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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

MAY/JUNE 1990
VOL. 73, NO. 3

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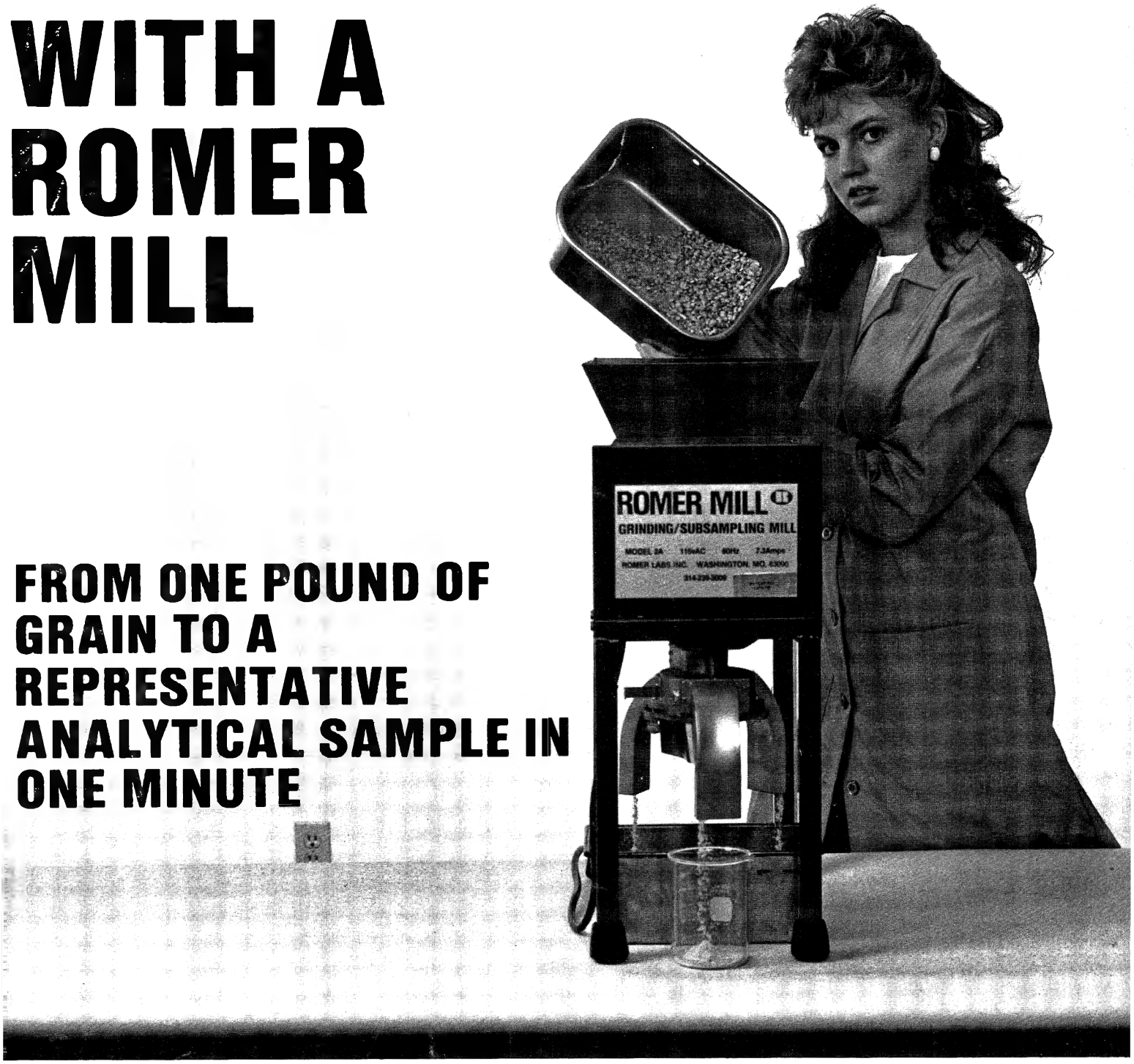

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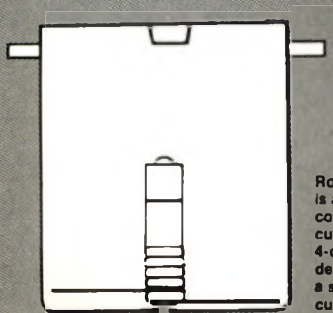

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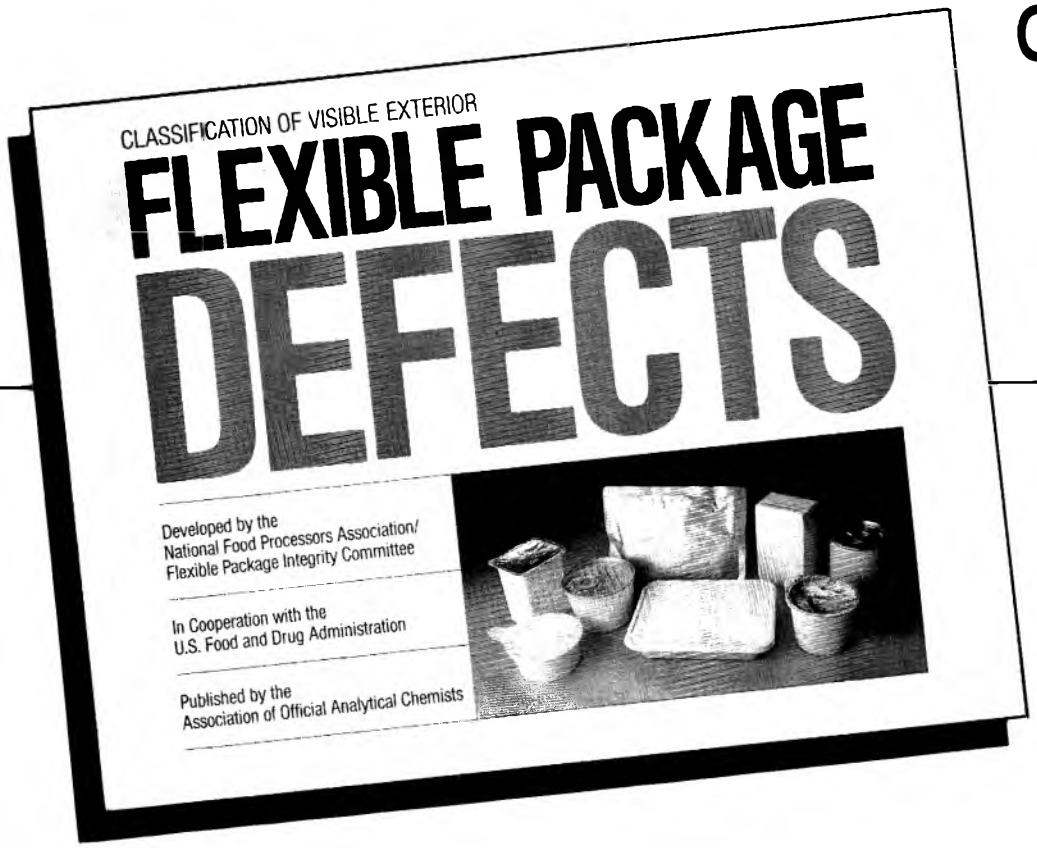

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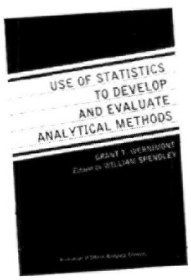

☐ **Use of Statistics to Develop and Evaluate Analytical Methods**

By G.T. Wernimont. Ed. by W. Spendley. 1985. 199 pp. Index. Figures. Tables. Glossary. Softbound. ISBN 0-935584-31-5

Price—Members: $49.55 in U.S., $54.55 outside U.S. Nonmembers: $54.50 in U.S., $59.50 outside U.S.

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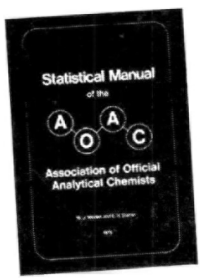

☐ **Statistical Manual of the AOAC**

By W.J. Youden and E.H. Steiner. 1975. 96 pp. Softbound. Illustrations. ISBN 0-935584-15-3.

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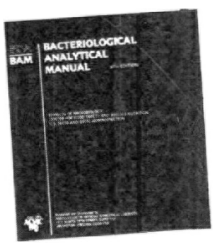

☐ **FDA Bacteriological Analytical Manual, 6th Ed.**

1984. 448 pp. Looseleaf. ISBN 0-935584-29-3.

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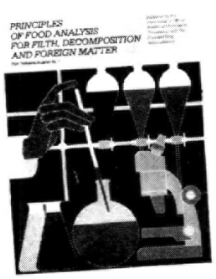

☐ **Principles of Food Analysis for Filth, Decomposition, and Foreign Matter— FDA Technical Bulletin No. 1**

1981. 286 pp. 2nd printing 1985. Illustrated. Softbound.

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Comprehensive laboratory manual/text on basic concepts of food sanitation analysis.

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J A O A C

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Books in Brief

Flow Injection Analysis: A Practical Guide. By B. Karlberg and G. E. Pacey. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1989. 372 pp. Price: US $117.00/Dfl. 240.00. ISBN 0-444-88014-3.

This is a practical guide for first-time and experienced users of flow injection analysis (FIA). It gives, not a detailed theoretical analysis, but a "nuts and bolts" approach to the description of the technique and how it can be utilized to solve analytical chemical problems. The advantages of flow injection, how, when, why, and where it works, are all fully explained. Criteria for the choice of hardware and useful hints for maintenance are provided. The large variety of detections suitable to combine with FIA are discussed, as are special modes of operation, their advantages and their limitations, and also conversion of batch methods to FIA methods. Numerous in-depth descriptions of applications of FIA techniques in water, soil, pharmaceutical, and industrial analysis are featured, and a complete bibliography is included.

Analytical Absorption Spectrophotometry in the Visible and Ultraviolet: The Principles. By L. Sommer. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1989. 312 pp. Price: US $129.25/Dfl. 265.00. ISBN 0-444-98882-3.

Although there are a number of comprehensive textbooks dealing with UV/VIS spectrophotometry, they tend to describe historical aspects or contain collections of detailed procedures for the determination of analytes and do not reflect sufficiently the present state of the method and stage of development reached. This book provides a concise survey of the actual state-of-the-art of UV/VIS spectrophotometry. Special attention has been paid to problems with the Bouguer-Lambert-Beer law, absorption spectra, present trends in instrumentation, errors in spectrophotometry, evaluation of analyte concentrations and calibration, optimization procedures, multicomponent analysis, differential spectrophotometric titration, the strong relations between complex formation and spectrophotometry, spectrophotometric investigation of complex equilibria and stoichiometry or automation in spectrophotometry.

Vogel's Quantitative Chemical Analysis, 5th edition. Revised by G. H. Jeffery, J. Bassett, J. Mendham, and R. C. Denney. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989, 877 pp. Price $87.95. ISBN 0-470-21517-8.

The fifth edition of this classic work reflects the profound changes that have taken place in chemical analysis over the last decade. Growth and development of the subject have now totally blurred the boundaries that existed between inorganic and organic chemistry, and consequently a much wider range of organic analyses has been incorporated, especially in areas related to environmental topics, such as heavy metals, dissolved oxygen, and chemical oxygen demand.

Care has been taken to maintain the balance, developed in previous editions, between traditional methods and up-to-date instrumental techniques; and sections have been extended to incorporate interesting and important modern analytical methods. The book has been carefully restructured to present the basic theory underlying chemical processes alongside coverage of more practical subjects, including the taking of representative samples, statistical treatment of data, and the types of equipment required. Its revised appendices present additional data routinely used by analysts, including statistical tables, pH values of buffer solutions, and solubilities of common reagents.

Fluorescence Analysis in Foods. Edited by Lars Munck. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1990. 289 pp. Price $147.00. ISBN 0470-21425-2.

Fluorescence analysis is an increasingly common technique in the food industry, mainly for quality control. The science and technology underlying the technique, and the scope of its applications, have undergone a period of rapid development in recent years, with the emergence of ultrasensitive optical detectors, image analyzers, and related electronics.

Written by leading international experts in the field, this book provides an authoritative overview for food scientists wishing to apply these new and versatile methods of analysis and in-line process control. Following an introduction to the physical basis of fluorescence, and to the use of fluorometers, microscopes, and image analyzers, applications cover the full range of food products of plant and animal origin, such as cereals, fish, and meat. Applications include the important areas of detection of bacteria and mycotoxins in food products, and the marking of protoplasts in plant tissue culture. Especially useful is a chapter dealing with the new instructive 'UNSCRAMBLER' data-evaluation programs, which are needed to make use of information from complex fluorescence spectra.

National Products Isolation: Separation Methods for Antimicrobials, Antivirals, and Enzyme Inhibitors. Edited by Gerald H. Wagman and Raymond Cooper. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1989. 618 pp. Price: US $139.00/Dfl. 285.00. ISBN 0-444-87147-0.

This new book encompasses the most recent progress made in the isolation and separation of natural products. It covers antibiotics, marine and plant-derived substances, enzyme inhibitors, and interferons. The most recent separation methodology is described. Although there is a bias toward antibiotics, this is because it is still the largest natural products area of research.

The 14 chapters are written by experts in their respective fields. The first 2 chapters are largely devoted to new methodology applied to purification of a variety of compounds. They include an extensive review and new applications of counter-current chromatography and the newly emerging LC-photodiode array technology. Chapter 3 provides a review of affinity chromatography applied to the separa-

tion of antibiotics for the first time. Next are chapters on antimicrobials with an update on all the most recent β-lactam (after 1976) discoveries. A comprehensive review of a very important class of antiparasitic agents—the avermectins—follows. An update of isolation and purification of a variety of marine-derived compounds is next. The succeeding chapter is a comprehensive review of the most recent developments in isolation and purification of interferons. This is followed by a discussion of enzyme inhibitors and their isolation and purification and ties in with a chapter on plant-derived natural products, some of which are also in this same category. The final chapter is a futuristic essay indicating the isolation of minute amounts of natural products and the fascinating biological properties they possess.

The book has extensive isolation schemes, tables, figures, and chemical structures. In many instances, a short summary of the producing organism, brief chemical description, and structure and biological activity of the compounds is presented. Detailed information of extraction, separation, and purification techniques follow. Each chapter has an extensive bibliography and, where applicable, an appendix showing sources of materials and equipment. A detailed subject index is included at the end of the book.

Dictionary of Food Ingredients, 2nd edition. By Robert S. Igoe. Published by Van Nostrand Reinhold/AVI, 115 5th Ave, New York, NY 10003, 1989. 222 pp. Price: $29.95. ISBN 0-442-31927-4.

This revised and enhanced new edition of the most comprehensive guide to food ingredients now features definitions of more than 50 new ingredients, more than 100 expanded definitions of previously listed ingredients, and new and expanded sections.

Encompassing more than 1,000 approved food ingredients, the dictionary details the functions, chemical properties, and applications of each ingredient. It covers currently used food additives, including natural ingredients, FDA-approved artificial ingredients, and compounds used in food processing.

The expanded ingredient categories section groups principle food ingredients by function and provides descriptive information about the characteristics and applications of each group. Informative new tables pinpoint key features and properties of ingredients and allow quick comparisons to aid in ingredient selection. Food technologists, researchers, and workers in the food processing industry will also appreciate the new section on "Substances for Use in Foods Listed Under Table 21 of the Code of Federal Regulations," which lists food ingredients according to their U.S. approved status.

A Handbook on How to Set Up or Improve Laboratory Quality Assurance Programs

Quality Assurance Principles for Analytical Laboratories

by Frederick M. Garfield

former Assistant Administrator
U.S. Drug Enforcement Administration

This handbook provides essential information needed to design, document, implement, or improve a laboratory quality assurance program . . . A program that can enable you to document the credibility of your laboratory's analytical data.

The handbook also provides a rational and solidly based justification for commitment of resources to improved laboratory operation.

Drawing from published principles, practices, guidelines, and procedures, the book brings together the experiences of experts who have developed and implemented *successful* "QA" programs.

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A. Quality Assurance Publications and Programs
B. Forms Used by U.S. Federal Agencies
C. Instrument Performance Checks
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1984. 224 pp. Softbound. ISBN 0-935584-26-9.

Price—Members: $43.25 in U.S., $48.25 outside U.S.
Nonmembers: $47.50 in U.S., $52.50 outside U.S.
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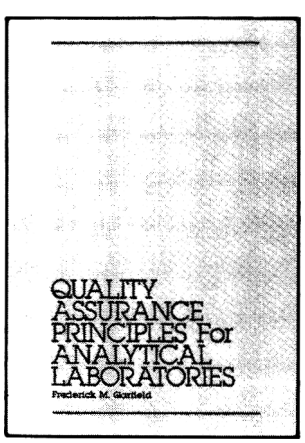

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Transparent reaction vessels (TRV) are designed to interface with their EZE-seal laboratory scale stirred autoclaves. The transparent vessels are manufactured of borosilicate glass and are available in 100 to 1000 mL capacities and pressures to 50 psi. The glass vessel package increases the operational flexibility of EZE-seal stirred autoclaves by allowing visual observation of pressurized chemical reactions and mixing studies. Autoclave Engineers.

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Automated sample pretreatment and delivery, minimum sample and reagent consumption, and the speed of up to 180 high-precision analyses/h are some of the major capabilities brought to atomic absorption (AA) by the new FIAS-200 flow injection system. The first commercial flow-injection system for AA, the self-contained FIAS-200 system includes two multichannel peristaltic pumps for maximum flexibility in handling samples, diluents, and reagents for mercury/hydride and flame AA analysis. When used with the Perkin-Elmer AS-90 autosampler on specific AA instruments, the FIAS-200 unit provides a completely automated sample handling system that is faster and more cost efficient than

continuous-flow systems. Perkin-Elmer Corp.
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Indicating Trap Alarm for Gas Chromatography Systems

The Alarm alerts the laboratory when an indicating gas trap—commonly used to protect GC columns from the damaging effects of oxygen—becomes saturated with gas. The Alarm senses the change in the trap's indicating material color, then emits a beeping alarm while a flashing red light gives a visual signal. J&W Scientific.
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New Chromatography Workstation

Spectra Station is a new generation chromatography workstation capable of collecting up to eight channels of data and controlling up to four Spectra-Physics LC systems simultaneously. Based on the new IBM OS/2 Version 1.2 Standard Edition operating system, Spectra Station features a state-of-the-art graphical user interface and advanced multitasking. The Presentation Manager graphical interface is intuitive, making it easy for new users to learn this system. Mouse-driven color graphics enable fast, simple setup of a complete LC or GC system. Spectra-Physics.
Circle No. 338 on reader service card.

EM Quant Test Strips

An instant test strip makes the detection of peroxide in ultra-high-temperature packaging and raw milk products easier than ever. EM Quant Test Strips provide a rapid screen for residues in milk, juice drinks, and other products in which hydrogen peroxide is used for sanitary processing. This simple "dip-and-read" test determines the presence of peroxide in concentrations from 0.5 to 50 ppm. The user simply dips the strip in the milk, juice, whey, or rinse water. In 5 s, the strip's special "reaction zone" turns from white to blue, in response to the amount of peroxide present. Comparison with a color chart gives the concentration range. EM Science.
Circle No. 339 on reader service card.

Disposable Pipet Tips

Bio-Rad's disposable pipet tips provide consistently accurate, reproducible pipetting. These tips are made to standards equal to or better than the standards specified by pipet manufacturers, and are a reliable replacement for OEM tips. Bio-Rad's tips are carefully molded of virgin polypropylene to assure smooth interiors and nonwettability. They thus prevent random hang-up of liquids, which reduce the precision of many other tips. Bio-Rad Laboratories.
Circle No. 340 on reader service card.

Cabinet with Mechanical Desiccant

AutoDry Desiccator is a convenient storage cabinet for laboratory applications requiring a dry environment. The Desiccator is designed for storage of chromatography plates, chemical standard, photo plates, and electronic parts. The cabinet features a self-contained, heat-driven component that automatically dehumidifies, recycling every 5½ h. A 15 watt incandescent light provides additional heat for moisture absorption. Labconco Corp.
Circle No. 341 on reader service card.

Basic Model Personal Air Sampler

A basic low-priced model universal constant-flow air sample pump, the 224-43XR is designed for personal air sampling applications. The pump features a built-in pressure regulator that permits multilow flow sampling (taking up to four samples simultaneously) and a built-in flow indicator for easy operation. Flow range is from 1 to 5000 mL/min. It has back pressure capabilities to 10 in. of water at 4 L/min for 8 h or compensates up to 40 in. of water over the most widely used flow rates. SKC Inc.
Circle No. 342 on reader service card.

PLSplus V 2.0

PLSplus V 2.0 is a completely redesigned version of the PLS application, and greatly enhances the features and speed of version 1.0. The new version of this application utilizes three of the most popular methods for multicomponent spectral quantitative analysis: PLS-1, PLS-2, and PCR. The speed of PLSplus is unrivaled in PC-based multicomponent analysis software. PLSplus is capable of using a variety of preprocessing algorithms including variance scaling, mean centering, multiplicative scatter correction (for samples with indeterminate path length) baseline correction, and path length correction. Users can even include their own preprocessing algorithms. Galactic Industries Corp.
Circle No. 343 on reader service card.

Unisphere-Alumina Columns

A new series of LC columns with internal diameters of 4.6, 9.4, and 21.2 mm and a length of 250 mm are now available. Each column is prepackaged with Unisphere-PBD alumina—a spherical, polybutadiene (PBD) coated particle composed of fused porous plates. This new reverse-phase packing offers a unique morphology and uniform PBD coating that is directly scalable for microgram- to gram-scale separations. SGM & Co.
Circle No. 344 on reader service card.

Sulfur Chemiluminescence Detector

The Model 350B sulfur chemiluminescence detector for GC and SFC is based on the same proven chemistry as the Model 350, but incorporates new electronics to provide wider linear range of six orders of magnitude, reducd background noise, and no measurable peak broadening. The Model 350B can be retrofit to any GC or SFC equipped with a flame ionization detector. Sievers Research, Inc.
Circle No. 345 on reader service card.

Preparative Chromatography System

The Delta Prep 4000's high performance and versatility combine to make preparative LC methods development, scale-up, and purification easy. The system's wide flow rate range of 0.5 to 150 mL/min enables the chromatographer to develop preparative LC methods using analytical-size columns with small amounts of sample and solvent, and then scale-up to purify milligram to multigram quantities on the same system. Millipore Corp.
Circle No. 346 on reader service card.

For Your Information

Meetings

June 4, 1990: Southeast AOAC Regional Section Meeting. Contact: M. Sher Ali, USDA, FSIS Eastern Laboratory, Russell Research Center: PO Box 6085, Athens, GA 30604, telephone 404/546-3571.

June 18–20, 1990: Midwest AOAC Regional Section Meeting. Contact: Max L. Foster, Kansas State Board of Agriculture, Division of Laboratories, 2524 W 6th St, Topeka, KS 66606, telephone 913/296-3301.

June 20, 1990: Mid-Canada AOAC Regional Section Meeting. Contact: Ezzat A. Ibrahim, Manitoba Agriculture, University of Manitoba, Feed Analysis Agriculture Service Co., Winnipeg, MB R3T 2X7, Canada, telephone 204/945-7675.

June 21–22, 1990: Pacific Northwest AOAC Regional Section Meeting. Contact: Steve Pope, Environmental Protection Agency, PO Box 549, Manchester, WA 98353, telephone 206/442-0370.

September 10–13, 1990: 104th AOAC Annual International Meeting and Exposition. The Clarion Hotel, New Orleans, LA. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

October 11–12, 1990: AOAC/Europe Regional Section Meeting, Brussels, Belgium. Contact: Ellen Jan de Vries, Duphar B.V., PO Box 900, NL 1380 DA Weesp, The Netherlands, telephone (31)-2940-79296.

November 8–9, 1990: Central AOAC Regional Section Meeting. Contact: Georgia Markakis, Michigan Dept of Agriculture, 1615 S Harrison Rd, East Lansing, MI 48823, telephone 517/337-5040.

New Sustaining Members

AOAC welcomes the following new private sustaining members: W. M. Ward Technical Services Laboratory, Winnipeg, Manitoba, Canada, and JEM Laboratory Services, Rome, GA.

Laboratory Occupation Safety and Health Standards Workshop

A workshop on laboratory occupation safety and health standards will be held June 6–7, 1990, in Washington, DC. Presented by the American Association for Laboratory Accreditation, the workshop will not only interpret the new OSHA occupational safety and health standards for laboratories but will guide attendees through the development of a Chemical Hygiene Plan and a Waste Disposal Plan. Topics will include Developing a Chemical Hygiene Plan for Your Laboratory, Material Safety Data Sheets, General Principles for Safe Handling of Laboratory Chemicals, Clearly Delineating Responsibility of Key Professionals, Facility Engineering Design and Control Considerations, Other Safety Considerations, and Developing a Waste Disposal Plan for Your Laboratory. The new OSHA occupational and health standards, which became effective on May 1, 1990, require laboratories using hazardous chemicals to develop and implement a written Chemical Hygiene Plan no later than January 31, 1991. For more information, contact CEEM, PO Box 200, Fairfax Station, VA 22309.

Interim First Action Methods

The following methods have been reviewed by the appropriate General Referee, Committee Statistician, and Methods Committee, and have been approved interim official first action by the Chairman of the Official Methods Board:

Methods Committee on Foods I—(*1*) Determination of total nitrogen content of milk by Kjeldahl analysis, which provides for use of a copper catalyst and either traditional or block digestor/steam distillation equipment, submitted by D. M. Barbano and J. L. Clark (Cornell University, Department of Food Science, Ithaca, NY) and C. E. Dunham and J. R. Fleming (Texas Milk Market, Carrollton, TX); (*2*) Fluorometric determination of alkaline phosphatase in fluid milk products, submitted by R. M. Rocco (Advanced Instruments, Inc., Needham Heights, MA); (*3*) Determination of phosphorus (colorimetry) and calcium and magnesium (atomic absorption spectrophotometry) in cheese, submitted by R. M. Pollman (State of New York, Department of Agriculture and Markets, Albany, NY); (*4*) Gas chromatographic-thermal energy analyzer method for determination of volatile *N*-nitrosamines in minced fish-meat and surimi-meat frankfurters, submitted by J. W. Pensabene, W. Fiddler, and J. G. Phillips (U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA); (*5*) Sulfuric acid/hydrogen peroxide digestion and colorimetric determination of phosphorus in meat and meat products, submitted by D. K. Christians, T. G. Aspelund, S. V. Brayton, and L. L. Roberts (Hach Co., Loveland, CO); (*6*) Immunoaffinity column coupled with solution fluorometry or with liquid chromatography-postcolumn derivatization for determination of aflatoxins in corn, raw peanuts, and peanut butter, submitted by M. W. Trucksess, M. E. Stack, S. Nesheim, S. W. Page, and R. H. Albert (Food and Drug Administration, Washington, DC) and T. J. Hansen and K. F. Donahue (Vicam, Somerville, MA); (*7*) Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison extraction procedure, submitted by J. F. Lawrence, C. F. Charbonneau, and C. Ménard (Health and Welfare Canada, Health Protection Branch, Ottawa, Ontario, Canada).

Methods Committee on Foods II—In vivo rat assay for true protein digestibility, submitted by F. E. McDonough (U.S. Department of Agriculture, Beltsville, MD), F. H. Steinke (Protein Technologies International, St. Louis, MO), G. Sarwar (Health and Welfare Canada, Ottawa, Ontario), B. O. Eggum (National Institute of Animal Science, Tjele, Denmark), R. Bressani (Institute of Nutrition of Central America and Panama, Guatemala City, Guatemala), P. Huth (Kraft Inc., Glenview, IL), W. Barbeau (Virginia Polytechnic and State University, Blacksburg, VA), G. V. Mitchell (Food and Drug Administration, Washington, DC), and J. G. Phillips (U.S. Department of Agriculture, Philadelphia, PA).

Methods Committee on Microbiology and Extraneous Materials—Two modifications of **989.15**, Fluorogenic Monoclonal Enzyme Immunoassay

Screening (Q-trol) Method for *Salmonella* in Foods, to substitute a colorimetric substrate for the fluorescent substrate used in **989.15**, and to provide for reading results visually or photometrically, submitted by M. S. Curiale and M. J. Klatt (Silliker Laboratories, Chicago Heights, IL) and W. Gehle and H. Chandonnet (Dynatech Laboratories, Chantilly, VA).

The methods will be recommended to the membership for adoption official first action at the 104th AOAC Annual International Meeting, September 10–13, 1990, at New Orleans, LA. Copies of the methods are available from AOAC Technical Services.

AOAC/Europe to Meet in Brussels, October 11–12

The first international symposium organized by the AOAC/Europe Regional Section will be held in Brussels, Belgium, October 11–12, 1990. The topic will be "Analytical Science in Europe: the Elimination of Barriers."

The increase in European legislation and the single European market in 1992 demands the interlinking of national measurement systems. Intended for analytical chemists, microbiologists, and laboratory managers from government, industry, international organizations, scientific institutes, and trade and consumer organizations, the symposium aims to bring together both providers and users of measurements from throughout Europe and the Mediterranean countries to discuss ways to improve quality and establish mutual confidence in test data. The symposium language will be English only.

"People want to know what EC 92 will bring in food and drug control," says Paul Beljaars, AOAC/Europe Regional Section President. "They want to know how national food laboratories will operate and what will be the status of each laboratory."

"The issue is trading, really, and AOAC can be a platform for these discussions because AOAC is neutral, and not interested in politics," he says.

Beljaars says that one issue, for example, is reference materials, which may be recognized inside the European Community, but not outside. The European Community is also interested in these problems, he says.

The Symposium will open on Thursday, October 11, with introductions and remarks by Beljaars, R. R. Christensen, AOAC Executive Director, Arlington, VA, U.S.A.; and J. Bessemans, CERIA/COOVI, Director-General, Brussels, Belgium.

The first session of the symposium will be on an international measurement system, chaired by B. King, Government Analyst, Teddington, U.K. Topics will include:

- EC policy developments affecting official chemical laboratories: P. Gray, DG III European Commission, Brussels, Belgium.
- EURACHEM: R. Kaarls, Netherlands Measurement Institute, van Swinden Laboratory, Delft, The Neth-

erlands.

■ The use of reference materials to establish measurement traceability: P. J. Wagstaffe, Community Bureau of Reference, European Commission, Brussels, Belgium.

■ Interlaboratory comparison as a means of assessing laboratory proficiency: C. Maas, Inspectorate for Health Protection, Food Inspection Service, Zutphen, The Netherlands.

■ Laboratory accreditation: J. Summerfield, National Measurement Accreditation Service, U.K.

On Thursday evening, a reception hosted by the AOAC/Europe Regional Section will be held at the City Hall of Brussels.

On Friday, October 12, the session on Good Laboratory Practices will be chaired by R. Battaglia, Federation of Migros Cooperation, Central Laboratory, Zurich, Switzerland. Topics will include:

■ Knowledge of measurement uncertainty is essential to the production of sound measurements, which are fit for their purpose: M. Thompson, University of London, London, U.K.

■ Different means of assessing and monitoring the analytical quality in a group of laboratories—an experience: B. Carton, National Institute for Security Research INRS, Nancy, France.

■ The use of chemometrics and LIMS as an aid to quality: B. G. M. Vandeginste, Unilever Research Laboratories, Vlaardingen, The Netherlands.

■ Trends in laboratory economics: moving from the public to the private sector: J. Faugere, Municipal Laboratory, Bordeaux, France.

The education and training of future analytical scientists will also be discussed during this session and an Open Forum discussion will be held.

Members of the AOAC/Europe Regional Section constitute the organizing committee for the symposium. They are:

■ President—Beljaars, Inspectorate for Health Protection, Food Inspection Service, Maastricht, The Netherlands.

■ President-elect—J. Sabater, Laboratorio Dr. J. Sabater, Barcelona, Spain.

■ Secretary-treasurer—E. J. de Vries, Duphar BV, PO Box 900, 1380 DA Weesp, The Netherlands.

■ Members—C. Bourgeois, Societe Produits Roche, Fontenay Sous Bois, France; B. King, Laboratory of the Government Chemist, Teddington, U.K.; A. L. Malskaer, Vanlose, Denmark; T. Rihs, Swiss Federal Research Station for Animal Production, Posieux, Switzerland; M. C. Walsh, State Laboratory, Dublin, Ireland; and R. Wood, Ministry of Agriculture, Fisheries, and Food, London, U.K.

The registration fee is Bf (Belgian Francs) 3.500 before September 1, 1990; Bf 4.000 after.

For AOAC members, the fee is Bf 3.000 before September 1; Bf 3.500 after.

For students, the fee is Bf 2.500 before September 1; Bf 3.000 after.

Fees include lunch on first and second day, symposium reception, coffee, tea, and beverages, information pack, and abstracts. Payment must be made in the form of a check in Belgian Francs, payable to the Symposium Secretariat. Refund of fees, less a 20% administrative charge, will be made until September 1, 1990. Substitutions will be accepted at any time.

All inquiries concerning the symposium should be addressed to: AOAC/Europe Regional Section Symposium, Food Science and Technology Service CERIA/COOVI, Institute of Fermentation Industries, Avenue E. Gryzon 1, B-1070 Brussels. Contact: Patrick Dysseler, Tel: (32)-2-52 67 342; FAX: (32)-2-52 67 042.

COMING IN THE NEXT ISSUE

FOCUS ON IMMUNOAFFINITY SEPARATIONS

- High-Performance Immunoaffinity Chromatography for Drug Residue Analysis—*Stanley E. Katz and Marietta Sue Brady*

TOBACCO

- Cigarette Smoke Composition. Part 1. Limitations of FTC Method When Applied to Cigarettes that Heat Instead of Burn Tobacco—*Michael F. Borgerding, Richard D. Hicks, James E. Bodnar, Dennis M. Riggs, Edward J. Nanni, George W. Fulp, Jr, William C. Hamlin, Jr, and J. A. Giles*
- Cigarette Smoke Composition. Part 2. Method for Determining Major Components in Smoke of Cigarettes that Heat Instead of Burn Tobacco—*Michael F. Borgerding, L. Arthur Milhous, Jr, Richard D. Hicks, and J. A. Giles*

and

75 YEARS OF REPORTING ANALYTICAL SCIENCE
1915–1990

J A O A C

Instructions to Authors

Scope of Articles and Review Process

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

General Information

Contributed manuscripts accepted for publication after peer review are subject to a charge of US$40 per printed page. Payment is not a condition of publication, however, and waivers are granted on receipt of a written request to the Managing Editor by an administrative officer of the author's institution.

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

1. Write in clear, grammatical English.
2. To Managing Editor, AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm]) of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); 4. Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.
3. Suggest in a covering letter the names of at least 4 qualified reviewers, i.e., individuals engaged in or versed in research of the type reported.
4. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.
5. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused.

Format and Style

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

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

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

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

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

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

   JOURNAL ARTICLE REFERENCE

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

   BOOK CHAPTER REFERENCE

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

   BOOK REFERENCE

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

   OFFICIAL METHODS REFERENCE

   (4) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 29.070–29.072

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

Miscellaneous

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

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Journal Information

THE ASSOCIATION

The primary objective of the Association of Official Analytical Chemists (AOAC) 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.

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

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

European Representatives For information about AOAC and its publications, 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; Lars Appelqvist, Swedish University of Agricultural Sciences, Dept of Food Hygiene, S 750 07 Uppsala, Sweden, telephone 46-18-172398.

THE JOURNAL

The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. Each volume (one calendar year) will contain about 1200 pages. The scope of the *Journal* encompasses the development and validation of analytical procedures pertaining to the physical and biological sciences related to foods, drugs, agriculture, and the environment. Emphasis is on research and development of precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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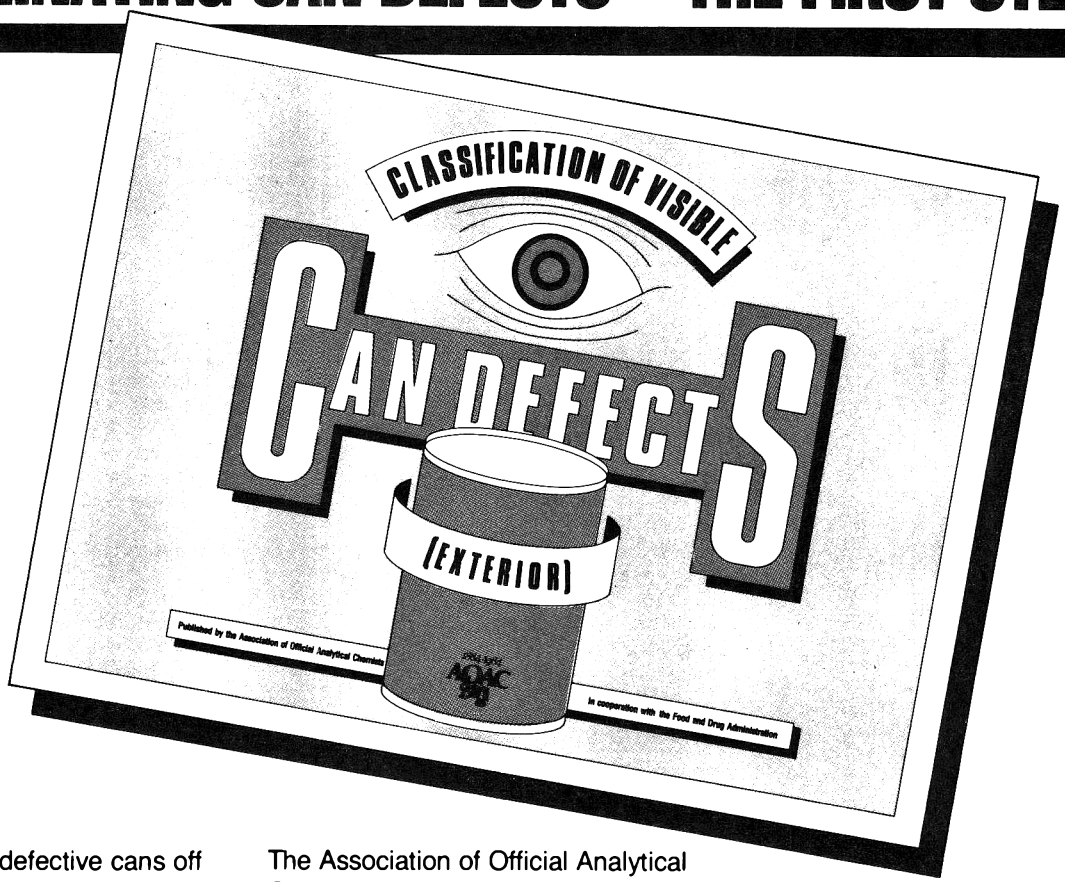
1/89

ELIMINATING CAN DEFECTS—THE FIRST STEP



How can we keep defective cans off the market?

Botulism and other forms of food poisoning can sometimes be traced to defective cans that have leaked and thereby allowed micro-organisms to enter food. Recognizing a can defect, doing something to correct the cause, and removing the defective cans from commerce will help prevent food poisoning outbreaks. The first step is to ensure that responsible personnel know how to identify defective cans.

The Association of Official Analytical Chemists, in cooperation with the Food and Drug Administration, has published a pamphlet that unfolds to a 24″ × 36″ chart, suitable for wall display, to help food industry personnel learn to identify can defects quickly. The chart uses a combination of photographs, easy-to-follow explanations, and color coding to illustrate can defects, classify them according to their degree of potential hazard, and show what to look for in routine inspection of the finished product.

The chart is a valuable reference resource for food processors, salvage operators, retail food personnel, wholesalers and state and local government sanitarians.

For copies, send order form and check to:

Association of Official Analytical Chemists
2200 Wilson Blvd., Suite 400-J
Arlington, VA 22201-3301 USA
Telephone: (703) 522-3032
FAX: (703) 522-5468

I enclose $_____ for the following quantity of Can Defects charts:

(US Funds drawn on US Banks only)

_____ One package of 10 charts @ $45.00 in U.S., $50.00 outside U.S. $_____
_____ Additional packages of 10 charts @ $35.00 in U.S., $40.00 outside U.S. _____
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PAPERS THAT MADE A DIFFERENCE

Emergence in the 1960s of aflatoxin as a potential threat to the health of both human and animal consumers of fungal-contaminated products precipitated a new era of method development for analytical chemists. A section was reserved in the AOAC *Journal* strictly for mycotoxins. The mycotoxin of greatest concern was aflatoxin. All research efforts on the potential cancer-causing aflatoxin demanded reliable measurement of that toxin. No particular method stood out as a single bench mark before which was "darkness" and after which "light." Methods relied on extractions of the mycotoxin in a solvent that solubilized the toxin with a minimum of extraneous material, cleanup of the extract with minimum loss of toxin, and, finally, quantitation. It is in the quantitation of aflatoxin that the spectacular "plateaus of excellence" emerged as various newly developed chromatographic techniques were applied. This report reprints abstracts of some of the important *Journal* papers that introduced these new concepts.

Aflatoxins are intensely fluorescent when exposed to ultraviolet light. The fortuitous circumstance permits the detection of these compounds at extremely low levels and provided the basis for practically all the physicochemical methods for quantitation. Early procedures utilized a visual comparison of the fluorescence intensity of the toxins with those of standards applied to thin-layer chromatographic plates. Plates were prepared in the laboratory. Comparisons were subjective. Quantitation was compromised by observer acuity and was time-consuming because such a large number of visual observations were necessary. A within-laboratory average coefficient of variation of 45% was often reported. The human error was removed with the introduction of instrumental quantitation. The first bench mark in instrumental quantitation of aflatoxin was the introduction of fluorodensitometry by A. C. Bechwith and L. Stoloff [*J. Assoc. Off. Anal. Chem.* **51,** 602–608 (1986)] and W. A. Pons, Jr [*J. Assoc. Off. Anal. Chem.* **54,** 870–873 (1971)].

Densitometry quantitation was applied to aflatoxins separated on thin-layer plates. The former paper describes transmission densitometry in which both excitation energy and the emitted energy transversed the entire gel layer. The latter paper compared results from a typical reflectance densitometer to those obtained by transmission densitometry and determined that comparable results were obtained on both types of instruments. Aflatoxin analysis was firmly projected into the realm of reliability based on unbiased instrumentation.

Use of liquid chromatographic (LC) quantitation was a second bench mark in aflatoxin analysis. Again, unbiased instrumentation was used. The concept was introduced in 1973 by J. N. Seiber and D. P. H. Hsieh [*J. Assoc. Off. Anal. Chem.* **56,** 827–830 (1973)]. The advantage was the by-pass of thin-layer plates.

This early paper described UV detection at 254 nm, rather than fluorescence detection. As a consequence, sensitivity was compromised; 1000 ng B_1 was required to elicit an adequate instrument response. But these authors adventured into application of this new technique to aflatoxin separation. Later, W. A. Pons, Jr, refined LC detection of aflatoxin. Using a UV detector set at 360 nm, he reported baseline separation and sharp peaks for the 4 aflatoxins B_1, B_2, G_1, and G_2 following 10 ng injections [*J. Assoc. Off. Anal. Chem.* **59,** 101–105 (1976)].

Development of a flow cell packed with silica gel allowed the fluorescent property of the toxins to be used in quantitation by LC and provided another step in the ever-improving instrumental analysis [*J. Assoc. Off. Anal. Chem.* **60,** 583–589 (1977)].

That important paper was authored by J. Panalaks and P. M. Scott (both Scott and Pons were AOAC Wiley Award recipients). Sensitivity increased dramatically; 0.6 ng of each of the 4 aflatoxins was detected easily. Pons incorporated LC quantitation into methods specifically developed for estimation of aflatoxins in various agriculture products. His last report, published after his death in 1979, utilized the flow cell and fluorescence detection for quantitation of aflatoxin in corn [*J. Assoc. Off. Anal. Chem.* **62,** 586–594 (1979)].

This report was a real bench mark and established LC as the instrumentation procedure preferred by most chemists for quantitation of aflatoxin.

THEN CAME BIOLOGY. The concept of immunology was applied to quantitation of mycotoxins. Immunochemical assay procedures are common in medical laboratories. Real analytical creativity adapted the techniques for mycotoxin analysis. F. S. Chu is considered one of the

founding fathers of ELISA, enzyme-linked immunosorbent assays. He and his students continue very meaningful research in this area. Chu's 1987 report [*J. Assoc. Off. Anal. Chem.* **70,** 854-857 (1987)] describes an improved ELISA for aflatoxin B_1.

ELISA assays utilize the specificity of antibodies for the particular mycotoxin. Extract cleanup is eliminated. Methods are rapid and usually require a little over an hour to complete. The burgeoning number of commercial kits available for assays for a number of mycotoxins attest to the surge of successful applications of the ELISA technique. Chu's former student, J. J. Pestka, is the current AOAC Associate Referee for immunochemical methods for mycotoxins. His recent special report [*J. Assoc. Off. Anal. Chem.* **71,** 1075-1081 (1988)] offers an excellent overview of the theory and application of ELISA.

In the summary, Pestka states that "the evolution and implementation of mycotoxin immunoassays in the field of analytical chemistry and their use as research tools offer a model for... pesticides, growth promoters, antibiotics and industrial wastes. . . ." Truly biology has made vast inroads into traditional analytical chemistry. (Many chemists are more than a little nervous.)

Louise S. Lee
Journal Co-Editor for Food Contaminants and Biological Methods
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Fluorodensitometric Measurement of Aflatoxin Thin Layer Chromatograms

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The precision limit of visual comparison procedures used in aflatoxin assay methods can be no better than ±20% for a single observation and, under operating conditions, it is probably close to ±28%. Fluorodensitometric procedures described in the literature can improve this precision to ±9% for the average of multiple observations. A possible source of inaccuracy in the published densitometric procedures is pointed out and a method using internal standards to avoid the inaccuracy is presented. Tested with spiked extracts of "clean" peanut butter, the procedure gave an average 101 ± 3% recovery of added aflatoxin B_1 and 89 ± 6% recovery of aflatoxin B_2 with a precision for individual assays equal to the precisions reported for the other procedures. The major source of error was shown by elimination to reside in the thin layer chromatography and aflatoxin instability.

Ref.: *J. Assoc. Off. Anal. Chem.* **51,** 602-608 (1968).

Evaluation of Reflectance Fluorodensitometry for Measuring Aflatoxins on Thin Layer Plates

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A reflectance fluorodensitometer employing illumination of chromatograms with longwave UV light at 45° angles to the plate surface and measurement of reflected fluorescence at 90° was found to be suitable for measuring aflatoxins on silica gel-coated thin layer plates. The relationship of peak area vs. concentration was linear for 1-20 ng aflatoxins B_1 and G_1/spot. Degradation of aflatoxins was slight. Five repetitive scans of the same chromatogram containing 5 ng each of B_1 and G_1 reduced the recorded areas an average of 1% per scan. Consecutive scans of 8 identical standard chromatograms containing 5 ng each of B_1 and G_1 and 1.5 ng each of B_2 and G_2 showed a reproducibility, as measured by coefficients of variation, of ±4-5% (B_1 and G_1) and ±5-9% (B_2 and G_2), representing the combined errors of standard application, TLC development, and scanning. Analysis of aflatoxins in purified sample extracts from 6 contaminated oilseed meals, 3-500 μg aflatoxins/kg, in which the same TLC plates were scanned by a transmission densitometer and the reflectance densitometer yielded essentially equivalent values.

Ref.: *J. Assoc. Off. Anal. Chem.* **54,** 870-873 (1971).

Application of High-Speed Liquid Chromatography to the Analysis of Aflatoxins

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Partial resolution of aflatoxins B_1, B_2, G_1, G_2, and P_1 was achieved by high-speed liquid chromatography (HSLC) on a porous layer silica adsorbent, using chloroform-isooctane as the eluting solvent and a 254 nm UV monitor for detection. The resolution was somewhat less than, although comparable with, that obtained by thin layer chromatography, using Adsorbosil-1 adsorbent and fluorodensitometric detection. The HSLC response to B_1 and G_1 was linear in the 400–3000 ng range, allowing application of the technique to the quantitative analysis of B_1 and G_1 in crude extracts of *Aspergillus parasiticus* cultures. The coefficients of variation (precision) were 4.2% for B_1 and 23.2% for G_1 in a series of 4 replicate injections. The advantages and limitations of the technique for quantitative analysis and isolation are compared with those of more conventional chromatographic methods.

Ref.: *J. Assoc. Off. Anal. Chem.* **56,** 827–830 (1973).

Resolution of Aflatoxins B_1, B_2, G_1, and G_2 by High-Pressure Liquid Chromatography

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Aflatoxins were completely resolved as sharp peaks in the order B_1-B_2-G_1-G_2 by high-pressure liquid chromatography on a small particle (10 μm) porous silica gel column in 7–13 min (B_1 through G_2) by a water-saturated chloroform-cyclohexane-acetonitrile elution solvent (25 + 7.5 + 1.0), with detection by ultraviolet absorbance at 360 nm. The relationship between peak height and amount injected was linear over a 5–400 ng range for each aflatoxin. Both retention times and peak heights were highly reproducible, multiple injections of mixed standards giving coefficients of variation of 1.0–1.4% (retention time) and 1.6–2.8% (peak height) for the 4 aflatoxins. Detection was highly sensitive, with mean peak height, mm/ng, of 7.1 (B_1), 6.4 (B_2), 4.5 (G_1), and 4.1 (G_2), allowing detection of 1–2 ng of each aflatoxin.

Ref.: *J. Assoc. Off. Anal. Chem.* **59,** 101–105 (1976).

Sensitive Silica Gel-Packed Flowcell for Fluorometric Detection of Aflatoxins by High Pressure Liquid Chromatography

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Aflatoxins B_1, B_2, G_1, and G_2 were quantitatively detected by high pressure liquid chromatography on a 5 μm Lichrosorb column, using a Lichrosorb-packed flowcell in the fluorometric detector. The relationship between peak height and the amount injected was linear only up to about 2 ng but showed a linear log-log relationship. Methods for constructing and packing the flowcell are given. A guard column and venting valve were used to minimize deterioration of the analytical column and the adsorbent-packed flowcell. The method was applied to a peanut butter extract, although with the cleanup procedure used, the life expectancy of the flowcell is limited.

Ref.: *J. Assoc. Off. Anal. Chem.* **60,** 583–589 (1977).

High Pressure Liquid Chromatographic Determination of Aflatoxins in Corn

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A high pressure liquid chromatographic (HPLC) method is proposed for determining aflatoxins in corn. The sample is extracted with methanol-10% NaCl (4 + 1), pigments are precipitated with zinc acetate, and the extract is cleaned up on a small (2 g) silica gel column. Aflatoxins in the purified extract are resolved by normal phase HPLC on a microparticulate (10 μm) silica gel column with water-saturated chloroform-cyclohexane-acetonitrile solvent, and detected by fluorescence on a silica gel-packed flowcell. The method was compared with chloroform-water extraction of the official CB method on 15 samples of contaminated corn. In 5 of the 6 samples containing aflatoxins B_1, B_2, G_1, and G_2, methanol-10% NaCl extracted more aflatoxin than did chloroform-water, as measured both by HPLC and by thin layer chromatography. In samples containing only B_1 and B_2, the 2 extraction solvents were virtually equivalent. Agreement was good between HPLC and TLC for each extraction solvent. Average recovery of aflatoxins B_1, B_2, G_1, and G_2 added to yellow cornmeal at 3 levels was >90%.

Ref.: *J. Assoc. Off. Anal. Chem.* **62**, 586–594 (1979).

Improved Enzyme-Linked Immunosorbent Assay for Aflatoxin B_1 in Agricultural Commodities

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An improved enzyme-linked immunosorbent assay (ELISA) for aflatoxin B_1 in cornmeal and peanut butter was developed. Aflatoxin B_1 in cornmeal and peanut butter samples was extracted with 70% methanol in water containing 1% dimethylformamide diluted with assay buffer to a final concentration of 7.0% methanol, and directly subjected to an ELISA procedure that took less than 1 h for quantitative analysis and less than 30 min for screening tests. Analytical recoveries for 5–100 ppb B_1 added to the cornmeal and peanut butter were 91 and 95.4%, respectively. The interwell and interassay coefficient of variation was 10% or less at the 20 ppb level and above. Agreement for B_1 levels in more than 30 naturally contaminated corn, mixed feed, and peanut butter samples was excellent between the ELISA data and the data obtained from different independent laboratories using TLC or other analytical methods.

Ref.: *J. Assoc. Off. Anal. Chem.* **70**, 854–857 (1987).

Enhanced Surveillance of Foodborne Mycotoxins by Immunochemical Assay

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Mycotoxins are a chemically diverse group of fungal secondary metabolites with a wide range of toxic effects. Conventional thin-layer and instrumental methods of mycotoxin analysis are time-consuming and make routine safety and quality control screening of these compounds in agricultural commodities difficult. As an alternative, specific polyclonal and monoclonal antibodies have been raised against mycotoxin-protein conjugates and used in sensitive radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs). One of the simplest ELISA approaches involves competition for a solid-phase antibody between a mycotoxin-enzyme conjugate and an unconjugated mycotoxin in the sample extract. ELISAs have been developed for aflatoxins B_1 and M_1, zearalenone, T-2 toxin, and deoxynivalenol, which are highly specific, rapid (10 min), easily adaptable for analyzing large numbers of samples, and directly applicable to assaying methanol-water extracts of a wide range of foods. Several commercial mycotoxin ELISAs using this approach (most typically for aflatoxin B_1) are currently being marketed. Since ELISAs will be used in large part by personnel with limited technical expertise, individual kits must be critically evaluated by analytical chemists for suggested sampling procedures, efficiency of extraction, cross-reactivity, mycotoxin recovery, assay reproducibility, and product shelf-life prior to routine use in food safety and quality control screening.

Ref.: *J. Assoc. Off. Anal. Chem.* **71**, 1075–1081 (1988).

SPECIAL REPORT

Liquid Chromatographic Approaches to Antibiotic Residue Analysis

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Liquid chromatography has been widely used for determination of antibiotics and other drugs in formulations and in biological fluids for clinical tests. Application to residue analysis is more difficult because of the need for higher sensitivity and isolation from complex biological substrates. Numerous LC methods for chloramphenicol and sulfonamides have been described. LC methods are beginning to be described for residues of other antibiotics. Several approaches have been used for extraction of residues from food substrates. Residues have been cleaned up and concentrated by using partitioning and/or solid-phase extraction. With some residues, cleanup and analysis can be done in the LC system. LC offers considerable potential for rapid, automated residue analysis.

Many liquid chromatographic (LC) methods have been described for determination of antibiotics. Most of these are intended for determination in formulations, fermentation broths, or biological fluids for clinical applications. The use of LC methods in residue analysis has been more limited. Since the subject was reviewed in 1986 (1), considerable progress has been made in development of LC methods for residues of some antibiotics. Residue analysis generally requires greater sensitivity and isolation from more complex substrates than is the case for other applications. LC approaches have been used primarily for confirmation of residues rather than as direct screening tests. However, with recent improvements in methodology, LC procedures can approach the speed and simplicity of screening tests. Confirmatory tests must equal or exceed the sensitivity of screening tests if they are to be of any value.

A major advantage of LC over other chromatographic approaches for antibiotic residue analysis is that frequently little or no sample preparation is required. Another advantage is that procedures can be partially or completely automated. Aerts and coworkers (2) recently described a fully automated procedure for determination of a variety of sulfonamide residues in food substrates.

At the simplest, samples can be injected directly for LC analysis. Direct injection of blood serum is being increasingly used in clinical applications for determination of drug levels in blood (3, 4). The serum is either injected directly into the LC column or injected onto a precolumn with column switching. We have successfully used direct injection for determination of oxytetracycline in honey (5). The honey was diluted with 0.1M HCl and injected directly into the LC system. The procedure requires about 10 min and is sensitive to about 0.5 ppm. It is therefore comparable in speed and simplicity to rapid screening methods.

For determination of antibiotic residues in milk and tissues, some type of extraction and/or deproteinization is required. Some examples of approaches that have been used are summarized in Table 1. Strong mineral acids have been used mainly for extraction of the tetracycline group (6, 7). Tungstic acid has been used for deproteinization of tissues (8). Trichloroacetic acid has also been used as a protein precipitant (9–11). Buffers have been used for extraction of tetracycline (12, 13). Shaikh and coworkers (14) used buffer extraction and heat to extract neomycin from tissues. Ultrafiltration in the presence of organic solvent has been used for serum and also tissues (15, 16). Strong alkali has been used to extract tobramycin from kidney tissue (10). Aerts et al. (2) used saline solution for extraction followed by dialysis. Water-immiscible organic solvents have been used to extract a variety of antibiotics from tissue homogenates and milk (17–22). Water-miscible organic solvents have also been used for extraction/deproteinization; a few examples are listed (23–26). The addition of organic solvents is helpful in dissociating drug-protein complexes. Solid-phase extraction of adsorbent cartridges or pre-columns has been used with substrates such as blood serum (27), milk (28, 29), whole blood (30), or even tissue that has been liquefied enzymatically (31).

After trying a number of these approaches, we concluded that extraction/deproteinization with water-miscible organic solvents was simple, effective, and broadly applicable. Some examples of the use of this approach in our own labora-

Table 1. Extraction and/or deproteinization methods used for antibiotic residue analysis

Approach	Compound/substrate
Mineral acids (HCl, $HClO_4$)	tetracyclines/meat, fish (6)
	tetracyclines/blood, tissue (7)
Tungstic acid	penicillin G/tissues (8)
Trichloracetic acid	kanamycin/tissue (9)
With HCl	oxytetracycline/fish (10)
With NaOH	tobramycin/kidney (11)
Buffer	tetracyclines/liver (12)
	oxytetracycline/fish (13)
Buffer extract/heat	neomycin/tissues (14)
Ultrafiltration	ampicillin/serum (15)
	doxycycline/tissue (16)
Strong alkali (NaOH)	tobramycin/kidney (10)
Saline solution/dialysis	sulfonamides/milk, meat, egg (2)
Water-immiscible organic solvents	doxycycline/tissues (17)
	tetracyclines/tissues (18)
	chloramphenicol/milk (19)
	chloramphenicol/tissues (20, 21)
With ultrasound	sulfonamides/tissues (22)
Water-miscible organic solvents	several (23, 24)
	penicillin G/milk (25)
With acid	many/tissues (26)
	tetracyclines/tissue (7)
Solid-phase extraction	trimethoprim/serum (27)
	chloramphenicol/milk (28)
	penicillins/milk (29)
	hydrophilic drugs/blood (30)
	drugs/enzymatically liquefied tissue (31)

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Table 2. Extraction/deproteinization with water-miscible organic solvents

Antibiotic/substrate	Buffer	Solvent
Penicillins/milk (32)	none	acetonitrile 2:1
Penicillins/tissue (33)	none	acetonitrile 4:1
Tylosin/tissue (34)	blended with 3 v/w pH 2.2 buffer	acetonitrile 4:1
Tetracyclines/tissue (35)	blended with 3 v/w 1N HCl	acetonitrile 4:1
Novobiocin/tissue, milk (36)	blended with 3 v/w 0.1M $NH_4H_2PO_4$	methanol 1:1 methanol 2:1[a]
Virginiamycin/tissue (37)	blended with 3 v/w 0.1M $NH_4H_2PO_4$	methanol 1:1
Lincomycin/tissue, milk (unpublished data)	blended with 3 v/w 0.1M $NH_4H_2PO_4$	2-stage: methanol 1:1; acetonitrile 3:1

[a] Liver, kidney.

tory are shown in Table 2. In some cases, the samples were buffered to improve recoveries.

As was also observed in other laboratories (7, 26), mixing the sample with strong acid greatly improved recovery of tetracyclines. When 1:1 methanol was used, the mixture was buffered to pH 4.6 with $NH_4H_2PO_4$ to facilitate filtration. A 2-stage procedure was used with lincomycin, because a low concentration of organic solvent did not adequately precipitate interferences and direct use of high organic solvent concentrations gave low and erratic recoveries.

The advantages of extraction/deproteinization with water-miscible organic solvents are: (*1*) The procedure is rapid and simple and, with pH adjustment, appears to be universally applicable. (*2*) Recoveries are generally consistent and high (near 100%). (*3*) Because residues are uniformly distributed in the melange formed by addition of organic solvents, an aliquot of filtrate can be taken as equivalent to a given amount of original sample. Lengthy multiple extractions of the filter cake are therefore unnecessary.

There are also some disadvantages with the use of organic solvents: (*1*) They are somewhat toxic. (*2*) Organic solvents must usually, but not always, be removed from extracts prior to reverse-phase chromatography. (*3*) Water-insoluble interferences coextract. It is therefore desirable to keep the organic solvent concentration as low as possible. Use of a 2-stage treatment can reduce carryover of water-insoluble interferences and still give efficient deproteinization.

In general, the simplicity, efficiency, and broad applicability of procedures using water-miscible organic solvents outweigh any disadvantages.

The amount of further treatment of sample extracts required depended on the characteristics of the compound to be analyzed. The simplest approach was to concentrate the analyte directly from the filtrate on the LC column and then to elute with a solvent gradient for analysis. For this approach to be successful, the sample must be injected in a solvent with no eluting strength at all. Most compounds were not retained on reverse-phase packings from the filtrates because of the high organic solvent concentrations. An exception was novobiocin which was retained from 50% methanol. The 1:1 methanol filtrates could therefore be injected directly. When 2:1 methanol was used, the filtrates were diluted 1:1 with water and injected. Novobiocin was eluted with an acetonitrile gradient for analysis. This provided a simple, sensitive analytical procedure (36).

A similar approach could be used with a number of other compounds after the organic solvent was removed from the filtrate. The organic solvent could be removed by evaporation or by extraction with a water-immiscible organic solvent. Petz (23) and Malisch and Huber (24) found that many compounds could be recovered in the organic layer formed after salt (and sometimes methylene chloride) was added to acetonitrile filtrates. This procedure will not work with very polar compounds, however. Tetracyclines were recovered in the water layer formed when methylene chloride (added as a solvent bridge) and hexane were added to the filtrate. Considerable interfering material was removed with the organic layer. The tetracyclines were then concentrated directly on the analytical column, as with novobiocin, and eluted with a methanol–acetonitrile gradient (35).

Some published procedures for tetracycline residues inlude a separate cleanup step with some type of adsorbent cartridge. The residues must then be eluted and concentrated by evaporation. Onji et al. (6) found that considerable losses of tetracyclines could occur during evaporation of eluates. Direct concentration on the analytical column is not only simpler but avoids losses inherent when a separate cleanup step is used.

Some compounds such as tylosin and virginiamycin preferentially partitioned into the organic layer when water-immiscible organic solvents were added to the filtrates. The analytical procedure for tylosin (34) is based on a complex retention mechanism involving binding to the silica support of a bonded reverse-phase packing at high organic solvent concentration (38). With this retention mechanism, which is really normal-phase rather than reverse-phase, tylosin must be injected in acetonitrile rather than water.

Unlike tylosin, virginiamycin must be injected in water for reverse-phase LC determination. The filtrate was prepared using 1:1 methanol to minimize coextraction of lipids. The organic layer was separated by a simple partitioning procedure and evaporated in the presence of a small amount of water to transfer the virginiamycin into water for LC analysis. Virginiamycin, which is actually a mixture of compounds, was concentrated on the LC column from the water layer and eluted with an acetonitrile gradient for analysis. The principal component, designated M, was determined since the minor components are difficult to detect (37).

The 4 compounds for which methods have just been described have fairly strong UV absorptions and thus can be determined by LC procedures with little sample preparation. Other compounds may require more rigorous cleanup. The LC system is a powerful cleanup tool. When extracts contain too much interference for direct LC determination, the analytes can be concentrated on the LC column from sample extracts as previously described after organic solvents are removed. The compounds of interest can be eluted as narrow bands by using a solvent gradient. Narrow fractions can be collected by a procedure sometimes termed "heart-cutting" (39) and rechromatographed under different conditions. The process can be repeated if necessary to obtain the desired concentration and cleanup.

Concentration directly on the LC column offers some advantages over the use of disposable cartridges sold for this purpose: (*1*) Results are reproducible since the same column is used repeatedly. (*2*) No activation is required. (*3*) There is no contamination from the column. (*4*) Column efficiency is much greater so that more precise fractionation is possible.

We have successfully used this approach with lincomycin.

Lincomycin is a weakly basic compound with a pka of 7.6 Because there is no simple way to derivatize the compound, detection was based on the weak UV absorption at 200 nm. This requires rigorous cleanup to separate the compound from interferences. A 2-stage extraction/deproteinization procedure was used to efficiently recover lincomycin from milk and tissue. Lincomycin could not be partitioned completely into either the organic or the water layer. Solvent was therefore removed by evaporation. The salt and free-base forms had markedly different retentions on reverse-phase packings. The extract was chromatographed at pH 4.6 (salt form), and a narrow fraction containing the lincomycin was collected. This fraction was rechromatographed at pH 7.5 (free base), and the lincomycin was separated from interferences in the fraction collected. This approach appears promising for use with other difficult cleanup situations such as determination of β-lactam antibiotics at the low ppb levels. The LC cleanup can be automated using an autosampler and fraction collector.

The methods discussed to this point have used UV absorption for detection of analytes. This approach is simple and UV detectors are universally available. Other detection modes may also be used to improve sensitivity or specificity, especially with compounds with little or no UV absorbance. Native fluorescence has been used for determination of virginiamycin S (40) and sulfonamides (41). A method for chloramphenicol in milk has been described (42), which uses electrochemical detection. However, this method of detection has not been used much for residue analysis, perhaps because of poor specificity.

Derivatization of analytes, both before and after chromatographic analysis, has been done to improve sensitivity and specificity. Some examples of pre-column derivatization include formation of fluorescent derivatives of monensin (43) and other ionophores (44) with 9-anthryldiazo methane and formation of a fluorescent derivative of kanamycin with *o*-phthalaldehyde (11). β-Lactam antibiotics have been determined in blood serum after conversion to fluorescent degradation products (45) and in milk by conversion to the penilloaldehydes with mercury salts after hydrolysis to the penicilloate (46). Post-column derivatization has also been used and has the advantages that a separate sample treatment step is not required and also that the analytes are better separated from interferences prior to derivatization. Neomycin has been determined after treatment with *o*-phthalaldehyde (14), and streptomycin in meat has been determined by derivatization with ninhydrin (47). For β-lactam antibiotics in biological fluids, methods include derivatization of amoxicillin with fluorescamine (39), fluorescent labeling with *o*-phthalaldehyde (48), and degradation of ampicillin and metabolites with sodium hypochlorite (49) and conversion to the mercuric mercaptide derivative of penicillenic acid (50). Aerts et al. (2) used dimethylaminobenzaldehyde to derivatize sulfonamides.

The presence of a chromatographic peak of the proper retention time may not adequately confirm the presence of a suspect residue. However, the absence of any chromatographic peak at or near the retention time of the analyte clearly establishes that the analyte is not present above the detection limit of the method. Since the actual incidence of residues is quite low, the usual result will be negative, i.e., to establish that no detectable residue is present.

Although equipment is now available commercially for interfacing LC equipment with mass spectrometers, this is still quite expensive. Furthermore, special techniques using volatile buffers may be required. Diode array detectors are only slightly more expensive than UV detectors and can be substituted for conventional UV detectors. These provide information as to both the identity and purity of chromatographic peaks and can be readily used routinely. Methods employing this approach have been described by Malisch and Huber (24) and Riond et al. (16). With β-lactam antibiotics, a portion of sample extract can be treated with β-lactamase and the disappearance of any peaks noted (8, 25). Peak height ratios taken at 2 different wavelengths have also been used to confirm the identity and purity of chromatographic peaks (14).

In summary, liquid chromatographic approaches are effective both for analysis and concentration and cleanup of antibiotic residues in sample extracts. A major advantage of LC analysis over other chromatographic approaches is that frequently little sample preparation is required and procedures can be automated to a high degree. Some examples of simplified and automated approaches to sample preparation and analysis are described that appear to be broadly applicable for determination of antibiotic residues in various substrates.

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REVIEW OF PROTEIN QUALITY EVALUATION METHODS

Evaluation of Protein Digestibility-Corrected Amino Acid Score Method for Assessing Protein Quality of Foods

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The current concepts of protein quality evaluation were reviewed. A detailed examination of existing animal assays and more promising amino acid scoring methods has been carried out by an Ad Hoc Working Group on Protein Quality Measurement for the Codex Committee on Vegetable Proteins during the last 5 years. Several factors such as inadequacies of protein efficiency ratio (PER, the poorest test) and other animal assays, advancements made in standardizing methods for amino acid analysis and protein digestibility, availability of data on digestibility of protein and individual amino acids in a variety of foods, and reliability of human amino acid requirements and scoring patterns were evaluated. On the basis of this evaluation, amino acid score, corrected for true digestibility of protein, was recommended to be the most suitable routine method for predicting protein quality of foods for humans. Amino acid scores corrected for true digestibility of protein (as determined by rat balance method) were termed "protein digestibility-corrected amino acid scores." A detailed method for the determination of the protein digestibility-corrected amino acid score was proposed, and information about the range of scores to be expected in foods or food products was provided in the present investigation. The protein digestibility-corrected amino acid score method is a simple and scientifically sound approach for routine evaluation of protein quality of foods. Accuracy of the method would, however, be confirmed after validation with growth or metabolic balance studies in humans.

The only true measurement of protein quality for human use is growth and/or metabolic balance evaluation carried out in suitable subjects of the target population. Recognizing that such studies cannot be done on a routine basis, it is necessary to develop in vitro or animal assay techniques that correlate closely with data from human experiments undertaken for each food product. For accuracy and wide applicability, the routine methods must measure all the basic parameters that determine the quality of a protein, including quantities of essential amino acids, digestibility of protein, and bioavailability of amino acids (1).

A major review and evaluation of protein quality methods was done at the Airlee Conference in 1981 (2). It was generally agreed at this conference that the protein efficiency ratio (PER) method, which is the official method in Canada and the United States, should be replaced by a more appropriate and precise method. Although the relative net protein ratio (RNPR) method was considered to be an improvement over the PER method, a method based on comparison of amino acid content of a food with human amino acid requirements (amino acid scoring system) was considered to be the most suitable approach for evaluating protein quality of foods (1). It was further recommended that amino acid score should be adjusted for incomplete digestibility of protein, and for unavailability of individual amino acids, especially those that are susceptible to damage by processing (1). This meeting recognized the need for further research to standardize amino acid analysis methods, to improve methods for the determination of digestibility of protein and bioavailability of amino acids, and to further investigate human amino acid requirements for developing an accurate amino acid scoring pattern (1).

The recommendations of the Airlee Conference were followed up by the Codex Committee on Vegetable Proteins (CCVP), which was established to develop international standards (including protein quality requirements) for vegetable protein products. The formation of an Ad Hoc Working Group on Protein Quality Measurement for CCVP provided the impetus for conducting cooperative research on the most promising methods for assessing protein quality. In a collaborative study organized by the U.S. Department of Agriculture (USDA) (3), 17 protein sources were studied for amino acid composition, for protein and amino acid digestibility (by rat balance and in vitro methods), amino acid availability (by rat, *Escherichia coli*, and *Streptococcus zymogenes* growth methods and chemical methods), and for protein quality indices based on PER, net protein ratio (NPR), RNPR, net protein utilization (NPU), and biological value (BV). Collaborative studies on protein digestibility determinations were also organized by USDA to test the suitability of in vitro methods (4), and to standardize the rat balance method (5). Results of these and other relevant studies were discussed at the Fifth Session of CCVP held in 1989 (Ottawa, Canada) (6).

Amino acid score, corrected for true digestibility of protein (as determined by the rat balance method), was recommended to be the most suitable routine method for evaluating protein quality of vegetable protein products (and other food products) at the Fifth Session of CCVP (6). Amino acid score was based on the amount of the single most limiting amino acid, and its calculation included the use of the requirement pattern suggested by the Food and Agriculture Organization/World Health Organization/United Nations for pre-school children (7). Although CCVP accepted the protein digestibility-corrected amino acid score as the best available method for routine evaluation of protein quality, it recognized the need for further input of the wider scientific community because of the broad implications, beyond the purview of CCVP, of the protein quality methodology (6).

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The Committee, therefore, recommended that an FAO/WHO expert consultation should be held to evaluate the usefulness of the protein digestibility-corrected amino acid score method for evaluating protein quality for humans (6).

The objectives of the present manuscript are to discuss the background information leading to the development of the protein digestibility-corrected amino acid score, and to describe the method in detail. The discussion includes a brief review of information on rat growth assays for predicting protein quality, determination of digestibility of protein and bioavailability of amino acids in foods, and amino acid scoring procedures including reference patterns and amino acid methodology.

Rat Growth Methods

Rat growth assays are widely used for predicting protein quality in foods, and numerous workers have discussed the appropriateness of these methods (8–14). The protein efficiency ratio (PER = weight gain of test group/protein consumed by test group) is the official method in Canada and the United States for assessing protein quality of foods. But the PER method is the poorest of current animal tests in meeting criteria of a valid routine test (6, 8–14). The most serious criticism of the PER assay is its inability to properly credit protein used for maintenance purposes. A protein source may not support growth and have a PER near zero, yet may be adequate for maintenance purposes. Due to the error introduced by not making allowance for maintenance, the PER values of proteins of differing quality are not proportional (in protein quality) to each other, i.e., a PER of 2.0 cannot be assumed to be twice as good as a PER of 1.0. The lack of proportionality to protein quality makes the PER method unsuitable for the calculation of utilizable protein such as in protein rating (protein in a reasonable daily intake, g × PER), which is the official method of evaluating protein claims of foods sold in Canada (15).

The NPR method [(weight gain of test group + weight loss of nonprotein group)/protein consumed by test group] (16) credits the protein used for both growth and maintenance. This method assumes, however, that the protein required to prevent weight loss of the rats fed the nonprotein diet is equivalent to the protein required for maintenance purposes. As normally carried out, NPR values are uncorrected. Relative NPR method (RNPR = NPR of test protein expressed relative to a value of 100 for NPR of reference protein) (17) has a scale 1 to 100. The BV method (retained nitrogen/absorbed nitrogen × 100) and NPU method [{(body nitrogen of test group-body nitrogen of group fed nonprotein diet)/nitrogen consumed by test group} × 100] also rate proteins on a scale of 1 to 100.

Hegsted and coworkers (11, 18, 19) recommended a multidose slope assay using a reference protein for assessing protein quality. Two versions of the slope assay were introduced, i.e., the relative nutritive value (RNV) and the relative protein value (RPV). The RNV method includes the measurement of the slope of the linear portion of the line relating growth response to nitrogen intake of rats fed zero and 3 or more levels of dietary protein, expressed on a scale relative to a value of 1.00 or 100 for a standard protein (18, 19). The RPV method is the same as the RNV method except that data for the nonprotein group are omitted when the slope of the response lines is calculated (11). The RPV modification of the slope assay appeared to overcome the problem (excessive downward curvature) found with the RNV procedure when proteins severely limiting in lysine are assayed. However, the RPV was reported to yield erroneously high values for some proteins due to parallelism, i.e., lines having similar slopes but different intercepts on the weight gain axis (9). Parallelism is frequently encountered with mixtures of proteins limiting in threonine or co-limiting in lysine and threonine. The RNV method is superior to the RPV method in terms of agreement with amino acid scores, and is probably the best assay for predicting protein quality by rat growth (8).

The RNPR method, which is a modified NPU method (based on body weight), provides results similar to the RNV method (8). The RNPR method is more economical and does not require complex statistical analyses of data as is necessary for the multi-dose assays such as RPV and RNV. The RNPR method (2 weeks) is shorter than the standard PER method (4 weeks) and therefore is less expensive, and RNPR values (unlike PER values) are proportional to each other in protein quality within reasonable limits. Recent collaborative studies (14, 20, 21) have confirmed that RNPR is more accurate and reproducible than PER, and the adoption by AOAC of the NPR method (expressed as RNPR) as official first action as an alternative to the PER method has been recommended (21). Although the RNPR method overestimates the quality of lysine-deficient proteins for the rat, it may in fact be a better predictor of protein quality for human infants than some other methods based on rat growth (8, 13).

In the standard PER and NPR methods, foods are tested at 10% dietary protein, and unsupplemented casein is used as the reference protein. However, diets containing 8–10% protein from unsupplemented casein do not meet rat growth requirements for sulfur amino acids (17). Therefore, methionine-supplemented casein was used as the reference protein in the determination of RNPR. The RNPR method includes the use of 8% protein level because high quality proteins such as egg and casein (normally used as reference proteins) show peak PER or NPR values at about 8% compared to dietary protein levels of 10, 12, or 16% (17). Similarly, the BV and NPU values for egg and casein were higher at 8% than at 12, 16, or 20% dietary protein.

It is well known that the requirements of rats for methionine + cystine are much higher than those of humans and that any rat growth assay (especially those which do not credit protein used for maintenance such as PER) will underestimate the protein quality for humans of any protein product limiting in sulfur amino acids such as soybean protein products, peanuts, and grain legumes or pulses, i.e., peas, beans, and lentils. Modifications for higher essential amino acid requirements of rats compared with humans have been suggested for accurate prediction of protein quality for humans by rat bioassays (22). Sarwar et al. (23) compared essential amino acid requirements of rats and humans. The rat requirement for sulfur amino acid was about 50% higher than that of humans, whereas the differences between the requirements of other essential amino acids were relatively small (23). Based on these comparisons, a factor of 1.5 was selected to correct the RNPR values (casein + methionine = 100) of food products deficient in sulfur amino acids for rat growth. The resultant data were called corrected RNPR (CRNPR) values (23). The use of the correction factor would be appropriate in predicting protein quality (CRNPR) for adults and children but not for infants because of higher essential amino acid requirements of infants compared to adults and children (7).

Table 1. RNPR and CRNPR values of some common vegetable foods or products limiting in sulfur amino acids for rat growth[a]

Product	RNPR, %	CRNPR, %
Soybean protein concentrate	65	97
Soybean protein isolate	61–63	91–94
Peas, field & chick	60–67	90–100
Pea protein concentrates	50	75
Peanut meal	49	73
Beans, pinto, kidney, & black	38–49	57–73
Lentils	28–35	42–52
Yeast (*S. cerevisiae*)	57–69	85–100
Yeast (*C. utilis*)	40–59	60–85
Beef, ground	91	100
Tuna	82	100
Chicken franks	75	100
Pork sausage	63	94
Casein	83	100
Mixtures (50:50 protein basis) of beef & vegetables proteins:		
Casein	86	100
Soybean protein products	73–76	100
Pea protein concentrate	72	100
Peanut meal	73	100
Rapeseed protein products	86–88	100
Sunflower protein isolate	78	100
Wheat gluten	77	100

[a] Abstracted from Sarwar (44) and Sarwar et al. (66). The CRNPR values of above 100 were considered to be 100.

The RNPR and CRNPR values of some common foods or food products limiting in sulfur amino acids for rat growth are shown in Table 1. The CRNPR values were obtained by multiplying the RNPR values with a factor of 1.5. The CRNPR values for the soybean protein products (91–97%) and field and chick peas (90–100%) were higher than those for lentils, beans, and peanut meal (42–73%) (Table 1). Processing of field peas into flour and protein concentrate had negative effects on CRNPR values (75–81%). The CRNPR values for yeasts (*Saccharomyces cerevisiae* and *Candida utilis*) grown on different substrates (sulfite waste liquor, cane and/or beet molasses or ethyl alcohol) were 60–100% (Table 1).

The CRNPR values for most animal protein products and beef-vegetable proteins (including soybean protein concentrate and isolate) mixtures were 100% (Table 1). The high CRNPR values (up to 97%) of the soybean protein products and their mixtures with beef (100%) (Table 1) are in close agreement with protein quality data for these products obtained in experiments with humans. Several studies (24–26) with young men have demonstrated that the protein quality of soybean protein isolate is comparable to that of milk or beef. Bodwell (27) reviewed data on "efficiency score" and "requirement score" of a number of protein sources for children and adults. The scores for different kinds of soybean protein isolates for children and adults were 79–115% compared to 100 for milk, lean beef, fish, and egg (27).

Unlike other rat growth assays for assessing protein quality, the CRNPR method would not discriminate against proteins deficient in sulfur amino acids. However, the CRNPR method has been criticized for using a constant factor of 1.5 regardless of degree of deficiency in sulfur amino acids (28). It was suggested that the correction factor should be (in part) based on the total sulfur amino acid content of the test proteins (28). According to this suggestion, the CRNPR method would not be very efficient, because its calculation would also require determination of total sulfur amino acids. Moreover, the accuracy of the sulfur amino acid requirement of growing rats used in the CRNPR method can be questioned (28).

In Vitro Methods for Predicting Protein Quality

In theory, the most logical approach for evaluating protein quality is to compare amino acid content of food with human amino acid requirements. Resulting amino acids scores may be calculated from the content of the single most limiting, amino acid or from 2, 3, 4, or 5 key essential amino acids (which are likely to be deficient in mixed human diets) such as lysine, methionine plus cysteine/cystine, threonine, and tryptophan (27). The validity of amino acid scoring procedures has been limited by lack of standardized and reproducible procedures for determining tryptophan and sulfur amino acids, by insufficient data on digestibility of protein and bioavailability of amino acids in foods, and by uncertainty about human amino acid requirements to be used for the scoring pattern (28).

During the last few years, significant advancements have been made in standardizing amino acid methodology, in reaching a consensus about human amino acid requirements, and in obtaining information about digestibility of protein and bioavailability of amino acids in a number of protein sources (6). These developments have facilitated the use of an amino acid scoring procedure that is a better predictor of protein quality for humans than rat growth methods (6).

Amino Acid Analyses

Amino acids in foods have been commonly analyzed by ion-exchange chromatography of protein hydrolysates (8). Hydrolysis procedures for determining amino acids in food proteins have been standardized in collaborative studies (29–32). Hydrolysis with 6N HCl at 110°C for 24 h is routinely used for all amino acids except tryptophan and sulfur amino acids. A shorter acid hydrolysis (4 h) at high temperature (145°C) has, however, been demonstrated to yield results (for most amino acids) comparable to the classical acid hydrolysis (110°C for 24 h) (33, 34). Since sulfur amino acids and tryptophan are destroyed to varying extents by 6N HCl hydrolysis, a preoxidation with performic acid followed by the HCl hydrolysis, and an alkaline (4.2N NaOH) hydrolysis have been widely used for accurate determinations of sulfur amino acids (methionine as methionine sulfone and cysteine/cystine as cysteic acid) and tryptophan, respectively (35). The 3 hydrolysis procedures and the ion-exchange chromatography of amino acids in the hydrolysates were standardized by Satterlee et al. in a collaborative study (29), and have been adopted official by AOAC (36) as a part of the calculated PER method based on the essential amino acid composition of test protein or on both essential amino acid composition and enzymatic digestibility of test protein.

Intra- and interlaboratory variations (expressed as CV) for most amino acids in foods determined by ion-exchange chromatography of 6N HCl hydrolysates have been reported to be less than 3 and 10%, respectively (30, 35). The interlaboratory variations for tryptophan (up to 24%), cystine (up to 18%), and methionine (up to 16%) were, however, large (35). The relatively high interlaboratory variations for tryptophan, cystine, and methionine may be due to the failure to follow the same detailed conditions for preparation of hydrolysates

(performic acid + 6N HCl for sulfur amino acids, and 4.2N NaOH for tryptophan) by the participating laboratories. Interlaboratory variation due to chromatographic or analytical measurement is considerably lower than that produced by hydrolysate preparation in different laboratories (33). Improvements in interlaboratory variation for tryptophan (CV = 4–16%), cystine (CV = 7–17%), and methionine (CV = 5–12%) in foods and food/feed ingredients were noted by meticulous attention to details of prechromatographic handling of samples, including preparation of hydrolysates (32).

Although ion-exchange chromatography of amino acids has been in use for over 20 years, several laboratories are now switching to liquid chromatography. Promising methodology for analyzing amino acids (including methionine and cysteine/cystine) in protein hydrolysates using phenylisothiocyanate (PITC) derivatization and liquid chromatography has been developed (37–39). The PITC derivatization results in the formation of relatively stable derivatives of amino acids (both primary and secondary), which are easily separated on reverse-phase columns with UV detection at 254 nm (37). The PITC derivatization method has been reported to be rapid (12 min compared to 60–90 min for ion-exchange) and reproducible (within a laboratory), and to provide amino acid data similar to those obtained by conventional ion-exchange procedures (38). A simple chromatographic method (requiring no derivatization) for the determination of tryptophan in alkaline hydrolysates of foods and feces has also been developed (38).

Amino acid profiles of food/feed and/or feces samples as determined by ion-exchange chromatography and by liquid chromatography using precolumn PITC derivatization have been compared (37–39). The differences for most amino acids due to the analytical procedures were less than 10% (37–39). The within-laboratory variation expressed as coefficients of variation for the determination of most amino acids, in the food and feces samples, by the liquid chromatographic method was not more than 4% which compared favorably with the variation of ion-exchange methods (38). To further standardize and to obtain information on the between-laboratory variation for the liquid chromatographic amino acid analysis method, there is an urgent need to organize interlaboratory studies.

Amino Acid Scoring Patterns

With the more recent (1985) publication of the FAO/WHO/UNU (7) suggested patterns of amino acid requirements for infant, preschool child (2–5 years), school child (10–12 years), and adult, there is now reason for confidence in the reliability of scoring patterns. The FAO/WHO/UNU (7) suggested pattern of amino acid requirements for the preschool child is similar to the National Research Council (40) scoring pattern. An earlier FAO/WHO (41) scoring pattern suggested a significantly higher methionine + cystine value, but extensive studies (42, 43) on essential amino acid requirements of children (21–27 months) have indicated that the value was too high, and that the true requirement is almost identical to the values contained in the NRC (40) pattern and the FAO/WHO/UNU (7) suggested pattern for preschool child. Therefore, the use of the FAO/WHO/UNU (7) suggested pattern for preschool child has been recommended as the reference for calculating amino acid scores. These scores would apply to children and adults (including a margin of safety) but not to infants. The FAO/WHO/UNU (7) suggested pattern of amino acid requirements of infants (which is based on amino acid composition of human milk) should be used as the reference in calculating amino acid scores of infant foods.

Digestibility of Protein and Bioavailability of Amino Acids

Data on digestibility of protein and/or bioavailability (true digestibility) of amino acids in diets of various areas of the world, and in common foods or food ingredients have been recently reviewed by Sarwar (44) and by Hopkins (45). The digestibility data discussed in these reviews were abstracted from human and/or rat balance experiments.

As reviewed by Hopkins (45), values for true digestibility of protein in diets from India (54–75%), Guatemala (77%), and Brazil (78%) were considerably lower than the values in North American diets (including vegetarians, 88–94%), suggesting that protein digestibility is of greater concern in diets of some developing countries. The poor digestibility of protein in the diets of developing countries is due to the use of less-refined cereals and pulses (such as beans and lentils) as major sources of protein (45). Low true protein digestibility values (63–65%) have also been reported in experiments with children fed millet- and ragi-based diets in India (45).

Values for true digestibility of protein in some common foods for human adults have also been reviewed (45). Animal protein sources (meat, fish, poultry, eggs, milk protein products) and flours or breads of low fiber wheats, wheat gluten, farina, peanuts, and soy protein isolates have high true protein digestibility of 94–99% (45). Whole corn (except high amylose containing opaque-2) and flour or bread of high-fiber wheat, polished rice, oat meal, triticale, cottonseed, soy flour, and sunflower have intermediate protein digestibility values of over 85% (45). The ready-to-eat cereals (corn, wheat, rice, or oat) had low protein digestibilities of 70–77%, caused probably by the heat involved in their processing (45). Various types of dry beans (*Phaseolus vulgarus*) have low true protein digestibility of about 75% (45). Millet also has a low protein digestibility of 79% (45).

