September / October 1994 Volume 77 Number 5

JOURNAL OF AOAC INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence®

AOAC INTERNATIONAL

JAINEE 77(5) 1061-1340 (1994)
ISSN 1060-3271

AOAC Short Course

December
15-16, 1994

Radisson Plaza
Lord Baltimore
Baltimore, Maryland

To register or
for information
call Short Course
Registration at
+1-703-522-3032
or fax
+1-703-522-5468

Laboratory Waste Disposal, Environmental Compliance & Safety

Who Should Take This Course:
Laboratory managers, supervisors, quality assurance coordinators, or anyone else responsible for the development and supervision of laboratory processes.

What You'll Learn:
How to prevent regulatory noncompliance action and guard yourself and your employees from health hazards. This course will cover the US EPA requirements for generators of hazardous waste, OSHA regulations for exposure of employees to health hazards, and the components of good laboratory waste management and hygiene programs, including record keeping, compliance reporting, and training.

Two-Day Program Covers:

- Introduction
- Hazard Communication, Labels, Tags, Signs, MSDs
  Substance Inventory, Information Sources
  Employee Information and Training
  Hazcom Exercise
- Basic Toxicology
- EPA Regulations and Inspections
- RCRA Training Plans
- Use of Spill Control Equipment
- Problem Session
- Rule 120 for RCRA Generators
- Laboratory Hygiene Plan
- Elements of a Lab Health and Safety Program
- DOT Regulations for Shippers of Hazardous Waste
- Use of Monitoring Equipment and Exercise
- Respiratory Protection
- Fire Protection and Control

Registration fee: Members $495.00
Nonmembers $560.00

Discounts are available if register more than one person.

AOAC® INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence®

JOURNAL OF AOAC INTERNATIONAL

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

Table of Contents

September/October 1994 VOL. 77, NO. 5 JAINEE 77(5)1061-1340 (1994)

The *Journal of AOAC INTERNATIONAL* (ISSN 1060–3271) is published bimonthly by AOAC INTERNATIONAL, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 77 (1994) are: Members, $130.00 in U.S., $171.00 outside U.S.; Nonmembers, $189.00 in U.S., $230.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.
POSTMASTER: Send address changes to AOAC INTERNATIONAL, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA.

DRUGS, COSMETICS, FORENSIC SCIENCES

FOOD BIOLOGICAL CONTAMINANTS

FOOD CHEMICAL CONTAMINANTS

FOOD COMPOSITION AND ADDITIVES

RESIDUES AND TRACE ELEMENTS

STATISTICAL ANALYSIS

TECHNICAL COMMUNICATIONS

Books in Brief

Scientific Evidence in the Courtroom: Admissibility and Statistical Significance After Daubert. By Robert P. Charrow and David E. Bernstein. Published by the Washington Legal Foundation, Publications Department, 2009 Massachusetts Ave, NW, Washington, DC 20036, 1994. Library of Congress Catalog Card No. 94-60916.

In 1992, the U.S. Supreme Court issued a landmark decision, *Daubert v. Merrell Dow Pharmaceutical*, which set general standards to govern the admissibility of scientific evidence. This monograph, which reviews the *Daubert* decision and the role of scientific evidence, was written by two of the nation's leading experts on science and the law, Robert Charrow and David Bernstein. It is an invaluable resource for all who must deal with science in the courtroom. The monograph offers an insightful review of the *Daubert* decision and some lower federal court opinions, which have applied the case to reject questionable science as evidence in tort and personal injury cases. What makes this monograph unique, however, is its original mathematical proof that sets a quantitative standard that a plaintiff must meet to show that this scientific evidence of causation is sufficient to satisfy his legal burden of proof.

ELISA-Mation. Version 1.0. By Jarrett N. Schmit. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431-9868, 1994. Price: $89.95. 32 pp. 3.5 in. diskette. ISBN 0-8493-0753-8.

ELISA-Mation is a unique, color-animated, easy-to-operate introductory tutorial for the ELISA test. The manual uses both text and animated sequences to clearly describe the steps and reactions occurring in an ELISA test. It also provides many educational uses, including a review for professionals, an autotutorial for students, an adjunct to formal lectures, an in-class demonstration, and a training tool for laboratory personnel. The software package includes: full-color animation to enhance the learning efficiency and enjoyment of today's students and professionals; comprehensive coverage of all topics required to understand ELISA; efficient learning principles that include clearly stated objectives, reviews, and quizzes; short, simple, and clearly written instructions for

hard-drive installation, and removal; system operation by use of four easy-to-find keys; and system compatibility with all IBM compatible computers, requiring only 235 kb free memory, an EGA or VGA monitor, and DOS 3.3 or greater.

