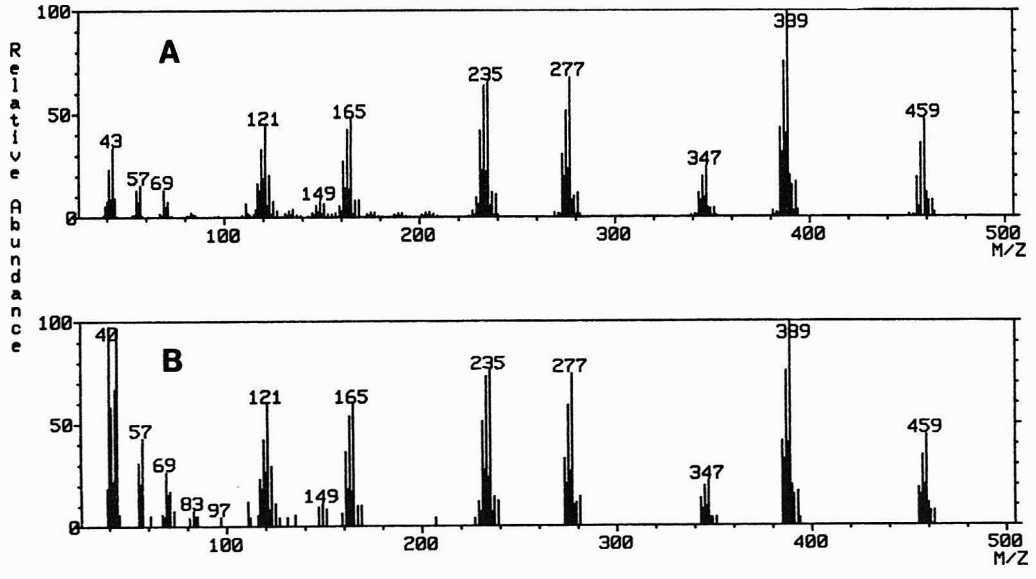

Figure 5. EI mass spectra of propyl-TOT: A, standard propyl-TOT; B, peak 3 in Figure 2.

Table 2. Frequency of OTCs in household commodities

Item	N[a]	n[b]	DBT	TBT	DOT	TOT	TPT
Diaper	10	0	0	0	0	0	0
Diaper cover	10	6	3	0	6	4	0
Bib	10	3	0	0	3	0	0
Sanitary panty	10	2	1	0	1	0	0
Underwear	10	0	0	0	0	0	0
Socks	5	1	0	0	1	0	0
Gloves	5	0	0	0	0	0	0
Outergarment	5	1	0	0	1	0	0
Stockings	5	1	0	0	1	1	0
Shoe polish	10	1	0	0	1	0	0
Adhesive	5	0	0	0	0	0	0
Paint	5	0	0	0	0	0	0
Wax	5	0	0	0	0	0	0

[a] Number of items surveyed.
[b] Number of items found OTC.

peaks at RTs 5.12 min (peak 1), 14.11 min (peak 2), and 18.23 min (peak 3) were observed for this sample. Peaks 1, 2, and 3 had the same RTs as those of dipropyl-DBT, dipropyl-DOT, and propyl-TOT, respectively. To confirm OTC of peaks 1, 2, and 3, GC/MS analysis was performed, and mass spectra are shown in Figures 3–5. All of their mass spectra were characteristic of OTC. A base peak at *m/z* 221 $[Sn(C_4H_9)(C_3H_7)]^+$ and fragment peaks at *m/z* 121 $[Sn]^+$, 165 $[Sn(C_3H_7)]^+$, 179 $[Sn(C_4H_9)]^+$, 207 $[Sn(C_3H_7)_2]^+$, 235 $[Sn(C_4H_9)_2]^+$, 263 $[Sn(C_3H_7)_2(C_4H_9)]^+$, and 277 $[Sn(C_3H_7)(C_4H_9)_2]^+$ and their isotope peaks were observed for peak 1, as shown in Figure 3. Because this mass spectrum was the same as that of dipropyl-DBT, we identified the OTC of peak 1 as DBT. Also, we confirmed OTC of peaks 2 and 3 as DOT and TOT, respectively, because mass spectra of these peaks were the same as those of corresponding propyl species (Figures 4 and 5).

Among 95 household commodities, 15 contained 1 or 2 OTCs. Detection frequencies of OTCs are summarized in Table 2. Diaper covers had the highest frequency among surveyed items, followed by bibs. No OTCs were found in diapers, underwear, gloves, adhesives, paint, and wax. Table 3 shows the amounts of OTCs in 15 items. Diaper cover A contained 3 kinds of OTCs at near 1000 ppm DOT, and a similar concentration was observed for diaper cover B. DOT was found in 14 samples except for sanitary panties B, where DOT ranged from 24.4 to 1666.7 μg/g (as its dichlorides). DBT in 4 items ranged from 3.7 to 33.7 μg/g. Five samples contained TOT, which resulted from impurities of commercially available DOT preparations and coexistence with DOT.

Disubstituted OTCs are mainly used in the plastic industry, particularly as stabilizers in poly(vinyl chloride). They are also used as catalysts in the productions of polyurethane forms and in the room-temperature vulcanization of silicones (9, 10). Origins of OTCs found in 15 samples can be classified as intentional addition to products for some effect (e.g., water resistivity) and carry over from materials such as poly(vinyl chloride). We assumed that OTCs detected in diaper covers A–E, bib A, outergarments, and shoe polish were used as catalysts with silicones to increase water resistivity because of the considerable amounts of DOT and the usage of their products. The amounts of DOT found are not astonishing, because textile products are silicone-coated using DOT as a catalyst at a percentage order. On other samples, OTC may come from carry over as catalysts and stabilizers for polyurethane and poly(vinyl chloride), respectively.

The toxicities of the OTCs are a concern for public health. Toxicological data of OTCs were stated in the literature (9–11). Barnes and Magee (12) and Barnes and Stoner (13) reported that disubstituted OTCs did not affect the central nervous system but they were potent irritants that could induce an inflammatory reaction in the bile duct. Also, DBTD was found to be the most toxic by studying the relationship of the chemical structure to the action of several disubstituted OTCs on the thymus and thymus-dependent lymphoid tissue (14–16). DOTD and TOTC are considered biologically unreactive when given

Table 3. Contents of organotin compounds in household commodities[a]

Item	DBT, μg/g	DOT, μg/g	TOT, μg/g	Materials or ingredients
Diaper cover A	33.7	902.8	58.3	Cotton, polyester
Diaper cover B	8.9	1666.7	—	Polyester
Diaper cover C	—	743.4	128.5	Cotton
Diaper cover D	—	509.6	91.3	Polyester
Diaper cover E	—	288.0	33.6	Wool
Diaper cover F	3.7	24.4	—	Polyester, polyurethane
Bib A	—	312.5	—	Cotton, polyester
Bib B	—	59.7	—	Cotton, poly(vinyl chloride)
Bib C	—	48.9	—	Cotton, poly(vinyl chloride)
Sanitary panty A	—	144.4	—	Cotton, polyurethane, nylon
Sanitary panty B	5.5	—	—	Nylon, polyurethane
Outergarment	—	388.9	—	Cotton
Stockings	—	136.1	14.2	Nylon, polyurethane
Socks	—	34.2	—	Cotton, polyurethane, nylon
Shoe polish	—	326.4	—	Wax, lipid, organic solvent

[a] Each value is the mean of duplicate determinations and expressed as its chloride form.

orally (11, 17–19). No skin lesions were observed for DOTD (10). We cannot determine dermal toxicity by the amounts detected in this study, because no examinations were performed on amounts contacting body surfaces and then being absorbed. However, our results offer little disturbance, judging from the above toxicological data. This project was a small-scale survey of OTC in household commodities and does not reflect the prevalence in most items designated under the Law (1). However, our results showed the occurrence of OTC compounds at high concentrations. We plan on constructing a sample preparation including DPT and continuing the survey of OTCs in household commodities for accumulation of further data.

Acknowledgment

We are much obliged to Ikai Y. for GC/MS operation.

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REGULATORY ANALYTICAL METHODS

Introduction

Regulatory agencies approve and promulgate methods of analysis that regulate commodities in their markets. In many instances, these internally approved methods are not widely disseminated outside the agencies. In order to give a method a wider audience, an agency may submit it for publication in the *Journal of AOAC International*. This "Regulatory Analytical Methods" section in the Journal will provide select methods submitted by an agency. Final decisions to publish a method rests solely with the Editor-in-Chief of the Journal.

The methods published under this section have received approval by a national, state/provincial, or international regulatory agency or body and are used to regulate commodities that fall within the scope of the Journal. Each regulatory analytical method published contains its own introduction that explains the origin, nature, and approval that the particular method has undergone.

Having been approved by the regulatory agency submitting the method, the methods published under this section do not undergo the peer review accorded AOAC Journal articles before being published. These methods are ***not*** AOAC Official Methods of Analysis, because they have not been subjected to the full AOAC collaborative study process.

The intent of publishing these regulatory methods in the Journal is to give them a wider distribution and provide them with a publication reference. It is hoped that this section will be used by regulatory bodies of the world to disseminate their methods to scientists everywhere. They are not, however, meant to supplant the AOAC collaborative study process, and AOAC encourages all regulatory bodies and associated industries to support that process.

REGULATORY ANALYTICAL METHODS

Animal Drugs

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The U.S. Food and Drug Administration (FDA) is required by the general safety provisions of sections 409, 512, and 706 of the Federal Food, Drug and Cosmetic Act to determine if each food additive, new animal drug, or color additive proposed for use in food-producing animals is safe. The pertinent regulations implementing the statutory provisions are found in 21 CFR Parts 70 and 500.

The sponsor of an application for use of an animal drug is required as part of the approval to submit scientific data to demonstrate that the use of the drug is safe for the animals proposed and any edible food to be used for consumption. To demonstrate the safety of the drug, the sponsor must submit acceptable analytical methods. These methods must be capable of determining and confirming the amount and presence of the animal drug or its metabolites in a variety of matrixes.

FDA typically requires analytical methods for finished pharmaceutical and medicated feed dosage forms and for residues of the drug in edible animal tissues. The sponsor must present data or information that demonstrates the method can perform what it purports according to sound analytical principles. In addition, FDA performs a trial of the method according to strict procedures to ensure the method is acceptable. The U.S. Department of Agriculture laboratories also participate with FDA in testing tissue residue methods.