A comparative review of protein digestibility of some common foods as determined by human and rat balance methods suggested that the abilities of rats and humans to digest a variety of food proteins are similar (44, 46). As noted in humans, high true protein digestibility values of 93–100% were obtained for animal foods or food products (casein, minced beef, beef salami, skim-milk, tuna, chicken franks, and sausage) and soy protein isolate by the rat balance meth-

Table 2. Values (%) for true digestibility of crude protein and amino acids in some diets as determined by human balance method[a]

Diet	Egg	Pork muscle	Peanut butter
Protein	97	98	95
Isoleucine	99	99	95
Leucine	99	100	98
Lysine	98	99	90
Methionine	99	98	90
Cystine	99	98	95
Phenylalanine	98	99	97
Tyrosine	97	97	96
Threonine	99	100	96
Tryptophan	97	97	95
Valine	98	98	93

[a] Recalculated from apparent digestibility data of Watts et al. (49), using fecal protein and amino data of subjects fed low-nitrogen diet as estimates for metabolic fecal protein and amino acids.

Table 3. Values (%) for true digestibility of protein and selected amino acids in peas, beans, and lentils as determined by rat balance method

Product	Protein	Lys	Met	Cys	Thr	Trp
Pea, Century (autoclaved)[a]	83	85	62	85	78	72
Pea, Trapper (autoclaved)[a]	84	85	61	83	75	73
Pea flour[b]	88	92	77	84	87	82
Pea protein concentrate[c]	92	92	73	87	90	91
Pea protein concentrate[d]	93	94	93	94	88	92
Chick pea (canned)[c]	89	89	74	88	84	82
Chick pea (canned)[d]	88	90	80	92	82	85
Lentil (canned)[c]	84	84	41	40	77	73
Lentil (autoclaved)[a]	85	86	58	66	77	90
Kidney bean (canned)[c]	81	80	44	0	74	76
Pinto bean (canned)[c]	79	78	45	56	72	70
Pinto bean (canned)[d]	73	75	51	46	66	69
Pinto bean (autoclaved)[a]	80	78	61	59	68	58
Seafarer bean (autoclaved)[a]	84	81	60	72	77	74
Black bean (autoclaved)[a]	72	72	51	46	62	47
Fababean (autoclaved)[a]	86	85	59	75	76	63

[a] Sarwar and Peace (51).
[b] Sarwar (50).
[c] Sarwar et al. (48).
[d] Eggum et al. (47).

od (47, 48). Intermediate true protein digestibility values of 86–92% were obtained for chick peas, beef stew, rolled oats, whole wheat cereal, and pea protein concentrate. Low true protein digestibility values (70–85%) were reported for different types of dry beans including pinto beans and kidney beans (*P. vulgaris*), and lentils (*Lens culinaris*) (47, 48).

Limited human studies have been reported on true digestibility (bioavailability) of individual amino acids in foods. In one study (49), the differences between digestibility of protein and individual amino acids in diets containing whole egg, pork muscle, or peanut butter were reported to be not more than 5% (Table 2).

Considerable rat data comparing true digestibility of protein and individual amino acids in various foods have been generated (47, 48, 50, 51). A recent review (44) of the digestibility data revealed that the differences between the digestibilities of protein and most individual amino acids in animal protein sources, and low-fiber cereals and oilseed products were less than 10%. However, digestibility of protein was not a good predictor of digestibility of limiting amino acids in grain legumes. In beans, peas, and lentils, values for true digestibility of methionine, cystine, and tryptophan were as much as 43, 44, and 25% lower than those of protein, respectively (Table 3).

Low protein and amino acid digestibilities of grain legumes or pulses (dried seeds belonging to the family Leguminosae and sub-family Papilionoideae) have been attributed to the presence of less-digestible protein fraction(s), residual levels of antiphysiological factors (such as trypsin inhibitors, amalyse inhibitors, hemagglutinins, etc.), and high concentrations of indigestible carbohydrates and tannins (52–54). The presence of significant amounts of fermentable carbohydrates that support maximum microbial growth in the large intestine would result in increased bacterial protein in the feces (55).

It has been argued that possible microbial modifications of undigested and unabsorbed nitrogenous residues in the large intestine may influence the determination of amino acid bioavailability by the balance method. To address this concern, amino acid digestibilities determined by the fecal and ileal recovery methods in pigs have been compared by several workers (56–58). For most amino acids, the fecal digestibility values were somewhat higher than the ileal values, suggesting disappearance in the large intestine; the disappearance being larger in the case of threonine and tryptophan than other essential amino acids. In the case of methionine, however, the fecal value was lower than the ileal value, suggesting synthesis of methionine by the microflora of the large intestine of pigs fed some cereal grains. The differences between the fecal and ileal methods of determining amino acid digestibility have been discussed in greater detail in a recent review (44).

Animal growth assays have been frequently used to evaluate bioavailability of amino acids. Although limited to the bioavailability determination of a single amino acid at a time, the bioavailability results of the properly processed protein sources obtained by the growth method (not affected by the modification in the large intestine) are considered (in theory) to be more accurate than those obtained by the balance method (28).

In a USDA-organized cooperative study, bioavailabilities of some key amino acids (such as lysine, methionine, and tryptophan) in the same batches of some foods were determined by the rat growth or balance method by different participating laboratories. The rat growth method was used by one laboratory (Beltsville, MD) while the rat balance method was used by 2 different laboratories (Ottawa, Canada; and Tjele, Denmark). The comparative amino acid bioavailability data for 7 food products are shown in Table 4. The differences in bioavailabilities of tryptophan, lysine, and methionine obtained by the 2 methods were 1–9, 1–13, and 3–15%, respectively. These differences may be regarded as small especially if consideration is given to the fact that the 2

Table 4. Comparison of amino acid bioavailability results (%) obtained by rat growth and balance methods[a]

	Lysine			Methionine			Tryptophan		
Product	Growth	Balance		Growth	Balance		Growth	Balance	
Casein	100	98	100	100	97	99	100	100	100
Tuna	100	97	99	99	92	95	98	92	97
NFDM[b]	101	94	96	100	92	92	105	97	98
NFDM (heated)	80	88	88	92	95	88	93	95	94
Pinto bean	87	75	78	58	51	45	61	69	70
Chick pea	95	90	89	89	80	74	86	85	82
Pea protein	93	94	92	88	93	73	97	92	91

[a] Abstracted from Bodwell et al. (3).
[b] Nonfat dry milk.

Table 5. Values (%) for digestibility of protein and selected amino acids in diets containing mixtures of protein sources as determined by rat balance method[a]

Mixture	Protein	Lys	Met	Cys	Thr	Trp
Wheat flour-casein	95	91	91	89	90	90
Wheat flour-egg white	95	90	93	95	90	91
Wheat flour-beef	93	88	89	89	87	88
Macaroni-cheese	94	92	90	88	83	94
Macaroni-cheese	95	95	93	98	92	98
Potatoes-beef	89	90	83	74	86	88
Potatoes-beef	86	89	83	89	83	86
Wheat flour-soy protein	92	86	85	90	86	90
Wheat flour-pea flour	92	89	84	93	88	88
Wheat flour-rapeseed	93	86	88	93	87	89
Rice-soybean	90	89	77	82	84	87
Wheat-soybean	93	91	87	96	87	93
Wheat-horsebean	90	94	93	93	90	93
Corn-pea	83	85	84	86	82	80
Corn-soybean	93	93	87	94	93	98
Corn-horsebean	91	94	88	92	91	96
Barley-soybean	92	95	86	99	92	92
Barley-horsebean	91	96	90	90	95	93
Oat-soybean	93	91	84	97	89	98
Oat-horsebean	91	96	93	93	93	94

[a] Abstracted from Sarwar (44).

methods were used by 2 different laboratories using their own analyzed amino acid data. The rat balance method remains the most widely used animal assay for determining digestibility of protein and of all individual amino acids at the same time (12).

Regardless of the method used, pinto beans had the lower values for bioavailability of methionine, lysine, and tryptophan (Table 4). As noted earlier, the differences between digestibility of protein and limiting amino acids (such as methionine, cystine, and tryptophan) in beans, lentils, and peas are large, suggesting that in these cases amino acid scores would have to be corrected for bioavailability of individual amino acids. The wide differences between digestibilities of protein and limiting amino acids in beans, lentils, or peas would, however, be of limited practical significance because a normal human diet is usually based on a mixture of protein sources.

Data on digestibility of protein and key essential amino acids (lysine, methionine, cystine, and tryptophan) in diets based on mixtures of protein sources have been obtained by the rat balance method (Table 5). In most cases, digestibility of protein was a good predictor of digestibility of individual amino acids; the differences being less than 10%. Considerable data on digestibility of amino acids in foods have been reported to suggest that correcting amino acid scores for true digestibility of protein would be sufficient and further correction for bioavailability of individual amino acids would not be needed for most fixed human diets (44).

The use of protein digestibility values for correcting amino acid composition data as an approach for assessing protein quality of mixed human diets has been suggested by FAO/WHO/UNU (7). Suitable and standardized methods for determining protein digestibility would have to be developed before it could be used in regulatory methods for evaluating protein quality.

Several in vitro protein digestibility methods utilizing 3 or 4 enzymes (porcine pancreatic trypsin, bovine pancreatic chymotrypsin, porcine intestinal peptidase, and protease from *Streptomyces geriseus*) have been developed (59-62). Among these, the pH-stat procedure of Pedersen and Eggum (62) appears to be the most promising in vitro method for predicting protein quality of foods. Unlike other methods (59, 60) in which digestibility is estimated by measuring the pH drop in the protein suspension caused by enzymatic digestion, the pH is kept constant during the incubation period by automated titration with NaOH and digestibility estimates are based on the amount of alkali used in the pH-stat procedure (62).

The pH-stat procedure of Pedersen and Eggum (62) as standardized by McDonough et al. (4) was recently tested in a collaborative study which included analysis of 17 protein sources by 6 laboratories. The method was found to have excellent reproducibility and repeatability; mean relative standard deviations for reproducibility and repeatability were less than 5 and 2%, respectively. To validate the pH-stat procedure, protein digestibility of the same batch of 7 protein sources (casein, NFDM-heated, tuna, macaroni/cheese, pea protein concentrate, rolled oats, and pinto beans) tested in the in vitro study were also determined by the rat balance method in an interlaboratory study which included the participation of 8 laboratories. As noted in the case of the pH-stat procedure, the mean relative standard deviations for reproducibility and repeatability for the rat balance method were less than 5 and 3%, respectively. In general, the pH-stat procedure gave higher digestibility values than the rat balance method, especially in the case of pinto beans where the in vitro value was 20% higher than the in vivo value. Since low protein digestibility of beans in humans is well known, the pH-stat in vitro procedure was considered unsatisfactory for predicting protein digestibility for humans. When compared with other existing methods, the rat balance method, as standardized by McDonough et al. (5), was considered to be the most suitable practical method for determining protein digestibility in foods (6).

Determination of Protein Digestibility-Corrected Amino Acid Score

A method for the determination of a protein digestibility-corrected amino acid score is proposed in this section. A description of analyses required to calculate the protein digestibility-corrected amino acid score is also outlined.

For determining protein digestibility-corrected amino acid score, a test food would have to be analyzed for proximate and amino acid compositions, and for protein digestibility as determined by the rat balance method.

(a) *Proximate composition.* Levels of moisture, total nitrogen, fat, and crude fiber should be determined according to the AOAC methods. Total protein should then be calculated by using a nitrogen-to-protein conversion factor of 6.25. Foods high in moisture (such as meats) should be dried before analyses. Similarly, foods high in fat (such as meat, nuts, whole milk powder, etc) may require a lipid extraction prior to analyses.

(b) *Amino acid profile.* Protein hydrolysates should be prepared and analyzed for amino acids by ion-exchange chromatography as specified in the AOAC methods (36). Amino acids in protein hydrolysates may also be determined by liquid chromatography of precolumn phenylisothiocyanate derivatives (38). Amino acids should be expressed as mg/g protein or g/100 g protein.

(c) *Amino acid score.* Amino acid ratios (mg of an essen-

Table 6. Protein digestibility-corrected amino acid scores for beans, peas, and lentils

Product	Protein (N × 6.25%)	True protein digestibility, %	Amino acid score, %	Protein digestibility-corrected score, %
Pinto beans (canned)[a]	23.6	73	78	57
Pinto beans (canned)[b]	23.7	79	80	63
Pinto beans (autoclaved)[c]	19.9	80	77	62
Kidney beans (canned)[b]	18.9	81	84	68
Seafarer beans (autoclaved)[c]	23.3	84	84	70
Black beans (autoclaved)[c]	21.7	72	74	53
Fababeans (autoclaved)[c]	27.9	86	55	47
Lentils (canned)[b]	28.0	84	62	52
Lentils (autoclaved)[c]	21.9	85	60	51
Chick peas (canned)[a]	21.2	88	81	71
Chick peas (canned)[b]	21.4	89	74	66
Peas (Century, autoclaved)[c]	13.9	83	82	68
Peas (Trapper, autoclaved)[c]	15.7	84	73	61

[a] Data for protein content, protein digestibility, and amino acids (used in calculating scores) were taken from Eggum et al. (47).
[b] Data for protein content, protein digestibility, and amino acids were taken from Sarwar et al. (48).
[c] Data for protein content, protein digestibility, and amino acids were taken from Sarwar and Peace (51).

tial amino acid in 1.0 g of test protein/mg of the same amino acid in 1.0 g of reference protein × 100) for 9 essential amino acids should be calculated by using the FAO/WHO/UNU (7) suggested pattern of amino acid requirements for preschool children (2–5 years) as the reference protein containing (mg/g protein): His, 19; Ile, 28; Leu 66; Lys, 58; Met + Cys, 25; Phe + Tyr, 63; Thr, 34; Trp, 11; and Val 35. The lowest amino acid ratio is termed amino acid score. For example, a pinto bean sample contained 30.0, 42.5, 80.4, 69.0, 21.1, 90.5, 43.7, 8.8, and 50.1 mg/g protein of His, Ile, Leu, Lys, Met + Cys, Phe + Tyr, Thr, Trp, and Val, respectively. The respective amino acid (His, Ile, Leu, Lys, Met + Cys, Phe + Tyr, Thr, Trp, and Val) ratios for the bean sample would be 158, 152, 122, 119, 84, 144, 128, 80, and 143%. This would then result in an amino acid score of 80% with tryptophan as the first limiting amino acid. The scores above 100 would be considered as 100.

(d) *Protein digestibility.* True protein digestibility should be determined using the rat balance method as standardized by McDonough et al. (5). Data on fat and crude filter in the test food should be used in adjusting the formulation of test and nitrogen-free diets to be equal in levels of total fat and crude fiber. The test and nitrogen-free diets should also contain equal amounts of moisture and lactose (in testing high lactose foods such as milk powder).

(e) *Protein digestibility-corrected amino acid score* (%) of a test food should then be calculated by the following equation: True protein digestibility × the lowest amino acid ratio. Protein digestibility-corrected amino acid scores above 100 would be considered as 100.

Protein Digestibility-Corrected Amino Acid Scores for Some Common Foods or Food Products

Availability of information on amino acid composition and protein digestibility of a number of foods or food products has enabled us to calculate protein digestibility-corrected amino acid scores for these products. The scores for various types of beans, lentils, and peas ranged from 47 to 71% (Table 6), and these products were first limiting in sulfur amino acids and/or tryptophan for human nutrition. The grain legumes (pulses) noted in Table 6 contained less than 30% total protein. Pin milling and air classification have been used to prepare protein concentrates of lima bean, navy bean, northern bean, chick pea, cow pea, lentil, field pea, mung bean, and fababean containing about 50–75% protein (63). Information on protein digestibility and accurate amino acid profiles of protein-rich products prepared from grain legumes is, however, limited.

Soybean protein products had high protein digestibility-corrected amino acid scores (89–99%, Table 7), and were marginally deficient in sulfur amino acids for human nutrition in some cases. The protein digestibility-corrected amino acid scores for pea protein concentrates were 68–78% (Table 7), based on tryptophan and/or sulfur amino acids as the first limiting amino acid(s). Because of improved protein digestibility, the scores for pea protein concentrates were higher than those for whole peas (Tables 6 and 7).

The rapeseed protein products had fairly high protein digestibility-corrected amino acid scores (83–93%, Table 7), with lysine being the first limiting amino acid. Wheat gluten

Table 7. Protein digestibility-corrected amino acid scores for some vegetable protein products

Product	Protein (N × 6.25%)	True protein digestibility, %	Amino acid score, %	Protein digestibility-corrected score, %
Soybean protein concentrate[a]	70.2	95	100	99
Soybean protein isolate[a]	92.2	95	94	89
Soybean protein isolate[b]	88.2	92	99	91
Soybean protein isolate[c]	86.4	98	100	99
Soy assay protein[d]	93.0	95	97	92
Pea protein concentrate[a]	57.0	92	79	73
Pea protein concentrate[b]	54.1	93	84	78
Pea protein concentrate[c]	52.1	92	74	68
Rapeseed protein concentrate[a]	68.3	95	98	93
Rapeseed protein isolate[a]	87.3	95	87	83
Sunflower protein isolate[a]	92.7	94	39	37
Wheat gluten[a]	87.0	96	26	25
Peanut meal[a]	61.2	94	55	52

[a] Data for protein content, protein digestibility, and amino acids (used in calculating scores) were taken from Sarwar et al. (23).
[b] Data for protein content, protein digestibility, and amino acids (used in calculating scores) were taken from Eggum et al. (47).
[c] Data for protein content, protein digestibility, and amino acids (used in calculating scores) were taken from Sarwar et al. (48).
[d] Data for protein content, protein digestibility, and amino acids (used in calculating scores) were taken from Sarwar (50).

and sunflower protein isolate were severely limiting in lysine and had low protein digestibility-corrected scores of 25 and 37%, respectively (Table 7). Breakfast cereals such as rice-wheat gluten, whole wheat, and rolled oats (first limiting in lysine) also had low protein digestibility-corrected amino acid scores of 26, 40, and 57%, respectively (44). A sample of peanut meal had protein digestibility-corrected score of only 52% (Table 7), and was co-limiting in several essential amino acids such as methionine + cystine, lysine, threonine, and/or tryptophan.

Animal protein products such as egg white powder, casein, ground beef, beef salami, skim milk powder, tuna, and chicken franks had protein digestibility-corrected amino acid scores of about 100% (97–100%) (44). A sample of pork sausage had, however, a relatively low protein digestibility-corrected score of 63% due to deficiency in tryptophan (44).

The low protein quality of a vegetable protein source can be improved by the addition of supplementary protein or limiting amino acid, and by protein complementation. Protein supplementation means to increase the protein quality of a deficient protein by the addition of a moderate amount of another protein having a high content of the essential amino acid which is limiting in the deficient protein such as the addition of milk proteins (rich in lysine) to cereals which

Table 8. Protein digestibility-corrected amino acid scores for some protein mixtures[a]

Mixture (50:50 protein basis)	True protein digestibility, %	Amino acid score, %	Protein digestibility-corrected score, %
Wheat flour (WW) + beef	93	91	85
WW + egg white	95	83	79
WW + casein	95	96	91
WW + rapeseed concentrate	93	72	67
WW + pea flour	92	89	82
WW + soy protein	92	78	72
Beef + rapeseed concentrate	95	100	100
Beef + rapeseed isolate	96	100	100
Beef + soybean concentrate	96	100	100
Beef + soybean isolate	98	100	100
Beef + peanut meal	95	80	76
Beef + pea concentrate	95	84	80
Beef + sunflower isolate	95	88	84
Beef + wheat gluten	95	81	77

[a] Abstract from Sarwar (44). True protein digestibility, amino acid score, and protein digestibility-corrected amino acid score for whole wheat flour were 90, 46, and 41%, respectively.

have a low content of lysine (64). Protein complementation means that quality of 2 proteins is higher than either of the 2 component proteins (64). Proteins that complement each other have different limiting amino acids. When blended in the right proportions, cereals and legumes complement each other because cereals are limiting in lysine but contain moderate amounts of sulfur amino acids, while legumes are limiting in sulfur amino acids but contain high amounts of lysine (64). Traditional combinations of vegetable proteins consumed in some countries (such as rice-chick pea in Asia, wheat-bean in the Near East, maize-bean in the Americas, etc.) have good protein quality because the amino acid compositions of the cereals and legumes complement each other, producing a balanced mixture of amino acids. The use of amino acid supplementation to increase protein quality of a protein source should only be considered when protein supplementation or complementation has proved impracticable (64). Benefits from amino acid supplementation have not been demonstrated consistently in humans (65). Furthermore, an excess of a supplementary amino acid such as synthetic methionine may have a deleterious effect on infants and children (64).

Data on the protein digestibility-corrected amino acid scores of some protein mixtures having supplementary and/or complementary effects are given in Table 8. The protein digestibility-corrected amino acid score of whole wheat flour of 46% was improved to 72–96% by the addition of rapeseed protein concentrate, soy protein, egg white, pea flour, beef, or casein (Table 8). Similarly, the addition of ground beef caused considerable improvement in the protein digestibility-corrected amino acid scores of wheat gluten (25 vs 77%), sunflower protein isolate (37 vs 84%), pea protein concentrate (68 vs 80%), and peanut meal (52 vs 76%) (Tables 7 and 8).

In conclusion, the protein digestibility-corrected amino acid score has been recommended to be the most suitable regulatory method for evaluating protein quality of foods (6). Since this method is based on human amino acid requirements, it could be considered more accurate than animal assays used for predicting protein quality of foods. There is, however, a need to obtain information on how well the protein digestibility-corrected amino acid score method predicts or correlates with growth or metabolic balance studies in humans.

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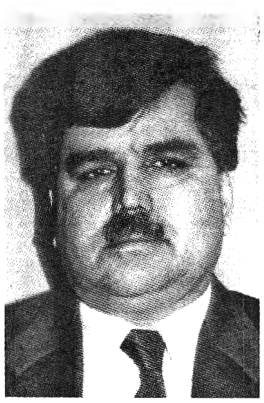

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CHEMICAL CONTAMINANTS MONITORING

Survey of Lead and Cadmium in Adult Canned Foods Eaten by Young Children

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A U.S. Food and Drug Administration survey of lead and cadmium in 10 adult canned foods commonly eaten by children less than 5 years old was conducted between October 1981 and September 1985. The survey, which included foods preserved by a commercial canning process and packaged in metal containers, found the highest mean levels of lead (0.32 μg/g) in tuna and of cadmium (0.02 μg/g) in tuna and tomatoes. Lead levels in foods packaged in lead-soldered cans were about 5 times as high as those in foods packaged in nonlead-soldered cans. Mean lead levels appeared to decline over the 4 years of the study. Cadmium levels were usually below the data reporting limit (0.01 μg/g).

The U.S. Food and Drug Administration (FDA) is committed to reducing the lead content of the diet of very young children, who are especially susceptible to lead toxicity. Lead in processed foods is due in part to the lead-containing solder used in the formation of can seams. The use of lead solder for making cans has declined in recent years because of efforts by can manufacturers to reduce this source of lead.

FDA has conducted a number of surveys of the lead level in infant foods. These surveys have produced information on the level of lead in infant formula and evaporated milk.

Another source of lead exposure to young children is canned foods normally produced for adult consumption. (In the present paper, the term "canned foods" refers to foods that are preserved by a commercial canning process and packaged in metal containers [not glass].) Young children eat adult foods because they are physically able to do so and because the foods cost less than those produced specifically for infants or young children. The survey described here was designed to provide background information on the levels of lead in these foods to allow assessment of this route of lead exposure.

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The analytical method used for the determination of lead in the survey foods allowed determination of cadmium at small additional cost, thereby accommodating the continuing need for information on the level of cadmium in foods.

Experimental

Sample Collection

Selection of canned food products.—The types of products in the survey were selected from a list of canned foods frequently consumed by children from 0 to 5 years old. This list was obtained from Market Research Corporation of America (1), which collected food consumption data during July through December 1977. From 27 items listed as eaten 5 or more times per child per year, the 10 products listed in Table 1 were selected for inclusion in the FDA survey. Products such as infant formula and evaporated milk were not included in the survey because FDA already had sufficient information on these products. Data for the most closely related foods in the FDA Total Diet Study (TDS) (Table 1) indicated that these 10 foods provide about 5.5 and 10% by weight of the average diet for the age groups 6–11 months and 2 years, respectively (2, 3).

Collection of canned foods.—The survey called for collection of 4 brands for each of the 10 food products listed in Table 1 (i.e., 40 samples) from each of 25 (20 in fiscal year 1985) different retail markets throughout the United States each fiscal year (i.e., 1000 samples per year, except 800 in fiscal year 1985). The retail markets were randomly selected from Standard Metropolitan Statistical Areas (4) each year. Brands and can sizes were selected on the basis of their percentages of the market share according to 1978–1980 data (5–7). Twelve cans were collected for each sample and

Table 1. Survey products and consumption values (2) for foods included in FDA Total Diet Study

Survey product	Food	Food consumption value, g/day	
		6–11 mo	2 yr
Tuna	032 tuna, canned in oil, drained	0.300	1.471
Orange juice	098 orange juice, frozen, reconstituted	18.115	59.370
Applesauce	084 applesauce, canned, sweetened	9.057	5.687
Apple juice	099 apple juice, canned, unsweetened	17.094	17.594
Fruit punch	104 orange drink with added vitamin C, canned	9.447	24.954
String beans	122 beans, snap green, canned	1.585	3.234
Baked beans	039 pork and beans, canned	1.024	4.593
Tomatoes	120 tomatoes, canned	0.000	0.152
Chicken noodle soup	155 chicken noodle soup, canned, reconstituted with water	6.835	17.808
Vegetable soup	157 vegetable beef soup, canned, reconstituted with water	5.792	16.196
Products' total		69.249	151.06

the contents were composited for the analysis. Collections were often incomplete because of unavailability of some products in a particular market area. For example, canned applesauce did not appear to be marketed in the northeastern part of the country. If a particular size of can could not be found, an alternative size was usually collected. The size and type of can used to package each sample were identified. The type of can was identified as one of the following: (a) conventional lead-soldered, (b) lead jet-soldered, (c) wire-welded, (d) forge-welded, (e) drawn/redrawn aluminum, (f) triple drawn, (g) drawn/redrawn 2-piece, (h) cemented, or (i) other.

Sample Preparation

The contents of the 12 cans collected for a sample were composited by blending in a glass or stainless steel blender. For some of the larger cans (e.g., 46 oz), the contents of only 6 cans were composited because of the difficulty in handling the large volume. Because the presence of lead solder particles in the food would have made it impractical to obtain homogeneity by mechanical blending, the composite was homogenized in the presence of dilute nitric acid. A 500 g aliquot of the composite and 500 g 2N nitric acid were weighed into a clean vessel and blended by using a Polytron homogenizer. This mixture was allowed to stand for at least 16 h to dissolve metal particulates (8, 9). The mixture was then reblended by using the Polytron homogenizer, and a 20.0 g aliquot was immediately taken for analysis.

Analysis

The 20.0 g aliquot of homogenate (equivalent to 10.0 g original composite) was dry ashed with potassium sulfate ash aid, and the diluted ash was analyzed by anodic stripping voltammetry (10, 11). Drying times given in the method had to be extended because of the large amount of residual nitric acid from the homogenization procedure. For some foods a 10.0 g aliquot of the homogenate was analyzed to shorten the drying time and avoid splattering. For quality assurance, at least 1 reagent blank analysis, 1 recovery analysis, and 1 duplicate analysis were performed per 10 test samples.

Results

Thirteen FDA laboratories (Atlanta, Baltimore, Boston, Buffalo, Cincinnati, Dallas, Detroit, Los Angeles, Minneapolis, New Orleans, New York, Philadelphia, and Seattle) participated in the survey; the total number of test samples analyzed was not evenly distributed among the laboratories. The 3× standard deviation detection limits of the method as developed (10) are 0.0014 μg Cd/g and 0.0045 μg Pb/g. However, the participating laboratories could not achieve these limits based on historical information. The participating laboratories were instructed to achieve reagent blanks of ≤0.005 μg Cd/g and ≤0.020 μg Pb/g and to report findings of less than twice their reagent blank as zero. After preliminary review of the survey findings, analytical data for a particular batch of test samples were accepted if the average reagent blank for the batch was <0.01 μg Cd/g or <0.02 μg Pb/g and the average lead or cadmium recovery was between 80 and 120%. These values were somewhat arbitrarily chosen but reflect the minimum capabilities of a majority of the participating laboratories. In retrospect, it would have been preferable to analyze a sufficient number of reagent blanks with each batch of test samples and to determine the detection limit of each batch from the standard deviation of the

Table 2. Mean, median, and maximum lead findings (μg Pb/g) for all can types by fiscal year[a]

	Fiscal year 1982				Fiscal year 1983			
Product	n	Mean	Median	Max.	n	Mean	Median	Max.
Tuna	72	0.48	0.31	4.2	71	0.29	0.03	2.9
Orange juice	65	0.08	0.04	0.63	67	0.04	0.03	0.25
Applesauce	66	0.20	0.12	0.99	52	0.11	0.10	0.6
Apple juice	61	0.11	0.06	0.68	58	0.07	0.03	0.51
Fruit punch	63	0.08	0.06	0.75	54	0.08	0.05	1.1
String beans	69	0.21	0.18	0.70	67	0.16	0.10	1.0
Baked beans	71	0.26	0.22	1.1	72	0.19	0.10	1.3
Tomatoes	66	0.20	0.13	1.1	69	0.24	0.15	2.2
Chicken noodle soup	70	0.14	0.07	1.2	72	0.09	0.07	0.47
Vegetable soup	69	0.17	0.13	0.74	70	0.19	0.10	2.0
All products	672	0.20	0.10	4.2	652	0.15	0.07	2.9
	Fiscal year 1984				Fiscal year 1985			
Product	n	Mean	Median	Max.	n	Mean	Median	Max.
Tuna	89	0.30	0.06	2.6	68	0.21	0.03	5.3
Orange juice	86	0.04	0.03	0.29	66	0.04	0.03	0.13
Applesauce	75	0.11	0.08	1.4	53	0.09	0.06	1.2
Apple juice	82	0.03	0.02	0.19	59	0.03	0	0.12
Fruit punch	77	0.06	0.03	1.4	58	0.05	0.04	0.41
String beans	91	0.11	0.04	1.1	70	0.09	0.03	1.0
Baked beans	92	0.13	0.04	1.7	71	0.08	0.02	1.1
Tomatoes	84	0.19	0.10	1.6	66	0.22	0.05	1.5
Chicken noodle soup	87	0.13	0.09	0.97	72	0.16	0.10	1.3
Vegetable soup	93	0.17	0.10	4.3	72	0.13	0.07	1.0
All products	856	0.13	0.06	4.3	655	0.11	0.04	5.3

[a] A value of zero was used for findings below the data reporting limit of 0.02 Pb/g in computing statistics.

Table 3. Mean lead findings (μg Pb/g) for lead-soldered and nonlead-soldered cans by fiscal year[a]

	Fiscal year 1982				Fiscal year 1983			
	Lead-soldered		Nonlead-soldered		Lead-soldered		Nonlead-soldered	
Product	n	Mean	n	Mean	n	Mean	n	Mean
Tuna	41	0.78	31	0.08	23	0.85	48	0.03
Orange juice	34	0.12	31	0.03	23	0.10	44	0.01
Applesauce	34	0.31	32	0.10	27	0.16	25	0.06
Apple juice	37	0.16	24	0.04	23	0.13	35	0.03
Fruit punch	55	0.09	8	0.02	50	0.09	4	0
String beans	51	0.28	18	0.02	39	0.22	28	0.06
Baked beans	66	0.28	5	0.06	44	0.30	28	0.03
Tomatoes	58	0.22	8	0.09	59	0.28	10	0.04
Chicken noodle soup	70	0.14	0	—	72	0.09	0	—
Vegetable soup	69	0.17	0	—	70	0.19	0	—
All products	515	0.24	157	0.06	430	0.21	222	0.03

	Fiscal year 1984				Fiscal year 1985			
	Lead-soldered		Nonlead-soldered		Lead-soldered		Nonlead-soldered	
Product	n	Mean	n	Mean	n	Mean	n	Mean
Tuna	34	0.65	55	0.08	14	0.83	54	0.05
Orange juice	47	0.07	39	0.02	34	0.05	32	0.02
Applesauce	16	0.25	59	0.08	4	0.37	49	0.07
Apple juice	29	0.08	53	0.01	10	0.05	49	0.02
Fruit punch	58	0.07	19	0.03	45	0.06	13	0.03
String beans	38	0.24	53	0.03	21	0.23	49	0.02
Baked beans	47	0.24	45	0.02	18	0.28	53	0.01
Tomatoes	63	0.25	21	0.01	38	0.37	28	0.02
Chicken noodle soup	87	0.13	0	—	71	0.17	1	0
Vegetable soup	93	0.17	0	—	64	0.14	8	0.01
All products	512	0.19	344	0.04	319	0.20	336	0.03

[a] A value of zero was used for findings below the data reporting limit of 0.02 μg Pb/g in computing statistics.

reagent blanks. For all the accepted findings, the means ± standard deviation of the reagent blanks were 0.0011 ± 0.0024 μg Cd/g and 0.009 ± 0.008 μg Pb/g. The 3× standard deviation approximations of the survey's detection limits based on these reagent blanks were 0.0073 μg Cd/g and 0.024 μg Pb/g. In addition, data reporting limits of 0.01 μg Cd/g and 0.02 μg Pb/g were applied to the accepted findings, below which a value of zero was assigned for statistical analysis. A few exceptions to these criteria were allowed if the reagent blanks were slightly high and the levels of lead and cadmium in the respective test samples were all above the reagent blank level, or if aberrant recoveries could be explained by low spike levels. The average recovery (approximately 330 recoveries each of lead and cadmium) for the accepted analyses was about 94%, with a relative standard deviation (RSD) of about 14% for both lead and cadmium.

Table 4. Mean and 90th percentile lead findings (μg Pb/g) for fiscal years 1982–1985 by product and lead/nonlead-soldered cans[a]

	All cans			Lead-soldered cans			Nonlead-soldered cans		
Product	n	Mean	90th percentile	n	Mean	90th percentile	n	Mean	90th percentile
Tuna	300	0.32	0.90	112	0.76	1.5	188	0.06	0.14
Orange juice	284	0.05	0.12	138	0.08	0.17	146	0.02	0.04
Applesauce	246	0.13	0.28	81	0.25	0.59	165	0.08	0.12
Apple juice	260	0.06	0.15	99	0.12	0.24	161	0.02	0.07
Fruit punch	252	0.07	0.13	208	0.08	0.14	44	0.03	0.06
String beans	297	0.14	0.38	149	0.25	0.50	148	0.03	0.08
Baked beans	306	0.17	0.49	175	0.27	0.59	131	0.02	0.04
Tomatoes	285	0.21	0.55	218	0.27	0.63	67	0.03	0.07
Chicken noodle soup	301	0.13	0.27	300	0.13	0.27	1	0	—
Vegetable soup	304	0.17	0.32	296	0.17	0.33	8	0.01	0.08
All products	2835	0.15	0.37	1776	0.21	0.50	1059	0.04	0.09

[a] A value of zero was used for findings below the data reporting limit of 0.02 μg Pb/g in computing statistics.

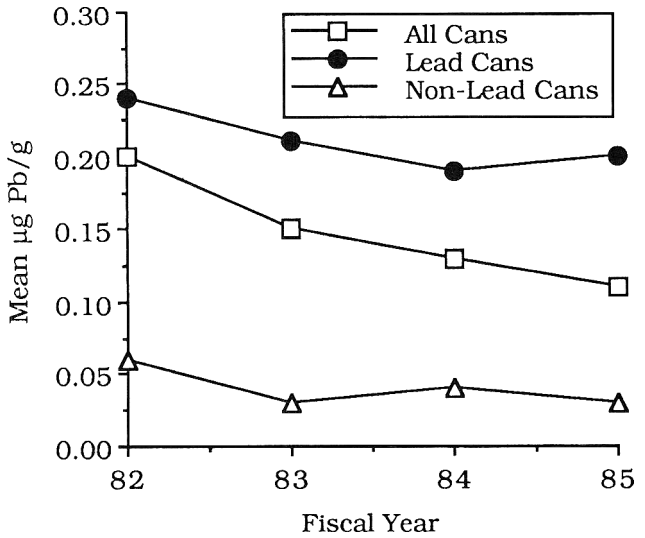

Figure 1. Mean lead levels in canned foods by fiscal year.

The average RSDs for all duplicate analyses were 15% for lead (n = 318) and 22% for cadmium (n = 313). The average RSDs for duplicates above the reporting limits were 12% for lead (n = 234) and 17% (n = 80) for cadmium.

Descriptive statistics were computed for all the acceptable data. There were 2835 acceptable lead analyses and 2896 acceptable cadmium analyses. For the statistical analysis all values below the data reporting limits (0.02 µg Pb/g and 0.01 µg Cd/g) were assigned a value of zero. Table 2 lists the mean, median, and maximum lead findings for each of the 10 products for each fiscal year. Tuna and tomatoes had the highest mean levels of lead in each fiscal year except one. Table 3 lists the mean lead findings for each of the 10 products for each fiscal year and separates the data for lead-soldered and nonlead-soldered cans. Figure 1 is a plot of the mean lead level of all products (Tables 2 and 3) vs fiscal year.

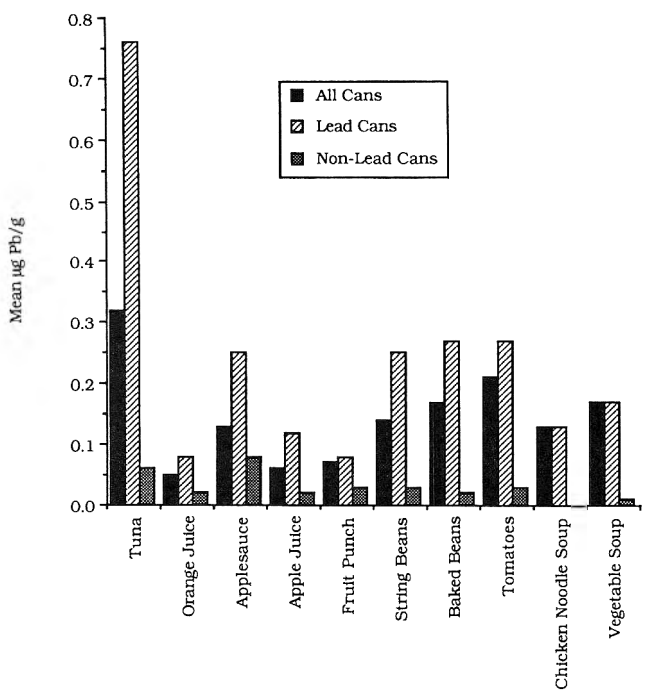

Figure 2. Mean lead levels in canned foods by product over fiscal years 1982–1985.

Table 5. Lead levels in foods for different can types for fiscal years 1982–1985

Product	Can type[a]	n	Mean,[b] µg Pb/g
Tuna	A	112	0.76
	C	1	0
	D	5	0
	E	39	0.05
	G	143	0.06
Orange juice	A	135	0.08
	B	3	0.02
	C	137	0.02
	E	4	0
	H	4	0.01
	I	1	0
Applesauce	A	80	0.25
	B	1	0.10
	C	159	0.08
	D	3	0.13
	E	1	0
	F	1	0
Apple juice	A	97	0.12
	B	2	0.04
	C	153	0.02
	F	1	0.02
	H	3	0
	I	4	0.02
Fruit punch	A	208	0.08
	C	29	0.02
	F	2	0.18
	G	2	0
	I	11	0.02
String beans	A	144	0.25
	C	77	0.04
	D	12	0.02
	E	19	0.04
	F	28	0.02
	G	12	0.02
Baked beans	A	138	0.32
	B	37	0.12
	C	128	0.02
	F	2	0.01
	G	1	0
Tomatoes	A	206	0.28
	B	12	0.14
	C	64	0.03
	D	3	0.01
Chicken noodle soup	A	112	0.16
	B	188	0.11
	C	1	0
Vegetable soup	A	116	0.20
	B	180	0.15
	C	8	0.01

[a] A = conventional lead-soldered; B = lead jet-soldered; C = wire-welded; D = forge-welded; E = drawn/redrawn aluminum; F = triple drawn; G = drawn/redrawn 2-piece; H = cemented; I = other.

[b] A value of zero was used for findings below the data reporting limit of 0.02 µg Pb/g in computing statistics.

As expected, lead levels in foods from lead-soldered cans were higher than those in foods from nonlead-soldered cans. Table 4 lists the mean and 90th percentile lead findings for all products and also lists the data separately for lead-soldered and nonlead-soldered cans. The mean level for foods in lead-soldered cans was about 5 times as high as that for foods in nonlead-soldered cans. The 90th percentile for foods in lead-soldered cans was about 10 times as high as that for

Table 6. Mean, median, and maximum cadmium findings (μg Cd/g) for all can types by fiscal year[a]

	Fiscal year 1982				Fiscal year 1983		
Product	n	Mean	Median	Max.	n	Mean	Median	Max.
Tuna	80	0.03	0.02	0.20	69	0.03	0.02	0.30
Orange juice	76	0.008	0	0.12	68	0.005	0	0.05
Applesauce	74	0.01	0	0.17	56	0.007	0	0.07
Apple juice	73	0.002	0	0.06	59	0.001	0	0.01
Fruit punch	73	0.002	0	0.06	54	0	0	0.01
String beans	77	0.01	0	0.16	67	0.001	0	0.02
Baked beans	79	0.01	0	0.37	72	0.001	0	0.02
Tomatoes	74	0.02	0.02	0.26	69	0.01	0.01	0.05
Chicken noodle soup	77	0.004	0	0.04	72	0.005	0	0.07
Vegetable soup	80	0.01	0	0.17	70	0.01	0	0.07
All products	763	0.01	0	0.37	656	0.01	0	0.30

	Fiscal year 1984				Fiscal year 1985		
Product	n	Mean	Median	Max.	n	Mean	Median	Max.
Tuna	86	0.02	0.02	0.07	68	0.02	0.02	0.07
Orange juice	81	0.004	0	0.02	66	0.005	0	0.04
Applesauce	72	0.003	0	0.05	52	0.004	0	0.03
Apple juice	80	0.002	0	0.04	60	0.002	0	0.03
Fruit punch	71	0.002	0	0.04	60	0.001	0	0.05
String beans	88	0.001	0	0.03	68	0.001	0	0.02
Baked beans	88	0.001	0	0.02	69	0.001	0	0.02
Tomatoes	82	0.02	0.01	0.07	69	0.02	0.02	0.43
Chicken noodle soup	86	0.003	0	0.05	72	0.003	0	0.06
Vegetable soup	87	0.003	0	0.02	72	0.003	0	0.03
All products	821	0.01	0	0.07	656	0.006	0	0.43

[a] A value of zero was used for findings below the data reporting limit of 0.01 μg Cd/g in computing statistics.

foods in nonlead-soldered cans. Figure 2 is a graph of the mean lead level of each product (Table 4) over all fiscal years. Tuna in lead-soldered cans had the highest levels of lead. Table 5 lists the mean lead findings for each product by can type over all fiscal years.

Two types of lead-soldered cans were identified: conventional lead-soldered and lead jet-soldered. The lead jet-soldered process uses much less lead in making the can, and consistently lower levels of lead were found in the foods in these cans, compared to the levels in the same products packaged in conventional lead-soldered cans. Lead levels found for applesauce in forge-welded cans and for fruit punch in triple drawn cans were anomalous in that lead levels were higher than expected, that is, they were similar to those found for foods in lead-soldered cans. However, since only a few test samples were analyzed in each category, misidentification of can type is a possibility.

Table 6 lists the mean, median, and maximum cadmium findings for each of the 10 products for each fiscal year. Most cadmium levels were below the data reporting limit (0.01 μg/g). Tuna and tomatoes had the highest mean levels of cadmium each fiscal year. Table 7 lists the mean and 90th percentile cadmium findings for the 10 products over all fiscal years. Although not shown in the table, there was no overall difference in cadmium levels between lead-soldered and nonlead-soldered cans.

Table 7. Mean and 90th percentile cadmium findings (μg Cd/g) for all cans types for fiscal years 1982–1985[a]

Product	n	Mean	90th percentile
Tuna	303	0.02	0.04
Orange juice	291	0.005	0.02
Applesauce	254	0.007	0.02
Apple juice	272	0.002	0.01
Fruit punch	258	0.001	0
String beans	300	0.003	0.01
Baked beans	308	0.003	0.01
Tomatoes	294	0.02	0.04
Chicken noodle soup	307	0.003	0.01
Vegetable soup	309	0.007	0.02
All products	2896	0.008	0.02

[a] A value of zero was used for all findings below the data reporting limit of 0.01 μg Cd/g in computing statistics.

Discussion

Tin Interference

A few of the participating laboratories reported an unidentified peak, which they suspected was due to tin in the voltammetric cell. This peak was identified as tin by Holak and Specchio (12). The extent to which tin interfered with the findings reported in the present survey is unknown, but, in view of the ensuing discussion, appears minimal.

In their study, Holak and Specchio failed to investigate the behavior of tin in foods taken through every step of the analytical method, e.g., the first part of the procedure (dry ash treatment). In addition, they did not use both the AOAC official method (13) and their proposed method to analyze foods with known tin levels. As a consequence of these omissions, they failed to demonstrate that the interference occurs when a sample is dry ashed.

Tin at 200 μg/g was studied during the development of the

Table 8. Comparison of mean lead and cadmium levels from Total Diet Study and adult canned food survey[a]

Product	Fiscal year 1982		Fiscal year 1983		Fiscal year 1984		Fiscal year 1985	
	TDS MB 1–4	ACF	TDS MB 5–8	ACF	TDS MB 9–12	ACF	TDS MB 13–16	ACF
				Lead, μg/g				
Tuna	0.13	0.48	0.19	0.29	0.22	0.30	0.13	0.21
Orange juice[b]	0.02	0.08	0.03	0.04	0.01	0.04	0	0.04
Applesauce	0.14	0.20	0.05	0.11	0.07	0.11	0.04	0.09
Apple juice	0.04	0.11	0.05	0.07	0	0.03	0.01	0.03
Fruit punch	0.03	0.08	0.07	0.08	0.02	0.06	0.01	0.05
String beans	0.16	0.21	0.06	0.16	0.03	0.11	0.04	0.09
Baked beans	0.18	0.26	0.09	0.19	0.16	0.13	0.01	0.08
Tomatoes	2.16	0.20	0.22	0.24	0.18	0.19	0.28	0.22
Chicken noodle soup[c]	0.06	0.14	0.12	0.09	0.05	0.13	0.02	0.16
Vegetable soup[c]	0.10	0.17	0.20	0.19	0.09	0.17	0.05	0.13
				Cadmium, μg/g				
Tuna	0.020	0.03	0.046	0.03	0.027	0.02	0.026	0.02
Orange juice[b]	0.005	0.008	0	0.005	0	0.004	0	0.005
Applesauce	0.001	0.01	0	0.007	0	0.003	0.001	0.004
Apple juice	0.001	0.002	0.001	0.001	0.003	0.002	0.001	0.002
Fruit punch	0.001	0.002	0	0	0	0.002	0	0.001
String beans	0.012	0.01	0.005	0.001	0	0.001	0	0.001
Baked beans	0.009	0.01	0.003	0.001	0.002	0.001	0.002	0.001
Tomatoes	0.015	0.02	0.023	0.01	0.019	0.02	0.019	0.02
Chicken noodle soup[c]	0.007	0.004	0.007	0.005	0.003	0.003	0.004	0.003
Vegetable soup[c]	0.010	0.01	0.007	0.01	0.007	0.003	0.013	0.003

[a] TDS MB = Total Diet Study market basket; trace findings between the detection limit and the quantitation limit for MB 1–8 were given value of 1/2 TDS quantitation limit (1/2 limit = 0.01 μg Pb/g and 0.005 μg Cd/g), whereas for MB 9–16 an estimate of the trace level was used in the calculations. ACF = adult canned food survey; findings below the data reporting limits of 0.02 μg Pb/g and 0.01 μg Cd/g were given a value of zero. This difference in data treatment had little effect on the reported levels.

[b] Total Diet Study values were multiplied by 4 to account for water reconstitution.

[c] Total Diet Study values were multiplied by 2 to account for water reconstitution.

analytical method used in the present survey (10); this level of tin did not affect the results for lead and cadmium. It was found that properly controlled dry ashing renders tin electro-inactive. If the dry ash procedure is not used correctly, a small tin peak may appear in the voltammogram. However, the supporting electrolyte used in our survey provides adequate separation of the lead, cadmium, and tin peaks so that the potential tin interference can be recognized and appropriate action taken by the analyst. Tin does not affect lead results because the peak potentials of tin and lead are substantially different (by approximately 100 mV). Tin may interfere with cadmium determinations because the peak potentials of cadmium and tin are only slightly different (by approximately 40 mV).

By employing standard quality control procedures (monitoring peak potentials and regression correlation coefficients), our analysts were able to recognize any problems and either solved them or did not report the results. Further support for the accuracy of the survey findings is their agreement with results obtained by graphite furnace-atomic absorption spectrometry for foods in the FDA Total Diet Study as discussed later.

Although more than 2000 samples were analyzed for this survey, relatively few occurrences of a tin peak were reported. When tin appeared in the voltammogram, the interference was eliminated by adding more tartaric acid to the supporting electrolyte (to mask tin) or by more closely controlling the nitric acid treatment during dry ashing (to render tin electro-inactive). These method modifications were reported previously (14). Some laboratories reporting this problem also reported poor recoveries; their results were subsequently eliminated from the statistical calculations.

Comparison with Other Studies

FDA conducted a preliminary survey of the levels of lead, cadmium, and zinc in selected canned foods during fiscal years 1980 and 1981 (15) to get an indication of the general levels of lead in canned foods before beginning the survey described here. The preliminary survey collected 39 different canned food products, but the type of can was not reported and the sampling was not statistically balanced. The 10 products reported here were represented in the preliminary survey, although for 4 products, fewer than 10 samples were analyzed. In addition, there were slight differences in product type between the survey for fiscal years 1980–1981 and the present survey: citrus juice vs orange juice, fruit juice (noncitrus) vs apple juice, and green beans vs string beans. The mean levels of lead and cadmium in products analyzed in the preliminary survey were compared with the fiscal year 1982 findings from the present survey for all can types. The findings are in good agreement for both lead and cadmium except for the level of lead in applesauce, which was about 5 times as high for fiscal year 1982 as the level reported for fiscal years 1980–1981. However, because the preliminary survey reported the mean of only 5 analyses, the earlier result probably does not accurately represent the product.

Table 8 compares the mean lead and cadmium levels from this adult canned food (ACF) survey with the findings from

Table 9. Daily intakes of lead and cadmium from 10 food products by 2 age groups[a]

Fiscal year	μg Pb/day				μg Cd/day			
	6–11 mo		2 yr		6–11 mo		2 yr	
	TDS	ACF	TDS	ACF	TDS	ACF	TDS	ACF
82	4.1	8.6	8.0	17.7	0.27	0.40	0.75	0.96
83	4.7	5.9	10.9	12.8	0.14	0.27	0.37	0.66
84	2.2	5.1	5.0	11.5	0.11	0.20	0.26	0.48
85	1.2	4.7	2.3	10.6	0.14	0.22	0.35	0.52
Mean	3.1	6.1	6.5	13.1	0.17	0.27	0.43	0.66

[a] TDS = Total Diet Study; ACF = adult canned food survey; see Table 1 for list of food products; see footnote a in Table 8 for treatment of data below the quantitation or reporting limit.

the FDA TDS for samples collected during approximately the same time period (16–18) and analyzed by graphite furnace-atomic absorption spectrometry. Each TDS finding is the mean of 4 samples, whereas the ACF findings represent from 52 to 93 samples (Table 2). Generally, the findings of the 2 studies agree, considering the number of samples in each study. Where differences occur, the ACF lead findings are usually higher than the TDS findings. Since the TDS program does not specify the type of can to collect (i.e., lead- or nonlead-soldered), the lead differences may be explained by the differences in can type. Also, the ACF canned orange juice was compared to the TDS frozen orange juice, which is commonly packaged in paperboard containers, and the ACF canned fruit punch was compared to the TDS canned orange drink. The lead level in TDS tomatoes in fiscal year 1982, which was 10 times as high as the ACF finding, is an exception to the higher findings in the ACF survey. One of the 4 TDS findings for tomatoes in fiscal year 1982 was about 8 μg Pb/g, which caused the mean to be higher than in the other 3 years.

The mean lead and cadmium levels for each product/fiscal year (Table 8) were used to compute daily intakes of the elements based on the consumption values used by the TDS for the age groups 6–11 months and 2 years (2). These results are listed in Table 9 and are graphically displayed in Figures 3 and 4 for lead and cadmium, respectively. Daily intakes based on ACF survey findings are slightly higher than TDS intakes for both lead and cadmium. The average total daily intakes of lead and cadmium based on all of the TDS market baskets collected for fiscal years 1982–1986 (234 foods/market basket; 20 market baskets) are 11.6 μg Pb/day and 4.04 μg Cd/day for the 6–11 month age group and 15.6 μg Pb/day and 7.20 μg Cd/day for the 2 year age group. On the basis of the ACF means from Table 9, the contributions of the adult canned foods to these total daily intakes are 53% of the lead and 7% of the cadmium for the 6–11 month age group and 84% of the lead and 9% of the cadmium for the 2 year age group.

During the sampling period for this survey, many canned food processing manufacturers began changing from lead-soldered to nonlead-soldered cans. The number of domestically manufactured lead-soldered cans for foods dropped from 90% of all cans made in 1979 to 24% in 1986. This change is evident in the number of samples collected in each category over the 4 years of the survey (Table 3). The mean lead level for all products changed little for each can type during the 4 years of the survey, but the mean lead level for all can types (Table 2) declined from 0.20 to 0.11 μg Pb/g.

FDA has reported the results of a national survey of raw agricultural products (19), which included tomatoes, one product also associated with the ACF survey. The 231 samples of raw tomatoes collected in the survey (1980–81) had mean levels of 0.002 μg Pb/g and 0.017 μg Cd/g, minimum levels of <0.0001 μg Pb/g and 0.002 μg Cd/g, and maximum levels of 0.025 μg Pb/g and 0.048 μg Cd/g. The mean cadmium level of the raw tomatoes agrees very closely with the

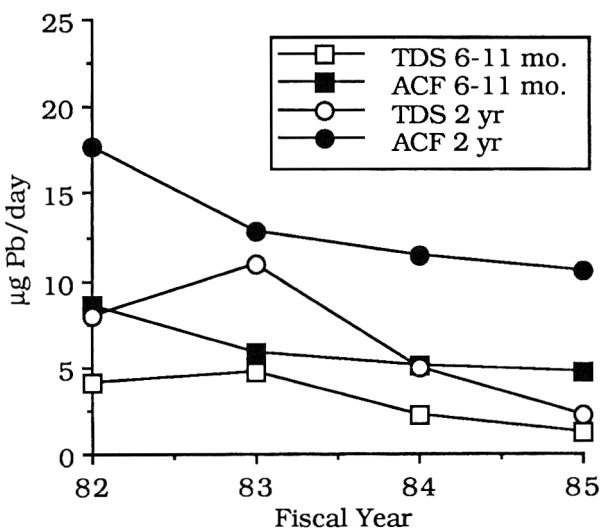

Figure 3. Lead daily intakes from 10 food products.

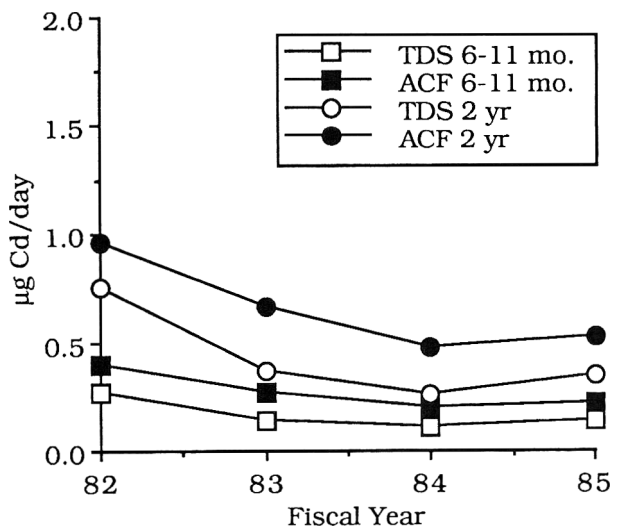

Figure 4. Cadmium daily intakes from 10 food products.

ACF mean of 0.02 μg Cd/g for tomatoes (Table 7). However, the mean lead level of ACF tomatoes, 0.21 μg Pb/g (Table 4), is about 100 times that of the raw tomatoes, and even the mean lead level of ACF tomatoes from nonlead-soldered cans, 0.03 μg Pb/g (Table 4), is 10 times that of the raw tomatoes.

Conclusions

Levels of lead and cadmium were determined in samples of 10 canned adult foods commonly eaten by young children. The mean levels ranged from a high of 0.32 μg Pb/g in tuna (minimum = <0.02 μg Pb/g; maximum = 5.3 μg Pb/g) and 0.02 μg Cd/g in tuna (minimum = <0.01 μg Cd/g; maximum = 0.03 μg Cd/g) and tomatoes (minimum = <0.01 μg Cd/g; maximum = 0.43 μg Cd/g) to a low of 0.05 μg Pb/g in orange juice (minimum = <0.02 μg Pb/g; maximum = 0.63 μg Pb/g) and 0.001 μg Cd/g in fruit punch (minimum = <0.01 μg Cd/g; maximum = 0.06 μg Cd/g). The mean level of lead in foods packaged in lead-soldered cans was 5 times as high as the level in foods packaged in nonlead-soldered cans. The mean level of lead over all the products was highest during the first year of the study (0.20 μg Pb/g) and lowest during the last year of the study (0.11 μg Pb/g). During this period can manufacturers were reducing the use of lead-soldered cans, which may explain the reduction in mean lead levels. Cadmium levels did not show a similar reduction, but they were usually below the data reporting limit (0.01 μg/g). Comparisons to the level of lead in raw tomatoes indicate that lead may be indirectly added to processed foods even if they are packaged in nonlead-soldered cans.

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Sulfamethazine (Sulfadimidine) Residues in Canadian Consumer Milk

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A survey on the presence of sulfamethazine (sulfadimidine) residues in consumer milk has been conducted in 10 cities across Canada. In each city, homogenized milk was purchased at 3 different retail outlets, each supplied by different processing plants. A total of 30 samples was analyzed by a liquid chromatographic method. The limit of quantitation was 5 ppb. In addition to automatic integration, visual inspection of the chromatograms was required to distinguish between low concentrations of sulfamethazine and 2 unknown interfering peaks. Two samples, from different cities, contained 11.4 and 5.24 ppb of the drug. Drug identity was confirmed by mass spectrometry. All other samples appeared to be free of the drug.

Sulfamethazine (sulfadimidine) is commonly used as a growth promoter and as an antimicrobial agent effective against a number of infections in many agriculturally important animals. Although Canadian regulations permit its use in cattle, poultry, and swine, sulfamethazine has never been approved for intramammary use. The safety of this drug to the consumer has recently been questioned because of its apparent toxicity (1). A recent survey that used a radioreceptor assay (Charm Test II) with a claimed sensitivity of 5 ppb (2) revealed the presence of sulfonamide residues, assumed to be sulfamethazine, in 82 of 174 milk samples from the United States and in 12 of 40 milk samples from Canada. Because the method is specific for classes of antimicrobial agents, it may be unable to distinguish among the different sulfa drugs or give exact quantitative data on the residue concentration.

Weber and Smedley (3) recently developed a sensitive assay by liquid chromatography (LC), which is specific for sulfamethazine in milk. Using their method, a survey of consumer milk across the United States confirmed that 11 of 49 samples contained sulfamethazine residues; the highest level was 40 ppb (c.f., "Answers," April 22, 1988). The present survey was undertaken to find whether possible violations in the use of sulfamethazine occur in Canada.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Kratos Spectroflow 400 solvent delivery system, Spectra-Physics 8780XR autosampler, and Kratos Spectroflow 783 detector set at 265 nm. Column: Supelcosil-C18-DB, 5 μm, 4.6 × 250 mm. Mobile phase: methanol–0.1M potassium dihydrogen phosphate (3 + 7).

(b) *Diazomethane generator.*—Aldrich, as described by Fales et al. (4).

(c) *Gas chromatograph (GC).*—Hewlett-Packard 5840A, using helium carrier gas at 30 cm/s linear velocity and temperature program of 200°C for 1 min followed by linear increase at 5°/min to 250°C. Capillary column: J&W Scientific, Inc., 15 m × 0.25 mm, coated with 0.25 μm DB-1.

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(d) *Mass spectrometer.*—Hewlett-Packard 5985 in electron impact mode, ion source 200°C, scanning from m/z 50 to 400.

Reagents

Authentic drug-free control milk was obtained from Agriculture Canada, Ottawa. Standard sulfamethazine was a U.S. Pharmocopoeia reference standard. All other chemicals were reagent grade or better, purchased from local suppliers.

Test Samples

A total of 30 samples of homogenized milk in 1 L cartons was collected at 3 different retail outlets, each supplied by different processing plants, in 10 cities across Canada: Vancouver, Edmonton, Saskatoon, Winnipeg, Toronto, Ottawa, Montreal, Quebec City, Halifax, and Moncton. Samples were shipped in cooled containers by courier and frozen and stored at −30°C until use. Just before analysis, cartons were thawed by immersion in water at 30–35°C, an aliquot was taken for analysis, and the remainder was distributed in 50 or 100 mL portions and frozen for extended storage at −80°C.

LC Analysis

The method of Weber and Smedley (3) was applied without modification. Whole milk is extracted with chloroform, the solvent is evaporated, and the residue is partitioned between hexane and aqueous 0.1M potassium dihydrogen phosphate. An aliquot of the water phase is chromatographed, using UV detection at 265 nm.

MS Confirmation

For positive samples, three 15 mL portions of milk were extracted and chromatographed in several portions, using 30% methanol in water as mobile phase. The fractions that eluted at the retention time of the sulfamethazine standard were collected, combined, and evaporated in a rotary evaporator at 40°C. The residue was redissolved in 100 μL methanol and reacted with diazomethane (4). The reaction mixture was again evaporated, redissolved in 10 μL methanol, and subjected to GC-MS analysis.

Results

LC Method

Between-run variations of the slope and the intercept were determined by daily injections of standard solutions containing, 5, 10, and 20 ppb sulfamethazine in water, which were injected at the beginning, middle, and end of each run. The results are summarized in Table 1. During 9 runs on 8 different days, the correlation (R^2) between concentration and peak was 0.991 or higher; the slope of the regression of peak height on concentration ranged from 3.18 to 3.89 (mean 3.43, SD 0.22), and none of the intercepts were significantly different from 0.

The control milk (BDR-131) gave slight but consistently positive instrumental readings (mean 0.559 ppb, SD 0.159). Visual inspection of the chromatograms indicated that this

Table 1. Between-run statistics on standard solutions used in LC determinations of sulfamethazine

Date	N	Y-intercept	Std error	Slope	Std error	R^2
880920	12	0.9656	0.4012	3.4878	0.0152	0.9997
880921	12	0.7377	0.4853	3.5357	0.0201	0.9996
880921	12	−2.1430	0.5036	3.8948	0.0190	0.9996
880922	12	0.8289	0.4833	3.2652	0.0183	0.9995
880923	12	−0.6250	2.0545	3.1781	0.0952	0.9910
880926	12	0.3411	0.3574	3.3075	0.0117	0.9997
880927	12	−0.0060	0.3155	3.5421	0.0120	0.9998
880928	12	1.2569	0.6307	3.2902	0.0413	0.9994
880929	12	−0.2710	0.1250	3.3626	0.0082	0.9999
Mean		0.1255		3.4293		
SD		1.0480		0.2159		
CV		—		6.2947		

interference was due to 2 small endogenous peaks (Rt 1.50 and 1.68 min), which were poorly resolved from sulfamethazine (Rt 1.55 min, Figure 1). All test samples exhibiting chromatograms visually judged free of the drug gave instrumental readings equivalent to 0.18–1.77 ppb drug. For this reason, the limit of detection was set at 2 ppb or higher, and all chromatograms were carefully inspected. The limit of determination was set at 5 ppb, which was the lowest standard concentration used. The recovery of sulfamethazine in control milk fortified with 5 ppb drug was 87.2% (SD 4.6).

Residues Found in Milk

Only 2 of the 30 milk samples tested contained sulfamethazine above the limit of determination: sample BDR-106 from Toronto and BDR-123 from Montreal had 11.4 and 5.24 ppb, respectively; all other samples appeared to be free of the drug.

Confirmation by MS

GC-MS analysis confirmed the identity of sulfamethazine in the 2 samples containing over 5 ppb by the presence of characteristic ions. The molecular ion of methylated sulfamethazine at 292 could not be seen; however, ions 108, 227, and 228 were found to be characteristic for the analyte both from standard solutions and incurred samples (Table 2). The nearly identical relative ratios of the 3 diagnostic ions in the standard solution and in the 2 positive samples was further evidence that they all originated from sulfamethazine. Selected ion monitoring of these 3 ions gave a good response to the methylated drug with a retention time of 8.9 min. None of the 4 ions could be detected in extracts from an authentic drug-free milk sample.

Discussion

The method used in the present paper has been shown to be quantitative to 5 ppb (3). Two of the 30 samples tested contained over 5 ppb sulfamethazine, and this was confirmed by mass spectrometry. An additional 7 samples appeared on the integrator output to contain between 0.9 and 1.7 ppb of the drug. Visual inspection of the chromatograms, however, indicated that this was due to interference by 2 endogenous substances.

Electronic integrators are widely used for automated data reduction of detector output. While many integrators are capable of storing the time sampled response and plotting a true chromatogram for each run, often the scale adjustments

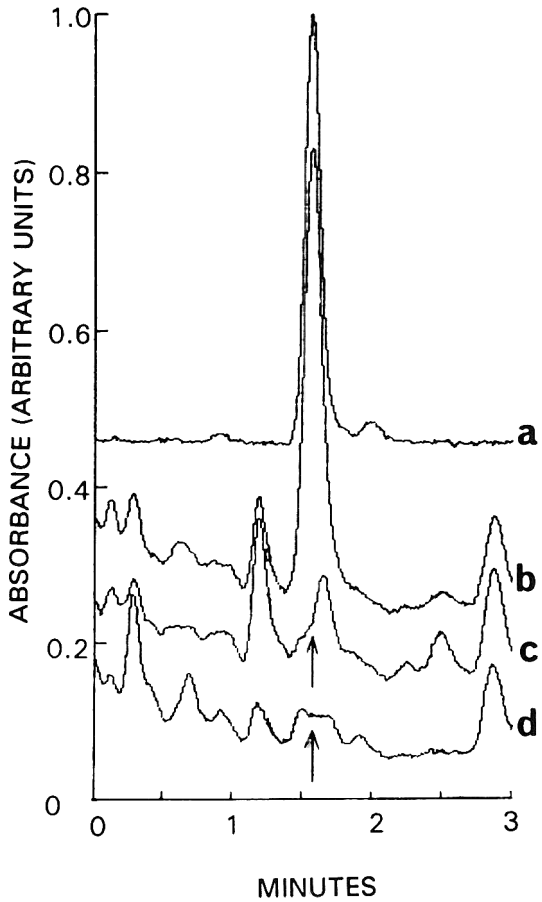

Figure 1. Chromatograms of (a) standard sulfamethazine (5 ppb); (b) positive sample (BDR-123, Rt 1.55 min, 5.24 ppb); (c) test sample judged to be negative (BDR-125, instrumental reading, Rt 1.60 min, 1 ppb); and (d) control milk (BDR-131). Arrows indicate expected elution of sulfamethazine.

of these plots preclude good resolution of the curve. In addition, very small numeric variations in retention time may be ascribed to slight variations in conditions, e.g., temperature or flow rate, and the printed values accepted without question. Our present experience emphasizes the need for plotting every chromatogram with adequate resolution and visually inspecting them.

In a similar survey, Brady and Katz (5) found that about 40% of a total of 64 consumer milk samples from the New York region contained sulfonamides above the 12.5 ppb limit of detection. No attempt was made by these authors to identify the sulfonamides, or quantitate their concentration.

The 2 samples that tested positive for sulfamethazine contained 5.24 and 11.4 ppb of the drug. These amounts found may seem harmless to the consumer. At present, the limit of

Table 2. GC-MS confirmation of sulfamethazine positive milk samples

Sample	Retention, min	Area counts at m/z			Ratio	
		227	228	108	228/227	108/227
Standard	8.9	6038	4002	1522	0.66	0.25
BDR-106	8.9	9978	6914	2527	0.69	0.25
BDR-123	8.9	2164	1409	571	0.65	0.26
BDR-131[a]	—	—	—	—	—	—

[a] Authentic drug-free control milk.

sulfamethazine residues in pork in Canada is set at 100 ppb. However, milk consumption far outstrips the daily intake of pork, especially in infants. For example, an infant who weighs 10 kg and consumes milk, containing 100 ppb sulfamethazine, at a rate of 1 L/day would be subjected to a daily intake of 0.01 mg/kg body weight.

An FAO/WHO expert committee recently proposed a recommended maximum residue level (MRL) of 25 ppb sulfamethazine in milk, on the basis of an acceptable daily intake (ADI) of 0–0.004 mg/kg (6). In Canada, the drug is not approved for intramammary use and no acceptable limit is in force. Oral preparations for cows require a 96 h withdrawal period in order to ensure that when milk from treated cows enters the consumer market it is free of harmful residues. Thus, it is important, for the purpose of meaningful inspection, to establish a level that could be considered safe to the consumer.

The milk samples in the present study came from industrial sources; each sample represented a pool of milk coming from a large number of individual farms. The 2 mildly positive samples found in this study may have been due to a few highly contaminated farm gate lots, or they may represent a more widespread, but low level contamination. In either case, contamination could have been caused by short-term administration to a few cows under active treatment for a specific condition; it could also have been due to unintentional contamination by residual drug in the equipment used for preparing the feed, or the routine use of sulfamethazine for lactating cows by a few delinquent dairy farms. The presence of up to 100 ppb sulfamethazine has been reported in the liver of untreated pigs housed in pens previously occupied by medicated animals (7). Dairy husbandry practices make similar contaminations among dairy cows extremely unlikely (M. S. Brady, Rutgers University, New Brunswick, NJ, personal communication, 1989).

A survey of milk collected at farm gate lots, now being organized, should shed more light on the presence of sulfamethazine in some of the consumer milk.

Acknowledgment

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DRUG RESIDUES IN ANIMAL TISSUES

Determination of Thiabendazole, 5-Hydroxythiabendazole, Fenbendazole, and Oxfendazole in Milk

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The liquid chromatographic determination previously developed for benzimidazoles in cattle liver has been slightly modified and applied to the determination of 4 benzimidazoles in milk. Recoveries of fenbendazole (FBZ), oxfendazole (OFZ), and thiabendazole (TBZ) from milk fortified at the 10 ppb level were 80% or greater with an intralaboratory coefficient of variation of 11% or less. Recovery of 5-hydroxythiabendazole (5-OH-TBZ) at the 30 ppb level averaged 56% with an intralaboratory coefficient of variation of 5%. Limited data on the depletion of FBZ, OFZ, TBZ, and 5-OH-TBZ in milk were also generated.

Benzimidazoles are used as anthelmintics. Fenbendazole (FBZ) and thiabendazole (TBZ) are approved for use in food-producing animals, but oxfendazole (OFZ) and mebendazole (MBZ) are not yet approved in the United States for this use. LeVan and Barnes conducted a collaborative study of LeVan's liquid chromatography procedure for determining FBZ, OFZ, TBZ, 5-hydroxythiabendazole(5-OH-TBZ), and MBZ in beef liver and muscle (C. J. Barnes (1989) *J. Assoc. Off. Anal. Chem.* **73**, 91-92). The target sensitivity for this procedure in cattle tissue was 100 ppb for OFZ, MBZ, TBZ, and 5-OH-TBZ, and 800 ppb for FBZ. This report describes the application of the procedure, with a few modifications, to the determination of FBZ, OFZ, TBZ, and 5-OH-TBZ in milk.

The procedure exploits the weakly basic nature of the target benzimidazoles for effective extraction and cleanup. The milk sample is mixed with sodium carbonate and homogenized in ethyl acetate. The ethyl acetate extract is evaporated to dryness and the residue is partitioned between phosphoric acid and hexane. The acid fraction containing the benzimidazoles is neutralized and then extracted with ethyl acetate. The ethyl acetate is evaporated to dryness, and the residue is dissolved in methylene chloride and applied to a silica Bond-Elut column. The benzimidazoles are eluted with methanol–methylene chloride (1 + 3, v/v). The eluate is then evaporated to dryness and redissolved in the mobile phase, and the benzimidazoles are determined by liquid chromatography.

LeVan's determinative procedures were used except for the following modifications: For FBZ, OFZ, and TBZ, 100 g sodium sulfate was used in the homogenization and extraction steps instead of 80 g, and a tissumizer was used instead of a blender. For 5-OH-TBZ, the amount of butylated hydroxytoluene solution added to the ethyl acetate extracts, prior to the rotary evaporation step, was doubled to increase the recoveries of the 5-OH-TBZ.

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METHOD

Reagents

(a) *Butylated hydroxytoluene (BHT).*—10 mg/mL solution. Dissolve 1 g crystalline BHT (Sigma Chemical Co., St. Louis, MO 63178) in 100 mL LC grade ethyl acetate.

(b) *Phosphoric acid solution.*—1M. Dilute 68.3 mL reagent grade H_3PO_4 (Mallinckrodt, St. Louis, MO) to 1 L with deionized water.

(c) *Sodium carbonate solution.*—1M. Dissolve 106 g anhydrous Na_2CO_3 (ACS certified, Fisher Scientific, Columbia, MD 21046) in 1 L deionized water.

(d) *Potassium hydroxide solution.*—10N. Dissolve 560 g KOH pellets (ACS certified, Fisher Scientific) in deionized water and dilute to 1 L.

(e) *Ammonium phosphate buffer, pH 7.0.*—0.01M. Dissolve 1.15 g $NH_4H_2PO_4$ (LC grade, Fisher Scientific) in ca 950 mL water, adjust pH to 7.0 with dilute NH_4OH (ACS grade, Fisher Scientific), and dilute to 1 L.

(f) *Sodium sulfate.*—Anhydrous, granular, 12–60 mesh (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(g) *n-Hexane.*—Nanograde, glass-distilled (Mallinckrodt).

(h) *Ethyl acetate.*—LC grade (Burdick and Jackson, McGaw Park, IL 60085).

(i) *Methanol.*—LC grade (Fisher Scientific).

(j) *Methylene chloride.*—Nanograde (Mallinckrodt).

(k) *Mobile phase.*—Prepare methanol–ammonium phosphate buffer solutions in 3 different proportions, (70 + 30), (53 + 47), and (40 + 60), using appropriate volumes of methanol and pH 7.0 ammonium phosphate buffer. Filter through 0.2 μm filter before use.

(l) *Fenbendazole (FBZ; Panacur) analytical standard.*—Lot RC 2550, Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, NJ 08876.

(m) *Oxfendazole (OFZ) analytical standard.*—Lot B3-JK-058, Syntex Animal Health, Inc., West Des Moines, IA 50265.

(n) *Thiabendazole (TBZ) analytical standard.*—Lot L-585, 216-000S141, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.

(o) *5-Hydroxythiabendazole (5-OH-TBZ) analytical standard.*—Lot L-588, 072-000A012, Merck Sharp & Dohme Research Laboratories.

Standard Solutions

(a) *Stock solutions (1 mg/mL).*—(*1*) FBZ and OFZ: Dissolve 10 mg in 10 mL dimethyl sulfoxide. (*2*) TBZ and 5-OH-TBZ: Dissolve 10 mg in 10 mL methanol.

(b) *Intermediate standard solutions of FBZ, OFZ, and TBZ (1 μg/mL).*—Combine 100 μL of each stock solution in a 100 mL volumetric flask and dilute to volume with methanol.

(c) *Intermediate standard solution of 5-OH-TBZ (2 μg/mL).*—Dilute 200 μL 5-OH-TBZ stock solution to 100 mL with methanol.

(d) *Working standards.*—(*1*) FBZ, OFZ, and TBZ: Prepare mixed standards of 0.025, 0.050, 0.10, and 0.20 μg/mL by placing 25, 50, 100, and 200 μL portions of 1 μg/mL mixed intermediate standard solution into separate 15 mL centrifuge tubes, evaporating to dryness under nitrogen stream at 30–35°C, and adding 1 mL mobile phase (53 + 47). (*2*) 5-OH-TBZ: Prepare standards of 0.15, 0.30, and 0.45 μg/mL by placing 75, 150, and 225 μL of the 5-OH-TBZ 2 μg/mL intermediate standard solution into separate 15 mL centrifuge tubes, evaporating to dryness under nitrogen stream at 30–35°C, and adding 1 mL mobile phase (53 + 47).

Apparatus

(a) *Liquid chromatograph.*—Variable wavelength UV absorbance detector Model 116, with Model 302 pump (Gilson, Middleton, WI 53562); 50 μL Rheodyne loop injector or WISP 710 B autosampler (Waters, Milford, MA 01757); chart recorder (Perkin-Elmer, Norwalk, CT 06859); integrator, Model 3392 A (Hewlett-Packard, Palo Alto, CA 94304).

(b) *Columns.*—(*1*) TBZ and 5-OH-TBZ: 10 μm Lichrosorb RP-18 column, 25 cm × 4.6 mm id (Alltech/Applied Science, Deerfield, IL 60015). (*2*) FBZ and OFZ: 5 μm Hypersil ODS column, 25 cm × 4.6 mm id, or 10 μm Lichrosorb RP-18 column (Alltech/Applied Science). Conditions: flow rate, 1.0 mL/min; wavelengths, 298 nm (FBZ, OFZ, and TBZ) and 318 nm (5-OH-TBZ); attenuation, 0.0025–0.005 AUFS; column temperature, ambient; guard column, 3 cm × 4.6 mm id, packed with pellicular C-18 (Alltech/Applied Science).

(c) *Silica columns.*—3 mL Bond-Elut (No. 601303, Analytichem International, Harbor City, CA 90710).

(d) *Nylon membrane filters.*—0.2 μm, Nylaflo (No. 66602, Gelman Sciences, Ann Arbor, MI 48106).

(e) *Filters.*—0.45 μm (Acro LC 3S, No. 4441, Gelman Sciences).

(f) *Blender.*—Tekmar® tissumizer (Tekmar Co., Cincinnati, OH 45222).

Extraction

To 10 g milk in Erlenmeyer flask, add 5 mL 1M Na_2CO_3 and mix. Add 150 mL ethyl acetate and 1 mL BHT solution. Blend 5 min at high speed with tissumizer. Add 100 g anhydrous sodium sulfate and blend 1 min. Let sodium sulfate settle 2–3 min to avoid plugging filter paper in the next step. Transfer ethyl acetate to 500 mL round-bottom flask through funnel filter fitted with No. 41 paper. Add another 150 mL ethyl acetate to sample in Erlenmeyer flask and blend 2 min. Transfer ethyl acetate to same round-bottom flask. Evaporate filtrate to dryness on rotary evaporator. (*Caution:* Watch for bumping).

Add 10 mL hexane to 500 mL sample flask, swirl to dissolve residues, and transfer to 125 mL separatory funnel. Rinse flask with additional 10 mL hexane and transfer rinse to separatory funnel. Rinse flask in same manner with two 10 mL portions of 1M phosphoric acid and transfer rinses to separatory funnel containing hexane. Stopper and shake vigorously 2 min. Let layers separate ca 10 min and drain lower aqueous phase into second 125 mL separatory funnel. Re-extract hexane layer twice more with 10 mL portions of 1M H_3PO_4, each time draining aqueous phase into second separatory funnel. Wash pooled acid layers by shaking with 5 mL hexane for 30 s.

Drain lower aqueous phase into 100 mL beaker. Adjust pH to 8 or 9 by slowly adding, with stirring, ca 9 mL 10N KOH. Keep beaker in ice or cold water bath during neutralization. Check pH with meter or indicator paper. Transfer contents of beaker into 250 mL separatory funnel and rinse beaker with several portions of ethyl acetate (total of 50 mL), adding rinses to separatory funnel. Shake vigorously 2 min. Let the phases separate; then drain lower (aqueous) layer into the 100 mL beaker. Drain ethyl acetate into 250 mL round-bottom flask through funnel plugged with glass wool and containing ca 40 g sodium sulfate. Pour beaker contents back into separatory funnel and re-extract with another 50 mL ethyl acetate. Discard lower aqueous layer and drain ethyl acetate through funnel containing sodium sulfate into same 250 mL round-bottom flask. Rinse sodium sulfate with 25 mL ethyl acetate, adding rinse to round-bottom flask. Add 0.2 mL BHT solution (2 mg) to round-bottom flask and evaporate to dryness on rotary evaporator. (*Caution:* Watch for bumping.)

Cleanup

Add 3 mL methylene chloride to round-bottom sample flask and swirl to dissolve residue. Condition silica column with 2 mL methylene chloride. Perform following operations by gravity flow: Apply sample to conditioned Bond-Elut, and rinse sample flask two times with 3 mL portions of methylene chloride, applying each rinse to Bond-Elut. Wash Bond-Elut with 5 mL methylene chloride, and discard the wash. Elute the benzimidazoles with 5 mL 25% methanol in methylene chloride, collecting eluate in conical tube of appropriate size. Evaporate eluate to dryness under nitrogen and reconstitute residue in 1.0 mL mobile phase. Mix on vortex-type mixer to dissolve residue; then filter through 0.2 μm filter into liquid chromatography injection vial, and proceed with liquid chromatographic determination.

Construct standard curves by plotting peak heights vs concentrations: 0.025, 0.050, 0.10, and 0.20 μg/mL for TBZ, OFZ, and FBZ; and 0.15, 0.30, and 0.45 μg/mL for 5-OH-TBZ. Curves should be linear with correlation coefficients greater than 0.997–0.999 range. Calculate recoveries for fortified milk, except those that were analyzed along with incurred milk, by peak height comparison. Calculate results for incurred milk by using equation of standard curve, summarized as follows:

$$\text{Recovery from fortified milk, \%} = \frac{(\text{peak ht of sample})(\text{equiv. ppb std})(100)}{(\text{peak ht of std})(\text{fortification level})}$$

$$\text{Benzimidazole in milk containing incurred residues, ppb} = (\mu\text{g drug/mL from the standard equation})(1000)/10$$

Because of the broad range of retention times of these 4

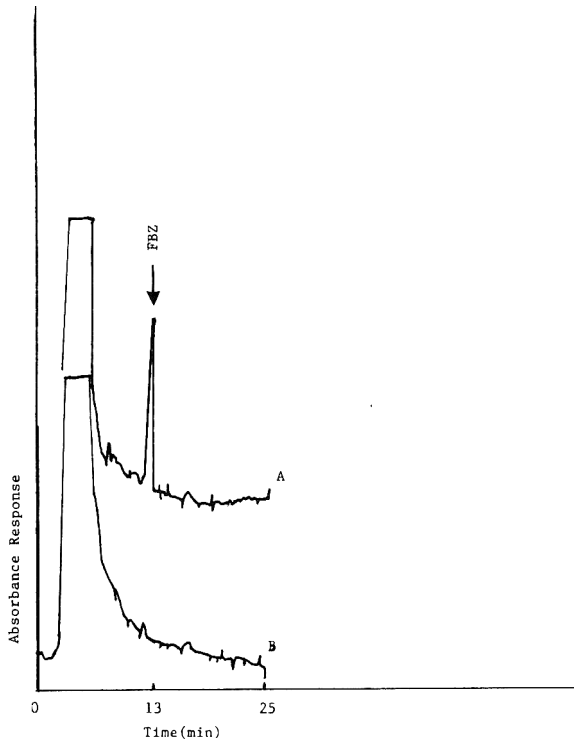

Figure 1. Liquid chromatography of FBZ (A) from extracts of milk fortified with 20 ppb FBZ, OFZ, TBZ, and 5-OH-TBZ and (B) from control milk (Hypersil ODS 5 μm column).

compounds in the mobile phase specified in the original method, 3 separate liquid chromatography analyses were performed with different proportions of methanol and aqueous buffer. A mobile phase of methanol–0.01M ammonium phosphate buffer (70 + 30, v/v) was used for FBZ analyses (Figures 1 and 2); the retention time was approximately 13 min. For OFZ and TBZ, the (53 + 47) mobile phase was used, which resulted in retention times of 10 and 17 min, respectively (Figures 3 and 4). Figure 5 shows the chromato-

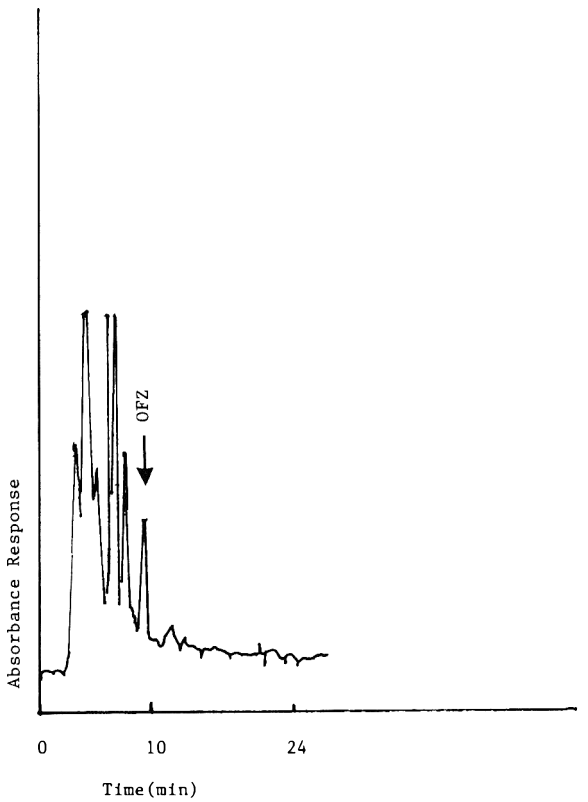

Figure 3. Liquid chromatography of OFZ from extracts of milk of an animal dosed with FBZ (Hypersil ODS 5 μm column).

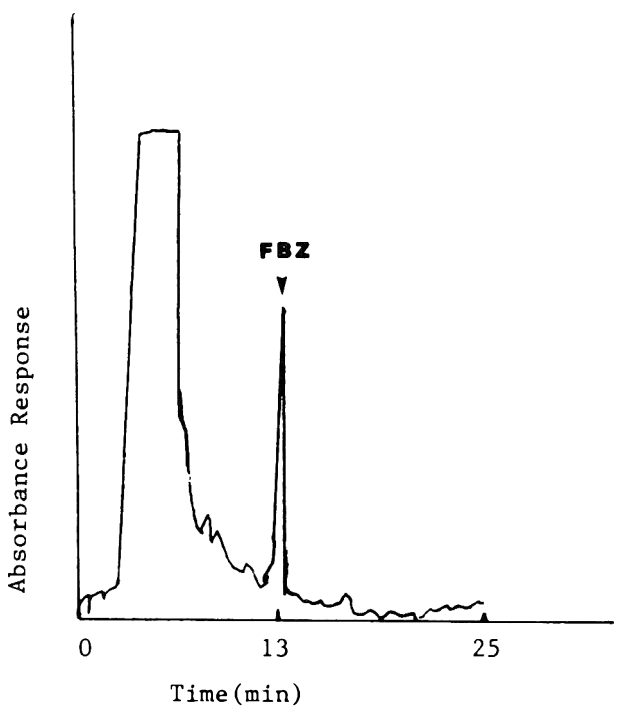

Figure 2. Liquid chromatography of FBZ from extracts of milk from an animal dosed with FBZ (Hypersil ODS 5 μm column).