Perfumery: Practice and Principles. By Robert R. Calkin and J. Stephan Jellinek. Published by John Wiley & Sons, 605 Third Ave, New York, NY 10158-0012, 1994. 296 pp. Price: $74.95. ISBN 0-471-58934-9.

Perfumery, once an inexact process steeped in secrecy and dependent on subjective sensor perception and guesswork, has become a highly developed science. Over the past 30 years, gas chromatography, mass spectrometry, and other technological innovations have made it possible to analyze, study, and teach the formulas of perfumes. These developments have profoundly altered the perfume industry, making it more efficient and far more competitive. Modern perfumers now need to not only master the changing technology of perfume manufacturing, but also to understand what, in terms of chemical structure and perception, makes a particular material aesthetically pleasing. *Perfumery: Practice and Principles* presents a comprehensive, easy-to-use guide to the basic techniques and evolving technology of manufacturing perfumes as well as providing guidelines for actual formulation and analysis. It includes real-life case studies and practical applications of instrumentation, offering valuable insights into the use of fragrance in a myriad of products such as detergents, soaps, cosmetics, and designer perfumes.

Directory of Capillary Electrophoresis. Edited by D. Coleman. Published by Elsevier Science B.V., PO Box 211, 1000 AE Amsterdam, The Netherlands, 1994. 156 pp. Price: Dfl. 137.00/US 70.00. ISBN 0-444-81798-0.

This directory of researchers in capillary electrophoresis not only contains the names and address of most leading scientists in this rapidly developing field, it also specifies their main interests in developing and/or applying the technique. Additionally, the papers that each listed scientist considers as his or her most significant contribution in this area are given. A geographical index with techniques and applications areas shows who is doing what in each country. Instrument manufacturers and suppliers are given on a country by country basis, along with the products or services they offer. A subject index is also included, which lists scientists alphabetically according to their individual specialties.

Microcomputers and Electronic Instrumentation: Making the Right Connection. By Howard Maimstadt, Christie Enke, and Stanley Crouch. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1994. 464 pp. Price: US $75.00, Export $90.00. ISBN 0-8412-2861-2.

This book explores the new wave of microcomputer instrumentation. It contains clear, concise descriptions and diagrams of concepts, devices, circuits, and systems. Readers will find out how microcomputers operate and explore the use of microcomputer fundamentals, including digital data formatting, transmission and storage, and converting and acquiring data in scientific instrumentation. The book explains how to measure and control analog and digital signals, along with the basics of supplying power to instruments and computers. Readers will find out how to manipulate analog data with operational amplifiers and servo systems and receive an introduction to the principles of servo control and measurement systems. Later chapters describe methods for processing electronic signals and sensing and controlling in automation and robotics. Readers will learn about analog-to-digital and digital-to-anolog converters, signal-to-noise ratio enhancement, and more. The final chapter explains how to troubleshoot instruments. It begins with the basics and from there describes the troubleshooting process; finding a faulty unit; and "fixing it yourself." *Microcomputers and Electronic Instrumentation: Making the Right Connections* is an integral component of a unique electronics learning program that also includes two companion videotapes and a laboratory electronics kit and manual.

WHO Expert Committee on Biological Standardization: 43rd Report. Published by the World Health Organization, Distribution and Sales, 1211 Geneva 27, Switzerland, 1994. 218 pp. Price: Sw. fr.31.-/US $27.90, in developing countries: Sw. fr. 21.70. ISBN 92 4 120840 6.

This book records the work of an expert committee commissioned to coordinate a range of research and other activities needed to ensure that biological products conform to international standards of purity, potency, safety, and stability. The book has two parts. The first opens with a brief discussion of selected issues relevant to the production and quality testing of biologicals, followed by comments on the status and development of international biological standards and reference reagents for various antibiotics, antibodies, antigens, blood products, cytokines and growth factors, and endocrinological substances. The main part of the report issues detailed requirements for the manufacturing and control of selected biologicals. New requirements are provided for Vi polysaccharide typhoid vaccine, and for live combined measles, mumps, and rubella vaccines.