The types of methods described above, because they are used for regulatory purposes by FDA in its compliance programs, are available for release to the general public. These methods are releasable under the Freedom of Information Act. 21 CFR 514.11(e)(6) specifically permits the public disclosure of assay methods after approval of the animal drug has been published in the *Federal Register*.

Because of the volume and types of feeds containing approved animal drugs and used for food-producing animals, the FDA Center for Veterinary Medicine (CVM) has made it a policy to make available to the public methods for animal drugs in Type A Medicated Articles (premixes) and the corresponding Type B and C Medicated Feeds and tissue residues. CVM is the unit responsible for evaluating the effectiveness and safety of animal drugs.

Before 1973, the methods were incorporated into a manual called the *Food Additives Analytical Manual*. This manual, which is no longer available, included methods for direct and indirect food additives and animal drugs. In 1985, CVM issued a new manual called *Animal Drug Analytical Manual*. This manual, often referred to as ADAM, contains only methods for animal drugs. The 1985 edition contained only 8 methods. The manual is published and distributed by AOAC International. Information for the manual is furnished by CVM.

Since 1985, there have been no updates to ADAM. CVM, in cooperation with AOAC International, is now introducing a new procedure for making methods more readily available. It is the intention of CVM in conjunction with AOAC International to publish the methods in the *Journal of AOAC International* first and then incorporate the same in a later update to ADAM.

Tiamulin Hydrogen Fumarate

The following methods are approved regulatory methods to determine and confirm residues of tiamulin hydrogen fumarate.

Method I. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Feed Premixes

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Tiamulin, 14-desoxy-14-[(2-diethylaminoethyl)mercaptoacetoxy]mutilin hydrogen fumarate (CAS No. 55297-96-6), is used for the treatment of swine dysentery and pneumonia and for weight gain and feed efficiency. Denagard™ is the trade name for a 45% (as tiamulin) water-soluble formulation.

Regulation Information

21CFR520.2455 provides for approved use of tiamulin in the treatment of swine as a 45% water-soluble powder to make medicated drinking water (227 mg tiamulin/gal) for treatment of swine dysentery (1983) and for treatment of swine pneumonia (1987).

21CFR558.600 provides for approved use of tiamulin in a Type A medicated article containing 5, 10, or 113.4 g tiamulin (as hydrogen fumarate) per pound to be used in the manufacture of a Type C medicated feed at 35 g/ton (for use in the control of swine dysentery) and at 10 g/ton (for use in weight gain and feed efficiency for swine).

When used at 35 g/ton, the feed must be withdrawn 2 days before slaughter. The drug must not be used in conjunction with other polyether ionophores such as lasalocid, monensin, narasin, and salinomycin.

21CFR558.4 provides for the following assay limits.

Type A medicated articles.—5 and 10 g/lb, 90–115% label content; 113.4 g/lb, 100–108% label content.

Type C medicated feeds.—35 g/ton, 70–130% label content.

Tolerances: 21CFR556.738

Marker Residue: 8-α-Hydroxymutilin

A tolerance of 0.4 ppm was established for tiamulin in swine for 8-α-hydroxymutilin (the marker residue) in liver (target tissue).

A marker residue concentration of 0.4 ppm in liver corresponds to a concentration of 10.8 ppm tiamulin. The safe concentrations for total residues in uncooked edible tissues of swine are 3.6 ppm in muscle, 10.8 ppm in liver, and 14.4 ppm in kidney and fat.

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Method I. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Feed Premixes

Scope

Tiamulin hydrogen fumarate is determined in feed premixes (Type A medicated articles). The method is applicable to the analysis of tiamulin hydrogen fumarate feed premixes prepared with rice hulls (or wheat middlings) and mineral oil. Applicability of the method to other types of premix carriers or diluents must be demonstrated, as needed, before use.

Principle

Premix samples are treated with a solution of sodium carbonate to remove the tiamulin hydrogen fumarate from the carrier matrix and convert the fumarate to the free base. The tiamulin base is then extracted into an organic solvent mixture and subsequently back-extracted into an aqueous solution of tartaric acid, a process that converts the base to the tartrate. Tiamulin (as the tartrate) is then analyzed by reversed-phase liquid chromatography (LC). Under the LC conditions specified, the analyte determined is the tiamulin free base.

Apparatus

All references to commercial apparatus and chemicals used in this method are for descriptive purposes only and do not constitute an endorsement by the U.S. Food and Drug Administration (FDA) or the U.S. Government. Equivalent products may be used.

(a) *Liquid chromatograph.*—Equipped with a 254 nm UV detector.

(b) *Precolumn.*—Adsorbosil silica, 200–425 mesh (Alltech Associates).

(c) *Guard column.*—CO:PELL ODS (Whatman Inc.).

(d) *Analytical column.*—Hypersil ODS, 5 μm, 250 × 4.6 mm id.

(e) *Analytical balance.*—Capable of weighing to ±0.001 mg.

(f) *Jars.*—One-half gallon wide-mouth, with caps.

(g) *Platform shaker.*

Reagents

(a) *Acetonitrile and methanol.*—LC grade.

(b) *Ammonium carbonate, sodium carbonate, tartaric acid, ethyl acetate, hexane, and glacial acetic acid.*—ACS reagent grade.

(c) *Tiamulin hydrogen fumarate.*—Analytical standard of known purity (Fermenta Animal Health Company, Kansas City, MO 64190).

(d) *Sodium carbonate solution, 1.0% w/v.*—Weigh sodium carbonate (40 g), and dilute to 4000 mL with United States Pharmacopeia (USP) purified (or distilled) water.

(e) *Extraction solvent.*—Mix 3000 mL hexane with 1000 mL ethyl acetate (*Caution*: flammable solvent).

(f) *Tartaric acid solution, 0.1% w/v.*—Weigh 1.0 g tartaric acid, and dilute to 1000 mL with USP purified (or distilled) water.

(g) *Ammonium carbonate solution, 1% w/v.*—Add 10 g ammonium carbonate to 1 L volumetric flask, and dilute to volume with USP purified (or distilled) water.

(h) *Acetic acid solution, 1% v/v.*—Add 10 mL glacial acetic acid to 1 L volumetric flask, and dilute to volume with USP purified (or distilled) water.

(i) *LC mobile phase.*—Methanol–acetonitrile–1% ammonium carbonate (60 + 30 + 25, v/v/v). Filter mixture through 0.45–0.50 µm disc (or equivalent filter system), and then degas by ultrasonic vibration and vacuum (ca 3 min) (*Caution*: flammable solvent).

(j) *Regeneration solution.*—Mix equal volumes of methanol and 1% acetic acid. Filter and degas as with *LC Mobile Phase*.

Note: Unless otherwise specified, mixed reagents are stable for ca 7 days.

(k) *Tiamulin standards.*—Prepare stock solutions containing 0.075, 0.150, and 0.180 mg/mL tiamulin hydrogen fumarate as follows: Accurately weigh (to the nearest 0.001 mg) 7.5, 15, and 18 mg tiamulin hydrogen fumarate into three 100 mL volumetric flasks, and dilute to volume with 0.1% w/v tartaric acid solution. Calculate the concentration of tiamulin hydrogen fumarate in each solution from the exact weights while correcting for the purity of the standard.

Analysis

Safety precautions.—Safe laboratory practices should be observed during the method when using all chemicals.

Isolation Procedures

Sample preparation.—Accurately weigh sufficient premix to contain ca 110 mg tiamulin hydrogen fumarate into a 1/2 gal. wide-mouth jar. For a 10 g/lb premix, the sample weight should be 5.000 g.

Sample extraction.—Add 250 mL 1% sodium carbonate solution to each wide-mouth jar, and cap jar tightly. Place jars on a platform shaker and shake 30 min. Add 250 mL (accurately measured) extraction solvent of hexane–ethyl acetate (3 + 1, v/v), and shake an additional 60 min. Remove jars from the shaker, and shake vigorously by hand 15–20 s. Let jars stand until layers separate. (*Note*: The separation process may be accelerated by centrifugation. However, the loss of the organic solvent must be minimized to avoid false high results.)

Volumetrically pipet 10.0 mL organic (upper) layer into a suitable separatory funnel. Volumetrically add 30.0 mL 0.1% tartaric acid solution to the funnel, cap, and extract gently in a horizontal position for 30 s. (*Note:* The funnel must be shaken hard enough to obtain adequate mixing between the phases but not hard enough to create emulsions.) Let phases separate, and then draw off a portion of the lower layer and analyze as described in *Determinative Procedures*.

Determinative Procedures

LC parameters.—Detection system, ca 254 nm; sensitivity, 0.02 AUFS; mobile phase flow rate, 1.5 mL/min; injection volume, 50 µL. Under these conditions, the retention time of tiamulin (as the free base) is ca 7 min. (*Note*: If necessary, adjust the mobile phase ratio and flow rate to obtain the optimum chromatographic results. Adjust the injection volume to give a response of ca 80% full scale deflection.)

System suitability check.—After the system reaches equilibrium, make repeated injections of the 0.150 mg/mL standard preparation until reproducible peak areas (or heights) are obtained. Then, inject the lower concentration suitability standard (0.075 mg/mL) in triplicate, followed by the 0.180 mg/mL standard (also in triplicate). Using peak areas or heights, calculate the concentrations of the 0.075 and 0.180 mg/mL standards, and compare with the theoretical concentrations. The results must be within ±5% of the theoretical concentration of both standards to continue.

Analysis of samples.—Following a satisfactory system suitability check, inject samples and standards in the following order: 1 injection of standard, 2 injections of sample, 1 injection of standard, 2 of sample, etc.

The suggested standard and sample concentrations were chosen to provide corresponding detector responses approximately equal to each other when the premix is at 100% of label claim. If responses differ by more than 20%, adjust the standard accordingly. If necessary, assay a graduated series of calibration standards to check detector linearity.

The tiamulin free base peak should be baseline-resolved from front-running interferences. If it is not, column regeneration may be indicated. Regeneration is recommended after overnight operation of the system. Pump at least 60 mL regeneration solvent through the system, followed by at least 30 mL methanol, followed by mobile phase until the system stabilizes. Back pressure of the acetic acid–methanol regeneration solvent may necessitate a flow rate of ca 1 mL/min.