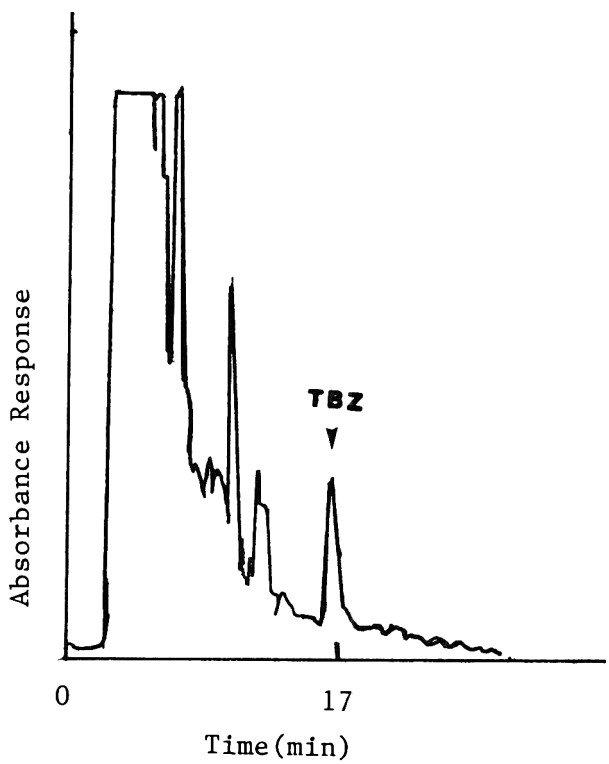

Figure 4. Liquid chromatography of TBZ from extracts of milk from an animal dosed with TBZ (Hypersil ODS 5 μm column).

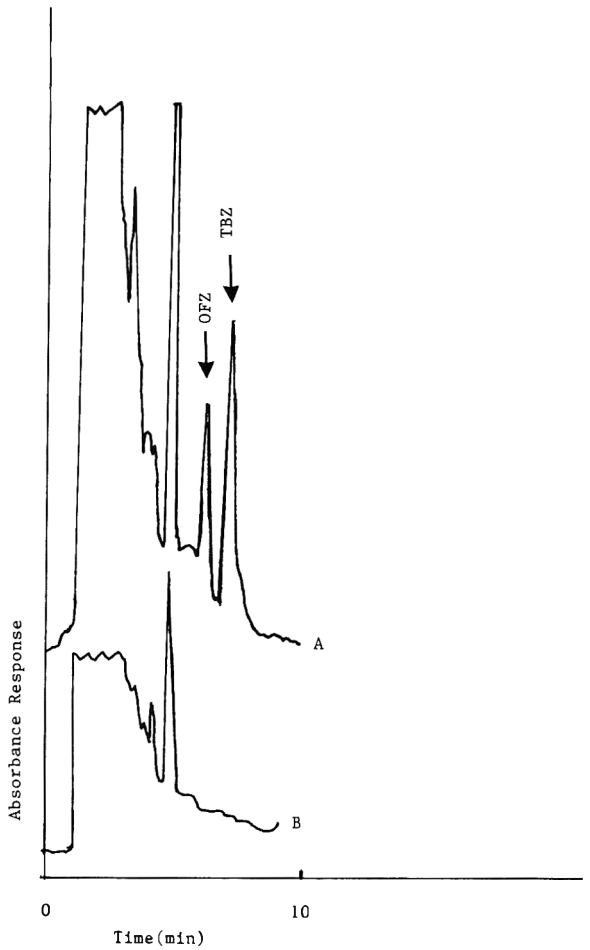

Figure 5. Liquid chromatography of OFZ and TBZ (A) from extracts of milk fortified with 20 ppb FBZ, OFZ, TBZ, and 5-OH-TBZ and (B) from control milk (Lichrosorb RP-18 10 μm column).

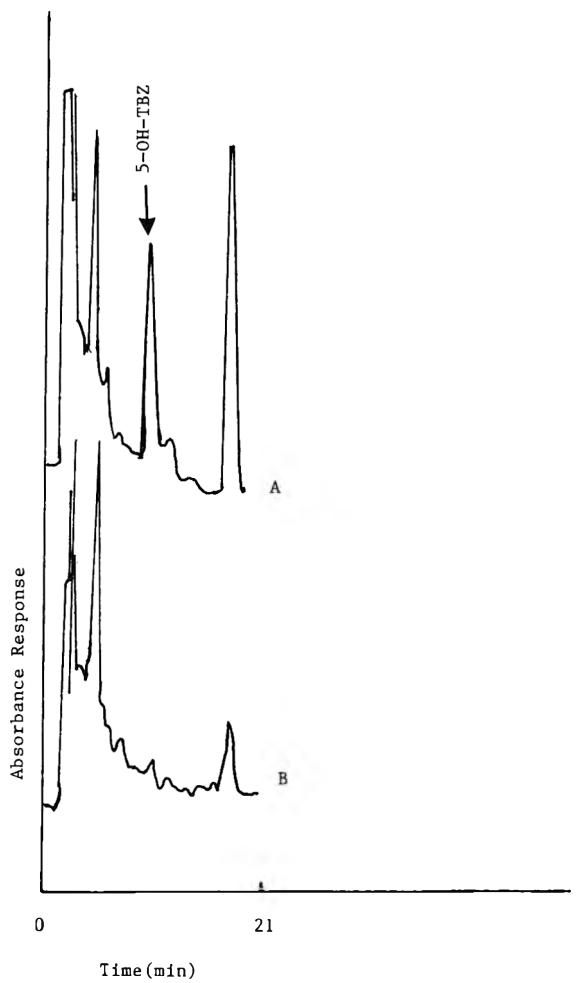

Figure 6. Liquid chromatography of (A) extracts of milk fortified with 30 ppb 5-OH-TBZ, and (B) from control milk (Lichrosorb RP-18 10 μm column).

graphic patterns for OFZ and TBZ with a shorter retention time. Since 5-OH-TBZ was insufficiently resolved from background when the (53 + 47) mobile phase was used, a weaker mobile phase (40 + 60) was used (Figure 6) and resulted in a retention time of approximately 15 min. Probably all 4 compounds could be quantitated in a single analysis by using a gradient system, but this was not studied.

Milk Samples

(a) For FBZ, OFZ, TBZ, and 5-OH-TBZ, 10.0 g commercially available homogenized milk was fortified with 5, 10, or 20 ppb of each chemical, with 5 replicates at each level.

(b) Because of the initial poor results, some of the recoveries for 5-OH-TBZ were repeated under different conditions. Five 10.0 g samples of commercially available homogenized milk were fortified at 30 ppb with 5-OH-TBZ.

(c) Raw milk dosed with FBZ or TBZ was provided by Bioon Inc., Rockville, MD 20850. The FBZ-dosed milk was obtained from a cow that received FBZ paste as a single oral dose of 10 mg active ingredient/kg of body weight. This milk was used to generate data for recoveries of incurred residues of FBZ and OFZ from milk (Table 1). The TBZ-dosed milk was obtained from a cow that received TBZ paste as a single dose of 110 mg active ingredient/kg of body weight. This milk was used to generate the data for recoveries of incurred residues of TBZ and 5-OH-TBZ from milk (Table 2). The first dosed-milk sample was collected 4 h after dosing; the rest of the samples were collected at 12 h intervals for 7 days. These milk samples were frozen promptly and kept frozen until analyzed. The control milk was obtained from an untreated cow receiving the same feed as the treated cows.

(d) For controls (0 ppb), 10.0 g homogenized milk without fortification was extracted and analyzed.

Table 1. Levels of FBZ and OFZ in milk from a cow dosed with FBZ

	Residue found, ppb[a]	
Hours after dosing	FBZ	OFZ
4	ND	ND
16	ND	ND
28	105	414
40	47.1	372
52	16.9	240
64	3.71	103
76	ND	28.6
88	ND	6.3
100	ND	3.7
112	ND	0.1
124	ND	ND
136	ND	ND
148	ND	ND

[a] Not detectable (<0.5 ppb).

Table 2. Levels of thiabendazole and 5-OH-thiabendazole in milk from a cow dosed with thiabendazole

Hours after dosing	Residue found, ppb[a] TBZ	5-OH-TBZ
4	ND	ND
16	6.83	44.6
28	1.1	8.2
40	ND	ND
52	ND	ND
64	ND	ND
76	ND	ND
88	ND	ND
100	ND	ND

[a] Not detectable (<0.5 ppb).

Results

Data in Table 3 show recoveries from fortified milk when LeVan's procedures were used with the modifications described. Good recoveries were obtained for all compounds except 5-OH-TBZ for which the recovery ranged from 16.7 to 69.9%. The average recoveries for FBZ, OFZ, and TBZ were 80.1, 93.8, and 86.0% at 5 ppb, 88.2, 88.9, and 83.3% at 10 ppb, and 78.9, 86.1, and 81.4% at 20 ppb, respectively. The results were very consistent, with 3.1–14.4%, 4.1–11.2%, and 2.0–6.5% intralaboratory coefficients of variation for FBZ, OFZ, and TBZ, respectively. Most control values for FBZ, OFZ, and TBZ did not exceed the 0–2 ppb range (see Figures 1 and 5).

We speculated that 5-OH-TBZ was not as stable as the other 3 compounds and that the long time required to extract all 20 samples might be responsible for the low recovery of 5-OH-TBZ. Thus, shortening the sample preparation time or increasing the amount of antioxidant (BHT) added to the ethyl acetate extracts might increase the recovery of 5-OH-TBZ. When sample analyses were conducted with twice the amount of BHT prescribed in the method, the recovery of 5-OH-TBZ increased approximately 10%. With this modification, 5-OH-TBZ was determined in a second series of control samples that had been fortified only with 5-OH-TBZ: 5 milk samples fortified with 30 ppb 5-OH-TBZ, and 2 controls. Samples were prepared and analyzed as quickly as possible, with the results given in Table 4. The recovery of 5-OH-TBZ increased to an average of 55.7%; the results are very consistent, with a coefficient of variation of 5.3%. An interference

Table 3. Recovery of 4 benzimidazoles from fortified milk, using modifications described for FBZ, OFZ, and TBZ

Fortification level, ppb	Recovery, % FBZ	OFZ	TBZ	5-OH-TBZ
5	95.0	88.8	77.8	28.0
	74.6	90.8	90.4	30.2
	69.4	95.4	87.4	57.2
	89.8	97.4	91.2	32.6
	71.9	96.8	83.2	29.2
$\bar{x}$, %	80.1	93.8	86.0	35.4
SD	11.5	3.82	5.56	12.3
CV, %	14.4	4.1	6.5	34.7
10	97.1	99.7	85.5	38.6
	80.0	96.5	76.1	69.9
	99.0	91.1	85.5	33.6
	84.5	76.6	86.9	31.3
	80.6	80.7	82.5	33.3
$\bar{x}$, %	88.2	88.9	83.3	41.3
SD	9.15	9.97	4.33	16.2
CV, %	10.4	11.2	5.2	39.2
20	79.0	87.0	81.0	32.1
	77.5	88.0	79.0	19.0
	76.5	75.5	81.0	39.9
	78.5	90.0	83.0	16.7
	83.0	90.0	83.0	18.8
$\bar{x}$, %	78.9	86.1	81.4	25.3
SD	2.48	6.07	1.67	10.2
CV, %	3.1	7.0	2.0	40.3

Table 4. Recovery of 5-OH-thiabendazole from milk fortified at 30 ppb, using modifications described for 5-OH-TBZ

Replicate	Found, ppb	Recovery, %
1	15.8	52.7
2	18.1	60.3
3	16.6	55.3
4	16.9	56.3
5	16.1	53.7
$\bar{x}$, %	16.7	55.7
SD	0.89	2.94
CV, %	5.3	5.3

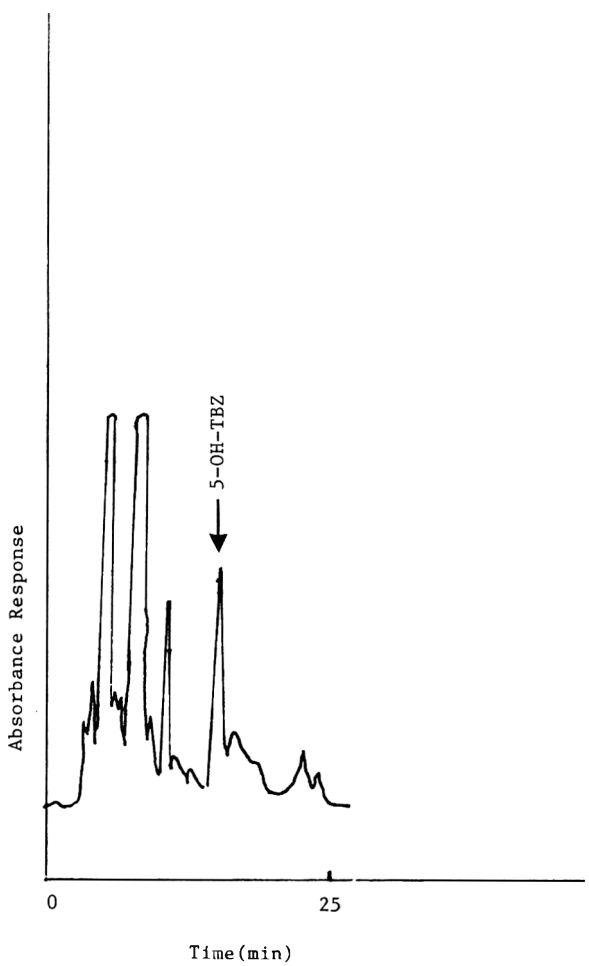

Figure 7. Liquid chromatography of 5-OH-TBZ from extracts of milk from an animal dosed with TBZ (Lichrosorb RP-18 10μm column).

of approximately 3 ppb occurred at the retention time of 5-OH-TBZ (Figure 6).

The residue levels in dosed milk were corrected for predosing blanks but not for the percent recoveries obtained from the fortified samples. Sample chromatograms of the dosed milk containing FBZ and OFZ are given in Figures 2 and 3. Data for OFZ and FBZ are presented in Table 1. No FBZ or OFZ was detected in the milk at 4 or 16 h after dosing. The highest concentrations of FBZ and OFZ (105 and 414 ppb, respectively) appeared in the milk 28 h after dosing. Residues of FBZ and OFZ had declined below detectable levels (<0.5 ppb) by 76 and 124 h after dosing, respectively; approximately 80% of the compound found in the milk was present as OFZ. A small amount of OFZ (up to 15%) was detected in the FBZ-fortified samples which were analyzed concurrently with the dosed milk. This suggested that some FBZ residues may convert to OFZ during the preparation of the dosed extracts.

TBZ and 5-OH-TBZ levels in dosed milk are given in Table 2, and sample chromatograms of the dosed milk are shown in Figures 4 and 7. The highest concentrations of TBZ and 5-OH-TBZ (6.8 and 44.6 ppb, respectively) appeared in the milk 16 h after dosing. The residues were not detectable (<0.5 ppb) in the milk 40 h after the cow was given TBZ.

Data developed in this study indicate that LeVan's method as conducted in our laboratory can be used for determining FBZ, OFZ, and TBZ at the 10 ppb level and above and 5-OH-TBZ at the 30 ppb level and above in cow's milk. Recoveries for FBZ, OFZ, and TBZ are about 85%. Recoveries of 5-OH-TBZ at 56% are a little less than the FDA criterion of 60% for drug residues at 100 ppb and below. Additional work is needed to improve the chromatography and to perform exhaustive extraction or radiolabel studies to definitively establish that these benzimidazoles are being adequately isolated from dosed milk. Hazleton Laboratories has already completed exhaustive studies for these drugs in cattle tissues.

Acknowledgments

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Capillary Gas Chromatographic Method for Determination of Benzylpenicillin and Other Beta-Lactam Antibiotics in Milk

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A capillary gas chromatographic method is described for determining residues of beta-lactam antibiotic residues in milk, with specificity for benzylpenicillin (penicillin G), phenoxymethylpenicillin, methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin. Residues are extracted from milk with acetonitrile. Samples are cleaned up by partitioning between aqueous and organic phases at different pH values. The penicillin residues are methylated with diazomethane to render them amenable to determination by gas chromatography on a methyl silicone fused silica column. Samples are introduced by split/splitless injection using a programmed temperature vaporization injector and are detected by nitrogen-selective thermionic detection. Internal standardization is used for quantitation. The limits of detection for all penicillins are well below 1 μg/kg. Recoveries of spiked samples at 3 and 10 μg/kg are in the range of 42–85% (coefficients of variation 2–5%) and 41–92% (coefficients of variation 3–7%), respectively.

Beta-lactam antibiotics and especially benzylpenicillin (penicillin G) are very widely used in veterinary medicine, thus causing concern about residues occurring in food derived from treated animals. Despite their low toxicity, beta-lactam antibiotics are the only group of drugs reliably documented to harm human health; residual concentrations of benzylpenicillin in food lead to allergenic reactions (1).

Microbiological assays are commonly used for the determination of penicillins. Like immunological or receptor tests, they are well suited as screening procedures, but their lack of specificity does not allow unambiguous identification and quantitation of a particular penicillin.

A few papers describe instrumental methods for more specific analysis of penicillin residues in food, using liquid chromatography (2–8) or gas chromatography on packed columns (9). They are reviewed by Moats (10). These methods are not sensitive enough to allow control for the maximum limits established in FRG and EEC regulations: 4 μg/kg of penicillin in milk (EEC) (11), 3 μg/kg of benzylpenicillin, 3 μg/kg of phenoxymethylpenicillin, and 10 μg/kg of the isoxazolylpenicillins (oxa-, cloxa-, dicloxacillin) in meat of slaughtered animals (FRG) (12).

The present paper describes a specific and sensitive method for determination of benzylpenicillin, phenoxymethylpenicillin, methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin by capillary gas chromatography at quantitation levels as low as 3 μg/kg and detection limits in the sub-ppb range. Because of the ease of interfacing gas chromatography and mass spectrometry, the method could serve as a confirmatory method for unambiguous verification of results obtained by other assays.

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METHOD

Reagents

(a) *Solvents.*—Acetonitrile (Merck 800015, distilled over phosphorus pentoxide, 8 g/L); dichloromethane; petroleum ether and cyclohexane [distilled over sodium, 0.5 g/L of sodium in paraffin (45%, Fluka 7910, Neu-Ulm, FRG)]; diethyl ether (distilled over potassium hydroxide, 30 g/L); acetone and deionized water (distilled over potassium permanganate, 0.5 g/L).

Acetonitrile, dichloromethane, and cyclohexane were distilled using a rotating strip column (Normag 8101, 6238 Hofheim, FRG); all other solvents were treated using a standard column (60 cm, 3 cm id) packed with Raschig rings.

(b) *Phosphate buffer.*—pH 2.2 (0.2M and 0.5M). Prepare from 0.2M (0.5M) solutions of potassium dihydrogen phosphate and *o*-phosphoric acid.

(c) *Phosphate buffer.*—pH 7. Prepare from 0.2M solutions of potassium dihydrogen phosphate and disodium hydrogen phosphate.

(d) *o-Phosphoric acid.*—1M.

(e) *Sodium sulfate.*—Dehydrate at 600°C overnight in muffle furnace and dry at least 1 h at 130°C before use.

(f) *Cotton wool.*—Defat by extraction with dichloromethane for 3 days in a Soxhlet extractor. Dry at 50°C in an oven.

(g) *Standards.*—Benzylpenicillin and phenoxymethylpenicillin (both as potassium salts); methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin (all as sodium salts) (Sigma, 8024 Deisenhofen, FRG). Prepare aqueous stock solutions of 1 mg/mL each. Stock solutions are stable at least 1 week at 3°C. Prepare dilutions daily.

(h) *Internal standards.*—2,4,6,-Triphenoxy-1,3,5-triazine ("Triazine") and 2-(4-biphenylyl)-5-(4-*tert*-butylphenyl)-1,3,4-oxadiazole ("Oxadiazole") (Aldrich, 7924 Steinheim, FRG). Prepare stock solution of 1 mg/mL each in acetone–cyclohexane (1 + 1; v/v) and dilute to 1 μg/mL with cyclohexane. Methyl red (Merck, 6100 Darmstadt, FRG). Using 25 mL volumetric flask, dissolve 10 mg in 5 mL 0.1M sodium hydroxide and 15 mL water. Add 2.5 mL 0.2M phosphate buffer, pH 2.2, and dilute to volume with water. Dilute with water to 0.2 μg/mL.

(i) *Reagents for diazomethane generation.*—*N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide (Merck), diethylene glycol monoethyl ether, potassium hydroxide solution, 50% (w/w). Place ca 200 mg *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide into flask of diazomethane generator, dissolve in 5 mL diethyl ether–diethylene glycol monoethyl ether (2 + 1, v/v), and add 1 mL potassium hydroxide solution. Transfer produced diazomethane with gentle stream of nitrogen into reagent tube (20 mL) that contains 10 mL acetone and is immersed in an ice bath. Reaction is completed after ca 30 min. Yellow solution is stable for 2 days at 5°C. [*Caution:* The micro scale device does not eliminate the explosive and health hazards of diazomethane. Proper precautions must be observed (i.e., fume hood, safety screen, avoidance of strong light, sharp edges).]

Apparatus

(a) *Tissue homogenizers.*—Moulinette (Moulinex, 5000 Köln, FRG) and Ultra-Turrax, Type KG 18/10 (Janke & Kunkel, 7813 Staufen, FRG).

(b) *Centrifuges.*—Universal centrifuge for 150 mL centrifuge tubes; stainless steel centrifuge tubes, 150 mL; Piccolo Type 702 tabletop centrifuge (all available from Heraeus Christ, 3360 Osterode, FRG).

(c) *Vortex mixer.*—Model VF (Janke & Kunkel).

(d) *Heating module.*—Reacti-Therm (Pierce Chemical Co., Rockford, IL (61105).

(e) *Centrifuge tubes.*—8 and 13 mL, capped with polytetrafluoroethylene (PTFE, Teflon®) liner.

(f) *Diazomethane generator.*—(Pierce 28131, or equivalent).

(g) *Gas chromatograph.*—Varian Model 3700 (Varian Inc., Sunnyvale, CA 94086) equipped with cooled injection system KAS 2 for cold sample injection (Gerstel, 4330 Mülheim/Ruhr, FRG) and thermionic phosphorus/nitrogen detector (PND). Column, fused silica, 25 m × 0.2 mm id, 0.4 mm od, 0.11 μm film thickness, methyl silicone Ultra-1 (Hewlett-Packard, 6000 Frankfurt, FRG).—Capillary column connector, for 0.8 to 0.4 mm od connections (Gerstel).

Operating conditions.—Carrier gas: nitrogen 1.4 bar inlet pressure; carrier gas venting (front pressure control KAS 2) 20 mL/min; solvent venting 30 mL/min; venting at split mode 6 mL/min. Purge gas: nitrogen 25 mL/min. Detector gases: Hydrogen 1.4 bar inlet pressure; air 180 mL/min. Injection program for KAS 2: split/splitless mode with solvent venting; injection volume 3 μL; injection temperature 48°C; first heat-up phase 2°C/s; first retention phase 50°C/10 s; second heat-up phase 12°C/s; second retention phase 300°C/180 s. Detector 310°C; bead current 3 pA, bias voltage 4 V. Oven program: 1 min at 70°C; then maximum heating to 220°C; increase from 220 to 295°C by temperature program (6°/min). Electrometer 10^{-12} amp/mV; attenuation 4–16.

(h) *Glassware.*—Clean all glassware by successively using detergent, tap water, deionized water, and methanol. Let air-dry. Deactivate glassware used for evaporation (round-bottom flasks, pear-shape flasks, centrifuge tubes, and GC glass vaporization tube): Fill containers with a solution of 5 mL dimethyldichlorosilane in 100 mL toluene, let stand 30 min, and rinse successively with toluene and absolute methanol.

Procedure

Extraction.—Weight 25.0 ± 0.2 g into 100 mL stainless steel centrifuge tube (add standard solutions at this point for spiked samples, mix, and let stand for 10 min at room temperature). Hold tube in ice bath, add 25 mL acetonitrile, and homogenize 1 min. Add 5 mL 0.5M phosphate buffer, pH 2.2, while continuing to homogenize, add an additional 65 mL acetonitrile, and homogenize 1 min more. Centrifuge 10 min at 4000× *g* and decant supernate into 250 mL separatory funnel containing 7 g sodium chloride and 50 mL dichloromethane. Shake separatory funnel 2 min and let stand ca 30 min until phases have separated.

Discard aqueous (lower) layer and decant organic layer into 250 mL Erlenmeyer flask (with ground-glass joint and stopper) containing ca 5 g anhydrous sodium sulfate (do not run off residual drops of aqueous layer). Shake 30 s, filter organic layer through cotton wool plug into 250 mL round-bottom flask, and rotary-evaporate extract at 30°C to ca 10 mL. Transfer residue to 25 mL pear-shaped flask and continue rotary-evaporation. When extract is evaporated to ca 4 mL, add 3 mL dichloromethane, evaporate again, repeat

addition and evaporation of dichloromethane, add 3 mL petroleum ether and, evaporate until ca 0.5 mL residue is left.

Cleanup.—Suspend residue by ultrasonication with 3 portions of 3 mL petroleum ether and transfer solutions to 13 mL centrifuge tube. Rinse pear-shape flask 3 times with 2 mL portions of phosphate buffer, pH 7, and add rinsings to tube. Vortex-mix for 30 s and separate layers by centrifuging. Transfer lower (aqueous) layer into second centrifuge tube and repeat this partitioning twice.

Wash combined aqueous layers twice with 1.5 mL diethyl ether followed by two 1.5 mL portions of dichloromethane. Vortex-mix and centrifuge at each step for separation of layers. Remove organic layers (diethyl ether = upper layer, dichloromethane = lower layer) by Pasteur pipet and discard.

Acidify aqueous solution with 1.5 mL *o*-phosphoric acid (add here 0.5 mL methyl red solution, 0.2 μg/mL) and extract 4 times with 1.5 mL dichloromethane by vortex-mixing and centrifuging. Combine organic layers (lower) in third centrifuge tube (8 mL), centrifuge again to separate residual water, and evaporate to ca 200 μl at 35°C under nitrogen.

Methylation

Add 1 mL diazomethane solution to centrifuge tube and let react 2 h at room temperature. Evaporate to near dryness at 35°C under nitrogen. Before completely dry, remove tube from heating module and evaporate last 2–3 μL under observation, holding centrifuge tube in hand (gloves, fume hood!).

Dissolve residue in 250 μL diluted internal standard solution and store at −20°C if gas chromatographic analysis is not performed immediately.

Preparation of Methylated Standard Solutions

Pipet 0.5 mL of appropriately diluted standard solution and 2 mL 0.2M phosphate buffer, pH 2.2, into 8 mL centrifuge tube; add 1.5 mL dichloromethane and vortex-mix 30 s. After phase separation by centrifuging, transfer organic (lower) layer into second centrifuge tube and wash aqueous phase with three 1.5 mL portions of dichloromethane. (*Note*: Do not transfer any aqueous phase.) Evaporate combined dichloromethane layers to ca 200 μL at 35°C under nitrogen and continue as described under *Methylation.*

Calculation

Calculate correction factor, F_c, from standard chromatograms for each penicillin from areas and concentrations of internal standard and the corresponding penicillin by following formula:

$$F_c = (C_p \times A_s)/(C_s \times A_p)$$

where C_p = concentration of penicillin (μg/mL); C_s = concentration of internal standard (μg/mL); A_s = area of penicillin peak (integrator units); A_p = area of internal standard peak (integrator units).

Calculate concentration, C (μg/kg), of a particular penicillin in milk from the chromatogram of a residue-containing sample by following formula:

$$C = (F_c \times S \times A_p \times 1000)/(A_s \times W)$$

where W = sample weight (g); S = amount of internal standard added (μg).

Results and Discussion

Extraction and Cleanup

The pH value for extracting those penicillins included in the method (Figure 1) had to be optimized. Adding 0.5M phosphate buffer, pH 2.2, to milk resulted in a pH of 4.1–4.3 in the aqueous layer after the salting-out procedure. Lower pH values led to losses of acid-labile penicillins (penicillin G, methicillin), higher values led to lower overall recoveries because of poorer extractability of the carboxylate ion compared to the free acid.

The extraction procedure (Figure 2) follows a scheme discussed in some detail for residue analysis of macrolide antibiotics (13). The acid/base partitioning had to be reversed because of the basic nature of macrolides and the acidic nature of penicillins. Working at concentrations at and below 10 μg/kg, we observed considerable losses in the recovery of the penicillins when evaporations were taken to dryness without special precautions. When a standard of 0.25 μg penicillin G (corresponding to 10 μg/kg in milk) was added to acetonitrile/dichloromethane and cleanup was performed free of matrix (with each evaporation step taken to complete dryness), recovery was only about 15%. The most crucial step was the primary evaporation of the crude extract. Its evaporation to 0.5 mL was facilitated by repeated additions of dichloromethane and petroleum ether at the final stage of evaporation. Besides special precautions with the technique of evaporation, it was essential to use silylated glassware when working at low concentration levels.

To avoid stable emulsions in subsequent partitioning steps, it was necessary to suspend the residue of the crude extract in

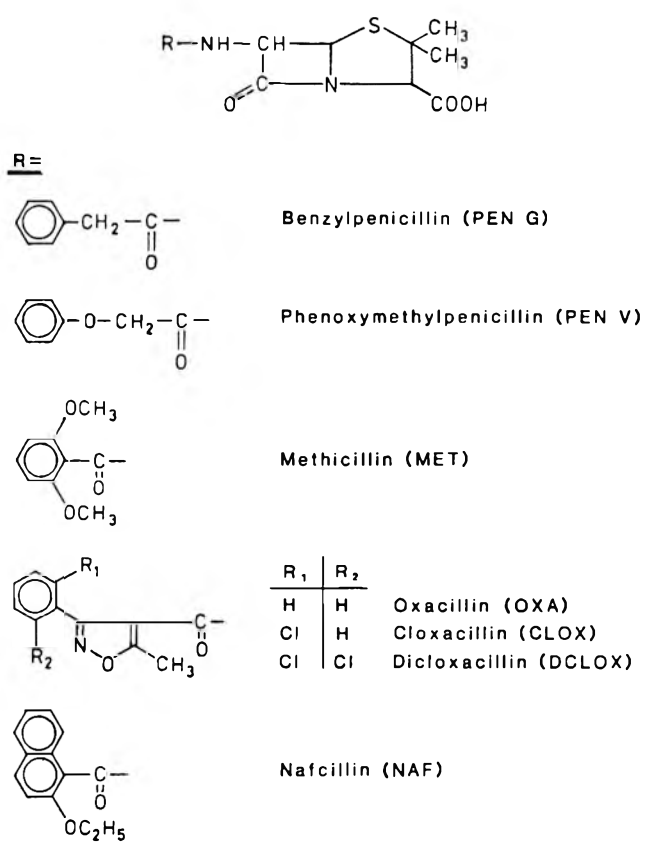

Figure 1. Structures of penicillins.

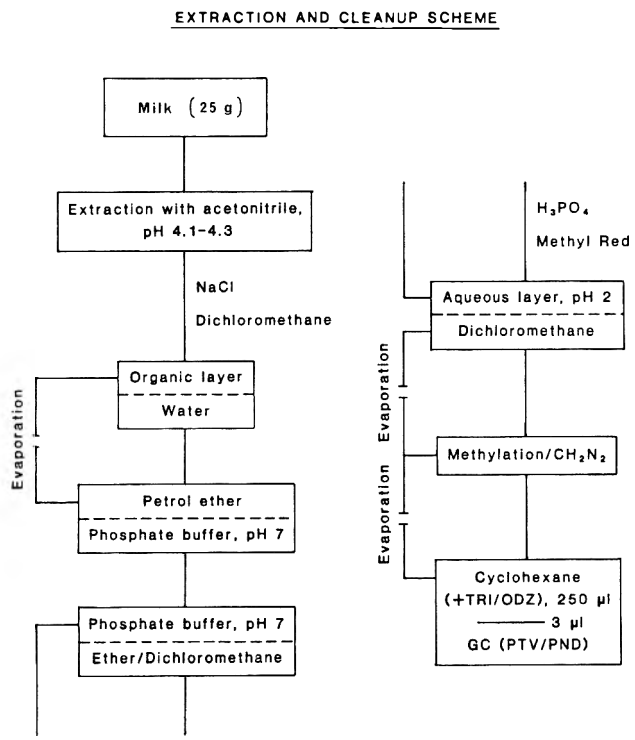

Figure 2. Scheme of extraction and cleanup.

petroleum ether first and then to partition successively between petroleum ether, diethyl ether, dichloromethane, and phosphate buffer (pH 7), while always discarding the organic layer.

Derivatization

Methyl red (2-[[4-(dimethylamino)phenyl]azo]-benzoic acid) was added just before derivatization to serve as indicator for correct methylation.

Preparation of methyl esters was also successful when methyl iodide was used with finely ground potassium carbonate as catalyst. However, an additional filtration step would have been necessary because, without filtration, unavoidable fines of potassium carbonate entered the GC injection port and led to poor chromatograms. We therefore preferred methylation with diazomethane, which eliminated the need of filtration.

At room temperature, derivatization rates were maximum after 2 h; methicillin and nafcillin reacted most slowly, while penicillin G and phenoxymethyl penicillin reached complete derivatization after 20 min.

Gas Chromatography

With fused silica capillary columns coated with very thin films (0.1 µm) of 100% methyl silicone, separation of the methylated penicillins was excellent (Figure 3). Both columns tested (Ultra-1, Hewlett-Packard; DB-1, J&W) gave

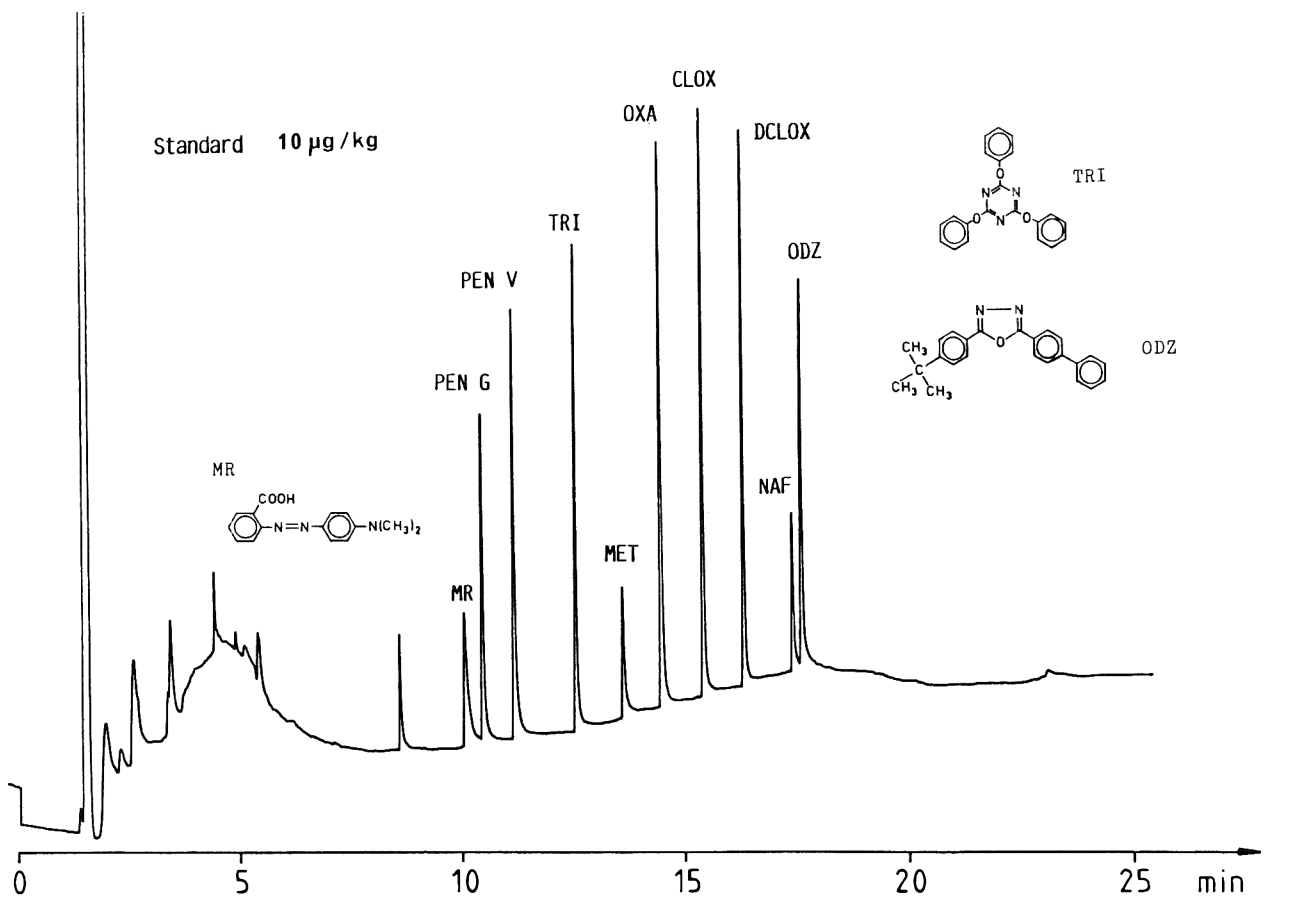

Figure 3. Capillary GC separation of various penicillins as methyl esters on methyl/silicone fused silica column. Nitrogen-selective thermionic detection (amount of each penicillin injected: 1 ng, corresponding to 10 µg/kg in milk). Peak identification: MR = methyl red, PEN G = benzylpenicillin, TRI = "Triazine" (internal standard), MET = methicillin, OXA = oxacillin, CLOX = cloxacillin, DCLOX = dicloxacillin, NAF = nafcillin, ODZ = "Oxadiazole" (internal standard).

Table 1. GC determination of penicillins in milk: reproducibility for injections and derivatizations of standards and recovery of standards added at 2 levels

Penicillin	Reproducibility CV, %		Fortification study			
			10 μg/kg		3 μg/kg	
	Injections (n = 10)	Derivatizations (n = 6)	Rec., % (n = 4)	CV, %	Rec., % (n = 3)	CV, %
PEN G	0.9	2.8	72	6.8	68	4.3
PEN V	2.2	2.9	92	3.0	85	2.6
MET	3.6	2.9	41	3.6	42	4.8
OXA	2.5	4.3	83	6.4	81	2.0
CLOX	2.3	4.6	84	5.6	80	3.2
DCLOX	2.8	4.4	70	11.7[a]	70	1.7
NAF	4.4	4.2	61	2.6	62	4.2

[a] Another set of 4 fortified samples resulted in a coefficient of variation of 3.8% (average recovery 65%).

comparably good results. With a 5% phenyl column (DB-5), we observed increased tailing of peaks; with a cyanopropyl-modified coating (OV-1701), column bleeding was unacceptable when the nitrogen-selective detector was used and in addition penicillins were strongly retained.

In capillary GC analysis, split injection onto a hot injection port is commonly used with injection volumes restricted to about 1 μL. Determination of derivatized penicillins at a split ratio of 1:10 was disadvantageous; in residue analysis it is too costly to lose 90% of injected penicillins because of the split. Furthermore, reproducibility decreased considerably with decreasing amounts of injected penicillins probably because of losses due to adsorption and/or thermal degradation.

Both sensitivity and reproducibility were markedly improved by mounting a cooled injection system with a septum-free sampling head (KAS 2, Gerstel, FRG) for cold sample injection onto our gas chromatograph. This system is similar to other programmed temperature vaporization (PTV) injectors.

For penicillin residue analysis, optimization of operating conditions resulted in injecting a volume of 3 μL onto an injector at 48°C, and venting off the injection solvent (cyclohexane) during 10 s at 50°C. After automatic closing of the venting valve, the penicillins are quantitatively transferred onto the column by a heat-up rate of 12°/s and holding at 300°C for 3 min. After that period, the split valve is opened and chromatography continues in the split mode. With a holding period briefer than 3 min at 300°C, those penicillins that elute last (dicloxacillin, nafcillin) are not quantitatively transferred.

After some weeks of food sample injections, smaller peaks (especially for methicillin and nafcillin) and increased tailing was observed. To avoid contamination of the analytical column, we therefore used a 1 m uncoated but deactivated fused silica column to serve as precolumn and retention gap. Chromatography without increasing tailing could be maintained when the retention gap was shortened by about 5 cm each 14 days.

We found it most reliable as regards chromatographic results and ease of leak-tight connection to insert the analytical column (0.4 mmod) into the precolumn (0.53 mmid) for a few millimeters and to connect both with a low weight column-connecting union for which ferrules of correct size are available. We also tried butt connections and disposable "press fit" glass connectors but found them less reliable. With the latter, we either had problems of leaks or broken glass.

The alkali bead of the nitrogen-selective detector had an average lifetime of about 3 months. At the end of a working period, selectivity decreased, which became obvious by the change from a negative to a positive solvent peak. For each new bead, selectivity had to be optimized separately as regards flow rates of detector gases and bead current by using a test mixture of tridecane and azobenzene.

The internal standards "Triazine" and "Oxadiazole" were selected because they are both very inert, nitrogen-containing compounds, which fit well into the chromatographic pattern. They do not have to be derivatized so they were also used to find optimal derivatization conditions. All results are calculated using "Triazine" as internal standard. An interferring peak at the retention time of the "Triazine" could be identified by a difference in the regular peak ratio of both internal standards. The addition of "Oxadiazole" thus serves as an additional control of quantitative results.

Standard and Fortification Analyses

Repeated injections and derivatizations of standards resulted in reproducibilities expressed as coefficient of variation of 1–4% and 3–5%, respectively (Table 1).

Milk was fortified at levels of 3 and 10 μg/kg by adding aqueous standard solutions and mixing. The samples were analyzed after a holding period of 15 min at room temperature. Results were similar for both fortification levels. The average recoveries were generally well above 60% with coefficients of variation between 2 and 7% even at the 3 μg/kg level except for methicillin (Table 1). We do not have an explanation for the high coefficient of variation with dicloxacillin at the 10 μg/kg level, especially in the light of the very good reproducibility at the 3 μg/kg level. To check this, another set of 4 samples was analyzed, resulting in a coefficient of variation of 3.8% (average recovery 65%) that fit much better into the whole picture.

A typical chromatogram for milk fortified with all 7 penicillins at 3 μg/kg, and control milk processed through the procedure is shown in Figure 4.

Limits of detection for all penicillins in milk are well below the μg/kg range (Table 2). These limits are calculated by the mean of the measured signal of 5 blank samples of different origin at the retention times of the individual penicillin plus

Table 2. Calculation of limits of detection for GC determination of penicillins in milk[a]

Penicillin	ES	B (n = 5)	s	B + 3s	Limit of detection, μg/kg
PEN G	9947	448	128	832	0.3
PEN V	10265	138	85	393	0.1
MET	5857	19	8	43	0.1
OXA	10745	118	108	442	0.1
CLOX	9072	67	93	346	0.1
DCLOX	8007	160	140	580	0.2
NAF	4455	148	145	583	0.4

[a] ES = area counts for external penicillin standard (corresponding to 10 μg/kg); B = average area count for signals in milk blank samples (n = 5) at retention times of individual penicillins; S = standard deviation of B.

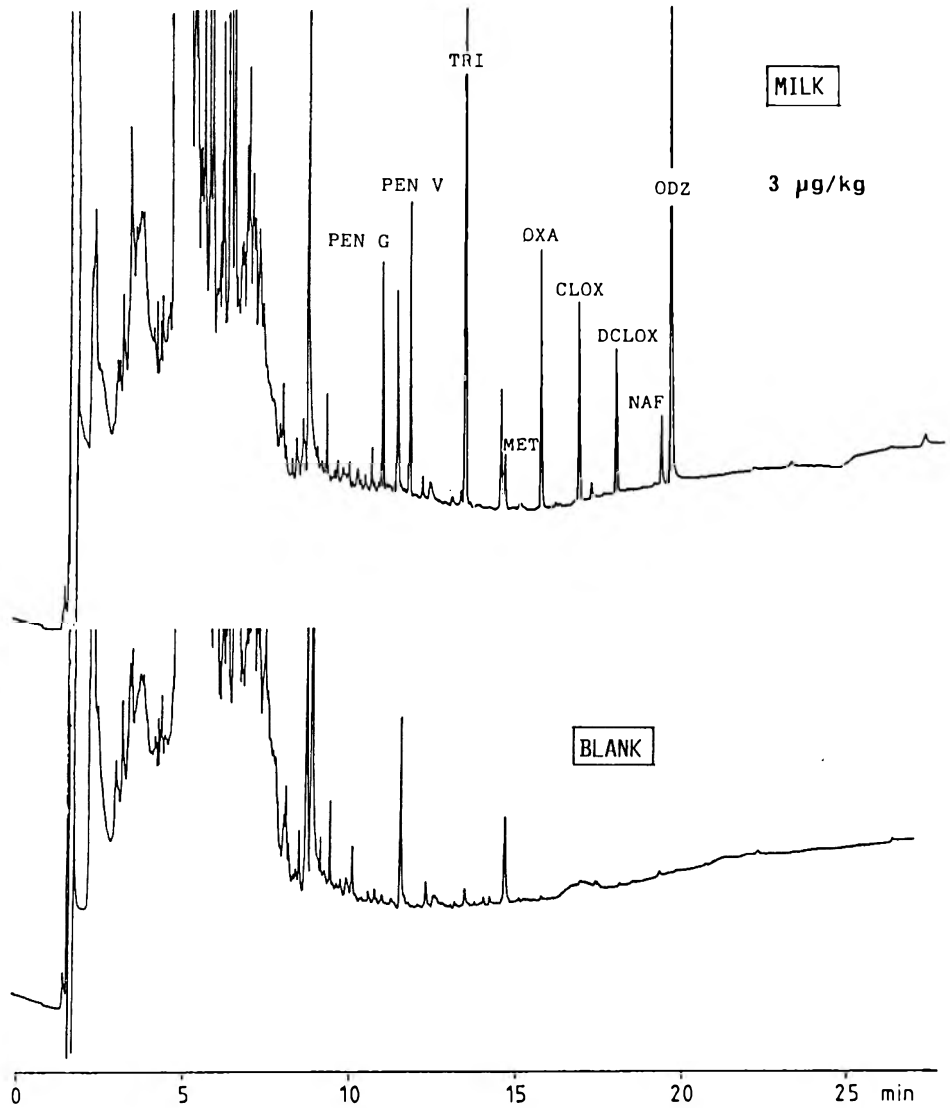

Figure 4. Chromatogram of milk blank and milk fortified with 3 µg/kg of each penicillin.

3× the standard deviation of the mean. This procedure is similar to the obligatory procedure for establishing limits of detection in evaluation of residue-detection methods for official analysis of thyrostatics and hormones in the EEC (14).

The method was also applied to various milk samples with incurred residues at different concentration levels (4000–3 µg/kg). The results were in excellent correlation to those that were independently obtained by another laboratory using the Charm test and the microbiological brilliant black reduction test. Thus study will be published separately.

The gas chromatographic method is implied to confirm positive results obtained by microbiological, immunological, or receptor binding tests. Further confirmation should be possible by applying on line GC-mass spectrometric techniques. This is a focus of our present work. Tissues can be analyzed when additional cleanup steps using anion exchange and diol cartridges are included (Meetschen, U., and Petz, M., unpublished data).

Acknowledgments

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Matrix Solid-Phase Dispersion (MSPD) Isolation and Liquid Chromatographic Determination of Oxytetracycline, Tetracycline, and Chlortetracycline in Milk

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A multiresidue method for the isolation and liquid chromatographic determination of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) antibiotics in milk is presented. Blank and tetracycline (OTC, TC, and CTC) fortified milk samples (0.5 mL) were blended with octadecylsilyl (C18, 40 μm, 18% load, endcapped, 2 g) derivatized silica packing material containing 0.05 g each of oxalic acid and disodium ethylenediaminetetraacetic. A column made from the C18/milk matrix was first washed with hexane (8 mL), following which the tetracyclines were eluted with ethyl acetate–acetonitrile (1 + 3; v/v). The eluate contained tetracycline analytes that were free from interfering compounds when analyzed by liquid chromatography with UV detection (photodiode array, 365 nm). Correlation coefficients of standards curves for individual tetracycline isolated from fortified samples were linear (from 0.982 ± 0.009 to 0.996 ± 0.004) with average percentage recoveries from 63.5 to 93.3 for the concentration range (100, 200, 400, 800, 1600, and 3200 ng/mL) examined. The inter-assay variability ranged from 8.5 ± 2.4% to 20.7 ± 13.0% with an intra-assay variability of 1.0–9.3%.

The tetracyclines are antibacterial compounds commonly used for the prevention and/or treatment of diseases in livestock production. As a feed additive in subtherapeutic doses, tetracyclines contribute to the maintenance of optimal health and thus promote growth in food-producing animals. For these and other reasons, such antibacterial compounds have become an integral part of the livestock-producing industry.

However, the use of these compounds may result in residues being present in animal-derived food products, especially if proper withdrawal times for treated animals have not been practiced. These residues may pose a health threat to consumers, depending on the compound, the type of food, and the amount of residue present. For this reason, regulatory agencies have established maximum legal tolerance levels for these drugs in animal-derived food products (1 and references therein). Maximum legal levels vary depending on the food type and/or the animal species from which it originated. The maximum tolerance levels for tetracyclines in the form of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) range from zero for CTC in milk and eggs to 4 μg/g for CTC in the kidney of chickens, turkeys, ducks, swine, calves, and cattle. The maximum allowable levels of OTC, TC, and CTC for other foods fall within this range.

Tetracycline residues that exceed the maximum level may be of toxicological concern. Some individuals may have an allergic reaction to these compounds and/or resistance by some bacteria may be induced (2).

Safety evaluations of these drugs take into account more than the aforementioned concerns. A critical part of such safety evaluations depends on the assay method used to determine the quantity of residue present. Methods used to determine tetracycline residue levels include, but are not limited to, microbiological (3, 4) and chromatographic techniques such as thin-layer (5), gas (6), and liquid (7–10) chromatography. However, microbiological methods lack specificity, and thin-layer chromatographic methods lack sensitivity (5). Extensive sample preparation steps for biological matrixes have been required to facilitate GC and LC analyses and may include extractions of tetracyclines by heating in acid solutions in the presence of EDTA (11). These extracts are then deproteinated by chemical treatment with trichloroacetic acid (TCA) and additional heating. The anhydrotetracyclines that result are then extracted into an organic solvent and analyzed (11).

Isolation procedures as outlined above have certain deficiencies. Heating and acid treatment may result in compound degradations and/or incomplete conversion of the tetracyclines to their anhydro forms. Thus, the data acquired may not be truly representative of the sample and/or the results may be inconsistent. In addition, OTC forms anhydro derivatives that undergo further degradation, producing 2 isomeric apo-OTC (alpha and beta) forms in solution (10). Only the beta-apo-OTC is extracted into organic solvent (11). It may not be a valid assumption that a one-to-one ratio of the 2 isomeric apo-OTC forms results from the treatment. Other matrixes, such as serum, may require less exhaustive extraction protocols (8).

In addition OTC, TC, and CTC demonstrate different stabilities at different pH values and treatment (12). CTC, for example, is stable in acid solutions, TC is stable in neutral and alkaline solutions, and OTC is stable is aqueous solutions for prolonged periods. Rigorous extraction techniques utilizing acids may adversely affect recoveries of tetracyclines and time-consuming extraction methods may result in tetracycline losses due to photodecomposition (13–14). Tetracycline

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isolations from complex biological matrixes such as animal-derived foods can be difficult, and classical isolation techniques that require many sample manipulations and rigorous extraction procedures, as described above, are too labor- and materials-intensive.

Multiresidue isolation methods that minimize time and materials requirements mark the trend in residue isolations. Ideally, residue isolation techniques, in addition to minimizing time and materials requirements, should result in extracts that contain the target residue(s) with high recoveries and minimal background interferences. Recently, we have developed a multiresidue extraction technique, named matrix solid-phase dispersion (MSPD) (15-21), that overcomes many of the problems associated with classical isolation techniques. The MSPD method has been successfully used for the isolation of sulfonamides, benzimidazoles, chlorsulfuron, and chloramphenicol in milk, as well as sulfonamides, benzimidazoles, organophosphates, and beta-lactams from animal-derived matrixes (15-21). We report here the first application of MSPD methodology used in conjunction wtih matrix modifiers to facilitate the isolation and liquid chromatographic determination of oxytetracycline, tetracycline, and chlortetracycline as residues in milk.

Experimental

Apparatus and Reagents

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

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

(c) *Liquid chromatographic analysis.*—Analyses of fortified sample extracts and standard tetracycline solutions were conducted on a Hewlett Packard (HP1090/HP 79994A HPLC Chemstation) instrument equipped with a photodiode array detector set at 365 nm with a bandwidth of 30 nm and a reference spectrum range of 100-450 nm. Liquid chromatography mobile phase [0.01M aqueous oxalic acid-acetonitrile (7 + 3; v/v)] at an isocratic flow rate of 1 mL/min and a reverse-phase octadecylsilyl (ODS) derivatized silica column (10 μm, 30 cm × 4 mm id, Micro Pak, Varian, Sunnyvale, CA) maintained at 40°C was used for all determinations.

Standard curves were generated by plotting peak areas of injected standard tetracyclines (Part (h)), and fortified sample extracts for the concentrations examined. A comparison of extracted fortified sample tetracycline areas to areas of pure standards run under identical conditions gave percent recoveries. Inter-assay variability was determined in the following manner: The peak areas for 5 replicates of each concentration (100, 200, 400, 800, 1600, and 3200 ng/mL, 20 μL injection volume) were averaged, which resulted in a mean ± standard deviation (SD). This SD was divided by its respective mean, which resulted in a coefficient of variation (CV). The CVs determined for each concentration were then averaged, which resulted in a mean ± SD. This was defined as the inter-assay variability. Intra-assay variability was defined as the coefficient of variation for the mean of 5 replicates of the same sample and represents the variability associated with the analytical instrumentation used.

(d) *Tetracyclines.*—Oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) (Sigma Chemical Co., St. Louis, MO). One should wear rubber gloves and protective clothes when handling.

(e) *Matrix modifiers.*—Oxalic acid (Mallinckrodt, Inc., Paris, KY) and disodium ethylenediaminetetraacetate (EDTA) (Sigma Chemical Co.)

(f) *Column material.*—Bulk C18 (40 μm, 18% load, end-capped; Analytichem International, Harbor City, CA) was cleaned by making a column (50 mL syringe barrel) of the bulk C18 material (22 g) and sequentially washing with 2 column volumes each of hexane, methylene chloride (DCM), and methanol. Washed C18 was vacuum-aspirated until dry.

(g) *Stock tetracycline solutions.*—1.0 mg/mL prepared daily by dissolving standard compounds with LC grade methanol and diluting to desired μg/mL levels with methanol.

(h) *Preparation of standard stock solutions for standard curves.* 10 μL of 5, 10, 20, 40, 80, and 160 μg/mL stock solutions were added to individual LC vials. A volume (490 μL) of LC mobile phase [0.01M aqueous oxalic acid-acetonitrile (7 + 3; v/v)] was added to the vials. A portion (20 μL) of each vial was injected into an LC and analyzed.

(i) *Sample extraction columns.*—Ten mL syringe barrels, thoroughly washed and dried before use.

Extraction Procedure

Whole raw milk samples were obtained from the Louisiana State University dairy farm. Two g C18 and 0.05 g each of disodium ethylenediaminetetraacetate (EDTA) and oxalic acid were placed into a glass mortar. An aliquot (0.5 mL) of milk (thoroughly mixed) was placed directly onto the C18 material. Standard tetracyclines (10 μL of 5, 10, 20. 40, 80, and 160 μg/mL stock solutions) were added to the milk and the samples were allowed to stand for 1 min. Conversely, milk samples could be placed into the mortar and fortified, and the C18 and modifiers could be added and blended to obtain identical results.

Milk placed on top of the C18 forms a bead of solution that is not absorbed by the C18, and the bead is then fortified. Mixing of the milk and C18 can only be accomplished by mechanical blending. Blank milk samples were prepared similarly except that 10 μL methanol containing no tetracyclines was added to the sample.

The samples were then gently blended for 30 s into the C18 with a glass pestle until the mixture was homogeneous in appearance. A gentle circular motion with very little pressure was required to obtain a homogeneous mixture. The resultant C18/milk matrix was placed into a 10 mL plastic syringe barrel that was plugged with a filter paper disc (Whatman No. 1).

The column head was covered with a filter paper disc, and the column contents were compressed to a final volume of 4. 5 mL with a syringe plunger that had the rubber end and pointed plastic portion removed. A pipet tip (100 μL) was placed on the column outlet to increase residence time of the eluting solvents on the column. The resulting column was first washed with 8 mL LC grade hexane. Flow through the column was gravity controlled in all cases. If the initial flow through the column was hindered, positive pressure was applied to the column head (pipet bulb) to initiate gravity flow. When flow had ceased, excess hexane was removed from the column with positive pressure as described above.

The tetracyclines were then eluted with a 8 mL of ethyl acetate-acetonitrile (1 + 3; v/v) as described above for hexane. The resultant extract was dried under a steady stream of dry nitrogen gas maintained at 40°C in a water bath. Then, 0.5 mL LC mobile phase (0.01M aqueous oxalic acid-acetonitrile, 7 + 3; v/v) was added to the dry residue.

Table 1. Correlation coefficients, percentage recoveries, inter- and intra-assay variabilities of tetracyclines isolated from fortified milk samples

	Recovery ± SD, %		
Concn, ng/mL[a]	Oxytetracycline	Tetracycline	Chlortetracycline
100	98.4 ± 6.4	64.0 ± 8.2	88.3 ± 25.7
200	88.4 ± 9.2	40.4 ± 17.3	66.7 ± 4.1
400	93.0 ± 6.0	41.2 ± 11.9	69.9 ± 5.9
800	95.3 ± 7.8	72.8 ± 9.4	84.7 ± 7.4
1600	93.3 ± 11.5	90.3 ± 17.5	64.6 ± 8.2
3200	91.4 ± 6.6	72.3 ± 5.6	89.0 ± 8.3
Correl. coeff. std curves, %	0.996 ± 0.004	0.994 ± 0.006	0.982 ± 0.009
Inter-assay variability, %	8.5 ± 2.4	20.7 ± 13.0	12.4 ± 8.4
Intra-assay variability, %	1.4	1.0	9.3

[a] n = 5 at each concentration.

The sample was sonicated (5–10 min) to disperse the residue, which resulted in a suspension. This was transferred to a micro-centrifuge tube and centrifuged (IEC Centra-M, International Equipment Co., Needham, MA) at 17 000 × *g* for 5 min. The resultant clear supernatant liquid was filtered through a 0.45 μm filter (Micro-Prep Disc, Bio Rad Inc., Richmond, CA), and a portion (20 μL) was analyzed by LC.

Results

Table 1 gives the tetracycline examined, concentrations analyzed (100–3200 ng/mL), standard curve correlation coefficients (±SD), percentage recoveries, and inter- and intra-assay variabilities of the tetracyclines (OTC, TC, and CTC) isolated from fortified milk samples. Representative chromatograms of extracted blank and fortified milk samples are shown in Figures 1A and 1B, respectively. Tables 2 and 3 give the percentage recoveries of OTC, TC, and CTC isolated from samples treated with different ratios of the matrix modifiers oxalic acid (OA) and disodium ethylenediaminetetraacetate (EDTA) and eluted from the MSPD columns with different ratios of ethyl acetate to acetonitrile.

Discussion

Classical techniques utilized for tetracycline isolations can be labor- and materials-intensive. Multiple sample manipulations and chemical modifications to the sample and/or sample extract can lead to inconsistent assays. OTC, TC, and CTC vary in their stability at specified conditions of pH and temperature and demonstrate different degradation characteristics in aqueous solutions. Thus, optimizing extraction conditions to facilitate multiresidue isolations of OTC, TC, and CTC can be difficult. Classical isolation techniques that require heating in acid solutions and protein precipitations utilizing trichloroacetic acid (11) may contribute to experimental error as a result of tetracycline degradations. In addition, the conditions necessary for conversion of tetracyclines to their anhydro forms (10, 11) by heating may be different from matrix to matrix and from extract to extract, as a result of interactions with other constituents present in the extract. Optimizing conditions for equilibrium formation of the 2 isomeric anhydro forms of OTC may adversely affect TC and CTC recoveries, resulting in erroneous multiresidue determinations.

A further difficulty associated with tetracycline isolations is the propensity of tetracyclines to complex with inorganic ions. This complexation makes isolation from biological matrixes difficult. This has been overcome, with varying degrees of success, by the addition of ethylenediaminetetraacetate (EDTA) to the extracting solutions (11). EDTA chelates inorganic ions and apparently releases the tetracyclines from metal ion complexes. Tetracycline extractions can also be facilitated by using acidulants, such as hydrochloric, phosphoric, and citric acids, which alter pH and function to change the extraction characteristics of the tetracyclines. By in large, these techniques work but require the use of large sample sizes, large volumes of extracting solvents, and/or separations by preparative-scale column chromatography (7). Such multi-step procedures have historically been required to isolate tetracyclines with reasonable recoveries.

Recently, we have developed a method we named matrix solid-phase dispersion (MSPD) that overcomes many of the complications associated with classical residue isolation procedures. MSPD isolations are accomplished by blending the sample, in this case milk, with C18 (octadecylsilyl derivatized silica) packing material. A column made from the C18/milk matrix is then eluted with an experimentally determined solvent sequence. In the MSPD technique, the sample is dispersed over a large surface area (1000 sq. m/2 g C18) and exposes the entire sample to the extraction process. Even though the volume of extracting solvent is only 8 mL, the process is an exhaustive extraction whereby a large volume of solvent is passed over an extremely thin layer of sample.

We theorize that the dispersion of the sample onto the C18 packing material involves mechanical and hydrophobic forces. Nonpolar materials such as lipids and other membrane components associate with the nonpolar C18 polymer and facilitate the disruption and unfolding of the structural components of the milk matrix. The hydrophilic components and more polar protein ends preferentially orient themselves away from the nonpolar lipid C18 inner regions and extend outward. Water and more polar constituents would associate with these hydrophilic ends. The theoretical aspects of the MSPD technique have been published (15–20). Scanning and transmission electron micrographs of uncoated and tissue coated C18 support this hypothesis (unpublished observations).

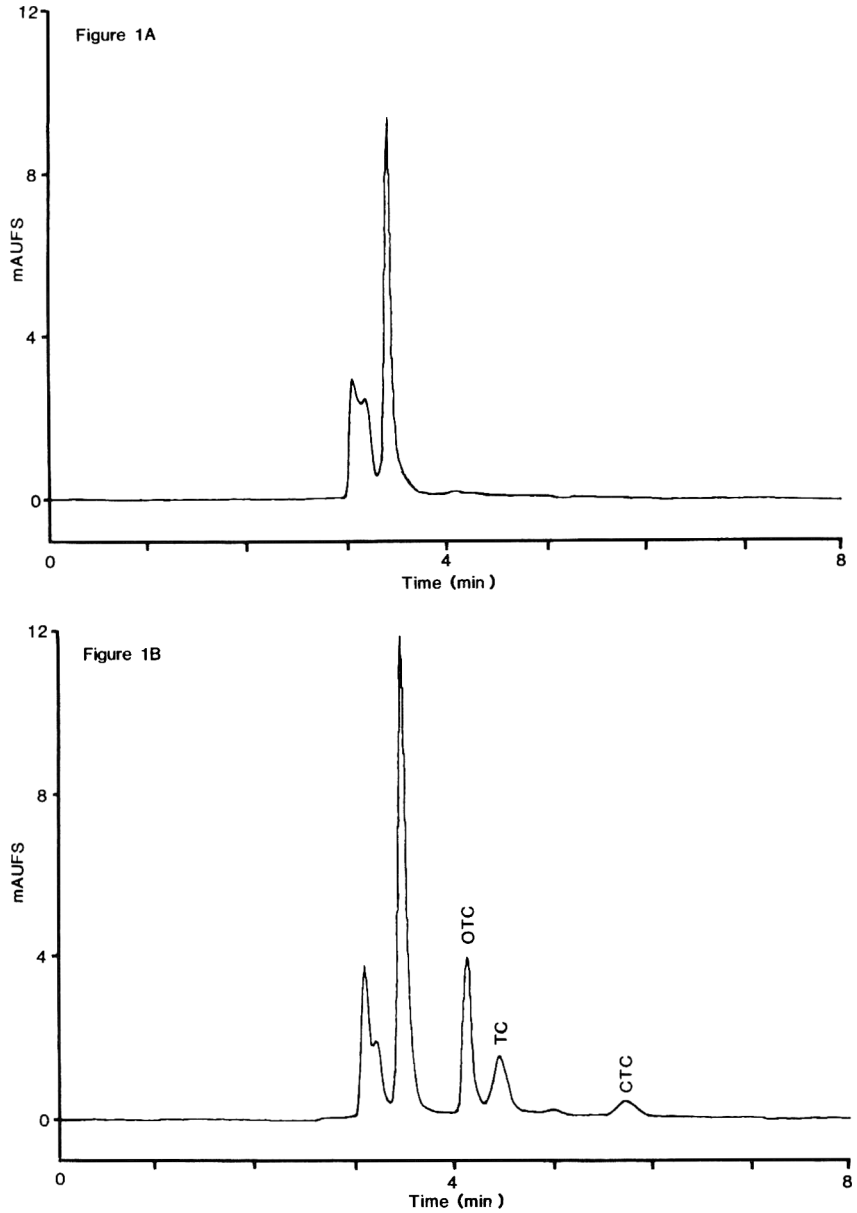

Figure. 1. Representative liquid chromatograms (UV, photodiode array, 365 nm) of the ethyl acetate–acetonitrile (1 + 3; v/v) extract of blank (1A) oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) fortified (400 ng/mL) milk (1B) samples. Order of elution is OTC, TC, and CTC.

MSPD requires the mechanical blending of the milk sample, or other matrix, into the octadecylsilyl derivatized silica (C18) bonded phase packing material. This results in a thin layer of sample dispersed evenly on the individual C18 beads. A uniform dispersion of milk cannot be accomplished by simply passing the milk through a column containing C18 packing material, as is usually done in classical solid-phase extraction (SPE) techniques. An inconsistent elution profile results, as was experienced in this laboratory for benzimidazole isolations from milk (17) and sulfonamide isolations from infant formula (19).

Tetracycline isolation from milk used the basic MSPD approach and was facilitated by the use of the matrix modifiers EDTA and oxalic acid. Prior to blending the milk samples into the C18, the modifiers were admixed to the C18. It was experimentally determined that by adding EDTA and oxalic acid (OA) to the C18, tetracycline recoveries could be enhanced (Tables 2 and 3).

When milk was prepared without EDTA or OA added to the C18, no tetracyclines were eluted from the MSPD column. Modifying (Tables 2 and 3) the milk with EDTA or OA singly resulted in increased recoveries of tetracyclines compared to using no modifiers. An equal ratio of OA to EDTA gave the best recovery for the 3 tetracyclines examined. EDTA chelates inorganic ions and decomplexes the tetracyclines from matrix constituents. OA serves a dual purpose because it will complex inorganic ions and also lower the pH of the milk (pH 3.0), which enhances the extractability of the tetracyclines into the eluting solvent.

The best extracting solvent sequence and ratios were experimentally determined as well (Tables 2 and 3). When matrix modified C18/milk was extracted with 100% ethyl acetate, the recoveries were approximately 50%. Elution of the column with 100% acetonitrile resulted in higher recoveries but provided a sample extract that required additional preparative time prior to analysis by liquid chromatography.

Table 2. Effect of different ratios of matrix modifiers Na_2EDTA and oxalic acid (OA) on percentage recoveries of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) in milk extracts eluted from MSPD columns[a]

Na_2EDTA + OA Ratio, g/g	OTC		TC		CTC	
	Mixed	CH_3CN	Mixed	CH_3CN	Mixed	CH_3CN
0.09 + 0.01	65	67	65	68	39	45
0.08 + 0.02	70	72	69	72	47	63
0.07 + 0.03	76	80	79	78	57	77
0.06 + 0.04	65	72	30	39	63	75
0.05 + 0.05	94.5 ± 8.5[b]	83	87.5 ± 9.1[b]	60	85.5 ± 7.2[b]	85

[a] Average of duplicate samples at 2 ppm. Mixed = ethylacetate–acetonitrile (1+3); CH_3CN = 100% acetonitrile.
[b] Average of 4 replicates.

Various ratios of ethyl acetate to acetonitrile resulted in different percentage recoveries for the tetracyclines examined. In general, as the percentage acetonitrile was increased, an increased percentage recovery was obtained. A 1 + 3 ratio of ethyl acetate to acetonitrile was chosen because extracts had a minimal amount of background interferences, evaporated readily, were easily filtered through a 0.45 μm filter, and resulted in reasonable recoveries.

When the extracts were reduced to dryness, a considerable amount of white residue remained in the tube. This residue was easily solubilized with LC mobile phase. When the tube contents were sonicated, a homogeneous suspension resulted; when this suspension was centrifuged (17 000 × *g*), the supernatant liquid was clear and easily filtered through a 0.45 μm filter. Representative LC chromatograms (UV, photodiode array, 365 nm) show that elutions with a 1 + 3 ratio of ethyl acetate to acetonitrile (0.05 g + 0.05 g of EDTA to OA, wt/wt) resulted in blank milk extracts that contained no interferences (Figure 1A). An LC chromatogram of a tetracycline (OTC, TC, and CTC) fortified milk extract is shown in Figure 1B. The peak at 4.9 min in the fortified milk extract was present as an impurity in the CTC standard.

During the early developmental stages of this method, we experienced considerable variability in terms of TC recoveries, while OTC and CTC recoveries were consistent. We attribute this variability to several factors. A primary factor responsible for the variability noted for TC may be its particular ionization characteristics. TC recoveries were greatly reduced when a 0.06 + 0.04 ratio of EDTA plus OA (g + g; wt/wt) (Table 3) was used. This pattern was observed for many trials. Apparently, the conditions necessary for the optimal recovery of OTC and CTC are very near the transitional pKa of TC, and this contributes to the variability observed. This decrease in recoveries for tetracyclines at increasing concentrations has been observed in other studies (10).

In addition, we found that when the tetracycline-fortified milk extracts were reduced to dryness in a glass tube, the recovery of TC was reduced while the recoveries of OTC and CTC were not adversely affected. We attributed this to the binding of TC to the glass.

Why OTC and CTC were not affected cannot be completely explained because, when pure working solutions of OTC, TC, and CTC, were reduced to dryness in a glass tube and subsequently prepared for LC analysis in the same manner as sample extracts, the recovery of the individual tetracyclines was reduced to less than 20%. Evidently, other constituents in the extract prevent the binding of OCT and CTC to the glass but not TC. This phenomena may again be related to the pKa of TC in the sample extract. In either case, the variability was reduced when plastic tubes were used to collect and prepare the extracts for LC analysis. Photodecomposition of TC could also be a contributing factor to the variability noted but was not studied in sufficient detail to note its total effect.

Results (Table 1) indicate that the isolation of tetracyclines by using the matrix modified MSPD method gave extracts that were linear with respect to increasing concentrations of OTC, TC, and CTC in fortified milk samples. Inter-assay variability for TC was greater than that associated with OTC or CTC. Correspondingly, the percentage recovery of TC was less than those of OTC and CTC.

This extraction procedure was developed to facilitate the simultaneous multiresidue extraction of OTC, TC, and CTC. Because the chemical characteristics of OTC, TC, and CTC are different, it was difficult to optimize conditions to affect the simultaneous isolation of these 3 tetracyclines from a complex biological matrix. If TC were to be isolated singly, a modification of this procedure would be in order. However, if one were to simultaneously screen for OTC, TC, and CTC, an extraction method as outlined here may be optimal.

The low percentage recovery and larger variability for TC

Table 3. Effect of matrix modifiers Na_2EDTA and oxalic acid (OA) on percentage recoveries of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) in milk extracts eluted from MSPD columns by different ratios of ethyl acetate to acetonitrile[a]

Ethyl acetate and acetonitrile	0.1 g OA			0.1 g EDTA			0.05 + 0.05 g (OA + EDTA)		
	OTC	TC	CTC	OTC	TC	CTC	OTC	TC	CTC
1 + 1	28	40	58	38	37	25	34	31	43
1 + 2	72	67	73	52	52	37	69	54	75
1 + 3	78	74	74	49	58	37	94.5 + 8.5[b]	87.5 ± 9.1[b]	85.5 ± 7.2[b]

[a] Average of duplicate samples.
[b] Average of 4 replicates.

compared to OTC and CTC at the 200 and 400 ng/mL levels is believed to be associated with its ionizing characteristics in the presence of the matrix modifiers OA and EDTA, a concentration-dependent effect. We are presently examining the extraction characteristics of TC individually in an attempt to determine specific interactions that may be occurring between the milk, matrix modifiers, and extracting solvent. The use of an appropriate internal standard may serve to reduce variabilities and increase the calculated recoveries of OTC, TC, and CTC. This should enable one to further optimize matrix-modified MSPD isolations of tetracyclines from milk and allow the extension of this methodology to tetracycline isolations from other biological matrixes.

The LC mobile phase (0.01M oxalic acid–acetonitrile, 7 + 3; v/v) proved to be suitable for the separation of OTC, TC, and CTC (Figure 1B) on a reverse-phase (C18, octadecylsilyl derivatized silica) column and is a modification of the method of Martinez and Shimoda (9). The column was conditioned (20–30 min) with the mobile phase until a steady baseline was observed.

The OA appears to effectively strip or mask any metal ions that may affect tetracycline analyses. This was evidenced by OTC, TC, and CTC peaks that were consistently symmetrical and well resolved. The column should, however, be dedicated to tetracycline analyses because the column selectivity for other compounds was reduced in other solvent systems. Also, the column conditioning step was shortened when the column was dedicated to tetracycline analyses.

When the column was to be stored for more than one day, it was flushed with 0.017M H_3PO_4–acetonitrile (7 + 3; v/v). After several hundred sample analyses, a slight loss of peak symmetry was observed, but this was easily corrected by washing the column with 0.017M aqueous H_3PO_4–acetonitrile (2 + 8; v/v) until a steady baseline was observed and then reconditioning the column with the tetracycline mobile phase. The use of mixed mode reverse-phase/ion-exchange LC columns (Alltech Associates, Inc., Bulletin No. 168) designed for tetracycline separations may obviate the need for extensive column handling as described above, but these columns were not available to us during this study.

The types and amounts of matrix modifiers and eluting solvents chosen were dictated by the type of compounds and the matrix from which we were attempting to isolate them. EDTA has been used in tetracycline isolations (9–11) to facilitate their decomplexation from metal ions and facilitate their extraction from biological matrixes. The use of oxalic acid to facilitate tetracycline decomplexations and extractions from MSPD columns represents the first known use of this compound for this purpose. Oxalic acid has been shown to facilitate tetracycline separations by LC (9) when used in the mobile phase and may function similarly to EDTA by complexing with metal ions. Other matrixes may require different matrix modifiers and solvent elution systems for tetracycline isolations and may provide an extract containing higher recoveries and/or different levels of coextractants. In the case of immunoassay techniques, maximizing recoveries may be of paramount importance provided there are no cross reacting compounds in the extract; thus, this extraction method may enhance screening protocols.

The minimal detectable limit was 2 ng on column (100 ng/mL, 20 μL injection volume from a 0.5 mL final volume), and coextracted interferences were eliminated when the extract was monitored at 365 nm. The matrix-modified MSPD isolation of tetracyclines uses small sample sizes (0.5 mL), uses 8 mL each of washing and extracting solvents, and does not require a heating step to convert the tetracyclines to their anhydro froms. Conversely, classical tetracycline isolations have required use of larger sample sizes, extractions with large volumes of acidified aqueous solutions, protein precipitation with trichloroacetic acid, heating of the acidified extracts to form anhydro derivatives, extraction of anhydro derivatives into a suitable organic solvent, and evaporation of large volumes of extracting solvents.

We have demonstrated that MSPD isolations of tetracyclines can be facilitated by matrix modifiers and that these modifiers can be varied for specific applications. MSPD may be valuable for tetracycline isolations from other food matrixes and could be enhanced by the use of carefully selected matrix modifiers and solvent elution systems.

Acknowledgments

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DRUGS

Automation of Dihydroxyaluminum Sodium Carbonate Analysis in Antacid Tablets by Energy Dispersive X-Ray Fluorescence

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A sensitive, specific, automated energy dispersive X-ray fluorescence (EDXRF) method for determination of anhydrous dihydroxyaluminum sodium carbonate in antacid tablets has been developed. The compound was quantitated by impact grinding, pelletizing at 10 tons pressure, and monitoring the aluminum by using a rhodium anode X-ray tube, high resolution thermoelectrically cooled Si(Li) detector with sample spinning, and computer data processing. The assay procedure was validated with spiked laboratory-prepared samples at 100 ± 20% levels. The average recovery was 100.6% with a relative standard deviation of 1.6% (n = 14). Instrument precision was determined and found to have an average relative standard deviation of 1.0% (n = 16). In addition, analysis precision by the EDXRF method was compared to that for titration and autoanalyzer methodologies and found to be statistically comparable. The sample precision had an averaged relative standard deviation of 2.7% (n = 16) by X-ray methodology. The advantages of this EDXRF method include increased sample throughput with excellent precision and accuracy, no solvent usage, and automated data handling.

Because of its neutralization capacity, dihydroxyaluminum sodium carbonate is commonly used in antacid tablets (1). Methods for the determination of this compound usually involve an EDTA titration (2). Atomic absorption spectroscopy (AAS) or inductively coupled plasma spectroscopy (ICP) can also be used to quantitate this ingredient (3). An unpublished method involving autoanalyzer instrumentation has also been used for routine quality control testing. These methods are time consuming, require various solvents and reagents, and are destructive to the sample.

Energy dispersive X-ray fluorescence (EDXRF) techniques have been commonly used to determine aluminum in metallurgical applications (4–5). Although this is an established technique, it has rarely been used for the quantitative determination of metal ions in foods, drugs, or cosmetics. The established applications and technology principles formed the bases for developing a rapid EDXRF quality control procedure (6). This method has been shown to be precise and accurate with increased sample throughput, less sample preparation, no solvent usage, and automated data handling while being nondestructive to the sample. The EDXRF method was compared to titration and autoanalyzer methodologies and found to be statistically comparable.

The basic steps of the method involve impact grinding the sample to a consistent particle size throughout; the sample is then precisely pelletized to give a smooth, uniform surface. The energy dispersive spectrometer irradiates the sample with X-rays under vacuum to a depth of about 15 μm, inducing the atoms present to emit characteristic fluorescence. Detection is achieved by a solid-state peltier cooled detector which sends a digitized signal for computer applications.

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METHOD

Reagents

(a) *Pulverized sucrose.*

(b) *Magnesium stearate.*

(c) *Dihydroxyaluminum sodium carbonate (hydrous).*—Determine anhydrous DASC content by USP XXI titration methodology.

(d) *Food starch.*

(e) *Corn syrup, 42DE.*

Equipment

(a) *Mixer mill MM2.*—Brinkmann Cat. No. 27-16-700-4.

(b) *Mortar.*—Stainless steel, 10 mL, Brinkmann Cat. No. 27-16740-3.

(c) *Ball.*—Stainless steel, 9 mm diameter, Brinkmann Cat. No. 27-16-743-8; 12 mm diameter, Brinkmann Cat. No. 27-16-744-6.

(d) *Glassine paper.*

(e) *Semi-automatic pelletizing press.*—Herzog, Model HPT 20, equipped with 32 mm chrome steel (64 HRC) press tooling. 31 mm pre-flared Spec-Cap, Spex Cat. No. 3619A, or 31.2 mm tapered plastic sample cups, Chemplex Cat. No. 552.

(f) *Spectrometer.*—Tracor X-ray Spectrase 5000E energy dispersive X-ray fluorescence spectrometer with 50 kV, 0.35 mA (maximum output) rhodium anode X-ray tube, high resolution thermoelectrically cooled Si(Li) detector, 6-position filter wheel, 10-position sample changer with spinner. Data acquisition and processing done by IBM PS/2 Model 50Z with Tracor X-ray software and acquisition board.

EDXRF acquisition parameters: tube voltage 8 kV; tube current 0.28 mA; no filter; livetime 20 s; maximum energy 10 keV; atmosphere vacuum; warmup 0 s; sample spinner on.

Impact Grinding

Place sample or standard material into clean 10 mL stainless steel mortar. Add one 9 mm and one 12 mm stainless steel ball. Place 10 mL stainless steel mortar cap on mortar and clamp onto mixer mill MM2. Set potentiometer to maximum level (1500 rpm) and timer between 3 and 5 min.

When grinding sets have stopped, remove mortar, uncap, and, with aid of spatula, place powder onto glassine paper. Remove stainless steel balls from powder while observing that powder consistency is lump-free with apparent uniform particle size.

If powder appears lumpy or nonuniform, repeat grinding steps. If powder appears lump-free and uniform, proceed as directed under *Pelletizing.*

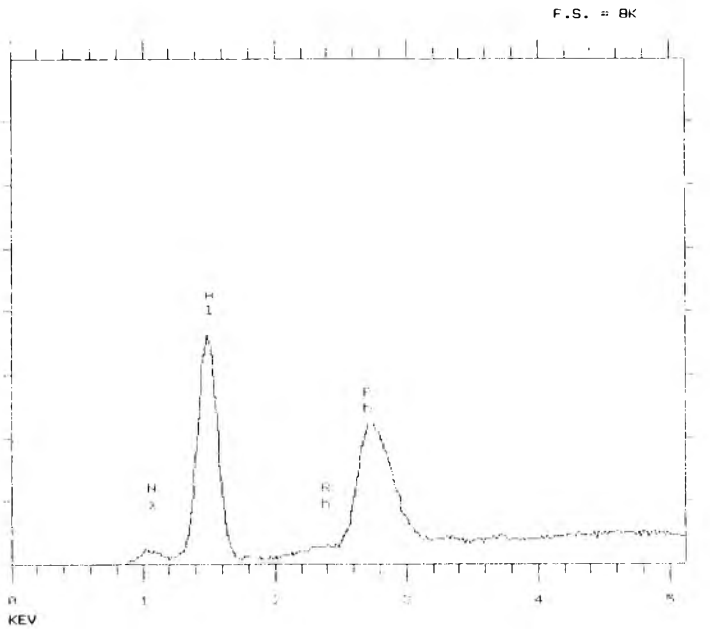

Figure 1. X-ray spectrum of antacid tablet: A, dihydroxyaluminum sodium carbonate.

Pelletizing

Use these press operating elements: press force 10 tons; ejection force 0.5 tons; time interval 7 s.

Preparation of Standard Pellets

(**a**) *Pelletizing medium formula A.*—92.5% pulverized sugar (wt in g); 5.8% food starch (wt in g); 1.7% magnesium stearate (wt in g).

(**b**) *Pelletizing medium formula B.*—87.8% pulverized sugar (wt in g); 5.6% food starch (wt in g); 4.9% corn syrup—42 DE (wt in g); 1.7% magnesium stearate (wt in g).

(**c**) *Multilevel seven-point calibration.*—(*1*) Prepare zero level calibration standard by impact grinding and pressing 2.8 (± 0.1) g of pelletizing medium as directed under impact grinding and pelletizing procedures.

(*2*) Prepare two 80% theory level calibration standards by impact grinding and pressing 530 (±40) mg DASC reference material into 2300 (±60) mg of pelletizing medium as directed under impact grinding and pelletizing procedures.

(*3*) Prepare one 90% theory level calibration standard by impact grinding and pressing 600 (±50) mg DASC reference material into 2240 (±50) mg of pelletizing medium as directed under impact grinding and pelletizing procedures.

(*4*) Prepare one 100% theory level calibration standard by impact grinding and pressing 670 (±50) mg DASC reference material into 2170 (±50) mg of pelletizing medium as directed under impact grinding and pelletizing procedures.

(*5*) Prepare one 110% theory level calibration standard by impact grinding and pressing 735 (±50) mg DASC reference material into 2105 (±50) mg of pelletizing medium as directed under impact grinding and pelletizing procedures.

(*6*) Prepare two 120% theory level calibration standards

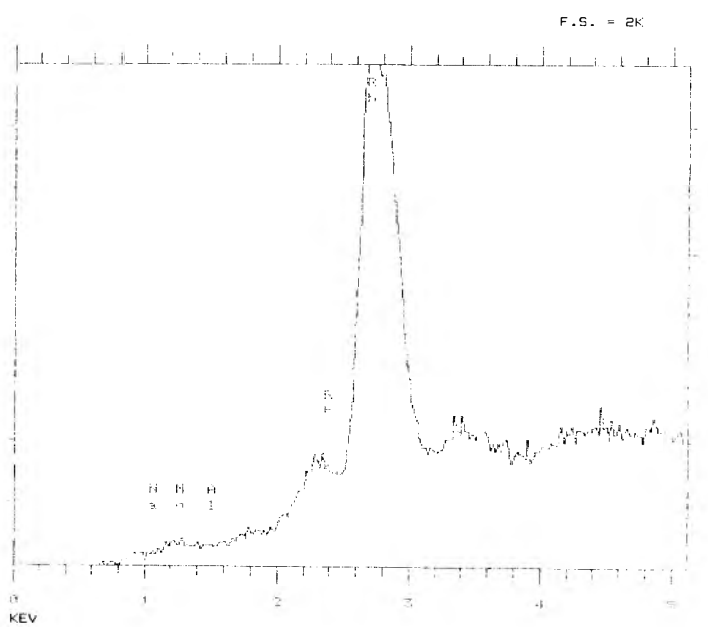

Figure 2. X-ray spectrum of excipient mixture tablet.

Table 1. EDXRF recovery of DASC in formula A

Theory, %[a]	Added, mg/tab.	Found, mg/tab.	Rel. error, %	Recovery, %
80	232.70	237.21	1.9	101.9
80	243.05	239.76	−1.4	98.6
90	278.08	274.32	−1.4	98.6
100	299.38	301.45	0.7	100.7
110	327.44	328.38	0.3	100.3
120	349.89	346.67	−0.9	99.1
120	357.06	357.86	0.2	100.2
			$\bar{X}$	99.9
			% RSD	1.2

[a] Theory = 300 mg/tab.

Note: Since standard curve is composed of spiked placebos, precision and recovery are as good as the fit of the line.

The quadratic curve equation was found to be: Intensity = $-0.03448X^2 + 116.1X + 14.55$; X = amount in mg anhydrous DASC per tablet; correlation (R^2) = 0.9997.

by impact grinding and pressing 900 (±50) mg DASC reference material into 2040 (±50) mg of pelletizing medium as directed under impact grinding and pelletizing procedures.

(d) *Standard value calculation.*—Express standards as mg anhydrous dihydroxyaluminum sodium carbonate per 1.42 g tablet:

$$\% \text{ Anhydrous DASC} = (W \times P)/(W + W_1)$$

Anhydrous DASC, mg/1.42 g tablet

$$= (\% \text{ anhydrous DASC}/100) \times 1420$$

where W = weight of DASC, mg as is; P = % purity of DASC (by USP procedure); W_1 = weight of pelletizing medium, mg.

Procedure

(a) *Preparation of sample pellets.*—Break 2 antacid tablets into several pieces and proceed as directed under impact grinding and pelletizing procedures.

(b) *Spectroscopy.*—See Figure 1 for typical spectrum of the sample or standard. Figure shows sodium peak at ca 1.0 keV and aluminum peak at about 1.5 keV, present from DASC in pellet. Rhodium at 2.4–3.0 keV is present from X-ray source.

Table 2. EDXRF recovery of DASC in formula B

Theory, %[a]	Added, mg/tab.	Found, mg/tab.	Rel. error, %	Recovery, %
80	241.84	250.32	3.5	103.5
80	247.15	247.45	0.1	100.1
90	270.50	277.80	2.7	102.7
100	299.97	293.00	−2.3	97.7
110	330.92	338.68	2.3	102.3
120	353.63	355.54	0.5	100.5
120	362.60	371.29	2.4	102.4
			$\bar{X}$	101.3
			% RSD	2.0

[a] Theory = 300 mg/tab.

Note: Since standard curve is composed of spiked placebos, precision and recovery are as good as the fit of the line.