For Your Information

Meetings

September 29–30, 1994: AOAC Europe Section Meeting, Nyon, Switzerland. Contact: T. Rihs, Swiss Federal Research Station for Animal Production, CH-1725 Posieux, Switzerland, telephone +41 37 877 111.

October 24–26, 1994: AOAC Central Section Meeting, Lexington, KY. Contact: Robert L. Beine, University of Kentucky, Kentucky Agricultural Experiment Station, Regulatory Services Building, Lexington, KY 40506, telephone +1 (606) 257-4834.

October 25–26, 1994: AOAC Central Europe (proposed subsection) Section Meeting, Smolenice, Slovakia. Contact: Pavel Farkas, Food Research Institute, Priemyselna 4, PO Box 25, Bratislava, Slovakia, telephone +41 7 61355.

October 27, 1994: AOAC MidAtlantic USA Section Meeting, College Park, MD. Contact: Charles P. Lattuada, U.S. Department of Agriculture, Building 322, BARC-East, 10300 Baltimore Ave, Beltsville, MD 20733, telephone +1 (717) 656-2301.

November 10, 1994: AOAC New York/New Jersey Section Meeting, New Brunswick, NJ. Contact: Paul D. Duke, Hoffman La Roche, 340 Kingsland St. Nutley, NJ 07110, telephone +1 (201) 235-3364.

November 1994: AOAC MidCanada Section Meeting, Winnipeg, Manitoba, Canada. Contact: Jane M.L. Weitzel, Province of Manitoba, Department of Energy and Mines, 745 Logan Ave. Winnipeg, MN, R3E 3L5, Canada, telephone +1 (204) 945-2590.

December 15–16, 1994: AOAC Short Courses, Baltimore, MD. Topic: Laboratory Waste Disposal. Contact AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

February 8–9, 1995: AOAC Southeast USA Section Meeting, Atlanta, GA. Contact: Thomas W. Brooks, South Carolina Department of Agriculture, 1101 Williams St, Columbia, SC 29211, telephone +1 (803) 737-2070.

April 26–28, 1995: AOAC Pacific Southwest Section Meeting, South Lake Tahoe, CA. Contact: Gordon Burns, ETS Laboratories, 1204 Church St, St.

Helena, CA 94574, telephone +1 (707) 963-4806.

September 17–21, 1995: The 109th AOAC INTERNATIONAL Annual Meeting and Exposition, Nashville, TN. Contact: AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

Methods Development in the Field of Food Irradiation

The following article is based on information provided by AOAC Associate Referee for Irradiated Food Leslie G. Ladomery of the International Atomic Energy Agency. It gives the current status of detection methods developed through international cooperative efforts for irradiated food.

The literature abounds with references to published papers on various analytical approaches to the detection of irradiated food. In 1991, the International Atomic Energy Agency (IAEA) published a literature review citing some 500 references (IAEA-TECDOC-587, IAEA, March 1991). Since then, this number has probably doubled. Vast sums of money are being spent by institutes and governments on research in this field.

Among the international organizations actively engaged in this field are IAEA and the Food and Agriculture Organization (FAO), through the Coordinated Research Program on the Detection of Irradiation Treatment of Food (ADMIT), the European Union, through the European Standards Committee and the European Bureau of Reference (BCR), and AOAC INTERNATIONAL.

Food irradiation is a relatively new technology and the identification of irradiated food represents a new and challenging field of research. More importantly, individuals, activist groups, and authorities under the political influence of anti-nuclear action groups claim that food irradiation cannot be authorized in the absence of suitable analytical methods. Some even claim that a general method that would identify all irradiated food and determine the absorbed radiation dose with sufficient accuracy for regulatory purposes is required.

Although several methods are already available with application to a wide range of irradiated food, a "super general" method does not exist and, chances are, will never exist. However, the expectations of a single general method helps explain the intensity of research in the field of food irradiation. Still not a widely accepted technology, food irradiation has only been authorized in some 37 countries and only on a food-by-food basis. It is subject to mandatory maximum, and occasionally minimum, limits for absorbed dose of radiation. Furthermore, irradiated food is subject to rigorous labeling requirements.

Specifically, government authorities require detection (qualitative) methods for (*1*) monitoring their food supply to check compliance with food irradiation regulations, and (*2*) verifying labeling of irradiated or unirradiated food in trade. Some authorities and interest groups also consider quantitative methods essential for the determination of absorbed dose of radiation.