Calculations.—Calculate the amount of tiamulin hydrogen fumarate in the premix by using peak heights (or areas) according to the following formula:

$$\text{tiamulin hydrogen fumarate (g/lb)} = (R_u/R_s) \times (C_s/V_i) \times (V_o/W) \times (V_f/1000) \times 454$$

Table 1. Recovery (g/lb) of tiamulin from spiked samples for one instrument

Parameter	Rec., g/lb		
	A[a]	B	C
	7.36	10.76	13.93
	7.35	10.78	14.01
	7.37	10.75	13.93
	7.36	10.67	13.98
	7.44	10.68	14.09
	7.57	10.54	14.01
	7.65	10.70	—
Mean, g/lb	7.44	10.70	13.98
SD, g/lb	±0.112	±0.075	±0.060
CV, %	1.50	0.70	0.43
Av. rec., %	100.2	100.9	101.3

[a] Theoretical values (g/lb): A = 7.43, B = 10.60, C = 13.80.

Table 2. Recovery (%) of tiamulin and coefficient of variation (%) from FDA laboratories

Sample	Beltsville	Kansas City
Unmedicated premix	NDI[a]	NDI
Fortified premix		
50X[b]	88 ± 3.9	96 ± 1.0
CV[c]	4.5	1.0
100X	75 ± 4.0	97 ± 4.5
CV	5.3	4.6
150X	76 ± 4.5	96 ± 2.5
CV	5.9	2.6
Medicated premix		
5 g/lb tiamulin	4.4 ± 0.3	5.4 ± 0.3
CV	6.9	5.5

[a] NDI, no detectable interferences.
[b] X, amount equivalent to that in the medicated premix.
[c] CV, coefficient of variation.

where R_u = response (area or height) of sample peak, C_s = concentration of standard in mg/mL, V_o = volume of organic solvent extraction solvent (250 mL), V_f = volume of final tartaric acid solution used for back extraction (30 mL), 454 = conversion factor for grams to pounds, R_s = response (area or height) of standard peak, V_i = aliquot volume of organic solvent taken from initial extraction (10 mL), W = weight of sample taken (g), and 1000 = conversion factor for milligrams to grams.

Recovery procedures.—Analyze spiked samples or samples with known tiamulin content to determine recovery levels.

Confirmation procedures.—No confirmation procedures were provided.

Method Reliability

Validation.—*(1) Method evaluation (sponsor's comments).*—Two separate 5-point calibration curves were constructed on 2 instruments by 2 analysts. Concentrations of standards used were as follow: 39.0, 77.9, 154.8, 169.8, and 193.8 μg/mL. Each standard was run in triplicate, and 2 separate calibration curves were constructed (one by using peak heights and one by using peak areas). The curve using peak heights showed a correlation coefficient of 0.9999, a y-intercept of 3.32 mm, and a slope of 0.907. The curve using peak areas showed a correlation coefficient of 0.9998, a y-intercept of 580.4 nominal area units, and slope of 697.1.

System suitability checks for a typical run (2 instruments, 2 analysts, areas, and heights) showed 100.8 and 101.5% of theoretical values for lower and higher standards, respectively, using peak areas; using peak heights, the values were 96.8 and 95.7% of theoretical values for lower and higher standards, respectively.

(2) *Recovery data (sponsor's information).*—Spiked samples were prepared with both wheat middlings and rice hulls as carriers at 3 concentrations. These were assayed over a 5 day period using 2 different instruments by 2 different analysts. Calculations for recoveries were performed using either peak heights or peak areas. Results are summarized in Table 1.

(3) FDA evaluation.—The method was run on samples containing rice hulls, rice mill by-products, or wheat middlings and tiamulin at 5 g/lb. The method was tested at FDA's Beltsville and Kansas City laboratories. Results are shown in Table 2.

FDA concluded that the method was acceptable for regulatory purposes of determining tiamulin in premixes (Type A medicated articles).

The method (PMS-689/85:Rvsn 1/91) was submitted by Fermenta Animal Health, Co., Kansas City, MO, in conjunction with the approval of the New Animal Drug Applications, NADAs 134-644 and 139-472.

REGULATORY ANALYTICAL METHODS

Method II. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Tiamulin–Poly(vinyl chloride) Formulations

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Tiamulin, 14-deoxy-14-[(2-diethylaminoethyl)mercapto-acetoxy]mutilin hydrogen fumarate (CAS No. 55297-96-6), is used for the treatment of swine dysentery and pneumonia and for weight gain and feed efficiency. Denagard™ is the trade name for a 45% (as tiamulin) water-soluble formulation.

Regulation Information

21CFR520.2455 provides for approved use of tiamulin in the treatment of swine as a 45% water-soluble powder to make medicated drinking water (227 mg tiamulin/gal) for treatment of swine dysentery (1983) and for treatment of swine pneumonia (1987).

21CFR558.600 provides for approved use of tiamulin in a Type A medicated article containing 5, 10, or 113.4 g tiamulin (as hydrogen fumarate) per pound to be used in the manufacture of a Type C medicated feed at 35 g/ton (for use in the control of swine dysentery) and at 10 g/ton (for use in weight gain and feed efficiency for swine).

When used at 35 g/ton, the feed must be withdrawn 2 days before slaughter. The drug must not be used in conjunction with other polyether ionophores such as lasalocid, monensin, narasin, and salinomycin.

21CFR558.4 provides for the following assay limits.

Type A medicated articles.—5 and 10 g/lb, 90–115% label content; 113.4 g/lb, 100–108% label content.

Type C medicated feeds.—35 g/ton, 70–130% label content.

Tolerances: 21CFR556.738

Marker Residue: 8-α-Hydroxymutilin

A tolerance of 0.4 ppm was established for tiamulin in swine for 8-α-hydroxymutilin (the marker residue) in liver (target tissue).

A marker residue concentration of 0.4 ppm in liver corresponds to a concentration of 10.8 ppm tiamulin. The safe concentrations for total residues in uncooked edible tissues of swine are 3.6 ppm in muscle, 10.8 ppm in liver, and 14.4 ppm in kidney and fat.

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Method II. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Tiamulin–Poly(vinyl chloride) Formulations

Scope

Tiamulin hydrogen fumarate is quantitatively determined in poly(vinyl chloride) (PVC) formulations containing, by weight, about 25% tiamulin.

Principle

Tiamulin is removed from the PVC matrix by extraction with water. An aliquot of the aqueous extract is then analyzed by reversed-phase liquid chromatography (LC).

Apparatus

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(a) *Liquid chromatograph.*—Equipped with a 254 nm UV detector.

(b) *Analytical column.*—Stainless steel, 30 cm × 3.9 mm id, packed with octadecyl (C_{18}) stationary phase, 10 μm particle size.

(c) *Filters.*—For disposable tips, 44 μm porosity (Supelco Inc.)

Reagents

(a) *Methanol and acetonitrile.*—LC grade.

(b) *Tiamulin hydrogen fumarate.*—Analytical standard of known purity (Fermenta Animal Health Company, Kansas City, MO 64190).

(c) *Ammonium carbonate solution, 1% w/v.*—Add 10 g ammonium carbonate (ACS reagent grade) to 1 L volumetric

Table 1. Recovery (%) of tiamulin and coefficient of variation (%) from FDA laboratories

Sample	Beltsville	Denver
Unmedicated premix	NDI[a]	NDI
Fortified premix		
50X[b]	102 ± 1.47	106 ± 6.2
CV[c]	1.4	5.9
100X	100 ± 0.75	100 ± 1.9
CV, %	0.7	1.9
150X	101 ± 1.2	102 ± 3.1
CV	1.1	3.1
Medicated premix		
25% triamulin	26.0 ± 0.3	26.3 ± 0.2
CV	1.2	0.9

[a] NDI, no detectable interferences.
[b] X, amount equivalent to that in the medicated premix.
[c] CV, coefficient of variation.

flask, and dilute to volume with United States Pharmacopeia purified (or distilled) water.

(d) *LC mobile phase.*—Methanol–acetonitrile–1% ammonium carbonate (60 + 30 + 25, v/v/v). Filter mixture through a 0.65 or 0.50 μm disc, and then degas by ultrasonic vibration and vacuum (*Caution*: flammable solvent).

(e) *Tiamulin stock solution, 2.2 mg/mL.*—Accurately weigh 0.22 g tiamulin hydrogen fumarate analytical standard into 125 mL Erlenmeyer flask. Add 100.0 mL water, stopper flask, and shake or place in an ultrasonic bath for 1 h. Calculate concentration of tiamulin in the standard solution from the exact weight while correcting for the purity of the standard.

Analysis

Safety precautions.—Safe laboratory practices should be observed during the method when using all chemicals.

Isolation Procedures

Sample preparation.—Accurately weigh 0.66 g sample into 125 mL Erlenmeyer flask. Add 100.0 mL water, stopper flask, and shake or place in an ultrasonic bath for 1 h. (*Note*: At the end of the shaking period, undissolved material will be observed in the sample flask. This is normal and results from the PVC granules, which do not dissolve in water.)

Take aliquots of the sample extract using a disposable pipet fitted with an Eppendorf plastic pipet tip and 45 μm filter.

Determinative Procedures

LC conditions.—Using the column and mobile phase specified above and a flow rate of 1.5 mL/min, the retention time for tiamulin should be ca 7 min.

Analysis of samples.—Inject aliquots of sample and standard in the following sequence: 1 injection of standard, 2 injections of sample, 2 of standard, 2 of sample, 2 of standard, etc. Initially, make several injections of standard to ensure repeatability.

The suggested standard and sample concentrations were chosen to provide approximately equal corresponding detector responses when the sample concentration is at 100% of the label guarantee. If the responses differ by more than 20% or before beginning a group of assays, a graduated series of calibration standards should be run. This will check detector linearity and allow for assay of various sample concentrations.

Calculations.—Calculate the percent by weight of tiamulin in the feed as follows:

$$\text{tiamulin (\%w/w)} = [(R_{sx})(C_{std})(V)/(R_{std})(1000)(W)] \times 100$$

where R_{sx} = peak height of sample, R_{std} = peak height of standard, C_{std} = concentration of tiamulin in the standard (mg/mL), V = volume of sample (mL), W = weight of sample (g), 1000 = factor for converting grams to milligrams.