The quadratic curve equation was found to be: Intensity = $-0.018882X^2 + 117.43X - 75.385$; X = amount in mg anhydrous DASC per tablet; correlation (R^2) = 0.9932.

Table 3. Instrument precision[a]

Observation	Formula A, mg/tab.	Formula B, mg/tab.
1	302.3	285.6
2	302.4	285.3
3	300.2	288.1
4	296.1	292.5
5	300.8	289.9
6	296.9	293.7
7	297.2	286.6
8	298.1	287.4
$\bar{X}$	299.3	288.6
% RSD	0.84	1.08

[a] Theory = 300 mg/tab.

Spectrum of excipient mixtures of formula A is shown in Figure 2. Spectrum show traces of magnesium at about 1.25 keV, present from magnesium stearate in the pellet. There are no detectable levels of sodium or aluminum in this sample and, therefore, no interference in the DASC methodology.

Results

Recovery studies.—Placebo formula A was prepared by blending pulverized sugar, starch, and magnesium stearate in appropriate concentrations as outlined in the antacid formula. Dihydroxyaluminum sodium carbonate was added at levels equivalent to 80–120% of dosage theory. Recovery was 99.9% with an RSD of 1.2% (Table 1). Placebo Formula B was prepared by blending pulverized sugar, starch, corn syrup, magnesium stearate, and water in appropriate concentrations as outlined in the antacid formula. Dihydroxyaluminum sodium carbonate was added at levels equivalent to 80–120% of dosage theory. Recovery was 101.3% with an RSD of 2.0% (Table 2).

Since standard curve and recovery samples are prepared from spiked placebos, the precision and recovery is reflective of the fit of the curve and sample manipulation.

Precision studies.—Instrument precision was evaluated by reading a single preparation of formulas A and B 8 individual times. Relative standard deviations of 0.84% and 1.08%, respectively, were obtained (Table 3). Method precision was determined and compared to titration and autoanalyzer methods. For both formulas A and B, acceptable RSD values were obtained and were statistically comparable to titration and autoanalyzer precision (Tables 4 and 5).

Evaluation of alternative pelletizing plastic support cup.—A study was conducted to determine possible differ-

Table 4. Formula B precision data[a]

Observation	X-Ray	Titration	Autoanalyzer
1	302.6	297.4	330.9
2	293.3	301.0	333.9
3	297.8	319.6	318.7
4	276.5	303.3	330.9
5	287.8	301.2	300.5
6	276.5	305.3	310.0
7	297.5	302.3	300.5
8	297.5	299.1	318.7
$\bar{X}$	291.2	303.6	318.0
% RSD	3.4	2.3	4.2

[a] Theory = 300 mg/tab. Units = mg anhydrous DASC/1.42 g tab. Spec cap reinforcement used.

Note: Batch content uniformity is affecting these data.

Table 5. Formula A precision data[a]

Observation	X-Ray	Titration	Autoanalyzer
1	302.6	304.5	312.6
2	302.1	306.0	318.7
3	308.7	317.7	330.9
4	307.7	301.8	321.7
5	310.5	316.3	318.7
6	299.5	319.0	318.7
7	294.3	325.0	306.6
8	311.4	310.3	333.9
$\bar{X}$	304.6	312.6	320.2
% RSD	2.0	2.6	2.8

[a] Theory = 300 mg/tab. Units = mg anhydrous DASC/1.42 g tab. Spec cap reinforcement used.
Note: Batch content uniformity is affecting these data.

Table 6. Plastic cup precision data for formula A[a]

Observation	Anhydrous DASC, mg/tab.
1	295.1
2	297.3
3	296.6
4	297.6
5	286.6
6	299.8
7	304.0
8	289.7
$\bar{X}$	295.8
% RSD	1.9

[a] Theory = 300 mg/tab.
Note: Batch content uniformity is affecting these data.

ences between plastic and aluminum pelletizing support cups. Table 6 summarizes results of 8 individual analyses of Formula A in plastic. The recovery and precision are comparable with results obtained in aluminum (Table 5).

Evaluation of single tablet assay.—A study was conducted to determine if a single tablet could be pelletized for assay vs the 2 tablets presently used. The results indicate that single and double tablet assay results are comparable (Table 7).

Table 7. Single tablet pellet precision data for formula A[a]

Observation	Reinforcement	Anhydrous DASC, mg/tab.
1	spec cap	291.9
2	plastic cup	284.7
3	spec cap	287.1
4	plastic cup	292.7
5	spec cap	306.3
6	plastic cup	287.2
7	spec cap	291.8
8	plastic cup	285.6
$\bar{X}$		291.9
% RSD		2.4

[a] Theory = 300 mg/tab.
Note: Batch content uniformity is affecting these data.

Discussion

The EDXRF automated procedure described for the determination of DASC in antacid tablets shows excellent precision and accuracy. The new methodology is a substantial improvement with time savings, less sample preparation, no solvent usage, and automatic data handling, and is nondestructive. These advantages should translate into considerable opportunity for cost savings in manufacturing.

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ENVIRONMENTAL ANALYSIS

Slurries Introduction in Flow Injection Atomic Absorption Spectroscopic Analysis of Sewage Sludges

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The direct introduction of slurries in flow injection-flame atomic absorption spectroscopy has been studied using a single-channel manifold. The influence of flow injection parameters on the sensitivity and accuracy of this procedure has been established. A method has been developed for the determination of copper, manganese, and lead in sewage sludge where batch digestion of the samples in a microwave oven is carried out before dilution. Both sensitivity and limit of detection of the flow injection analysis procedure are adequate for the analysis of actual samples (the limit of detection was 0.06, 0.05, and 0.3 ppm for copper, manganese, and lead, respectively). The accuracy of the proposed method, determined from the analysis of 2 reference samples from the European Community Bureau, is better than that obtained by the continuous aspiration of the samples.

The introduction of slurries in atomic spectroscopy allows a direct analysis of solid samples, and, moreover, problems arising from the dissolution step can be avoided. This approach is especially suitable for the analysis of organic samples, because of problems related to the destruction of these matrixes.

It is well established that slurries can be analyzed by electrothermal atomic absorption spectroscopy (1–4). However, when flame atomic spectroscopy or inductively coupled plasma emission is used, accuracy of results depends on the particle size of the dispersed samples (5–7).

The development of rapid acid extraction procedures, used before sample dilution, makes possible the solubilization of metallic elements without a complete destruction of the matrix (8, 9). When a slurry that has been treated by acid extraction is introduced into the flame, atomization of the sample is based on atomization of water-solubilized elements, not the slurry. This fact reduces the problems associated with particle size. In addition, slurry introduction avoids the need for filtration steps, which are time-consuming and could cause losses of the elements to be determined.

The determination of metallic elements in sewage sludges and in soils treated with these products is very important to determine their possible effects on crops and their introduction in the trophic chain (10).

Slurries of solid samples have been analyzed by electrothermal atomic absorption (1–3, 11–13) and by flame atomic absorption (5, 14, 15). In the latter case, the particle size of the suspended material is critical in the direct determination of the total content of metallic elements in soil and rocks. Therefore, a grinding time between 7 and 8 h in a ball-mull micronizer (5) and 0.5 h in a Fritsch pulverizer, using an agate ball-mull (15), is required to obtain an adequate particle size.

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We have developed a fast and simple method for the determination of a series of elements in sewage sludge samples that is independent of the particle size and requires only previous digestion of the sample with nitric acid in a microwave oven (16). The method presents only 2 drawbacks: problems from the use of strong acid samples, which could damage the nebulizer system, and problems due to clogging. Using a Perkin Elmer 3030, no clogging problems have been found. However, when thin nebulizer systems are used, clogging could be a serious drawback and thus the discrete introduction of slurries could be more advantageous.

The use of flow injection flame atomic absorption spectroscopy (FI-AAS) allows good precision and high frequency in the introduction of discrete samples (17–19). If the suspended material does not cause plugging in the manifold, the procedure avoids the problem of acid attack of the nebulizers.

So far, the introduction of slurries for FI-AAS analysis of metallic elements has not been reported. In the present work, copper, manganese, and lead have been determined by introducing aqueous slurries of the samples, after a treatment with nitric acid in a microwave oven. The effects of the experimental and FIA parameters have been studied and the results obtained are compared with those found by the continuous aspiration of slurries into the flame.

Experimental

Apparatus

(a) *Flame atomic absorption spectrometer.*—Pye Unicam SP 1900, equipped with hollow cathode lamps of copper, manganese, and lead. Experimental parameters were optimized to obtain best sensitivity for all elements considered. (Table 1 summarizes these parameters.)

(b) *Manifold.*—Monochannel FIA (flow injection analysis) manifold (Figure 1), with Gilson Minipuls 2 HP-4 peristaltic pump, Rheodyne injection valve type 50 with peristaltic pump, Rheodyne injection valve type 50 with different fixed loops and PTFE tubes with 0.8 mm internal diameter. In the experiment carried out at low pump flow rate, a custom-made PTFE "T" piece interface was used to provide air, water, or ethanol compensation of aspiration flow of nebulizer.

(c) *Microwave oven.*—To carry out pre-analysis digestion of sewage sludge samples: Balay BAHM-100 microwave oven, frequency 2450 MHz, maximum power 650 watts. Pyrex glass containers, with Teflon joint and heat-resistant cap (Schott Duran), used for pressurized acid extractions.

Reagents

Standards.—1000 ppm copper, manganese, and lead stock solutions were prepared from pure analytical reagents. Standard solutions were prepared by dilution. HNO_3 was added at the same concentration as in the sample slurries.

Table 1. Instrumental parameters

Parameter	Element		
	Cu	Mn	Pb
Wavelength, nm	324.75	279.48	283.31
Lamp current, mA	3	6	3
Slit width, mm	0.18	0.15	0.18
Burner height, mm	9	9	8
C_2H_2 flow, L/min	0.9	1.2	1.2
Air flow, L/min	4.5	4.5	4.5

Procedure

Sewage sludge samples were treated with HNO_3 in sealed vessels, using microwave oven to provide fast acid extraction of metallic elements. Afterward, deionized water was added to samples, and slurries were prepared by manual shaking.

Discrete volumes of the slurries were injected in the FIA manifold and analyzed by flame atomic absorption.

The effect of FIA parameters on the sensitivity and accuracy of the sewage sludges analysis was checked.

Results and Discussion

FIA Parameters

Using the manifold indicated previously, the experimental FIA parameters such as pump flow rate, reaction coil length, and sample loop volume were modified to obtain a better sensitivity and accuracy.

Standards containing 15% (v/v) HNO_3, 10 ppm lead, and 3 ppm manganese and others containing 4 ppm copper and 3% (v/v) HNO_3 were used to check sensitivity. At the same time, discrete volumes of a sewage sludge sample were introduced to note the effect on the values for sample concentration obtained when FIA parameters are modified.

Figures 2 and 3 show the effect of pump flow rate on sensitivity and accuracy for each element. When the pump flow rate increases, the peak height corresponding to samples and standards increases; the highest sensitivity is obtained using the maximum possible flow rate for the nebulizer.

The concentrations of these elements were compared to those found in a previous study. Horizontal lines in Figures 2A, 2B, and 2C indicate the confidence level of the previous established values.

Except at lower flow rates, accurate values were obtained in all the experimental conditions studied. However, peak definition was better (Figure 2B) and the sensitivity was maximum at high pump flow rates. Thus, pump flow rates higher than 8 mL/min can be recommended.

In some cases, however, lower pump flow rates may be necessary. Some discussions have dealt with the most appropriate way to ensure adequate experimental conditions and better sensitivity in all cases (20–22).

Some of the proposed ways to increase sensitivity at low flow rates are the use of an air (21) or organic solvent (22) compensation. In the determination of lead at a pump flow rate of 3 mL/min, the slopes of the calibration line obtained using water, ethanol, and air compensation were respectively, 2.55, 3.78, and 4.5 mm/ppm. Thus, air or ethanol compensation can improve sensitivity of FI-AAS analysis at lower pump flow rates.

An increase in the coil length increases the dispersion of the samples and thus reduces the sensitivity obtained for the 3 elements studied (Figures 4A, 4B, and 4C). Accuracy does not seem to be affected by this parameter.

The sample injection volume affects both the sensitivity and accuracy of the analysis by FI-AAS (Figures 5A, 5B, and 5C). A large sample injection volume provides high sensitivity and accuracy in all cases.

From these studies, we selected a pump flow rate of 8.4 mL/min, a coil length of 80 cm, and a sample loop of 500 μL.

Recommended Method

Weigh 0.5 g dried and homogenized sample in 100 mL Pyrex glass reactor, add 5 mL concentrated HNO_3 and 2 or 3 drops of isoamyl alcohol (as antifoam agent), seal reactor and place in microwave oven 3 min at 80% maximum power.

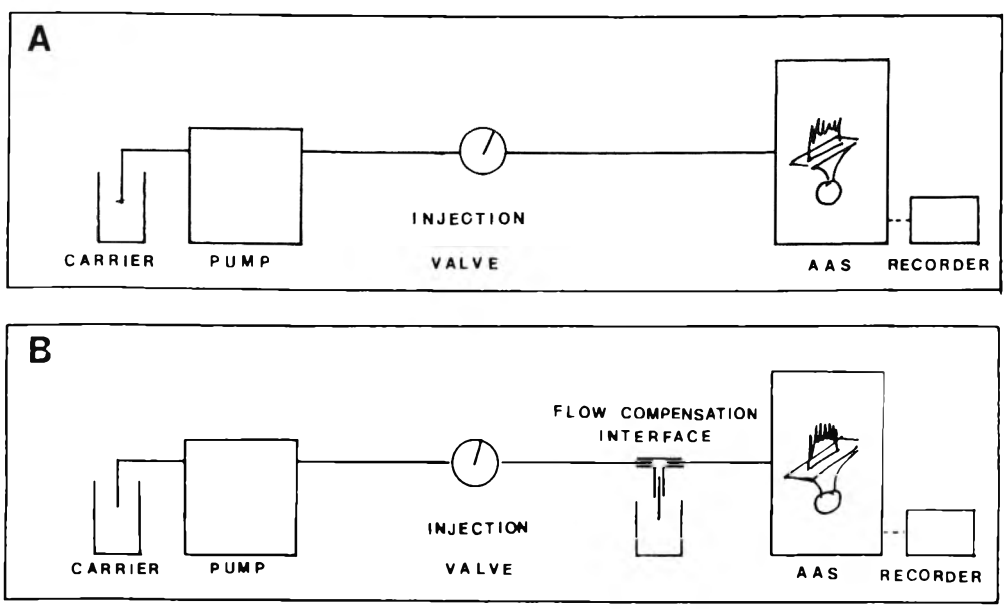

Figure 1. FIA manifold used in determination of copper, manganese, and lead in sewage sludges: A, directly coupled FIA-AAS system; B, FIA-AAS coupling by means of connector interface.

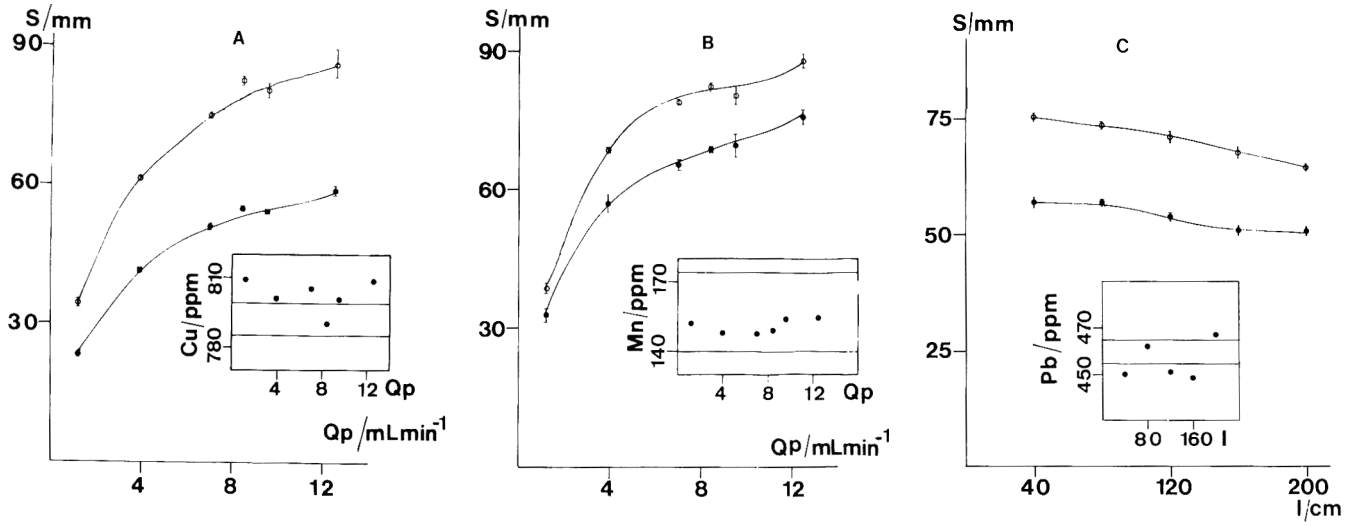

Figure 2. Effect of pump flow rate (Qp) on sensitivity and accuracy in determination of copper (A), manganese (B), and lead (C) by FI-AAS. S/mm-height in mm of transient peaks obtained. O = Standard of copper (3 ppm), manganese (4 ppm), and lead (10 ppm). ● = Slurry of a sewage sludge sample.

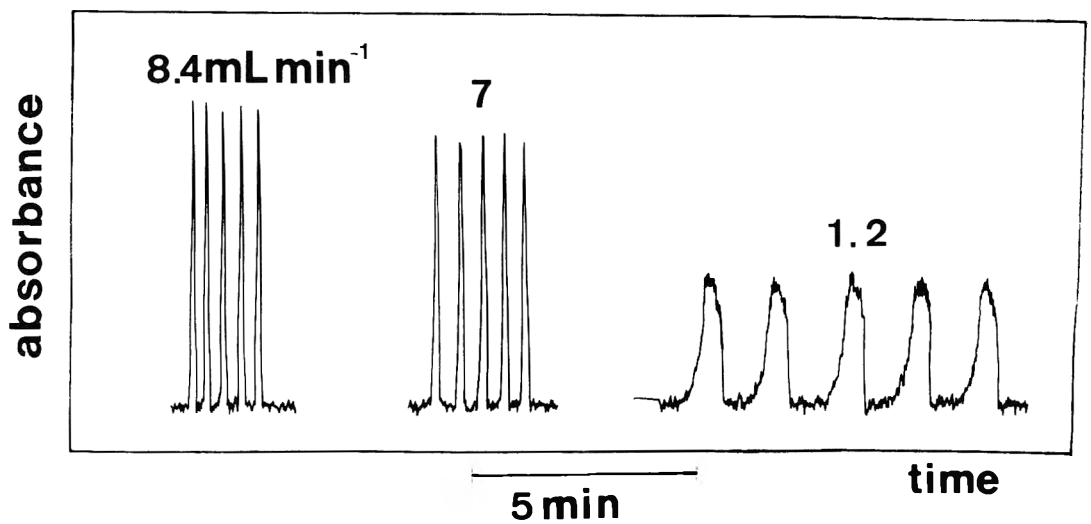

Figure 3. Lead standard at different pump flow rates.

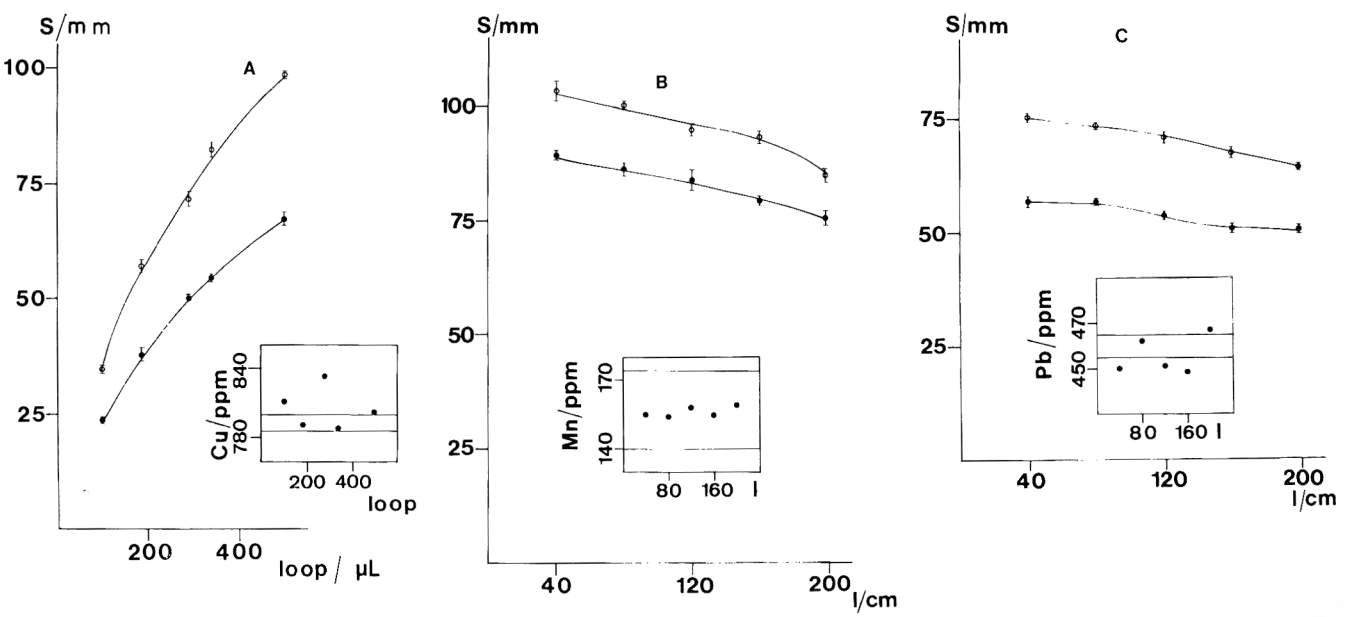

Figure 4. Effect of coil length (l) on sensitivity and accuracy in analysis of copper (A), manganese (B), and lead (C) by FI-AAS. O = Standard of copper (3 ppm), manganese (4 ppm), and lead (10 ppm). ● = Slurry of a sewage sludge sample.

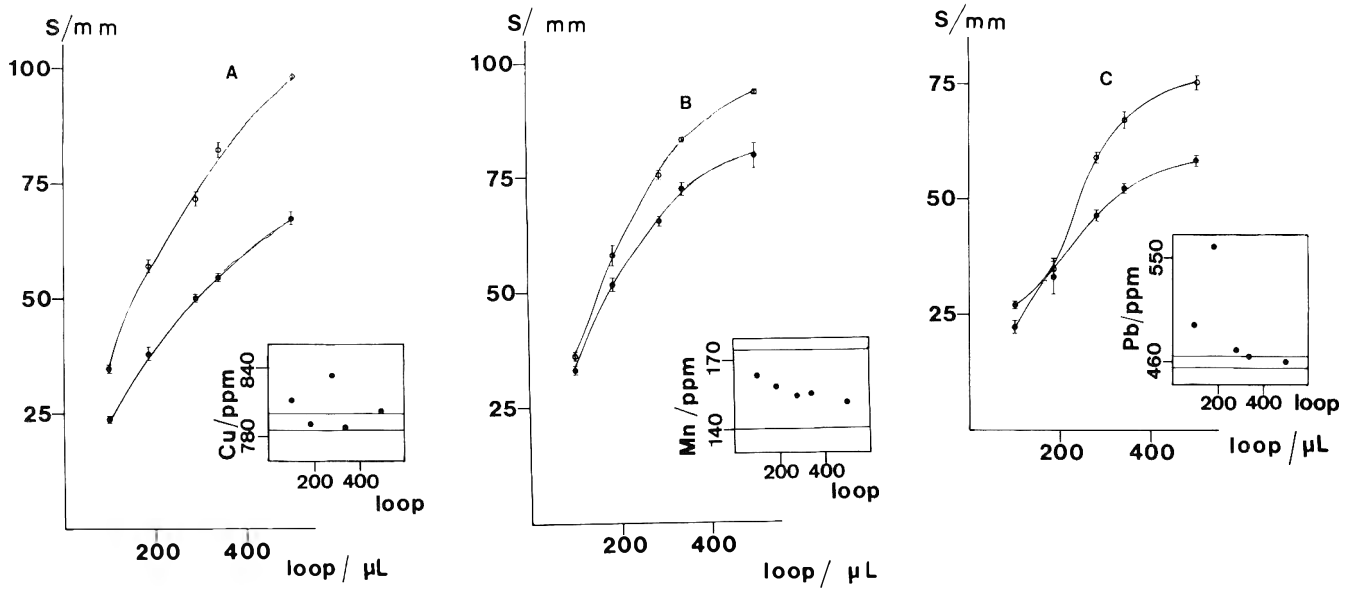

Figure 5. Effect of injected volume on sensitivity and accuracy in analysis of copper (A), manganese (B), and lead (C) by FI-AAS. O = Standard of copper (3 ppm), manganese (4 ppm), and lead (10 ppm). ● = Slurry of a sewage sludge sample.

Table 2. Analytical parameters[a]

	FIA			Batch		
Element	Analytical curve	r	LD/ppm	Analytical curve	r	LD/ppm
Cu	0.015 + 0.078 C	0.9998	0.06	0.015 + 0.078 C	0.99994	0.06
Mn	0.007 + 0.101 C	0.9998	0.05	0.006 + 0.105 C	0.9998	0.05
Pb	0.006 + 0.011 C	0.9995	0.3	0.001 + 0.011 C	0.99992	0.3

[a] r = regression coefficient; LD = limit of detection.

Table 3. Analysis of sewage sludges[a]

	Certified, ppm			FIA, ppm			Batch, ppm		
Sample	Cu	Mn	Pb	Cu	Mn	Pb	Cu	Mn	Pb
Sewage sludges	—	—	—	790 ± 10	144 ± 5	446 ± 5	765 ± 16	136 ± 3	419 ± 7
Domestic origin: (CRM 144)	713 ± 26	449 ± 13	495 ± 19	700 ± 2	437 ± 1	462 ± 19	643 ± 12	401 ± 3	440 ± 30
Industr. origin: (CRM 146)	934 ± 24	588 ± 24	1270 ± 28	944 ± 16	532 ± 9	1349 ± 14	857 ± 70	500 ± 20	1260 ± 50

[a] Standard deviation is used to indicate experimental variability of data.

Let cool and open reactor. Dilute sample with 25 mL water. Determine lead and manganese directly in slurry. For copper determination, take 5 mL slurry and dilute to 50 mL with water.

Inject 500 µL slurry, without separating dispersed solid, in FIA manifold and determine the concentration of analytes, using aqueous standards with the same content of HNO_3.

The results were compared to those obtained for batch and the continuous aspiration system, after sample digestion in the microwave oven. Table 2 summarizes the typical regression lines obtained for each element considered, both in FIA and in batch. Absorbance units per ppm have been used in the analytical curves for both procedures. As can be seen, comparable sensitivity and limit of detection have been obtained.

Analysis of Sewage Sludge Samples

One actual sample and 2 reference samples of sewage sludges were analyzed; results are summarized in Table 3. The FIA method provides results comparable to the certified values, within the confidence levels established. On the other hand, results for batch analysis are in all cases lower than those found for the FIA procedure. This contrasts with the accurate results previously found by using another instrumentation, and could be due to problems arising when dispersed samples with large particle sizes are introduced in nebulizers with different designs and sizes.

The studies carried out demonstrate that use of flow injection methodology can be used without loss of sensitivity to avoid problems originating when slurries of solid sample are

introduced in flame atomic absorption spectroscopy. The direct injection of a high acid slurry minimizes clogging of the nebulizer and its attack by the acids. When sewage sludge slurries are introduced by flow injection, a previous acid extraction of the metallic elements is necessary. No significant absorbance values were obtained when samples without previous treatment were introduced in the flame.

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FEEDS

Amino Acid Analysis of Feeds in The Netherlands: Four-Year Proficiency Study

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To improve accuracy and precision of amino acid analysis in 12 Dutch feed laboratories, a proficiency study was organized twice annually over a 4-year period. The method used included reflux acid hydrolysis for 22 h followed by evaporation, separation on a cation-exchange resin in an amino acid analyzer, and photometric detection after post-column derivatization with ninhydrin. For the determination of sulfur-containing amino acids, samples were oxidized prior to hydrolysis. For the determination of tryptophan, samples underwent an alkaline hydrolysis excluding oxygen, were separated by liquid chromatography on a Hypersil ODS analytical column, and were assayed by UV or fluorescence detection. The average relative standard deviations within (CV_r) and between (CV_R) laboratories were 3 and 7%, respectively. For some mixed feed samples, the results from the proficiency study were compared with those obtained by the International Analytical Group. For those samples, the relative standard deviation of the difference between IAG and Dutch groups was only 1.9%. For samples that were analyzed twice during this 4-year period, relative standard deviation of between-series differences was only 2.1%.

Much time and effort is spent on laboratory accreditation to assure that laboratories produce results under high quality conditions. To demonstrate and control accuracy and precision over time, participation in collaborative studies is desirable. For feed laboratories performing amino acid analysis in The Netherlands, a national proficiency study (1) has been organized twice annually.

For amino acid analysis by ion-exchange chromatography, the most widely used procedures are based on the work of Schram et al. (2) and Moore et al. (3). Using the method as described by the former, Slump (4) showed that the optimal hydrolysis time was about 22 h with 6 mol/L of HCl under reflux at boiling temperatures (110°C). Correction factors were introduced for the thermo-labile amino acids threonine and serine, and the hydrolysis-resistant amino acids valine and isoleucine.

After hydrolysis, acid is removed in a vacuum evaporator at 45°C until 2–3 mL water is left. Then the residue is mixed with citrate buffer, pH 2.2. Bech-Andersen et al. (5) and Mason et al. (6) have shown that evaporation of the acid could be replaced by neutralization. In practice, the resulting high salt load on the ion-exchange column caused peak broadening and cannot be applied on all ion-exchange resins (7).

The sulfur-containing amino acids methionine and cysteine are oxidized prior to hydrolysis (8). Chloride and water interfere in the determination of methionine and cysteine as methionine sulfone and cysteic acid, respectively (9).

Sample size depends on the crude protein content (10), although Bech-Andersen et al. (5) showed that this is of little practical importance for recovery.

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Most methods for determining tryptophan specify defatting the samples followed by oxygen-free alkaline hydrolysis with lithium hydroxide in vacuum tubes. No significant difference was found with the use of barium hydroxide (unpublished results). Crucial for good results is the use of a vessel from which oxygen can be easily removed (7).

Although no methods are prescribed in a proficiency study, participants mainly applied the procedure as described by Schram et al. (2), using Biotronic LC-2000 or LKB-Alfa Plus amino acid analyzers. To evaluate accuracy and precision during the 4-year study period, a standard mixture was circulated to study recovery, and some samples were offered more than once. There was also the opportunity to compare precision and accuracy of the Dutch group with the results on the same materials by the International Analytical Group (IAG) of the "Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungs-anstalten" (VDLUFA) in Germany.

In the 4-year period, a common method (11) for determination of tryptophan, specifying liquid chromatography with fluorescence or UV detection, was studied and prepared for adoption as a national standard.

Results of the studies were discussed annually and contributed to a better control of the analytical process and an improved comparability among laboratories.

Experimental

Apparatus and Conditions

(a) *Amino acid analyzer.*—LKB-Alfa Plus or Biotronic LC 2000. Micro columns, 4 × 200 mm, containing cation-exchange resins (sulfonated crosslinked polystyrene, particle size 8 μm) were obtained via analyzer manufacturers. Amino acids were separated, depending on analyzer, with 3 or 4 citrate/borate pH buffers (pH range from 2.8 to 10; flow rate 35 mL/h, temperature range 55 to 80°C). Amino acids were derivatized post-column with ninhydrin. Photometric detector wavelengths were 570 nm for alpha amino acids, 440 nm for proline. Volume of sample injected ranged between 20 and 100 μL. Analysis time for one run was ca 90 min. After each run, column was regenerated with 0.2 mol/L of NaOH containing 1% EDTA. All elution buffers were filtered through 0.2 μm filters (Millipore) and preserved with thymol.

Participants calibrated their systems with a mixture (Ultrapure) of amino acids that included norleucine as an internal standard.

(b) *Hydrolysis and evaporation apparatus.*—Heating device with temperature control for 400 mL conical flasks or destruction bloc (Tecator), capacity 20 glass hydrolysis vessels, 40 × 220 mm. All vessels were provided with coolers. Rotary evaporators at 45°C were used for evaporation.

(c) *Alkaline hydrolysis apparatus.*—Oven at 120°C for 24 mL screw-cap tubes with Teflon valve (20 × 100 mm).

(d) *Liquid chromatograph.*—With UV or fluorescence detector. Tryptophan was separated with 0.06 mol/L of ace-

Table 1. Mean recoveries and relative standard deviations for chromatography of amino acid standard mixture and meat meal hydrolysate

	Meat meal hydrolysate			Standard mixture		
Amino acid	Mean rec., %	CV_r, %	CV_R, %	Mean, g/kg	CV_r, %	CV_R, %
Lys	96.7	1.3	5.6	34.1	1.9	6.0
His	97.3	1.0	5.4	15.3	1.6	8.5
Arg	97.4	1.0	6.4	33.8	1.6	3.2
Cys	98.5	1.5	6.6	4.9	7.1	13.1
Asp	97.5	1.0	6.1	46.1	1.1	7.1
Thr	98.7	1.6	4.0	21.0	1.3	3.0
Ser	100.7	0.9	3.9	25.5	1.3	2.4
Glu	100.6	2.3	5.3	66.4	1.1	4.2
Pro	92.9	3.0	11.7	38.2	1.6	6.7
Gly	96.5	1.6	5.4	52.9	1.9	2.7
Ala	98.9	1.5	6.1	39.5	1.8	3.8
Val	100.9	1.2	3.7	31.4	1.7	6.3
Met	98.8	2.0	5.5	7.8	1.7	12.3
Ile	99.7	1.6	4.2	16.6	1.2	5.3
Leu	101.3	2.3	7.4	42.4	1.0	3.4
Tyr	96.2	1.5	6.8	14.8	1.5	9.6
Phe	98.2	1.0	5.5	23.4	2.2	6.1

tate buffer (pH 4.8) containing 80 mL acetonitrile/L as mobile phase over Hypersil ODS (C18) analytical column (Microbore 20 × 2.1 mm; 40 000 plates per meter) and assayed by UV detection at 217 nm or fluorescence detection (excitation at 283 nm, emission at 343 nm).

Reagents

(a) *Hydrochloric acid.*—6 mol HCl/L (Merck, p.a.) in deionized water containing 1 g phenol/L.

(b) *Performic acid.*—Prepared by adding 3.0 mL 30% H_2O_2 to 27 mL 88% formic acid containing 4.73 g phenol/L. The solution was allowed to stand at room temperature for 1 h and then was cooled to 0°C in ice before use.

Sample Preparation

Samples included in the proficiency studies were as follows: a standard solution (1985), meat meal (1985-1/2), mixed feed (1984, 1985), soybean meal (1984, 1986), fish meal (1987), maize meal (1987), and pig feces (1985). In the years 1985, 1986, and 1987, mixed feed samples from IAG (VDLUFA) studies were also included in the proficiency studies and were used for the study on the estimation of tryptophan. The standard solution used for calibration contained 5 μmol/mL of each of the individual high grade amino acids (Pierce). A hydrolysate for meat meal prepared in one laboratory was also circulated for analysis.

According to recommendations of ISO 6498 (12), samples of fish meal, meat meal, and pig feces were freeze-dried and stored at 4°C; the other samples were air-dried at 70°C. All dry samples were separated into portions over an electronically driven conical sample divider.

Preparation of Hydrolysates

The procedure as described by Schram et al. (2) as applied by most participants; 2 of them neutralized the hydrolysates instead of evaporating (6).

(a) *Unoxidized samples.*—A feed sample, containing 5–15 mg N/100 mL HCl (6 mol/L) were accurately weighed into the hydrolysis vessel; norleucine was added as internal standard. Samples were hydrolyzed 22 h in boiling acid on reflux.

The hydrolysate was quantitatively filtered through paper and either the complete hydrolysate or an aliquot was evaporated to 5 mL. Twice the volume of water was added and solvent was again evaporated. The final solution was transferred to a 100 mL volumetric flask, diluted to volume with citrate buffer at pH 2.2, and stored in a refrigerator.

Before use, the hydrolysates were brought to room temperature and a suitable aliquot was filtered through a 0.2 μm membrane filter. Between 40 and 200 μL was injected onto the cation-exchange column.

(b) *Oxidized samples.*—A sample containing ca 30 mg N was accurately weighed into a 250 mL round-bottom flask and cooled 30 min in ice water. Fifteen mL freshly prepared and cooled oxidation mixture was added to the sample and the mixture was refrigerated in an ice bath for 16 h. After oxidation, 4.5 mL of 48% HBr and a few drops of octanol

Table 2. Mean (g/kg), relative standard deviation (%), repeatability, and reproducibility (g/kg) of amino acid analysis in meat meal in 2 tests[a]

Amino acid	Mean (1)	Mean (2)	CV_r (1)	CV_R (1)	CV_r (2)	CV_R (2)	r (1)	R (1)	r (2)	R (2)
Lys	35.5	36.3	2.4	4.0	1.5	4.7	2.4	4.0	1.6	4.8
His	17.0	18.4	2.1	15.0	3.9	8.8	1.0	7.2	2.0	4.6
Arg	36.0	37.0	3.1	4.4	0.8	3.6	3.2	4.5	0.9	3.7
Cys	5.5	5.4	3.2	9.6	2.0	19.3	0.5	1.5	0.4	2.9
Asp	48.9	51.0	1.3	2.9	1.1	4.3	1.8	4.0	1.6	6.1
Thr	22.8	22.9	2.8	5.4	1.1	4.8	1.8	3.5	0.7	3.1
Ser	27.2	28.4	2.2	8.6	1.2	5.8	1.7	6.6	0.9	4.7
Glu	71.2	70.3	1.4	3.0	1.1	1.4	2.8	6.1	2.2	2.7
Pro	40.7	39.4	4.9	8.9	2.1	6.8	5.6	10.2	2.5	7.6
Gly	58.0	57.0	1.7	4.5	1.1	7.7	2.8	7.4	1.8	12.4
Ala	41.7	41.6	1.2	4.0	1.3	4.1	1.4	4.7	1.5	4.8
Val	33.0	33.9	1.5	8.4	1.8	3.3	1.4	7.8	1.7	3.1
Met	8.4	9.2	2.1	17.2	4.1	6.9	0.5	4.1	1.1	1.8
Ile	18.0	18.2	1.6	12.2	1.8	4.6	0.8	6.2	0.9	2.4
Leu	45.4	46.4	1.8	3.1	3.2	5.1	2.3	4.0	1.6	6.7
Tyr	15.9	16.1	2.4	4.2	1.8	6.2	1.1	1.9	0.8	2.8
Phe	24.3	25.3	1.6	4.1	1.8	4.8	1.1	2.8	1.3	3.4

[a] (1) = test 1985-1; (2) = test 1985-2.

Table 3. Comparison of mean (g/kg), relative standard deviation (%), repeatability, and reproducibility (g/kg) of amino acid analysis in soybean meal[a]

Amino acid	Mean (1)	Mean (2)	CV_r (1)	CV_R (1)	CV_r (2)	CV_R (2)	r (1)	R (1)	r (2)	R (2)
Lys	27.8	28.2	2.1	2.3	1.6	5.5	1.7	1.8	1.3	4.4
His	11.8	11.7	4.4	4.9	4.5	6.1	1.5	1.7	1.5	2.0
Arg	31.9	30.8	1.6	4.6	3.2	6.1	1.5	2.9	2.8	5.3
Cys	7.2	6.6	3.1	10.7	2.1	10.6	0.6	2.2	0.4	2.0
Asp	51.7	50.4	2.2	6.0	1.9	6.9	3.3	8.8	2.7	9.9
Thr	18.1	17.9	2.5	6.4	2.6	9.2	1.3	1.5	1.3	4.7
Ser	24.5	25.4	3.0	6.5	1.9	5.1	2.1	4.5	1.3	3.6
Glu	81.8	79.5	1.1	2.1	1.1	4.8	2.6	4.8	2.6	10.8
Pro	23.4	22.6	3.4	17.1	3.1	9.4	2.3	11.2	2.0	6.0
Gly	19.9	19.5	1.5	2.0	2.6	4.5	0.8	1.1	1.4	2.5
Ala	18.7	18.9	1.2	9.6	2.1	4.8	0.7	5.1	1.1	2.6
Val	20.7	22.2	2.1	8.2	1.6	9.7	1.2	4.8	1.0	6.1
Met	6.5	6.3	5.1	15.4	2.4	9.4	1.0	2.8	0.4	1.7
Ile	19.8	21.3	3.0	8.8	1.7	6.5	1.9	4.9	1.0	4.0
Leu	33.9	33.4	2.1	2.9	1.4	6.6	2.1	2.8	1.3	6.3
Tyr	15.8	16.0	2.0	8.2	2.8	7.5	0.9	3.7	1.3	3.4
Phe	21.6	21.3	2.7	4.8	2.6	8.2	1.7	2.9	1.6	5.0

[a] (1) = test 1984; (2) = test 1986.

were added while the flask was swirled in an ice bath. When the flask was clear of bromine vapors, the HCl solution was added and the samples were hydrolyzed as described under (a).

(c) *Tryptophan.*—A 200 mg sample containing less than 20 mg moisture was accurately weighed into a 24 mL screw-cap tube; 1 drop of octanol, 100 mg starch, and 8 mL solution of 4 mol/L of LiOH was added. The solution was placed in a freezer for 30 min and then evaporated during mixing on a vortex mixer. Then the tube was placed in an oven at 120°C for 12 h.

After hydrolysis, the mixture was cooled and transferred to a 50 mL volumetric flask, 2 mL acetic acid (98%) was added, and the solution was diluted to volume with double-distilled water. The samples were stored in a refrigerator. Before use, the hydrolysates were brought to room temperature and a suitable aliquot was filtered through a 0.2 μm membrane filter. Between 4 and 20 μL was injected onto the LC column.

Correction Factors

Slump (4) used the method of Schram et al. (2) and studied the effect of hydrolysis time (up to 150 h) on the recovery of individual amino acids on a large number of feeds. From the destruction rate for a 22 h hydrolysis period, correction factors derived for threonine and serine were 1.05 and 1.10, respectively. Similarly, correction factors for incomplete hydrolysis of isoleucine and valine were 1.07 and 1.08, respectively. These factors have been applied systematically in this proficiency study.

Collaborative Studies

In general, the proficiency studies have been organized according to the International Recommendations (1, 13).

Calculations and Statistics

Variance analysis and other statistical calculations were done with the Genstat program (14). Terminology for accuracy is used according to ISO 5725 (15) and Horwitz (1): r = repeatability ($2.83 \times s_r$) and R = reproducibility ($2.83 \times s_R$) in g/kg; coefficients of variation (relative standard deviations) are $CV_r = (s_r/m) \times 100\%$ and $CV_R = (s_R/m) \times 100\%$, where m is the amino acid content averaged over laboratories after elimination of outlier results.

To evaluate between series and between research group standard deviations (SED), a test of paired data was used. The average difference d and SED were calculated and Student's t-test was applied to test for the zero hypothesis, H_0: $[d/(SED/\sqrt{n})] > t_{0.05,n}$, where n = number of paired values.

Results and Discussion

Recoveries

The recovery obtained through the chromatographic system was measured with a standard mixture. Mean recoveries and relative standard deviations (CV) are given in Table 1. The average recovery was 98%. Mean recovery of proline, which was measured at 440 nm, was 93%; all other mean recoveries were between 96 and 101%. Although repeatability is adequate, reproducibility is poorer for proline than for the other amino acids. Isoleucine may be inadequate as an internal standard for proline.

To determine the effect of hydrolysis on the precision of analysis, a meat meal hydrolysate (same as in Table 1) was circulated and the results were compared with those for meat meal samples (Table 2) hydrolyzed in the participating laboratories. The coefficients of variation (CV_r = 1.5% and CV_R = 5.2%) of the circulated *hydrolysate* were only slightly better than those of the circulated *samples* (CV_r = 1.9%, CV_R = 5.6%). The hydrolysis procedure was well standardized.

Tables 3–6 show the proficiency results for the individual samples. In general, the average precision was similar for all feeds, including the Dutch mixed feeds. The average CV_r was 3% and the average CV_R was 7% with no significant differences between sample materials.

For the mixed feeds obtained from IAG in 1985 and 1986, reproducibility was not as good (CV_r = 3.5 and 3.0%, respectively). When proline, methionine, and cysteine results were excluded from the calculation, CV_r was 2.1% (±1) and CV_R was 6.7% (±4). Although the precision is much improved compared with that reported by Cavins et al. (10), the present oxidation procedure might be inadequate, considering the relatively poor reproducibility values for methionine

Table 4. Comparison of mean (g/kg), relative standard deviation (%), repeatability, and reproducibility (g/kg) of amino acid analysis in mixed feed[a]

Amino acid	Mean (1)	Mean (2)	CV_r (1)	CV_R (1)	CV_r (2)	CV_R (2)	r (1)	R (1)	r (2)	R (2)
Lys	9.0	8.8	1.6	3.5	1.6	5.3	0.4	0.9	0.4	1.3
His	4.6	4.8	1.9	5.1	3.4	19.7	0.2	0.7	0.5	2.7
Arg	11.9	11.1	2.0	3.3	2.6	6.1	0.7	1.1	0.8	1.9
Cys	2.7	2.6	8.6	15.1	2.3	9.0	0.7	1.2	0.2	0.7
Asp	17.3	16.9	2.3	5.6	1.4	3.4	1.1	2.7	0.7	1.6
Thr	6.7	6.8	1.5	4.1	0.9	3.6	0.3	0.8	0.2	0.7
Ser	8.8	9.1	0.4	7.3	2.3	4.0	0.1	1.8	0.4	1.7
Glu	30.2	29.6	1.1	2.9	1.1	2.7	0.9	2.4	1.0	2.3
Pro	9.9	9.5	3.0	7.6	3.6	5.2	0.8	2.1	1.0	1.4
Gly	7.7	7.5	1.2	2.6	1.8	3.1	0.3	0.6	0.4	0.7
Ala	8.7	8.6	1.6	1.6	2.7	4.9	0.4	0.4	0.7	1.2
Val	8.5	8.7	0.8	7.1	1.4	6.7	0.2	1.7	0.4	1.7
Met	3.2	2.9	4.1	13.7	2.3	9.9	0.4	1.2	0.2	0.8
Ile	6.9	7.0	0.9	10.7	2.0	6.7	0.2	2.1	0.4	1.3
Leu	13.3	13.5	1.6	1.6	0.7	2.2	0.6	0.6	0.3	0.8
Tyr	5.7	5.6	2.6	9.0	3.1	10.0	0.4	1.4	0.5	1.6
Phe	7.7	7.7	2.0	11.1	2.5	4.2	0.4	2.4	0.5	0.9

[a] (1) = test 1984; (2) = test 1985.

and cysteine compared with those for the other amino acids.

However, the absolute values for repeatability and reproducibility were similar to those for other amino acids. The relative low contents are probably the main reason. In general, integration is a source of variation because of variable separation of the peaks allowed in routine performances such as these.

Between-Series Effects

Some samples (meat meal, soybean meal, mixed feed) were analyzed in 2 test series (Tables 2, 3, and 4). As stated before, the average precisions were similar in different test series. The average relative standard deviation of the differences between series is 2.1%, which is similar to the average value of CV_r. The average difference between series is not significant ($P < 0.05$).

Comparison Between International and Dutch Tests

In Table 6, the Dutch group results are compared with those of the International Analytical Group (IAG) for the same mixed feed samples. The average CV_r values for the Dutch group for the years 1985, 1986, and 1987 were 3.5, 3.0, and 2.1% (±1.7%) respectively. Mean CV_R averaged over the same 3 years for the Dutch group was 7% (±2.9); for IAG the average was 6.4% (±3.2). On average, the Dutch group values were lower by 0.3 g/kg (d) for the individual amino acid contents, which is not significantly ($P < 0.05$) different from zero. The relative standard deviation of the differences (SED) between research groups averaged over the years 1986 and 1987 was 1.9%, which is of the same level as CV_r. In 1985, this standard deviation was still 3%.

Relative Standard Deviations vs Concentration

The relation between CV and concentration is discussed for lysine. The relative standard deviations for lysine in the different sample materials in simple feed samples are on average 2% for CV_r and 4% for CV_R. For maize meal (Table 5, lysine content 2.7 g/kg), the variation is about twice as

Table 5. Mean (g/kg), relative standard deviation (%), repeatability, and reproducibility (g/kg) of amino acid analysis in fish meal, maize meal, and pig feces

Amino acid	Fish meal					Maize meal					Pig feces				
	Mean	CV_r	CV_R	r	R	Mean	CV_r	CV_R	r	R	Mean	CV_r	CV_R	r	R
Lys	54.5	0.9	2.8	1.4	4.3	2.7	1.9	4.1	0.1	0.3	8.5	4.8	13.9	1.2	3.3
His	15.1	1.9	18.6	0.8	8.0	2.9	1.7	6.6	0.1	0.6	3.2	3.9	37.8	0.4	3.4
Arg	43.9	0.9	5.3	1.2	6.6	4.5	2.7	17.6	0.3	2.2	7.5	4.3	12.5	0.9	2.7
Cys	5.9	2.0	9.5	0.3	1.6	2.1	2.4	14.8	0.1	0.9	2.6	4.6	16.9	0.3	1.2
Asp	63.6	0.8	3.0	1.4	5.5	6.4	1.7	6.4	0.3	1.2	15.0	2.4	5.0	1.0	2.1
Thr	30.1	1.1	3.2	0.9	2.7	3.5	1.4	8.3	0.1	0.8	7.5	0.7	3.1	0.2	0.7
Ser	32.2	1.2	5.4	1.1	5.0	4.8	1.0	8.8	0.2	1.2	7.4	4.2	6.6	0.8	1.4
Glu	99.7	0.7	1.8	1.9	5.2	17.6	1.1	4.3	0.5	2.2	17.4	1.3	3.2	0.7	1.6
Pro	30.8	1.3	7.5	1.1	6.5	8.3	2.1	4.8	0.5	1.1	6.3	3.9	8.7	0.7	1.6
Gly	50.7	1.1	4.7	1.6	6.8	3.5	2.9	6.3	0.3	0.6	8.2	3.5	4.8	0.8	1.1
Ala	46.6	0.9	3.9	1.2	5.2	7.1	1.7	5.1	0.3	1.0	9.5	3.1	8.2	0.8	2.2
Val	36.3	1.0	2.2	1.0	2.3	5.1	2.0	9.0	0.3	1.3	8.8	3.9	8.2	1.0	2.0
Met	20.0	1.6	3.8	0.9	2.2	1.9	3.7	17.9	0.2	1.0	2.9	2.9	14.8	0.2	1.2
Ile	30.1	1.2	4.0	1.0	3.4	3.4	2.7	10.6	0.3	1.0	7.6	2.4	3.3	0.5	0.7
Leu	51.9	1.0	3.6	1.5	5.3	11.6	1.5	3.5	0.5	1.2	11.4	1.2	4.3	0.4	1.4
Tyr	18.8	2.2	10.0	1.2	5.4	4.0	1.8	10.3	0.2	1.2	6.2	1.6	16.4	0.3	2.9
Phe	26.7	1.2	4.9	0.9	3.7	4.6	3.0	5.4	0.4	0.7	7.2	2.8	6.6	0.6	1.4

Table 6. Comparison of means (g/kg) and relative standard deviations (%) found in international (IAG) and Dutch studies of amino acid analysis in mixed feeds MF 85, MF 86, and MF 87

	MF 85				MF 86				MF 87			
Amino acid	Mean	Mean (IAG)	CV_R	CV_R (IAG)	Mean	Mean (IAG)	CV_R	CV_R (IAG)	Mean	Mean (IAG)	CV_R	CV_R (IAG)
Lys	7.3	7.8	5.2	4.3	7.2	7.6	7.9	6.4	10.2	11.0	6.1	3.8
His	4.9	4.7	10.4	11.9	4.4	4.7	11.8	7.7	5.0	4.8	9.2	5.2
Arg	10.9	11.0	9.3	6.9	10.4	10.6	8.8	9.3	11.3	11.5	7.7	4.5
Cys	2.7	2.7	17.4	14.9	2.8	3.3	12.9	16.4	3.0	3.1	9.3	9.3
Asp	15.0	14.9	5.7	5.8	13.9	14.2	5.7	3.9	16.7	17.0	6.4	7.8
Thr	6.7	6.6	3.0	4.0	6.3	6.5	7.5	8.4	7.6	7.1	5.3	5.5
Ser	9.0	8.3	4.6	4.9	8.4	8.3	8.5	8.7	9.2	9.3	4.9	5.5
Glu	29.5	30.1	3.1	8.7	28.9	29.6	4.8	4.6	35.4	35.6	4.9	2.9
Pro	10.2	11.6	3.1	8.7	11.8	11.5	11.2	3.7	13.2	13.2	6.4	0.9
Gly	8.5	8.5	6.7	6.0	8.1	8.3	3.6	3.1	9.0	9.0	4.9	5.8
Ala	10.5	10.7	5.4	4.0	10.3	10.6	5.4	3.3	8.8	9.2	4.9	4.7
Val	9.1	8.5	6.0	6.8	8.7	8.4	9.8	5.4	10.1	9.9	7.3	4.3
Met	4.6	4.7	8.3	14.8	4.4	4.5	8.9	14.0	3.7	4.1	12.7	10.6
Ile	6.9	6.7	5.5	5.8	6.5	6.5	6.5	8.6	7.8	7.7	5.1	4.8
Leu	16.2	15.8	3.9	6.2	15.5	15.9	5.4	3.6	14.8	14.5	3.6	4.5
Tyr	6.1	6.1	9.2	3.7	6.0	6.2	9.0	6.5	6.3	6.5	4.3	6.5
Phe	8.2	8.0	3.5	3.9	7.6	8.2	6.3	7.3	8.7	9.0	3.6	2.2

high as for fish meal (Table 5, lysine content 54.5 g/kg). However, the absolute values for repeatability and reproducibility for maize meal are very good. In the IAG mixed feed MF 87 showed a precision similar to the simple feeds, precision for MF 85 and MF 86 was less good.

In the feces sample analyzed (Table 5), with a concentration of lysine similar to the mixed feed samples, CV_r and CV_R were 4.8 and 13.9%, respectively. Also, reproducibility for histidine, arginine, methionine, cysteine, and tyrosine in this material was poor. It is known that this type of sample is more sensitive to hydrolysis conditions. This needs further investigation.

Tryptophan

The average values for tryptophan found by IAG and the Dutch group were in good agreement in mixed feed MF 87 [2.2 g/kg] but not in MF 85 (IAG = 1.5, and Dutch group = 1.9 g/kg). Because oxygen is the main reason for low values, it is assumed this was the case for the IAG data. In 1985, the equipment used for hydrolysis in both groups had not yet been optimized. Relative standard deviations between laboratories for the IAG test in 1985 (10 participants; CV_R = 16%) was better than for the Dutch test (5 participants; CV_R = 22%), although still much higher than for the other amino acids. By using the same device for oxygen-free hydrolysis, the reproducibility of the 1987 test of mixed feed was improved to CV_R = 11% and for IAG to CV_R = 8%.

Conclusions

The traditional method of Schram et al. (2) is a good basis for the development of a national standard routine method for determining amino acid content of feeds. The procedures for hydrolysis have been standardized; oxidation, calibration, and integration procedures are the remaining sources of between-laboratory variation. In general, the precision of ion-exchange chromatography for amino acid analysis is adequate for application in feed science, manufacturing, and trade. From this study, precision criteria for laboratory quality control and trade can be derived.

The results of the Dutch group on mixed feeds in 3 successive years are in good agreement with the results obtained by IAG on the same samples. A slightly different level was observed. The analysis of feces showed poor precision for several of the amino acids. For tryptophan, the standardized procedure improved the precision considerably.

The organization of proficiency studies on feeds has been taken over by the Dutch Quality Service for Agricultural Laboratories.

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Karl Fischer Determination of Moisture in Soft-Moist Pet Foods: Collaborative Study

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Collaborators: D. E. Florini; F. Frias; D. E. Gamblin; A. V. Gardner; B. Hsiek; C. E. Jones; G. W. Latimer; J. P. Minyard, Jr; J. M. Padmore; M. M. Sharpe; N. Thiex; W. R. Windham; and Daniel H. Mowry (*Committee Statistician*)

Twelve collaborators participated in a study of a Karl Fischer method for determination of moisture in soft-moist, commercially available pet foods. The method avoids the erroneous high results noted in other methods (vacuum drying, toluene distillation, and drying at 135°C) when volatile components other than moisture, such as propylene glycol, are present. The standard deviations for repeatability (s_r) and reproducibility (s_R) were 0.3 and 0.4, respectively. The corresponding relative standard deviations (RSD_r and RSD_R) were 1.1 and 1.7%, respectively. The method has been approved interim official first action by AOAC.

No applicable AOAC method or standard technique exists to determine moisture in soft-moist pet foods that also contain other volatile components such as propylene glycol. The presence of propylene glycol causes erroneous high results when moisture is determined by a drying technique. The following exemplifies the significance of the error: Test rations were prepared in which the ratio of propylene glycol/water was varied but the total combination was kept constant, i.e., the combination was maintained at 25% of the ration, but the ratios were varied at 40/60, 50/50, 70/30, etc. When the samples were analyzed for moisture using AOAC method **930.15** (1), in which samples are dried 2 h at 135°C, the results were the same for all samples.

A method for measuring the moisture content in various soft-moist pet foods, which avoids this problem, has been developed and collaboratively studied. Samples are extracted with anhydrous methanol and analyzed using the Karl Fischer titration technique.

AOAC method **950.02** specifies grinding to prepare feed samples for analysis or, if the sample cannot be ground, it should be reduced to as fine a condition as possible. The soft, pliable nature of soft-moist pet foods makes grinding through a screen unsuitable because the material either blinds the screen or, if forced through, produces extruded strands. The particle size of such samples can be reduced by using a household-style food blender and shaking it back and forth to move the material into the blades.

The results of the collaborative study are reported in the present paper.

Collaborative Study

Thirteen laboratories agreed to participate in the collaborative study. Twelve samples (6 matched pairs as blind duplicates) of ground, commercially available soft-moist dog food and cat food products from 4 different manufacturers were distributed. Each laboratory was asked to perform a single analysis on each sample.

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

Moisture in Soft-Moist Pet Foods
Karl Fischer Method
Interim First Action

(Applicable to samples containing 20–30% moisture and other volatile materials)

Method Performance:

$$s_r = 0.3;\ s_R = 0.4;\ RSD_r = 1.1\%;\ RSD_R = 1.7\%$$

A. Principle

Water is extracted with methanol from pet food that contains other volatile components, and aliquot is titrated with Karl Fischer reagent.

B. Apparatus and Reagents

(a) *Karl Fischer titration assembly.*—Manual or automatic, with stirrer.

(b) *Karl Fischer reagent.*—Stabilized, single solution (Fisher Scientific Co., SK3), or equivalent. (*Caution:* If solution contains pyridine, see safety note on pyridine.) To standardize reagent, add 100 mg H_2O from weighing pipet, or other suitable device, to 30–50 mL pretitrated CH_3OH, and titrate with Karl Fischer reagent. C = mg H_2O/mL reagent.

(c) *Methanol.*—ACS reagent grade. ≥99.8% CH_3OH. ≤0.1% H_2O. ≤0.001% acetone.

C. Sample Preparation

Reduce sample to as fine condition as possible. Household-style blender may be used; shake blender back and forth to move sample into blades.

D. Determination

Accurately weigh 8–10 g prepared sample into erlenmeyer that contains magnetic stirring bar. Add 200 mL CH_3OH, and stopper flask. Stir magnetically 15 min. Let solids settle. Transfer 10 mL aliquot to titration vessel containing pretitrated methanol and titrate with Karl Fischer reagent. Determine blank on 10 mL CH_3OH as above and subtract from sample determination.

$$\text{Water, \%} = [2 \times (\text{mL reagent} \times C)]/\text{g sample}$$

Ref.: JAOAC, May/June issue (1990).

Results and Discussion

A complete set of results was received from each of 12 collaborators (Table 1). Collaborator 7 was unable to participate after the samples were received. Collaborators used a

Table 1. Collaborative results for Karl Fischer determination of moisture (%) in soft-moist pet foods[a]

	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5		Pair 6	
Coll.	1	E	2	F	3	C	4	B	5	D	6	A
1	24.93	26.18	20.33	20.28	24.31	24.12	27.41	26.28	24.79[b]	27.51	24.42	23.86
2	26.27	25.84	21.12	20.92	24.89	24.72	27.04	26.07	26.16	25.92	23.76	23.56
3[c]	24.74	25.84	18.56	20.62	23.25	24.94	24.33	26.61	24.14	26.31	21.77	24.09
4	27.00	26.71	21.00	21.61	25.10	25.70	27.30	27.05	27.10	27.40	24.60	24.54
5	26.17	26.26	21.23	21.37	25.19	25.17	26.65	26.49	26.46	26.48	23.87	23.37
6	26.90	26.90	21.30	21.40	25.20	25.20	27.20	27.30	26.50	26.70	23.90	24.00
8[c]	31.30	39.80	25.10	34.00	29.30	37.90	42.00	38.30	30.90	39.00	28.50	37.30
9	26.03	25.96	20.88	21.36	24.86	25.21	26.31	26.65	26.53	26.65	23.60	23.97
10[c]	27.32	27.36	21.69	22.53	26.52	26.25	28.13	28.22	27.59	28.09	25.40	24.65
11	25.86	25.50	20.88	20.67	24.60	24.79	26.20	26.25	26.16	25.86	23.10	23.35
12	27.30	27.00	22.40	22.00	25.90	26.30	28.00	27.40	27.60	27.30	24.50	24.40
13	26.96	26.72	21.69	21.88	25.52	25.02	26.81	27.06	26.92	26.78	24.47	24.27
Mean[d]	27.12		21.87		25.83		27.96		27.29		24.72	
SD	2.97		2.82		2.80		3.88		2.78		2.92	
CV, %	10.94		12.90		10.84		13.87		10.21		11.80	
Mean[e]	26.36		21.24		25.10		26.86		26.71		23.97	
SD	0.62		0.56		0.53		0.53		0.54		0.46	
CV, %	2.36		2.61		2.13		1.98		2.01		1.92	
RSD_r, %	1.30		1.06		0.94		1.48		0.59		0.92	
RSD_R, %	2.02		2.46		1.97		1.36		1.98		1.74	

[a] Each value is the result of a single determination on a blind duplicate sample.
[b] Outl er by Grubbs test. Data for pair 5 excluded.
[c] Data rejected. See text.
[d] All data included in evaluation.
[e] Outlying and rejected data omitted.

variety of Karl Fischer reagents to perform the titration: the type specified in the method and 2 reagents, Hydranal® and Ericsen®, which do not contain pyridine. Results were comparable for the 3 types. Because collaborators used equipment from different manufacturers, the weight of water used to standardize the Karl Fischer reagent, the amount of pretitrated methanol, and the volume of the aliquot titrated also varied among the participants. However, results were comparable for these variations as well.

The results from collaborators 3, 8, and 10 were indicated to be outliers on the basis of the ranking test (2). Before the data were rejected, the collaborators were contacted and, in each case, an explanation accounted for the result. The expiration date had passed for the Karl Fischer reagent used by collaborator 3. In the laboratory of collaborator 8, one technician analyzed the numbered set of samples, and a second technician analyzed the lettered set. The second technician had no experience in performing Karl Fischer analyses. Collaborator 10 had problems with the air conditioner in the laboratory during the week that the samples were analyzed; the humidity was so high that, "It was literally raining from the ceiling." Results from those 3 collaborators were not rejected as outliers but because of assignable causes.

The result for sample 5 from collaborator 1 was omitted as an outlier on the basis of the Grubbs test (3). The collaborator knew no reason for the value obtained. Table 1 shows statistical data for all values as well as with rejected and outlying values excluded.

Recommendation

The Associate Referee recommends that the Karl Fischer method for determination of moisture in soft-moist pet foods be adopted official first action.

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METALS AND OTHER ELEMENTS

Influence of Automatic Dishwashings and Scrubbings on Release of Lead from Glazed Ceramicware

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Use of lead glazes on ceramicware and the release of lead from finished glazes are reviewed. Single units of ceramicware with initial lead leach levels from <0.1 to 470 μg/mL were subjected to multiple automatic dishwashings with intermediate scrubbing of the ware by plastic fiber pads. Ware was periodically leached with 4% acetic acid solution at room temperature for 24 h, and the solutions were analyzed for lead. Release of lead under these conditions is discussed. Results indicated that neither passive leachings nor multiple dishwashings and scrubbings can predict future lead-release characteristics of glazes and decorations.

The World Health Organization's Committee on Ceramic Foodware Safety (1) recognizes that lead intoxication can occur from improperly prepared lead glaze on ceramicware used for food storage or preparation. The Committee apparently assumes that lead will continue to be used in the glazing of ceramicware because its report states that "...an unglazed pottery presents a different problem due to the fact that it can permit the growth of pathogenic bacteria which potentially may constitute a more serious health hazard than that resulting from the possible exposure to lead and cadmium." They further suggest that glazes are actually hygienically beneficial.

The use of lead compounds in glaze formulations provides the following advantages that have not been totally obtained by other less toxic compounds: (*1*) lead acts as a flux for melting the glaze ingredients (2–4); (*2*) lead imparts a high brilliance to the glaze (2–6); (*3*) because lead causes a lower surface tension in the glaze at lower temperatures, a smoother finished surface results, which covers blemishes (2, 3, 7); (*4*) leaded glazes extend the maturation temperature range and yield a uniform product (2, 3, 6); (*5*) colors tend to be more brilliant under leaded glazing (4, 6, 8, 9); (*6*) the variability of the coefficient of expansion available from lead glazes allows the thermal expansion of the glaze to match the body (i.e., bisque) and eliminates glaze crazing (2, 4); and (*7*) lead tends to prevent devitrification of the glaze (4).

The release of lead from glazes on ceramicware depends on the chemical composition of the glaze and the temperature and length of time that the glaze is fired. There appears to be no empirical means of reliably predicting the lead-release characteristics of a new glaze formulation. Prepared glaze formulations must be fired onto the ware, and the ware must be tested to determine whether excessive lead is released (3, 10). Standard tests for lead release use an acetic acid solution at room temperature to leach (i.e., extract) the interior of the ware for 24 h, and then the lead in the solution is determined by flame atomic absorption spectrophotometry (11, 12). The Food and Drug Administration (FDA) uses this method in regulatory analysis of domestic and imported ceramicware for excessive lead release.

The most serious manufacturing error that can cause excessive lead release is glaze underfiring. This frequently occurs in commercial ware produced by "cottage industry." These potters often use nonfritted, raw lead compounds in their glaze formulation and fire their ware by burning wood on a short heating cycle. Wood fires do not reach temperatures high enough to fix the lead in many glaze formulations (13). For example, ware that releases excessive amounts of lead (908 μg Pb/mL) was refired at a high temperature. After refiring, the ware released substantially less lead (0.1 μg Pb/mL) (13).

The body (i.e., bisque) of ceramicware will often dissolve in the glaze as the glaze becomes molten, thereby changing the chemical composition of the glaze in a layer that has been estimated to be about 3 mils (1 mil = 1/1000 in.) thick (4, 14, 15). Such changes in chemical composition can alter the lead-releasing properties of the glaze in this layer. This effect is of little consequence if the finished thickness of the glaze is greater than 3 mils. Glaze applications of less than 3 mils or thicker glazes that have worn to this level may show changes in lead-release characteristics over time that are quite different from that of the same glaze applied in thicker layers (14). Therefore, a ceramic glaze that has been analyzed to be lead safe by a single initial leaching may not ensure that a product will be free from excessive lead release over its useful life.

There is concern that the use of automatic dishwashers may adversely affect the release of lead from glazed ceramicware. The detergents employed in these machines are more alkaline, and the water temperatures are generally higher than those used for handwashing. These conditions may cause the glaze to deteriorate more rapidly and release excessive amounts of lead. Hellsten (16) found a positive correlation between lead content and corrosion of overglaze colors as a result of dishwashing. Evidently, some components of dishwashing detergents formed complexes with components of the applied colors. These complexes extracted the lead and left behind a soft and easily damaged silica matrix.

Young et al. (17) studied multiple dishwashing in an automatic dishwasher of a "corrosion resistant" and a "less corrosion resistant" glazed enamel. The dishwashing caused surface corrosion of both enamels, and components of the detergent were found incorporated into the surfaces of both eroded enamels. After washing, more lead was leached from the "corrosion resistant" than from the "less corrosion resistant" enamel. Young postulated that during the dishwashing cycle the "corrosion resistant" enamel retained lead that was released during the lead leach test. The "less corrosion resistant" enamel continuously released lead, which left less lead available for extraction in the lead leach test.

Wallace (18) performed 3 dishwashing cycles on an underfired ceramic plate. Dishwashing enhanced the lead release by about 10% relative to the initial leaching. Wallace also

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Table 1. Effect of multiple dishwashings and scrubbings on release of lead (μg/mL)[a] from ceramicware

Item	Ware	Type[b]	Fill vol., mL	Leach 1[c]	2	3	4	5	6	7	8
1	Mug	SHW	315	16.3	2.2	1.7	1.9	1.0	1.0	1.4	1.6
2	Mug	SHW	360	9.8	3.4	4.3	4.6	2.2	3.4	5.4	10.2
3	Bowl	SHW	625	0	(0.2)	1.2	2.3	(0.1)	1.2	1.5	1.5
4	Bowl	LHW	1500	4.1	(0.3)	(0.7)	(0.7)	0	(0.5)	(0.5)	(0.6)
5	Plate	FW	300	110	(0.7)	(0.5)	(0.6)	0	5.3	(0.7)	(0.9)
6	Mug	SHW	340	0	(0.1)	(0.1)	(0.1)	0	0	(0.1)	0
7	Plate	FW	85	2.4	(0.9)	3.7	5.1	2.8	2.0	6.9	9.4
8	Bowl	SHW	630	7.8	1.0	3.1	3.3	(0.4)	2.0	3.0	3.1
9	Plate	FW	160	13.6	5.2	8.5	10.1	3.8	4.9	5.3	8.6
10	Plate	FW	50	470	162	146	110	4.6	5.0	5.1	4.2
11	Plate	FW	500	0	(0.1)	(0.1)	0	0	0	0	0
12	Plate	FW	275	0	(0.1)	0	(0.1)	0	(0.1)	0	0
13	Saucer	FW	35	43.2	17.1	123	117	13.3	18.8	41	18.2
14	Pan	SHW	500	0	(0.1)	(0.2)	(0.2)	0	(0.2)	(0.2)	(0.1)
Av., μg/mL[d]				48.4	13.6	20.8	18.2	2.0	3.1	4.9	4.1

[a] Findings less than detection limit (0.1 μg Pb/mL) are reported as zero; findings between detection limit and quantitation limit (1 μg Pb/mL) are given in parentheses.
[b] SHW, LHW, and FW indicate small hollow-, large hollow-, and flatware, respectively (19).
[c] Leach 1 analyzed in the laboratories of the FDA San Francisco District during routine surveillance.
[d] Average values were determined with findings less than 1 μg Pb/mL considered as zero.

observed the same decreasing lead release in subsequent repeated leachings that is often observed in underfired ware (13). When the ware that had been repeatedly leached was scrubbed with a plastic fiber pad and subjected to additional dishwashing cycles, the lead released was 94% greater than that in the initial analysis.

The present paper describes the investigation of the validity of a single (initial) leaching to determine the stability and safety of glazed ceramicware over extended use as represented by a series of automatic dishwashings and scrubbings.

Experimental

Apparatus

(a) *Automatic dishwasher.*—Undercounter/overcounter washer Model 8658 (Forma Scientific, Marietta, OH).

(b) *Atomic absorption spectrometer.*—Video 22, Model 975 (Thermo Jarrell Ash Corp., Franklin, MA) equipped with lead hollow cathode lamp; absorption monitored at 217.0 nm.

(c) *Inductively coupled plasma atomic emission spectrometer.*—Model Plasma II (Perkin-Elmer Corp., Norwalk, CT). Lead emission was monitored at 220.353 nm with 500 ms sampling time, 0.075 nm survey window, and background correction at ± 0.025 nm.

Sample Preparation

Ceramicware was collected as part of FDA's monitoring program for lead in domestic and imported hollowware. Single units of the ware listed in Table 1 were studied. The initial leach was analyzed in the laboratories of the FDA San Francisco District Office during the routine safety inspection of ceramicware. The lead content of the glaze was unknown. The following automatic dishwashing and scrubbing protocols were used:

(a) *Protocol A.*—(*1*) Scrub food contact surface of ware briskly for ca 30 s with scrub pad and rinse with distilled water; (*2*) wash 3 cycles in automatic dishwasher.

(b) *Protocol B.*—(*1*) Scrub food contact surface of ware briskly for ca 30 s with scrub pad and rinse with distilled water; (*2*) wash 1 cycle in automatic dishwasher.

(c) *Protocol C.*—(*1*) Wash 5 cycles in automatic dishwasher; (*2*) scrub food contact surface of ware briskly for ca 30 s with scrub pad and rinse with distilled water; (*3*) wash 1 cycle in automatic dishwasher.

Extraction Series

Each piece of ware was subjected to the following 8-step regimen. Leach solutions were obtained after each step by the standard method (11, 12). Leach 1—Handwash ware with detergent, rinse with distilled water, and dry; Leach 2—Air-dry for unknown length of time, handwash with detergent, rinse with distilled water, and dry; Leach 3—Protocol A; Leach 4—Protocol A; Leach 5—Protocol A, air-dry 21 days; Leach 6—Protocol B; Leach 7—Protocol C, and Leach 8—Protocol C.

Analysis

Lead was determined in the leach solutions by either flame atomic absorption spectrometry (AAS) (11, 12) or inductively coupled argon plasma atomic emission spectrometry (ICP). AAS and ICP gave equivalent results.

Results and Discussion

Lead findings for all leach solutions are presented in Table 1. In averaging the data, a value of zero was used for all findings below the quantitation limit of the analytical method (1 μg Pb/mL = 10 × detection limit of 0.1 μg Pb/mL).

No automatic dishwashing or scrubbing occurred between Leach 1 and Leach 2, and lead release decreased or remained below the quantitation limit of the analytical method (1 μg Pb/mL) for all ware. The average of Leach 1 of all items was

48.4 μg Pb/mL (n = 14). The average of Leach 2 of all items was 13.6 μg Pb/mL (n = 14), a decrease of about 72%. This illustrates the well known phenomenon of cleansing the glaze by the initial leaching (13, 20), which also frequently occurs during multiple leachings of items that release excessive lead.

Lead levels for items 3, 6, 11, 12, and 14 were below the detection limit for Leach 1 and remained below or near the quantitation limit throughout the automatic dishwashing and scrubbing regimen. The glazes on the ware either did not contain lead or were formulated and fired properly, and the automatic dishwashing and scrubbing had no effect. Of these, only item 3 fairly consistently released lead (1–2 μg/mL) after automatic dishwashing and scrubbing, which indicates that it probably had a lead-based glaze. This ware, a small green glazed bowl with a textured inner surface, developed concentric bands of efflorescence on the outer surface during leaching. These bands are probably the result of stress cracking of the body, which sometimes occurs during manufacturing when textured patterns such as this do not permit ware to properly release from the mold as it dries. The cracks may have allowed the loss of acetic acid through and into the body of the ware.

For items 1, 2, 4, 5, and 8, the lead levels of Leaches 2–8 remained fairly constant throughout the automatic dishwashing and scrubbing regimen. However, there were 2 deviations. The lead level for Leach 8 of item 2 (10.2 μg Pb/mL) slightly exceeded its Leach 1 value and was about 2.4 times the average value for Leaches 2–7. Also, the lead level for Leach 6 of item 5 was 5.3 μg Pb/mL, whereas the findings for its other leaches after Leach 1 were all below the quantitation limit. A qualitative test (21) found the petals of the flower on item 5 to be the source of the lead. These petals had dissolved completely before Leach 2. Thus, automatic dishwashing and scrubbing may cause enhanced release of lead. However, decorations may not always be quickly depleted of lead. A qualitative test (21) found many spots of color on item 8 to be the source of lead. These spots did not visibly change during the study, and the lead levels were relatively constant. During leaching, item 4, a large green glazed bowl with a textured inner surface similar to that of item 3, also developed concentric bands of efflorescence on the outer surface, probably as a result of stress cracking of the body. The acetic acid may have leached from the body as well as the glazing, but the data indicate that lead was not released.

The lead levels of Leaches 2–8 for items 7, 9, and 13 did not decrease to a constant level after Leach 1 as expected, but instead fluctuated below and above the Leach 1 lead level. It appears that automatic dishwashing and scrubbing interact with the ware surface, allowing lead to be released from deeper layers. With item 7, a highly decorated plate, a qualitative test (21) on each color indicated that only the red glaze released lead. Little or no visible deterioration of the appearance of this piece occurred throughout the automatic dishwashing and scrubbing regimen. Item 9, a plate with a heavily textured surface, developed a thick efflorescence on the underside of the ware, probably from stress cracking of the body. As a result, the acetic acid was probably lost into and through the body of the ware. The color on item 13, a highly decorated saucer, was completely removed by contact with the leach solution. Evidently, the clear glazing under the colored decoration continued to release lead throughout the leachings.

The leach solutions of item 10 did not reach a constant lead level until Leach 5. This ware, a highly decorated small plate, also lost its colors completely during the dishwashing and scrubbing regimen. The underlying glaze appears to have released relatively high amounts of lead until the ware was air-dried for 21 days between Leach 4 and Leach 5. The extended period of drying apparently stabilized the glaze. This finding is similar to Smith's report (22) that air-drying and then leaching a second time reduced the lead release from lead crystal glass by about one-third.

Table 2. Current FDA action levels for maximum permitted lead release from earthenware

Category	Action basis	Action level, μg/mL
Flatware (≤25 mm deep)	av. 6 units	7
Small hollowware (<1.1 L)	any one of 6 units	5
Large hollowware (>1.1 L)	any one of 6 units	2.5

The air-drying between Leach 4 and Leach 5 appears to have affected the lead-release characteristics of all lead-releasing ware. The average Leach 5 lead release was about 89% less than that of the Leach 4 value. The average lead level of Leach 6, although slightly higher than that of the average Leach 5 value, was still about 83% less than the Leach 4 average. The apparent stabilization of the glaze continued for Leach 7 and Leach 8 with average lead levels less than Leach 4 by about 73 and 78%, respectively. Hellsten (16) suggested that sodium metasilicate (Na_2SiO_3) in automatic dishwasher detergent forms a gelatinous silicate layer on the surface of the ware. This layer reduces ionic diffusion including that due to ionic lead. These experimental results indicate that if such a barrier is formed, then air-drying is necessary to consolidate the silicate layer and reduce lead diffusion. The hysteresis of the dehydration/hydration of silica gel, which was discussed by Ephraim (23), may also account for the stabilization of the diffusion layer.

Under FDA guidelines (Table 2) (19), items 1, 2, 4, 5, 8, 9, 10, and 13 would not have been acceptable on the basis of the lead results for Leach 1. On the basis of Leach 2, no automatic dishwashing and scrubbing, only items 10 and 13 remained unacceptable. However, by Leach 8, after the dishwashing and scrubbing regimen, item 10 became acceptable and items 2 and 9 were again unacceptable; in addition, item 7, which was acceptable on the basis of Leaches 1 and 2, became unacceptable. Item 13 remained unacceptable throughout the study.

Conclusions

These limited findings indicate that single or multiple passive leachings or multiple dishwashings and scrubbings cannot reliably predict the future lead-release characteristics of typical glazes and decorations. In general, however, the lead levels released by ware with initially low leach levels did not increase, and the lead levels released by ware with initially high leach levels decreased after multiple automatic dishwashing and scrubbings.

Air-drying of ceramicware after detergent washing appears to stabilize or reduce lead release. Further investigation is needed to document this effect, which may increase protection from lead exposure for the consumer.

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Continuous Flow Vapor Generation for Inductively Coupled Argon Plasma Spectrometric Analysis. Part 1: Selenium

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Total selenium is determined by inductively coupled plasma (ICP) atomic emission using hydride vapor generation. A 1 g sample is wet ashed in a 16 × 150 mm 10 mL volumetric test tube on a programmed heating block with nitric, sulfuric, and perchloric acids at up to 310°C. After treatment with hydrochloric acid, the selenium is reduced by sodium borohydride to hydrogen selenide in a simplified continuous flow manifold. A standard pneumatic nebulizer effects the gas-liquid separation of H_2Se, which is quantified by ICP atomic emission at 196.090 nm. The instrument detection limit for the method has been determined to be 0.4 μg/L. For a 10:1 dilution of a nominal 1 g sample, the detection limit is 4 μg/kg and the linear range is up to 4 mg/kg. The method has demonstrated statistical control for samples of biological and environmental interest and is especially well suited to analysis of small samples.

The accurate and precise determination of total selenium in samples of biological interest has been complicated by the natural partitioning of this metalloid into organic and inorganic forms. These forms, which include selenate, selenite, selenide, alkyl-selenium, and seleno-amino acids, all maintain a distinct set of physical-chemical properties that preclude a casual approach to analysis. Furthermore, there may be severe chemical or spectroscopic interferences to analysis.

Selenium in biological matrixes has been routinely determined by several methods including hydride generation atomic absorption spectrometry (HG-AAS) (1-3), graphite furnace atomic absorption spectrometry (GF-AAS) (3-6), fluorometry (7-10), and gas chromatography (11-13). HG-AAS has been highly successful in most applications within the limits of well studied matrix interferences (14-16). Analysis of selenium by HG-AAS is complicated by a narrow working concentration range, which can require dilution and reanalysis for some samples.

Significant effort has been placed on selenium analysis by inductively coupled argon plasma (ICP) with atomic emission detection (17, 18) and mass spectrometric detection (19). Samples have been introduced using both pneumatic nebulization and vapor generation (20-24). The gain in analytical sensitivity using hydride vapor generation with ICP-atomic emission spectroscopy has made it a most desirable technique, however, the application usually requires specialized apparatus (25-27).

The essential elements of all hydride generation methods for selenium determination are first to release the selenium from the matrix and oxidize it to selenate, second to reduce the selenate to selenite, third to reduce the selenite to hydrogen selenide, and fourth to separate the H_2Se vapor for

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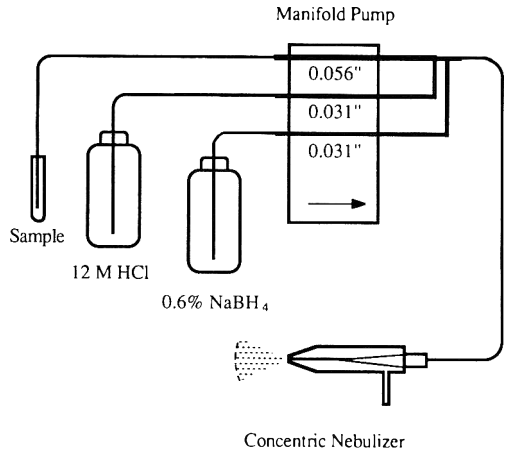

Figure 1. Continuous flow manifold for H_2Se vapor generation.

quantitation (1–3). Kaiser et al. (28) and Welz et al. (29, 30) have shown that digestion of selenium at a high temperature in nitric, sulfuric, and perchloric acids completely destroys the organic matrix and liberates selenium from even the most resistant complexes. We developed modifications to allow for small sample analysis, sample matrix variation, and automation of sample preparation and analysis. We describe a simple approach to continuous selenium vapor generation in which a standard pneumatic nebulizer becomes the gas-liquid separator.

METHOD

Equipment and Apparatus

(a) *Inductively coupled argon plasma atomic emission spectrometer.*—Model 3520 sequential ICP (Applied Research Laboratories, Dearborn, MI) with DPS 4.1 instrument and data analysis software.

(b) *Nebulizer.*—Meinhard TR-30-A2 concentric, with flush tip.

(c) *Autosampler.*—ISIS (ISCO, Inc., Lincoln, NE).

(d) *Mixing manifold for H_2Se vapor generation.*—Four-channel peristaltic pump (Minipuls 2, Gilson, Middleton, WI) with Viton pump tubing, 1.4 mm id, one each; 0.76 mm id, two each (Elkay Products, Inc., Shrewsbury, MA, Cat. Nos. LK-116-0651-120 and LK-116-0651-070) and polypropylene barbed tees 1.4 mm id (Cole-Parmer, Chicago, IL; Cat. No. YB-06365-77). Tubing to autosampler, reagent reservoirs, and nebulizer is 1.6 mm PTFE. Small pieces of Viton tubing connect tees to each other and to PTFE tubing. Figure 1 shows connections.

(e) *Temperature-controlled sample digester.*—Digestion System 40, 1016 Digester, and Autostep 1012 Controller (Tecator, Sweden). Fit an adapter plate of 3.2 mm aluminum with 17 mm holes on top of heater block to hold digestion tubes.

(f) *10 mL digestion tubes.*—Kimax 10 ± 0.12 mL graduated culture tube, 16 × 150 mm (No. 45071, Kimble Science Products, Toledo, OH).

Instrument Operating Parameters

(a) *Sample pump speed.*—Sample uptake about 3 mL/min; HCl and $NaBH_4$ uptake rates about 0.8 mL/min at maximum pump speed. Ratios of sample to acid and sample to reductant are determined by diameters of manifold tubing. Flow rate is not critical as long as it is stable throughout run.

(b) *Argon flows.*—Carrier 1.0 L/min; coolant 12.0 L/min; plasma gas 0.8 L/min. These are standard settings recommended by manufacturer.

(c) *ICP operating conditions.*—Pre-integration time 60 s; three integrations 10 s each; sampler rinse time 15 s; wavelength 196.090 nm; off-peak background correction at +0.032 and −0.040 nm; photomultiplier bias 110; RF forward power 1150 W at 27 MHz; reflected power <10 W.

(d) *Calibration points.*—0.0, 10.0, and 300 μg/L; blank subtraction was not used.

Reagents

(a) *Concentrated hydrochloric, nitric, and perchloric acids.*—Trace-metal analysis grade (Instra-Analyzed, J. T. Baker, St. Louis, MO).

(b) *Concentrated sulfuric acid.*—Ultrapure (Seastar Chemicals, Ltd, Seattle, WA). Selenium is common contaminant in sulfuric acid and manufacturers do not routinely analyze for it; test each new lot against lot of known performance.

(c) *Selenium stock standard.*—1000 mg/L of Se as SeO_2 in water (EM Science, Cherry Hill, NJ).

(d) *5M Hydrochloric acid.*

(e) *Sodium borohydride solution.*—0.6%. Dissolve four 0.3 g pellets of $NaBH_4$ (98%, pellet, Alfa/Ventron, Danvers, MA) in 200 mL 0.5% (w/v) sodium hydroxide. Prepare fresh solution daily.

(f) *Liquid argon.*—Welding grade.

(g) *Water.*—Prepare in NANOPure II (Sybron/Barnstead, Boston, MA) deionizer with charcoal filtration.

(h) *Fe, Zn, Cu, Cd, Pb, and Hg stock solutions.*—1000 mg/L atomic absorption standards (EM Science, Cherry Hill, NJ).

(i) *Selenium compounds.*—Selenomethionine (Sigma Chemical Co., St. Louis, MO); sodium selenate (Aldrich Chemical, Milwaukee, WI); trimethylselenonium iodide (Organometallics, Inc., East Hampstead, NH) dried in vacuum.

(j) *Reference materials.*—(*1*) Bovine Liver SRM 1577a [National Institute of Standards and Technology (NIST), Gaithersburg, MD]. (*2*) Trace Elements in Water SRM 1643b (NIST). (*3*) Dogfish muscle reference material for trace metals, DORM-1 [National Research Council of Canada (NRCC), Ottawa, Ontario, Canada]. (*4*) Dogfish liver reference material for trace metals, DOLT-1 (NRCC). (*5*) Lobster hepatopancreas marine reference material for trace metals and other elements, TORT-1 (NRCC).