Qualitative Methods

Qualitative methods verify label declarations and monitor the food supply to detect unauthorized applications of food irradiation, either in domestic or import trade. In comparison with the availability of analytical methods for other food, the availability of analytical methods for irradiated food is very good. The actual status of development and availability of detection methods developed through international cooperative efforts is given in Table 1.

Quantitative Methods

Quantitative methods are required because legal maximum limits are set for absorbed energy (from gamma, x-rays, and electrons) based on the dose range required to achieve the intended technological purpose. This practice is similar to setting legal maximum limits for thermal energy in the canning industry (or other forms of heat processing), whereby accurate and collaboratively studied quantitative methods are then required to determine heat absorbed by the food. Chemical, physical, or other changes that the food undergoes would be used as indicators.

In reality, the minimum dose of ionizing energy imparted to the food during irradiation is important for commercial (extension of shelf-life) and public health (elimination of food-borne pathogens) reasons. Furthermore, dose distribution in the consignment (dose uniformity) is more important than absorbed dose in the sample. Although radiation dose determined analytically in the finished product can be used to verify minimum or maximum legal limits, governments only tend to set maximum limits. Quantitative methods of analysis, as well as appropriate sampling plans, are required to enforce legal limits for absorbed radiation dose.

Dose distribution and dose uniformity can only be determined by proper dosimetry, as required by the Codex General Standard. Furthermore, good irradiation practices can only be assured by proper regulatory control and facility inspection, which is required by the Codex General Standard, as well.

Self-Enforcing Nature of Technical Dose Limits

The effectiveness of the process and possible deleterious effects on product

quality are based on the minimum and maximum doses. The maximum limit of 10 kGy overall average (1 kiloGray = 1 Joule radiation energy/kg) set by the Codex Alimentarius Commission for any good is based on the results of years of wholesomeness testing. The vast majority of food products are damaged by doses of radiation far below 10 kGy. This limit is, therefore, inherently self-enforcing, except with dry food ingredients such as spices, herbs, and other such products. Doses higher than 10 kGy are also tolerated by food products if very low temperatures are used during irradiation in the preparation of sterile, shelf-stable products for astronauts, hospitals, etc. Such products are not yet available on the market and wholesomeness recommendation will have to be obtained from the FAO/IAEA/WHO experts for the general marketing of such products.

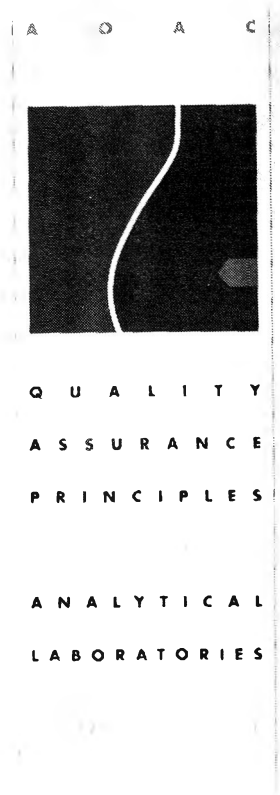
Possibilities for International Standardization of Methods

International standardization of existing detection methods is possible. The

Table 1. Status of research and collaborative trials on identification methods for irradiated food