Recovery procedures.—Analyze spiked samples or samples with known tiamulin content to determine recovery levels.

Confirmation procedures.—No confirmation procedures were provided.

Reliability of the Method

Validation.—*(1) Method assessment (sponsor's comments).*—The assay was performed on a single formulation lot by 2 analysts in quadruplicate on each of 3 days. The average value was 26.2% (w/w) tiamulin, with a range of 0.6%, standard deviation of 0.18%, and CV of 0.7%.

(2) FDA evaluation.—The method was tested on a 25% tiamulin–PVC formulation at FDA's Beltsville and Denver district laboratories. Results are given in Table 1.

FDA concluded that the method was acceptable for regulatory purposes of determining tiamulin in premixes (Type A medicated articles).

The method (PMS-677/83:Rvsn 2/86) was submitted by Fermenta Animal Health Co., Kansas City, MO, in conjunction with the approval of the New Animal Drug Applications, NADAs 134-644 and 139-472.

REGULATORY ANALYTICAL METHODS

Method III. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Complete Swine Meal Feeds

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When used at 35 g/ton, the feed must be withdrawn 2 days before slaughter. The drug must not be used in conjunction with other polyether ionophores such as lasalocid, monensin, narasin, and salinomycin.

21CFR558.4 provides for the following assay limits.

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Type C medicated feeds.—35 g/ton, 70–130% label content.

Tolerances: 21CFR556.738

Marker Residue: 8-α-Hydroxymutilin

A tolerance of 0.4 ppm was established for tiamulin in swine for 8-α-hydroxymutilin (the marker residue) in liver (target tissue).

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Method III. Liquid Chromatographic Determination of Tiamulin Hydrogen Fumarate in Complete Swine Meal Feeds

Scope

The method is applicable to the determination of tiamulin hydrogen fumarate in complete swine meal feeds. The method is not to be used for protein supplements and concentrates.

Principle

Feed is treated with a sodium carbonate solution to remove tiamulin from its carrier and to convert tiamulin to the free base. The tiamulin base is extracted into an organic solvent mixture and then concentrated by back-extraction into an aqueous tartaric acid solution. Tiamulin is then analyzed by reversed-phase liquid chromatography (LC).

Apparatus

All references to commercial apparatus and chemicals used in this method are for descriptive purposes only and do not constitute an endorsement by the U.S. Food and Drug Administration (FDA) or the U.S. Government. Equivalent products may be used. The equipment needed is listed in *Apparatus* of Method I.

Reagents

The same reagents and solutions in Method I are required, except that the tiamulin standard solutions are prepared as follows: (*1*) *Stock solution, 600 μg/mL.*—Accurately weigh 60 mg tiamulin hydrogen fumarate into 100 mL volumetric flask. Dilute to volume with 0.1% tartaric acid solution. Calculate the concentration of tiamulin in the stock solution from the exact weight, and correct for standard purity. The solution is stable for one week. (*2*) *Working solution, 60* μg/mL.—Pipet 10.0 mL stock solution into 100 mL volumetric flask, and di-

Table 1. Recovery (%) of tiamulin and coefficient of variation (%) from FDA laboratories

Sample	Beltsville	Denver	Kansas City
Unmedicated feed	NDI[a]	NDI	NDI
Fortified feed			
50X[b]	88 ± 1.8	85 ± 4.5	79 ± 11.2
CV[c]	2.1	5.3	14.2
100X	83 ± 4.8	81 ± 9.3	88 ± 4.2
CV	5.7	11.5	4.8
150X	81 ± 2.8	93 ± 0.002	73 ± 14.3
CV	3.5	0.0002	19.5
Medicated feed			
10 g/ton tiamulin	6.2 ± 0.3	9.1 ± 0.3	9.6 ± 0.4
CV	5.3	3.4	4.4

[a] NDI, no detectable interferences.
[b] X, equivalent amount to that in the medicated premix.
[c] CV, coefficient of variation.

lute to volume with 0.1% tartaric acid. Prepare a fresh solution for each assay.

Analysis

Safety precautions.—Safe laboratory practices should be observed during the method when using all chemicals. Be sure that the centrifuge bottle, bottle holder, and rotor are all rated for use under the conditions of the procedure.

Isolation Procedures

Sample preparation.—Accurately weigh a feed sample into a 1/2 gal. wide-mouth jar. For 10 g/ton tiamulin feeds, weigh 200 g sample; for 35 g/ton tiamulin feeds, weigh a 50 g sample.

Sample extraction.—To the jar with feed, add 250.0 mL extraction solvent followed by 250 mL 1% sodium carbonate. Cap jar and shake 5 s. (*Note*: Only glass containers should be used for solutions of tiamulin. Jars should have Teflon lid liners.)

Place jars on a mechanical shaker in an upright position, and shake 1 h. Good extraction is obtained using an orbital shaker at 250 rpm.

Let extracted samples settle 60 min. Transfer liquid layer from the settled feed in each jar to a centrifuge bottle. Balance the centrifuge, and spin 15 min at ca 1500 rpm. Let cool to room temperature.

Pipet 75.0 mL organic (upper) layer into a separatory funnel. Add 5.0 mL 0.1% tartaric acid solution to the separatory funnel. Hold the stoppered separatory funnel in a horizontal position, and gently shake 30 s. Let phases separate, and then draw off the aqueous (lower) layer into a glass-stoppered container.

Repeat the extraction with 2 additional 5.0 mL portions of tartaric acid solution. Combine these additional extracts with the first 5.0 mL portion. This is the sample solution to be chromatographed.

Determinative Procedures

LC conditions.—Use the same LC parameters given in Method I.

Analysis of samples.—Use the same sample analysis procedure given in Method I. Establish a flow rate of 1.5 mL/min through the LC system. Back pressure should be on the order of 1500 psi. Inject a standard solution until the peak areas (or heights) are reproducible. The retention time of the tiamulin peak should be ca 7 min. Allow at least 13 min per injection for adequate peak area (height) integration and for the system to return to a stable baseline. Inject sample and standard in the following order: 1 injection of standard, 2 injections of sample, 1 injection of standard.

Calculations.—Calculate the tiamulin concentration in the feed sample by using the peak heights of the standard and sample in the following equation:

$$\text{tiamulin (g/ton)} = \frac{(R_u)(C_s)(250)(V_2)(0.908)}{(R_s)(W)(V_1)}$$

where R_u = average response (peak height) for feed sample (or unknown), R_s = average response for standard, C_s = concentration of standard (μg/mL), W = weight of feed (g), V_1 = aliquot volume (75 mL), V_2 = volume of 0.1% tartaric acid (15 mL).

Recovery procedures.—Recovery is determined by analysis of fortified samples or samples with a known tiamulin content.

Confirmatory procedures.—No confirmatory procedures were provided.

Reliability of the Method

Validation.—Results given in Table 1 were obtained on samples of fortified feed, control (unmedicated) feed, and manufactured feed as analyzed by FDA. Because of emulsions encountered in the original samples, the Kansas City laboratory analyzed another sample using the method presented in the *Animal Drug Analytical Manual* and designated as PMS-690/85-Rvd 2/87. Results obtained by the Kansas City laboratory are also presented in Table 1.

FDA concluded that the method is acceptable for regulatory purposes of determining tiamulin in complete feeds (Type C medicated feeds).

The method (PMS-690/85:Rvd 2/87) was submitted by Fermenta Animal Health Co., Kansas City, MO, in conjunction with the approval of the New Animal Drug Applications, NADAs 134-644 and 139-472.

REGULATORY ANALYTICAL METHODS

Method IV. Gas Chromatographic Determination of Tiamulin Residues in Swine Liver

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21CFR558.4 provides for the following assay limits.

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Type C medicated feeds.—35 g/ton, 70–130% label content.

Tolerances: 21CFR556.738

Marker Residue: 8-α-Hydroxymutilin

A tolerance of 0.4 ppm was established for tiamulin in swine for 8-α-hydroxymutilin (the marker residue) in liver (target tissue).

A marker residue concentration of 0.4 ppm in liver corresponds to a concentration of 10.8 ppm tiamulin. The safe concentrations for total residues in uncooked edible tissues of swine are 3.6 ppm in muscle, 10.8 ppm in liver, and 14.4 ppm in kidney and fat.

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Method IV. Gas Chromatographic Determination of Tiamulin Residues in Swine Liver

Scope

Residues derived from tiamulin hydrogen fumarate are determined in swine liver.

Principle

The method includes alkaline hydrolysis of tiamulin metabolites and subsequent derivatization of a major hydrolytic product, 8-α-hydroxymutilin (the marker of tiamulin residues, X) with pentafluoropropionic anhydride (PFPA). After column chromatographic cleanup, the 8-α-hydroxymutilin–PFP derivative (XP) is determined by gas chromatographic (GC)/electron capture detection. Two GC column conditions are presented for use.

Limit of Reliable Measurement

The determinative method was validated by the sponsor at 200, 400, and 800 ppb. The determinative and confirmatory methods were validated at 400 ppb by a U.S. Food and Drug Administration/U.S. Department of Agriculture (FDA/USDA) interlaboratory study.

Apparatus

All references to commercial apparatus and chemicals used in this method are for descriptive purposes only and do not constitute an endorsement by the FDA or the U.S. Government. Equivalent products may be used.

(a) *Acetone–dry ice bath.*

(b) *Blender.*—1 qt stainless steel Waring Blender equipped with swivel clamps and a variable transformer.

(c) *Chromatographic column.*—400 × 20 mm id, with disc.

(d) *Filter paper.*—11 cm, Whatman No. 42.

(e) *Flat-bottom joint flasks (24/40).*—125 and 250 mL.

(f) *Folded filter paper.*—Whatman 2V (24 cm).

(g) *Powder funnels.*

(h) *Gas chromatograph.*—Varian GC 3700 equipped with a ^{63}Ni electron capture detector.

(i) *GC columns.*—Column A: 100 cm × 1/4 in. (6.1 mm) id glass, packed with 3% SP-2250 on 80–100 Supelcoport (1-1980, I-1541) and conditioned 48 h at 270°C. Column B: 100 cm × 1/4 in. (6.1 mm) id glass, packed with 3% SP-2100 on 80–100 mesh Supelcoport and conditioned 48 h at 270°C.