(k) *Standards.*—Intermediate standards are 1.00 and 10.0 mg Se/L 0.25M HCl. Prepare standards in 10 mL digestion tubes by pipetting 0.100 mL of 1.00 mg/L intermediate for 10.0 μg/L standard and 0.300 mL of 10.00 mg/L intermediate for 300 μg/L standard. Also prepare a blank. Digest blanks and standards in same manner as samples.

Sample Preparation

(a) Weigh 1.0–1.5 g wet tissue, or weigh 0.25–0.50 g desiccated tissue, or pipet 1 mL blood into 10 mL digestion tube. Label tubes with black permanent marking pen. For desiccated tissue samples, add about 0.5 mL water and vortex-mix. Add 3.0 mL concentrated nitric acid and one PTFE boiling stone to each tube. Let mixture react at room temperature for about 1 h, with occasional gentle swirling. Using the digester, heat by ramp from ambient to 70°C in 1 h.

Check that dissolution is complete, then heat by ramp from 70 to 175°C in 30 min and hold 90 min or until all solutions are reduced to about 2–3 mL. Cool to room temperature. Add 1.0 mL sulfuric acid and 1.0 mL perchloric acid and mix gently. A program found to minimize bumping and boilovers is outlined below:

Temp.,°C	Ramp, min	Hold, min
190	30	60
210	10	20
250	20	20
310	20	25

Since sample heating and cooling rates vary significantly with the exact type of digester heating block, hold times and ramps will require empirical adjustment.

Cool to room temperature, and slowly add 8 mL 5M hydrochloric acid. Add first few drops of acid very slowly with swirling to prevent spattering. Vortex-mix solution, then return tubes to digester to reduce at 95°C for 15 min. Cool to room temperature, and dilute to 10 mL mark with deionized water. Vortex-mix tubes thoroughly.

(**b**) Modify above procedure for other samples as follows: For water analysis, add 1.0 mL sulfuric acid and one PTFE boiling chip to a 10 mL sample. Heat at 105°C overnight or until volume is reduced to about 2–3 mL. Add 1.0 mL perchloric acid and continue digestion procedure. For analysis of skimmed or homogenized milk, add 1 mL concentrated nitric acid and one PTFE boiling chip to a 3 mL sample. Heat by ramp to 175°C in 30 min, and hold until volume is reduced to about 2 mL and continue with digestion procedure. For specimens with more than 4 mg/kg and less than 40 mg/kg of Se, use 0.5–1.0 g sample. After nitric acid dissolution, dilute to 10.0 mL with water. Transfer 1.00 mL aliquot of digested solution to 10 mL digestion tube and continue with digestion procedure incorporating dilutions into calculations. Starchy plant matter dissolves rapidly in nitric acid, but tends to boil dry at this stage; after initially vigorous dissolution, add additional 1.0 mL nitric acid.

Interference Study

Prepare set of 100 μg Se/L solutions by pipetting 0.100 mL of 10.0 mg Se/L intermediate standard into series of digestion tubes. Pipet in appropriate volumes of 1000 mg/L stock metal solution to yield desired concentrations of interfering ion. Add 1.0 mL $HClO_4$ and 1.0 mL H_2SO_4 to each. Continue with sample preparation procedure beginning with 190°C digestion. Prepare blanks, controls, and calibration standards in same manner. Analyze samples in normal fashion. Metals tested in this way were Fe and Zn up to 150 mg/L; Cu up to 15 mg/L; and Pb, Cd, and Hg up to 5 mg/L.

Sample Analysis

Connect hydride generation manifold with concentrated hydrochloric acid introduced at first mixing tee and sodium borohydride solution introduced at second tee. Ignite torch, and then start manifold pump. Adjust reflected power to below 10 W. Let system warm up and stabilize for 20 min.

Calibrate instrument with 0.0, 10.0, and 300 μg Se/L standards at beginning of each run. Root-mean-square relative deviation (automatically computed by DPS software) should be less than 4% for an acceptable calibration. Analyze a 10.0 μg Se/L standard after every 20 samples to verify that drift is within acceptable limits.

Results and Discussion

We have applied this procedure to a variety of biological matrixes, and found it satisfactory in nearly every case. In practice, vapor-generation ICP (VGICP) maintains a linear working range of 0.004 to 4 mg/kg of Se, assuming a 1 g sample and a nominal 10-fold dilution. A 3-order of magnitude working range minimizes the analytical difficulties found with the relatively narrow concentration range of HGAA and GFAA analysis. Use of the Meinhard nebulizer as a gas–liquid separator proves to be an accurate and efficient method of sample introduction into the ICP system. The hydride generation manifold displayed in Figure 1 is simple, inexpensive, and easily maintained in routine operation. The sample preparation process requires only a modest amount of sample, needs little supervision during the digestion, requires no quantitative liquid transfers, and allows batch sizes of 40 tubes.

The instrument detection limit is calculated as 3× SD of the intensity of the blank standard (which is integrated thrice) times the slope of the calibration line. In practice, this value is 0.4 to 0.6 μg/L of Se. Replicate analysis of samples is used in each batch to evaluate the repeatability of the procedure. The relative standard deviations for 82 pairs of blood samples determined over a period of 5 months gave a log-normal distribution where 90% of the pairs have <12% RSD and the geometric mean was 1.6% RSD. Forty-five pairs of liver samples had <8% RSD at the 90th percentile and a geometric mean of 1.5% RSD. These statistics include the uncertainties of both sampling and determination.

No chemical interferences were observed in the hydride generation process for iron up to 150 mg/L, copper up to 15 mg/L, zinc up to 150 mg/L, lead up to 5 mg/L, and cadmium up to 5 mg/L, which were the highest levels tested. This is consistent with previous work (14). Mercury caused an 8% depression at the lowest level tested, 1 mg/L, and a 16% depression at 5 mg/L. Response of the digestion procedure to the chemical form of the selenium moiety was tested using solutions of sodium selenate, trimethylselenonium iodide, and selenomethionine; Se recoveries were respectively 97–100%, 101–105%, and 99–100%, which is consistent with Welz et al. (29). The off-peak background correction points at +0.032 and −0.040 nm were selected after scanning a prepared Se standard under VGICP conditions.

It is important to note that use of the $NaBH_4$ reducing agent contaminates the ICP nebulizer and torch assembly with substantial boron residue. Apparently, volatile boron compounds in the waste trap are slowly released and cause an unstable background in subsequent boron determinations. Hence, boron analysis will require a separate nebulizer, torch, and waste assembly.

Memory effects have not been seen in routine sample analysis, but can occur under some circumstances. Samples that contain Se greater than 10 mg/kg will have a memory effect. Metal deposition in the manifold occurred rarely with samples containing very high levels of copper or mercury that caused fluctuating memory effects. The hydride generator and nebulizer can be cleaned in situ by substituting concentrated nitric acid for the sodium borohydride solution; dilute aqua regia will remove metal deposits in a few minutes.

The nebulizer is not being used as a means of introducing liquid into the plasma, but rather as a means of stripping

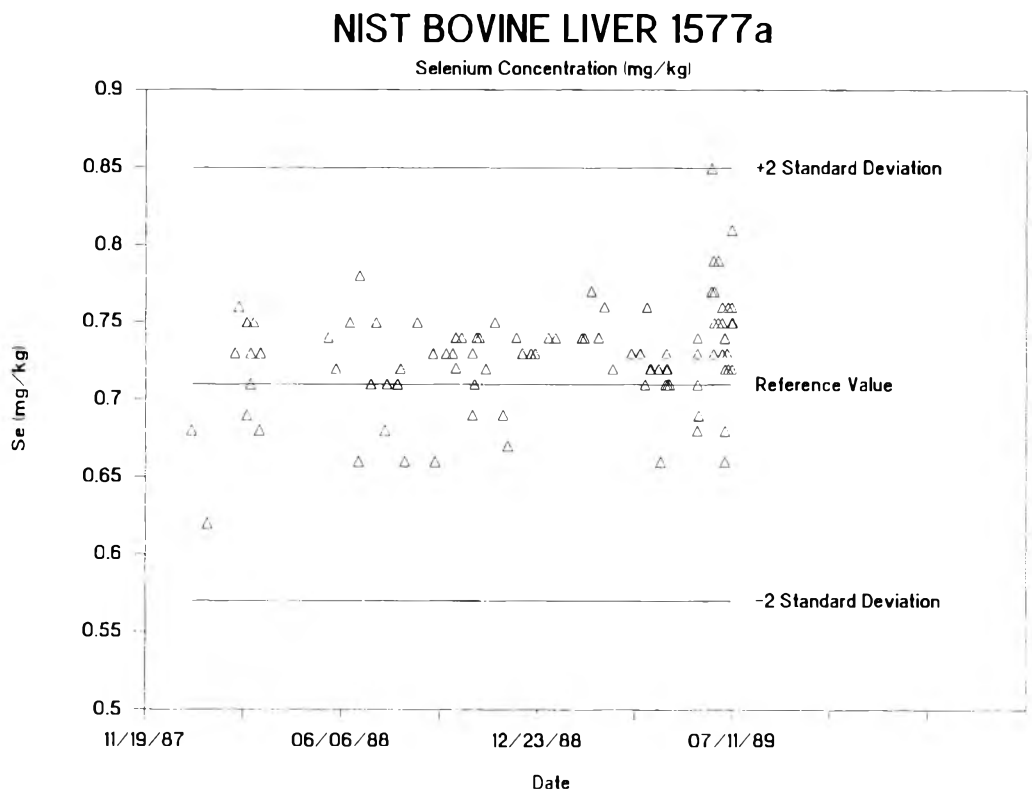

Figure 2. NIST Bovine Liver 1577a control chart for VGICP selenium method.

volatile H_2Se from the liquid and introducing that into the plasma. We operate the nebulizer in near-standard conditions so that switching between VGICP and pneumatic nebulization requires no adjustment of the carrier flow. Salt deposition in the nebulizer and torch has not been a problem; this is likely due to the automatic tip flush and the water presaturation of the carrier gas that are standard features of this ICP system.

The digestion programs were optimized empirically. The main criteria for optimizing were (*1*) to minimize loss of Se at

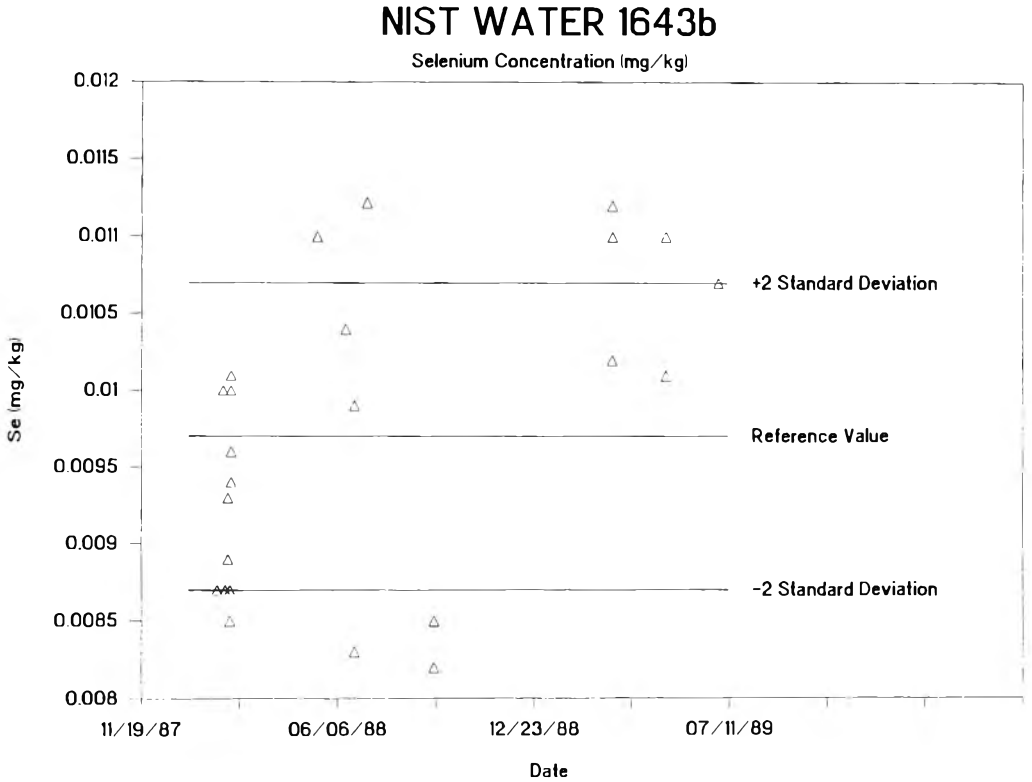

Figure 3. NIST Trace Elements In Water 1643a control chart for VGICP selenium method.

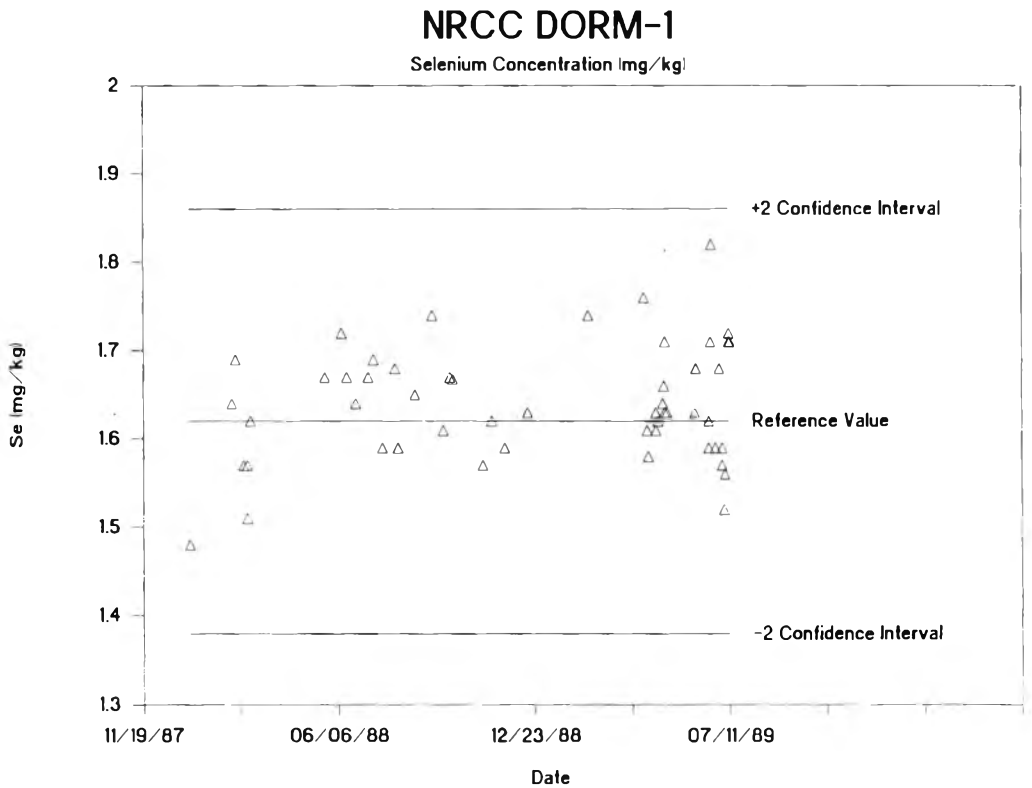

Figure 4. NRCC DORM-1 control chart for VGICP selenium method.

the highest temperature, (*2*) to prevent the tubes from bumping and boiling over, and (*3*) to reduce foaming at the start of digestion. The heating period at 310°C must be long enough to expell all the $HClO_4$, but not so long as to volatilize the selenium. When dense white fumes first form in the upper portion of the digestion tubes, the digestion is complete. Overheating will cause poor repeatability and the calibration curve will become nonlinear.

The ruggedness of the VGICP selenium method was tested in the analysis of numerous standard reference materials over an 18-month period. These included SRM 1577a Bovine Liver, SRM 1643b Trace Elements in Water, DORM-1

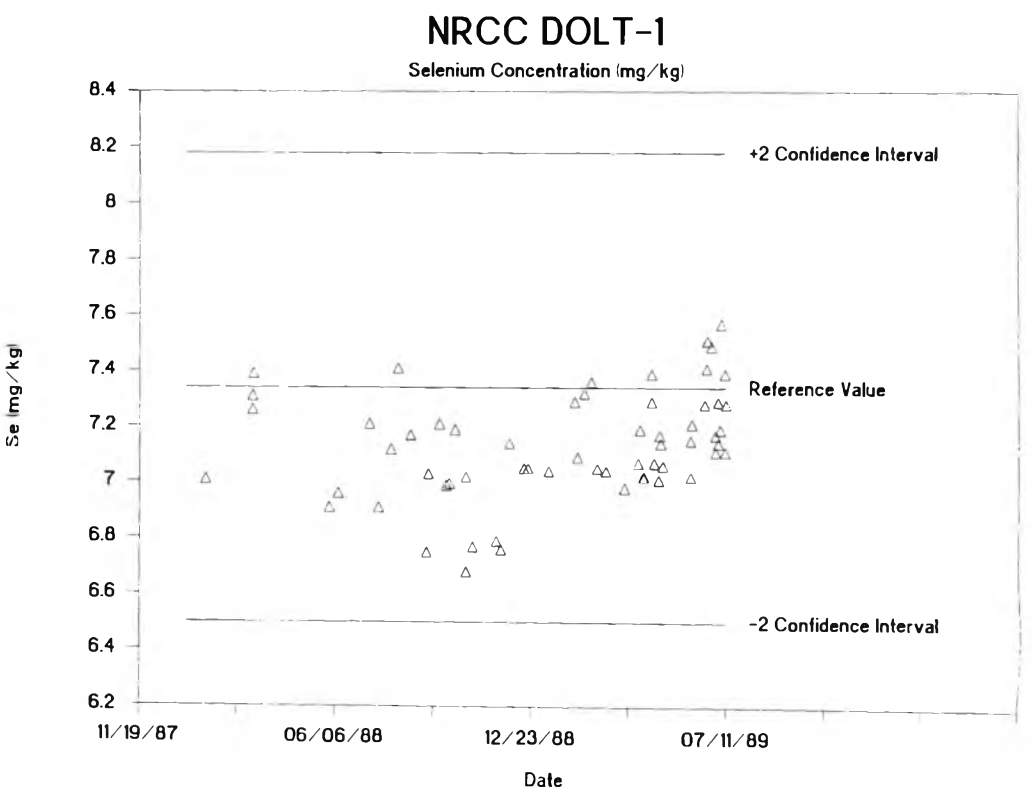

Figure 5. NRCC DOLT-1 control chart for VGICP selenium method.

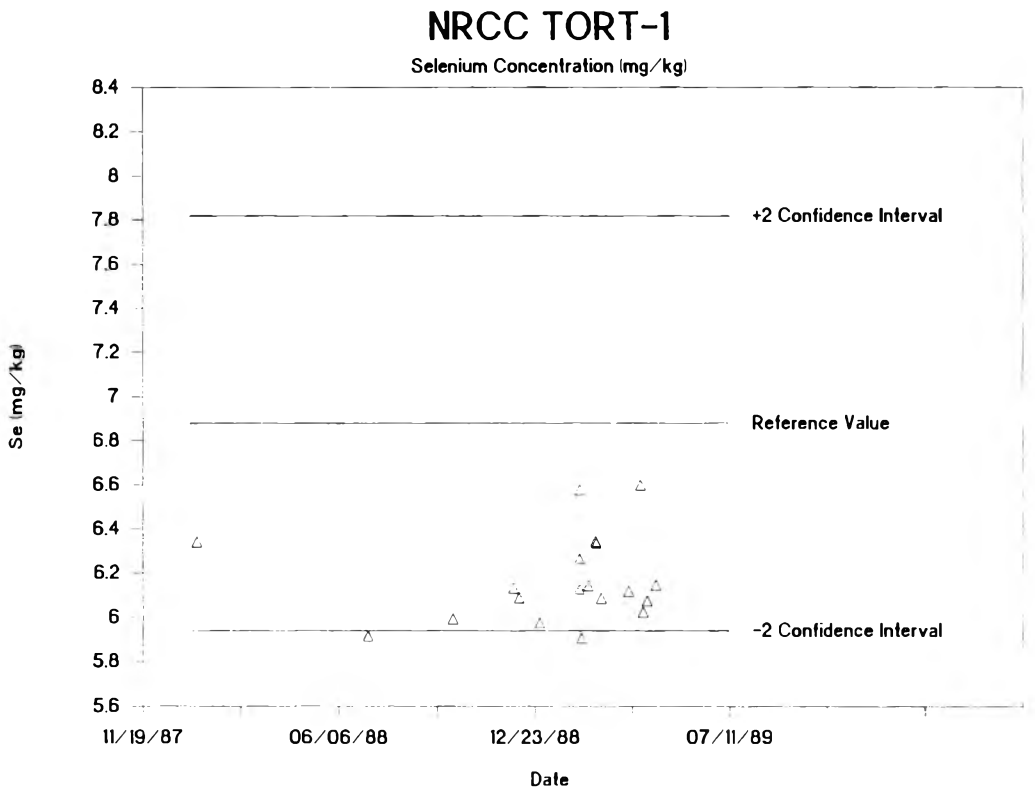

Figure 6. NRCC TORT-1 control chart for VPICP selenium method.

dogfish muscle, DOLT-1 dogfish liver, and TORT-1 lobster hepatopancreas. The results of the analyses over the study time period are presented in Figures 2 through 6. The mean values and standard deviations are listed in Table 1. Correlation with published values is excellent for all reference materials except NRCC TORT-1, which shows a negative deviation from the accepted value. We cannot account for the TORT-1 results, but it is not a matrix interference because we get 97% recovery of Se spiked in this matrix.

An interlaboratory method comparison was performed using 3 other accepted methods of analysis. The selenium analytical methods (31) included: (*1*) hydride generation atomic absorption following dry ashing with magnesium nitrate, (*2*) Zeeman-corrected graphite furnace atomic absorption following nitric acid digestion, and (*3*) neutron activation analysis following selenium precipitation of digested samples by hypophosphorous acid and Nucleopore membrane filtration. The field-collected specimens consisted of 16 fish liver and mixed tissue samples, 12 soft tissue invertebrate samples, and 47 bird liver and muscle tissue samples. The results of the study are presented in log-log plots (Figure 7). Examination of the figure shows uniform good correlation of the analysis results. Small amounts of scatter can be observed in the low concentration range. However, relative deviation throughout the entire data range was less than 10%. This is especially encouraging in consideration of the varied sample preparation and analysis techniques.

Table 1. Reference material selenium concentrations (mg/kg) by VGICP analysis

Reference material	Reference value	Vapor generation ICP value	Count
NIST			
Bovine Liver 1577a	0.71 ± 0.07[a]	0.73 ± 0.03[a]	106
Trace Elements in Water 1643b	0.0097 ± 0.0005[a]	0.0098 ± 0.0011[a]	26
NRCC			
DORM-1	1.62 ± 0.12[b]	1.64 ± 0.02[b]	58
DOLT-1	7.34 ± 0.42[b]	7.15 ± 0.01[b]	66
TORT-1	6.88 ± 0.47[b]	6.16 ± 0.09[b]	21

[a] Standard deviation.
[b] 95% confidence interval.

Conclusions

The VGICP method for selenium determination demonstrates the statistical control, accuracy, and precision required for routine analysis. The digestion and analysis protocols are fully amenable to programmed operation and autosampling. The analysis requires minimal sample, a strong consideration in biomedical analysis. The 3-order of magnitude working range as well as substantial freedom from matrix and chemical interference make VGICP a rigorous method for selenium analysis.

Acknowledgments

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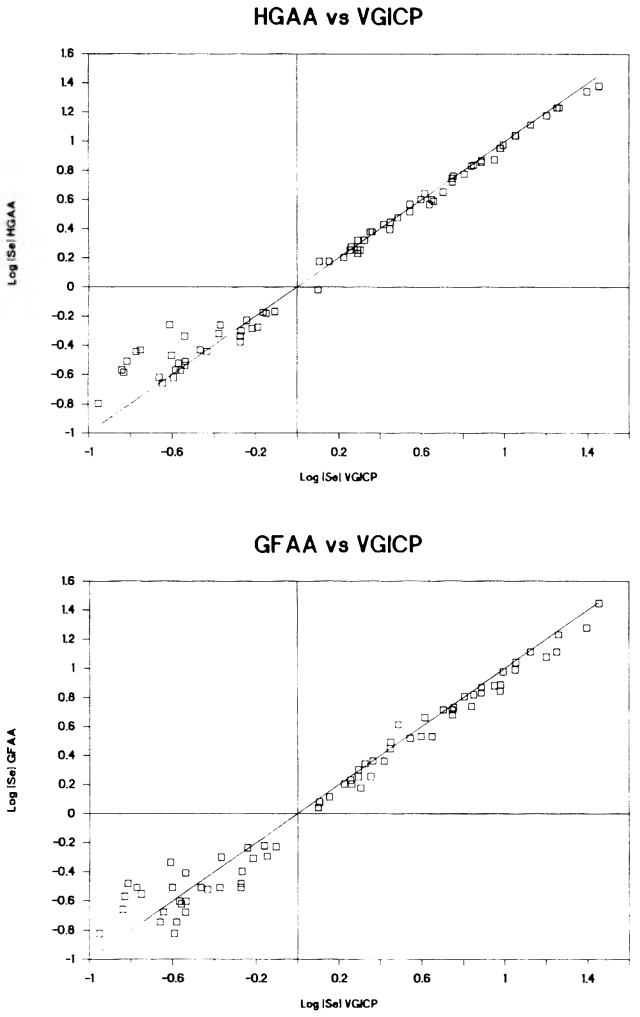

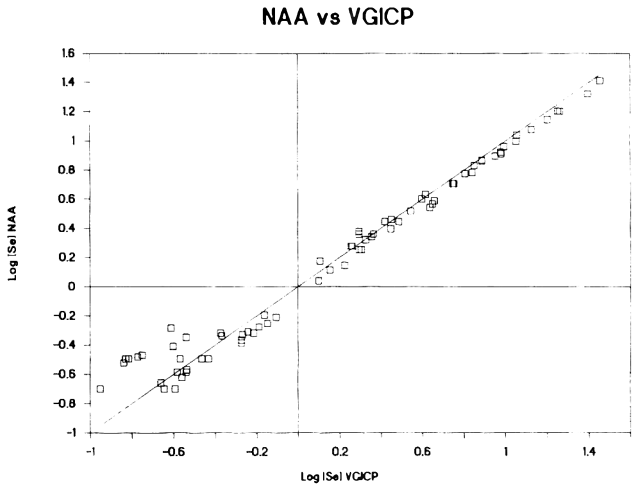

Figure 7. Correlation of selenium analysis by HGAA, GFAA, and NAA methods with VGICP analysis. Fish, invertebrate, and bird tissue, log(Se, mg/kg, wet weight). The ideal x = y line is drawn for comparison.

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METHOD PERFORMANCE

Diagnostic Data Evaluation. Part III. Collaborative Study Evaluation: Standard Deviation Considered To Be A Constant

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The diagnostic evaluation of the crude protein collaborative study identified possible problems with a standardized interval for the Kjeldahl digestion, with sample preparation, and with excess sample weights. The standard deviations for the ham and the beef samples cannot be considered to be representative of the method because of these problems. The standard deviations for the frankfurter and the pork sausage samples for all analysts met the performance standard of 0.24. There was no evidence of analyst bias.

The need for diagnostic data evaluation is well demonstrated by subjecting a crude protein collaborative study (1, 2) to the techniques described. For the purposes of diagnostic data evaluation, the study was well designed because there were sufficient replicate analyses by each analyst on each sample to identify possible problem areas. The diagnostic evaluation will be limited to the basic Kjeldahl portion of the study. At the time, the study was considered to be successful, although the standard deviations for the different meat products were not as consistent as they should have been for well homogenized samples.

Protein determination by the Kjeldahl method is a classic definitive procedure. The method is extremely simple, and it is possible to obtain a high degree of accuracy in all of the measurements. However, to increase productivity and simplicity, the digestion time appears to have been standardized to a finite time by the laboratories rather than the 30 min after clearing of the digest as specified in the official final action method (3). The standardized time may not have been evaluated adequately for all samples of the study.

Historically, the determination of protein in meat products was first performed for regulatory purposes on emulsified products like the cooked sausage. Such products are more homogeneous before sample preparation for analysis. For increased productivity with the "ganged digestion units," the units are usually controlled with a common automatic timer. For the emulsified meat products, it was possible to standardize the digestion time to approximately 1 h and still achieve very acceptable standard deviations for repeatability.

But for other products such as hams and dried and ground beef that are not so finely divided before sample preparation, homogeneity problems confound the analytical problems. The diagnostic analysis of the collaborative study data was performed to determine if additional method evaluation or sample preparation was necessary.

Performance Standards

The proposed standards of performance that will be used to evaluate the data are based on a well homogenized sample. Data were derived from the Meat Industry Technical Services (MITS) Check Sample Series. The second set of values for the performance is based on error analysis of the method (manuscript in preparation). The "tighter" standard from the error analysis of the procedure is attainable and possibly should be lower. The standard deviation for reproducibility (s_x) for the error analysis was assumed to be 150% times the standard deviation for analyst repeatability (s_a). The standards were also considered to be preliminary standards because the MITS samples did not cover the concentration range of the collaborative study.

	Check sample	*Error analysis*
Standard deviations for:		
Analyst repeatability (s_a)	0.24	0.21
Method repeatability (s_o)	0.24	
Method reproducibility (s_x)	0.32	0.315
Critical differences for:		
Analyst (r_a)	0.68	0.59
Repeatability (r)	0.68	
Reproducibility (R)	0.90	0.89
Analyst (r_{a99})	0.876	0.77
Repeatability (r_{99})	0.876	
Reproducibility (R_{99})	1.17	1.15
Normalized difference		
Single value should not exceed		
3 times s_x (99.7% confidence interval)	0.96	
2.58 times s_x (99% confidence interval)	0.83	
2.0 times s_x (95.5% confidence interval)	0.64	

Experimental

Six samples, 1 pork sausage, 1 frankfurter, 1 ham, 2 ground beef, and 1 dried beef, with a protein range from 10.7 to 30.30%, were studied. Quadruplicate analysis was performed on different days. The data are listed in Table 1 with the statistics from the collaborative study. The statistical evaluation was completed by the method of Steiner (4).

Diagnostic Evaluation

The pork sausage and the frankfurter were the only samples that met the proposed standards. The standard deviations of 0.277 (s_o) and 0.328 (s_x) could be considered marginally acceptable for ground beef 1. The standard deviations for the remaining 3 samples were accepted at that time because the collaborative study was done for the purpose of determining method equivalence. The higher variability was attributed to the problem of achieving a homogeneous sample.

With the development of the diagnostic evaluation tech-

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Table 1. Protein collaborative study data

Anal.	Pork sausage	Frank-furter	Ham	Ground beef 1	Ground beef 2	Dried beef
1A	10.60	11.20	15.80	17.60	20.90	30.70
	10.40	11.20	15.90	17.20	20.70	29.70
	11.10	11.30	15.90	17.20	21.10	30.60
	10.60	11.30	15.90	17.30	20.40	29.50
1B	10.60	11.00	16.30	17.40	20.30	30.60
	10.40	10.90	15.50	17.50	20.90	31.00
	10.80	11.10	16.50	17.10	20.40	30.80
	10.50	11.10	15.80	17.10	20.50	31.10
2A	10.79	11.35	15.14	17.83	21.30	30.51
	10.80	11.44	15.20	17.90	20.77	30.67
	10.72	11.40	15.16	17.82	21.38	30.50
	10.73	11.41	15.28	17.90	20.84	30.54
2B	10.89	10.89	15.72	17.62	20.37	29.82
	10.78	11.09	15.16	17.12	21.08	29.76
	10.87	10.87	15.67	17.64	20.35	29.79
	10.70	11.13	15.17	17.14	21.14	29.76
3A	10.70	11.10	16.10	17.20	20.70	29.80
	10.80	11.20	16.40	17.70	22.00	30.60
	10.60	11.10	16.30	17.20	20.60	29.70
	10.50	11.30	16.40	17.90	21.90	30.70
3B	10.70	11.10	15.30	17.20	20.50	29.80
	11.00	11.10	16.10	17.50	20.90	30.00
	10.40	11.20	15.40	17.40	20.50	29.30
	10.80	11.10	16.10	17.50	20.50	30.20
4A	11.10	11.40	15.90	17.60	20.60	30.30
	10.90	11.30	15.90	17.30	21.80	29.80
	10.70	11.50	15.40	17.60	21.10	30.60
	10.70	11.60	16.60	18.00	22.10	29.60
4B	10.80	11.00	16.10	17.20	21.30	30.40
	10.40	11.10	16.20	16.90	21.30	30.40
	10.50	11.10	16.00	17.70	21.70	30.70
	10.50	11.30	16.20	17.20	20.90	30.60
5A	10.49	10.54	16.57	17.04	20.94	31.00
	10.64	11.03	16.89	17.02	20.47	29.87
	10.89	10.90	16.57	17.20	21.72	30.91
	10.99	11.24	16.87	16.73	20.44	30.06
5B	10.59	10.94	16.72	17.35	20.90	30.30
	10.50	10.88	16.14	17.36	21.02	30.70
	10.76	11.20	16.84	17.34	21.23	30.55
	10.76	11.14	16.40	17.52	20.84	30.84
$\bar{x}$	10.70	11.15	15.99	17.40	20.96	30.30
s_o	0.160	0.183	0.346	0.277	0.489	0.496
s_x	0.195	0.201	0.554	0.328	0.500	0.496

niques for more in-depth data analysis, this study was an excellent choice to determine if other factors influenced and caused the higher variability. The data were normalized by subtracting the average value for each sample from the individual value reported by each analyst. The normalized values are listed on Table 2 with each analyst's normalized averages ($\bar{x}_a$) and each analyst's standard deviations (s_a). All analysts have higher values for s_a than the proposed standard of performance of 0.24. Analyst 4 with $\bar{x}_a = 0.102$ may have a high bias because $\bar{x}_a$ does exceed $\pm 0.092(0.32*2/48^{0.5})$.

The 4 normalized replicates for each day were averaged and the averages are listed in Table 3. With Table 3, the differences for the averages that exceed the critical difference $r_a = 0.32$ are listed. The normalized averages that exceed $\bar{x}_a$ for 4 samples are also identified. The critical differences for the averages that exceed the critical differences are also listed with Table 3.

The design of the study with 8 replicate analyses on each sample was ideal for diagnostic purposes because it was possible to determine a normalized average ($\bar{x}_a$) and the s_a value for each sample. They are listed in Table 4. The values that do not meet the purposed standards of performance are also identified. The combination of the higher standard deviations, the excessive number of critical differences between laboratories for the normalized averages that exceed the standard, and the bias for many of several of the samples suggest the sample homogeneity was the most probable cause for the higher standard deviations for the ham and the beef samples.

Diagnostic Evaluation of Specific Samples

Ham.—Sample averages for the 2 different days for analysts 2 and 5 exceed the 99.7% confidence interval of ±0.48 ($s_x*3/4^{0.5}$). The exclusion of the values for both analysts reduced s_x to 0.35, which would have been a marginally acceptable level at the expense of eliminating 40% of the data. The problems of obtaining a homogeneous meat sample are exemplified by this sample. The bias exhibited by the results from the 2 analysts compensate with regard to the sample average but are responsible for the higher than acceptable standard deviation.

Ground beef.—Ground beef 1 was initially marginally acceptable. Analyst 2 with an average of 0.4625 for day 1 exceeds the 99% confidence interval of 0.45. Analyst 5 with an average of −0.405 exceeds the 95.5% confidence interval of 0.32. Three analysts (2, 4, and 5) have critical differences that exceed the performance standard of 0.32. The s_a values of analysts exceed the method repeatability performance standard of 0.24. For 8 samples, the standard deviations of 0.256 and 0.254 for analysts 3 and 5 could be considered marginally acceptable. Sample nonhomogeneity is the most probable cause for the higher standard deviations and the bias. This sample, however, is more consistent in homogeneity because the higher standard deviations are evidenced by the data from 4 of the 5 analysts.

The results from ground beef 2 are very similar to those from ground beef 1. The standard deviations for s_a exceed the performance standard for all 5 analysts, and bias was exhibited for the data of analysts 1 and 4 (Table 4). Sample homogeneity was a major cause of the unacceptable performance.

Dried beef.—The diagnostic data evaluation for this sample was essentially the same as for ground beef 2.

Discussion

The method and the data were subjected to a critical path analysis to identify causes for the higher than expected standard deviations (manuscript in preparation). For the emulsified product and the pork sausage, the performance standards were determined from the Check Sample Series and by the error analysis.

Because the data from the samples that did not meet the performance standards were obtained from product that had not been cominuted or had only been ground through a coarse plate, the first process that should be investigated is the sample preparation. It is imperative that food choppers have well sharpened knives and plates. The sample preparation procedure should include requirements that knives and plates be sharpened regularly or replaced.

One process often overlooked in the cutting and mixing actions in a meat chopper is the action between the worm and the barrel. Worms and barrels should be maintained as a matched set and replaced when excessive wear is observed.

Table 2. Protein collaborative study normalized data[a]

Anal.	Pork sausage	Frank-furter	Ham	Ground beef 1	Ground beef 2	Dried beef	$\bar{x}_a$	s_a
1A	−0.10	0.05	−0.19	0.20	−0.06	0.40		
	−0.30	0.05	−0.09	−0.20	−0.26	−0.60		
	0.40	0.15	−0.09	−0.20	0.14	0.30		
	−0.10	0.15	−0.09	−0.10	−0.56	−0.80[b]		
1B	−0.10	−0.15	0.31	0.00	−0.66[b]	0.30		
	−0.30	−0.25	−0.39	0.10	−0.06	0.70[b]		
	0.10	−0.05	0.51	−0.30	−0.56	0.50		
	−0.20	−0.05	−0.19	−0.30	−0.46	0.80[b]	−0.056	0.342
2A	0.09	0.20	−0.85[c]	0.43	0.34	0.21		
	0.10	0.29	−0.79[b]	0.50	−0.19	0.37		
	0.02	0.25	−0.83[b]	0.42	0.42	0.20		
	0.03	0.26	−0.71[b]	0.50	−0.12	0.24		
2B	0.19	−0.26	−0.27	0.22	−0.59	−0.48		
	0.08	−0.06	−0.83[b]	−0.28	0.12	0.54		
	0.17	−0.28	−0.32	0.24	−0.61	−0.51		
	0.00	−0.02	−0.82[b]	−0.26	0.18	−0.54	−0.085	0.407
3A	0.00	−0.05	0.11	−0.20	−0.26	−0.50		
	0.10	0.05	0.41	0.30	1.04[d]	0.30		
	−0.10	−0.05	0.31	−0.20	−0.36	−0.60		
	−0.20	0.15	0.41	0.50	0.94[c]	0.40		
3B	0.00	−0.05	−0.69[b]	−0.20	−0.46	−0.50		
	0.30	−0.05	0.11	0.10	−0.06	−0.30		
	−0.30	0.05	−0.59	0.00	−0.46	−1.00[d]		
	0.10	−0.15	0.11	0.10	−0.46	−0.10	−0.040	0.386
4A	0.40	0.25	−0.09	0.20	−0.36	0.00		
	0.20	0.15	−0.09	−0.10	0.84[c]	−0.50		
	0.00	0.35	−0.59	0.20	0.14	0.30		
	0.00	0.45	0.61	0.60	1.14[e]	−0.70[b]		
4B	0.10	−0.15	0.11	−0.20	0.34	0.10		
	−0.30	−0.05	0.21	−0.50	0.34	0.10		
	−0.20	−0.05	0.01	0.30	0.74[b]	0.40		
	−0.20	0.15	0.21	−0.20	−0.06	0.30	0.102	0.360
5A	−0.21	−0.61	0.58	−0.36	−0.02	0.70[b]		
	−0.06	−0.12	0.90[c]	−0.38	−0.49	−0.43		
	0.19	−0.25	0.58	−0.20	0.76	0.61		
	0.29	0.09	0.88[c]	−0.67[b]	−0.52	−0.24		
5B	−0.11	−0.25	0.73[b]	−0.05	−0.06	0.00		
	−0.20	−0.27	0.15	−0.04	0.06	0.40		
	0.06	0.05	0.85[c]	−0.06	0.27	0.25		
	0.06	−0.01	0.41	0.12	−0.12	0.54	0.080	0.407

[a] Data normalized by subtracting average value for each sample from individual analyst value.
[b] Value exceeds the 95.5% confidence interval of 0.64.
[c] Value exceeds the 99% confidence interval of 0.83.
[d] Value exceeds the 99.7% confidence interval of 0.96.
[e] Value exceeds the 99.9% confidence interval of 1.05.

Passing the product 3 times through a chopper with sharp cutting edges and well matched parts as specified in *Official Methods of Analysis*, section **24.001** (section **983.18**, 15th Ed.), will provide a sample with the necessary homogeneity to meet performance standards.

The digestion time coupled with the sample weight may also have been causes for the failure to meet the performance standards. The AOAC Kjeldahl method (3) specifies that the sample be digested an additional 30 min after clearing. Unfortunately, there is a high probability that the digestion units used in most laboratories have an automatic timer. The emphasis on productivity prevents the analyst from observing each digestion. Widely different sample weights, if the extreme values that are acceptable in the method are used, may require different total digestion times. The method as presently written should be revised for the laboratories of the present. It may be necessary to develop a matrix according to sample composition. Conversely, only samples of similar matrixes should be digested at the same time so that automated equipment will maintain analytical quality.

Recommendation

It is recommended that method refinement be performed so that the automated analysis can be performed with the precision and accuracy inherent in the method.

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Table 3. Tabulation of normalized replicate daily averages ($\bar{x}_a$) and differences between days

Anal.	Pork sausage	Frank-furter	Ham	Ground beef 1	Ground beef 2	Dried beef
1A	−0.025	0.100	−0.115	−0.075	−0.185	−0.175
1B	−0.125	−0.125	0.060	−0.125	−0.435[a]	0.575[b]
[DIFF]	0.100	0.025	0.175	0.050	0.250	0.750[c]
2A	0.060	0.250	−0.795[b]	0.463[a]	0.113	0.255
2B	0.110	−0.155	−0.560[b]	−0.020	−0.225	−0.518[d]
[DIFF]	0.050	0.405[e]	0.235	0.483[f]	0.338	0.773[c]
3A	−0.050	0.025	0.285	0.010	0.340[g]	−0.100
3B	0.025	−0.050	−0.265	0.000	−0.360[g]	−0.475[a]
[DIFF]	0.075	0.075	0.550[f]	0.010	0.700[c]	0.375
4A	0.150	0.300	−0.040	0.225	0.440[a]	0.225
4B	−0.150	−0.025	0.135	−0.150	0.340[g]	0.225
[DIFF]	0.300	0.325	0.175	0.375	0.100	0.000
5A	0.052	−0.222	0.735[b]	−0.405[a]	−0.068	0.160
5B	−0.048	−0.110	0.535[b]	−0.008	−0.038	0.298
[DIFF]	0.100	0.332	0.200	0.397	0.030	0.138

[a] Exceeds the 99% confidence interval of 0.413 for $\bar{x}_a$.
[b] Exceeds the 99.9% confidence interval of 0.526 for $\bar{x}_a$.
[c] Exceeds the $\bar{r}_{a99.9}$ of 0.559
[d] Exceeds the 99.7% confidence interval of 0.48 for $\bar{x}_a$.
[e] Exceeds r_a of 0.34.
[f] Exceeds r_{a99} of 0.438.
[g] Exceeds the 95.5% confidence interval of 0.32 for $\bar{x}_a$.

Table 4. Protein collaborative study tabulation of analyst standard deviations (s_a) and normalized sample averages ($\bar{x}_a$)

Anal.	Pork sausage	Frank-furter	Ham	Ground beef 1	Ground beef 2	Dried beef
1 s_a	0.231	0.141	0.292[a]	0.185	0.293[a]	0.586[a]
1 $\bar{x}_a$	−0.075	−0.013	−0.028	−0.100	−0.310[b]	0.200
2 s_a	0.069	0.234	0.240	0.320[a]	0.394[a]	0.416[a]
2 $\bar{x}_a$	0.085	0.0475	−0.678[c]	0.221	−0.053	−0.131
3 s_a	0.188	0.029	0.429[a]	0.256[a]	0.632[a]	0.470[a]
3 $\bar{x}_a$	−0.125	−0.012	0.022	0.050	−0.010	−0.288[d]
4 s_a	0.233	0.210	0.342[a]	0.350[a]	0.496[a]	0.396[a]
4 $\bar{x}_a$	0.000	0.138	0.048	0.038	0.390[c]	0.000
5 s_a	0.180	0.227	0.261[a]	0.254[a]	0.411[a]	0.414[a]
5 $\bar{x}_a$	0.003	0.166	0.635[c]	−0.205	−0.015	−0.229[d]

[a] Exceeds the performance standard for the method s_a of 0.24.
[b] Exceeds the 99% confidence interval of ±0.291 for $\bar{x}_a$.
[c] Exceeds the 99.9% confidence interval of ±0.372 for $\bar{x}_a$.
[d] Exceeds the 99.5% confidence interval of 0.226 for $\bar{x}_a$.

Diagnostic Data Evaluation. Part IV. Evaluation of A Check Sample Series To Determine Analyst Performance

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Data from a check sample series were subjected to diagnostic data evaluation. The chemical analyses that were performed were moisture, protein, and fat in meat products. Four performance outliers were identified (1 protein, 1 moisture, and 2 fat values). With the performance outliers excluded from the statistical evaluation, the analyst standard deviation ranged from 0.088 to 0.218 for protein, 0.145 to 0.393 for moisture, and 0.271 to 0.378 for fat. No bias was present. All laboratories were able to meet the established standards. With the limited number of samples it was not possible to attribute any reasons for the outliers.

The evaluation of a series of check samples to determine if analysts or laboratories are competent and have met the performance standards of the method is a very simple procedure when the mean difference or the mean ratio is used to perform the evaluation. The evaluation process with normalization described by the author (1) can be used for determining the equivalence of methods or analysts. The determination from the normalized data of an analyst's repeatability (s_a) and bias as expressed by the normalized average ($\bar{x}_a$) is basically a determination of equivalence.

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The determination of equivalence for different methods can be done and has been reported by the author (2). The use of the normalization technique is independent of method. The s_a and $\bar{x}_a$ are identified as pertaining to the analyst; however, when the same analyst uses different methods, the descriptors could also be identified with the method. In fact, with the normalization technique, comparisons can be made of different methods when used by different analysts.

In any multi-method/multi-analyst study, the number of analysts participating is critical. Ideally, the study should have 6 analysts who analyze 12–15 samples. The minimum number of analysts needed to evaluate method performance is three. If there are only 3 analysts, the number of samples analyzed by each analyst should be doubled.

This check sample series evaluation demonstrates the ease with which individual analyst performance can be monitored. The use of either normalization technique can be performed with a statistical calculator or with a personal computer using appropriate software. With a functioning laboratory quality control program, all analysts in a laboratory should obtain equivalent results.

The data for this example will be taken from the Meat Industry Technical Services (MITS) 1985 Check Samples Series. The analyses that were performed in the series were moisture, protein, and fat. The average values reported for MITS, as the best estimate of the analyte present, will be used in the normalization of the individual values.

Performance Standards

The standards of performance from the *Chemistry Quality Assurance Handbook* (3) (CQAH), as determined by error analysis (EA) (manuscript in preparation) and target standards (TARSTD) are:

Protein			
Standard deviations for:	*CQAH*	*TARSTD*	*EA*
Analyst repeatability (s_a)	0.24	0.22	0.18
Method repeatability (s_o)	0.24	0.24	
Method reproducibility (s_x)	0.32	0.30	0.27
Critical differences for:			
Analyst (r_a)	0.68	0.62	0.51
Method repeatability (r)	0.68	0.68	
Method reproducibility (R)	0.90	0.84	0.76
Normalized average ($\bar{x}_a$)*:*			
Acceptable range (n = 9)		±0.20	
Acceptable range (n = 10)		±0.19	

Moisture			
Standard deviations for:	*CQAH*	*TARSTD*	*EA*
Analyst repeatability (s_a)	0.46	0.32	0.111
Method repeatability (s_o)	0.46	0.46	
Method reproducibility (s_x)	0.65	0.47	0.166
Critical differences for:			
Analyst (r_a)	1.30	1.20	0.314
Method repeatability (r)	1.30	1.30	
Method reproducibility (R)	1.83	1.36	0.47
Normalized average ($\bar{x}_a$)*:*			
Acceptable range (n = 9)		±0.31	
Acceptable range (n = 10)		±0.29	

Fat			
Standard deviations for:	*CQAH*	*TARSTD*	*EA*
Analyst repeatability (s_a)	0.63	0.36	0.197
Method repeatability (s_o)	0.63	0.63	
Method reproducibility (s_x)	0.66	0.54	0.295
Critical differences for:			
Analyst (r_a)	1.78	0.94	0.557
Method repeatability (r)	1.78		
Method reproducibility (R)	1.87	1.53	0.834
Normalized average ($\bar{x}_a$)*:*			
Acceptable range (n = 9)		±0.36	
Acceptable range (n = 10)		±0.34	

The CQAH performance values are general performance standards for all analysts. The TARSTD values are the performance standards that an experienced analyst should meet. The TARSTD values will be used in the evaluation. The EA values are shown to indicate the performance that many of the analysts should be able to attain when effective quality controls are used.

Diagnostic Evaluation

Protein.—Tables 1 and 1N are the tabulations of the raw and the normalized protein data. The averages and the standard deviations have been determined for each sample. Sample 4 is the only sample that does not meet the standard of performance for the method for reproducibility. The value of 1.0 for the difference between laboratories 3 and 5 exceeds the critical difference R = 0.84.

Laboratory 3 did not meet the performance standard for analyst repeatability. For the normalized values (Table 1N), the difference of 1.0 between Samples 3 and 4 exceeds the critical difference r_a = 0.62. The sample represented by the normalized value of 0.9 is the performance outlier. With the exclusion for Laboratory 3 of these values for Sample 4 from the raw and normalized data, the standards of performance are met.

Moisture.—Tables 2 and 2N are the tabulations of the raw and the normalized moisture data. The performance standards for s_x and s_a were not met for Sample 8 and Laboratory 6, respectively. The critical difference R = 1.36 was exceeded by the difference between Laboratories 1 and 6 (R = 1.6). From the normalized data (Table 2N), the difference of 1.6 between Samples 8 and 9 for Laboratory 6 exceeded the critical difference r_a = 1.20. The analytical value that is the performance outlier is the value for Sample 8 from Laboratory 6. When the value for this sample is excluded from the evaluation, the performance standards of the method are met (see Tables 2 and 2N).

Fat.—Tables 3 and 3N are the tabulations of the raw and the normalized fat data. Samples 6 and 8 and Laboratories 3 and 6 did not meet the standards of performance for s_x and s_a, respectively. The critical difference R = 1.53 of the extreme values is exceeded for Sample 6 (R = 1.6) and Sample 8 (R = 1.9). From the normalized data (Table 3N) the critical difference r_a = 0.94 of the extreme values is exceeded by Laboratory 3 (R = 1.7) and Laboratory 6 (R = 1.3). When the sample values represented by the normalized values for the 1.3 reported for Sample 6 by Laboratory 3 and the 1.0 for Sample 8 by Laboratory 6 are excluded from the evaluation, the performance standards of the method are met (see Tables 3 and 3N).

Two of the performance outliers from Laboratory 6 were for the same sample. The possible cause of the performance outliers may have been caused by fat separation in the sample during transit and storage. The separated fat may not have been reincorporated evenly before sample analysis. The 2 performance outliers from Laboratory 3 for Samples 4 and 6 may be due either to the sample nonhomogeneity or to a problem in the laboratory. There is not sufficient data to assign the most probable cause of the performance outliers. For laboratories that have quality control programs, the samples would have been available for reanalysis and would have been reanalyzed when the critical values for R or r_a were exceeded. A review of the Tables 1N, 2N, and 3N indicates that the laboratories do not have a measurable bias, because all of the $\bar{x}$ are within the 95.5% confidence range for the different analytical methods.

Conclusions

Diagnostic data evaluation with normalization of the individual results with the averages provides a quantitative measure of the analyst's repeatability with single sample analysis. It also provides a quantitative measure of the analyst bias when the analyst average is compared to the overall average.

References

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Table 1. MITS Check Sample protein data

	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	17.9	—	18.0	15.4	11.0	11.5	17.6	15.5	14.7	17.0
2	18.3	16.4	17.7	15.4	11.2	11.4	17.6	15.5	15.2	16.9
3	18.3	16.7	17.8	(16.2)	11.1	11.3	17.7	15.6	15.1	17.0
4	18.5	16.4	17.6	15.3	11.1	11.4	17.6	15.8	15.1	16.8
5	18.2	16.4	17.7	15.2	10.9	11 5	17.6	15.7	15.0	17.0
6	18.1	16.3	18.1	15.4	11.4	11.6	17.4	15.6	15.0	—
All values: 1										
$\bar{x}$	18.2	16.4	17.8	15.5	11 1	11.4	17.6	15.6	15.0	16.9
s_x	0.204	0.152	0.194	0.360	0.171	0.105	0.098	0.117	0.172	0.089
With 16.2 excluded:										
$\bar{x}$				15.3						
s_x				0.089						

Table 1N. MITS Check Sample protein data normalized with MITS average

MITS averages used for normalization:										
$\bar{x}$	18.3	16.6	17.8	15.3	11.2	11.5	17.7	15.7	15.1	17.1
	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	−0.4	—	0.2	0.1	−0.2	0.0	−0.1	−0.2	−0.4	−0.1
2	0.0	−0.2	−0.1	0.1	0.0	−0.1	−0.1	−0.2	0.1	−0.2
3	0.0	0.1	0.0	(0.9)	−0.1	−0.2	0.0	−0.1	0.0	−0.1
4	0.2	−0.2	−0.2	0.0	−0.1	−0.1	−0.1	0.1	0.0	−0.3
5	−0.1	−0.2	−0.1	−0.1	−0.3	0.0	−0.1	0.0	−0.1	−0.1
6	−0.2	−0.3	0.3	0.1	0.2	0.1	−0.3	−0.1	−0.1	—

All individual normalized averages ($\bar{x}$) and standard deviations (s_a):

	Laboratory					
	1	2	3	4	5	6
$\bar{x}_a$	−0.122	−0.070	0.050	−0.070	−0.110	−0.033
s_a	0.205	0.116	0.310	0.149	0.088	0.218
With 0.9 excluded:						
$\bar{x}_a$			0.044			
s_a			0.088			

Table 2. MITS Check Sample moisture data

	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	74.7	—	63.9	63.9	51.8	50.4	62.4	56.1	53.1	68.5
2	74.8	57.6	63.8	63.9	52.3	50.5	62.7	57.2	53.9	68.8
3	75.1	58.0	64.0	63.9	52.0	50.6	62.8	57.1	54.0	68.8
4	74.7	57.5	62.9	63.4	51.7	50.7	62.4	56.7	53.9	68.8
5	75.0	57.8	63.9	63.7	51.9	50.5	62.3	56.7	53.8	68.6
6	74.8	58.0	63.3	64.0	52.4	50.5	61.9	(55.5)	53.2	—
All values: 1										
$\bar{x}$	74.9	57.8	63.3	63.8	52.0	50.5	62.4	56.6	53.7	68.7
s_x	0.164	0.228	0.437	0.219	0.279	0.103	0.319	0.644	0.394	0.134
With 55.5 excluded:										
$\bar{x}$								56.8		
s_x								0.434		

Table 2N. MITS Check Sample moisture data normalized with MITS average

MITS averages used for normalization:										
$\bar{x}$	74.7	57.8	63.5	63.7	51.9	50.3	62.5	56.6	53.7	68.7
	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	0.0	—	0.4	0.2	−0.1	0.1	−0.1	−0.5	−0.6	−0.2
2	0.1	−0.2	0.3	0.2	0.4	0.2	0.2	0.6	0.2	0.1
3	0.4	0.2	0.5	0.2	0.1	0.3	0.3	0.5	0.3	0.1
4	0.0	−0.3	−0.6	−0.3	−0.2	0.4	−0.1	0.1	0.2	0.1
5	0.3	0.0	0.4	0.0	0.0	0.2	−0.2	0.1	0.1	0.1
6	0.1	0.2	−0.2	0.3	0.5	0.2	−0.6	(1.10)	−0.5	—

All individual normalized averages ($\bar{x}$) and standard deviations (s_a):

	Laboratory					
	1	2	3	4	5	6
$\bar{x}_a$	−0.089	0.210	0.290	−0.070	0.100	0.122
s_a	0.318	0.208	0.145	0.291	0.170	0.519
With 1.10 excluded:						
$\bar{x}_a$						0.000
s_a						0.393

Table 3. MITS Check Sample fat data

	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	1.6	—	14.9	17.1	27.9	28.8	15.9	24.9	28.2	8.3
2	1.8	22.3	15.0	16.5	28.2	29.1	16.2	24.1	27.7	8.2
3	1.6	22.9	15.1	16.7	29.1	(30.4)	16.2	24.7	27.3	8.3
4	1.5	22.7	15.4	16.8	28.5	29.5	15.8	25.1	27.5	8.8
5	1.7	22.3	15.5	16.8	28.4	29.4	16.1	24.4	27.6	8.4
6	1.5	22.6	14.8	17.3	28.1	29.4	16.0	(25.9)	28.1	—
All values: 1										
$\bar{x}$	1.6	22.5	15.1	16.9	28.4	29.4	16.0	24.9	27.7	8.4
s_x	0.117	0.261	0.279	0.289	0.418	0.539	0.163	0.625	0.350	0.234
With 30.4 and 25.9 excluded:										
$\bar{x}$						29.2		24.6		
s_x						0.288		0.398		

Table 3N. MITS Check Sample fat data normalized with MITS average

MITS averages used for normalization:										
$\bar{x}$	1.7	22.4	15.1	17.1	28.4	29.1	16.0	24.9	27.7	8.2
	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	−0.1	—	−0.2	0.0	−0.5	−0.3	−0.1	0.0	0.5	0.1
2	0.1	−0.1	−0.1	−0.6	−0.2	0.0	0.2	−0.8	0.0	0.0
3	−0.1	0.5	0.0	−0.4	0.7	(1.3)	0.2	−0.2	−0.4	0.1
4	−0.2	0.3	0.3	−0.3	0.1	0.4	−0.2	0.2	−0.2	0.6
5	0.0	−0.1	0.4	−0.3	0.0	0.3	0.1	−0.5	−0.1	0.2
6	−0.2	0.2	−0.3	0.2	−0.3	0.3	0.0	(1.0)	0.4	—

All individual normalized averages ($\bar{x}$) and standard deviations (s_a):

	Laboratory					
	1	2	3	4	5	6
$\bar{x}_a$	−0.067	−0.150	0.170	0.100	0.000	0.144
s_a	0.278	0.314	0.533	0.309	0.271	0.413
With 1.3 and 1.0 excluded:						
$\bar{x}_a$			0.044			0.038
s_a			0.378			0.274

MICROBIOLOGICAL METHODS

Comparative Study of Colorimetric DNA Hybridization Method and Conventional Culture Procedure for Detection of *Salmonella* in Foods

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A second generation nucleic acid hybridization assay has been developed and evaluated against the conventional culture method for detection of salmonellae in foods. The assay involves a liquid hybridization with *Salmonella*-specific oligonucleotide probes, capture of probe:target hybrids onto a solid support (plastic dipstick), and a colorimetric end point detection. The assay can be completed in 2.5 h, following approximately 44 h of culture enrichment. One thousand samples representing 20 food types were analyzed in parallel by both methods. Samples included uninoculated test product, and product inoculated with *Salmonella* at 2 levels. Eighteen *Salmonella* serotypes were used as inocula. The data demonstrate that the colorimetric hybridization method and the conventional culture method are equivalent in their ability to detect *Salmonella* contamination of foods.

The recent application of nucleic acid hybridization technology to the detection of salmonellae in foods and food ingredients has resulted in the development of a method that is significantly more rapid than standard culture methods (1–3), and is directly comparable in accuracy (4, 5). The assay is based on isolation and identification of regions of chromosomal DNA that are unique to *Salmonella*. Detection of *Salmonella* results from a solid-phase (filter membrane) hybridization of highly specific ^{32}P-labeled nucleic acid probes with these unique regions of DNA.

This first generation DNA hybridization (DNAH) method for detection of *Salmonella* in foods has been subjected to interlaboratory collaborative study (5) and obtained AOAC official final action status in 1988. The method provides results within 48 h, including the culture enrichment steps prior to performing the 4 h DNAH assay procedure. The reduction in analysis time is derived from the ability to definitively identify negative cultures after only 48 h of enrichment and assay, vs the 4–7 days required by the BAM/AOAC conventional culture method (6, 7). The high degree of accuracy is a result of the exceptional specificity that resides in regions of the genetic material. This specificity has been demonstrated by the low rate of false positive reactions experienced across a wide variety of food types (4).

It is clear that analytical methods offering time savings while maintaining accuracy can result in considerable benefit to the food industry by reducing labor and inventory costs, and by lessening the response time in the event of a contamination problem. However, to achieve the necessary level of accuracy, the first generation DNAH format required the use of a radioisotope, ^{32}P, for detection. For this reason, the use of this test has been limited to those sites that are amenable to the handling of radioisotopes.

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To broaden the accessibility of this technology, we have developed a second generation DNAH *Salmonella* assay that utilizes an enzymatic detection method and a colorimetric end point. This assay is based on the identification of regions of ribosomal RNA (rRNA) that are unique to *Salmonella*. The rRNA is present in 5000–10 000 copies/cell, and this natural amplification of target allows the use of a non-isotopic detection method. The assay takes approximately 2.5 h to perform, following a standard culture enrichment as described for the first generation assay (5).

When *Salmonella* rRNA (target) is present in the sample, hybridization takes place between this target, a polydeoxyadenosine (polydA)-tailed oligonucleotide "capture" probe, and a fluoresceinated oligonucleotide "detector" probe. These target:capture probe:detector probe complexes are then captured on the surface of a polydeoxythymidine (polydT)-coated plastic dipstick via hybridization of the polydA and polydT moieties. Detection is accomplished by the binding of an anti-fluorescein-horseradish peroxidase conjugate to the fluorescein-labeled detector probe and enzyme-mediated conversion of a chromogen to a colored product. The reaction is stopped after a specific time, and the colorimetric end point is read in a simple photometer at 450 nm. Details of the development of this assay and comparison to the first generation DNAH test will be reported elsewhere (Wilson et al., in press).

The objective of this study was to compare the performance of the colorimetric hybridization method to the BAM/AOAC culture procedure for the detection of *Salmonella* in a variety of foods.

Experimental

Test Organisms

Eighteen *Salmonella* species previously isolated from foods were employed for inoculation (Table 1).

Preparation of Inoculated Foods

For preparation of inoculated dry-powdered, granulated, and semisolid test products, freeze-dried cell pellets of *Salmonella* strains to be used as inocula were ground by mortar and pestle and mixed with test product to produce primary "seed" stocks as described previously (4). "Seeds" for dry powdered and granulated products were prepared by dry-blending ground freeze-dried cell pellets into 500 g uninoculated product. "Seeds" for semisolid products (chocolate and peanut butter) were prepared by blending ground freeze-dried cell pellets into 500 g melted (45°C) test product. The seeds were held 4–14 days at 25°C to allow stabilization of the levels of viable salmonellae. The number of viable *Salmonella*/g of "seed" was estimated by plate count on selective media. After stabilization and estimation of contamina-

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

Test product	Test organism	MPN of seed, cells/g	Inoculation level	Calcd inoc. level, cells/g[a]
Pork	*Salmonella anatum*	17	high	0.68
			low	0.07
Roast beef	*Salmonella agona*	23	high	0.92
			low	0.09
Turkey	*Salmonella weltevreden*	194	high	>2.4
			low	0.78
Peanut butter	*Salmonella cubana*	23	high	0.80
			low	0.08
Shrimp	*Salmonella muenster*	21	high	0.84
			low	0.08
Coconut	*Salmonella rubislaw*	0.4	high	0.02
			low	<0.02
Pecans	*Salmonella typhimurium*	23	high	0.92
			low	0.09
Fish	*Salmonella derby*	20	high	0.28
			low	0.03
Nonfat dry milk	*Salmonella bovismorbificans*	4.3	high	0.18
			low	0.02
Milk chocolate	*Salmonella senftenberg*	23	high	0.92
			low	0.09
Pepper	*Salmonella rubislaw*	93	high	>2.4
			low	0.37
Dry casein	*Salmonella havana*	43	high	1.72
			low	0.17
Gelatin	*Salmonella derby*	9.3	high	0.37
			low	0.04
Dry eggs	*Salmonella infantis*	23	high	0.92
			low	0.09
Yeast (inactive)	*Salmonella siegberg*	15	high	0.60
			low	0.06
Soy flour	*Salmonella amsterdam*	9.3	high	0.37
			low	0.04
Dry pasta	*Salmonella drypool*	23	high	0.92
			low	0.09
Bone meal	*Salmonella mbandaka*	15	high	0.60
			low	0.06
Cheese powder	*Salmonella meleagridis*	23	high	0.92
			low	0.09
Cake mix	*Salmonella montevideo*	9.3	high	0.37
			low	0.04

[a] Calculated inoculation level = MPN of "seed" divided by 25.

tion levels in the seeds, the seeds were mixed with appropriate amounts of the test products to obtain the desired levels of contamination as defined below.

For all dry-powdered, granulated, and semisolid food types, secondary seed stocks were prepared by serial dilution (in test product) of the primary seed. Twenty 25 g test samples were then prepared at the desired inoculation levels (0.04–0.2 cells/g and 0.4–2.0 cells/g) by blending 1 g of the prepared seed stocks with 24 g uninoculated test product. To determine actual inoculation levels, a *Salmonella* MPN determination was performed on the seed stocks on the day seed was mixed with uninoculated product to produce the test samples.

Moist products (pork, turkey, shrimp, and fish) were inoculated with an appropriate dilution of a 24 h broth culture of the selected test organism. A single colony of the test organism was inoculated into gram-negative (GN) broth (Difco, Detroit, MI) and incubated 24 h at 35°C. The cell count was determined by direct microscopic count, and 0.1 mL of the serially diluted culture was added to 25 g samples of the ground moist test product to obtain the desired levels of inoculation. A list of food products, test organisms, and inoculation levels is shown in Table 1.

Analysis of Test Products

The 25 g samples were added to 225 mL of the appropriate enrichment broth and analyzed by both the AOAC (6) or *Bacteriological Analytical Manual* (7) culture procedure and by the colorimetric DNA hybridization method. In addition, 10 uninoculated control samples of each food type were enriched as described and analyzed. Samples employed as controls were from the same production lots as the inoculated samples. In cases where uninoculated product was demonstrated to contain *Salmonella* by either method, the isolated *Salmonella* was serotyped to ensure that it was derived from natural contamination.

BAM/AOAC Methods

The following products were pre-enriched by AOAC procedure **46.117**, 14th Ed. (**967.26**, 15th Ed.): dried whole egg, milk chocolate, nonfat dry milk, and casein (6). All other test

Table 2. Comparison of colorimetric DNA hybridization and BAM/AOAC procedures for detection of *Salmonella* in inoculated test products

Product	Samples analyzed	Total samples pos.[a]	Samples positive		
			BAM/AOAC	Colorimetric DNA hybridization	
				Assay[b] (χ^2)[d]	Confirmed[c] (χ^2)
Pork	40	32	32	32 (****)[e]	32 (****)
Roast beef	40	37	37	37 (****)	37 (****)
Turkey	40	30	25	30 (2.29)	29 (1.50)
Peanut butter	40	40	40	40 (****)	40 (****)
Shrimp	40	29	28	30 (0.50)	29 (0.00)
Coconut	40	17	17	17 (****)	17 (****)
Pecans	40	37	37	37 (****)	37 (****)
Fish	40	28	28	28 (****)	28 (****)
Nonfat dry milk	40	34	34	34 (****)	34 (****)
Milk chocolate	40	39	39	39 (****)	39 (****)
Pepper	40	12	12	12 (****)	12 (****)
Casein	40	27	27	25 (0.50)	25 (0.50)
Gelatin	40	22	21	22 (0.00)	21 (0.50)
Dry egg	40	39	39	39 (****)	39 (****)
Yeast (inactive)	40	40	40	40 (****)	40 (****)
Soy flour	40	28	27	28 (0.00)	28 (0.00)
Dry pasta	40	40	40	40 (****)	40 (****)
Bone meal	40	34	34	34 (****)	34 (****)
Cheese powder	40	30	26	29 (0.80)	29 (0.80)
Cake mix	40	35	33	35 (0.50)	35 (0.50)
All products	800	630	616	628 (5.50)	625 (3.37)

[a] Samples positive by BAM/AOAC method plus additional samples positive by cDNAH assay and confirmed from culture media.
[b] Results of colorimetric assay not considering subsequent confirmation.
[c] Results of colorimetric assay including confirmation by isolation of *Salmonella* from culture media.
[d] $\chi^2 = (|\text{samples positive by cDNAH and negative by BAM/AOAC} - \text{samples positive by BAM/AOAC and negative by cDNAH}| - 1)^2 \div (\text{samples positive by cDNAH and negative by BAM/AOAC} + \text{samples positive by BAM/AOAC and negative by cDNAH})$.
[e] Statistical analysis not applicable because cDNAH and BAM/AOAC results agreed for all samples analyzed.

products were pre-enriched according to BAM methods (7), except raw fish, raw pork, raw shrimp, and raw turkey. These products were pre-enriched in lactose broth as described for processed meats (4, 7). Selective enrichment, isolation, and preliminary screening of typical *Salmonella* colonies were performed by AOAC methods (6). Presumptive positive colonies were chosen from the selective/differential agars and isolates identified according to AOAC methods (6).

Colorimetric DNA Hybridization (cDNAH) Method

The same sample pre-enrichments described above for the BAM/AOAC methods were employed for the colorimetric DNA hybridization assay. Selective enrichments of all samples except raw flesh foods (fish, poultry, pork, shrimp) used for the hybridization assay were incubated 6 h at 35°C. Following incubation, the selective enrichments were mixed using a vortex mixer, and 1 mL was transferred from each selective enrichment to separate 10 mL tubes of GN broth. The selective enrichments were returned to 35°C for an additional 18 ± 2 h. The GN cultures were incubated 12–16 h at 35°C prior to performance of the colorimetric DNA hybridization assay. Selective enrichments of raw flesh foods were incubated 16–18 h at 35°C prior to transfer to GN. The GN cultures were then incubated 6 h prior to assay. In addition, the selective enrichments were reincubated for an additional 6 h.

GN cultures were analyzed for *Salmonella* using the colorimetric Gene-Trak® *Salmonella* assay (Gene-Trak Systems, Framingham, MA). Reagents and equipment are available from the manufacturer and are as described previously (8). The method was performed as follows: Sample GN cultures (0.25 mL from each of the 2 GN broths per sample) were added to 12 × 75 mm glass tubes. The bacteria were lysed, and nucleic acids were denatured for 5 min at room temperature by addition of 0.1 mL 0.75N NaOH. Samples were neutralized with 0.1 mL 2M TRIS·HCl (pH 7.5); the tubes were covered and incubated 15 min in a 65°C water bath. One-tenth mL of a solution containing the fluorescein-ated reporter oligonucleotides and the dA-tailed capture oligonucleotides was added to each tube, and the tubes were mixed briefly and returned to the water bath.

Following 15 min hybridization at 65°C, a polydT-coated plastic dipstick was placed into each sample tube. Tubes were shaken and returned to the water bath for an additional 60 min of incubation at 65°C. This incubation allows for capture of the target:capture probe:detector probe complexes by hybridization of the dA-tailed capture probe to the polydT on the surface of the dipstick. During this incubation, the concentrated anti-fluorescein-horseradish peroxidase conjugate was diluted 1:100 in 1× wash buffer, and 0.75 mL aliquots were transferred to a clean set of 12 × 75 mm test tubes.

After capture of the hybridization complex, dipsticks were removed, blotted briefly onto adsorbent paper, and washed 1 min at 65°C in prewarmed 1× wash buffer. The dipsticks were then washed a second time for 1 min at room temperature in 1× wash buffer. The dipsticks were removed from the wash basin, blotted, transferred to the tubes containing conjugate, and incubated 20 min at room temperature. This allows the anti-fluorescein-horseradish peroxidase conjugate to bind to the fluorescein. During the 20 min incubation, 0.75

Table 3. Comparison of colorimetric DNA hybridization and BAM/AOAC procedures for detection of *Salmonella* in uninoculated test products

Product	Samples analyzed	Total samples pos.[a]	Samples positive		
			BAM/AOAC	Colorimetric DNA hybridization	
				Assay[b] (χ^2)[d]	Confirmed[c] (χ^2)
Pork	10	0	0	0 (****)[e]	0 (****)
Roast beef	10	0	0	0 (****)	0 (****)
Turkey	10	2	2	2 (****)	2 (****)
Peanut butter	10	0	0	0 (****)	0 (****)
Shrimp	10	0	0	0 (****)	0 (****)
Coconut	10	0	0	0 (****)	0 (****)
Pecans	10	0	0	0 (****)	0 (****)
Fish	10	0	0	0 (****)	0 (****)
Nonfat dry milk	10	0	0	0 (****)	0 (****)
Milk chocolate	10	0	0	0 (****)	0 (****)
Pepper	10	0	0	0 (****)	0 (****)
Casein	10	0	0	0 (****)	0 (****)
Gelatin	10	0	0	0 (****)	0 (****)
Dry egg	10	0	0	0 (****)	0 (****)
Yeast (inactive)	10	0	0	0 (****)	0 (****)
Soy flour	10	0	0	0 (****)	0 (****)
Dry pasta	10	0	0	0 (****)	0 (****)
Bone meal	10	0	0	0 (****)	0 (****)
Cheese powder	10	0	0	0 (****)	0 (****)
Cake mix	10	0	0	0 (****)	0 (****)
All products	200	2	2	2 (****)	2 (****)

[a] Samples positive by BAM/AOAC method plus additional samples positive by cDNAH assay and confirmed from culture media.
[b] Results of colorimetric assay not considering subsequent confirmation.
[c] Results of colorimetric assay including confirmation by isolation of *Salmonella* from culture media.
[d] $\chi^2 = (|\text{samples positive by cDNAH and negative by BAM/AOAC} - \text{samples positive by BAM/AOAC and negative by cDNAH}| - 1)^2 \div (\text{samples positive by cDNAH and negative by BAM/AOAC} + \text{samples positive by BAM/AOAC and negative by cDNAH})$.
[e] Statistical analysis not applicable because cDNAH and BAM/AOAC results agreed for all samples analyzed.

mL aliquots of a mixture of substrate/chromogen (2:1 v/v) were transferred to a new set of 12 × 75 mm test tubes.

At the end of the conjugate incubation, the dipsticks were removed, washed twice for 1 min each at room temperature in 1× wash buffer to remove unbound conjugate, blotted, transferred to tubes containing substrate/chromogen, and incubated 20 min at room temperature to allow color development. At the end of this incubation, the dipsticks were removed and discarded and the color development process terminated with the addition of 0.25 mL 4N sulfuric acid.

Optical density (OD) was determined with a spectrophotometer at 450 nm. The positive cutoff value was generated by the addition of 0.10 to the negative control OD. Test samples producing an OD greater than the negative control OD + 0.10 were considered to be presumptively positive.

Samples producing positive hybridization results were further analyzed to confirm the presence of *Salmonella* by streaking corresponding GN and selective enrichment broth cultures to xylose lysine desoxycholate (XLD), Hektoen enteric (HE), and bismuth sulfite (BS) agar plates. Initial attemps at confirmation were made using the GN cultures; the selective enrichments were used for confirmation only in the event that confirmation was not obtained from the GN cultures. All subsequent steps in identification of suspicious colonies were performed as described above for the BAM/AOAC method.

Data Analysis

A pair-wise statistical analysis of the methods was performed for each food group, using the method of McNemar (9). An χ^2 value of >3.84 was indicative of a significant difference at the 5% probability level.

Results and Discussion

Inoculated Foods

Test products, inoculated organisms, and calculated inoculation levels of the test products determined on the day comparative analyses were initiated are presented in Table 1. The MPN data indicate that, for most products, the desired inoculation levels were achieved. For a few products, inoculation levels were higher than expected, but in no case did the MPN for the low level inoculation exceed 1 cell/g.

The comparative data associated with each inoculated test product are presented in Table 2. In all cases, 40 samples were analyzed (20 low and 20 high level inoculated samples). The number of samples positive by BAM/AOAC, the cDNAH assay not considering subsequent culture confirmation, and cDNAH assay and confirmed by isolation of *Salmonella* from the GN or selective enrichments are reported (Table 2).

The cDNAH assay and confirmatory data were statistically compared to the results of the BAM/AOAC analyses. A total of 800 inoculated samples were analyzed. Positive results were obtained on 628 samples using the cDNAH assay of which 625 were confirmed by isolation of *Salmonella* from the GN or selective enrichments. Six hundred sixteen samples were positive by BAM/AOAC. There was perfect agreement between cDNAH confirmed and BAM/AOAC data for 13 of the 20 foods: pork, roast beef, peanut butter, coco-

Table 4. Comparison of colorimetric DNA hybridization and BAM/AOAC procedures for detection of *Salmonella* in all test products

Product	Samples analyzed	Total samples pos.[a]	Samples positive		
			BAM/AOAC	Colorimetric DNA hybridization	
				Assay[b] (χ^2)[d]	Confirmed[c] (χ^2)
Pork	50	32	32	32 (****)[e]	32 (****)
Roast beef	50	37	37	37 (****)	37 (****)
Turkey	50	32	27	32 (2.29)	31 (1.50)
Peanut butter	50	40	40	40 (****)	40 (****)
Shrimp	50	29	28	30 (0.50)	29 (0.00)
Coconut	50	17	17	17 (****)	17 (****)
Pecans	50	37	37	37 (****)	37 (****)
Fish	50	28	28	28 (****)	28 (****)
Nonfat dry milk	50	34	34	34 (****)	34 (****)
Milk chocolate	50	39	39	39 (****)	39 (****)
Pepper	50	12	12	12 (****)	12 (****)
Casein	50	27	27	25 (0.50)	25 (0.50)
Gelatin	50	22	21	22 (0.00)	21 (0.50)
Dry egg	50	39	39	39 (****)	39 (****)
Yeast (inactive)	50	40	40	40 (****)	40 (****)
Soy flour	50	28	27	28 (0.00)	28 (0.00)
Dry pasta	50	40	40	40 (****)	40 (****)
Bone meal	50	34	34	34 (****)	34 (****)
Cheese powder	50	30	26	29 (0.80)	29 (0.80)
Cake mix	50	35	33	35 (0.50)	35 (0.50)
All products	1000	632	618	630 (5.50)	627 (3.37)

[a] Samples positive by BAM/AOAC method plus additional samples positive by cDNAH assay and confirmed from culture media.
[b] Results of colorimetric assay not considering subsequent confirmation.
[c] Results of colorimetric assay including confirmation by isolation of *Salmonella* from culture media.
[d] χ^2 = (|samples positive by cDNAH and negative by BAM/AOAC − samples positive by BAM/AOAC and negative by cDNAH| − 1)^2 ÷ (samples positive by cDNAH and negative by BAM/AOAC + samples positive by BAM/AOAC and negative by cDNAH).
[e] Statistical analysis not applicable because cDNAH and BAM/AOAC results agreed for all samples analyzed.

nut, pecans, fish, nonfat dry milk, milk chocolate, pepper, dry egg, yeast, dry pasta, and bone meal. There were some discrepancies between the number of positive samples by BAM/AOAC and the cDNAH confirmed data for 7 of the foods tested, but in no cases were these differences statistically significant (i.e., $\chi^2 > 3.84$). Considering the data for all inoculated products, the cDNAH assay was statistically equivalent to BAM/AOAC for detection of *Salmonella* in the 20 foods tested ($\chi^2 = 3.37$).

Uninoculated Control Foods

Comparative data for uninoculated control products are presented in Table 3. There was perfect agreement between cDNAH confirmed and BAM/AOAC data for all 20 foods. Two samples of one food type (turkey) were found to be naturally contaminated. *Salmonella* was isolated and characterized serologically as a different serovar (group B) compared to the inoculated serovar (group E).

All Products

A summary of the data for all samples analyzed is presented in Table 4. A total of 1000 samples was analyzed. Six hundred thirty samples were positive by the cDNAH assay of which 627 were confirmed positive by isolation of *Salmonella* from the GN post-enrichment cultures or selective enrichments. Six hundred eighteen samples were positive by the BAM/AOAC culture method. Defining a false negative as a negative reaction on a sample found positive by isolation of *Salmonella* by any method, there were 14 false negatives by BAM/AOAC (2.2%) and 5 false negatives (0.8%) by cDNAH confirmed.

There were 17 samples positive by the cDNAH assay, which were negative by BAM/AOAC, but 3 of these were not confirmed by isolation of *Salmonella* from associated culture media (tetrathionate broth, selenite cystine broth, and GN broth cultures). Assuming these unconfirmed positives to be false positives indicated a false-positive rate of 0.8% (3 unconfirmed positive assays/368 samples negative for *Salmonella*). It is noted that of the 627 samples positive by cDNAH confirmed, confirmations were obtained from the GN post-enrichment cultures alone in all but 9 cases (2 on raw shrimp, 7 on raw pork). The remaining 9 samples could not be confirmed from the GN broths, but confirmations were obtained using the associated tetrathionate broth and selenite cystine broth selective enrichment cultures. While this indicates that confirmation can be obtained from the GN broths alone in the vast majority of cases, it is advisable to retain the selective enrichment cultures from samples producing positive hybridization assay results for additional attempts at confirmation when this is not accomplished from the GN cultures. As is indicated by the results of this study, failure to achieve confirmation from the GN cultures is more likely to occur for samples containing a heavy burden of competitor microflora, such as raw meat products.

Summary

The performance of a recently developed colorimetric DNA hybridization assay for *Salmonella* was compared to the standard BAM/AOAC culture method. A total of 1000 samples consisting of inoculated and uninoculated control samples representing 20 different foods was analyzed. Eigh-

teen *Salmonella* species were included in the study. *Salmonella* was inoculated into foods at levels similar to those that might be present in naturally contaminated foods. The results demonstrated that the cDNAH method was as effective as the BAM/AOAC culture method for all conditions evaluated. Considering all samples analyzed, false negative rates for the BAM/AOAC culture method and the confirmed cDNAH assay were 2.2% and 0.8%, respectively. Assuming all samples that could not be confirmed by isolation of *Salmonella* by the BAM/AOAC or cDNAH confirmation procedures negative, the false-positive rate for the cDNAH assay was 0.8%. On the basis of these results, the cDNAH assay appeared to be an effective procedure for rapid detection of *Salmonella* in foods.

An AOAC collaborative study involving 11 laboratories has recently been completed and the colorimetric DNA hybridization method for *Salmonella* in foods has been adopted official first action. The results of the collaborative study have been reported separately (8).

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MYCOTOXINS

Comparison of Two Immunochemical Methods with Thin-Layer Chromatographic Methods for Determination of Aflatoxins

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Three different methods were compared for the determination of total flatoxins in corn and peanuts naturally contaminated with aflatoxins and in corn, peanuts, cottonseed, peanut butter, and poultry feed spiked with aflatoxins B_1, B_2, and G_1. The 3 methods were an enzyme-linked immunosorbent assay (ELISA) screening test; a monoclonal antibody–affinity column–solid-phase separation method; and the AOAC official thin-layer chromatography (TLC) methods for all except poultry feed, for which Shannon's TLC method for mixed feed was used. The ELISA test is designed to provide only positive results for total aflatoxins at ≥20 ng/g or negative results at <20 ng/g. The affinity column separation is coupled with either bromination solution fluorometry to estimate total aflatoxins or liquid chromatography (LC) to quantitate individual aflatoxins. Fluorodensitometry was used to determine aflatoxins in commodities analyzed by the TLC methods. The LC and TLC results were in good agreement for all the analyses. The results for the affinity column using bromination solution fluorometry were similar except those for cottonseed, which were about 60% higher. The ELISA screening method correctly identified naturally contaminated corn and peanut positive samples. No false positives were found for controls. The correct response for spiked corn, raw peanuts, peanut butter, and cottonseed at ≥20 ng aflatoxins/g was about 90%. The correct response for spiked poultry feed at ≥20 ng aflatoxins/g was about 50%.

Biotechnology offers new tests for rapid, simple, and sensitive determination of aflatoxin contamination in grains and grain products. These tests are based on the affinity of the monoclonal or polyclonal antibodies for aflatoxins. The antibodies are covalently attached to Sepharose in an affinity column, to a membrane attached to a plastic or paper holder, or to wells in microtiter plates, as in several enzyme-linked immunosorbent assays (ELISA) (1–4). These immunochemical methods use specific antibodies to capture the aflatoxins in food extracts.

As the popularity of the immunoassays grows, the demand to replace some of the older methods with these faster methods continues to increase. Thus the effectiveness of some of these methods for aflatoxin analysis needs to be evaluated. Fifteen sets of test samples containing various levels of aflatoxins B_1, B_2, and G_1 in corn, peanuts, peanut butter, cottonseed, and poultry feed were prepared (5). Twelve sets of the test samples were used in a collaborative study of an ELISA test kit. One set was analyzed by the AOAC official thin-layer chromatography (TLC) methods for corn, peanuts, and peanut butter (AOAC **26.026–26.031,** 14th Ed.; **968.22,** 15th Ed.) and for cottonseed (AOAC **26.052–26.058,** 14th Ed.; **980.20A–G,** 15th Ed.) (6), and by Shannon's method for mixed feed (7). The remaining 2 sets were analyzed by the ELISA method used in the collaborative study (5) and an affinity column separation method (8). The ELISA method (5, 9) was designed to give positive results for commodities containing ≥20 ng aflatoxins (B_1, B_2, G_1)/g. In the affinity column method, the final test sample extract can be used for total aflatoxin estimation by solution fluorometry with bromine (Br_2 method) or for quantitation of individual aflatoxins B_1, B_2, G_1, and G_2 by liquid chromatography (LC) with a UV detector (LC method). Results obtained by the 2 immunochemical techniques were compared with those obtained by the AOAC official TLC methods. The effectiveness of these newer methods depends on their overall agreement with the official methods.

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Experimental

Test Samples

Portions of 25 lb each of ground corn, peanut butter, and raw peanuts were analyzed according to AOAC **26.026–26.031 (968.22)** and **26.058 (980.20G)** (6); cottonseed was analyzed according to AOAC **26.052–26.058 (980.20A–G)** (6). The method of Shannon et al. (7) was used for the analyses of poultry feed. All commodities were found to contain <2 ng total aflatoxins/g. Corn, raw peanuts, peanut butter, and poultry test samples were spiked in duplicate with total aflatoxins B_1, B_2, and G_1 at 30, 20, and 10 ng/g. The ratio of B_1:B_2:G_1 was 10:1:3. Test samples of cottonseed were spiked in the same manner but at 60, 20, and 10 ng/g. The artificially contaminated (spiked) test samples, controls (<2 ng/g), and naturally contaminated corn (101 ng/g) and peanut meal (69 ng/g) test samples (analyzed according to AOAC **26.026–26.031 (968.22)** were used in this study.

Apparatus and Reagents

See refs, 5, 6, 7, and 8 for the complete listings. An abbreviated list that includes the most important items is given below.

(a) *General equipment.*—High-speed blender; micropipet and tips; filter paper; test tube (12 × 75 mm, borosilicate); timer; syringe (20 mL).

(b) *Fluorodensitometer.*—366 nm excitation, 420 nm emission.

(c) *TLC plates.*—Silica Gel 60.

(d) *Fluorometer.*—Sequoia Turner Model 450 (360 nm excitation filter, 450 nm emission filter).

(e) *Affinity column.*—Aflatest-P (Vicam, Somerville, MA 02145).

(f) *LC system.*—Injector, pump, UV detector, and C_{18} column.

(g) *ELISA test kit.*—ImmunoDot Screen Cup (International Diagnostic Systems Corp., St. Joseph, MI 49085), consisting in part of the following: antibody-coated cup; aflatoxin-enzyme conjugate; phosphate-buffered solution (pH 7.2); tetramethylbenzidine aqueous solution (0.4 g/L); hydrogen peroxide solution (0.02% in 0.13% aqueous citric acid).

TLC Methods

Portions of corn, raw peanuts, and peanut butter were extracted with chloroform and water and the extracts were analyzed according to AOAC **26.026–26.031 (968.22)** (6). Cottonseed was extracted with aqueous acetone as described in Pons' method, AOAC **26.052–26.058 (980.20A–G)** (6). Poultry feed was blended with dichloromethane and citric acid as indicated by Shannon et al. (7). All final extracts were analyzed and quantitated by a TLC-fluorodensitometric technique.

Monoclonal Antibody Affinity Column Method (8)

Extraction of corn, cottonseed, and poultry feed.—Weigh 50 g test portion into blender. Add 100 mL methanol–water (8 + 2) and blend for 1 min at high speed. Filter, and collect 12 mL filtrate. Add 36 mL water, mix, and pipet 8 mL (equivalent to 1 g test sample). Proceed with column chromatography.

Extraction of peanuts and peanut butter.—Weigh 50 g test portion into blender. Add 5 g NaCl and 125 mL methanol–water (6 + 4). Blend 1 min at high speed. Add 125 mL water, and blend at low speed for 2 s. Filter, and collect 5 mL (equivalent to 1 g test sample). Proceed with column chromatography.

Column chromatography.—Couple monoclonal antibody column to 30 mL syringe barrel with connector. Add test extract to syringe barrel, and use syringe plunger to push extract through column. Flow rate is ca 6 mL/min. Remove syringe, take plunger out of barrel, and reconnect syringe to column. Add 20 mL water to barrel and apply pressure to push water through. Remove as much water from packing as possible. Place small test tube under column. Add 1 mL methanol directly to column and use syringe to push it through column (1–2 drops/s). Use fluorometer cuvet as receiver to collect eluate and continue with quantitation by either bromination solution fluorometry or LC (eluate is equivalent to 1 g test sample).

Estimation of total aflatoxins by bromination solution fluorometry.—Set instrument excitation at 360 nm and emission at 450 nm. Insert blank and adjust zero knob to display 0. Insert standard solution (34 mg quinine sulfate/mL 0.1N H_2SO_4; this solution gives same fluoroscence intensity as 20 ng B_1/2 mL) and adjust span knob to display 20. Add 1 mL 0.001% bromine solution to eluate (total volume, 2 mL). Mix, and place test tube in instrument. Wait exactly 1 min and record reading (ng total aflatoxin/g test sample).

Quantitation of individual aflatoxins by liquid chromatography.—Add 0.2 mL water to eluate, mix, and evaporate under stream of nitrogen to 100–200 μL. Apply entire test extract to C_{18} column. Mobile phase is methanol–water (45 + 55) and flow rate is 1 mL/min. Set UV detector at 365 nm and 0.01 AUFS. Approximate retention times for aflatoxins B_1, B_2, and G_1 are 16, 12, and 10 min, respectively.

ELISA Screening Method (5)

Extraction of corn, raw peanuts, cottonseed, and poultry feed.—Weigh 50 g test portion in blender jar. Add 100 mL methanol–water (8 + 2). Blend 3 min at high speed, filter, and collect 5 mL. Measure 200 μL filtrate. Add 400 μL dilution buffer, mix, and save for immunoassay (test solution A).

Extraction of peanut butter.—Weigh 50 g test portion into blender jar. Add 250 mL methanol–water–hexane (55 + 45 + 100). Blend 3 min at high speed, filter, and separate layers. Place 15 mL aqueous methanol layer in beaker and heat on steam bath for 3 min to eliminate residual hexane. Pipet 500 μL test extract into test tube. Add 500 μL dilution buffer, mix, and save for immunoassay (test solution B).

Immunoassay procedure for corn, raw peanuts, cottonseed, and poultry feed.—Add 150 μL test solution A to ELISA cup, wait 1 min, and apply additional 150 μL test solution A to cup. Wait 1 min, and add 100 μL enzyme–aflatoxin conjugate solution. Wait 1 min, and add 1.5 mL wash solution. Add 1.0 mL substrate solution. Wait 1 min and observe color development.

Immunoassay procedure for peanut butter.—Add three 200 μL aliquots of extract (solution B) to cup at 1 min intervals and proceed as for corn.

Determination.—Observe center of cup for blue color or no color development exactly 1 min after adding substrate. Test samples containing ≥20 ng aflatoxins/g show no color development; test samples containing <20 ng aflatoxins/g develop blue or gray color in center of the cup.

Results and Discussion

The results for the comparison of the 3 methods and a summary of the results of the ELISA collaborative study (5) are given in Table 1. The ELISA test is designed to determine whether test samples contain more or less than 20 ng aflatoxins/g. The analysis may result in one of 2 outcomes: Positive results indicate test samples containing ≥20 ng aflatoxins/g, whereas negative results indicate test samples containing <20 ng aflatoxins/g. One set of test samples was analyzed in Trucksess's laboratory by the ELISA test before other test samples were sent to collaborators. All naturally contaminated corn and raw peanuts were found to be positive. At the 0 ng/g level, no false positives (all <20 ng/g) were found for all commodities. All test samples spiked at 30 ng/g were found positive, i.e, there were no false negatives. At 10 ng/g, 3 of the 10 samples analyzed were found positive. With the exception of poultry feed, all test samples at 20 ng/g were found positive. Only one of the 2 poultry feed test samples was found positive at 20 ng/g.

The validity of the method is supported by the data from the collaborative study (5). In this study, the ELISA test identified all controls (<2 ng/g) as negative and all naturally contaminated corn and raw peanuts as positive. The average positive rates for spiked test samples of corn, peanuts, peanut butter, and cottonseed at 10, 20, and ≥30 ng aflatoxins/g were 52, 86, and 96%, respectively, i.e., a 14% false negative rate was found at the 20 ng/g level. The ELISA test failed to identify 54% of the feeds containing aflatoxins added at 20 ng/g as positive. Results of the collaborative study do not agree completely with the results we obtained. We believe the discrepancy is most likely due to the inexperience of the collaborators in differentiating background color from the color change expected for aflatoxin.

Table 1. Results of ELISA, affinity column methods, and TLC methods for aflatoxins in corn, raw peanuts, peanut butter, cottonseed, and poultry feed[a]

Commodity	Total aflatoxins,[b] ng/g	ELISA positive		Affinity column				TLC	
		%[c]	%[d]	Br_2, ng/g	Av. rec., %	LC, ng/g	Av. rec., %	ng/g	Av. rec., %
Corn	NC[e]	100	100	146		140		101	
	30	100	92	27	90	26	87	29	97
	20	100	75	19	95	18	90	21	105
	10	0	33	7	70	8	80	11	110
	0	0	0	1		1		0	
					85		86		104
Raw peanuts	NC[f]	100	100	72		71		69	
	30	100	96	27	90	22	73	28	93
	20	100	83	19	95	19	95	19	95
	10	50	54	13	130	12	120	10	100
	0	0	0	2		1		<1	
					105		96		96
Peanut butter	30	100	100	33	110	31	103	27	90
	20	100	91	23	115	24	120	19	95
	10	50	61	12	120	12	120	10	100
	0	0	0	2		1		2	
					115		114		95
Cottonseed	60	100	96	77	128	50	83	69	115
	20	100	96	30	150	17	85	lost	
	10	50	58	9	90	7	70	8	80
	0	0	0	0		0		<2	
					123		79		98
Poultry feed	30	100	83	27	90	17	57	11	37
	20	50	46	8	40	6	30	7	35
	10	0	4	4	40	4	40	5	50
	0	0	0	0		0		<2	
					57		42		41

[a] Average of duplicate results for test samples containing aflatoxins; single results for test samples containing no aflatoxins.
[b] Ratio of aflatoxins in spiked test samples was B_1:B_2:G_1 = 10:1:3.
[c] Results obtained by authors in this study.
[d] Results of collaborative study (5).
[e] Corn naturally contaminated with aflatoxins (101 ng/g).
[f] Raw peanuts naturally contaminated with aflatoxins (69 ng/g).

The results obtained by the affinity column method and the TLC methods are shown in Table 1. Each data entry for the affinity column methods (Br_2 method and LC method) and the TLC methods represents the average of total aflatoxins found in duplicate analyses, except the 0 ng/g level, where only a single analysis was performed. The average recoveries of added aflatoxin, excluding poultry feed, were 107, 94, and 98% for the affinity column–Br_2 method, affinity column–LC method, and TLC methods, respectively. The low recoveries of aflatoxins from feeds could be due to interference of ingredients in the feed with the analysis by complexing or decomposing the aflatoxins. The recoveries of the individual

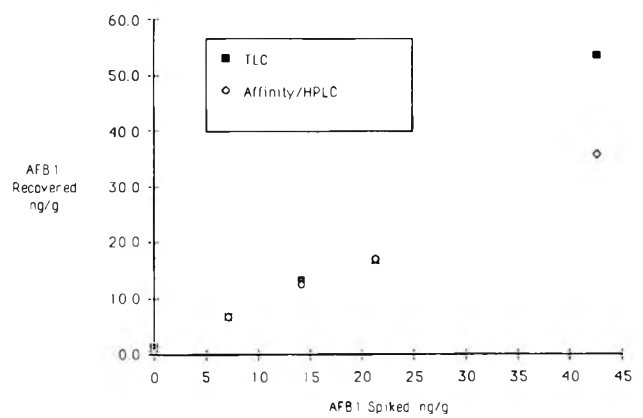

Figure 1. Recovery of aflatoxin B_1 from spiked test samples.

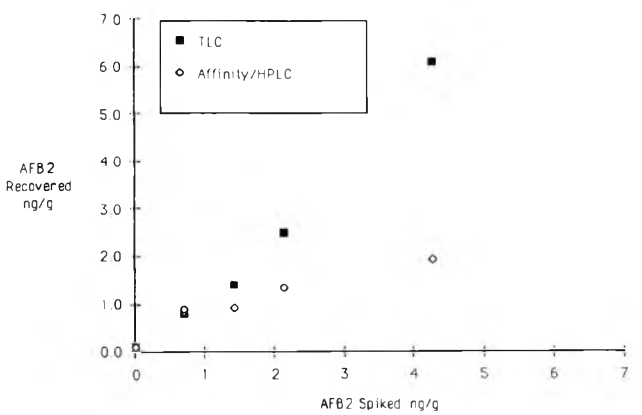

Figure 2. Recovery of aflatoxin B_2 from spiked test samples.

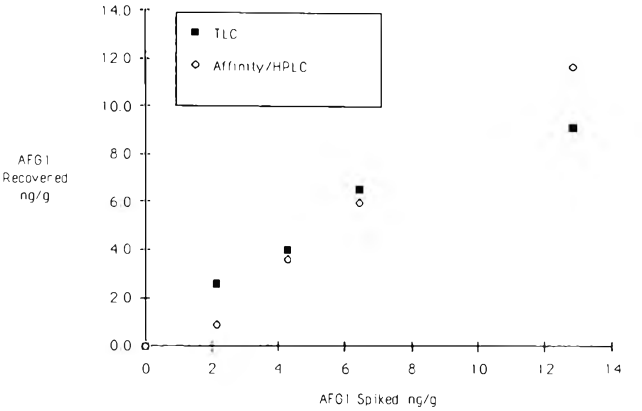

Figure 3. Recovery of aflatoxin G_1 from spiked test samples.

aflatoxins B_1, B_2, and G_1 from the spiked test samples for the TLC and the affinity column (LC) methods were also similar (Figures 1–3). The 2 methods compare very favorably.

In conclusion, the ELISA test was effective, i.e., 86–96% correct (when compared with results of the official TLC methods), at identifying test samples contaminated with ≥20 ng aflatoxins/g as positive. False positives and false negatives were observed for test samples spiked at 10–20 ng/g. The ELISA method has a low percentage of false negatives. It can be used to analyze a large number of test samples at the designated level of interest (above 20 ng/g) and eliminate negative test samples. The results of the affinity column methods and the TLC methods were in good agreement with one another. Solution fluorometry after affinity column cleanup can serve as a means to estimate total aflatoxin contamination; quantitation by LC with a UV detector can accurately determine the individual aflatoxins. The affinity column cleanup for aflatoxin analysis is as effective as the long-established CB method, AOAC **26.026–26.031 (968.22)** (6), and cottonseed method, AOAC **26.052–26.058 (980.20A–G)** (6).

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PESTICIDE FORMULATIONS

Liquid Chromatographic Determination of Bromadiolone in Rodent Baits

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A method is described for the determination of bromadiolone in rodent bait formulations. Samples are Soxhlet-extracted using methanol as extractant and analyzed by reverse-phase liquid chromatography with UV detection at 280 nm. Chromatography is performed using a ODS-Hypersil (5 μm) column, which enables separation of the 2 diastereoisomers of bromadiolone. The sum of the peak areas of the diastereoisomers is used for quantitation. The method was tested for precision, linearity, and recovery. Duplicate analyses of 10 formulation samples gave a mean relative standard deviation of 4.1%. Linearity was very good (correlation coefficient 0.9997) in the relevant concentration range. Recovery from spiked samples was 88.9 ± 2.3%. The method is applicable to rodent bait formulations with bromadiolone content 0.005% and 0.01%.

Bromadiolone, 3-[3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin, was introduced in 1977 as a rodenticide by Lipha (France) and is now widely used to control various vertebrate pests. Formulations consist predominantly of baits, where bromadiolone is impregnated on various grains. Products are formulated containing 0.005–0.01% bromadiolone. Technical grade bromadiolone exists as a mixture of 2 diastereoisomers; stated contents of formulations are total bromadiolone contents. Several analytical methods for determination of bromadiolone in various biological matrixes are described (1–5), all based on liquid chromatography (LC). No method on determination of bromadiolone in formulations has been published. For pesticide product control purposes by the national authorities in Denmark, such a validated method was needed.

An LC method is presented here for determination of bromadiolone in bait formulations consisting of impregnated grains. Samples are prepared by Soxhlet extraction. No further cleanup is required before analysis by reverse-phase LC with UV detection.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Model 510 pump (Waters Chromatography Div./Millipore, Milford, MA) equipped with Waters Model 712 WISP autosampler, Waters Model 490 programmable multiwavelength detector set at 280 nm and Hewlett-Packard Model 3393 A integrator (Hewlett-Packard Co., Avondale, PA), or equivalent.

(b) *LC column.*—250 × 4.6 mm ODS-Hypersil 5 μm (Bischoff, Leonberg, FRG), or equivalent. Column temperature must be maintained at 25.0 ± 0.1°C by LC column thermostat.

(c) *Extraction apparatus.*—100 mL Soxhlet extractor with 120 × 30 mm cellulose extraction thimbles (Whatman Ltd, Maidstone, England).

(d) *Grinding apparatus.*—Electric grinding mill capable of grinding grains to a fineness of 10–20 mesh (Regent Maskin AB, Stockholm, Sweden), or equivalent.

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Reagents

(a) *Solvents.*—Methanol and glacial acetic acid, both LC grade (May & Baker Ltd, Dagenham, England). Water was purified by Milli-Q purification system (Millipore).

(b) *Mobile phase.*—Methanol–water–glacial acetic acid (70 + 30 + 2). Adjustment of acetic acid concentration may be necessary to separate bromadiolone isomers from sample coextractants. Mobile phase flow rate 1.6 mL/min.

(c) *Bromadiolone standard solution.*—Accurately weigh ca 50 mg bromadiolone standard (Lipha, Lyon, France) into 100 mL volumetric flask and dilute to volume with methanol. From this solution, transfer 2.00 mL to 50 mL volumetric flask and dilute to volume with mobile phase to prepare bromadiolone standard solution.

Extraction and Liquid Chromatography

Sample and standard must be protected from light during preparation and analysis.

Accurately weigh amount of freshly ground sample equivalent to ca 0.5 mg bromadiolone into extraction thimble, plug with cotton, and extract with 250 mL methanol for 3 h on Soxhlet extraction apparatus. Let sample cool, evaporate almost to dryness on rotary evaporator (maximum temperature 40°C), transfer residue quantitatively to 25 mL volumetric flask and dilute to volume with mobile phase. Inject 10 μL into chromatograph. Bracket duplicate injections of each sample with 10 μL injections of standard solution.

Calculations

Use sum of 2 diastereoisomers peak areas as bromadiolone peak area. Sample bromadiolone response (R) is mean of duplicate injections and standard bromadiolone responses (R′) is mean of standards that bracket sample. Calculate sample content of bromadiolone as follows:

$$\text{Bromadiolone, \%} = (R \times W' \times V \times P)/(R' \times W \times V')$$

where W and W′ are weights of sample and standard, respectively; V and V′ are dilution volumes of sample and standard, respectively (here, V = 25 and V′ = 2500), and P is % purity of standard.

Results and Discussion

The isomeric ratio of bromadiolone diastereoisomers can be different from batch to batch, and is therefore often different from sample to standard. Experimental determination of response factors using pure isomers showed no difference between the 2 diastereoisomers. Thus, total content of bromadiolone is calculated by addition of the 2 diastereoisomer peak areas. Linearity was investigated by analyzing a series of dilutions (6 concentrations) of bromadiolone standard. The relationship between total peak area and concentration was linear (correlation coefficient = 0.9997) over the concentration range 5–50 μg/mL.

Figure 1A shows a typical chromatogram from a sample

Table 1. Results of duplicate LC analyses of bromadiolone in rodent bait formulations

Sample	Label claim, %	Found, %			
		Weight 1	Weight 2	Average	RSD, %
1	0.005	0.00502	0.00522	0.00512	2.8
2	0.005	0.00561	0.00512	0.00537	6.5
3	0.005	0.00661	0.00647	0.00654	1.5
4	0.005	0.00523	0.00484	0.00504	4.0
5	0.005	0.00579	0.00532	0.00556	3.5
6	0.01	0.00651	0.00616	0.00633	4.2
7	0.01	0.00877	0.00922	0.00900	5.5
8	0.01	0.00904	0.00852	0.00878	4.9
9	0.01	0.00757	0.00706	0.00732	2.1
10	0.01	0.01098	0.01066	0.01082	6.0
Mean					4.1

(label claim 0.01%) analyzed by the conditions given in *Method*. Effect of acetic acid concentration in mobile phase on the separation of bromadiolone isomers from coextracted sample components is demonstrated by Figure 1B. The chromatogram (same sample as Figure 1A) shows that adjustment of acetic acid concentration in the mobile phase is a possible way of eliminating interference from sample coextractants.

Table 1 shows the results of duplicate analyses of bromadiolone in 10 samples of rodent baits. The 10 samples were formulations with a claimed bromadiolone content of 0.005% (5 samples) and 0.01% (5 samples). A mean precision of 4.1% was found to be acceptable for samples with this level of analyte.

Extraction of bromadiolone from samples was performed by Soxhlet extraction for 3 h. Preliminary experiments had shown that increasing the extraction time gave no increase in extraction efficiency. Grinding of samples before extraction was chosen as a part of sample preparation although no significant difference was observed in a comparison of extraction efficiency from one sample prepared with and without grinding.

Due to susceptibility of bromadiolone to degradation when exposed to light, the extraction apparatus was wrapped with aluminum foil during extraction, and standard and sample solutions were protected from light during the analysis. Table 2 shows the results of bromadiolone analysis of samples to which known amounts of bromadiolone were added. The 2 samples with no initial content of bromadiolone were grain samples intended for preparation of baits by a production company. The mean recovery from these spiked samples was 88.9% with a standard deviation of 2.3% (RSD = 2.6%).

Table 2. Recovery of bromadiolone from spiked samples by LC analysis

Sample	Label claim, %	Found before spiking,[b] %	Added, %	Found after spiking,[b] %	Rec. %
1[a]	0	ND[c]	0.00441	0.00402	91.2
2[a]	0	ND	0.00879	0.00785	89.3
3	0.01	0.00738	0.01002	0.01596	85.8
4	0.01	0.00756	0.01002	0.01652	89.4
Mean					88.9
SD					2.3

[a] Grain sample not impregnated with bromadiolone.
[b] Duplicate determinations.
[c] Not detected.

The method presented here is simple and useful for the determination of total bromadiolone content in rodent baits. Chromatographic separation of the 2 bromadiolone diastereoisomers is good, so the method is also expected to be useful for determination of content of individual isomers in bait formulations.

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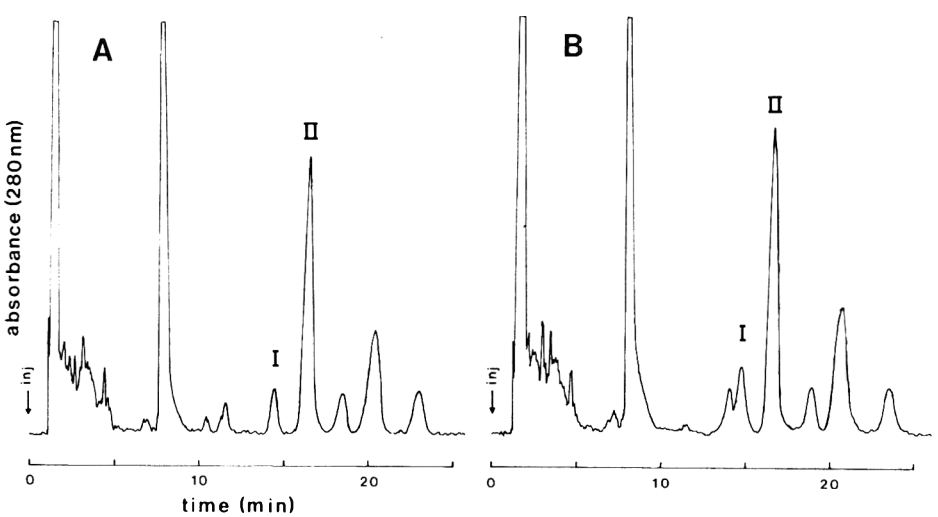

Figure 1. Chromatogram of typical rodent bait sample. Experimental conditions identical, except mobile phase composition: A, Methanol-water-glacial acetic acid (70 + 30 + 2); B, methanol-water-glacial acetic acid (70 + 30 + 1). I and II are bromadiolone stereoisomers.

Liquid Chromatographic Method for Determination of Oxydemeton-Methyl in Formulated Products: Collaborative Study

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A liquid chromatographic method has been developed for the determination of oxydemeton-methyl (Metasystox-R^R) in formulated products. Samples are dissolved in acetonitrile and analyzed by reverse-phase chromatography using methyl paraben as an internal standard. Twelve laboratories participated in a collaborative study of the method. Each collaborator was furnished with reference standard, internal standard, and blind duplicate samples of Metasystox-R 50% concentrate (MSR 50 Conc), Metasystox-R spray concentrate (MSR SC), and DYLOX^R/MSR 1.5–0.5 formulations. Collaborators were instructed to use peak area measurements for quantitation. Relative standard deviations for reproducibility (RSD$_R$) were 1.44, 2.14, and 3.63%, respectively. The method has been approved interim official first action by AOAC.

Oxydemeton-methyl (Metasystox-R^R), *S*-[2-(ethylsulfinyl)ethyl] *O,O*-dimethyl phosphorothioate, is available in 50% concentrate, spray concentrate, and DYLOX^R/MSR 1.5–0.5 formulations. Oxydemeton-methyl is a systemic insecticide typically applied to foliage, bark, and root systems. It is characterized by its selectivity toward aphids, leafhoppers, and other sucking pests.

A variety of gas chromatographic (GC) procedures have been applied for determination of oxydemeton-methyl. Most chromatographers have noted the extreme thermal lability of this material, which results in substantial decomposition during GC analyses (1–3). Others have performed oxidation reactions to convert the active ingredient to its sulfone (4–7). One group of chromatographers attempted to stabilize column performance with lecithin (8). Although hydrolysis has been used for routine analysis of technical and formulated products for manufacturing control, it is a nonspecific procedure (9).

A reverse-phase liquid chromatographic (LC) method that uses methyl paraben as an internal standard was developed, which separates oxydemeton-methyl from all known impurities, formulation excipients, and decomposition products (10). The method was submitted for collaborative study.

Collaborative Study

Twelve collaborators agreed to participate in the study, which was designed according to suggestions of Youden and Steiner (11). Each collaborator received a copy of the method, blind duplicate subsamples of each formulation, a reference standard, and an internal standard. Participants also received a material safety data sheet and were cautioned concerning the dermal absorptivity of the materials (12). A practice sample was supplied and collaborators were instructed to try a different manufacturer's column if the chromatograms exhibited a split analyte peak. Collaborators were also instructed to store study samples and the standard under refrigeration until the study was started and to allow the materials to come to room temperature before opening them. Peak areas were specified for quantitation.

Results and Discussion

A complete set of results was received from each of the 12 collaborators (Table 1). The collaborators used a variety of equipment to perform the analyses: 5 brands of chromatographs, 7 brands of injectors, 5 brands of detectors, 7 brands of data systems, and 7 brands of columns. The types of columns included Du Pont Zorbax ODS, Whatman Partisil ODS-2, Whatman Partisil ODS-3, E. Merck LiChrosorb RP18, Alltech Econosil C18, Alltech RSiL C18HL, and Alltech Versapack C18. Sample volumes injected varied from 10 to 25 μL. The pressures obtained varied from 4.9 to 22.4 MPa, and flow rates from 1.0 to 2.0 mL/min were used.

Collaborators were informed in advance of the possibility that a split analyte peak might occur occasionally, as shown

Table 1. Collaborative results of LC determination of oxydemeton-methyl (%) in formulations as blind duplicate samples

	MSR 50 Conc		MSR SC		DYLOX/MSR	
Coll.	A	B	A	B	A	B
1	47.5	46.9	25.5	25.1	4.69	4.58
2	47.8	48.1	26.2	25.7	4.69	4.56
3	47.5	46.1	24.7	25.1	4.61	4.56
4	47.6	47.6	25.1	25.7	4.60	4.67
5	47.7	47.4	25.7	25.7	4.46	4.46
6	48.5	48.0	25.7	26.1	5.71[a]	6.32[a]
7	48.0	48.0	26.1	26.2	4.92	4.87
8	46.6	46.5	25.0	24.8	4.47	4.47
9	46.5	46.1	24.7	25.2	4.50[a]	5.89[a]
10	47.0	47.3	25.8	25.6	4.72	4.72
11	47.9	47.2	25.8	26.1	4.63	4.71
12	45.5[a]	47.1[a]	24.3	25.1	5.01	4.96
Mean	47.35		25.46		4.67	
s_R	0.681		0.544		0.170	
s_r	0.399		0.300		0.049	
s_L	0.552		0.454		0.162	
RSD$_R$, %	1.44		2.14		3.63	
RSD$_r$, %	0.842		1.18		1.05	
N	11		12		10	

[a] Outliers rejected by the Cochran test.

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

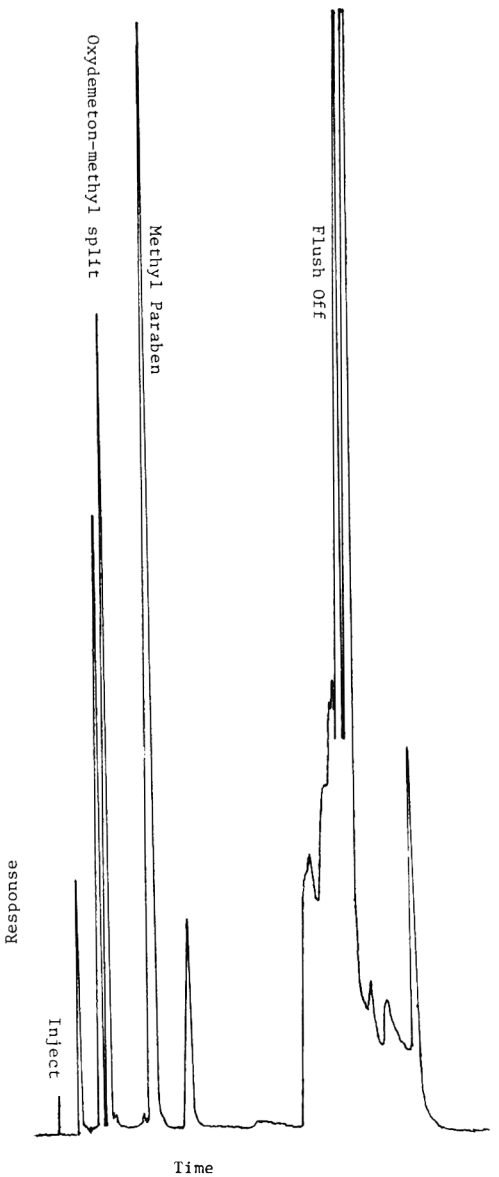

Figure 1. Abnormal chromatogram of Metasystox-R spray concentrate formulation showing unusual splitting of oxydemeton-methyl peak. Whatman Partisil 10 ODS column (5% carbon load).

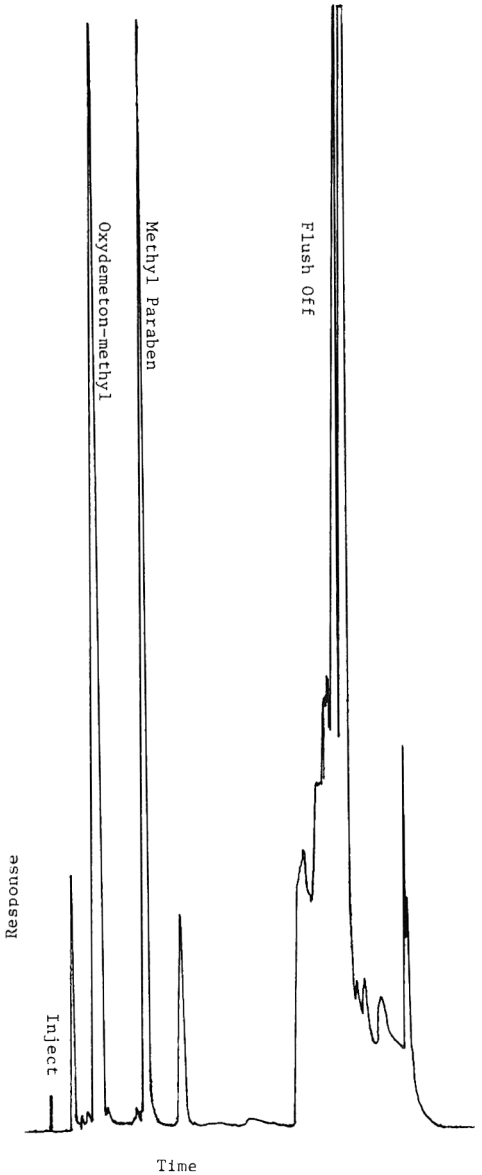

Figure 2. Typical chromatogram of Metasystox-R spray concentrate formulation showing correct peak shape. Whatman Partisil 10 ODS-2 column (15% carbon load).

in Figure 1. This abnormal event usually occurs only when an LC column of low carbon loading is used. A high carbon loading column (e.g., Whatman Partisil ODS-2) does not produce this effect. Figure 2 is a typical chromatogram obtained using a Whatman Partisil ODS-2 column.

To lessen variations between laboratory data systems, a slope sensitivity of ≤ 0.010 mV/min was recommended as a starting point to ensure proper integration of peak areas.

Collaborators' Comments

Comments were solicited but only a few were received. Two collaborators stated that they considered the method good and easy to perform. Another reported difficulties with peak tailing on their system and two others had difficulty meeting the ±1% repeatability requirements. Four collaborators performed the analyses over 2 day periods.

Recommendation

It is recommended that the LC method for determination of oxydemeton-methyl in 50% concentrate, spray concentrate, and DYLOX/MSR 1.5–0.5 formulations be adopted official first action as an AOAC-CIPAC method.

Oxydemeton-Methyl in Pesticide Formulations

Liquid Chromatographic Method

Interim First Action

AOAC-CIPAC Method

(Applicable to MSR 50% concentrate, MSR spray concentrate, and DYLOX/MSR formulations)

Method Performance:

MSR 50% concentrate

$s_r = 0.40$; $s_R = 0.68$; $RSD_r = 0.84\%$; $RSD_R = 1.44\%$

MSR spray concentrate

$s_r = 0.30$; $s_R = 0.54$; $RSD_r = 1.18\%$; $RSD_R = 2.14\%$

DYLOX/MSR

$s_r = 0.05$; $s_R = 0.17$; $RSD_r = 1.05\%$; $RSD_R = 3.63\%$

A. Principle

Oxydemeton-methyl is determined by liquid chromatography using an octadecyl bonded silica column with high

carbon loading. Peak areas of sample and analytical standard are compared using methyl 4-hydroxybenzoate (methyl paraben) as internal standard.

B. Apparatus

(a) *Liquid chromatography.*—Able to generate >7 MPa (>1000 psi) and measure absorbance at 225 nm. Operating conditions: column temperature ambient; flow rate ca 2 mL/min (ca 1000 psi); chart speed 0.5 cm/min; injection volume ca 10 μL; absorbance range 0.16 AUFS. Retention times: oxydemeton-methyl ca 2.8 min; internal standard ca 6.7 min. Pump LC mobile phase through column until system is equilibrated (flat baseline). After each injection, allow 1 min after internal standard elutes before flushing column with CH_3CN for 8 min. Return to mobile phase, reequilibrate, and continue injections.

(b) *Chromatographic column.*—250 mm × 4.6 mm id packed with ≤10 μm C18 bonded silica gel (Partisil ODS-2, or equivalent); equipped with a protective precolumn of similar packing. (*Note:* Use column with high carbon loading. If carbon loading is insufficient, e.g., <10%, oxydemeton-methyl peak may split and results will be erroneous.)

C. Reagents

(a) *Acetonitrile.*—CH_3CN. LC grade or distilled in glass.

(b) *LC mobile phase.*—CH_3CN–H_2O (30 + 70).

(c) *Internal standard.*—Methyl 4-hydroxybenzoate (methyl paraben) 0.8% w/v in CH_3CN.

(d) *Oxydemeton-methyl reference standard.*—Secondary standard ca 50% purity in methyl isobutyl ketone for stabilization (Mobay Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120-0013). Always store standard in freezer when not in use. Let warm to room temperature before opening. (*Caution:* See safety notes on mammalian toxicity, **H.**)

(e) *Water.*—LC grade or distilled in glass.

D. Preparation of Standard Solution

Accurately weigh ca 400 mg reference standard (ca 200 mg oxydemeton-methyl), **C(d)**, into a 100 mL volumetric flask. Pipet 5 mL internal standard, **C(c)**, into flask, dilute to volume with CH_3CN, and mix thoroughly.

E. Preparation of Sample

Accurately weigh amount of sample containing ca 200 mg oxydemeton-methyl into a 100 mL volumetric flask. Pipet 5 ml internal standard into flask, dilute to volume with CH_3CN, and mix thoroughly.

F. Determination

Adjust operating parameters so that oxydemeton-methyl elutes in 2.7–3.0 min. Adjust injection volume and attenuation to give largest possible on-scale peaks. Adjust integrator parameters to resolve oxydemeton-methyl peak from any closely eluting peaks. Make repetitive injections of standard solution and calculate response ratios by dividing oxydemeton-methyl peak area by peak area of internal standard. Peak ratios must agree within ±1%. Average duplicate response ratios obtained with standard solutions.

Inject duplicate aliquots of each sample solution. Response ratios must agree within ±1%. If not, repeat determination starting with standard injections. Reinject standard solution. Averages of response ratios of standards immediately preceding and following sample injections must agree within ±1%. Repeat determination on any samples not bracketed by agreeing ratios for standard solutions.

G. Calculation

For each injection, response ratio, R = (oxydemeton-methyl peak area/internal standard peak area).

$$\text{Oxydemeton-methyl, wt \%} = (R/R') \times (W'/W) \times P$$

where R and R' = average response ratios for sample and standard solutions, respectively; W' and W = weight, mg, of oxydemeton-methyl in standard and samples solutions, respectively; and P = purity of oxydemeton-methyl standard, %.

H. Safety Note

Mammalian toxicity for oxydemeton-methyl formulations is as follows (Mobay Corp. Product Material Safety Data Sheet):

MSR 50 Conc: Oral LD_{50} for male rats 105 mg/kg; for female rats 70 mg/kg. Dermal LD_{50} for male rats 350 mg/kg; for rabbits ca 225 mg/kg. Inhalation LD_{50} for male rats 1.5 mg/L/h.

MSR SC: Oral LD_{50} for male rats 125 mg/kg; for female rats 138 mg/kg. Dermal LD_{50} for male rabbits 359 mg/kg; for female rabbits 253 mg/kg. Inhalation LD_{50} for rats >1.69 mg/L/h.

DYLOX/MSR: Oral LD_{50} for male rats ca 524 mg/kg; for female rats 488 mg/kg. Dermal LD_{50} for rabbits >1000 mg/kg. Inhalation LD_{50} for rats >8 mg/L/h.

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CAS-301-12-2 (oxydemeton-methyl)

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Simplified Cleanup and Liquid Chromatographic Ultraviolet Determination of Linuron and Three Metabolites in Potatoes

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A simple and efficient method is presented for the extraction, cleanup, and liquid chromatographic (LC) determination of linuron and 3 of its metabolites, 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU), 3-(3,4-dichlorophenyl) urea (DCPU), and 3,4-dichloroaniline (DCA), in potatoes. Samples are extracted with acetone, partitioned into dichloromethane–hexane (1 + 1), and cleaned up using disposable silica cartridges. LC determination is performed using a LiChrosorb NH_2 5 μm column, with an isopropanol–isooctane gradient mobile phase and UV detection at 248 nm. Recoveries of linuron and 2 of the metabolites from untreated samples fortified at 0.02–2 μg/g ranged from 80 to 102%, while recoveries for the metabolite DCA ranged from 60 to 78%. The detection limit was 0.015 μg/g for linuron and each metabolite; the minimum quantitation level was 0.5 μg/g. The developed method was applied to potato samples from a field experiment.

Herbicides are widely used to protect agricultural production from weeds. Linuron, 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (I), is a selective herbicide used in Greece mainly in potato, carrot, and onion fields. In potato plants, linuron is applied after planting and before sprouting, at doses between 0.75 and 1.25 kg active ingredient/ha. Following application, linuron is taken up by the roots of the plant, translocated, and degraded to DCPMU, DCPU, DCA, and other metabolites (1).

Linuron tends to undergo thermal decomposition during gas chromatography. Therefore, determination of linuron in food by this technique is usually performed indirectly by determining its stable derivatives (2). In contrast, determination of linuron by liquid chromatography is performed directly with a UV detection system (3).

However, in the investigation of food contamination, it is not sufficient to determine only the parent herbicide; the metabolites should also be included in the toxicological and ecological assessment of the residues. An established method (3) for determination of linuron in potatoes and other vegetables, using a deactivated Florisil column for cleanup and a LiChrosorb Si 60 column for LC determination, was ineffective for the simultaneous determination of linuron and its metabolites. It was therefore decided to develop a simple and efficient method for the analysis of potato tubers for residues of linuron and its metabolites. The extraction procedure is based on that of Luke et al. (4), as modified by Lawrence (3), followed by a simplified and fast cleanup, using Sep-Pak silica cartridges and a new LC determination using a LiChrosorb NH_2 column with gradient elution.

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METHOD

Materials and Reagents

(a) *Solvents.*—Dichloromethane, acetone, and hexane, GR grade. Isopropanol and isooctane, spectroscopy grade (Merck).

(b) *Sodium sulfate.*—Anhydrous GR, ACS, ISO (Merck).

(c) *Sodium chloride.*—GR, ACS, ISO (Merck). Prepare saturated solution.

(d) *Linuron and metabolites.*—Linuron analytical standard (Hoechst), DCPMU and DCPU analytical standards (Velsicol). 3,4-Dichloroaniline for synthesis (Merck, assay: 98%). For LC, prepare 250 μg/mL stock solution of each compound in dichloromethane. Dilute stock solutions as required with dichloromethane and prepare 2.5 μg/mL standard mixture working solution.

(e) *Silica cartridges.*—Sep-Pak (Waters Associates Inc.), containing 1 g silica.

(f) *Potato samples.*—Potato samples came from field experiments in the area of Marathon, near Athens. Linuron was applied at the recommended dose of 1 kg a.i./ha and at double the dose. Control potato samples were collected from untreated plots of the same field.

Liquid Chromatographic System

(a) *Solvent delivery system and injector.*—Microcomputer-controlled pump module, Series 3B (Perkin-Elmer, Norwalk, CT). Syringe-loading sample injector (175 μL), Model 7105 (Reodyne, Berkeley, CA).

(b) *LC column.*—LiChrosorb NH_2, 5 μm column, 4 mm × 250 mm (Merck), preceded by Spherisorb S 10 W, 5 cm guard column (Phase Separations, Queensferry, Clwyd, UK).

(c) *Detector.*—LC 75 variable wavelength UV detector and LC 75 autocontrol (Perkin-Elmer). Compounds are monitored at 248 nm. System is set up to obtain ½ FSD for 7 ng linuron.

(d) *Recorder.*—Model 023 (Perkin-Elmer).

(e) *Mobile phase.*—The following elution program is used at ambient temperature (25°C): 10% isopropanol in isooctane (0–12 min), linear gradient elution from 10 to 40% isopropanol in isooctane (12–13 min), and 40% isopropanol in isooctane (13–22 min). Peaks of linuron and the 3 metabolites are well resolved at these conditions ($R_s > 1.2$).

Extraction Procedure

Wash marketable tubers (those greater than 5 cm in diameter) to remove adhering soil and towel dry. Macerate unpeeled potato sample in vegetable mixer and mix thoroughly.

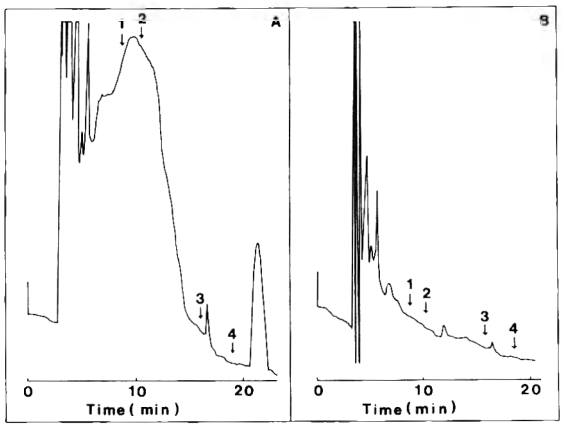

Figure 1. Typical LC chromatograms of potato tuber extracts. A, untreated sample without prior cleanup (10 μL); B, same sample, after cleanup (20 μL); 1, linuron; 2, DCA; 3, DCPMU; 4, DCPU. Detector range 0.01. Other conditions described in procedure.

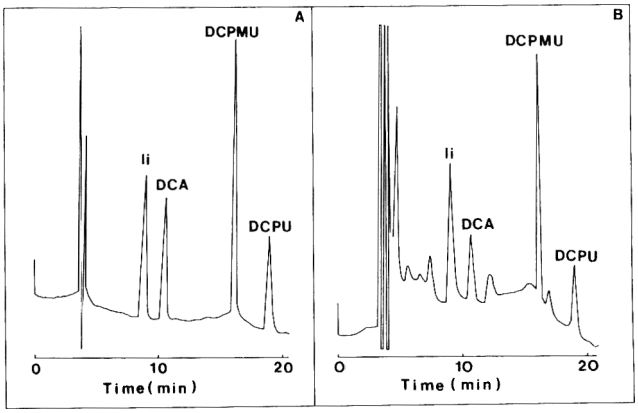

Figure 2. Typical chromatograms. A, 2.8 μL standard mixture of linuron and its metabolites (7 ng each); B, extract (20 μL) of potato fortified with linuron and its metabolites at 0.1 μg/g (7 ng). Detector range 0.01. Other conditions described in procedure.

Blend 35 g potato pulp in Sorvall Omni-mixer with 100 mL acetone at speed 6 for 4 min. Filter through Whatman No. 1 paper in Buchner funnel into 250 mL vacuum flask. Rinse cup with 20 mL acetone and pass rinse through same Buchner funnel. Transfer extract to 500 mL separatory funnel and partition with 200 mL hexane–dichloromethane (1 + 1). Transfer lower (aqueous) phase to 150 mL separatory funnel, add 15 mL saturated NaCl solution, and extract with three 70 mL portions of dichloromethane.

Collect organic extracts and combine with that in first separatory funnel. Add 7.5 g Na_2SO_4, shake until solution clears, and let stand 5 min. Filter through Whatman No. 1 paper into 1 L round-bottom flask. Evaporate solvent carefully to ca 2 mL, using rotary evaporator at 50°C and vacuum 2.5×10^4 N/m^2. Continue solvent evaporation with gentle stream of air to ca 0.5 mL. Transfer concentrate into 5 mL volumetric flask and dilute to volume with several 0.5 mL washings of hexane.

Cleanup Procedure

Condition silica cartridge with 10 mL hexane. Add, by means of 10 mL syringe, 1 mL sample in hexane, at rate of 3 mL/min. At the same rate, rinse with 10 mL dichloromethane–hexane (1 + 9); then elute linuron and metabolites from the cartridge with 2 mL dichloromethane–isopropanol (1 + 1), collecting in 5 mL vial. Stopper vial and shake to mix contents.

Analysis

Equilibrate LC system with mobile phase of 10% isopropanol in isooctane at flow rate of 1 mL/min. Inject 10 μL sample or 0.8–2.8 μL working standard solution. Determine linuron and metabolite concentrations in sample by external standard method, using standard solutions containing linuron and metabolites in similar concentrations to that of sample. Calculate concentration of linuron and each metabolite in sample (μg/g) using calibration curve. With each series of samples, carry at least 3 standards and one control sample through same procedure. After each day of running samples, wash analytical column with 100% isopropanol for 20 min and leave overnight to remove any strongly adsorbed material from column.

Results and Discussion

The developed method consists of 3 main steps: extraction, cleanup, and LC analysis. The extraction procedure used by Lawrence (3) was satisfactory. Acetone was a suitable solvent for the extraction of linuron and its metabolites from potato tubers. Partitioning between the dichloromethane and aqueous phase resulted in a clear solution. The critical step in obtaining satisfactory recovery levels and good reproducibility was the evaporation of the organic solvent on the rotary evaporator. DCA and DCPU were subject to loss when a high vacuum was applied or when the evaporation was allowed to proceed to dryness. Evaporation conditions were therefore optimized as described in the method for minimizing DCA and DCPU losses.

The proposed cleanup step with the silica cartridge was fast, effective, and economical because of the small solvent volumes used. The final eluate used for LC analysis was a clear, slightly yellow solution. Figure 1 illustrates chromatograms of a potato control sample before and after cleanup and clearly demonstrates the efficiency of the cleanup procedure.

In chromatogram A, prior to cleanup, the region of linuron and DCA is completely masked by a large interfering peak from the dichloromethane extract. The region of DCPMU is close to an interfering peak and the region of DCPU is clear.

Chromatogram B was obtained with the same extract as above, but after cleanup. The injection volume in chromatogram B is 20 μL, double that in chromatogram A (10 μL), to

Table 1. Recovery of linuron and metabolites DCA, DCPMU, and DCPU ($\bar{x} \pm$ cv, %) from spiked potato samples[a]

Concn, μg/g	Linuron, %	DCA, %	DCPMU, %	DCPU, %
0.02	91 ± 6	62 ± 5	97 ± 16	93 ± 12
0.05	102 ± 11	68 ± 5	89 ± 11	81 ± 16
0.1	96 ± 7	60 ± 9	90 ± 8	80 ± 10
0.2	83 ± 20	60 ± 13	94 ± 9	94 ± 8
0.5	93 ± 3	70 ± 9	89 ± 7	83 ± 14
1	94 ± 5	78 ± 10	82 ± 7	80 ± 12
2	93 ± 7	62 ± 9	81 ± 4	83 ± 15

[a] Each sample was analyzed in triplicate.

have the same quantity of coextracted material. The background is greatly reduced after cleanup, and there are no interferences.

Transfer of the sample prior to cleanup into hexane solution was necessary to obtain proper solvent polarity so that, by passing through the silica cartridge, linuron and its metabolites were retained on the cartridge. Linuron and metabolites remained on the cartridge during rinsing and were totally eluted by 2 mL dichoromethane–isopropanol (1 + 1). The more polar coextracted material remained on the cartridge.

The mechanism of LC separation occurring with the LiChrosorb NH_2 column is polar/anion exchange. A LiChrosorb Si 60 (5 μm) normal-phase column was ineffective in separating the linuron and DCA peaks. Linuron and DCA were separated from each other, as well as from peaks of extracted interferences, by using the amino bonded phase column and 10% isopropanol in isooctane mobile phase. Gradient elution to more polar mobile phase (40% isopropanol in isooctane) was used for eluting DCPMU and DCPU. With this gradient program, DCPMU and DCPU are eluted more quickly than with the isocratic elution and are detected as sharp peaks with higher sensitivity.

Peak heights were measured for quantitation. Studies on LC quantitation indicate no significant difference between measurements of peak height and peak area (5, 6). Figure 2 shows chromatograms of a standard mixture solution and of a spiked sample. Chromatogram A comes from the working standard mixture solution (2.8 μL) and represents 7 ng linuron and each metabolite. Chromatogram B (20 μL) comes from an untreated potato sample spiked at 0.1 μg/g of linuron and each metabolite (7 ng each).

Recovery of linuron and metabolites from potato tubers was evaluated. Seven samples, each of 35 g pulp of untreated potato tubers, were prepared by adding, respectively, 0.7, 1.75, 3.5, 7.0, 17.5, 35, and 70 μg linuron and each metabolite from standard mixture solutions. The corresponding spiking levels of linuron and each metabolite were 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/g. The spiked samples were extracted, cleaned up, and analyzed immediately according to the method. The spiking procedure and analysis were performed in triplicate. Recoveries (Table 1) ranged from 80 to 102% for linuron, DCPMU, DCPU, which is satisfactory for residue analysis (7). Recovery of DCA ranged from 60 to 78% because of losses during the concentration procedure. The coefficient of variation (CV) is less than 20%, as shown in the table, and within the accepted range (7).

A minimum detectable concentration of 0.015 μg/g was established for linuron and each metabolite in potato tubers. This concentration represents a peak height equivalent to 3 times the average noise level. The minimum quantitation level was 0.5 μg/g. The detector response to linuron and each metabolite was linear through the origin to at least 7 ng (equation of line for linuron: $y = 1.2x + 0.045$, $r = 0.999$).

No linuron or any of the 3 metabolites was detected in 14 potato samples from different plots treated with linuron at the recommended dose or in one sample from a plot treated with double the dose. Potato tubers were sampled 108 days after treatment, at which time linuron was detected in soil samples at the 0.05 μg/g level, for the recommended dose treatment, and at the 0.2 μg/g level, for the double dose treatment (8).

The method outlined here provides an efficient and simple cleanup, designed so that 2 mL solvent is sufficient to elute linuron and its metabolites. This miniaturization facilitates a rapid and economic analysis. The method also allows independent detection of linuron and its metabolites at the 0.015 μg/g level and determination at the 0.5 μg/g level in potatoes.

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Solid-Phase Extraction of Carbofuran, Atrazine, Simazine, Alachlor, and Cyanazine from Shallow Well Water

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Extraction of several nitrogen-containing pesticides from water on solid-phase C_{18} cartridges was rapid and accurate. One analyst can extract >48 samples/day. Recovery efficiencies were 77, 95, 92, 90, and 99% with detection limits of 0.20, 0.05, 0.05, 0.20, and 0.10 μg/L for carbofuran, atrazine, simazine, alachlor, and, cyanazine, respectively. Extraction of the atrazine and simazine dealkylation products (deethylatrazine and deethylsimazine) was less efficient, e.g., 26 and 9%, respectively. Comparisons with 10 U.S. Geological Survey samples gave similar results.

A recent report (1) stated that when pesticides were found in groundwater, an overwhelming number of cases could be attributed to normal use of these pesticides. If so, the importance of rapidly and accurately assaying for normal-use pesticides in groundwater is imperative, because of the large number of well water samples that may have to be assayed.

Several methods exist for extracting pesticides from water (2-4). Most are liquid-liquid extractions [e.g., U.S. Environmental Protection Agency Method 1 (1)]; however, solid-phase extraction has recently become the method of choice when the analytes of interest are known (5-8). Solid-phase extraction is faster and does not require large amounts of organic solvent, thereby purchasing costs and waste solvent disposal costs are reduced. Solid-phase extraction also avoids the emulsions often encountered in liquid-liquid extraction (2).

Some solid-phase extraction methods use C_{18} cartridges (1, 7, 9, 10). Davoli et al. (6) demonstrated that for 1 L water samples atrazine recovery using C_{18} cartridges was linear over the range of 0-50 ppt. Brooks et al. (5) demonstrated that alachlor, atrazine, and metolachlor could be extracted from groundwater with efficiencies >90%. Junk and Richard (10) reported recovery efficiencies of 91, 80, 92, and 92% for alachlor, atrazine, cyanazine, and carbofuran, respectively, when 100 mL water volumes were extracted.

The present paper describes use of solid-phase cartridges for routine large-scale extraction of several nitrogen-containing pesticides from well water. The pesticides are: carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate (CAS-1563-66-2)]; atrazine [6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine (CAS-1912-24-9)]; simazine [6-chloro-*N*,*N'*-diethyl-1,3,5-triazine-2,4-diamine (CAS-122-34-9)]; alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide (CAS-15972-60-8)]; cyanazine [2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile (CAS-21725-46-2)]; deethylatrazine [2-chloro-4-amino-6-(isopropylamino)-*s*-triazine]; and deethylsimazine (deisopropylatrazine) [2-chloro-4-amino-6-(ethylamino)-*s*-triazine]. Two hypotheses were made about this method: Recovery for different fortifications should be the same, and recovery should be 100%.

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Mention of proprietary products does not imply endorsement or approval by the U.S. Department of Agriculture to the exclusion of other suitable products.

METHOD

Apparatus

(a) *Gas chromatograph.*—Equipped with nitrogen-phosphorus (NP) detector. Operating conditions: 60 m × 0.75 mm id glass capillary column coated with 1 μm SPB-20; oven temperature—initial 210°C for 1 min, program rate 5°/min to 230°C, and final time of 21 min; He carrier gas at 4-4.5/min.

(b) *C_{18} solid-phase cartridges.*—0.4 g (Part No. 51910, Waters Associates).

(c) *Teflon tubing.*—0.5 cm id.

(d) *Funnels.*—For C_{18} cartridges.

(e) *Pipets.*—1 mL, for use as suction tubes.

(f) *Glassware.*—Graduated test tubes, 13 mL (Kontes); disposable Pasteur pipets, 23 cm; solvent dispensing units, 5 and 10 mL repeating pipets with 500 mL flasks.

(g) *Other equipment.*—Vacuum chamber; aspirator suction; pipettors, 1 and 5 mL; balance.

Reagents

(a) *Solvents.*—Methanol, ethyl acetate, and purified water (distilled in glass, or equivalent).

(b) *Anhydrous sodium sulfate.*

(c) *Water samples.*—<1 L actual, controlled, or fortified.

(d) *^{14}C-atrazine.*—Specific activity 62 μCi/mg (purchased from commercial source).

Sample Extraction

Samples were extracted in sets of 16, with both control and fortified samples included in most sets.

Weigh sample with bottle and store 24-48 h at 5°C. Remove sample 1/2 h before extraction. Join two C_{18} cartridges, using 1 cm piece of Teflon tubing, and funnel in series to vacuum chamber. Adjust suction to >5 kPa, and consecutively add 5, 5, and 10 mL ethyl acetate, methanol, and water, respectively, to funnel. Remove funnel, attach 30 cm piece of Teflon tubing between 1 mL pipet and top cartridge, insert pipet into water sample, and clamp pipet so end is ca 1 cm from bottom of bottle. Initiate suction to vacuum chamber and adjust suction to obtain ca 7-10 mL water/min through cartridge. After sample has passed through cartridge, continue suction >10 min. Disconnect Teflon tubing from cartridge, attach funnel to top cartridge, and add 0.5 mL ethyl acetate. Release suction and place rack with 13 mL tubes into vacuum chamber. Initiate suction of <10 kPa and place 2.3 mL ethyl acetate in funnel. Continue suction ca 1 min after solvent has passed through cartridge. Add ethyl acetate to bring extract in 13 mL tube to 2 mL above any water, and then add very small amount of anhydrous sodium sulfate to 13 mL tube, and shake.

Sample Fortification

In a preliminary extraction experiment, 175 μg ^{14}C-atrazine in ethanol was placed in a 4 L bottle, ethanol was evaporated with N_2 gas, and 3.5 L filtered well water was added. The bottle was shaken and stored at <5°C. After

Table 1. Pesticide concentrations (μg/L) used to fortify well water samples

Solution	Deethylatrazine	Deethylsimazine	Atrazine	Simazine	Carbofuran	Alachlor	Cyanazine
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.02	0.02	0.03	0.02	0.07	0.06	0.04
2	0.10	0.12	0.13	0.07	0.35	0.30	0.22
3	0.62	0.70	0.64	0.24	1.77	1.52	1.09

about 1 week, treated water was divided among seven 500 mL bottles. A 1 mL aliquot of treated water was taken from each bottle for ^{14}C-counting on 67th day of storage, and 2 of the 7 samples were extracted. The remaining 5 samples were extracted after 150 days. The extracts were assayed by both liquid-scintillation counting (LSC) and NP gas chromatography (GC). ^{14}C-Counts in each extract were compared with mean of counts from the seven 1 mL aliquots taken on 67th day.

Routinely, well water samples were fortified with pesticides dissolved into 3 ethyl acetate solutions. Glass bottles (1 L) with Teflon-lined caps were treated with one of the solutions (0, 1, 2, or 3) shown in Table 1. Ethyl acetate was allowed to evaporate by leaving cap off. Well water (ca 1 L) was pumped directly into the treated bottle. To make certain the pesticides desorbed from the bottle and went into solution, bottles were shaken and stored >2 weeks at <5°C before extraction.

To ascertain that loading capacity of cartridges was well above expected residue amounts, well water was fortified with 187 and 430, 436 and 939, and 145 and 348 μg/L of atrazine, alachlor, and cyanazine, respectively, in same manner as before.

Gas Chromatography

Perform analysis under conditions described in *Apparatus*. Use relative retention time vs standards for qualitative analyses.

For quantitative results, use peak areas for calculation as follows:

$$\text{Pesticide, } \mu g/kg = (A_S/A_{STD}) \times (C_{STD}/R_E) \times (I_{STD}/I_S) \times (V_{EX}/WT) \times 100$$

where A_S = peak area of sample; A_{STD} = peak area of standard (from curve); C_{STD} = concentration of standard, ng/μL; R_E = recovery efficiency; I_{STD} = volume of standard injected, μL; I_S = volume of sample injected, μL; V_{EX} = volume of extract, mL; and WT = amount of water extracted, g.

Statistical Analysis

An SAS GLM (general linear model) (SAS Institute, Inc., Cary, NC) was used to test the statistical hypotheses. A *t*-test was used for comparison between sample pairs.

Results

The method is simple and rapid. A C_{18} cartridge is used for sample extraction. Water is drawn through the cartridge under suction, the pesticides are eluted, and the extract is assayed (4). We extracted 16 samples simultaneously. One analyst can extract at least 3 sets of 16 samples (<1 L) per day. Using pipets as suction tubes frees the analyst to carry out other parts of the procedure during sample extraction.

Although most well water samples are clear, an occasional sample may contain particulate matter. Particulates seemed to reduce recovery efficiency in our earlier extractions, so we let the samples stand >20 h before extraction. Using a second cartridge in series filtered out any remaining particulate matter and assured that all pesticides were adsorbed from the large volumes (<1 L) of water extracted. The additional cartridge possibly overcame the need for 1.6% methanol in the water sample, as suggested by Brooks et al. (5), to prevent the formation of a hydrophobic envelope around the adsorbent. Under the conditions presented here, addition of methanol had no effect on recoveries (results not presented).

In the preliminary extraction experiment, atrazine recovery from well water, measured by both LSC and GC, was 97 and 103%, respectively (Table 2). The difference between the results for the 2 methods was almost uniform at 5.5%, indicating a discrepancy between the GC standard and radioactivity. A *t*-test confirmed that the 2 methods were statistically different. Nevertheless, the recoveries confirmed the results of other studies (1, 10) that the C_{18} cartridges were efficient for the extraction of atrazine from 500 mL water. During routine extractions of well water, atrazine recovery was 95% (Table 3).

Simazine, alachlor, and cyanazine recoveries were 92, 90, and 99%, respectively, during routine extractions of well water (Table 3) and confirm the results of Junk and Richard (10) for concentrations from 0.1 to 1 μg/L and of Brooks et al. for concentrations from 1 to 5 μg/L (5). Carbofuran recovery was the lowest (77%) of the parent pesticides, but was statistically uniform. Recoveries of deethylatrazine (26%) and deethylsimazine (9%) were poor because of the large volumes of water extracted. Deethylatrazine and deethylsimazine recoveries improved when <100 mL water was extracted.

Detection limits on routine groundwater samples were 0.20, 0.05, 0.05, 0.20, and 0.10 μg/L for carbofuran, atrazine, simazine, alachlor, and cyanazine, respectively. Samples of <1 L had correspondingly higher detection limits.

Recoveries of fortification 1 were statistically different from fortifications 2 and 3, because fortification 1 was at or near the limit of detection (Table 3). Recoveries from both

Table 2. Recovery (50 μg/L) of ^{14}C-atrazine from fortified water samples[a]

	Rec., %	
Days at 5°C	^{14}C	GC
67	98.2	103.9
67	100.1	105.8
150	98.7	104.4
150	97.9	103.5
150	100.4	106.1
150	95.8	101.3
150	88.4	93.4
Mean	97.1	102.6
CV, %	3.9	3.9

[a] The 2 methods for measuring atrazine were significantly different by a *t*-test.

Table 3. Recovery (%) of several pesticides from fortified water samples

Solution	Deethylatrazine	SE[a] CV, %	Deethylsimazine	SE CV, %	Atrazine	SE CV, %	Simazine	SE CV, %
0	ND[b]	—[c]	ND	—	0.8	—	ND	—
0	ND	—	ND	—	ND	—	ND	—
0	ND	—	ND	—	ND	—	ND	—
Mean	—	—	—	—	0.2	—	—	—
1	25.3	3.4	NM[d]	—	99.7	3.8	101.6	5.2
1	6.5	2.5	NM	—	68.1	5.7	82.1	4.3
1	NM	—	NM	—	ND	—	—	—
1	NM	—	NM	—	147.0	—	—	—
Mean	8.0	150.5	—	—	78.7	78.4	91.9	15.0
2	30.1	0.1	37.6	8.3	93.2	5.5	90.1	7.2
2	27.8	1.6	0.0	—	84.5	0.6	64.1	0.3
2	24.6	7.5	0.0	—	120.2	6.1	119.6	4.7
2	25.6	11.2	8.8	3.8	92.5	10.4	98.9	9.9
2	NM	—	NM	—	98.8	0.9	131.9	0.2
Mean	21.6	56.8	9.3	175.5	97.8	13.8	100.9	26.1
3	8.2	1.9	2.4	0.4	89.9	5.9	62.1	4.9
3	33.1	0.7	NM	—	85.5	1.7	81.2	1.7
3	29.7	—	23.7	—	102.5	—	109.6	—
3	66.7	5.9	15.1	2.7	89.6	3.6	81.7	2.2
3	16.1	1.5	1.8	0.5	87.9	6.5	78.1	15.9
Mean	30.8	73.1	8.6	120.4	91.1	7.3	82.5	20.7
Mean 2–3	26.2	92.6	8.9	212.8	94.5	15.6	91.7	33.3

Solution	Carbofuran	SE CV, %	Alachlor	SE CV, %	Cyanazine	SE CV, %
0	ND	—	ND	—	ND	—
0	ND	—	ND	—	ND	—
0	ND	—	ND	—	ND	—
Mean	—	—	—	—	—	—
1	97.9	0.6	103.9	17.9	111.6	7.7
1	49.9	5.5	ND	—	75.6	4.6
1	ND	—	ND	—	ND	—
1	ND	—	ND	—	ND	—
Mean	36.9	3.9	26.0	17.9	46.8	8.9
2	89.8	3.6	88.9	5.5	95.4	15.1
2	78.5	0.1	66.0	0.1	97.5	0.4
2	—	—	112.5	3.6	130.3	5.3
2	54.9	6.9	104.9	12.6	103.3	17.1
2	86.6	0.1	82.9	0.7	99.6	4.8
Mean	77.4	7.8	91.0	14.2	105.2	23.9
3	62.6	5.9	94.6	6.6	92.2	10.2
3	94.5	1.3	71.7	1.2	85.6	1.9
3	70.3	0.0	103.1	0.0	108.8	0.0
3	64.8	4.7	86.7	1.5	98.2	6.2
3	92.0	18.4	93.2	3.7	81.3	5.0
Mean	76.8	19.9	89.9	7.8	93.2	13.1
Mean 2–3	77.1	21.4	90.4	16.2	99.2	27.3

[a] SE = standard error of mean following each value and CV = coefficient of variation following each mean.
[b] ND = not detectable.
[c] One sample only, if not measurable.
[d] NM = not measurable.

fortifications 2 and 3 were statistically the same, except for simazine, by the SAS GLM procedure. Statistically, recoveries from all 3 fortifications (1, 2, and 3) were 100%, except for deethylatrazine and deethylsimazine, and carbofuran for fortification 3. The exceptionally large coefficients of variation (Table 3) was the reason recoveries from fortification 1 were statistically 100%.

Figure 1 shows chromatograms of standards and extracts

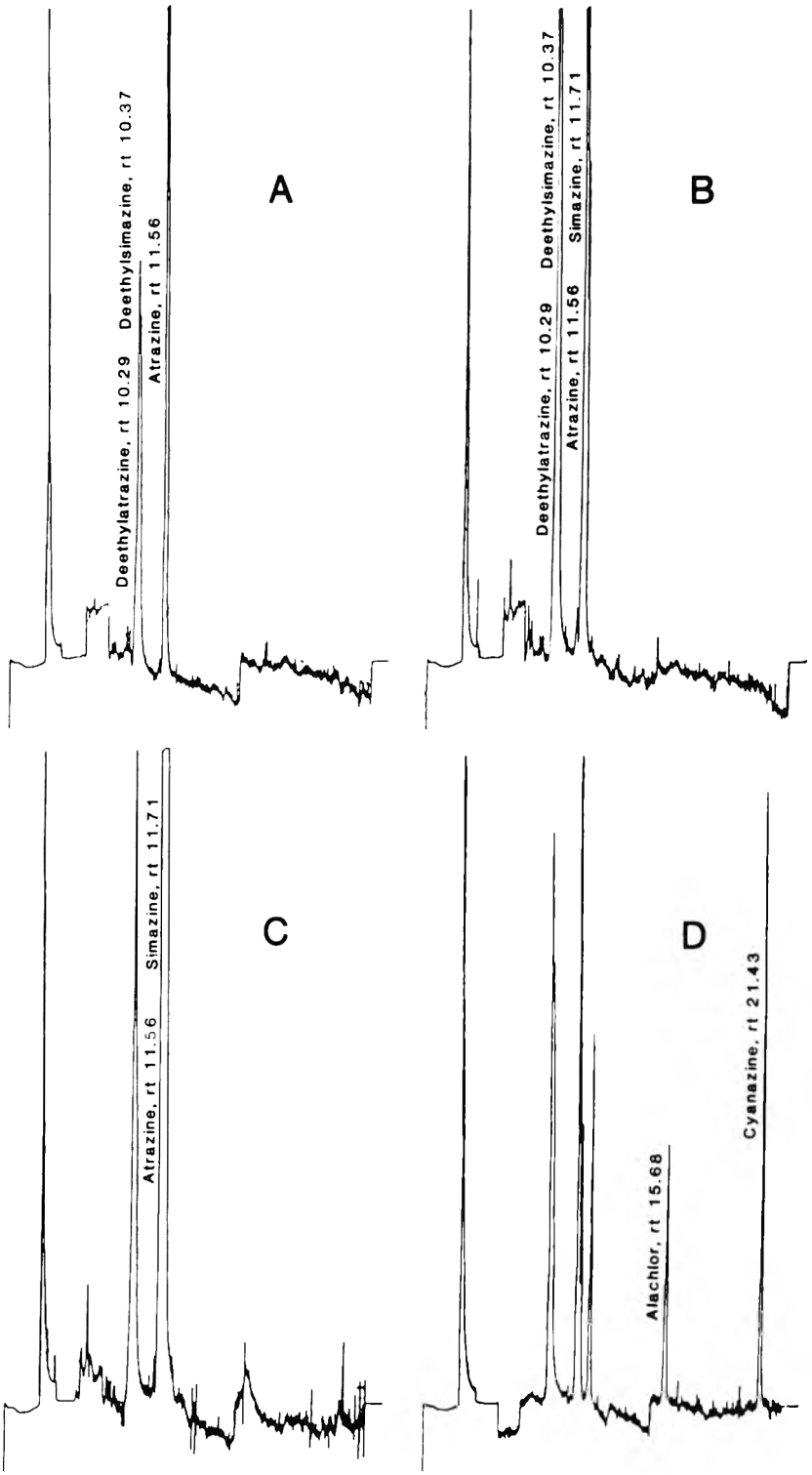

Figure 1. Chromatograms of nonfortified well water extracts (A, B, and C) and deethylatrazine, deethylsimazine, atrazine, simazine, carbofuran, alachlor, and cyanazine (D). Standard amounts were 0.52, 0.58, 0.64, 0.35, 1.77, 1.52, and 1.09 ng at retention times of 10.29, 10.37, 11.56, 11.71, 12.18, 15.68, and 21.43 min, respectively. Chromatogram A shows deethylatrazine, deisopropylatrazine (deethylsimazine), and atrazine. Chromatogram B shows deethylatrazine, deethylsimazine, atrazine, and simazine. Chromatogram C shows deethylsimazine, atrazine, and simazine. Any deethylatrazine in chromatogram C was not separated from the deethylsimazine.

of water from shallow wells. Deethylatrazine and deisopropylatrazine (deethylsimazine) usually were separated on the SPB-20 column, when the concentration of one was no more than twice that of other product. Separation of the 2 dealkylation products was good on an SPB-5 column (same length, diameter, and similar conditions, except initial temperature was 175°C). Separation of atrazine and simazine posed no problems unless their concentrations were less than 5 times that of other products. (Simazine and carbofuran were not separated on the SPB-5 column.)

Table 4. Concentration (μg /L) of atrazine in well water samples provided by the U.S. Geological Survey (USGS) (December 1987)

Sample	USDA[a]	USGS
GS01	0.29	0.30
GS02	1.17	1.0
GS03	4.5	3.1
GS04	22.4	16
GS05	0.54	0.4
GS06	3.08	2.7
GS07	1.08	0.6
GS08	0.28	0.2
GS09	0.53	0.5
GS10	1.50	1.0

[a] U.S. Department of Agriculture. Student's *t*-test indicated no significant differences between the 2 laboratories' results.

The U.S. Geological Survey (USGS) provided 10 well water samples from the Midwest in December 1987. For determination of atrazine, a *t*-test showed no differences between USGS results by their method and our results by the present method (Table 4).

Overloading the C_{18} cartridges was not a problem. Recoveries of 95 ± 15, 119 ± 20, and 106 ± 21 were obtained for atrazine, alachlor, and cyanazine, respectively, at concentrations between 145 and 939 μg/L. Most (99%) of our well water samples contained <100 μg/L of atrazine; 81% contained <1 μg/L of atrazine.

In conclusion, the method is accurate for atrazine, alachlor, carbofuran, cyanazine, and simazine, but not for the dealkylation products of atrazine or simazine. The method is rapid when conducted in sets of 16 samples, can be applied to water samples of <1 L, required no prefiltering and only small amounts of organic solvents, and let the analyst conduct other portions of the procedure during extraction.

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Improved Extraction of Atrazine and Metolachlor in Field Soil Samples

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A method was developed for extraction of weathered residues of atrazine and metolachlor from field soils; soils had last been treated with commercial formulations of the herbicides 8–15 months prior to sample collection. Maximum yields were obtained by batch extraction at 75°C for 2–16 h with methanol–water (80 + 20) in a sealed vial. Hydrolysis or other decomposition reactions were minor or negligible, depending on the extraction time. This method is an improvement over published methods that are validated by spike recoveries; the proposed method gives 1.7–1.8 times higher yields compared to shaking for 2 h at room temperature, and 1.3–1.8 times higher yields compared to Soxhlet extraction. The reproducibility of the method was better than 12%. The results underscore the impact of nonequilibrium sorption of organic compounds on analytical methodology and emphasize the need to validate extraction methods with field samples.

The customary validation of analytical methods for organic compounds in soils involves extraction of analyte from fortified samples, i.e., the spike recovery. There is growing evidence, however, that time-dependent nonequilibrium sorptive processes can render some of the analyte more resistant to extraction (1–4). Consequently, actual recovery from weathered field samples could give values lower than those predicted on the basis of a freshly added spike.

There have been few attempts to systematically optimize the extraction of contaminant residues in field samples, although discrepancies with spike recovery have been acknowledged (2, 3). Complete extraction of residues of the soil fumigant 1,2-dibromoethane (EDB) in field soils required hot solvent for prolonged periods (e.g., methanol, 75°C, 24 h) (4). The EDB residues in these samples consisted mainly of less mobile fractions that were resistant to leaching and microbial degradation (5). Common extraction methods, such as Soxhlet, sonication, and purge-and-trap were inefficient, despite satisfactory recoveries of spikes (4). A hot solvent technique similar to that used for EDB was also needed for complete recovery of the less mobile fractions of several other halogenated solvents and soil fumigants (6).

It is important to optimize the extraction of weathered residues of organic contaminants in order to predict their fate in the environment. We describe here an improved method for extracting the herbicides atrazine (2-chloro-4-ethylamino-6-isopropyl-1,3,5-triazine) and metoachlor (2-chloro-*N*-[2-ethyl-5-methylphenyl]-*N*-[2-methoxyl-1-methylethyl]-acetamide) in field soils to which the herbicides had last been applied several months prior to collection. These herbicides are widely used, often in combination, and have been associated with surface and groundwater contamination (7, 8).

Typically, atrazine and metolachlor are extracted from soil by shaking at room temperature with methanol, acetonitrile, or dichloromethane for 2–24 h (9–15), or by sonication with acetone–hexane (16). McGlamery et al. (17) found that a 2 h Soxhlet extraction with methanol or chloroform gave the highest recovery of spiked atrazine. Xu et al. (18) used Soxhlet extraction with methanol for 24 h. In the only study involving field residues, Mattson et al. (19) showed that a 1 h reflux with acetonitrile–water (90+10) gave the highest yields of atrazine residues in 2 field samples, when compared to other solvents (methanol–water (90+10); chloroform) or conditions (Soxhlet extraction; room temperature shake).

Even very mild conditions, such as a 2 h shake with methanol–water (90+10), gave satisfactory recoveries of spiked atrazine and metolachlor (9). However, the method reported here is more efficient than the above methods for field samples.

Experimental

Apparatus and Reagents

All solvents used were Optima grade from Fisher Scientific Co., Springfield, NJ. Standards of atrazine and metolachlor were obtained from the U.S. Environmental Protection Agency, Research Triangle Park, NC. Standards of ^{15}N,^{13}C-alachlor (MSD Isotopes, Montreal, Canada) and $^{2}H_5$-atrazine (Cambridge Isotope Laboratories, Inc., Woburn, MA) were >98% isotopically pure.

Determinations of atrazine and metolachlor were carried out with either Hewlett-Packard (HP) 5988A GC/MS or HP 5970 GC/MS instrumentation (9).

Procedure

(a) *Sample collection and handling.*—Soil 1 (Merrimac fine sandy loam, Typic Dystrochrept) was collected using a hand auger from the top 15 cm of a corn plot at the University of Connecticut experimental farm in Coventry, CT. The soil contains 53% sand, 33% silt, 14% clay, and 2.4% organic carbon. The clays are mainly illitic. Metolachlor had been applied to this site at 2.2 kg active ingredient (AI) per ha (2 lb/A) in 1986, 1987, and 1988. Atrazine had been applied at 1.1 kg AI/ha (1 lb/A) in 1986 and 1987, and 1.4 kg AI/ha (1.25 lb/A) in 1988. These rates are typical for pre-emergent weed control. The last application was 8 months prior to sampling.

Soil 2 (Merrimac sandy loam, Typic Dystrochrept) was collected from the top 15 cm of a corn plot at the Valley Laboratory of the Connecticut Agricultural Experiment Station in East Windsor, CT. The soil contains 61% sand, 38% silt, 1.6% clay, and 0.91% organic carbon. The clays in this soil are mainly illitic. This plot was treated with metolachlor at 9 kg AI/ha (8 lb/A) 17 months prior to sampling. An untreated control soil (Merrimac fine sandy loam) was collected from the same area.

Soils were sieved at field moisture (ca 10–15% water) through a 2 mm screen and mixed to homogeneity by tumbling. The moisture in the soil kept particles from segregating by size. The soils were stored frozen.

(b) *Soil extraction.*—For batch extractions, soil (8.5 g moist weight) was combined with solvent (35 mL) in a 40 mL screw-cap vial with a Teflon-backed silicone rubber liner (Pierce Chemical Co., Rockford, IL). After ensuring that

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Table 1. Comparison of solvents for extraction of atrazine and metolachlor from soil 1[a]

Solvent	Atrazine, μg/kg[b]	Metolachlor, μg/kg[b]
Methanol	178 ± 2	334 ± 25
Acetone	165 ± 6	365 ± 22
Acetonitrile	170 ± 6	348 ± 12
Methylene chloride	137 ± 2	283 ± 15
Hexane	59 ± 2	109 ± 2

[a] 75°C for 5 h.
[b] Mean and range of duplicates.

vial lip and liner face were free of particles, the samples were incubated with agitation on a hematology mixer (Fisher Scientific) (21°C and 50°C experiments) or on an orbital shaker (75°C experiments), or without agitation in a thermostated oven (95°C experiments). As a precaution against injury that could result from breakage, the vials incubated at 75°C and 95°C were shielded within a box and were not handled until cool. The cooled vials were centrifuged for 15 min at 750 × *g* (IEC Model UV centrifuge) and the supernatant liquid was removed for analysis.

Soxhlet extraction was carried out using 20 g soil and 100 mL methanol.

(c) *GC/MS determination.*—Analyses were carried out as described previously (9) except that the cleanup of soil extracts from (b) involved a liquid-liquid extraction technique described below in place of solid-phase extraction.

Briefly, soil extract (10 mL) and water (100 mL) were placed in a separatory funnel along with known amounts of $^{2}H_{5}$-atrazine and ^{15}N,^{13}C-alachlor as internal standards for atrazine and metolachlor, respectively. The herbicides were phase-transferred to 10 mL dichloromethane, which was subsequently concentrated to about 2 mL for splitless injection to the GC/MS instrument. Quantitation of atrazine and metolachlor was based on the response of the isotope-labeled standards, which ensured good accuracy and precision (9).

(d) *Decomposition rate constant measurements.*—The untreated control soil (8.5 g) and methanol-water (80+20) (35 mL) were placed in 40 mL screw-cap vials and spiked with a small volume of stock herbicide in methanol to achieve 300 μg/L of herbicide. After shaking briefly, one replicate was sacrificed as the time zero point. The remaining replicates were incubated for various times at 75°C (with shaking) or 95°C (without shaking). Workup consisted of centrifugation followed by phase-transfer of analyte to dichloromethane, exactly as described above for the soil extracts.

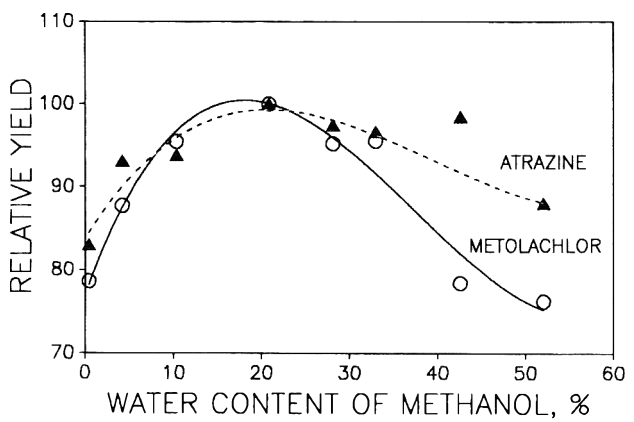

Figure 1. Relative efficiency of methanol-water solutions to extract herbicides from soil 1. Water content of solution includes soil moisture.

Table 2. Extraction yields from soil 1 using methanol-water (80+20)

Temp., °C	Yield, μg/kg[a]				
	2 h	4 h	10 h	16 h	24 h
	Atrazine				
21	139	152	143	139	NT[b]
50	213	215	189	191	NT
75	209	225	208	190	NT
95	236	233	198	170	NT
	Metolachlor				
21	281	316	361	402	406
50	325	390	443	465	476
75	363	438	453	510	470
95	321	437	505	498	408

[a] Duplicate analyses. Mean range: atrazine, ±3.4; metolachlor, ±9.8 μg/kg.
[b] NT = not tested.

Results

Solvent.—Methanol, acetone, and acetonitrile all gave high extraction yields of herbicides from soil 1 compared to the less polar and water-immiscible solvents dichloromethane and hexane (Table 1). Among the 3 water-miscible solvents, methanol was selected because chromatograms had fewer background peaks compared to acetone and acetonitrile. This effect has been noted previously (10).

The optimum water content of the methanol was 20% (v/v) for extraction of both herbicides (Figure 1). Cotterill (10) showed that approximately 20% water in methanol was optimum for simazine and also was more efficient than methanol alone for extracting fluometuron, lenacil, linuron, metribuzin, and propyzamide from air-dried soils. Others (2, 3) also have found that water improves the efficiency of the extractant.

Temperature and time.—Extraction yields from soil 1 with time at 4 selected temperatures between 21°C and 95°C are given in Table 2. In general, yields increased with temperature up to 75°C, with only slight (if any) improvement at 95°C. Although yields at the lower temperatures remained constant or increased modestly with time, those at the higher temperatures peaked at intermediate times and then declined. The optimum combination of temperature and time for metolachlor was 75°C, 16 h. The optimum for atrazine was 95°C, 2-4 h.

Soil 2, which had been obtained from a site with a different application history, gave similar results to soil 1 with respect to extraction of metolachlor (Figure 2). Atrazine had not been applied to this field. Again, the higher temperature leads to a considerable improvement in yield. With this soil, however, the yield peaked at 4 h compared to 16 h for soil 1.

The apparent decline in yields at longer times at 75°C and 95°C could be explained in part by hydrolysis or other decomposition reactions. Both atrazine and metolachlor are subject to solvolysis in methanol-water. Atrazine undergoes hydrolysis at the 2-position of the triazine ring with displacement of chloride to give the hydroxy derivative (20, 21). In water or moist soil, the reaction is very slow at room temperature and around neutral pH. The hydrolysis of metolachlor

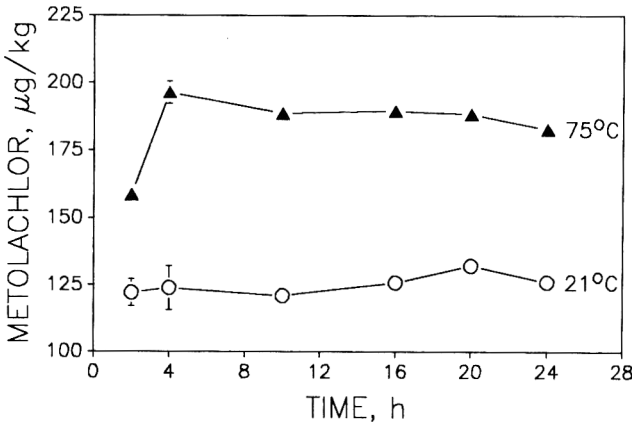

Figure 2. Yields of metolachlor from soil 2 using methanol–water (80+20). Triplicate analyses; error bar represents standard deviation.

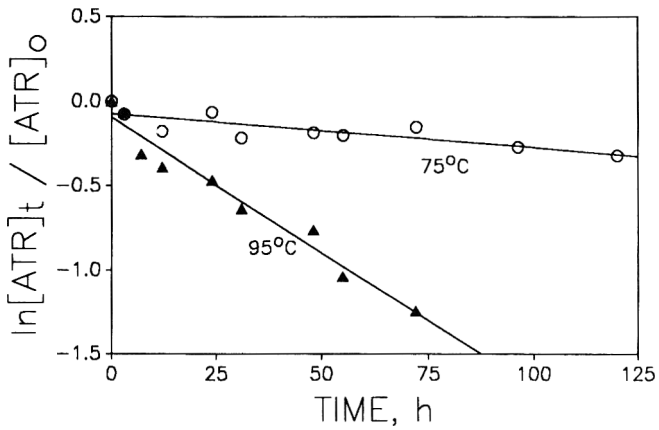

Figure 3. First-order decomposition plots for atrazine in methanol–water (80+20) in presence of herbicide-free control soil.

has not been reported but other chloroacetanilides are cleaved at the amide group (22).

Regardless of the nature of the decomposition reactions, herbicide disappearance can be modeled as a pseudo first-order process. Decomposition rate constants, k_{obs} (Table 3), were determined at 75°C and 95°C in methanol–water (80+20) from the slopes of the decay plots, as illustrated in Figure 3 for atrazine. Soil organic matter is believed to catalyze the hydrolysis of atrazine (21); therefore, to account for catalytic effects of the soil, the reactions were carried out in the presence of a surface soil that was similar to soil 1 except that it was free of herbicide.

The rate constants were used to generate correction factors to compensate for decomposition losses according to the following equation:

$$\text{Correction factor} = (\text{actual yield/observed yield}) = e^{k_{obs}t}$$

where t is the extraction time. The correction factor at each time is given in Table 3 and the corrected extraction data for soil 1 are shown in Table 4. The corrected values exceed the actual values by 5% or more only for atrazine at 95°C at times greater than 2 h, and for metolachlor at 95°C, 24 h. By comparison with Table 2 it can be seen that the optimum conditions are hardly changed by including a correction factor for decomposition. It is also evident that the correction does not totally compensate for the apparent extraction losses of herbicide at longer times. This may reflect catalytic differences between soil 1 and the control soil.

Table 3. First-order decomposition rate constants in methanol–water (80+20) in presence of soil, and resulting correction factors for extraction

Temp., °C	10^3 k_{obs} (h^{-1})[a]	Correction factor[b]				
		2 h	4 h	10 h	16 h	24 h
Atrazine						
75	2.0 ± 0.5	1.00	1.01	1.02	1.03	NT
95	16 ± 1	1.03	1.07	1.18	1.29	NT
Metolachlor						
75	1.2 ± 0.2	1.00	1.00	1.01	1.02	1.03
95	2.2 ± 0.4	1.00	1.01	1.02	1.04	1.05

[a] Mean ± standard error of estimate.
[b] Actual yield is observed yield multiplied by correction factor.

Reproducibility.—Replicate samples of soil 1 were extracted at 75°C for 16 h once per week over 4 weeks to check the reproducibility of the method. The results (Table 5) are uncorrected for decomposition. The relative standard deviations, which combine the analytical and extraction variabilities, were 6.7% for metolachlor and 11.8% for atrazine.

Comparison with Soxhlet extraction.—The 2 h Soxhlet extraction prescribed by McGlamery et al. (17) and the 24 h Soxhlet extraction prescribed by Xu et al. (18) using methanol were compared with the batch hot solvent method. Relative to the highest yields obtained from the batch hot solvent method, the yields obtained with the 2 h Soxhlet extraction were 60% for atrazine (soil 1), and 63 and 65% for metolachlor (soils 1 and 2, respectively). The relative yields obtained with the 24 h Soxhlet extraction from soil 1 were 56% for atrazine and 77% for metolachlor.