(j) *Ice–water bath.*

(k) *Meyer N-EVAP analytical evaporator.*—Model No. 111, Organomation Associates.

(l) *Pipets.*—Serological glass disposable, 0.01 mL.

(m) *Rotary flash evaporator.*—Buchler Instruments.

(n) *Separatory funnels.*—Pear-shaped, 125 mL, with Teflon stopcocks.

(o) *Syringes.*—5.0, 10.0, and 1000.0 μL.

(p) *TR-Vial system.*—5 mL TR-Vials and TR-block heater (Regis Chemical).

(q) *Vacuum filtering sidearm flask.*—1 L, with Buchner porcelain funnels (11 cm id) and Whatman No. 42 filter paper (11 cm).

Reagents

(a) *Acetone, hexane, benzene, and methylene chloride.*—Pesticide grade. *Note*: Pesticide grade toluene may be used to replace benzene for routine analysis but should not be used when sample solutions will be used for GC/mass spectrometric (MS) confirmation of XP (Method V) after completion of the determinative process. Methylene chloride may be replaced by pesticide grade chloroform.

(b) *Florisil.*—60–100 mesh PR, density = 0.42 g/cc (Floridin Co.). Activate at 100°C for 10 h before using and keep at 100°C.

(c) *Hydrochloric acid; sodium hydroxide; sodium sulfate, granular and anhydrous; and triethylamine (TEA).*—ACS reagent grade.

(d) *8-α-Hydroxymutilin.*—Analytical standard (Fermenta Animal Health Company, Kansas City, MO 64150).

(e) *Pentafluoropropionic anhydride.*—Purchase in 1 mL ampoules (Regis Chemical). Use a freshly opened ampoule for each analytical run, and discard any excess that is not used immediately.

(f) *Tiamulin hydrogen fumarate.*—Analytical standard (Fermenta Animal Health Co.).

(g) *Column eluting solution.*—Acetone–hexane (1 + 50, v/v).

(h) *Derivatizing solution.*—1% TEA in a mixture of acetone–benzene (1 + 2, v/v).

(i) *Extraction solution.*—0.5N HCl–acetone (1 + 60, v/v). Prepare immediately before use by mixing 10 mL 0.5N HCl with 600 mL acetone for extracting one sample twice.

(j) *Hydrochloric acid solutions.*—0.2 and 0.5N.

(k) *Sodium hydroxide solution.*—7.0N

(l) *Standard solution of 8-α-hydroxymutilin (X).*—2.0 μg X/mL in acetone. This solution can be stored at 4°C for 2 months.

Analysis

Safety precautions.—Safe laboratory practices should be observed during the method when using all chemicals. Special caution should be taken when using chemicals that are known to be potential carcinogens (e.g., benzene and chloroform).

Isolation Procedures

Preparation of liver samples.—After necropsy, remove the entire liver of a swine, and immediately rinse with deionized water, dry with paper towels, slice into pieces with a knife, and homogenize 1–2 min at high speed in a Waring blender. Label and store the homogenate in a 1 L plastic bottle at −30°C for future analysis or immediately analyze for the 8-α-hydroxymutilin residue content.

Extraction.—Extract 30.0 g liver homogenate in a blender by maceration with 300 mL freshly prepared extraction solution for 2 min at high speed. (*Note*: Place a piece of 5 cm^2 plastic sheet under the O-ring to avoid contamination of the O-ring and the cover of the blender with the extracting materials during maceration.) Filter the extract by vacuum through 2 Whatman No. 42 filter circles prewetted with water and layered in a Buchner funnel, and then wash with ca 30 mL acetone. Reextract the cake and refilter the extract.

Cool the filtrate by setting the filtering flask in an acetone–dry ice bath for 10 min. Filter the cooled extract again through a Buchner funnel as described above. (*Note*: The purpose of cooling is to remove the fat from the extract.)

Transfer the filtrate to a 1 L round-bottom flask. Evaporate acetone under reduced pressure at 45 ± 1°C using a rotary flash evaporator. (*Note*: To avoid contamination, wash the evaporator joint inside and outside with acetone before each evaporation.)

After the acetone is evaporated, continue evaporation for 5 min more at 65°C to ensure complete evaporation of acetone. In an ice–water bath, cool the flask, which contains ca 10–15 mL aqueous extract. All soluble metabolites derived from tiamulin are extracted in the aqueous phase.

Hydrolysis of tiamulin metabolites.—Filter the aqueous extract into a 250 mL flat-bottom joint flask through a folded filter paper circle supported in a powder funnel.

Wash the evaporation flask thoroughly with 20 mL 0.2N HCl 3 times, and filter the washings into the flask. Add washing solution to the funnel when the previous filtration is nearly drained or 1–2 mL is left in the filter paper. Add 12 mL 7.0N NaOH to the flask.

Stopper the flask tightly with a 3-prong clamp. Incubate the flask in a water bath at 45 ± 1°C for 20 min to hydrolyze the tiamulin metabolites.

(*Caution*: Conduct this step under a hood.) Remove the stopper, and acidify the extract with 7 mL concentrated HCl. Purge any HCl gas in the flask with air or nitrogen gas, and cool the flask in an ice–water bath.

Transfer the extract into a 125 mL separatory funnel. Extract the X residue produced with 20 mL methylene chloride (or chloroform), which is used for washing the flask. Repeat these washing and extracting processes 2 times more. For each extraction, shake the separatory funnel 10 s. Wait 5 min before

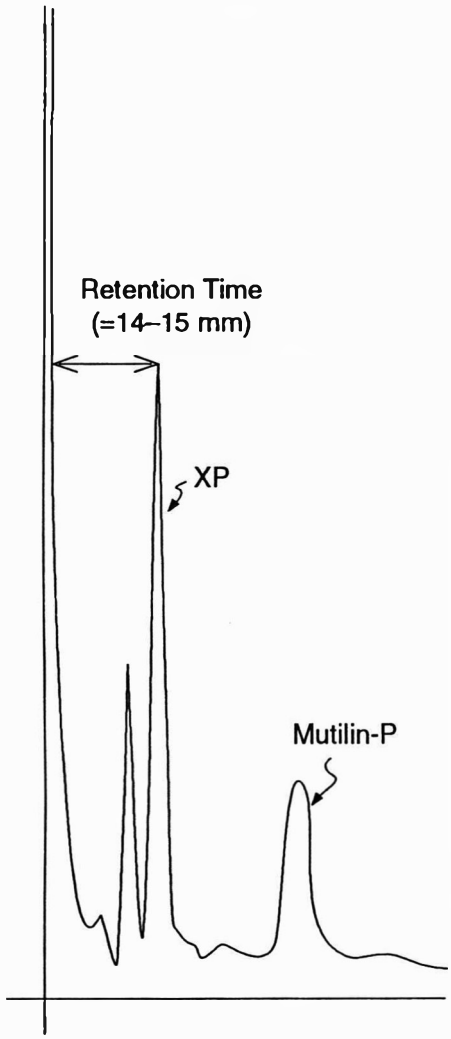

Figure 1. Chromatogram showing the retention time of 8-α-hydroxymutilin–P (XP) and mutilin–P with column A (P = PFP derivative).

partition separation. Collect the methylene chloride (or chloroform) phases in a 125 mL flat-bottom joint flask. (*Note*: The methylene chloride phase must not contain any entrained water. Use a longer separation time and a clean, dry separatory funnel to avoid traces of water.)

Evaporate the methylene chloride (or chloroform) to an oily residue, ca 50 µL, under reduced pressure at 45 ± 1°C by using the rotary flash evaporator. Purge the flask with air or nitrogen gas and cool with ice water.

Derivatization of X with PFPA to form XP.—Add 1.50 mL derivatizing solution to the flask with a 1.00 mL serological pipet (1.50 mL = 30.0 g liver). Stopper the flask, and dissolve all the residues on the glass wall of the flask by swirling the solution while cooling the flask in an ice–water bath.

Accurately pipet 1.00 mL (ca 20.0 g liver) from the flask into a 5 mL TR-Vial. Add 0.10 mL PFPA to the vial. Cap the vial tightly, and heat 20 min in a TR-block heater at 60°C for derivatization. (*Note*: Add 1 mL water to each hole of the block heater for more uniform heating.)

Cool the vial to room temperature before the cap is removed, and evaporate the solvent to an oily residue by purging with air or nitrogen gas with a Meyer N-Evap analytical evaporator at 60°C. (*Note*: Purge to cause minimum disturbance to the solvent surface.)

Cleanup of XP by column chromatography.—Pack a chromatographic column with 25 g hot activated Florisil. Immediately place 10 g sodium sulfate on top of the Florisil, and use the column as soon as it cools.

Dissolve the residue in the vial in 4 mL column eluting solution. Cap the vial tightly, shake 5 s, and pour the solution directly onto the column. Repeat this washing procedure 3 times more.

Elute the column with 200 mL eluting solution. Collect in a 250 mL flat-bottom joint flask. Evaporate the eluate to dryness under reduced pressure at 45 ± 1°C by using the rotary flash evaporator.

Purge the flask to dryness with air, and dissolve the residues in 20 mL toluene or benzene for GC determination. Further dilution may be needed depending on the concentration of X. (*Note*: If sample solutions are to be reserved for GC/MS confirmation, toluene should not be used.)

Determinative Procedures

GC conditions.—Before sample analysis, determine the linear operable range of the gas chromatograph under the following conditions (this will influence the dilution that should be made in the final sample extract): column temperature, 170–180°C; inlet temperature, 280°C; detector temperature, 330–350°C; flow rate for N_2 carrier gas, 60 mL/min; chart speed, 2.5 cm/min; attenuator, 8×10^{-11} (changeable depending on XP concentrations and instrument conditions; see *Discussion*).

The approximate retention times for XP using the 2 columns and the operating conditions above are 34 s for column A and 52 s for column B. See Figures 1 and 2 for representative chromatograms.

Preparation of internal processed standard curve of X.—Prepare a standard solution of X by dissolving X in acetone to yield a final concentration of 2.0 µg X/mL.

Transfer a 6.0 mL aliquot of the standard X solution (ca 12.0 µg X) to a 250 mL flat-bottom joint flask. After purging with air, add 10 mL 0.5N HCl, 60 mL 0.2N HCl, and 12 mL 7.0N NaOH solutions. Proceed with the instructions above from *Hydrolysis of tiamulin metabolites* starting with "Stopper the flask" through "Evaporate the eluate to dryness under reduced pressure at 45 ± °C by using the rotary flash evaporator..." in *Cleanup of XP by column chromatography*. Dissolve the XP residue in 160.0 mL benzene. (*Note*: Toluene should not be used if the XP will undergo GC/MS confirmation.)