Discussion

Extraction at 75°C in methanol–water (80+20) efficiently removes the herbicides from the soil matrix. Although extraction at 95°C gives slightly better yields of atrazine than extraction at 75°C, decomposition is more pronounced at this temperature. Therefore, to avoid the uncertainties associated with decomposition, it is recommended that the extraction be carried out at 75°C. The optimum time at this temperature is 4–16 h for metolachlor and 2–4 h for atrazine. In cases where both compounds are present in a sample, a relatively short extraction period (e.g., 4 h) may be used since acceptable yields of metolachlor (86% of maximum) were obtained from soil 1 after that time.

Table 4. Extraction yields in Table 2 corrected for decomposition

Temp., °C	Yield, μg/kg				
	2 h	4 h	10 h	16 h	24 h
Atrazine					
75	210	227	212	196	NT[a]
95	244	249	232	220	NT
Metolachlor					
75	364	440	458	520	484
95	322	440	516	516	430

[a] NT = not tested.

Table 5. Repeated analyses of soil 1 to test reproducibility of extraction method (75°C, 16 h)

Subsample extracted during:	Metolachlor, μg/kg[a]	Atrazine, μg/kg[a]
Week 1	510 ± 11	227 ± 0.4
Week 2	510 ± 20	227 ± 17
Week 3	450 ± 14	226 ± 9
Week 4	462 ± 18	199 ± 18
Mean	483 ± 32[b]	220 ± 26[b]

[a] Standard deviation of triplicate analyses of a single soil extract.
[b] Propagated error.

The batch hot solvent method is an improvement over published room temperature and Soxhlet extraction methods. This was demonstrated using 2 soils with different histories of herbicide application. Compared to the best yields at 75°C, only 55–62% yield was achieved by shaking for 2 h at 21°C. Furthermore, extension of the room temperature shake from 2 to 24 h resulted in only minor improvement. Soxhlet extraction achieved yields ranging from 56 to 77% compared to the best yields by the batch method. A likely explanation for the inefficiency of Soxhlet extraction is that, even though the solvent boils close to 65°C, its temperature in the thimble never exceeds about 53°C. It is also possible that the lack of agitation in the thimble impedes extraction.

In contrast to the need for high temperature extraction for the field residues, a 2 h shake with methanol–water (90+10) was sufficient to quantitatively recover herbicide spikes after a 1 h equilibration period (9). It is known that organic compounds undergo slow, diffusion-retarded sorption in soils as a consequence of the porous nature of soil particles (1). These slow sorptive processes, which may continue for months or years, can result in nonlabile fractions that are more resistant to extraction. With time, the nonlabile fractions can make up an increasingly larger proportion of total analyte as diffusion continues and as degradation and leaching processes in the field remove the labile fractions. Batch and column leaching studies showed that a large fraction of the total atrazine and metolachlor in these soils at the time of collection existed in a nonlabile sorbed state (23).

Due to convenience and the common assumption that sorptive equilibria are established rapidly, the equilibration period used for the determination of spike recovery is seldom more than 24 h. By contrast, equilibration times in the field can be years. Because the nonlabile fractions are believed to be retained mainly by diffusional resistance rather than by irreversible chemical reactions (1), the herbicide residues are ultimately leachable and need to be accounted for in monitoring programs and fate modeling.

In view of the findings here and elsewhere (1, 4, 6), a reevaluation of extraction methods for other pesticides and chemical contaminants is warranted.

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PLANT TOXINS

Determination of Total Gossypol in Cottonseed and Cottonseed Meals by Derivative UV Spectrophotometry

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A new method for the determination of total gossypol in cottonseed and cottonseed meals has been developed. The method involves oxalic acid hydrolysis of the bound gossypol in a methyl ethyl ketone–water azeotrope, partitioning the liberated gossypol into chloroform, and quantification by 2nd derivative UV spectrophotometry. The 2nd derivative transformation and measurement of the conventional analytical band around 300 nm permits direct quantification of all compounds containing naphthalene nuclei; chromogenic reaction is not required. The method was tested at concentration levels of total gossypol normally expected for cottonseed and cottonseed meal. Precision and accuracy data suggested an overall relative standard deviation of 4.0% and an overall recovery of 89.5%. Although results for cottonseed and solvent-extracted cottonseed meal analyses were comparable to those obtained by use of American Oil Chemists' Society methods, results were lower for screw-pressed meals. The lower results were attributed to the partial conversion of gossypol, during the cooking process of these meals, to compounds that differ in composition and structure from gossypol and which react with aniline to give false readings.

Cottonseed meal, the by-product of the cottonseed-oil processing industry, is a high protein material with valuable nutritive properties, which has found widespread application as animal feed. However, its utilization in animal nutrition is limited by the presence of gossypol, a well characterized polyphenolic binaphthyl aldehyde, and gossypol-related compounds. Gossypol and many of these compounds are toxic to nonruminants such as poultry (1–3) and swine (4–6), and young ruminants such as calves (7, 8) and lambs (9).

Gossypol and its related compounds are found primarily in the pigment glands of cottonseed. During cottonseed processing, moisture and heat convert most of the gossypol to the so-called "bound gossypol," a compound that has the aldehyde groups condensed with free amino groups of proteins to form a Schiff base. The formation of a Schiff base, while resulting in some detoxification of gossypol, also lowers the nutritive value of the cottonseed meal by reducing the availability of lysine, the limiting essential amino acid in cottonseed (10). Therefore, bound gossypol and, consequently, total gossypol (free plus bound) content is an important quality factor of cottonseed meal.

Several analytical methods for estimating total gossypol in cottonseed and cottonseed meals have been reported. Early methods, gravimetric procedures (11–13) that require hot aniline extractions and time-consuming handling, have been substituted by spectrophotometric methods. Pons et al. (14) first developed a spectrophotometric method based on the *p*-anisidine reaction of the gossypol liberated after oxalic acid hydrolysis of its bound form. This was essentially the Official American Oil Chemists' Society (AOCS) method Ba 8-55 for total gossypol (15), except aniline was substituted for *p*-anisidine as the coloring agent (16). Later, Smith (17) shortened the analysis time by omitting the acid hydrolysis step, and proposed treating the sample with a mixture of aniline–ethanol to convert bound gossypol to dianilinogossypol. Pons et al. (18) further shortened the analysis time by using 3-amino-1-propanol in dimethylformamide to hydrolyze and complex bound gossypol, and determined gossypol, after addition of aniline, as dianilinogossypol. This aminopropanol hydrolysis procedure was subsequently adopted by AOCS as official method Ba 8-58 (19), later Ba 8-78 (20). Fisher et al. (21) modified method Ba 8-78 to make it applicable to cottonseed containing less than 10 ppm total gossypol. Admasu and Chandravanshi (22), described an alternative method in which ferric nitrate is used in place of aniline, and reported identical results to those obtained by method Ba 8-78 for cottonseed and cottonseed meal presscakes.

However, it has been shown that spectrophotometric methods based on dianilinogossypol formation may lead to false readings (23, 24) due to the presence of extraneous compounds that react with the coloring agent. Therefore, a simple method not based on reaction with aniline might offer another approach to the accurate and precise determination of total gossypol in cottonseed materials, and, thus, the means of verifying the results for the AOCS methods.

The present report deals with the development of a new method for the determination of total gossypol in cottonseed and cottonseed meals by the use of 2nd derivative UV spectrophotometry (25–28) so that the reaction of gossypol aldehyde groups with aniline or other chromogenic reagents could be bypassed.

METHOD

Reagents

(a) *Aqueous acetone.*—Mix 700 mL ACS grade acetone with 300 mL water.

(b) *Methyl ethyl ketone–water azeotrope.*—Mix 1106 mL reagent grade methyl ethyl ketone with 110 mL water and distill, rejecting the first 100 mL of distillate. Azeotrope distills at 73.5°C. Store in brown bottle.

(c) *Oxalic acid solution.*—0.1M. Dissolve 12.6 g ACS grade oxalic acid dihydrate in methyl ethyl ketone–water azeotrope, and dilute to 1000 mL with azeotrope. Store solution in brown bottle.

(d) *Barium acetate solution.*—0.5M. Dissolve 136.73 g ACS grade barium acetate hydrate in water and dilute to 1000 mL with water.

(e) *Solvent mixture.*—Mix 715 mL ACS grade ethanol, 285 mL water, 200 mL ACS grade, peroxide free, diethyl ether, and 2 mL reagent grade glacial acetic acid. Store in brown bottle.

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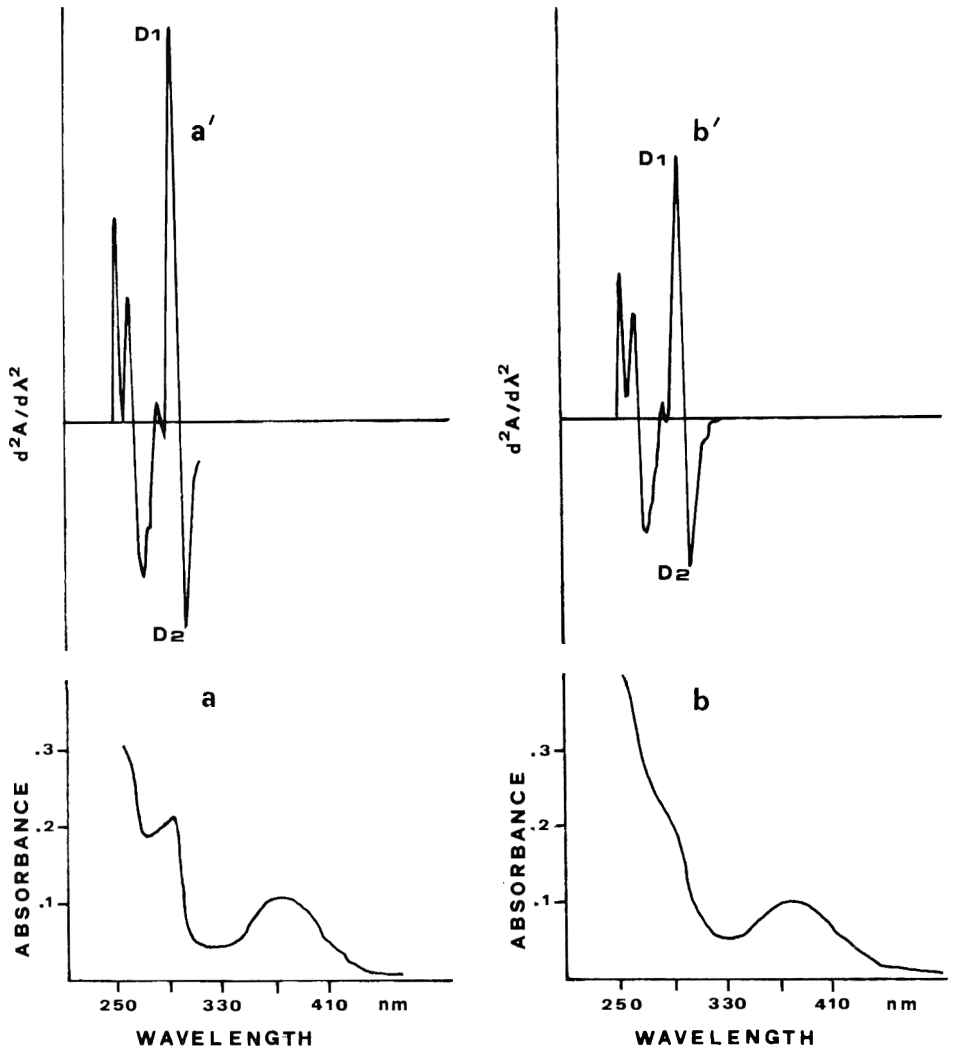

Figure 1. Normal (a, b) and corresponding 2nd derivative (a', b') UV spectra of gossypol working solution (3.4 μg/mL) and extract of cottonseed meal.

(f) *Other chemicals.*—ACS grade chloroform, sodium sulfate, and hydrochloric acid.

(g) *Gossypol standard solution.*—Weigh ca 12 mg gossypol acetic acid (89.62% gossypol; Makor Chemicals, Ltd), and dissolve in and dilute to 50 mL with solvent mixture. Dilute aliquots of this stock solution to prepare working solutions in range 1–4 μg/mL.

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer Model 512 double beam UV-Vis spectrophotometer with 10 mm quartz absorption cells. Derivative UV spectra produced by electronic differentiation of spectrophotometer output signal and monitored on Perkin-Elmer Model 165 chart recorder. Perkin-Elmer Model 200-0507 derivative accessory permits selection of six (1, 2, . . . , 6) time constants (sensitivities) for electronic differentiation of output signal. Positive as well as negative signals may be expected in derivative spectra; set recorder pen at 50% full scale deflection before scanning. Obtain 2nd derivative spectra in range of 250–340 nm at scanning speed of 240 nm/min with monochromator slit set at 3 nm and time constant 5.

(b) *Water bath.*—Tamson Model T.X.V.45 constant temperature control (accuracy ±0.1°C).

(c) *Rotary vacuum evaporator.*—Buchi, Model Rotavapor-R.

(d) *Laboratory mill.*—Retsch KG type SK1 mill equipped with 1 mm screen.

Sample Preparation

Grind ca 50 g cottonseed meal or dehulled cottonseed sample in laboratory mill through 1 mm screen. Grind cottonseed carefully to avoid overheating and oil expression.

Procedure

Accurately weigh ca 1 g sample material into 100 mL volumetric flask and add 25 mL oxalic acid solution. Place flask in 75°C water bath, allow to equilibrate, then stopper flask and heat 6 h. Remove flask from bath, cool to room temperature and add 25 mL aqueous acetone followed by 5 mL barium acetate solution. Mix contents of flask and dilute to volume with aqueous acetone. Let stand 10 min for complete precipitation of formed barium oxalate and filter through Whatman No. 41 paper into glass-stopper flask, discarding first 10 mL of filtrate. Pipet 25 mL aliquot of filtered extract into 250 mL separatory funnel and add to funnel 50 mL chloroform followed by 100 mL water acidified with 1 mL hydrochloric acid. Shake funnel 3 min to accom-

Table 1. Raw data and regression equation of calibration curve for gossypol determination by 2nd derivative spectrophotometry

Gossypol concn in std soln, μg/mL	Mean height,[a] mm (n = 7)	Std dev.	Rel. std dev., %
1.280	41.3	1.0	2.4
1.706	53.4	1.5	2.8
2.133	67.7	0.9	1.3
2.559	80.6	0.9	1.1
2.986	94.9	1.1	1.2
3.413	110.6	1.1	1.0

[a] Regression equation: $y = -0.74 + 32.2x$. $\bar{R}^2$ (adjusted for df) = 0.997.

plish partitioning, let stand 5 min, and filter lower organic layer through anhydrous sodium sulfate on Whatman No. 40 paper into 100 mL round-bottom, glass-stopper flask. Rinse sodium sulfate by filtering through paper 10 mL chloroform, and evaporate filtrates to dryness by using initially rotary vacuum evaporator at 40°C and, finally, nitrogen stream. Dissolve remaining residue with 50 mL solvent mixture and stopper flask.

Derivative Spectrophotometry and Calculation

Pipet appropriate aliquot of sample solution into 10 mL volumetric flask and dilute to volume with solvent mixture. Size of aliquot depends on total gossypol content of sample: for sample with expected total gossypol content <3000 ppm, use >1.0 mL aliquot; 3000–5000 ppm, 1.0 mL aliquot; 5000–7000 ppm, 0.7 mL aliquot; 7000–9000 ppm, 0.6 mL aliquot; 9000–11 000 ppm, 0.5 mL aliquot; 11 000–13 000, 0.4 mL aliquot. For maximum precision, resulting final solution should contain ca 2.5 μg gossypol/mL. Record 2nd derivative UV spectrum of final solution against solvent mixture, according to previously described conditions, and calculate its gossypol concentration (μg/mL) by measuring D_1D_2 height (Figure 1) and comparing it to heights obtained from spectra of gossypol standard solutions (calibration curve).

Calculate total gossypol content of cottonseed product as follows:

$$\text{Total gossypol, ppm} = (2000 \times C)/(V \times W)$$

where C = gossypol concentration, μg/mL, of final solution from standard curve; V = volume of aliquot taken, mL; W = weight of sample, g.

Results and Discussion

The performance of 2nd derivative vs normal UV spectrophotometry in the identification of gossypol is shown in Figure 1. The absorbance differentiation $d^2A/d\lambda^2$, where A = absorbance and λ = wavelength, resulted in transformation of the normal spectrum into a series of sharp maxima and minima that were used for qualitative purposes. When the derivative spectrum of a 3.4 μg/mL working solution of gossypol was recorded, a maximum (D_1) and a minimum (D_2) appeared, corresponding, respectively, to the inflection point and to the maximum of the conventionally recorded analytical band around 300 nm. The appearance of such extremes improved the resolution so that, while the normal spectrum of a cottonseed meal extract gave little information, its 2nd derivative spectrum permitted direct quantification of gossypol in the extracts by measuring the distance, in the ordinate direction, between the characteristic maximum D_1 and the adjoining minimum D_2, since the D_1D_2 height was in good linear relation to gossypol concentration (Table 1).

Table 2. Effect of hydrolysis time on the determination of total gossypol in various samples

Type of sample	Gossypol found,[a] ppm 0 h	6 h	16 h
1860 ppm gossypol soln	1770 (95.2%)	—	—
3719 ppm gossypol soln	3560 (95.7%)	3380 (90.9%)	1897 (51.0%)
Screw-pressed meal[b]	—	3444 (89.5%)	1887 (49.0%)
Screw-pressed meal[b] + 1921 ppm gossypol	—	5164 (89.5%)	—
Solvent-extracted meal[c]	—	6619 (89.5%)	3715 (50.2%)
Solvent-extracted meal[c] + 2293 ppm gossypol	—	—	5164 (53.3%)

[a] Values in parentheses are percent recoveries.
[b] Meal containing 3848 ppm total gossypol.
[c] Meal containing 7395 ppm total gossypol.

The hydrolysis process was that proposed by Pons et al. (14) and the partition process was that reported by Schramm and Benedict (29). Since it was found that 16 h hydrolysis leads to serious destruction of gossypol and/or gossypol-related compounds, 6 h hydrolysis was adopted instead. The data given in Table 2 indicate that a 5% loss of gossypol occurs during the partition process.

Interferences in extracted samples may cause inaccurate gossypol determination, so a standard addition method of analysis was evaluated. In this study, 9 of 12 samples from a solvent-extracted cottonseed meal were spiked at 3 fortification levels with standard gossypol and all samples were assayed for total gossypol content. Least-squares and regression analysis of the data (Table 3) based solely on the 3-level spiking showed that the relationship between "added" and "found" was adequately described by a linear regression ($\bar{R}^2$, adjusted for degrees of freedom, = 0.989). The intercept (6543) of the regression line ($y = 6543 + 0.907x$), which actually is the value in ppm predicted for the unspiked samples, was found by the *t*-test not significantly different from the arithmetic mean (6618.7 ppm) of the unspiked samples. This finding indicated that interferences were not present in extracted samples.

The lack of interfering bands of other absorbing compounds permitted accuracy evaluation using the data from both the spiked and unspiked samples (Table 3). Least-squares and regression analysis of these data showed that linearity was quite acceptable ($\bar{R}^2$, adjusted for df, = 0.995). Therefore, the slope (0.895 ± 0.043) of the regression line ($y = 6596 + 0.895x$) could be used as an estimate of overall recovery (89.5% ± 4.3) in the analysis for total gossypol in cottonseed products.

The precision of the proposed method was also studied by assaying, on each of 3 different days, 6 samples from a solvent-extracted cottonseed meal. To estimate the overall

Table 3. Accuracy data based on recovery of gossypol added to a cottonseed meal at 3 levels

Spiking level	Gossypol added,[a] ppm	Gossypol found, ppm
—	0	6591
—	0	6606
—	0	6659
1	1828	8485
1	1822	7973
1	1811	8198
2	3665	9844
2	3612	9848
2	3620	9674
3	5407	11508
3	5445	11588
3	5469	11412

[a] Concentration differences among samples of gossypol added are due to unavoidable variation in weights of samples taken for analysis.

Table 5. Total gossypol content of cottonseed products as determined by different methods

Type of product	Gossypol found,[a] ppm: AOCS method (Ba 8-55)	AOCS method (Ba 8-58)	Proposed method[b]
Solvent-extracted meal	7134	7767	7437
Solvent-extracted meal	5871	6093	6116
Cottonseed (local variety)	4751	5383	4991
Cottonseed (local variety)	3978	4314	4425
Screw-pressed meal	5324	4962	4416
Screw-pressed meal	4418	4490	3813

[a] Duplicate determinations.
[b] Values corrected for recovery.

precision, the raw data (Table 4) were subjected to "analysis of variance and expected mean squares for the one way classification-random effects model" (30). Within-day precision was 3.7%, between-day precision 1.3%, and overall precision was 4.0%.

Application of the proposed procedure to the analysis of total gossypol in cottonseed and solvent-extracted cottonseed meal samples gave results in substantial agreement with those obtained by using the AOCS methods Ba 8-55 and Ba 8-58 (Table 5). The considerably lower values observed when the proposed method was applied in the analysis of screw-pressed meals might be attributed to partial conversion of gossypol, during the cooking process of these meals, to compounds of various composition and structure (31, 32) that differ from that of gossypol. Based on these observations and on previous work on the structure of gossypol, we attempted to find what is exactly quantified by the proposed method.

According to Adams and Kirkpatrick (33), an examination of the normal spectra of gossypol (34, 35) and many of its related compounds shows them all to be characterized by a maximum at approximately 250 nm and a maximum around 300 nm with logarithms of molal extinction coefficients close to 5 and 4, respectively. A comparison, on the other hand, of the absorption spectrum of gossypol with those of substituted naphthalenes indicates that it is of the same general shape but with more intense peaks (36, 37). If this intensity were due merely to auxochromes in the molecule, the peaks of the normal spectra of the various gossypol-related compounds in which the functional groups differ should vary considerably from those of gossypol. However, this has been found (33) not to be the case; apogossypol hexamethyl ether, which has 2 aldehyde groups less than gossypol, shows only minor differences in the 2 main peaks from that of gossypol. These findings led Adams and Kirkpatrick (33) to the conclusion that the enhancement of the intensity in the gossypol spectrum was due to the presence of 2 naphthalene nuclei in the gossypol molecule. Based on these spectroscopic investigations, it may be considered that the proposed 2nd derivative method that involves transformation and measurement of the analytical band around 300 nm measures, in fact, not only gossypol per se, but also those compounds that contain naphthalene nuclei.

Table 4. Precision data for determination of total gossypol in cottonseed meal

Day	Gossypol found, ppm	Mean value, ppm	Std dev.	Rel. std dev., %
1	6699, 6342, 6610 6643, 6701, 6852	6641	168.4	2.5
2	6551, 6658, 6651 6908, 7572, 6721	6843	376.0	5.5
3	6703, 6637, 6579 6527, 6749, 6359	6592	139.8	2.1
	Overall	6692	261.0	3.9

Variance estimates:

Source	Rel. std dev., %
Within day	3.7
Between day	1.3
Overall	4.0

In conclusion, the present study shows that the use of 2nd derivative spectrophotometry provides a selective, accurate, and precise method for the determination of total gossypol at concentration levels normally expected for cottonseed and cottonseed meals. One analyst can easily process 6 samples in a 8 h work day. Although this method does not offer improved speed over AOCS methods, it does give to the analyst an alternative way of measuring total gossypol in cottonseed material.

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Enzyme-Linked Immunosorbent Assay for Microcystins in Blue-Green Algal Blooms

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A direct competitive enzyme-linked immunosorbent assay (ELISA) for the freshwater blue-green algal toxin microcystin (MCYST) in algae and water was developed. The assay involves coating anti-MCYST-variant leucine-arginine (LR) antibody to the ELISA plate and the use of MCYST-LR-peroxidase as the enzyme marker. The linear portion of the standard curve for MCYST in phosphate buffer containing saline (PBS) was 0.5-10.0 ng/mL (25-500 pg/assay). The minimum detection level for MCYST-LR was 0.20 ng/mL (10 pg/assay). Contaminated water could be directly used in the ELISA. The overall analytical recoveries for MCYST-LR added to water at levels of 1-20 ng/mL was 83.4%. For analysis of cellular MCYST, the toxin was first extracted from the algae with 0.1M ammonium bicarbonate, diluted with PBS to less than 0.5 mg dried algae/mL (<5.0 mg wet weight/mL) and directly used in the ELISA. C-18 reverse-phase Sep-Pak cartridges effectively adsorbed MCYST from the toxin-containing solutions. The toxin could be recovered from the cartridge by eluting with 60% methanol. Using this approach, an algae extract that was relatively free of MCYST was prepared and was used in a recovery study. The overall analytical recovery of MCYST added to the algae extract in the range of 0.25-20 ppm was 83% with a coefficient of variation of 11.9%. The detection limit for MCYST in dried algae was about 0.25-0.5 μg/g (0.25-0.5 ppm) lyophilized algae sample. This method was applied for the analysis of several naturally occurring algal blooms. Limited samples were also analyzed for MYCST by liquid chromatography. ELISA data were in general agreement with those obtained by liquid chromatography. MCYST concentrations from 0.006 to 2.9 μg/g (6 to 2900 ppb) and from 26 to 5200 μg/g (26 ppm to 5200 ppm) were found in water and algae (dried weight), respectively.

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Microcystins (MCYST), nodularin (NODLN), and related hepta- and pentacyclic peptides are a group of toxins produced by certain freshwater blue-green algae (cyanobacteria), including *Anabaena flos-aquae*, *Microcystis aeruginosa*, *Oscillatoria agardhii*, and *Nodularia spumigena* (1-5). These toxins cause intermittent but repeated poisonings of wild and domestic animals in many parts of the world as well as liver damage, gastroenteritis, diarrhea, and dermatitis in humans (6). Thus, the presence of these toxins in the environment is considered to be potentially hazardous to human and animal health. To decrease the risk of exposure to the toxins, monitoring the toxins in water, foods, and feedstuff is essential. Currently, liquid chromatography (LC) and mouse bioassay are most frequently used (7-10) to detect the toxins in cyanobacterial water blooms (2-5). LC analysis involves extensive cleanup before the assay and is very time consuming; the mouse assay, is nonspecific.

Investigations in our laboratory and in others have led to methods for the production of specific antibodies against several important MCYSTs, including monoclonal antibody against the leucine-alanine (LA) variant of MCYST (MCYST-LA) (11) and polyclonal antibodies against the leucine-arginine variant (MCYST-LR) (12). An indirect ELISA that involves coating of MCYST-LA directly to the microtiter plate (11) and a sandwich immunoassay format

(12) were reported; however, the sensitivity for the toxin analysis was low and the standard curve for the assay was in the microgram range.

Most recently, an effective method for the production of polyclonal antibodies with high affinity to the MCYST-LR variant was developed in our laboratory (13). The antibodies have good cross-reactivity with MCYST-arginine-arginine variant (MCYST-RR), MCYST-LR, MCYST-tyrosine-arginine (YR), and nodularin (NODLN), but less reactivity with variants MCYST-leucine-tyrosine (LY) and MCYST-leucine-alanine (LA). The antibodies did not cross-react with ozonolyzed MCYST-LR. We also tried to develop ELISA protocols for the analysis of microcystins in different matrixes. Details for the determination of microcystins in water and algae as well as application of this method for the analysis of several naturally occurring algal blooms are described in the present paper.

Experimental

Materials

Microcystin-LR, produced by *M. aeruginosa* PCC-7820, was supplied by W. W. Carmichael of Wright State University (Dayton, OH). Bovine serum albumin (BSA; RIA grade), *o*-phenylenediamine (OPD), and 30% hydrogen peroxide were from Sigma Chemical Co. (St. Louis, MO). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDPC) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Horseradish peroxidase (HRP) (ELISA grade) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). The HRP substrate, *o*-phenylenediamine, in tablet form (OPD, 4.0 mg/tablet), was supplied by Idexx (Portland, ME). Antibodies against MCYST-LR were prepared in rabbits after immunization with EDA-BSA-MCYST-LR conjugate according to the method of Chu et al. (13, 14). MCYST-LR was conjugated to peroxidase by a method using water-soluble carbodiimide (EDPC) as previously described (13). Sep-Pak C-18 reverse-phase cartridges were from Waters Associates (Milford, MA). All chemicals and organic solvents were reagent grade or better.

Apparatus

(a) *ELISA washer.*—Automatic (Dynatech Model B Miniwasher or equivalent).

(b) *ELISA reader.*—Dynatech MR 600 or equivalent.

(c) *ELISA plates.*—Nunc Microwell (high binding capacity, Nunc Co., Denmark).

(d) *Automatic pipets.*—Titertek 8 channel (50-200 μL) and single channel such as Gilson (50 and 100 μL).

Immunoassay Reagents

(a) *Phosphate-buffered saline (PBS).*—Sodium phosphate buffer, 0.01 mol/L (or 0.1 mol/L, as specified), containing 0.15 mol NaCl/L, pH 7.5.

(b) *Tween-PBS.*—0.1% (v/v) Tween 20 in PBS.

(c) *Standard solutions.*—Dissolve MCYST-LR standard in small amount of ethanol and then diluted to appropriate concentrations in PBS for ELISA. Ethanol concentrations were less than 1%.

(d) *BSA-PBS solution.*—0.1%. Dissolve 0.1 g BSA in 100 mL PBS (0.01M, pH 7.5).

(e) *Substrate buffer.*—Citric acid (0.05 mol/L) plus 0.1 mol NaH_2PO_4/L, pH 5.0.

(f) *Enzyme substrate solution (ESS).*—Dissolve 40 mg OPD or 10 OPD tablets plus 0.04 mL 30% H_2O_2 in 100 mL substrate buffer. Prepare fresh daily and use within 60 min after preparation.

(g) *Stopping reagent (SR).*—1N HCl.

Preparation of Samples

(a) *Water.*—Standard MCYST-LR was first dissolved in small amount of ethanol to approximately 2 μg/mL, diluted in distilled water, and then added to tap water for the recovery study. No cleanup of water samples before ELISA was necessary.

(b) *Algae.*—Algae collected from typical lake algal blooms generally was separated from water by centrifugation. The algae portion was lyophilized to dryness. Microcystins in the aqueous fraction were analyzed according to the method described above. For dry algae, appropriate amounts of lyophilized algae (≤0.5 g) were blended in a Waring blender (or homogenized in a homogenizer) with 10-20 mL 0.1M ammonium bicarbonate for 4 min. The homogenate was centrifuged 30 min at 16 000× *g*. One mL (25 mg dry algae/mL) of supernatant solution was then diluted with PBS to a final solution containing <0.5 mg algae/mL and used directly in the ELISA.

To ascertain the validity of the above procedure, 2 naturally occurring toxic algae samples and one *M. aeruginosa* PCC-7820 culture extract were further subjected to the following treatment. One mL of the above algae extract with pH adjusted to 7.0 was loaded onto a Sep-Pak cartridge. After washing with water, the cartridge was eluted with 5 mL each of 20, 60, and 100% methanol. Concentration of MCYST in each fraction was determined by ELISA. An analytical recovery study was carried out by adding 0.5-10 ng MCYST-LR to each mL of an algae extract (0.5-4.0 mg/mL) from which MCYST had been removed by cartridge treatment.

Direct Competitive ELISA

The protocol for the direct ELISA is essentially the same as we previously described for aflatoxin B_1 (15) except that the antibody was diluted in 0.01M PBS (1 to 1000, with 0.1 mL coated to each well). The coated plate was incubated overnight at 4°C, and the plate was washed with PBS-Tween (0.35 mL/well; 0.05% Tween 20 in PBS) followed by incubation with BSA-PBS (0.17 mL/well; 0.01% BSA in PBS) for 30 min at 37°C. The plate was washed again with PBS-Tween (0.35 mL/well) 4 times followed by incubation with standard MCYST-LR (0.05 mL/well) at different concentrations or blank buffer, or sample solution together with MCYST-LR-HRP conjugate (0.05 mL/well) for 60 min at 37°C. The plate was washed 4 times with PBS-Tween (0.35 mL) and incubated with 0.1 mL of freshly prepared OPD-substrate solution at room temperature for 15-20 min, and then 0.1 mL stopping reagent was added. The color was read at 490 nm. Samples were run in triplicate.

Standard Curve and Calculation of MCYST Concentration

The standard curve was established by plotting the % of binding of the enzyme, i.e., % of maximum absorbance, to the plate vs the concentration of standard MCYST-LR, in log scale, at various concentrations. The % of binding of the enzyme to the plate was calculated from the following formula: $(A/A_o) \times 100$, where A is the absorbance reading for the sample or standard and A_o is the absorbance reading for the blank in PBS. Thus, for an unknown solution, the concentra-

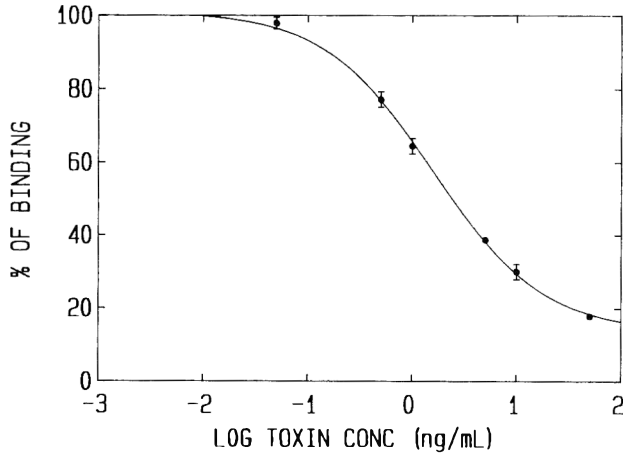

Figure 1. Standard curve for immunoassay of MCYST-LR in PBS. MCYST-LR-labeled HRP was used in the direct ELISA. Fifty μL portions of different concentrations of standard MCYST-LR in PBS were used in each assay. All data were obtained from the average of 3 sets of experiments. Concentrations (0.05–50 ng/mL or 2.5–2500 pg/assay) shown on the x-axis are in log scale.

tion could be directly estimated for the standard curve or calculated from any of the established computer programs directly after reading the absorbance.

Liquid Chromatography

Limited samples were subjected to LC analysis using the method of Harada et al. (7). All samples were analyzed in a Waters Associates LC system equipped with a Model 6000A pump and a Model 440 detector. A Waters μBondapak C-18 reverse-phase column (P/N 27324, 3.9 mm × 30 cm) was used. The column was equilibrated with a solvent system containing 4 parts of 0.05% trifluoroacetic acid in water and 6 parts of methanol and was run isocratically with the same solvent at a flow rate of 1 mL/min. The elution pattern was obtained by measuring absorbance at 254 nm. In general, 10–100 μL of sample in methanol was injected onto the column. Quantitation was performed by comparing the peak areas of the unknowns with a standard, which was established by injecting various concentrations of MCYST-LR (50–500 ng/injection) onto the column. To identify an extra peak in the unknown samples, a nodularin standard was also injected onto the column. All the algae samples were subjected to cartridge treatment, and only the 60% methanolic eluate was used in the LC analysis.

Results and Discussion

Standard Curves for ELISA of MCYST-LR

A standard curve for direct competitive ELISA of MCYST-LR in different matrixes is presented in Figure 1. Under the experimental conditions, the values for the blank (A_0, no toxin added) were generally about 0.9–1.2. It is apparent that the linear response of inhibition of binding by MCYST in the direct competitive ELISA was in the range of 0.5–10 ng/mL (25–500 pg/assay). Assuming the concentration of MCYST to cause a 10–15% inhibition of binding of MCYST-HRP to the coated antibody as the minimum detection level, the minimum detection level for MCYST-LR in the ELISA would then be about 10 pg/assay (0.20 ng/mL).

Table 1. Recovery by direct competitive ELISA of MCYST-LR added to water

Amt added, ng/mL	Recovery ng/mL	Recovery %	SD	CV, %
20	17.9	89.5	1.82	10.2
10	8.2	82.0	0.65	7.9
5	3.9	78.0	0.24	6.2
1	0.84	84.0	0.10	11.9
Overall		83.4		9.05

Recovery of MCYST-LR Added to Water

Recovery results for MCYST-LR added to water are given in Table 1. The overall recoveries, between 1 ng and 20 ng/mL (1–20 ppb), were 83.4% with a CV of 9.05%. At the 1 ng/mL level, the solution was diluted one-fold before ELISA; thus, as low as 0.5 ng/mL of the toxin in water could be determined without any treatment of the sample.

Recovery of MCYST-LR Added to Algae

Investigation of the analytical recovery of MCYST in algae was a very difficult task because practically all the algae collected from the naturally occurring blooms contained toxic *Microcystis*. Cartridge cleanup of the algae extract effectively removed almost all of the MCYST. Our first task was to prepare "a toxin-free extract (TFE)" by passing a preparation of algae extract through the cartridge and then to study the effect of this extract on ELISA. Results for the effect of 2 different algae preparations on blank absorbance readings are shown in Figure 2. The blank readings for the solutions containing ≤0.3–0.6 mg dried (lyophilized) algae/mL, almost approached the standard deviations of the blank readings obtained in PBS, which suggests that algae at 0.5 mg/mL could be directly used in the ELISA. Since the 0.5 ng/mL was the lowest concentration for MCYST in PBS in

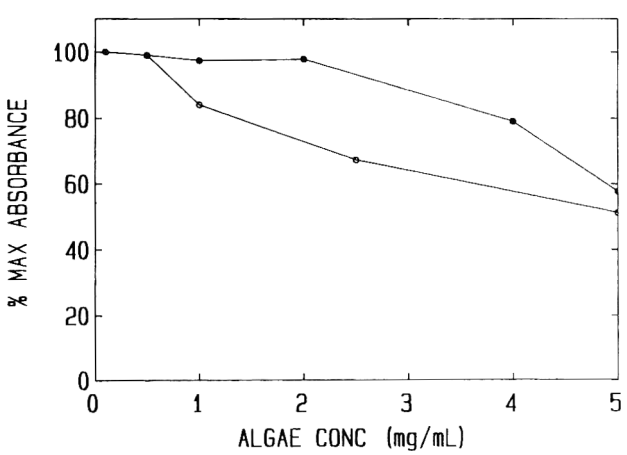

Figure 2. Effect of algae extract on ELISA of MCYST. Algae sample A (lower curve), containing primarily *Microcystis*, *Lyngbya*, and *Gloeotrichia* and sample B (upper curve), containing primarily *Microcystis*, were collected from Lake Mendota (Madison, WI) and Lake Winnebago (WI), respectively. Algae samples were lyophilized to dryness and then extracted with ammonium bicarbonate. Extracts were then cleaned up on a reverse-phase C-18 cartridge according to procedures described in the text. Unabsorbed material was used in the ELISA. Algae concentrations on x-axis are weight equivalent to original lyophilized algae before cleanup.

Table 2. Recovery by direct competitive ELISA of MCYST-LR added to algae extract[a]

Amt toxin added, ng/mL	Amt algae, mg/mL: 0.5 Rec., %	0.5 CV, %	0.5 μg/g	1.0 Rec., %	1.0 CV, %	1.0 μg/g	2.0 Rec., %	2.0 CV, %	2.0 μg/g	4.0 Rec., %	4.0 CV, %	4.0 μg/g
0.5	94.0	4.3	1	90.0[b]	10	0.5	86.0[b]	7.5	0.25	130.0[b]	13.6	0.125
1.0	86.0	19.6	2	83.0[b]	20.5	1	73.0[b]	7.6	0.5	101.0[b]	6.8	0.25
5.0	78.7	8.2	10	84.7	11.2	5	78.7	10.6	2.5	92.7	10.7	1.25
10.0	80.7	2.9	20	76.7	15.9	10	69.7	23.7	5.0	76.7	18.5	2.5

[a] MCYST-LR was added to an algae extract after cleanup. Amounts of algae shown are algae concentrations before cleanup.
[b] Data corrected for small amount of MCYST (0.1 ng/mg) present in algae extract.

the linear portion of standard curve (Figure 1), our results suggest that results would be best at toxin levels ≥1 μg/g (1 ppm). However, the limit of detection would be lower. Since the detection limit for the standard curve in buffer was about 0.20 ng/mL, the present method could detect as low as 0.4 μg/g (0.4 ppm) of MCYST in algae. The wet weight for the dried algae was approximately 10 times that of the dry weight. Thus, the detection limits for algae on a wet-weight basis were about 40 ppb.

For the analytical recovery study, an algal extract was subjected to Sep-Pak treatment. After such treatment, the absorbance for the blank solutions that contained 0.5, 1, 2, and 4 mg toxin-free algae/mL decreased 1.1, 2.7, 2.2, and 20%, respectively. From the data obtained in the 4.0 mg algae/mL set, the toxin concentration in the TFE was estimated to be 0.1 ng MCYST/mg extract. Various amounts of MCYST-LR were then added to the TFE, followed by ELISA. A wide range of toxin levels (0.125–20 μg/g) was covered in this recovery study (Table 2). Recoveries were good for solutions between 0.5 and 4.0 mg algae/mL except in one group that contained 0.5 ng toxin and 4.0 mg algae, with a recovery of 130%. The overall recovery and CV between 0.25 and 20 ppm were 83% and 11.9%, respectively. These data suggest that the detection limits in the presence of algae would be about 0.25 μg/g (0.25 ppm) for the lyophilized algae.

ELISA of MCYST-LR in Algae Extracts Before and After Cleanup

In recognizing the problems for carrying out a good analytical recovery study, a number of samples obtained from naturally occurring algal blooms were analyzed. We found much less interference than we expected because the toxin concentrations were above 1 ppm for the lyophilized algae cells in most cases. Thus, considerable dilution was needed for such analysis and a PBS standard curve was used in the studies. Nonetheless, we tested a cleanup procedure using the C-18 reverse-phase Sep-Pak cartridge. Three toxic algal samples were used. The first was an algae extract supplied by Dr. Carmichael's laboratory and was obtained from *M. aeruginosa* strain 7820 grown in the laboratory. The solution was found to contain 62.7 μg MCYST-LR/mL. The second (sample A) and third (sample B) samples were collected from Lake Mendota (Madison, WI) and Lake Winnebago (WI) in July 1989, and contained primarily *Microcystis*, *Lyngbya*, and *Gloeotrichia*. For the second and third samples, we first separated the algae from the water by centrifugation, and then the algae were lyophilized to dryness. The toxin was extracted from the algae with 0.1M ammonium bicarbonate and analyzed by ELISA. In these 2 samples, the toxin levels were 124 and 101 μg/g of dried sample A and B, respectively.

All 3 extracts were subjected to cleanup as described above. MCYST was recovered from the cartridge by elution with 5 mL each of 20%, 60%, and 100% methanol in water. The MCYST in each of these fractions was then determined. Part of the original solution (no cartridge treatment), diluted in PBS, was also subjected to ELISA. Results of different treatments are shown in Table 3. The results clearly demonstrate that the aqueous eluant contained ≤0.4% of the total original toxin. More than 90% of the MCYST present in the original extracts was detected in the fractions that were eluted with 20–100% of methanol; 88–99% was distributed in the 60% methanolic fraction. Two conclusions could be drawn from these data: (*1*) cartridge treatment of algal extracts is an effective method for removal of a considerable amount of matrix interference substances and could be used as a cleanup or partial purification method for MCYST; (*2*) no cartridge treatment for the analysis of algal extracts by ELISA is necessary because the ELISA data for the original extract are consistent with those after cartridge treatment. Since the toxin levels of lyophilized algae samples were very

Table 3. Recovery of MCYST from C-18 reverse-phase cartridge treatment[a]

Treatment	*M. aeruginosa* extract μg/mL	*M. aeruginosa* extract %	Algae bloom A μg/mL	Algae bloom A %	Algae bloom B μg/mL	Algae bloom B %
1. Original extract	62.70 (4)	100	2.85 (3)	100	1.43 (3)	100
2. Sep-Pak washing	0.08 (2)	0.1	0.008 (1)	0.3	0.006 (1)	0.4
3. 20% Methanol eluate	3.21 (3)	5.1	0.075 (2)	2.6	0.038 (2)	2.7
4. 60% Methanol eluate	56.20 (4)	89.6	2.5 (3)	88.4	1.40 (3)	98.6
5. 100% Methanol eluate	1.02 (2)	1.6	0.049 (1)	1.7	0.036 (1)	2.4

[a] One mL of original sample extract was applied to a Sep-Pak C-18 cartridge and then eluted with water, 20%, 60%, and 100% methanol as described in the text. Original solution and materials obtained from each fraction were diluted in PBS (dilution factors, in log scale, are shown in parentheses) and subjected to ELISA using standard curve established in PBS. See text for description of samples and sample preparation.

Table 4. ELISA of MCYST in algae and water of naturally occurring algal blooms

Sample	Water, μg/mL ELISA	Algae, μg/g ELISA	Algae, μg/g LC[a]
Algae 338[b]	0.19	26.3	—
Algae 339[b]	0.24	40.5	—
Algae 2[c]	0.006	32.4	—
Algae 3[d]	0.14	101	132
Algae 4[e]	2.15	76	72
Lake Mendota[f]	2.9	124	126
Algae 8969-001[g]	—[h]	2900	1150 (+1030)
Algae 8969-002[g]	—[h]	5200	1400 (+1500)
Algae 8969-003[g]	—[h]	2800	950 (+1250)
Algae 8969-004[g]	—[h]	1500	900 (−450)

[a] Two major peaks, one identified as MCYST-LR and the other, which eluted 1 min earlier than MCYST-LR and was also immunoreactive with the antibodies in ELISA, were observed for the 8969 series samples. Only MCYST-LR concentrations are reported in this column. Values in parentheses indicate the estimated concentration for the unknown peak using MCYST-LR as the standard.
[b] Lake Winnebago (WI) sample collected on 7/12/89.
[c] Big Long Lake (WI) sample collected on 7/13/89.
[d] Lake Winnebago sample collected on 7/17/89.
[e] Fox River sample collected on 7/31/89.
[f] Collected from Lake Mendota (Madison, WI) on 7/24/89. Microscopic examination revealed that the sample contained *Microcystis*, *Lyngbya*, and *Gloeotrichia*.
[g] Samples supplied by Milton Smith, National Wildlife Health Research Center. The original samples were supplied by Minnesota Dept of Nature Resources from a bloom that occurred in Lake Elysian, MN, in July 1989. The primary algae were *Microcystis* and *Anabaena*.
[h] No sample was available.

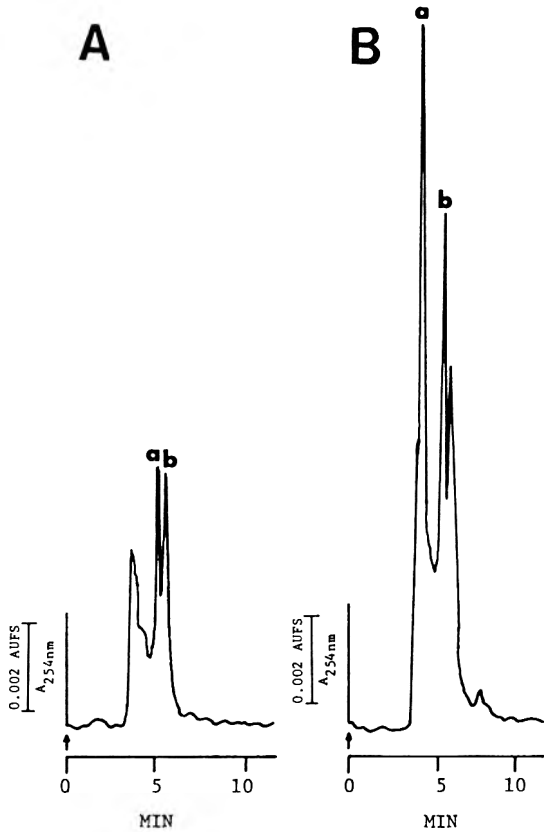

Figure 3. LC analysis of 2 algae extracts. Chromatograms A and B were obtained from sample extracts 8969-2 and 8969-3 of Table 4, respectively. Peak a was tentatively identified as NODLN. Peak b was identified as MCYST-LR. First peak was solvent front. Materials obtained from both peaks were immunoreactive with the antibodies.

high, considerable dilution of the samples was necessary to fit in the standard curve range. In most cases, less than 0.05 mg dried algae was used in each analysis.

ELISA of MCYST in Algae and Water of Naturally Occurring Blooms

To test the effectiveness of the analytical protocols developed in the present study, samples obtained from several naturally occurring blooms during the summer of 1989 in the United States were subjected to ELISA. Limited samples that were found to have concentrations within LC detection limits were also subjected to LC analysis. Results shown in Table 4 indicate that MCYST concentrations in water varied from 0.006 μg/g to as high as 2.9 μg/g. Levels in dried algae varied from 26 to 5200 μg/g. ELISA data are generally in good agreement with those obtained from LC analyses. However, in the 8969 series samples (Figure 3), 2 peaks were observed in the LC chromatogram. One was identified as MCYST-LR; thus, the concentration was accurately determined. The other, which had a retention time of 1 min earlier than MCYST-LR, was tentatively identified as NODLN. We also collected the materials obtained from both peaks and analyzed them by ELISA. Both materials were immunoreactive with the antibodies. The concentrations for the unknown LC peak of different samples were estimated from the peak areas of the MCYST-LR standard curve. Using such estimates, the concentrations of total MCYST (peak A + MCYST-LR peak) are consistent with those obtained from ELISA. In general, ELISA data were slightly higher than LC data except sample 8969-2, in which ELISA data were almost 153% higher than those obtained by LC analysis.

Conclusion and Comments

Investigations in our laboratory have led to a sensitive ELISA method for the detection of MCYST in water and algae. MCYST at a concentration of 1 ppb in water can be easily determined; however, a matrix interference was observed for the algae samples. Algal extracts in which MCYST had been removed by C-18 cartridge treatment interfered with toxin detection at a concentration above 1-2 mg dried algae/mL. Thus, the detection limits for MCYST in dried algae were only in the range of 0.25-0.5 μg/g (0.25-0.5 ppm; or 25-50 ppb for wet algae). Nonetheless, we feel that the present method is sensitive enough for detection of toxic algal blooms because the toxin level in algae in most toxic blooms was higher than 1000 μg/g (2-6). The detection limit for LC analysis was in the range of 50-100 μg toxin/g dry cells (7-10), which is almost 200 times less sensitive than the ELISA. Analyses of several naturally occurring algal blooms in the summer of 1989 confirmed that the ELISA is adequate for the determination of MCYST in water and algae. The ELISA data are generally in good agreement with the LC results.

In the present study, the C-18 reverse-phase Sep-Pak cartridge effectively removed most of the interference present in the sample matrixes. Thus, if high sensitivity for the detection of MCYST toxin in the algae is needed, we recommend

Sep-Pak treatment for cleanup before ELISA. The algal extract could first be passed through the cartridge, which could then be washed with water and 20% methanol to remove the interfering materials. The toxin could then be recovered from the cartridge with 100% methanol, concentrated, and finally subjected to ELISA.

Since the antibodies used in the present study have good cross-reactivity with MCYST-arginine-arginine variant (MCYST-RR), MCYST-LR, MCYST-tyrosine-arginine (YR), and nodularin (NODLN), the present method can detect most major MCYST variants (13). It is not surprising that in most cases, the ELISA values were generally higher than LC results for the naturally occurring algal blooms. Four algal samples analyzed in the present study contained another immunoreactive MCYST in addition to MCYST-LR. Although this toxin is tentatively identified as NODLN, the exact chemical nature remains to be studied. Thus, the ELISA data reflect total toxin concentration in the sample. It must be pointed out that the cross-reactivity with variants MCYST-leucine-tyrosine (LY) and MCYST-leucine-alanine (LA) in the ELISA was only 4 and 2% of MCYST-LR, respectively. Thus, the present method would be less sensitive for detection of these 2 MCYST variants.

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VETERINARY TOXICOLOGY

Ion-Exchange Liquid Chromatographic Determination of Nitrate and Nitrite in Biological Fluids

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A rapid, ion-exchange liquid chromatographic method for the determination of nitrate and nitrite in biological fluids is presented. Samples are deproteinated by ultrafiltration followed by removal of chloride using a silver form cation-exchange resin. Nitrate and nitrite are measured by ion-exchange liquid chromatography with conductivity detection. Recoveries from serum, ocular fluid, and water were determined for fortifications from 10 to 150 mg/L. Average recoveries ranged from 96 to 104% for nitrate and from 89 to 105% for nitrite. Pooled RSD values ranged between 1.5 and 1.9% for these analytes in all matrixes examined. The method of joint confidence hexagons was applied to the data to determine constant and relative bias of the method for each of the 3 matrixes in the study.

Several studies have established the role of nitrate and nitrite in mammalian acute toxicosis (1-4). Studies have included the biotransformation of the nitrate and nitrite moieties and resulting methemoglobinemia and anemic hypoxia. Analytical methods have included colorimetry (5-9), ion-specific electrode (10), chemiluminescence (11), and derivatization prior to gas chromatography (12, 13) and liquid chromatography (14). Each has its disadvantages. Colorimetric analysis in its various forms either has limited sensitivity, uses dangerous reagents, or requires a complex procedure. Gas chromatographic analysis methods determine only nitrate. Ion-specific electrodes yield satisfactory results only for high concentrations of nitrate in samples free of interfering species.

In recent years, liquid chromatography has demonstrated the reliable performance and low detection limits required for routine diagnostic analysis of nitrate and nitrite in biological fluids. The biological fluids of interest include serum, ocular fluid, urine, and abomasal fluid. Ion-exchange columns are used for separation, and the various methods of detection have included UV absorbance (15), UV vacancy (16), and conductivity (17-19). These LC methods have exhibited detection limits in aqueous solutions below 1 mg/L. Food and water samples have been analyzed by ion-pairing chromatography (20).

All these methods present difficulties in determining nitrate and nitrite concentrations in the presence of a large amount of the chloride ion as a result of column saturation and concomitantly insufficient resolution. This commonly occurs in the analysis of brines and biological fluids where levels of chloride routinely exceed 10^3 mg/L. Traditionally, chloride interferences are eliminated by precipitation as the silver salt, using silver reagents or silver-loaded cation-exchange resins (21-23). However, solubility product considerations warn against the application of silver precipitation for some common anion analyses such as bromide, sulfate, phosphate, and others.

In previous LC work on biological fluids, proteins were removed by precipitation. Recent advances in ultrafiltration technology permit a partitioning of the high molecular weight protein components in biological fluids. This process allows production of a protein-free fluid while maintaining the equilibrium concentrations of low formula weight analytes (24, 25). Since the partitioning, dilution, and ion chromatographic analysis proceeds quickly with a high degree of precision and accuracy, the method has been developed as a routine diagnostic procedure.

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METHOD

Reagents

(a) *Eluant.*—Distilled in glass, deionized 18 megohm water, 0.75 mM sodium bicarbonate (Fisher Scientific), USP, and 2.2 mM sodium carbonate (Mallinckrodt, Inc.), analytical reagent grade. Dilute stock solution of 75 mM bicarbonate and 220 mM carbonate to working strength daily.

(b) *Nitrite + nitrate mixed stock solution.*—20 000 mg/L. Dissolve 30.00 g sodium nitrite, analytical reagent grade (Fisher Scientific), and 27.42 g sodium nitrate, analytical reagent grade (Mallinckrodt, Inc.), in 1000 mL.

(c) *Working strength standards.*—Prepare 30.00, 20.00, 10.00, 6.00, and 2.00 mg/L by serial dilutions of 20 000 mg/L stock solution.

(d) *Ion-exchange resin.*—Convert Dowex 50W-X8 cation-exchange resin, 200-400 mesh (Bio-Rad Laboratories), from hydrogen form to silver form using Ag_2SO_4, 99.999% (Aldrich). Work under reduced light conditions, with foil-wrapped glassware if possible. Slurry-pack 150 mm × 15 mm low pressure capped column (Bio-Rad Laboratories) with resin. Place solid Ag_2SO_4, $1\frac{1}{2}$ × milliequivalents of resin bed capacity, at top of resin column. Use peristaltic pump to pass 1 mL/min of 18 megohm water through column. Occasionally monitor effluent pH, using indicator strips; neutral pH and uniform lightening of resin bed color indicate conversion to silver form. Wash resin bed with 4-8 L reagent water, using 1 mL/min flow. Completely dry converted resin by connecting column tip to vacuum line and pulling air through resin bed. Absolute dryness is necessary for long-term storage. Transfer resin to amber bottle for storage. Test silver form resin for residual anion contamination by using water extraction of 10 mg sample and ion chromatography.

(e) *Bovine serum.*—Sigma Chemical Co.

(f) *Bovine ocular fluid.*—Pooled from eyes collected from bovine postmortem diagnostic samples.

(g) *Spiking solutions.*—Prepare 15 000, 10 000, 5000, 3000, and 1000 mg/L solutions from 20 000 mg/L stock mixed standard by serial dilutions.

Apparatus and Materials

(a) *Centrifuge.*—Damon IEC HT Centrifuge with a 55° fixed angle rotor.

(b) *Ion chromatograph.*—Dionex Model 4000i equipped with conductivity detector. Conditions: Dionex Ionpac HPIC-AS4A column preceded by Dionex Ionpac HPIC-AG5 guard column; micro-membrane cation suppression; conductivity detector sensitivity 30 μS or 100 μS full scale. Eluant program: 2.2 mM carbonate + 0.7 mM bicarbonate at 1.5 mL/min for 6.2 min; 22 mM carbonate + 7.0 mM bicarbonate at 2.5 mL/min for 1.8 min; 2.2 mM carbonate + 0.7 mM bicarbonate at 1.5 mL/min for 3 min.

(c) *Data processing system.*—IBM PS-2 Model 50 personal computer and P.E. Nelson Turbochrom chromatography data system.

(d) *Analytical centrifuge.*—Clay Adams.

(e) *Touch vortex mixer.*—Fisher Scientific.

(f) *Ultrafiltration filters.*—Centrifree Micropartition filters, Amicon Corp., Danvers, MA.

(g) *Syringe filters.*—Cameo 0.45 μm LC nylon filters, Micron Separations, Inc., Honeoye Falls, NY.

Sample Storage and Preparation

Serum, ocular fluid, abomasal fluid, or urine should be frozen (−5°C) if immediate analysis is not possible. Studies in this laboratory have shown that frozen biological fluids have been stored for up to 4 weeks without deterioration of nitrate levels. Cases of nitrate/nitrite toxicosis found in this laboratory have shown high levels (>50 mg/L) of both nitrate and nitrite in urine; however, we have found most clinical postmortem samples of serum and ocular fluid to contain predominately the nitrate form. Fluids found in anterior and posterior chambers of the eye have been found acceptable for analysis (25). As a result of postmortem autolysis, examination of the total ocular fluids found in the eye is generally easier if more than 12 h has passed since death.

Ultrafiltration

Lightly centrifuge serum, ocular fluid, abomasal fluid, and urine to separate any gelled proteinaceous material and allow easy transfer by disposable Pasteur pipet. Transfer 1.0 mL fluid to micropartition filter for separation of proteins from sample. Centrifuge capped filter device at 2800 × *g* for 30 min. Filtrate recoveries should be 0.3–0.4 mL in standard applications. Filtrate can be conveniently stored by freezing at this point in sample preparation.

Removal of Chloride and Dilution

Pipet 200 μL ultra-filtrate to 10 mm × 76 mm polystyrene culture tube. Add ca 5 mg silver resin, cap, and vortex-mix for 30 s. Dilute mixture with 800 μL reagent water and mix to achieve 5-fold dilution of sample. Dilution after precipitation with silver minimizes formation of colloidal silver chloride. Centrifuge mixture ca 1 min, and draw supernatant liquid into plastic 1 mL tuberculin syringe. Inject sample through 3 mm diameter 0.45 μm syringe filter.

In routine sample analysis for nitrate alone, the chloride removal step, using silver ion-exchange resin, can be eliminated. It is sufficient to add 1.8 mL water for a total 10-fold dilution. The method retains good resolution and sensitivity for biological nitrate levels at this dilution.

Table 1. Typical calibration data, area (μV•sec) vs concentration (mg/L)[a]

	Concentration				
Ion	2	6	10	20	30
Nitrite	998 021	3 192 527	5 507 651	11 378 073	17 368 106
Nitrate	904 748	2 798 501	4 803 509	10 216 854	16 075 437

[a] Calibration equations:
Area = $[NO_2^-] \cdot 572800$
Area = $[NO_3^-]^2 \cdot 2729.7 + [NO_3^-] \cdot 454414$
Mean of 3 determinations.

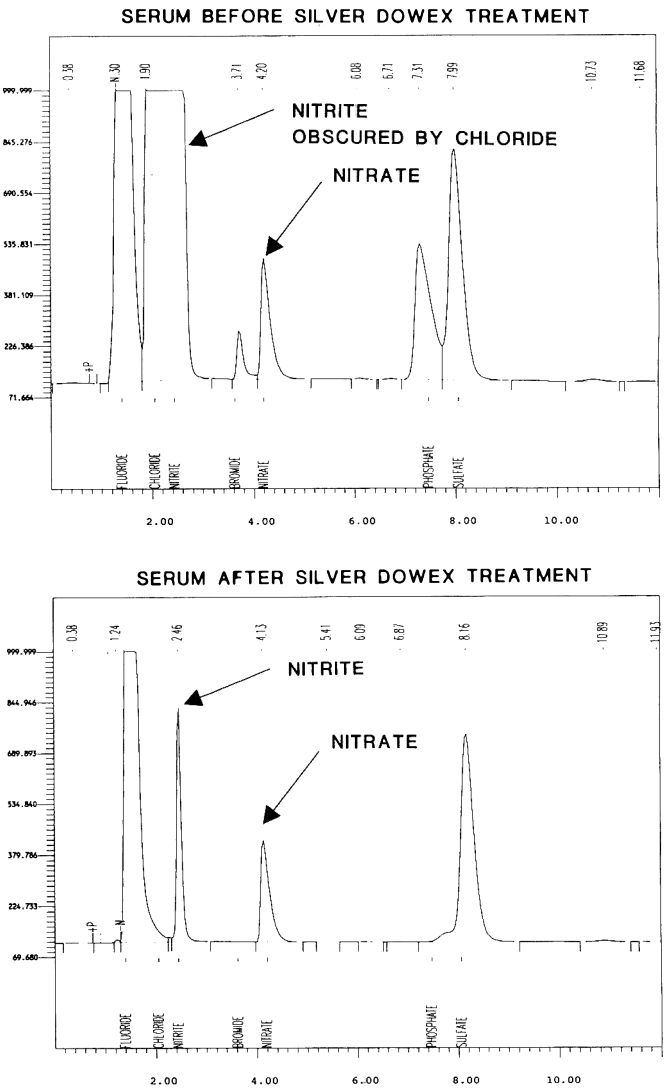

Figure 1. The major anionic components of bovine serum before and after treatment with silver form cation-exchange resin.

Calibration and Analysis

The analysis procedure was standardized for the recovery experiment by using aqueous solutions of 2.0, 6.0, 10.0, 20.0, and 30.0 mg/L each nitrite and nitrate. The calibration curve of integrated area vs concentration for nitrate ion was a second order polynomial with a forced y-intercept of zero. A simple linear calibration for nitrite ion using one standard at 10 mg/L and a forced intercept through zero was all that was required. More elaborate calibration equations did not yield any improvement in the goodness of fit for either ion. Typical calibration data are presented in Table 1. For routine work,

Table 2. Recovery of nitrate and nitrite from water

	Added, mg/L		Recovered,[a] mg/L		Mean deviation		Mean rec., %		RSD,[b] %	
Sample	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
W-1	10	10	9.1	9.5						
W-2	10	10	9.0	9.4	−0.8	−0.4	92.3	96.3	2.9	2.8
W-3	10	10	9.6	10.0						
W-4	30	30	28.3	29.3						
W-5	30	30	28.2	29.3	−2.0	−1.0	93.4	96.7	1.1	1.5
W-6	30	30	27.6	28.4						
W-7	50	50	48.6	50.2						
W-8	50	50	49.2	50.0	−0.9	0.1	98.1	100.1	0.8	0.3
W-9	50	50	49.4	50.0						
W-10	100	100	100.0	100.1						
W-11	100	100	100.6	100.3	0.2	0.0	100.2	100.0	0.3	0.4
W-12	100	100	99.9	99.5						
W-13	150	150	150.6	148.4						
W-14	150	150	151.7	149.2	2.0	−0.1	101.3	100.0	0.8	1.1
W-15	150	150	153.6	152.2						

[a] Recoveries corrected for blank nitrite (0.00 ± 0.00 mg/L) and nitrate (0.38 ± 0.07 mg/L).
[b] Includes uncertainty of blank measurement.

we have found that satisfactory calibration for nitrate can be obtained using a single 10 mg/L standard, but this restricts the dynamic range to below 10 mg/L.

Recovery Study

Triplicate 1.00 mL samples of pooled bovine serum, pooled bovine ocular fluid, and water were spiked with 10 μL spiking solutions to achieve final concentrations of 0, 10, 30, 50, 100, and 150 mg/L over endogenous levels. Endogenous levels of nitrate and nitrite in the ocular fluid and serum used in the study were determined by triplicate analyses and subtracted from the amounts determined after fortification.

Results and Discussion

Paired chromatograms of the major anionic components of nitrate- and nitrite-spiked bovine serum are shown in Figure 1. The lower chromatogram shows the chloride removal effects of the silver resin. Pretreatment with the silver form ion-exchange resin allows resolution of the formerly obscured nitrite peak. Besides nitrate and nitrite, it is apparent that ion-exchange chromatography can be used to analyze for bromide, sulfate, phosphate, and other low molecular weight ions in biological fluids. Bromide, sulfate, and phosphate determinations do not tolerate silver resin pretreatment or silver contamination of the analytical column. The peak preceding chloride in biological fluids contains acetate ion, a combination of other organic acids, and a trace of fluoride.

The silver chloride precipitation process causes a substantial reduction in the performance of the ion-exchange LC columns used in this study for anions with insoluble silver salts. This results from the small but not insignificant solubility of the silver salts. The dissolved and colloidal silver species in the injected sample bind to the analytical column. This precludes analysis of some common ions such as Cl^-, Br^-, PO_4^{3-}, and SO_4^{2-} on the contaminated column. Contaminated columns can be stripped of this silver contamination by using manufacturer-recommended metal stripping and regeneration washes.

Biological fluids contain many organic anions that can elute very slowly in an isocratic analysis. This can cause

Table 3. Recovery of nitrate and nitrite from bovine sera

	Added, mg/L		Recovered,[a] mg/L		Mean deviation		Mean rec., %		RSD,[b] %	
Sample	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
S-1	10	10	8.9	9.1						
S-2	10	10	9.2	9.8	−1.0	−0.4	90.4	95.4	1.7	4.4
S-3	10	10	9.0	9.8						
S-4	30	30	29.3	30.0						
S-5	30	30	29.7	30.9	−0.4	0.8	98.6	102.7	0.7	2.1
S-6	30	30	29.7	31.5						
S-7	50	50	51.4	51.2						
S-8	50	50	48.2	50.3	0.2	0.9	100.5	101.9	2.9	1.0
S-9	50	50	51.1	51.3						
S-10	100	100	101.1	100.4						
S-11	100	100	105.5	103.1	4.2	2.7	104.2	102.7	2.1	1.7
S-12	100	100	106.1	104.6						
S-13	150	150	157.3	150.7						
S-14	150	150	158.2	154.4	6.9	1.9	104.6	101.3	0.8	1.2
S-15	150	150	155.3	150.6						

[a] Recoveries corrected for endogenous nitrite (0.01 ± 0.02 mg/L) and nitrate (1.22 ± 0.30 mg/L).
[b] Includes uncertainty of endogenous measurements.

Table 4. Recovery of nitrate and nitrite from bovine ocular fluid

	Added, mg/L		Recovered,[a] mg/L		Mean deviation		Mean rec., %		RSD,[b] %	
Sample	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
O-1	10	10	8.8	9.9						
O-2	10	10	9.1	9.8	−1.1	−0.3	89.0	97.3	2.0	3.6
O-3	10	10	8.8	9.5						
O-4	30	30	27.0	29.2						
O-5	30	30	27.9	29.5	−2.4	−0.8	92.1	97.2	1.7	1.5
O-6	30	30	28.0	28.8						
O-7	50	50	46.9	51.0						
O-8	50	50	48.7	50.8	−2.2	0.1	95.5	100.1	1.6	2.4
O-9	50	50	47.7	48.4						
O-10	100	100	94.6	96.9						
O-11	100	100	98.3	102.4	−3.8	−0.6	96.2	99.4	1.6	2.4
O-12	100	100	95.7	98.8						
O-13	150	150	147.8	144.4						
O-14	150	150	150.7	146.9	1.0	−3.3	100.7	97.8	1.8	1.3
O-15	150	150	154.6	148.8						

[a] Recoveries corrected for endogenous nitrite (0.03 ± 0.00 mg/L) and nitrate (6.37 ± 0.32 mg/L).
[b] Includes uncertainty of endogenous measurements.

unstable baselines in later chromatograms. A gradient program with a 10-fold increase of eluant strength, after the analytes of interest are detected, elutes these ions and maintains flat a baseline for the next chromatogram. Gradient elution has proved essential for automated routine analysis.

Tables 2, 3, and 4 present the recovery trial results for water, serum, and ocular fluid, respectively. The pure water matrix experiment was designed to establish a point of comparison of the biological fluid analyses for the evaluation of nitrate or nitrate binding to proteins removed by ultrafiltration. Table 2 shows satisfactory recoveries of nitrite and nitrate from the fortified water samples. The average percent recoveries range from 92.3 to 101.3% for nitrite and from 96.3 to 100.1% for nitrate. The relative standard deviation (RSD) for nitrite recovery ranged between 0.3 and 2.9%; whereas the nitrate experiment maintained RSDs from 0.3 to 2.8% across the concentration range studied. To determine ultrafiltration membrane retention, an additional data set (not shown) was developed to compare recoveries of nitrite and nitrate from samples fortified before and after the ultrafiltration step. The results of this experiment do not suggest a statistical difference; hence, retention on the membrane is not indicated.

The results for the serum matrix, found in Table 3, also show satisfactory recoveries. The average recoveries for nitrite range from 90.3 to 104.6%, and the nitrate recoveries range from 98.2 to 103.6%. The serum nitrite and nitrate RSD values ranged from 0.8 to 2.9% and from 1.0 to 4.4%, respectively. In Table 4, the recoveries from fortified bovine ocular fluid display similarly satisfactory results. Nitrite average recoveries range from 88.9% at the 10 mg/L fortification level to 101.0% at the 150 mg/L level. The nitrate results are also encouraging. The values range from 97.1 to 100.1% for the average recovery across the fortification range examined. As well, the RSD values of the ocular fluid experiment demonstrated good precision with nitrite values between 1.6 and 2.0% and the nitrate values between 1.3 and 3.6%.

The accuracy and precision of the ultrafiltration and ion-exchange LC approach to analysis was studied using the AOAC recommended linear regression method of Linnig, Mandel, and Peterson (26), and Mandel and Linnig (27), later described by Wernimont (28). The notation used herein is taken from Wernimont. This method assumes that there are 2 forms of systematic error, constant bias and proportional bias, that are the intercept and slope of the regression of the quantity recovered vs the quantity known (or added). Ideally, the intercept (b_0) and slope (b_1) should be 0 and 1, respectively, but random errors make the values uncertain, and systematic biases change the best fit values. The confidence interval for the intercept and slope jointly is an ellipse with its centroid at the most probable value for the biases. The horizontal axis of the plot is the intercept of the recovery study linear regression data, and the vertical axis is the slope of that data. Hence if the ellipse contains the ideal point (0, 1), the method is free of bias within the precision of the method. Confidence hexagons have been shown to be a practical alternative to the ellipse generated by the statistical confidence interval between the slope and intercept of a recovery study (28). The utility of this method is that the recovery correction and confidence interval can be computed for any level of analyte. Since the magnitude and type of errors are evaluated by this procedure, analytical methods can be systematically evaluated for potential error sources.

Table 5 contains some statistical data from the recovery study. The repeatability measured by the pooled RSD is good, at 1.5–1.9%. Note that the pooled RSD does not include the variance due to the blank subtraction, since this would not be done in ordinary determinations. The standard error of the Y-estimate, $s_{x \cdot y}$, is an indicator of the overall precision of the recovery process, and it is used to calculate the confidence interval. The constant and proportional biases are also summarized in Table 5.

The joint 95% confidence hexagons for the recovery of nitrate from water, serum, and ocular fluid are plotted in Figure 2 according to the procedure of Mandel and Linnig. The ideal (0,1) point (intercept = 0 and slope = 1) is represented by a black dot at the center of the graph. The confidence regions for the water and ocular fluid contain the (0,1) ideal. The serum region, located above this ideal point represents a small positive proportional bias in that matrix. This can, in part, be ascribed to the molar volume of the dissolved protein and represents a maximum 3.6% positive

Table 5. Correction of nitrite recovery from bovine sera

Added, mg/L	Mean recd, mg/L	Corrected	Mean recovery corrected, %	Mean deviation corrected	C.I. 95%
[A]	[R]	[C]	100·[C]/[A]	[C]−[A]	
10	9.03	10.5	104.8	0.5	±2.1
30	29.6	29.9	99.5	−0.1	±2.0
50	50.3	49.4	98.7	−0.6	±1.9
100	104.2	100.3	100.3	0.3	±2.0
150	156.9	150.0	100.0	0.0	±2.3

[R] = mean of 3; endogenous nitrite (0.01 mg/L) subtracted.
[C] = $([R] - b_0)/b_1$, using $b_0 = -2.08$ and $b_1 = 1.06$.
[A] = Added above endogenous level.

relative deviation at a 95% confidence limit for the concentration range examined. Ocular fluid, as prepared, contains only small amount of dissolved protein. The experimental quantitation of nitrate demonstrates effective blanking, calculation of endogenous nitrate and instrument response factors as observed by proximity of the confidence hexagons to the zero intercept point.

The data for the nitrite ion in water, serum, and ocular fluid are graphed in Figure 3. Once again, water and ocular fluid areas are found in the same region of the graph. Comparison of the nitrate and nitrite results illustrate that the water matrix maintains a smaller confidence area than either of the biological fluids, hence a smaller range of deviation of experimental from actual concentration. The centroid of each of the confidence hexagons shows a negative constant bias and a positive relative bias. For the serum results, part of the positive relative bias is the effect of the molar volume of the dissolved protein. The negative constant bias, reflected in the negative intercept of the 95% confidence region of the 3 matrixes, is significant. This bias could result from reaction of the nitrite moiety during the sample preparation and analysis procedures. During the recovery study, standards were water solutions containing sodium nitrate and sodium nitrite at the required concentrations that were not subjected to the ultrafiltration and chloride removal steps of the sample preparation. The empirical values of slope and intercept from the recovery study should be used to correct analytical results when the best accuracy is needed.

The results of the confidence area analyses suggest that doing the sample preparation procedures to the calibration solutions is necessary. However, a recovery study and confidence hexagon analysis was performed for serum that was fortified after ultrafiltration and removal of chloride. Fortified reagent water that was neither ultra-filtered or treated for chloride removal was also analyzed. The negative intercepts of the recovery data, as described by the centroid of the 95% confidence hexagon, were decreased from about 2 mg/L to about 1 mg/L, indicating some loss during the sample preparation procedures. The sources of the small negative bias that remains is under investigation. As shown in Figure 1, the residual chloride peak and the nitrite peak are not resolved at the baseline. Elimination of integration error as a cause of the deviation requires study. A measurement error in the endogenous nitrite levels should not be involved because reagent water contains none.

Table 6 contains an example of the corrections to the recovered concentrations for the nitrite in bovine sera data set. The recovered concentration, [R], has been corrected using the equation: $[C] = ([R]-b_0)/b_1$, using the intercept value, $b_0 = -2.08$, and the slope value, $b_1 = 1.06$, obtained from the centroid of the 95% confidence hexagon for serum nitrite found in Figure 3. As shown in Table 4, the corrected percent recoveries and mean deviations are considerably improved following this approach. The confidence intervals in Table 4 were calculated with the precision statistics from the

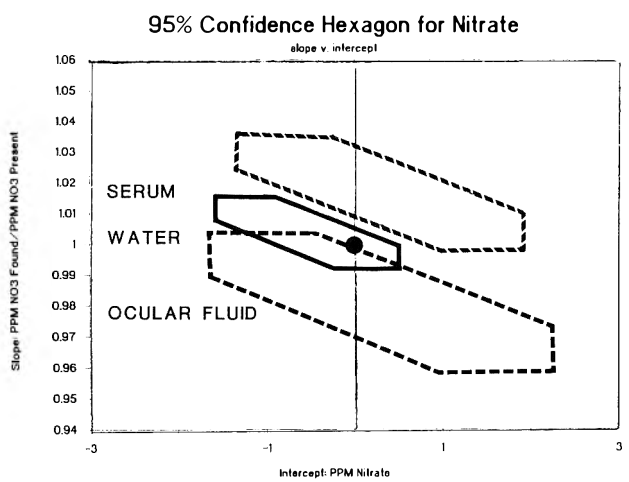

Figure 2. The 95% confidence hexagon plots for nitrate recovery from serum (top), water (middle), and ocular fluid (bottom). The ideal (0,1) point is shown by the large dot.

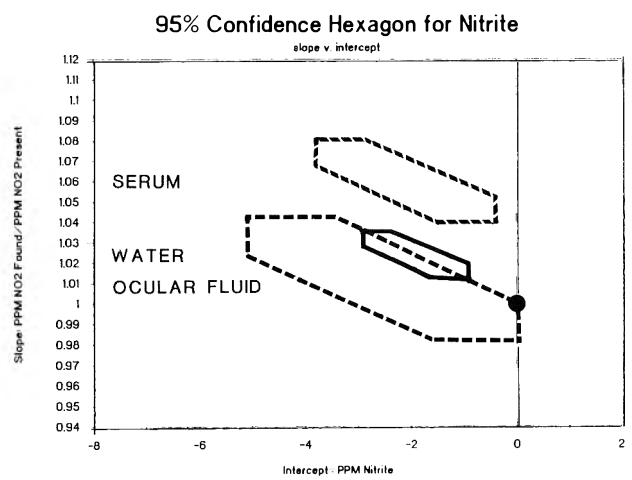

Figure 3. The 95% confidence hexagon plots for nitrite recovery from serum (top), water (middle), and ocular fluid (bottom). The ideal (0,1) point is shown by the large dot.

Table 6. Precision and bias statistics for recovery of nitrite and nitrate

Sample	RSD,[a] %	b_0	b_1	$s_{x\cdot y}$
Nitrite:				
Serum	1.8	−2.08	1.06	1.48
Water	1.5	−1.89	1.02	0.90
Ocular fluid	1.7	−2.51	1.01	2.25
Nitrate:				
Serum	1.9	0.02	1.02	1.43
Water	1.5	−0.54	1.00	0.91
Ocular fluid	1.8	0.30	1.00	1.70

[a] Pooled repeatability %RSD, not including variance of endogenous measurements.

recovery regression line thus (29):

$$\text{C.I.} = t_{95,13}(s_{x\cdot y}/b_1)(1/n' + 1/n + ([C]-\bar{X})^2/\sum xx)^{1/2}$$

where $n' = 3$ is the number of measurements of a value; $n = 15$ is the number of measurements in the recovery study; $\bar{X} = 68$ is the mean of the spike levels; and

$$\sum xx = (\sum X^2 - (\sum X)^2/n).$$

Conclusions

Generally good recoveries and precision are found for the determination of nitrate and nitrite in biological fluids by a method of ultrafiltration, silver resin dechloridation, and LC ion-exchange chromatography. Accurate determination of very low levels of nitrite will require elimination of, or correction for, a low level negative constant bias. A negative constant bias is not totally unexpected in consideration of the reactivity of nitrite. The method of confidence hexagons, as applied to this study, has shown the ability quantitatively to assess this small constant bias in the analysis of the nitrite ion.

The method has demonstrated the statistical control, minimal cost, and rapidity required for clinical diagnostics in veterinary and human medicine. This is of great value in consideration of the toxicological impact of increasing levels of ground water nitrate and airborne NO_x compounds (29, 30). The method has proven valuable in the rapid diagnosis of acute nitrate toxicosis in livestock and has been in routine use in this laboratory for 3 years. In addition, the method has allowed us to examine the role of nitrate and nitrite in bovine abortion.

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VITAMINS AND OTHER NUTRIENTS

Liquid Chromatography and Fluorescence Detection of Vitamin A in Animal Feeds, Finished Feeds, and Premixes

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A simple liquid chromatographic method for vitamin A (retinol) in animal feeds is described. The feed is saponified, diluted to minimize interferences, and extracted into petroleum ether with a single step. The analysis is sensitive and specific with liquid chromatography and a fluorescence detector. The minimum level of detection is 15 ng/mL, which is equivalent to 10 000 units/lb vitamin A. The method includes a stable and reproducible standardization of vitamin A that is used to calibrate standard peak heights in terms of units retinol/mL. Guarantees of 10 000 units/lb up to premix levels can be analyzed with good recoveries and precision.

Regulation of vitamin A in animal feeds by chemical analysis has been limited because of the lengthy and complex official methods available (1). With sophisticated liquid chromatographic (LC) equipment and fluorescence detectors, this task could become routine in well equipped laboratories. But a review of the literature showed the lack of a simple LC method for vitamin A in the difficult matrixes found in animal feeds (2–5).

The objective of the present study was to develop an LC method that would be simple, yet accurate and precise. This involved a stable and reproducible standardization of retinol; a single quantitative extraction of vitamin A from the alkaline digest; elimination of laborious cleanup steps; and an LC system that is sensitive, specific, and linear for vitamin A so that both low and high level guarantees (from 10 000 units/lb up to premix levels) can be analyzed.

Note: The term vitamin A is total vitamin A alcohol or retinol; 1 unit (IU) equals 0.300 μg retinol. The terms vitamin A and retinol are used synonymously in this manuscript. Concentrations of vitamin A are expressed in terms of units/mL or units/lb because most U.S. states legally require guarantees of vitamin A in animal feeds expressed as units (IU)/lb. Units/mL can be converted to μg/mL by multiplying by 0.300.

METHOD

(*Note:* Solutions of vitamin A are light-sensitive. Protect standards, feeds, and supplement extracts from direct sunlight or artificial light.)

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Model 510 LC pump equipped with Waters 740 data module (Waters Associates, Inc., Milford, MA 01757) and Kratos Spectroflow 980 fluorescence detector (Kratos Division, ABI Analytical, Inc., Ramsey, NJ 07446); automatic or manual loop injector. (Fixed loop injectors should be overfilled at 4–5 times loop volume.) General operating conditions: 100 μL injection; excitation wavelength 330 nm; emission wavelength 470 nm; 1.5 mL/min flow rate.

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(b) *Chromatographic column.*—10 cm × 2 mm id stainless steel guard column containing Corasil followed by Waters μPorasil, 3.9 mm × 30 cm, stainless steel column (Waters Associates, Inc.).

(c) *Mobile phase.*—Hexane–chloroform, LC grades (60 + 40). Filter through FH, 0.5 μm filter (Millipore Corp., Bedford, MA 01730) (6).

(d) *Alcohol.*—95%; methanol or ethanol.

(e) *Potassium hydroxide solution.*—Dissolve 70 g KOH in 70 mL water, mix, and cool. Prepare fresh each time.

(f) *L-Ascorbic acid.*

(g) *Petroleum ether.*—ACS grade.

(h) *2-Propanol.*—LC grade.

(i) *Vitamin A standard.*—All-trans-retinol, synthetic, crystalline, 85%, sealed ampule, or 70%, whichever is obtainable (Sigma Chemical Co.)

Preparation and Standardization of Vitamin A Standard

Transfer contents of 100 mg retinol ampule to 50 mL *fresh* cottonseed oil contained in 100 mL glass-stopper Erlenmeyer flash of actinic glass. Shake and let stand in refrigerator ca 2 weeks to ensure complete solution of retinol in oil. Retinol is stable under these conditions.

Weigh ca 0.5 ± 0.1 g into tared 5 mL beaker. Transfer to 100 mL volumetric flask with petroleum ether and dilute to volume with the same solvent.

Place 5.0 mL aliquot into 50 mL volumetric flask and dilute to volume with 2-propanol. Measure absorbance (*A*) of solution at 325 nm using 2-propanol as reference. Calculate concentration in units/mL by multiplying absorbance by 18.3, a factor derived from the extinction coefficient for retinol.

Place 5.0 mL aliquot into 50 mL volumetric flask and dilute to volume with petroleum ether. Immediately inject 100 μL into LC column and measure peak height as described under *Apparatus*. Convert peak height to 1 unit retinol by dividing peak height by units/mL from above data. This value will be referred to as "F," peak ht/unit, and will be used for calculating units/mL of sample concentrations. Carry out this standardization each day that samples are analyzed.

Preparation of Sample Extract

Grind coarse or pelleted feeds to pass 20 mesh sieve. Weigh 10.0 g sample (2.0 g sample for premixes) into 500 mL glass-stopper Erlenmeyer flask. Add ca 0.5 g ascorbic acid, and disperse sample with 10–15 mL water, taking care that all feed aggregates are absent and ascorbic acid is in solution. Add 1 g *fresh* cottonseed oil, 10 mL KOH solution, 50 mL alcohol, and magnetic stir bar. Attach water condenser to flask, place on magnetic stirrer hot plate, and reflux 30 min with stirring at ca 2 drops/s. Cool rapidly and transfer to 200 mL volumetric flask; dilute to volume with alcohol–water solution (3 + 1). Mix thoroughly and let particles settle.

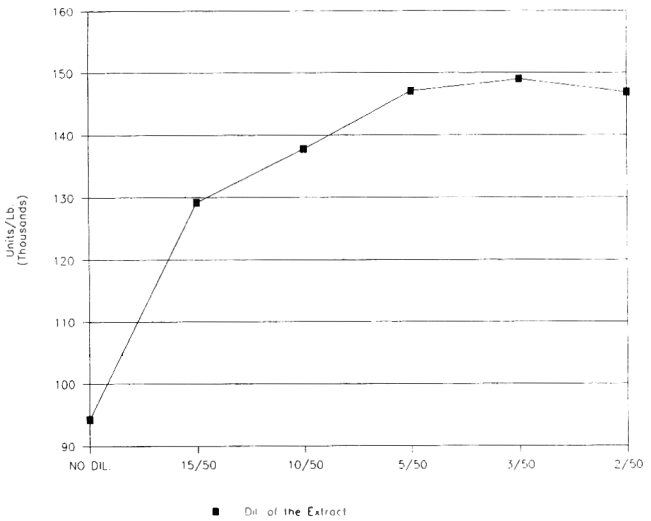

Figure 1. Recovery of vitamin A vs dilution factor.

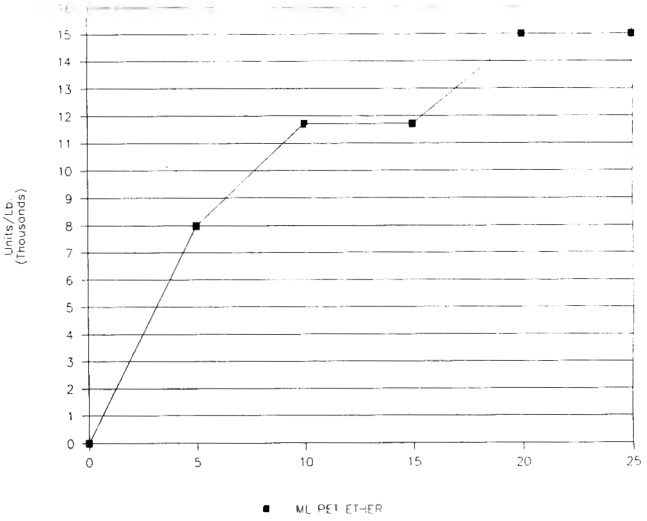

Figure 2. Recovery of vitamin A vs volume of extractant.