Because only 67% of the original 12.0 µg X is used in the derivatization step, the XP solution has a theoretical concentration corresponding to 50.0 ng X/mL. This XP solution is used as a standard solution of X for GC analysis. It can be stored at 4°C for 2 months. The procedure described above is designed to simulate a 400 ppb fortification of X to a control liver sample (12.0 µg X/30.0 g liver). Use a freshly derivatized standard for quantitation of tiamulin marker in samples.

Depending on the sensitivity of the GC system, the standard solution of X for GC analysis may be used directly for constructing standard curves of X, or it may be diluted 10-fold with benzene. The diluted standard solution of X for GC analysis has a concentration corresponding to 5.0 ng X/mL. Generate an internal processed standard curve of X by triplicate GC analysis of 1.0, 2.0, and 3.0 μL aliquots of the working standard solution corresponding to 5.0, 10.0, and 15.0 pg X, respectively. (*Note*: The linear range for the each instrument used must be determined, and samples must be diluted accordingly.)

Calculations.—Calculate X (ppb) according to the following equations:

$$X_{pg}/I_{\mu L} = X_{ng}/I_{\mu L}$$

$$\text{ppb of X} = \text{ng of X/g of sample} = (X_{pg}/I_{\mu L}) \times D(V_1/V_2) \times (1/W)$$

where X_{pg} = amount of X (pg) from an internal processed standard curve of X, $I_{\mu L}$ = sample solution (μL) injected into the GC system, D = final dilution of the sample solution (mL) before injection, V_1 = volume of derivatizing solution (mL) added in the first step of the derivatization procedure (1.50 mL), V_2 = volume of derivatizing solution (mL) taken in the second step of the derivatization procedure (1.00 mL), and W = liver sample (g) taken for extraction (30.0 g).

Using the chromatogram in Figure 3 as an example, the ppb of X may be calculated as follows: X_{pg} = 10.1 pg (from peak height and standard curve), $I_{\mu L}$ = 2.0 μL, D = 1600 mL; ppb X = ng X/g = 10.1 pg/2.0 μL × 1600 mL (1.50 mL/1.00 mL) × 1/30.0 g = 404 ppb.

The value of D (1600 mL) was the final volume of this 20 g sample before GC analysis. It was not prepared by dissolving the sample residue directly with 1600 mL benzene. It was diluted stepwise depending on GC tests. For instance, the sample residue was first dissolved in 20 mL benzene according to the final step in the column cleanup procedure. This 20 mL/20 g solution was tested by injecting 0.2 μL into the GC system, and it was found that the XP was too concentrated. Then, a 20-fold dilution was made by mixing 1.0 mL of this sample solution with 19.0 mL benzene.

Similarly, the diluted sample solution (400 mL/20 g) was also tested by GC, and the XP concentration was once more found to be too high. Therefore, another 20-fold dilution was made in the same way. The sample solution was continuously

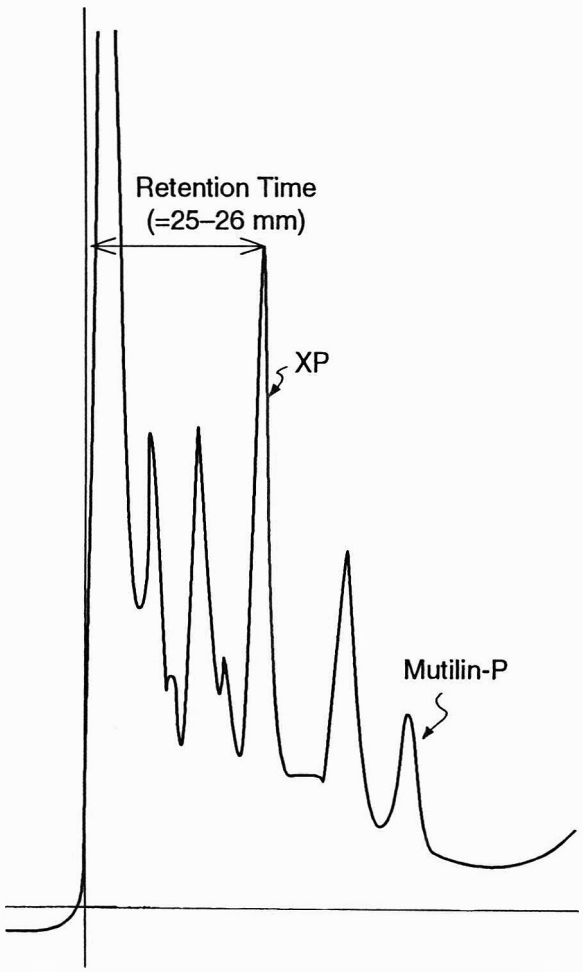

Figure 2. Chromatogram showing the retention time of 8-α-hydroxymutilin–P (XP) and mutilin–P with column B (P = PFP derivative).

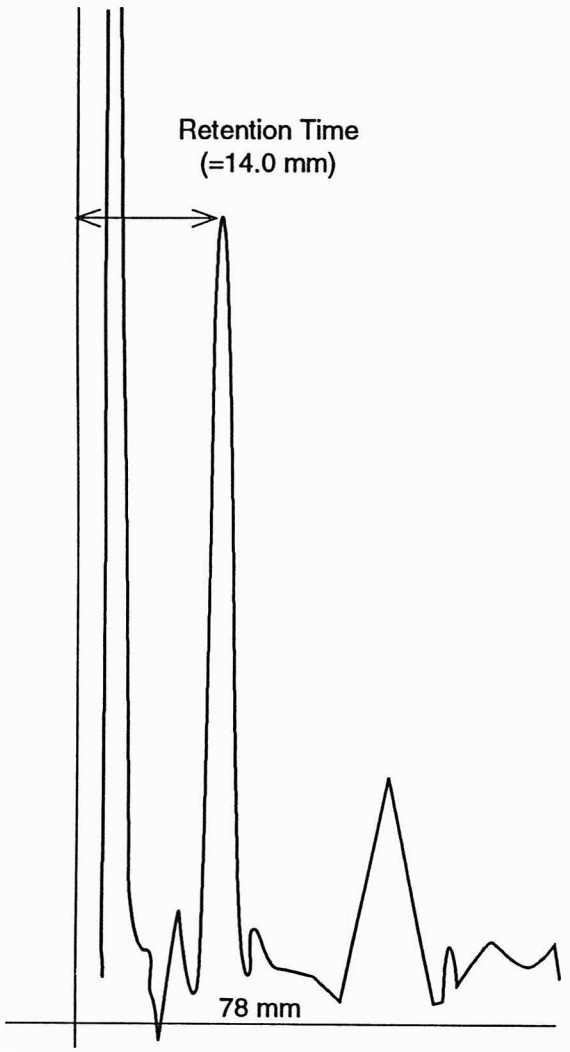

Figure 3. GC determination of X in treated liver using column A.

tested and diluted until the final solution, with a diluting concentration corresponding to 1600 mL/20 g, was obtained. The final volume of 1600 mL was chosen because when 2.0 µL of this solution was injected into the GC system, an XP peak with a height between the peak heights produced by 5.0 and 15.0 pg of X, as shown in Figure 4, was obtained.

The residue of X can be determined either by peak height or by peak area depending on the convenience of instrumentation. For the Varian GC 3700, measurement of peak heights is the most practical way to determine X residues. When nonideal linearity is obtained, generate data by interpolating the amount present from the 2 standards bracketing the response of the unknown. Use of a least-squares fit of the standard curve is not recommended. Injections should fall in the midrange of the standard curve.

Recovery procedures.—Analyze control swine liver fortified with X to determine recovery levels.

Confirmatory procedures.—See Method V for GC/MS confirmation of tiamulin fumarate residues.

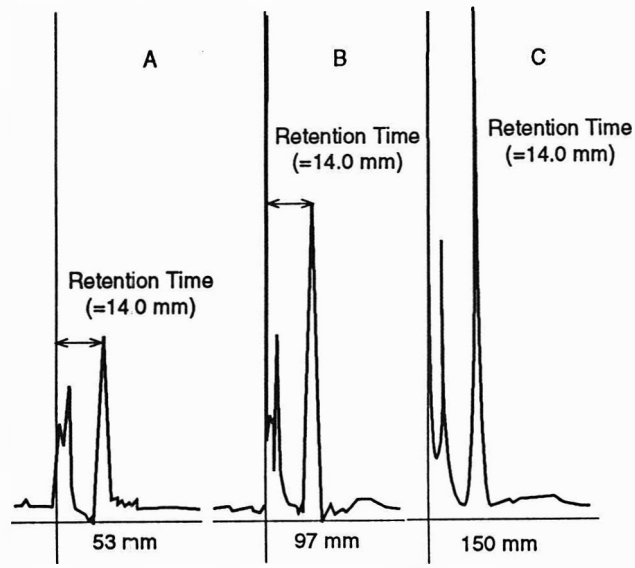

Figure 4. Retention times and peak height of external standard XP from 5.0 (A), 10.0 (B), and 15.0 pg X (C).

Reliability of the Method

Validation.—*(1) Recoveries of X fortified to control liver (sponsor's results).*—Thirty grams of a control swine liver (No. 33) were fortified in 5 replicates with 6000.0 ng X (200.0 ppb X), 12 000.0 ng X (400.0 ppb X), and 24 000.0 ng X (800.0 ppb X) and analyzed according to the procedure detailed above. Chromatograms of control and fortified samples are presented in Figure 5. On the basis of the internal processed standard curve of X, the average recoveries (±SD) of the fortified X were 180.4 ± 20.3 for the 200 ppb spike, 373 ± 22.2 for 400 ppb spike, and 710 ± 77.9 for 800 ppb spike (n = 5 in each analysis). The average percent recovery was 90.8%.

During the recovery study, the following were observed: *(1)* no significant interfering peaks appeared in the control samples, indicating the effective cleanup achieved in this procedure; (2) the recoveries of X varied from 88.8 to 93.4%, with an average of 90.8%, indicating the reproducibility of this procedure; and (3) the XP peak was the only major peak appearing on the chromatograms, indicating specific and sensitive determination of X by this procedure. After GC determinations, the samples were also sent for GC/MS confirmation (Method V). Results confirmed the existence of XP in all fortified samples but not in the control samples. Three to 5 samples could be

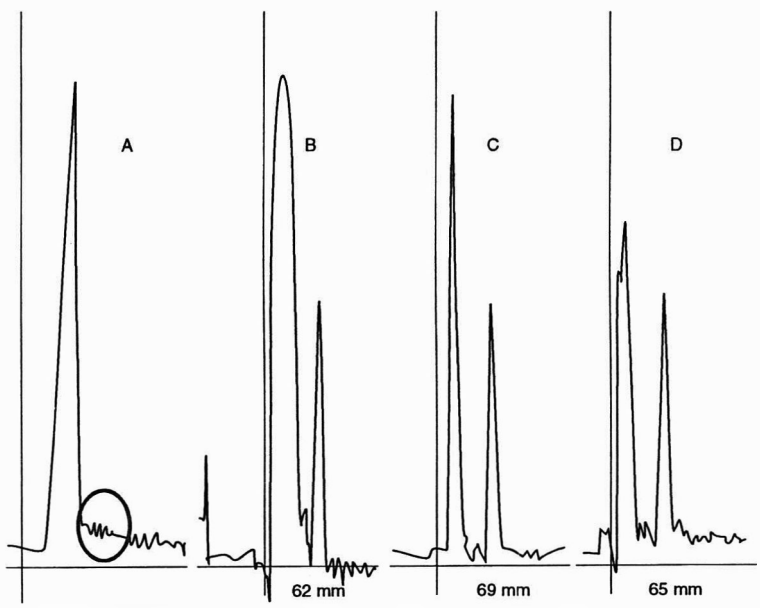

Figure 5. Chromatograms showing GC analysis of control swine liver No. 33 (A) and this liver fortified with X at 200 ppb (B), 400 ppb (C), and 800 ppb (D).

Table 1. Recovery (ppb) of tiamulin and coefficient of variation (%)[a]

Sample	Beltsville FDA	Beltsville USDA	Denver FDA
Control	ND[b]	ND[c]	ND[c]
Fortified controls, 440 ppb	335	400	370
	388	358	467
	320	368	432
	345	379	429
	371	333	485
	370		
$\bar{X}$	354.8 ± 25.7	367.6 ± 24.8	436.6 ± 44.1
CV, %	7.2	6.8	10.1
Dosed liver, composite A	490	344	204
	567	452	181
	414	213	157
	526	293	148
	497	215	181
$\bar{X}$	497.0 ± 50.5	303.4 ± 99.8	174.2 ± 22.2
CV, %	10.2	32.9	12.7

[a] Sponsor data (ppb): 448, 430, 448, $\bar{X}$ = 442.
[b] None detected, 2 samples.
[c] None detected, 5 samples.

analyzed per person per day by the method. On the basis of the validation results, the sponsor concluded that this method is of practical use as a standard procedure for routine and regulatory analyses of the residues of tiamulin in swine liver. For routine residue analysis and regulatory purposes, internal processed standard curves of X are suggested, because the experimental errors can be directly corrected.

(2) *Tissue residue validation study (FDA/USDA).*—Data from method trials conducted by the FDA and USDA are tabulated in Tables 1 and 2. Table 1 shows that the 3 laboratories in the study obtained satisfactory data for the fortified controls and control tissues. However, 2 of the laboratories reported unsatisfactory recovery data for the tiamulin marker in the dosed liver composite A. USDA Beltsville (Food Safety and Inspection Service) recoveries averaged 69% of that reported by the sponsor, with a 33% coefficient of variation. Denver FDA reported 39% recovery and a CV of 8.2%. These data raised questions about the stability of the tiamulin residues in the incurred liver samples, which at the time of analysis were 8 months old, and the adequacy of the blending, hydrolysis, and derivatization procedures. One analyst from the Beltsville FDA and one from USDA Beltsville independently and simultaneously analyzed a sample from the liver composite A. Both analysts obtained low recoveries: Beltsville FDA found 234 and 241 ppb, and Beltsville USDA found 166 and 172 ppb. The Beltsville FDA analyst previously obtained an average of 497 ppb for portions of composite liver A during the trial in Beltsville FDA laboratory.

Samples from a fresh liver composite, labeled B in Table 2, were analyzed independently and simultaneously by analysts from FDA, USDA, and the sponsor in the Beltsville USDA laboratory. Before the start of the sample analysis, USDA and

Table 2. Recovery (ppb) of tiamulin and coefficient of variation (%)[a]

	Beltsville FDA		Beltsville USDA		Sponsor	
Sample	Set I	Set II	Set I	Set II	Set I	Set II
Controls, 0 ppb	ND[b]	ND	ND	ND	—	—
Fortified controls, 400 ppb	390	355	438	312	336	133
Dosed liver, composite B	363	418	465	446	420	323
	500	430	455	449	412	252
	563	419	405	452	349	399
$\bar{X}$	448 ± 71		445 ± 20.7		359 ± 64.9	
CV, %	15.8		4.7		18.1	

[a] Sponsor data (ppb): 499, 489, 492, 475, $\bar{X}$ = 484.
[b] ND = none detected.

Table 3. Recovery of tiamulin in swine livers

Laboratory	Spike, ppb	Rec., %	CV, %
Beltsville FDA	448	93	15.8
Beltsville USDA	445	92	4.7
Sponsor	359	74	18.1

FDA analysts cross-checked their standards. Old and new working standards were checked. No significant differences were observed between standards previously prepared in each of the laboratories or between old and new standards.

As seen in Table 2, each analyst ran 2 sets of samples in the Beltsville USDA laboratory. Each set consisted of a control, a control fortified at 400 ppb with the marker, and 3 samples of dosed liver composite B. This composite was previously analyzed by the sponsor's analyst in the sponsor's laboratory. The results for this study are given in Table 3. For the combined dosed tissue data from all 3 analysts, the coefficient of variation was 11.2%. It was concluded that the method was satisfactory for determining tiamulin in swine livers.

Discussion

Sponsor's Comments

Florisil efficiency.—The same lot of Florisil should be used for cleanup when conducting the method. Use of different lots during the procedure can lead to erroneous results and is discouraged.

The efficiency of a given lot of Florisil can be checked by comparing the recovery of a fortified control swine liver sample, for example at 400 ppb of X, with an internal processed standard of X as described above. Because the fortified sample and the internal processed standard of X are prepared under identical conditions, satisfactory recovery of the fortified X indicates high efficiency of the Florisil.

Method continuity.—Laboratory work for the GC determination of the X residue in swine liver by this method can be suspended at any step except the hydrolysis and derivatization steps, because these are time-dependent. These 2 steps must be individually carried to completion.

Because both X and XP are quite stable at room temperature, especially in dilute acidic solutions, the unfinished sample solutions can be kept at room temperature for limited periods of time. However, if the suspension period is more than 5 h, the unfinished sample solutions should be stored at 4°C. During the suspension period, sample solutions should be stoppered and properly labeled.

Variable GC conditions and sensitivity.—For any given gas chromatograph, the sensitivity of an electron capture detector varies from time to time, especially when new columns are installed. For this reason, a new standard curve of X must be generated for each batch of samples, and the established sensitivity must be checked frequently with the standard solution during GC analysis.

Purging with nitrogen gas may be applied if proper facilities are available, and better results can be obtained. The conditions

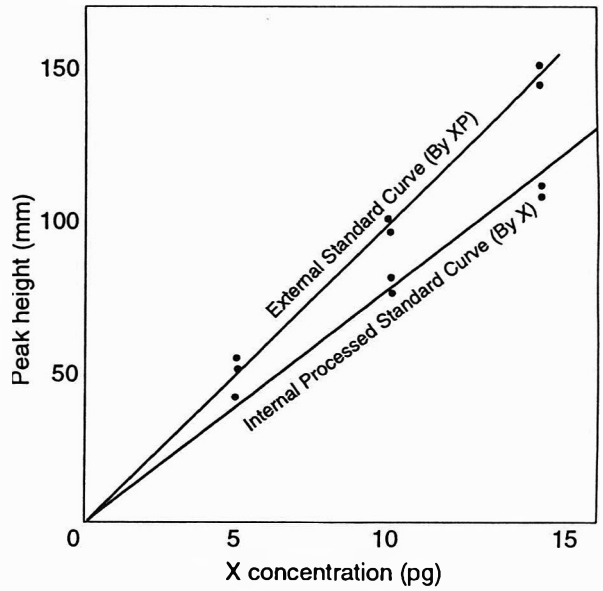

Figure 6. Typical standard curves of X.

for purging must be determined by the individual investigator. For any given batch of samples, identical GC conditions should be maintained and recorded.

A convenient linear range of the instrument in use should be established experimentally. Dilutions of the standard solution of X for GC analysis and sample extracts must be in accordance with an optimum linear range, which may vary with individual detection systems.

Extensive dilution of X residue solutions may be avoided by injecting concentrated X residue and setting the attenuator at 128 or higher. Under these conditions, the internal processed standard curve of X may not result in a straight line; therefore, the results may be subject to a higher percent error.

Estimation of experimental loss of X residue.—By using this procedure to determine the residue of X in swine liver,

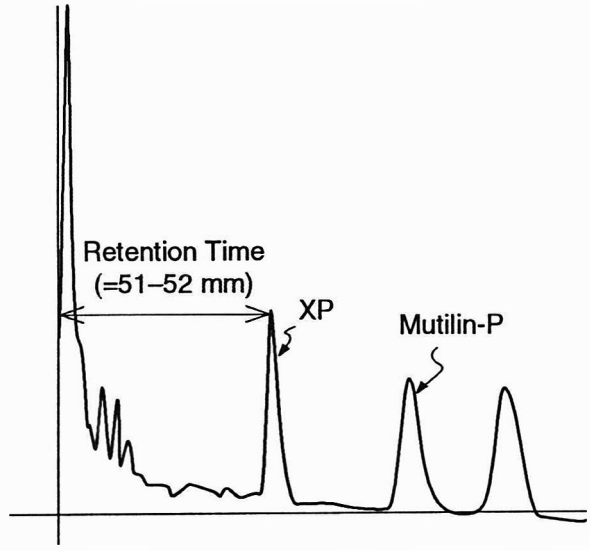

Figure 7. Chromatogram showing the retention time of 8-α-hydroxymutilin–P and mutilin–P with column C (P = PFP derivative).

experimental loss of X is inevitable, but this loss can be theoretically and directly corrected if an internal processed standard curve of X is used for quantitation. This is because the X residues, either in samples or in internal processed standard solutions, are subjected to identical processes and experimental losses. The experimental loss of X with this procedure may be estimated by comparing an internal processed standard curve of X with an external standard curve of X (a standard curve constructed by pure XP solution and not taken through the process).