(Vitamin A is stable in this alkaline digest if protected from light, and further steps can be carried out within the same week.)

Transfer 10.0 mL (5.0 mL for premixes) supernatant liquid to 100 mL volumetric flask and dilute to volume with alcohol–water solution (3 + 1). (This step—a 10-fold dilution—is necessary to obtain complete extraction of vitamin A into petroleum ether.) Pipet 10.0 mL of this dilution into 50 mL centrifuge tube, add 20.0 mL petroleum ether, stopper tightly, place on a tilt rocker platform, and extract 1 h. Centrifuge and inject 100 μL supernatant liquid into liquid chromatograph using conditions described under *Apparatus* (**a**). Use peak height for quantitation. Calculate concentration of vitamin A as follows:

$$\text{Units/lb} = \frac{\text{Pk ht}}{\text{F}} \times 20 \times \frac{\text{dilution}}{\text{wt sample}} \times 454$$

Discussion and Results

Information provided by Thompson (7) regarding problems of vitamin A analysis contributed to the design of this method: solid retinol standards are unstable and deteriorate rapidly once the container is opened; retinol is stable in cottonseed oil; concentrations of retinol standard solutions should be determined by the UV absorbance at 325 nm; dilute solutions of retinol are unstable; evaporations of retinol solutions and redissolving the residue cannot be done without losses of vitamin A; and diluted standards are less stable than extracts of samples where lipids and antioxidants provide protection.

Thompson (7) further emphasizes that alkaline hydrolysis is necessary for complete extractions of vitamin A. It releases the vitamin from protective coatings and converts esters to retinol; fats are converted to soaps that can be separated from vitamin A by extraction with organic solvents, and many other interferences are broken down and eliminated.

Vitamin A is stable for at least 1 week in this alkaline digest containing an antioxidant such as pyrogallol. The efficiency of the extraction with hexane and thus the minimum number of extractions needed is affected by the concentration of fatty acids in the digest, which must be controlled (7).

Our laboratory confirmed these problems, and the stability of retinol in cottonseed oil was used to prepare the vitamin A stock solution. The standardization procedure is based on the fact that all-trans-vitamin A alcohol or retinol has an absorption maximum at 325 nm and the extinction at this wavelength is directly proportional to the vitamin A concentration. Therefore, μg/mL of vitamin A can be converted to units/mL by multiplying by 18.3 if measurements are made in isopropanol using 1 cm quartz spectrophotometric cells (8).

Many workers (8) correct for irrelevant absorption by applying the Morton-Stubbs correction, that is, making 3 measurements, one at the maximum (325) and 2 at 6/7 of the maximum extinction (310 and 334 nm). We did not find that application of the Morton-Stubbs correction was justified, because errors are magnified at less than maximum absorption, interfering substances are absent in high quality retinol standards stabilized in cottonseed oil, and routine analyses of animal feeds for vitamin A favor simplicity.

Measurements by LC and UV absorption should be made immediately because dilute stock solutions are unstable. For example, when the LC standard was interspersed with samples, losses of standard potency ranged from 8 to 10% within a 1 h period and use of BHT did not improve the stability. Samples, however, did not appear to deteriorate within this time period, confirming Thompson's statement that this is due to the protection from the lipids and antioxidants present.

The efficiency of the vitamin A extraction from the saponified alkaline digest with petroleum ether is not affected by the amount of vitamin A present in the digest and is not affected significantly by multiple extractions, but is affected by the concentration of fatty acids present (7). This was easily controlled by dilution. The minimum dilution required for maximum recoveries was 10-fold (see Figure 1).

The ratio of extractant to diluted digest was another critical factor in obtaining maximum recoveries. A 2:1 ratio provided the most complete recovery (see Figure 2).

We attempted to increase the sensitivity of the method so that levels lower than 10 000 units/lb could be quantitated. This was done by pipetting 10.0 mL of the 20.0 extractant, evaporating under nitrogen just to dryness, immediately dissolving in 1.0 mL petroleum ether, and injecting into the LC column for vitamin A measurement. Losses ranged from 25 to 50%, confirming Thompson's observation that evaporation steps result in losses of vitamin A.

Table 1. Vitamin A analysis on 5 consecutive days from a single saponified extract per sample, showing stability of extract and precision

Guarantee, units/lb	Mean, units/lb	SD	CV, %
30 000	29 326	2678	9.13
60 000	59 700	2967	4.97
100 000	118 850	6326	5.32
2 000 000	2 155 562	82527	3.83
No guar.	7944	1532	19.28

Table 2. Data regression for peak height

Concn units/mL	Peak ht	F (peak ht/unit)
0.066	977	14803
0.132	1975	14962
0.197	2913	14787
0.263	3852	14646
0.329	4723	14356
0.987	13877	14060
1.645	22815	13869
Constant		168.1859
Std err or Y est.		70.92601
R squared		0.999935
No. of observations		7
Degrees of freedom		5
X coefficient		13803.45
Std err of coefficient		49.5711

The USP Reference Standard for Vitamin A (Vitamin A Acetate in Cottonseed Oil, contained in gelatin capsules) was carried through the proposed procedure: saponification, dilution, extraction, and LC measurement. Recoveries averaged 94.3% with a standard deviation of 2.07.

Table 1 shows stability of the saponified extract over a 5 day period and the precision of the method. The first 3 samples were formulated on the basis of initial results of the last 2 samples to contain about 30 000, 60 000, and 100 000 units/lb by spiking the feed with "no guarantee" with the premix guaranteed to contain 2 000 000 units/lb. Based on actual calculated formulations, recoveries averaged 102.7%.

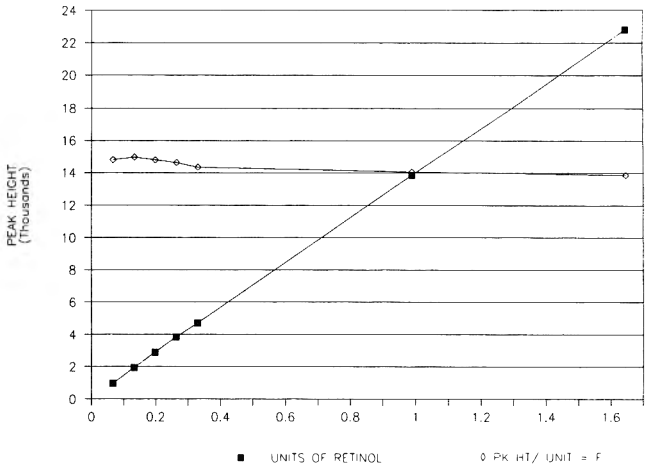

Figure 3. Units of vitamin A vs peak height for fluorescence detector.

By standardizing the method to specific sample weights (10 g for feeds and 2 g for premixes), volume of alkaline digest (200 mL), a minimum 10-fold dilution, and 2:1 ratio of extractant to extracted, the method cannot be modified to increase the sensitivity of the measured vitamin A in low level sample guarantees, 10 000 units/lb. To compensate for this lack of flexibility, the choice of the LC detector used for the final measurement becomes an important part of a successful method.

Reverse-phase systems with UV detection are neither as sensitive nor as specific as a normal-phase system with fluorescence detection (3). A simple 10-fold dilution step in the proposed method provides a suitable matrix solution so that a single extraction of the saponified digest can remove quantitatively the vitamin A. This dilution results in the concentra-

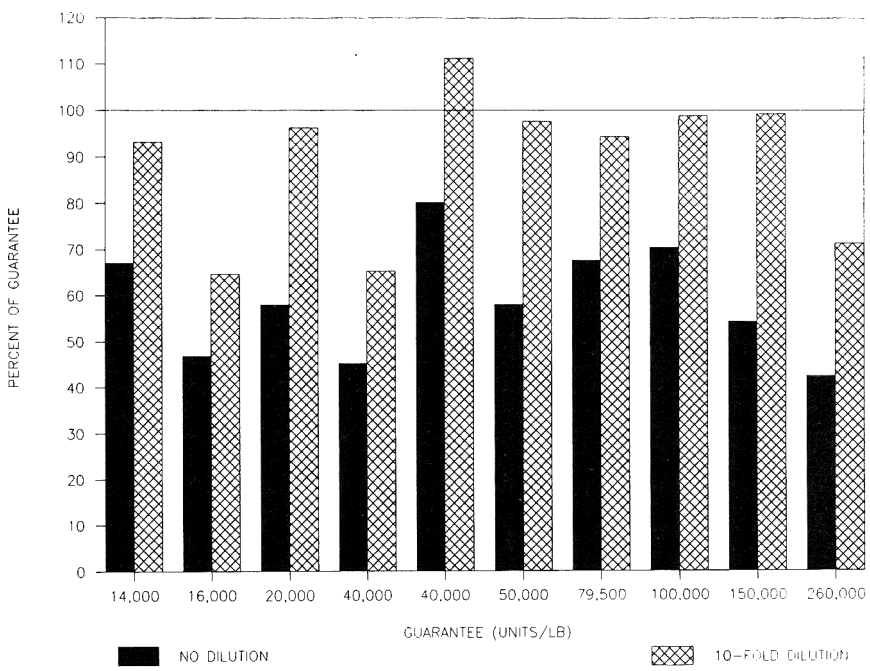

Figure 4. Guarantee vs percent of guarantee for determination of vitamin A in market samples.

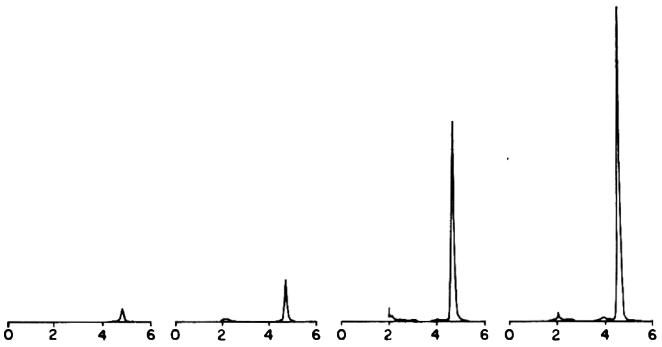

Figure 5. Chromatograms of 100 μL injections of samples with 10 900, 30 900, 153 000, and 240 000 units/lb of total vitamin A (retinol). Retention time 4.80 min.

tion of vitamin A in the ng/mL range; therefore, the detector must be extremely sensitive. Fluorescence provides this sensitivity as well as specificity which eliminates cleanup procedures and simplifies the chromatograms. Fluorescence response to vitamin A is linear over the entire range of guaranteed levels of vitamin A in finished feeds and premixes.

Comparisons showed peak heights to be superior to peak areas by regression analyses (Table 2). The constant and standard error was consistently lower and the use of a single factor (F) for calculation was shown to be linear over the entire concentration range. F is peak height/unit of vitamin A and is calculated by dividing peak height by units/mL standard. With this validation, it then becomes necessary to use only one LC standard per run as described in the method. Table 2 shows regression analysis of peak heights with graphic representation in Figure 3.

Figure 4 shows an average 34% increase in recoveries of vitamin A on market samples by the 10-fold dilution of the saponified extract prior to extraction.

Figure 5 shows chromatograms of samples containing 10 900; 30 900; 153 000; and 240 000 or 2 400 000 units/lb of total vitamin A (retinol). Retention time is 4.80 min.

Conclusion

In conclusion, the objectives of this study were met. The method is standardized to specific sample weights (10 g for feeds and 2 g for premixes), volume of alkaline digest (200 mL), a minimum 10-fold dilution, and a 2:1 ratio of extractant to extracted. The method describes a stable and reproducible standardization of retinol, a quantitative extraction of retinol from the saponified extract in a single step, elimination of laborious cleanup steps, and an LC system with sensitivity, specificity, and linearity that allows integration of peak heights as low as 15 mg/mL, equivalent to 10 000 units/lb. The method is applicable to guarantees from 10 000 units/lb up to levels in premixes, with good recoveries and precision.

Acknowledgment

Appreciation is extended to D. J. Mayers for his computer expertise on figures and data regression analyses.

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Liquid Chromatographic Determination of Total Niacin in Beef, Semolina, and Cottage Cheese

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A sensitive and precise liquid chromatographic method has been developed for the determination of total niacin in beef, semolina, and cottage cheese. The samples are extracted with $Ca(OH)_2$, cleaned up using C18 Sep-Pak cartridges, and chromatographed on a C18 column using a mobile phase consisting of 23% acetonitrile, 0.10% H_3PO_4, and 0.10% sodium dodecyl sulfate in water. Niacin is detected using an absorbance detector operating at 254 nm. This chromatographic system produces an unusually sharp peak for niacin, enabling a detection limit of approximately 0.05 mg/100 g. The mean recovery was 99.5% and the coefficients of variation for beef, semolina, and cottage cheese were 3.58, 1.02, and 10.0%, respectively.

The standard methods for the determination of niacin in foods are microbiological (1) and colorimetric (2). Both methods suffer severe drawbacks. The microbiological procedure requires maintaining a culture of viable microorganisms, and the assay requires an incubation period of 3 days. The colorimetric procedure requires the use of CNBr. To reduce the dangers of externally generated CNBr, the method has been modified by substituting CNCl for CNBr and by replacing the manual operations by a semiautomated flow system (3, 4).

Although several workers have been able to determine the niacin content of fortified foods (5–7) with reasonable precision, determination of niacin in unfortified foods is considerably more difficult. Skurray (8) used acidic and enzymatic digestion followed by treatment with NaOH and potassium ferricyanide in the sample preparation. The application of this method to meat samples (9) yielded a tiny niacin peak riding on the tail of a large interfering peak. Other workers have also experienced difficulties with interfering peaks (10–12). Van Niekirk et al. (13) developed a method similar to the column switching technique used by Snyder and Kirkland (14) and concluded that the niacin peak from a food sample extract could not be adequately separated from interferences on a single column.

In the method presented here, the niacin peak has been adequately resolved in 3 major food groups by using a single column and an isocratic mobile phase.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates Model 6000A pump, or equivalent, operating at 1.5 mL/min; Waters Associates WISP autosampler, or equivalent; Supelco Cat. No. 5-8348, LC-18-DB, 15 cm × 4.6 mm, 5 μm C18 analytical column, incorporating Waters Associates C18 Guard-Pak guard column; Waters Associates Model 441 fixed wavelength detector, or equivalent, operating at 254 nm and 0.02 AUFS and Waters Associates Model 730 data module, or equivalent.

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(b) *Cleanup cartridges.*—Sep-Pak Plus C18 cartridges (Waters Associates Part No. 11191).

(c) *Chemicals and reagents.*—Organic-free water prepared by passing reverse osmosis water through Milli-Q™ water purification system (Millipore Co., Bedford, MA 01730). LC quality acetonitrile, reagent grade *o*-phosphoric acid 85%, ethanol, calcium hydroxide, LC quality sodium dodecyl sulfate, and oxalic acid (Fisher Scientific Co., Medford, MA 02155) and niacin (Sigma Chemical Co., St. Louis, MO 63178).

(d) *Mobile phase.*—Dilute 1.00 mL phosphoric acid in 600 mL water and filter through 0.45 μm filter. Dissolve 1.00 g sodium dodecyl sulfate in solution composed of 120 mL water and 230 mL acetonitrile. Bring total volume to 400 mL with water, filter through 0.45 μm filter, and add to 600 mL acidified water. Mix well.

(e) *Stock standard solutions.*—Accurately weigh 200 mg niacin into 100 mL volumetric flask. Dissolve in ethanol–water (1 + 1), dilute to volume with ethanol–water (1 + 1), and mix well. This solution is stable at least 2 weeks at room temperature.

Preparation of Standard

Dilute niacin stock solution 10:100 × 10:100 × 10:100 with water to obtain niacin concentration of 0.200 mg/100 mL water. Pipet 20 mL final dilution into 500 mL erlenmeyer flask containing exactly 180 mL water, add ca 10 g $Ca(OH)_2$, and carry standard through *Sample Preparation I*, beginning with autoclave step.

Sample Preparation I

Calculate sample weight estimated to contain 0.04 mg niacin, using formula, SW = 4/C, where SW = sample weight in g and C = niacin claim in mg/100 g. Accurately weigh this quantity of sample into 250 mL blender jar. Estimate volume of water, W, in sample weight, add (200 − W) mL water to jar, and add 10 g $Ca(OH)_2$. Blend ca 30 s at high speed and transfer to 500 mL erlenmeyer flask.

Autoclave 15 min at 121°C. Cool in ice bath at least 30 min, and then filter cold solution through Whatman 2V fluted paper. Transfer ca 100 mL filtrate to 250 mL erlenmeyer flask containing 300–320 mg oxalic acid. Mix well and adjust final pH to 6.5–7.0 by dropwise addition of filtrate or by addition of a few crystals of oxalic acid. Filter slowly through 1 or 2 pieces of Whatman No. 42 paper so that clear filtrate is obtained. If desired, these filtrates may be stored overnight at refrigerator temperature before proceeding to next step.

Condition cleanup cartridge with ca 10 mL ethanol followed by ca 10 mL water. Slowly pass 10 mL clear sample filtrate through cartridge. Discard first 6 mL and collect next 3.5 mL in small sample vial. Add 1 drop of 85% H_3PO_4 and mix well.

Sample Preparation II

For many food samples, the calculated sample weight, 4/C, gives a value too small to yield a representative sample. In

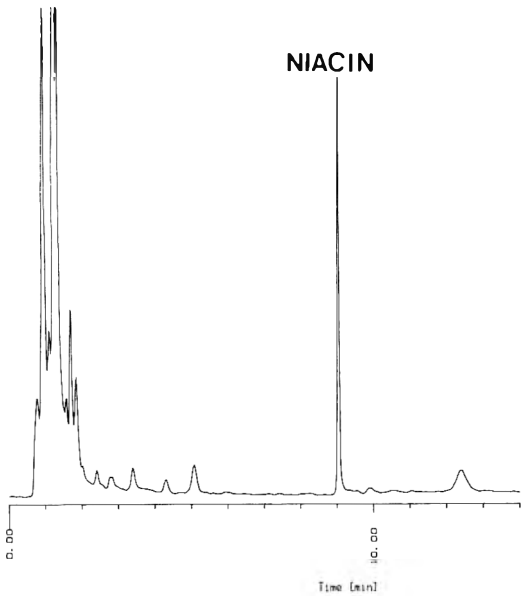

Figure 1. LC chromatogram of semolina sample.

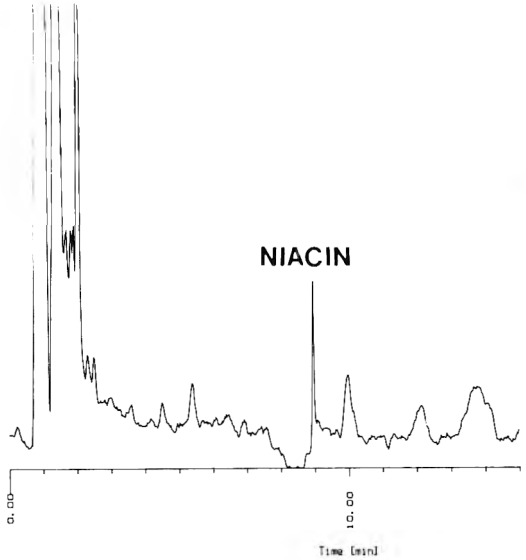

Figure 2. LC chromatogram of cottage cheese sample.

these cases, use sample weight, SW = 4F/C, where F = factor selected by analyst to give a reasonable sample weight. Carry SW through *Sample Preparation I*, but dilute sample solution with water by the factor F immediately before cartridge cleanup step.

Procedure

Inject 200 μL each of standard and sample solutions. Duplicate injections should differ by less than 2%. Calculate results, using following formula:

$$\text{Niacin, (mg/100 g)} = (4F/SW) \times (A/A')$$

where F = 1 for Sample Preparation I; F = dilution factor for Sample Preparation II; SW = sample weight in g; A and A′ = area of niacin peak in sample and standard, respectively

Results and Discussion

The key to this method is the unusually sharp peak obtained from the chromatographic system. The injection of niacin in a large volume (200 μL) of dilute phosphoric acid results in a plate count approximately 20 times greater than what would be expected for the column being used. This "peak-sharpening" effect increases with increasing injection volume and with increasing concentration of acid in injection solution. The effect is independent of the niacin concentration. A graph of peak response vs concentration in the usual working range of 0.004–0.040 mg niacin/100 mL yields a linear response for both peak area and peak height. The effect is shown by all columns that we have tested including the Waters 30 cm and 15 cm μBondapak columns and the Chemcopak Chemcobond 30 cm C18 column. The Supelco column was chosen for its high plate count combined with a low back-pressure.

The cleanup procedure following hydrolysis with calcium hydroxide uses oxalate to precipitate excess calcium and bring the solution pH to about 7. At this pH, niacin is not retained on the C18 column but a large number of colored compounds are retained. Cleanup following basic hydrolysis is more effective at pH 7 than at acidic pH. The chromatographic scans following this cleanup contain few interfering peaks because the system requires that the molecule be unretained from an aqueous mobile phase at pH 7 and strongly retained from 23% acetonitrile at acidic pH in the presence of an ion-pairing agent. Niacin, with its ability to assume a positive or a negative charge depending on pH, is one of the few molecules that fits into this category.

As seen from Figures 1–3, the cleanup is effective in removing interfering peaks. The semolina sample had to be diluted by a factor of 5 before the cartridge cleanup and showed niacin as the only significant peak beyond 3 min. The meat sample and the cottage cheese sample were cleaned up without dilution. The scan of the meat sample was clean but the cottage cheese sample indicated a large peak at about 6 min, which returned to baseline before the niacin eluted. In the case of samples of very low niacin content such as cottage cheese, it may be desirable to use less than the calculated sample weight to avoid overloading the cartridge. Overloading will not result in a loss of niacin but will result in leakage of potential interferences into the sample solution.

Certain batches of mobile phase show a slight drop in the baseline immediately before the niacin peak in both the standard and the sample. We suspect that this drop is due to unknown impurities in the acetonitrile or in the ion-pairing agent.

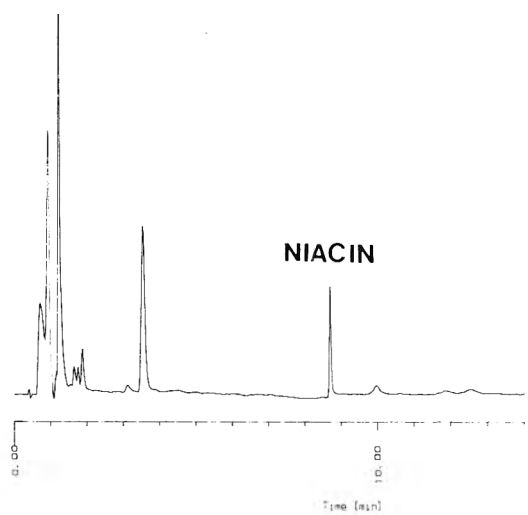

Figure 3. LC chromatogram of beef sample.

Table 1. Niacin assay values (mg/100 g)

	Beef	Semolina	Cottage cheese
	LC method		
	3.35	6.46	0.27
	3.29	6.62	0.31
	3.41	6.55	0.31
	3.32	6.62	0.32
	3.20	6.56	0.27
	3.54	6.64	0.25
Av.	3.35	6.58	0.29
SD	0.12	0.067	0.029
CV, %	3.58	1.02	10.0
Added, mg	0.0400	0.2000	0.0400
Recd, mg	0.0400	0.1998	0.0394
Recd, %	100.0	99.9	98.5
	Microbiological method		
	4.24	6.29	0.31
	3.89	5.93	0.24
Av.	4.07	6.11	0.28

No rapid deterioration of the LC columns was found using this detergent-based mobile phase. The buffer system yields a pH of about 2.8; at this pH, bonded silica columns are reasonably stable. After large sample runs, the column was washed with 60% methanol in water, the Guard-Pak was changed, and the column was washed with 100% methanol and allowed to stand in methanol. At start-up, the column was washed with acetonitrile and equilibrated with mobile phase.

The same samples that were assayed by the LC method were assayed by the microbiological method (1). As shown in Table 1, the 2 methods are in good agreement.

It should be noted that certain food samples (such as instant coffee) give results higher than those obtained by the microbiological method. Evidently, some interference is being carried along with the niacin peak to account for the high results. Thus, the method is not a general method for foods and can be used only with those food samples that have been tested against the microbiological method.

Acknowledgment

The authors thank Charles Williams for assaying these samples by the microbiological method.

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Rapid Analysis of Nutritionally Important Free Amino Acids in Serum and Organs (Liver, Brain, and Heart) by Liquid Chromatography of Precolumn Phenylisothiocyanate Derivatives

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An amino acid analysis method for protein hydrolysates, using precolumn phenylisothiocyanate (PITC) derivatization and liquid chromatography, was modified for its application in rapid analysis of commonly occurring free amino acids in serum and other physiological samples. The modifications included changes in column temperature (47.5°C compared to 25–35°C used in analyzing protein hydrolysates), method of preparing standard and test samples, and gradient conditions. By using a Waters Pico-Tag amino acid analysis 15 cm long column (which is also used for analyzing protein hydrolysates), separation of 27 PTC-amino acids in human serum and rat liver, brain, or heart was completed in 20 min by the modified method. The total time for analysis and equilibration was 30 min. The modified method was much faster than the traditional ion-exchange methods (2–3 h) or the existing liquid chromatographic methods using PITC derivatization (66–80 min) for determining nutritionally important free amino acids in physiological fluids and tissues. Variability of the method (expressed as coefficients of variation) for the determination (including deproteinization, derivatization, and liquid chromatography) of all amino acids was less than 5%, which compared favorably with the reproducibility of ion-exchange methods.

Sarwar et al. (1) recently modified the precolumn phenylisothiocyanate (PITC) derivatization liquid chromatography method (2–5) for accurate and rapid determination (12 min) of methionine (as methionine sulfone) and cystine/cysteine (as cysteic acid) and all other amino acids, except tryptophan, in hydrolysates of foods and feces. A simple liquid chromatographic method (requiring no derivatization) for the analysis of tryptophan in alkaline hydrolysates of foods and feces was also developed by Sarwar et al. (1).

The development of this rapid, accurate, and reproducible method for the determination of amino acids in protein hydrolysates provided the impetus for its application to physiological samples such as serum and liver, heart, and brain. Amino acid analysis of serum and organs is of ongoing interest in our laboratory and other centers conducting nutritional research.

In determining amino acids in protein hydrolysates by liquid chromatography of precolumn PITC derivatives, a Waters Pico-Tag amino acid analysis column (15 cm) is normally used. Limited studies reported in the literature (6–7), however, revealed that a longer (25–30 cm) and more expensive column is required for better resolution of all amino acids in plasma and other physiological fluids. Lavi et al. (6) demonstrated the separation of 23 PTC-amino acids from physiological samples in 80 min by using a 25 cm long column, while Cohen and Strydom (7) were able to separate 40 PTC-amino compounds in about 66 min by using a 30 cm long column. Since several of the amino compounds separated by Cohen and Strydom (7) have limited nutritional significance, there is a need to develop a rapid method for determining commonly occurring free amino acids in physiological fluids and tissues.

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Early and Ball (8) attempted to separate plasma amino acids using the faster-eluting Waters Pico-Tag amino acid analysis column for protein hydrolysates. They were able to achieve good separation of 17 PTC-amino acids, but failed to obtain adequate separation of some of the nutritionally important amino acids such as tryptophan (an essential amino acid), glutamic acid, glycine, glutamine, serine, and ornithine (8). In this preliminary report, Early and Ball (8) did not provide information about variability of the faster method, and did not study its application to determining free amino acids in biological tissues such as liver, brain, and heart.

The purpose of the present investigation was to modify the method of Sarwar et al. (1), used in amino acid analysis of protein hydrolysates, to permit its application in rapid analysis of free amino acids in biological samples. This was achieved by using the same column (Waters Pico-Tag amino acid analysis column, 15 cm) but by changing column temperature, altering method of drying standard and test samples, and using different gradient conditions than those used by Sarwar et al. (1). The PITC derivatization method reported in this investigation could be used for rapid (20 min) analysis of 27 nutritionally important amino acids in serum, liver, brain, and heart samples.

Experimental

Reagents and Apparatus

(a) *Reagents.*—The chemicals and solvents used were of analytical and chromatographic grade, respectively. Acetonitrile, methanol, glacial acetic acid, and phosphoric acid were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ 08865). Sodium acetate (anhydrous) was obtained from BDH Ltd (Poole, Dorset, UK) and disodium hydrogen phosphate was purchased from Mallinckrodt, Inc., Science Products Division (St. Louis, MO 63134). Triethylamine (TEA-99%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201) while phenylisothiocyanate (PITC) and amino acid standard H containing 17 amino acids and ammonia (representative of a protein hydrolysate) were obtained from Pierce Chemical Co. (Rockford, IL 61105). One mL of the amino acid standard H contained 1.25 μmoles of cysteine (Cys) and 2.5 μmoles of arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), threonine (Thr), valine (Val), alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), proline (Pro), serine (Ser), and ammonia.

Another amino acid standard (representative of a physiological sample) containing 27 amino acids and ammonia was prepared by adding 2.5 μmoles of α-aminobutyric acid (a-ABA), asparagine (Asn), citrulline (Cit), glutamine (Gln), hydroxyproline (OHPro), 1-methylhistidine (1-CH_3His), 3-

methylhistidine (3-CH_3His), ornithine (Orn), taurine (Tau), and tryptophan (Trp) to 1 mL of the commercial amino acid standard H. The individual amino acids were purchased from Sigma Chemical Co. (St. Louis, MO 63178-9916). Amino acid solutions were prepared in 0.1N HCl and were stored at −70°C until analyzed. The final concentration of amino acids in the standard used for derivatization was 150 nmoles/mL.

(b) *Apparatus.*—As described previously (1), high purity water generated by a Milli-Q-Water System from Millipore Corp. (Bedford, MA 01730) was used in the preparation of buffers and solutions. The PITC derivatization was carried out in 10 × 75 mm culture tubes from American Hospital Supply Canada, Inc. (Mississauga, Ontario). Samples were centrifuged by a Model IEC CENTRA-7R, 831a rotor from International Equipment Co., Division of Damon Corp. (Needham Heights, MA 02194) and dried by a Meyer N-Evap analytical evaporator from Organomation Assoc., Inc. (South Berlin, MA 01549). An LC gradient system consisting of 2 Model 510 pumps, a 712 Wisp autoinjector, a Model 490 multiwavelength detector, a 840 Data System, and a temperature control module purchased from Waters Chromatography Division Millipore Corp. (Milford, MA 01757) was used. A Waters Pico-Tag amino acid analysis column (3.9 mm × 150 mm, Cat. No. 88131) was used for the determination of all amino acids.

Preparation of Samples

(a) *Serum samples.*—The samples were deproteinized by adding an equal volume of acetonitrile, thoroughly mixing, and centrifuging, at 3000 × *g* for 15 min to remove the precipitated protein. Then 50 μL of the deproteinized serum (supernatant) was processed as described below.

(b) *Liver, brain, and heart samples.*—Free amino acids from these tissues were extracted by mixing (vortexing) 50 mg of freeze-dried and powdered (by mortar and pestle) sample with 2 mL of 0.1N HCl for 30 s. The mixture was then centrifuged at 3000 × *g* for 10 min. The supernatant liquid was filtered through a 0.2 μm filter (Millex-GS Millipore). Then 50 μL of the filtrate was processed as described below.

Drying and Derivatization

Samples of the deproteinized serum, liver, brain, and heart and amino acid standard (50 μL) contained in the culture tubes were evaporated to dryness at 35°C under nitrogen for 15 min on the analytical evaporator. The dried samples of the standard, serum, and tissues were mixed with 50 μL methanol + water + TEA (1 + 1 + 1) and redried 15 min at 35°C to remove ammonia that co-eluted with 1-CH_3His. Norleucine may be used as an internal standard because it elutes just after the reagent peak following leucine.

The derivatizing solution was prepared just before use, by mixing 200 μL methanol, 50 μL water, 50 μL TEA, and 20 μL PITC. All amino acids were derivatized by adding 50 μL derivatizing solution to the tubes containing dried samples of standard, serum, and tissues. The samples were mixed, and the tubes were covered with Parafilm and allowed to stand 20 min at room temperature for completion of derivatization. The excess reagent was evaporated under nitrogen for 15 min at 35°C, and then the derivatized dried samples were redissolved in 200 μL sample diluent [prepared by dissolving 710 mg disodium hydrogen phosphate in 1 L water-acetonitrile (19 + 1) and by adjusting the pH to 7.40 with phosphoric acid] for chromatography. A 20 μL (in the case of standard and organs) or a 40 μL sample (in the case of serum) was then injected into the chromatographic system. The addition of sample diluent to the dried-derivatized samples may result in the formation of a turbid solution. In this case, the samples should be centrifuged (3000 × *g* for 10 min) to obtain a clear supernate.

Chromatography

(a) *Solvents.*—Solvent A was prepared by mixing 11.45 g sodium acetate dissolved in 900 mL water with 46.5 g acetonitrile and 0.5 mL TEA. The pH of the solution was adjusted

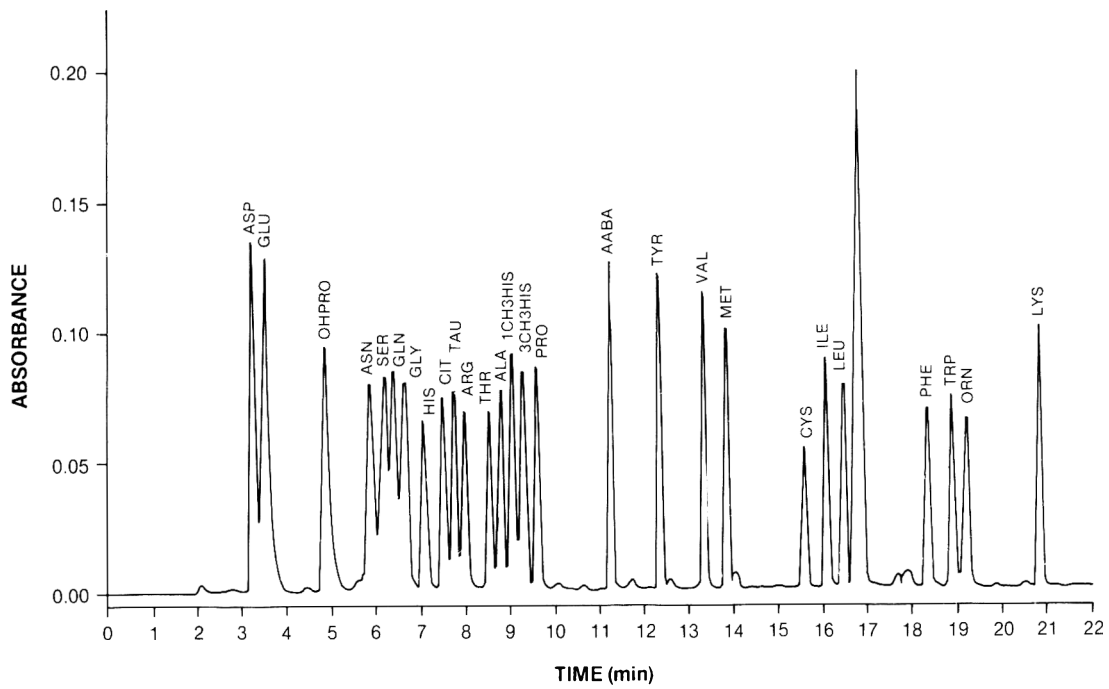

Figure 1. Elution profile of 27-amino acid standard showing resolution of each PTC-amino acid by liquid chromatography.

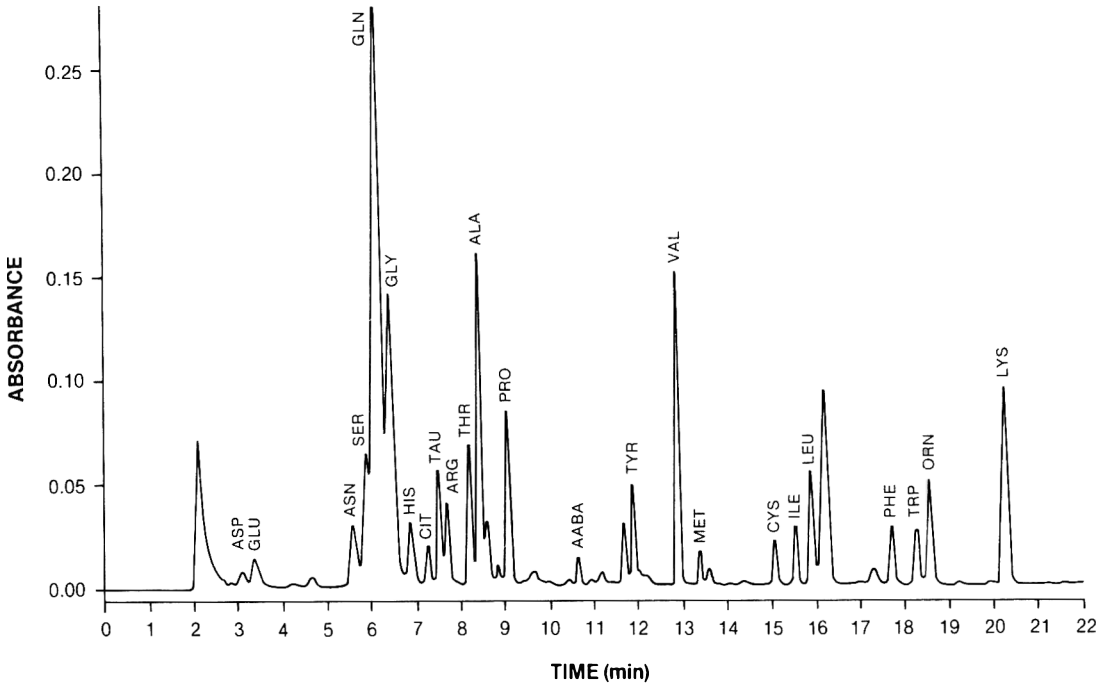

Figure 2. Elution profile of human serum showing resolution of PTC-amino acids by liquid chromatography.

to 6.40 with glacial acetic acid, and then diluted to a final volume of 1 L. The preparation of solvent B included the addition of 410 g water to 475 g acetonitrile followed by mixing and degassing.

(b) *Gradient conditions.*—Elution was commenced at 0.5 mL/min with 100% solvent A. A convex curve (curve 5) was used to increase solvent B to 10% over 6 min. The flow rate was increased linearly to 1 mL/min from 6 to 7 min. Then curve 4 was used to increase solvent B to 25% over the next 8 min. The solvent composition and flow rate were held at these levels for the next 2.5 min. Between 17.5 min and 20.9 min, both flow rate and solvent B were increased linearly to 1.6 mL/min and 40%, respectively. Solvent B was then increased linearly to 100% over the next 0.5 min and held at that level for 2.4 min. The column was then returned to 100% solvent A and equilibrated for 6.2 min. The column temperature was maintained at 47.5°C. The detection range was set at 0.2 AUFS and the UV absorbance at 254 nm.

Calculations

The amounts of free amino acids in the serum and tissue (liver, brain, and heart) samples were calculated as follows:

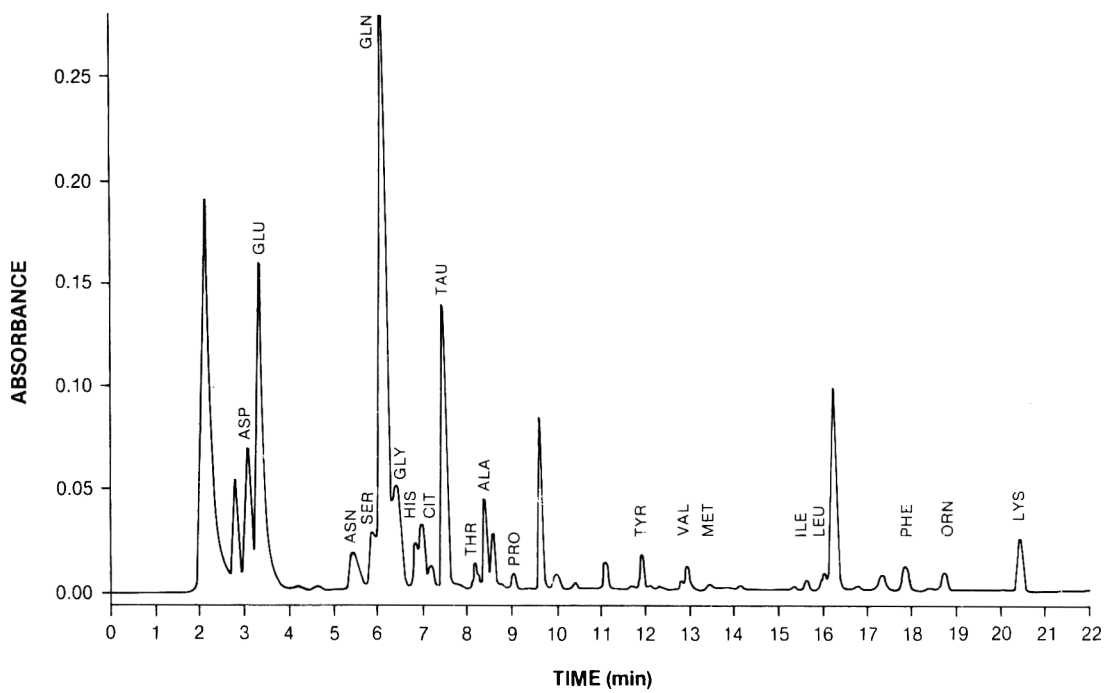

Figure 3. Elution profile of rat liver showing resolution of PTC-amino acids by liquid chromatography.

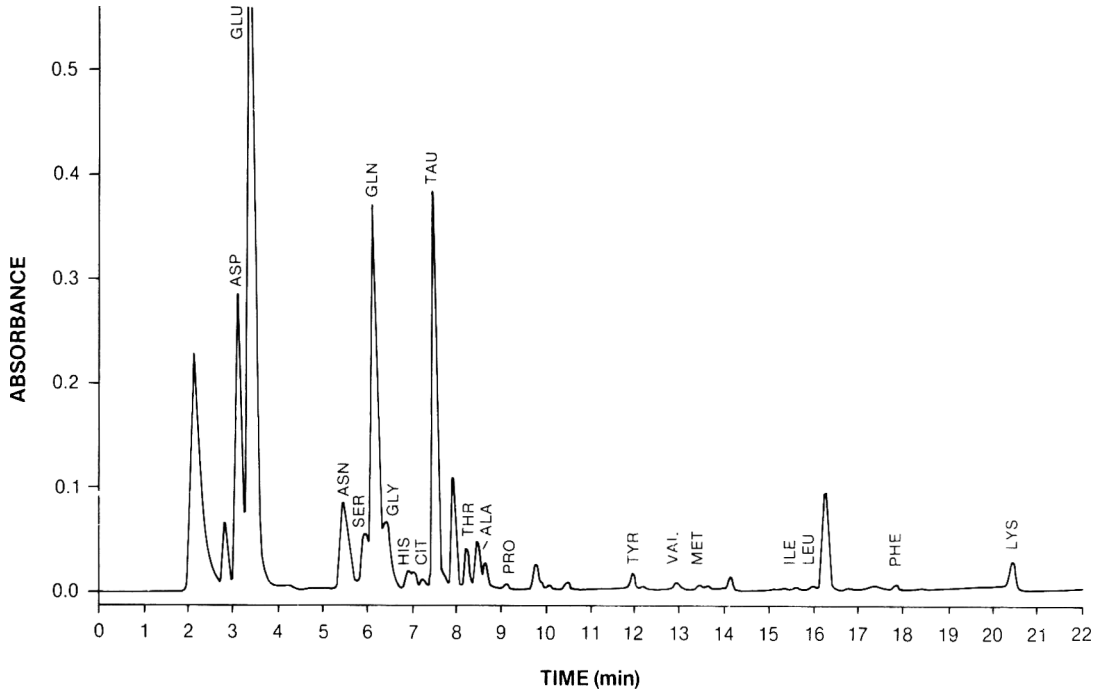

Figure 4. Elution profile of rat brain showing resolution of PTC-amino acids by liquid chromatography.

μmole amino acid/100 mL serum =
(PA/PA′) × (nmole/mL STD) × DF × (IV′/IV) × (1/10)

nmole amino acid/mg dried tissue =
(PA/PA′) × (nmole/mL STD ÷ W) × (IV′/IV) × V

where PA = area for an amino acid in sample chromatogram; PA′ = area for the same amino acid in standard chromatogram; DF = dilution factor for deproteinization, 1:1 (2); IV′ = injection volume of standard in mL; IV = injection volume of sample in mL; W = weight of dried tissue extracted (about 50 mg); V = volume in mL of extraction solution (2 mL).

Results and Discussion

The standard containing 27 amino acids revealed good separation of each amino acid (Figure 1). Free amino acids in several samples of human or rat serum, liver, brain, and heart were determined by using the procedure described in this investigation. The amino acid elution profiles of samples of human serum, rat liver, rat brain, and rat heart are shown in Figures 2, 3, 4, and 5, respectively. In general, all amino acids in the serum and organ samples were satisfactorily resolved. There were no major unknown peaks in the test samples; the peak between leucine and phenylalanine was also present in a

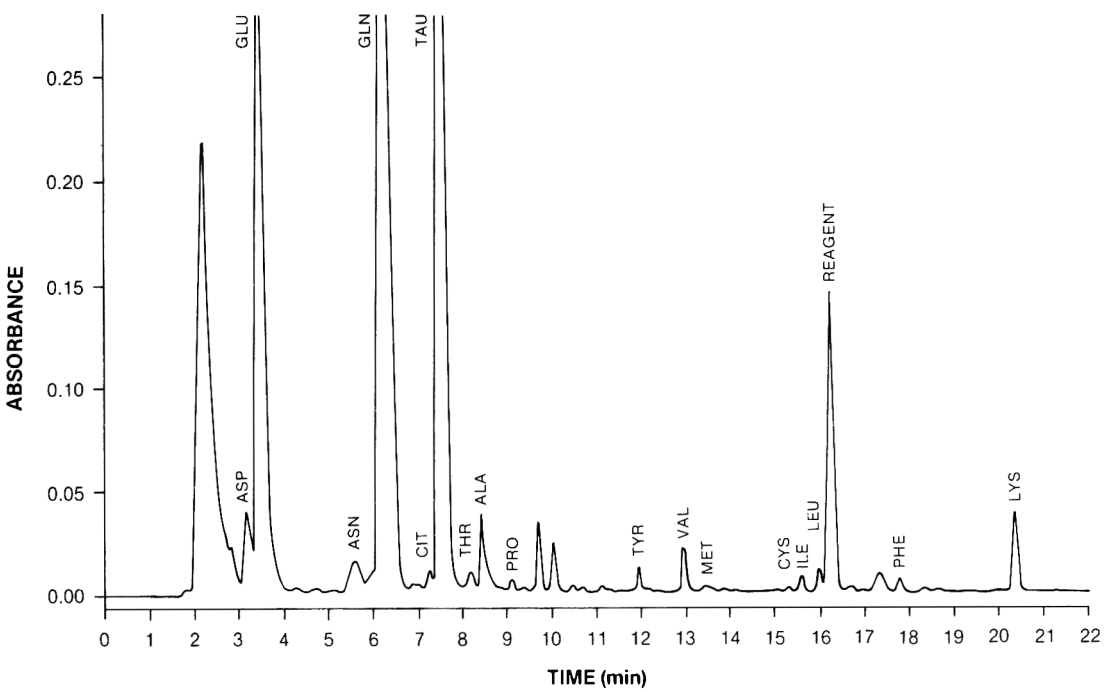

Figure 5. Elution profile of rat heart showing resolution of PTC-amino acids by liquid chromatography.

Table 1. Free amino acid concentrations (μmole/100 mL) in serum of rats fed 8% protein diet

Amino acid	Mean concn[a]	SD	CV, %
Asp	3.0	0.08	2.6
Glu	18.2	0.06	0.3
OHPro	5.6	0.13	2.3
Asn	5.4	0.14	2.6
Ser	38.6	0.45	1.2
Gln	68.6	1.54	2.2
Gly	34.1	0.42	1.2
His	11.4	0.14	1.2
Cit	11.9	0.10	0.8
Tau	27.2	0.24	0.8
Arg	11.9	0.10	0.8
Thr	30.3	0.22	0.7
Ala	88.3	0.88	1.6
Pro	24.5	0.60	2.4
α-ABA	1.8	0.05	2.7
Tyr	8.0	0.05	0.6
Val	11.2	0.05	0.4
Met	7.6	0.20	2.6
Ile	8.7	0.15	1.7
Leu	8.0	0.08	1.0
Phe	5.2	0.15	2.9
Trp	9.8	0.14	1.4
Orn	13.8	0.40	2.9
Lys	24.8	0.26	1.0

[a] n = 4.

Table 2. Levels (nmole/mg freeze-dried tissue) of free amino acids in liver of weanling pigs

Amino acid	Mean level[a]	SD	CV, %
Asp	11.3	0.21	1.8
Glu	55.6	1.08	1.9
OHPro	1.4	0.04	2.8
Asn	21.5	0.46	2.1
Ser	52.5	2.28	4.3
Gln	29.0	1.06	3.6
Gly	79.9	3.57	4.5
His	28.4	1.02	3.6
Cit	1.2	0.02	1.7
Tau	16.7	0.80	4.8
Arg	5.3	0.13	2.4
Thr	15.9	0.75	4.7
Ala	76.4	1.46	1.9
Pro	18.8	0.30	1.6
Tyr	11.4	0.50	4.4
Val	19.9	0.55	2.8
Met	9.1	0.30	3.3
Cys	0.9	0.02	2.2
Ile	12.7	0.31	2.4
Leu	31.2	0.88	2.8
Phe	12.3	0.30	2.4
Trp	3.0	0.09	3.0
Orn	20.8	0.80	3.8
Lys	5.0	0.20	4.0

[a] n = 7.

reagent blank. Although the resolution of Asn/Ser/Gln/Gly was not ideal, it was adequate for the estimation of these amino acids in most samples. The resolution of Asn/Ser/Gln/Gly obtained in this investigation was significantly better than that reported by Early and Ball (8). More research, however, is required to further improve separation of these amino acids in biological samples by using the faster-eluting Waters Pico-Tag amino acid analysis column for protein hydrolysates.

To estimate the variation of the liquid chromatography method, 4 samples of rat serum and 7 samples of pig liver were individually analyzed. The variability expressed as coefficient of variation (CV) of the entire analytical procedure, including deproteinization, derivatization, and chromatography, for all amino acids was less than 3 and 5% in the serum and liver samples, respectively (Table 1). To obtain information on the efficiency of the method used in the present investigation, a sample of rat serum or pig liver was analyzed with or without added amino acid standard. For example, a sample of rat serum or pig liver was divided into 2 portions. The amino acid standard (dried) was re-dissolved in one portion of serum or liver sample before deproteinization or extraction. Both the portions (i.e., with and without added standard) were then deproteinized/extracted, derivatized, and analyzed. The recovery of each amino acid listed in Table 1 or 2 was calculated by comparing data for serum or liver alone and serum or liver plus standard. The average values for the recovery of amino acids in the serum and liver samples were 100.4 and 96.6%, respectively.

Lavi et al. (6) reported some difficulty in determining lysine, threonine, cysteine, and histidine in physiological fluids by precolumn derivatization with PITC and separation of the derivatized amino acids by reverse-phase chromatography. These workers (6) used methanol and sulfosalicyclic acid as deproteinization agents that have been reported (7) to cause considerable losses and reduced yield of many PTC-amino acids. More recently, ultrafiltration has been used for deproteinizing samples prior to analysis by liquid chromatography. However, sample pretreatment (including acidification with HCl) and selection of appropriate membrane are important in obtaining recoveries of 90% or more. The acid of acetonitrile as a deproteinizing agent in the present investigation gave good results as clearly demonstrated by high yields of amino acids in the recovery studies.

A comparison of the liquid chromatography method with an established method of amino acid analysis was not considered necessary in this investigation because amino acid analysis by liquid chromatography of precolumn phenylisothiocyanate derivatives has been successfully validated by the ion-exchange chromatographic methods in our laboratory (1) and other research centers (3, 5).

In conclusion, the liquid chromatographic method reported in the present study can be used for accurate, rapid (20 min), and reproducible determination of most nutritionally important amino acids including taurine in serum and organs such as liver, heart, and brain. Taurine is the major free intracellular amino acid in animal tissues and it has become the focus of study by many as a conjugator of bile acids, as a protector of cell membranes by attenuating toxic substances and/or by acting as an osmoregulator, and as a possible neurotransmitter (9). Since the method developed in this investigation employs the same short column (15 cm) as the one used for protein hydrolysates, the analysis of biological and food samples can be conveniently switched back and forth, eliminating the need for the more expensive column (25-30 cm) for analyzing biological samples.

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TECHNICAL COMMUNICATIONS

Combined Sample Preparation and Inductively Coupled Plasma Emission Spectroscopy Method for Determination of 23 Elements in Solid Wastes: Summary of Collaborative Study

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An inductively coupled plasma (ICP) atomic emission spectroscopy method (EPA Solid Waste Method 6010) for the determination of 23 elements was subjected to collaborative study in 1986 and 1987 (C. L. Jones, V. F. Hodge, D. M. Schoengold, H. Biesiada, T. H. Starks, and J. E. Campana, EPA contracts 68-01-7159 and 68-01-7253, University of Nevada, Las Vegas, NV). The results of the study were reported for 7 waste samples and 2 check samples containing different known levels of each element submitted from 9 laboratories in the United States (NTIS PB88-124318, National Technical Information Service, Springfield, VA). Three of the waste samples were National Institute of Standards and Technology Standard Reference Materials River Sediment 1645, Coal Fly Ash 1633a, and Estuarine Sediment 1646. One sample was an EPA standard reference material designated as mine tailing. The 3 other waste samples were contaminated soil and 2 industrial sludges.

Since the ICP method is used after samples are digested with strong acid (SW-846 Method 3050), collaborators were provided with both predigested sample extracts as well as raw samples to assess the effect of the digestion method on the ICP determination. The predigested samples are designated as Method 6010. Samples digested by each collaborator are designated as Method 3050/6010. Each of the 7 samples was analyzed in duplicate. Each sample was also spiked with 19 of the 23 elements and analyzed in duplicate. An evaluation of the methods (Tables 1–3) shows that precision is very high.

Each collaborating laboratory also received 2 blind quality control samples, which were used to assess accuracy. An

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The recommendation was approved interim official first action by the General Referee, the Committee on Environmental Quality, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions are published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.

Table 1. Relative standard deviations (%) of collaborative study data for ICP determination of 23 elements in spiked bulk digests

Element	Hazardous waste 1	NIST SRM[a] River Sediment 1645	NIST SRM[a] Fly Ash 1633a	NIST SRM[a] Estuarine Sediment 1646	Industrial sludge	Electro-plating sludge	Mine tailing
Al	11	19	16	1.9	11	13	7.6
Sb	5.6	52	73	8.7	3.2	24	4.4
As	13	11	83	22	25	8.6	5.3
Be	5.8	5.8	57	4.8	6.4	9.9	8.5
Cd	11	6.6	5.7	7.6	3.1	9.8	12
Ca	8.8	9.4	5.6	5.3	8.5	7.0	7.9
Cr	6.2	5.5	36	7.6	5.8	7.8	39
Co	11	14	21	5.8	6.7	11	15
Cu	4.4	4.3	9.7	6.0	11	7.8	12
Fe	6.6	8.3	8.8	6.0	6.9	8.4	8.4
Pb	15	7.2	22	4.7	3.9	5.6	8.0
Mg	8.8	8.1	15	9.4	8.0	20	10
Mn	10	13	14	11	11	9.6	5.5
Mo	20	33	19	28	16	36	21
Ni	9.4	8.9	8.1	5.4	5.1	9.2	12
Se	7.5	13	16	6.2	13	13	19
Ag	44	23	17	46	47	19	27
Tl	19	13	22	29	30	20	29
V	12	58	7.5	7.3	5.5	11	18
Zn	9.1	6.7	7.6	15	10	2.5	16
Ba	11	10	8.7	6.4	8.0	20	11
Na	17	38	49	4.7	5.8	9.8	7.9
K	8.8	7.4	4.2	4.8	13	5.8	7.9
Median % RSD	10	10	16	6.8	8.0	11	11

[a] National Institute of Standards and Technology Standard Reference Material.

Table 2. Relative standard deviations (%) of collaborative study data for ICP determination of 23 elements in solids (not spiked)

Element	Hazardous waste 1	NIST SRM[a] River Sediment 1645	NIST SRM[a] Fly Ash 1633a	NIST SRM[a] Estuarine Sediment 1646	Industrial sludge	Electro-plating sludge	Mine tailing
Al	19	32	19	23	15	23	17
Sb	38	78	—	—	47	68	57
As	53	48	32	18	83	44	28
Be	31	27	27	35	42	70	41
Cd	37	17	57	52	17	22	59
Ca	9.0	13	10	11	10	17	8.6
Cr	11	19	28	22	12	12	90
Co	24	60	23	12	21	46	30
Cu	10	9.4	16	17	17	12	20
Fe	13	24	52	10	14	12	18
Pb	8.0	12	33	37	16	17	17
Mg	6.0	11	20	10	18	14	9.2
Mn	8.6	17	24	10	18	21	11
Mo	30	42	20	58	56	49	26
Ni	14	25	34	21	16	20	40
Se	42	61	—	30	43	74	77
Ag	41	43	47	1.4	38	54	60
Tl	31	30	—	—	38	45	120
V	21	72	15	17	28	35	47
Zn	14	12	20	8.6	12	9.2	20
Ba	7.4	11	4.3	14	24	38	8.8
Na	66	52	34	9.1	16	17	13
K	23	34	20	17	32	9.6	24
Median % RSD	21	27	23	17	18	22	26

[a] National Institute of Standards and Technology Standard Reference Material.

Table 3. Relative standard deviations (%) of collaborative study data for ICP determination of 23 elements in spiked solids

Element	Hazardous waste 1	NIST SRM[a] River Sediment 1645	NIST SRM[a] Fly Ash 1633a	NIST SRM[a] Estuarine Sediment 1646	Industrial sludge	Electro-plating sludge	Mine tailing
Al	17	24	20	22	14	18	26
Sb	27	56	25	62	28	40	58
As	13	26	16	22	20	20	22
Be	16	13	7.6	11	18	7.0	16
Cd	13	8.4	9.3	14	19	18	20
Ca	7.3	9.0	12	10	12	14	12
Cr	7.9	22	9.7	7.1	18	12	26
Co	18	22	12	9.2	18	13	18
Cu	12	14	10	9.7	19	9.4	12
Fe	14	19	44	16	18	14	18
Pb	15	6.4	9.6	11	20	19	5.8
Mg	5.9	8.4	17	9.0	16	10	10
Mn	14	9.0	11	10	16	18	9.4
Mo	19	31	24	18	18	43	20
Ni	13	20	9.7	10	20	15	17
Se	13	9.4	9.8	10	15	18	12
Ag	19	7.6	50	34	30	27	50
Tl	19	28	34	28	18	43	44
V	18	19	12	10	18	39	24
Zn	14	12	11	13	20	8.2	20
Ba	8.4	9.8	7.2	14	16	30	7.2
Na	14	40	32	9.4	20	15	12
K	19	17	18	18	22	5.7	16
Median % RSD	14	17	12	11	18	18	18

[a] National Institute of Standards and Technology Standard Reference Material.

Table 4. Accuracy estimates for ICP determination of 23 elements in QC solutions by Method 6010 and Method 3050/6010

	Concentration, mean/target	
Element	Method 6010	Method 3050/6010
Al	1.00	0.98
Sb	1.00	1.00
As	0.99	0.97
Be	1.02	0.98
Cd	0.98	1.03
Ca	1.02	0.99
Cr	1.01	0.99
Co	0.96	0.96
Cu	0.97	0.96
Fe	1.00	0.99
Pb	0.98	0.95
Mg	0.98	0.95
Mn	1.02	0.96
Mo	0.99	0.96
Ni	1.00	0.98
Se	1.04	0.97
Ag	1.00	0.53
Tl	1.02	0.93
V	0.95	0.96
Zn	0.95	0.93
Ba	1.00	0.96
Na	0.96	0.97
K	0.96	0.95
Mean	0.99 ± 0.03	0.97 ± 0.03

Table 5. Precision values for ICP determination of 23 elements in QC solutions by Method 6010 and Method 3050/6010

Method 6010[a]		Method 3050/6010[b]	
Element	RSD[c], %	Element	RSD[c], %
Ag	9.1	Ag	51
Tl	8.5	As	13
Zn	8.3	Cd	11
Cr	8.2	Se	10
Sb	7.7	Tl	9.5
Se	7.5	Mo	8.9
Ca	7.4	V	8.4
Cd	7.0	Sb	7.7
Mo	6.9	K	7.2
K	6.6	Zn	6.8
V	6.6	Ba	6.8
Mg	6.5	Ca	6.7
As	6.4	Ni	6.6
Al	6.3	Mg	6.2
Co	5.9	Na	5.8
Fe	5.9	Pb	5.6
Pb	5.9	Fe	5.3
Be	5.8	Cr	5.2
Ni	5.7	Mn	4.5
Cu	5.6	Co	4.3
Mn	4.3	Al	4.0
Na	4.2	Be	2.9
Ba	3.1	Cu	2.6

[a] Median RSD = 6.5%.
[b] Median RSD = 6.7%.
[c] RSD data are presented in order of increasing precision.

evaluation of these data (Tables 4 and 5) shows high accuracy and precision. The overall accuracy of Method 3050/6010, as shown by median percent recovery (Table 6), is good.

Summary of Methods

Method 6010 is a simultaneous, multielement ICP determination. The method measures element-emitted light by optical spectrometry. Samples are nebulized and the resulting aerosol is transported to the plasma torch. Metal-specific atomic-line emission spectra are produced by a radio-frequency inductively coupled plasma. The spectra are dispersed by a grating spectrometer, and the intensities of the lines are monitored by photomultiplier tubes.

Method 3050 describes the digestion of a 1–2 g (wet weight) sample in nitric acid and hydrogen peroxide. The digestate is refluxed with either nitric or hydrochloric acid, depending on the metals and instrumental techniques.

Recommendation

On the basis of the collaborative study, the General Referee recommended that the method be adopted official first action.

Table 6. Accuracy estimates for ICP determination (Method 3050/6010) of elements spiked into solids

Element	Median % recovery	Element	Median % recovery
Al[a]	—	Mn	95
Sb	51	Mo	88
As	84	Ni	90
Be	87	Se	87
Cd	88	Ag	68
Ca[a]	—	Tl	73
Cr	87	V	85
Co	81	Zn	97
Cu	93	Ba	97
Fe[a]	—	Na	45
Pb	92	K	48
Mg[a]	—		

[a] Not spiked.

Extraction Methods for Quantitation of Gentamicin Residues from Tissues Using Fluorescence Polarization Immunoassay

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Sodium hydroxide digestion of unhomogenized kidney and skeletal muscle for 20 min at 70°C was a superior method for extracting gentamicin from tissues, compared with simple homogenization, trichloroacetic acid precipitation of homogenized tissue, and sodium hydroxide digestion of homogenized tissue. Fluorescence polarization immunoassay was used to quantitate gentamicin. Sodium hydroxide digestion of unhomogenized tissue allowed for the recovery of 90.0 ± 5.9% ($\bar{X}$ ± SD) from renal cortex and 79.9 ± 3.5% from skeletal muscle. The limit of sensitivity was 17.4 ng/g kidney tissue, 15.8 ng/g digested muscle, and 39.0 ng/g digested heart. The within-assay coefficient of variation (CV) at 100 ng/g kidney was 9.2%; at 500 ng/g kidney, the CV was 2.5%; and at 2000 ng/g kidney, the CV was 1.5%. The between-assay coefficient of variation was <7.5% for all concentrations from kidney, and the 99% confidence interval at 100 ng/g kidney was 71.7–112.4 ng gentamicin/g kidney. The within-assay coefficient of variation (CV) at 100 ng/g muscle was 15%; at 500 ng/g muscle, the CV was 2.6%; and at 2000 ng/g muscle, the CV was 2.3%. The between-assay coefficient of variation was <15% for all concentrations from muscle, and the 99% confidence interval at 100 ng/g muscle was 72.5–136.8 ng gentamicin/g muscle. Gentamicin-free milk could be distinguished from milk containing gentamicin concentrations of 10 ng/mL milk with 95% confidence, and from milk containing concentrations of 30 ng gentamicin/mL milk with 99% confidence. Quantitative results at or below the tolerance level can be obtained within 90 min of sample acquisition using these extraction and assay methods.

Several methods have been used in the past to measure the amount of gentamicin sequestered in kidney as well as in other tissues. The method most frequently employed involves homogenization of the tissue with subsequent centrifugation and analysis of the supernatant liquid with a confirmed serum aminoglycoside assay (1-3). Other studies have used protein precipitation to enhance the recovery of aminoglycosides from renal tissue (4). Recently, in response to increasing evidence that aminoglycoside antibiotics bind to anionic tissue constituents (5-8), Gilbert and Kohlhepp developed a method using homogenization followed by tissue digestion in a heated sodium hydroxide (NaOH) solution, which improved recovery from 37 to 96% over a trichloroacetic acid (TCA) precipitation method (9). Preliminary studies from this laboratory further indicated that NaOH digestion without homogenization provided excellent recovery from renal tissue (10).

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The purpose of this study was to compare NaOH tissue digestion with other extraction methods for gentamicin using homogenization, and to demonstrate the usefulness of an automated fluorescence polarization immunoassay developed to quantitate gentamicin in serum for quantitating gentamicin in tissue extracts from kidney, skeletal and cardiac muscle, and milk.

Experimental

Apparatus

The following was used: automated fluorescence polarization immunoassay system (TDx) (Abbott Laboratories, Diagnostics Division, Irving, TX); high-speed stainless steel homogenizer (Tekmar Corp., Cincinnati, OH); ultrasonic disruption probe (Sonics and Materials, Inc., Danbury, CT). All centrifugations were conducted in a nonrefrigerated table-top centrifuge (Dynac, Clay Adams Division, Becton, Dickinson and Co., Parsippany, NJ).

Reagents and Chemicals

The following were used: gentamicin sulfate (524.38 μg active gentamicin/mg dry weight; Lot B No. 20150-146, AHFR No. 87-167) (gift from Schering Veterinary Corp., Kenilworth, NJ); gentamicin fluorescence polarization immunoassay kits (Cat. No. 0951220; Lot Nos. 01695AZ, 04748AZ, 05557AZ, and 08486AZ) (Abbott Laboratories, Abbott Park, IL). All other chemicals were reagent grade (Sigma Chemicals Co., St. Louis, MO).

Temperature and pH Stability Studies

Gentamicin sulfate was added to phosphate-buffered saline (PBS; 140mM NaCl, 1.72mM NaH_2PO_4, 9.05mM Na_2HPO_4; pH = 7.4 ± 0.1) to make a stock solution containing 8.00 μg gentamicin/mL. Three aliquots were incubated at 37, 56, 70, and 90°C in a shaking water bath for 15, 30, 60, 120, and 240 min. Three additional aliquots were adjusted to pH 3.4, 5.5, 7.4, 9.5, or 11.5 with either glacial acetic acid or 10N sodium hydroxide (NaOH). Then, 2 mL aliquots of these samples were tightly capped in polystyrene tubes and incubated at 70°C for 15, 30, 60, 120, and 240 min. After incubation, samples were readjusted to pH 7.5 ± 0.2 with either 1N NaOH or acetic acid (either glacial or 1%). All samples were then cooled to room temperature and analyzed in duplicate for gentamicin. If the analysis was not performed the same day, all samples were stored at −4°C until time of analysis.

Tissue Extraction Methods

Renal cortical samples were obtained from experimental sheep and cattle that had not been exposed to any aminogly-

cosides within the previous 6 months. Tissue samples were stored at −20°C until the time of the in vitro study. A sample of renal cortex or skeletal muscle (300–400 mg wet weight) was accurately weighed. Gentamicin sulfate diluted in PBS to a concentration of 500 μg/mL was added to achieve concentrations of approximately 0, 9, 45, or 90 μg gentamicin/g tissue. Samples were stored at −4°C until time of analysis. Five tissue samples were assayed after extraction using one of 4 extraction procedures below:

Homogenization method.—Six hundred μL PBS was added to the sample, and the mixture was incubated at room temperature for 1 h. The mixture was homogenized using a stainless steel ultrasonic homogenizer for 20 s at medium speed. The homogenizer probe was rinsed thoroughly with PBS, and the rinse was pooled with the original homogenate until the rinse appeared clear (1.8–2.4 mL PBS). The suspension was centrifuged at 1000 × *g* for 10 min, and the supernatant liquid was measured, and the specific gravity of the liquid was determined for final calculation.

Trichloroacetic acid (TCA) precipitation method.—Three hundred μL PBS was added to the tube and incubated; then 1 mL 50% TCA was added. The mixture was homogenized, the homogenizer probe was rinsed with 0.8 mL PBS, and the rinse was pooled with the homogenate. The suspension was centrifuged, and the supernatant liquid was transferred to another tube; the pH was adjusted to ca 7.5 with either 10N or 1N NaOH. The supernatant liquid was weighed and its density was determined for final calculations.

Sodium hydroxide homogenization/digestion method.—Three hundred μL PBS was added to the tube and incubated; the contents were homogenized, and the homogenizer was rinsed with 2 volumes of PBS, which were pooled with the original homogenate. An equal volume of 2N NaOH (w/v) was added to the homogenate, and the mixture was incubated 20 min at 70°C, and then cooled to room temperature. The pH was adjusted to 7.5 ± 0.2 with either 10% or 1% acetic acid and 1N NaOH. If the pH decreased below 7.0, the solution became cloudy. The tubes were reweighed and the density of the solution was measured.

Sodium hydroxide digestion method.—Twelve hundred μL PBS was added to the tissue sample and incubated. An equal volume of 2N NaOH (w/v) was added, and the mixture was incubated 20 min at 70°C. The digested homogenate was cooled to room temperature and the pH was adjusted to 7.5 ± 0.2. The tubes were reweighed and the density of the solution was measured. To increase the sensitivity of the assay, PBS was eliminated from the method, thus reducing dilution of the drug in the digested tissue.

Tissue Disruption Methods

Tissue disruption using a high-speed rotary stainless steel homogenizer was compared with tissue disruption using an ultrasonic cell disruption probe and minced tissue. The amount of drug recovered from tissue fortified with 31 μg/g tissue (using PBS homogenization method) as well as the bench time required for tissue disruption were compared.

Drug Assay Procedure

A liquid aliquot of each sample extract was assayed for immunoreactive gentamicin using an automated fluorescence polarization immunoassay system. Reagent kits designed to quantitate gentamicin in serum were obtained. The sample volume was increased from 1 to 20 μL to increase the sensitivity of the system. In all instances, standard tissue samples equivalent to 100, 250, 750, 1500, and 3000 ng gentamicin/g tissue were used in duplicate to create the calibration curve generated using the on-board computer. Fortified tissues at concentrations of 100, 500, and 2000 ng/g tissue served as controls to evaluate the standard curve, and were analyzed in quadruplicate on 4 successive days to evaluate both within-day variation from day-to-day variation. Standard curves were rejected (*1*) if the percent error between duplicate standards was greater than 3%; (*2*) if the residual mean square error between the standards and the calculated curve was greater than 1.00; or (*3*) if the control tissue samples were not predicted within 15% of their expected value.

Table 1. Comparison of recovery of gentamicin (μg/g) from renal cortex samples by various methods of extraction

Method	Calcd concn, μg/g[a]	Measured concn, μg/g[b]	Recovery, %
Homogenization[d]	0	0.76 ± 0.85	NA[c]
	9	5.80 ± 0.33	64.6 ± 3.7
	45	25.7 ± 1.50	57.2 ± 3.3
	90	49.8 ± 3.10	55.4 ± 3.5
TCA-precipitation[e]	0	0.74 ± 0.67	NA
	9	7.83 ± 0.37	86.9 ± 4.0
	45	37.4 ± 0.33	83.8 ± 0.8
	90	74.1 ± 2.53	82.4 ± 2.8
NaOH-digestion/ homogenization[e]	0	0.05 ± 0.10	NA
	9	8.42 ± 0.63	93.7 ± 6.9
	45	35.7 ± 2.10	79.4 ± 4.6
	90	76.0 ± 1.97	84.4 ± 2.2
NaOH-digestion[f]	0	0.37 ± 0.54	NA
	9	8.50 ± 0.40	94.5 ± 4.4
	45	40.7 ± 1.33	90.3 ± 2.9
	90	76.7 ± 3.90	85.3 ± 4.3

[a] Renal cortical homogenates to which gentamicin was added to achieve each concentration.
[b] n = 5.
[c] NA = not applicable.
[d,e,f] Methods with different superscripts significantly different ($P < 0.05$) using Student–Newman–Keuls multiple range test.

Statistics

The limit of sensitivity is defined as the upper bound of the 99% confidence interval of the predicted concentrations of the 0 ng/g standard, using the between-day variation (the concentrations that can be distinguished from 0 ng/g with 99% confidence). Drug recovery by the methods was compared by 2-way analysis of variance. Extraction methods were compared using Student–Newman–Keuls multiple range test. Significance was set at $P < 0.05$, unless otherwise stated. Data are reported as mean ± standard deviation ($\bar{X}$ ± SD).

Results

Temperature and pH Stability Studies

Gentamicin was not significantly affected by temperature or pH from pH 3.5 to 11.5.

In Vitro Tissue Assay

Extraction methods.—The recovery of gentamicin from renal cortical samples by NaOH digestion was 90.0 ± 5.9% (Table 1). Recovery using the NaOH digestion method was

Table 2. Comparison of recovery of gentamicin (μg/g) from skeletal muscle samples by various methods of extraction

Method	Calcd concn, μg/g[a]	Measured concn, μg/g[b]	Recovery, %
Homogenization[d]	0	0.079 ± 0.177	NA[c]
	10.8	5.29 ± 0.81	49.0 ± 7.5
	26.3	10.6 ± 1.00	40.4 ± 3.8
	54.0	26.0 ± 5.13	48.2 ± 9.5
TCA-precipitation[e]	0	0.00 ± 0.00	NA
	11.1	7.88 ± 0.38	71.0 ± 3.4
	27.7	18.3 ± 0.33	66.0 ± 1.2
	52.7	38.3 ± 2.53	72.7 ± 4.8
NaOH-digestion/ homogenization[e]	0	0.035 ± 0.079	NA
	10.8	8.39 ± 0.75	77.7 ± 6.9
	27.0	20.6 ± 0.68	76.3 ± 2.5
	52.7	40.8 ± 1.05	77.5 ± 2.0
NaOH-digestion[f]	0	0.20 ± 0.18	NA
	10.5	8.33 ± 0.35	79.3 ± 3.3
	27.7	21.9 ± 1.5	79.1 ± 5.4
	54.0	43.9 ± 0.38	81.3 ± 0.7

[a] Skeletal muscle homogenates to which gentamicin was added to achieve each concentration.
[b] n = 5.
[c] NA = not applicable.
[d,e,f] Methods with different superscripts significantly different ($P < 0.05$) using Student–Newman–Keuls multiple range test.

significantly higher ($P < 0.05$) than all other extraction methods (Table 1), whereas recovery using the simple homogenization method was significantly lower than that from the other extraction methods ($P < 0.01$). Recovery of gentamicin from skeletal muscle samples using the 2 NaOH-digestion methods were significantly higher ($P < 0.01$) than that from either simple homogenization or TCA-precipitation and homogenization (Table 2). Recovery of gentamicin from skeletal muscle using the simple homogenization method was significantly lower than that for the other 3 extraction methods ($P < 0.01$).

Tissue disruption methods.—The recovery of gentamicin from calf muscle by using ultrasonic probe tissue disruption was equivalent to recovery for the stainless steel rotary homogenizer tissue digestion (45.7 ± 4.78% vs 40.4 ± 3.80%, respectively). Use of the ultrasonic probe reduced the time required to disrupt the tissue samples by approximately 35% over the stainless steel rotary homogenizer.

Drug Assay Procedure

The limit of sensitivity of the FPIA method in kidney was 17.4 ng gentamicin/g kidney. Calculated concentrations of the fortified control kidney tissues were within 10% of expected concentrations (Table 3), the within-day coefficient of variations (CV) were less than 9.2% on each of 4 consecutive days, and between-day CVs were less than 7.5% at all concentrations. The 99% confidence interval at 100 ng gentamicin/g of kidney was 71.7–112 ng/g kidney tissue.

The limit of sensitivity of the FPIA assay in skeletal muscle was 15.8 ng gentamicin/g skeletal muscle. Calculated concentrations were within 15% of expected concentrations (Table 4), and between-day CVs were less than 15% at all concentrations. The 99% confidence interval at 100 ng/g digested muscle was 72.5–137 ng/g skeletal muscle.

The limit of sensitivity of the assay in heart muscle was 39.0 ng gentamicin/g digested heart. Except for the 500 ng/g control tissue, all calculated concentrations were within 10% of the expected values (Table 5), and between-day CVs were consistently less than 10%. The 99% confidence interval of 100 ng gentamicin/g digested heart muscle was 66.5–150 ng/g digested tissue.

Blank milk showed no gentamicin (consistent results of 0.00 ng/mL milk), and control milk samples of 30 ng gentamicin/mL were assayed at 32.5 ± 2.67 ng gentamicin/mL milk (99% confidence interval of 16.9–48.2 ng/mL milk) on day 0 and 29.6 ± 1.27 ng gentamicin/mL milk (99% confidence interval of 22.2–37.0 ng/mL or day 1 (Table 6). Stability of the standard curve was only marginal, remaining stable for only 24 h after calibration. In particular, the upper end of the standard curve drifted consistently over time (see Table 6).

Discussion

The results of the present study that compare the NaOH digestion procedure with and without homogenization show no advantage to homogenization prior to NaOH digestion. In every comparison, recovery of gentamicin was higher when the NaOH digestion method was used without homogenization. However, for more fibrous tissues, either homogenization or treatment with proteolytic enzymes may be needed to optimally extract aminoglycoside antibiotics from those tissues.

The FPIA method has never been pushed to these lower limits of detection because the assay was developed for monitoring therapeutic concentrations of gentamicin in human serum. Furthermore, the method has only been used for

Table 3. Analysis of gentamicin (ng/g) from digested kidney using FPIA calibrated from digested kidney samples fortified with gentamicin

Concn, ng/g	Day 0	Day 1	Day 2	Day 3	$\bar{X}$ ± SD[a]
0	0	0	2.54	5.55	2.02 ± 2.64
100	95.8	84.0	101	92.9	93.3 ± 6.97
250	232	225	241	232	232 ± 6.62
750	742	786	734	749	753 ± 99.0
1500	1480	1650	1490	1500	1530 ± 78.2
3000	2900	3210	3180	3180	3120 ± 142
100[a]	96.6 ± 8.87	90.2 ± 6.62	92.6 ± 5.83	89.0 ± 6.25	71.7–112[b]
500[a]	477 ± 9.21	507 ± 10.0	504 ± 11.6	495 ± 8.16	452–539[b]
2000[a]	2290 ± 33.2	2590 ± 125	2550 ± 42.2	2500 ± 86.4	2080–2890[b]

[a] n = 4.
[b] 99% confidence interval (n = 16) over all 4 days.

Table 4. Analysis of gentamicin (ng/g) from digested skeletal muscle using FPIA calibrated from digested muscle samples fortified with gentamicin

Concn, ng/g	Day 0	1	2	3	$\bar{X} \pm SD^a$
0	3.60	5.72	2.67	6.82	4.70 ± 1.90
100	95.4	97.3	99.2	103	98.7 ± 3.10
250	252	239	278	262	258 ± 16.9
750	752	730	840	838	790 ± 57.1
1500	1610	1530	1720	1750	1650 ± 104
3000	2890	2830	3600	3610	3230 ± 432
100[a]	102 ± 8.54	100 ± 10.5	112 ± 14.6	105 ± 9.59	72.5–137[b]
500[a]	540 ± 3.38	519 ± 13.5	586 ± 2.39	583 ± 11.2	467–647[b]
2000[a]	1900 ± 13.6	1970 ± 45.5	2150 ± 34.3	2120 ± 29.8	1710–2360[b]

[a] n = 4.
[b] 99% confidence interval (n = 16) over all 4 days.

Table 5. Analysis of gentamicin (ng/g) from digested heart muscle using FPIA calibrated from digested heart samples fortified with gentamicin

Concn, ng/mL	Day 0	1	2	3	$\bar{X} \pm SD^a$
0	13.1	8.14	18.9	13.1	13.3 ± 4.40
500	585	517	561	514	544 ± 34.8
1500	1480	1380	1500	1430	1450 ± 51.8
3300	3460	3220	3560	3460	3410 ± 144
6500	6560	6030	6840	6680	6530 ± 348
10000	9790	8800	9790	9510	9470 ± 470
100	110	98.4	115	107	67.4–150[b]
500	601	576	678	630	366–877[b]
2000	2030	1920	2120	2060	1530–2530[b]

[a] n = 4.
[b] 99% confidence limit (n = 4) for all 4 days.

tissue extracts in studies of gentamicin kidney depletion arising from this laboratory (11, 12). In those studies, the limits of sensitivity were not extended to the tolerance level for gentamicin in kidney because the concentrations observed in those studies was much greater than the tolerance level. From the present study, the limit of sensitivity for all of the samples was lower than the 99% confidence interval for tissues fortified at 100 ng gentamicin/g tissue. The limit of sensitivity in milk allowed for differentiation of milk concentrations of 50 ng/mL milk with 99% confidence and of 10 ng/mL with 95% confidence. Thus, the analytical method provides the sensitivity required for detection and quantitation of gentamicin residues in milk and edible tissues at or below the tolerance level.

The entire process, from the time of acquisition of tissue through the digestion procedure to reporting of results, can be done in less than 90 min. Furthermore, the digestion methods are easy and inexpensive. The process appears to have some application to the field situation if the automated FPIA system can be modified to provide more transportability. The FPIA method has been shown to provide a sensitive and expedient method of determination of gentamicin in serum and now in tissue extracts. Providing reagent kits can be created for other aminoglycosides used in food-producing animals, this technology can provide fast, quantitative results in the laboratory or field situation. This type of general extraction method for aminoglycosides may be useful for the surveillance of the meat industry for aminoglycoside residues in the future.

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Table 6. Quantitation of gentamicin from milk samples (ng/mL) using FPIA method

Concn, ng/mL	Day 0	1	2	3	$\bar{X} \pm SD^a$
0	0.00	0.00	0.00	0.00	0.00 ± 0.00
30	27.6	26.1	22.2	26.1	25.5 ± 2.31
100	100	89.7	90.1	93.1	93.2 ± 4.76
200	211	186	185	188	193 ± 12.5
600	530	455	473	486	486 ± 32.3
1500	1400	1210	1320	1480	1350 ± 112
30[a]	32.5 ± 2.67	29.6 ± 1.27	27.0 ± 2.98	29.3 ± 2.25	21.0–38.2[b]
250[a]	261 ± 3.13	236 ± 5.59	224 ± 4.42	241 ± 3.46	198–283[b]
1000[a]	931 ± 34.1	807 ± 40.4	720 ± 25.7	847 ± 25.2	580–1070[b]

[a] n = 4.
[b] 99% confidence interval (n = 16) over all 4 days.

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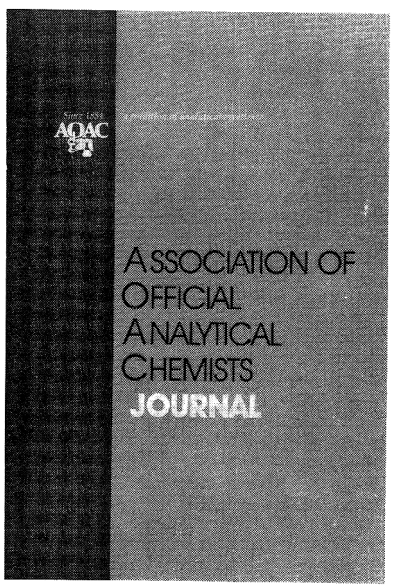

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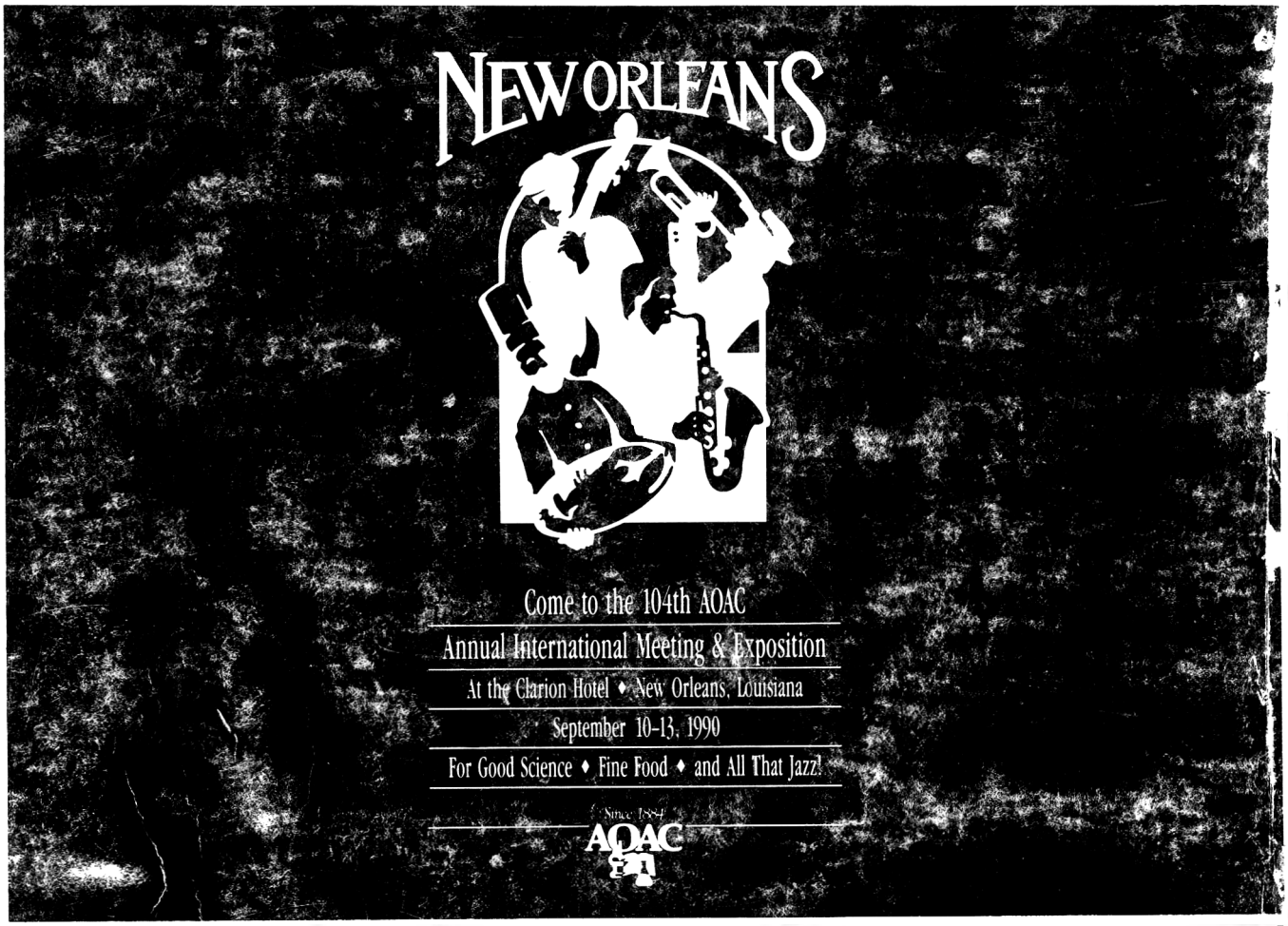

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