The external standard curve of X is not a requirement for routine analysis. It is not a true standard in the traditional sense. The external standard allows for measurement of peaks of XP in the absence of any losses, that is, 100% recovery.

Following the procedures detailed above in the derivatization and cleanup sections, standard XP can be prepared. The external standard curve of X is constructed by triplicate GC injections of a standard solution of XP corresponding to 5.0, 10.0, and 15.0 pg X (100.0 ng X = 230.36 ng XP). Examples of typical chromatograms are shown in Figure 4.

Figure 6 shows that the internal processed standard curve of X corresponds to approximately 75% of the external standard, indicating a 25% loss during extraction and cleanup. This 25% loss is corrected directly if an internal processed standard curve of X is used for quantitating X during residue analysis. If an external standard curve of X is used for quantitation, a 25% correction should be added to the quantities determined.

Interference of other animal drugs.—Liver samples collected from swine that were treated with Carbadox, Lincomycin, Tylan-Sulfa, CSP-250, Furacin, Bacitracin, and S.O.L. (Rabon) at approved levels were analyzed with this method. No interferences with tiamulin were observed.

GC columns.—Both GC columns give reproducible and essentially identical results. Column A is the column of preference, not because of improvement in the data but because of improvement in peak separation. The use of a third column, 5% OV-210 on 80–100 mesh Supelcoport under the same conditions as specified in the method, provides an additional means to determine any interference (see Figure 7).

The method (No. 371-3MD-80-0040-005) was submitted by Fermenta Animal Health Co., Kansas City, MO, in conjunction with the approval of the New Animal Drug Applications, NADAs 134-644 and 139-472.

REGULATORY ANALYTICAL METHODS

Method V. Gas Chromatographic/Mass Spectrometric Confirmation of 8-Hydroxymutilin, a Tiamulin Metabolite, in Swine Liver Extracts

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Tiamulin, 14-deoxy-14-[(2-diethylaminoethyl)mercapto-acetoxy]mutilin hydrogen fumarate (CAS No. 55297-96-6), is used for the treatment of swine dysentery and pneumonia and for weight gain and feed efficiency. Denagard™ is the trade name for a 45% (as tiamulin) water-soluble formulation.

Regulation Information

21CFR520.2455 provides for approved use of tiamulin in the treatment of swine as a 45% water-soluble powder to make medicated drinking water (227 mg tiamulin/gal) for treatment of swine dysentery (1983) and for treatment of swine pneumonia (1987).

21CFR558.600 provides for approved use of tiamulin in a Type A medicated article containing 5, 10, or 113.4 g tiamulin (as hydrogen fumarate) per pound to be used in the manufacture of a Type C medicated feed at 35 g/ton (for use in the control of swine dysentery) and at 10 g/ton (for use in weight gain and feed efficiency for swine).

When used at 35 g/ton, the feed must be withdrawn 2 days before slaughter. The drug must not be used in conjunction with other polyether ionophores such as lasalocid, monensin, narasin, and salinomycin.

21CFR558.4 provides for the following assay limits.

Type A medicated articles.—5 and 10 g/lb, 90–115% label content; 113.4 g/lb, 100–108% label content.

Type C medicated feeds.—35 g/ton, 70–130% label content.

Tolerances: 21CFR556.738

Marker Residue: 8-α-Hydroxymutilin

A tolerance of 0.4 ppm was established for tiamulin in swine for 8-α-hydroxymutilin (the marker residue) in liver (target tissue).

A marker residue concentration of 0.4 ppm in liver corresponds to a concentration of 10.8 ppm tiamulin. The safe concentrations for total residues in uncooked edible tissues of swine are 3.6 ppm in muscle, 10.8 ppm in liver, and 14.4 ppm in kidney and fat.

Method V. Gas Chromatographic/Mass Spectrometric Confirmation of 8-Hydroxymutilin, a Tiamulin Metabolite, in Swine Liver Extracts

Scope

The method is limited to obtaining mass spectra from a single gas chromatographic (GC) peak attributed to 8-hydroxymutilin–PFP, a pentafluoropropionic anhydride (PFPA) derivative of the tiamulin metabolite 8-hydroxymutilin.

Principle

Alkaline hydrolysis of tiamulin metabolites in swine liver yields a major hydrolytic product, 8-hydroxymutilin. Derivatization of the metabolite with PFPA, followed by column chromatographic cleanup, yields samples suitable for GC/mass spectrometric (MS) determination. For the derivative in which all 3 hydroxyl groups have reacted with PFPA, the molecular weight is 774. A mass spectrum of an authentic standard of the derivative can be used to confirm the presence of the material in treated swine liver samples.

Limit of Reliable Measurement

The method will produce a characteristic mass spectrum at 200 ng or below.

Apparatus

All references to commercial apparatus and chemicals used in this method are for descriptive purposes only and do not constitute an endorsement by the U.S. Food and Drug Administration or the U.S. Government. Equivalent products may be used.

(a) *Mass spectrometer.*—Instrument capable of unit resolution and having a mass range of at least 1000 amu.

Received January 14, 1992. Accepted December 4, 1992.
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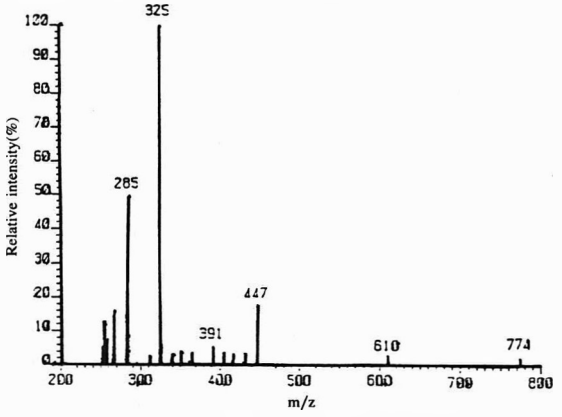

Figure 1

Figure 1. Mass spectrum for 8-hydroxymutilin–PFP standard.

(**b**) *Gas chromatograph.*—Interfaced with suitable separator.

(**c**) *GC column.*—Glass, 6 ft (1.8 m), packed with 3% SP2100 on 80–100 mesh Supelcoport.

(**d**) *Analytical balance.*—Capable of weighing to 0.1 mg.

(**e**) *Centrifuge.*

(**f**) *Graduated centrifuge tubes.*

Reagents

(**a**) *Acetone.*—Fisher certified (*Caution*: Acetone is a flammable liquid).

(**b**) *8-Hydroxymutilin–PFP standard.*—Dissolve 2 mg (weighed to nearest 0.1 mg) 8-hydroxymutilin–PFP standard in 1 mL acetone. This will give a solution equivalent to 2000 ng/μL.

Analysis

Determinative Procedures

GC/MS operating conditions.—The following parameters were used with a Varian-MAT CH-7 mass spectrometer, MAT 55–200 data system, and Varian 1740 gas chromatograph interfaced with a jet separator (some modifications may be required for other systems). (*1*) *GC parameters.*—Injection port temperature, 240°C; column temperature, 190°C; isothermal separator temperature, 280°C; helium carrier gas flow rate, 30 mL/min; divert valve to mass spectrometer, open 2 min after injection of the sample. (*2*) *GC/MS/data system parameters.*—Source temperature, 200°C; source pressure, 1×10^{-6} to 5×10^{-6} torr (with solvent bypass); emission current, 300 A; ionizing voltage, 70 eV; accelerating voltage, –3 kV; secondary electron multiplier, ca 2000 V; resolution, 1000; sample/mass peak, 8; scan rate, 4 s/decade; sampling rate, 5 kHz; mass range, 250–800 amu; and interscan time, 5 s.

Analysis of standard.—Inject 0.2 μL standard solution onto the column to determine the retention time of the GC peak for 8-hydroxymutilin–PFP (ca 5 min with the above conditions).

Determine the sensitivity of the compound with the particular system and associated conditions by injecting successive dilutions down to ca 100 ng/μL. This will indicate the level at which meaningful mass spectral data may be obtained from a particular MS/separator/GC combination. With the system and conditions specified in this method, 200 ng 8-hydroxymutilin will produce a molecular ion at *m/z* 774 and characteristic peaks at *m/z* 610, 447, 325, and 285.

Analysis of samples.—Analyze samples that were prepared according to the GC determinative procedure for tiamulin (Method IV). In general, all of the sample obtained is submitted for MS confirmation. (*Note*: At the 400 ppb level and with complete recovery, a total of ca 8000 ng will be available for GC/MS after solvent evaporation.) If samples are received in a dry state, add 0.5–1.0 mL acetone. Transfer the solution to a graduated centrifuge tube. After centrifuging, evaporate the solution to ca the 0.1 mL mark.

Withdraw 5–10 μL solution, and inject onto the SP-2100 column using the same parameters as were used for the standard. Obtain a spectrum (range, 250–800 amu) for the GC peak eluting at the same retention time as the standard of 8-hydroxymutilin–PFP. If a GC peak is not detected or a satisfactory mass spectrum is not obtained, reduce the volume of the solution and inject an additional sample from the more concentrated solution.

Compare standard and sample mass spectra for similarity and sample peak homogeneity.

Discussion

Sponsor's comments

Condition the GC column 48 h at 270°C before performing analyses.

If the sample solution is cloudy or contains suspended particles, centrifuge further or filter the solution before injection.

A mass spectrum (200–800 amu, Figure 1) for the standard of 8-hydroxymutilin–PFP showed the following characteristic peaks: *m/z* 774 (molecular ion), 610, 447, 325, and 285.

The method was submitted by Fermenta Animal Health Co., Kansas City, MO, in conjunction with the approval of the New Animal Drug Applications, NADAs 134-644 and 139-472.

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