Method	Food products	Status
Electron spin resonance (ESR)	Spices (paprika, black pepper, pistachio nuts), poultry (bones), fresh eggs (shells)	FAO/IAEA group carried out collaborative trials on spices and is carrying out trials on eggs (shells) and poultry (bones).
	Beef, trout, sardines (bones), pistachio nuts, grapes (seeds), papaya (seeds)	BCR group carried out interlaboratory trials and submitted methods to the European Standards Committee.
Thermo-luminescence (TL)	Fruit (strawberries, mangoes, papayas), vegetables (potatoes, mushrooms), spices, herbs, mixtures of spices and herbs	German group (Bogl et al.) published method and results of interlaboratory trial.
	Various food products (isolated minerals)	FAO/IAEA group is carrying out trials on improved method involving reirradiation normalization procedure.
Viscosity measurement	Black and white pepper	FAO/IAEA group is organizing collaborative trial.
	Black and white pepper, cinnamon, ginger, other spices, onion powder, dried food ingredients, vegetables, fruits, gum arabic, shrimp	German group (Bogl et al.) published method and results of interlaboratory trial.
Lipid volatiles (GLC)	Chicken, pork, beef	German group (Bogl et al.) is publishing method and results of collaborative trial in *J. AOAC*.
	Poultry, fish	FAO/IAEA group is organizing collaborative trial.
Cyclobutanones	Poultry, pork	FAO/IAEA group is organizing collaborative trial.
DNA fragmentation	Chicken (bone marrow cells)	FAO/IAEA group is organizing collaborative trial.
0-Tyrosine	Shrimp	FAO/IAEA group is studying this method.
	Chicken, pork, fish, shrimp	BCR group carried out interlaboratory trials.
Lipid hydroperoxides (LHP)	Meat	FAO/IAEA group is studying this method.
Carbon monoxide	Frozen food	FAO/IAEA group is studying this method.
Electrical impedence	Potatoes (several varieties)	FAO/IAEA group is studying this method in limited interlaboratory trials.
Near infrared (NIR)	Paprika, black and white pepper	FAO/IAEA group is studying this method.
Viscosity measurement	Black pepper	FAO/IAEA group is studying this method in interlaboratory trials.
	Black and white pepper, cinnamon, ginger, onion power	German group (Bogl et al.) is studying this method.
DEFT/APC (microbiological method)	Powered black pepper, whole white pepper, paprika, cut basilicum, cut marjoram, crushed cardamon	BCR group carried out collaborative trial and published method and results in *J. AOAC* (Vol. 76, No. 3, 1993); it should be accepted as an AOAC first action screening method.

ESSENTIAL FOR YOUR QUALITY ASSURANCE PROGRAM



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

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

Contents: Quality Assurance Planning. Statistical Applications and Control Charts. Personnel Considerations. Management of Equipment and Supplies. Sample and Record Handling. Sampling and Sample Analysis. Proficiency and Check Samples. Audit Procedure. Design and Safety of Facilities. Laboratory Accreditation.

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

192 pages. 2nd edition. May 1991. Softbound. ISBN 0-935584-46-3.
$65.00 In North America (USA, Canada, Mexico).
$73.00 Outside North America.
AOAC INTERNATIONAL Members: subtract 10% discount.

To order: Send your name and address and payment. AOAC INTERNATIONAL accepts checks (US funds on US banks only, please) and VISA, MasterCard, American Express, JCB, or Diners credit cards. When paying by credit card please include: type of credit card, card number, expiration date and your signature.

Mail to: AOAC INTERNATIONAL-J
1970 Chain Bridge Road, Dept. 0742
McLean, VA 22109-0742
or
Place credit card orders by phone:
+1-703-522-3032, or FAX: +1-703-522-5468.

The Scientific Association Dedicated to Analytical Excellence®

AOAC® INTERNATIONAL

European Union, through the European Standards Committee, is currently adopting several methods, most of which have been tested in interlaboratory trials and could also be considered by AOAC INTERNATIONAL.

AOAC INTERNATIONAL is also considering some methods with a view to their adoption, and several more methods are currently undergoing collaborative study within the FAO/IAEA Coordinated Research Program. These methods will also be submitted to the AOAC Official Methods Program.

Update Information: Food Irradiation

The Food and Agriculture Organization (FAO) and the International Atomic Energy Agency (IAEA), through the Coordinated Research Programme on the Detection of Irradiation Treatment of Food (ADMIT), involving 22 research institutes, are developing detection methods for irradiated food. During this 5-year research program, several promising methods have been investigated, and a number of them have been tested collaboratively. The final research coordination meeting of ADMIT took place in Belfast, Northern Ireland, June 20–24, 1994, in conjunction with an international meeting on the same subject organized by the Department of Agriculture for Northern Ireland. The joint event was attended by 57 scientists from 46 laboratories and 19 countries. The report from the ADMIT meeting is available from the Food Pre-servation Section of the Joint FAO/IAEA Division, IAEA, Wagramerstrasse 5, A-1400 Vienna, Austria. The proceedings of the joint international meeting will be published by the Royal Society of Chemistry by year end.

Validation Claims of SNAP Beta-Lactam Test Kit Expanded

The AOAC Research Institute expanded the validated performance claims of the IDEXX Laboratories, Inc., SNAP™ Beta-Lactam test kit to include a digital image reader instrument. The SNAP Beta-Lactam test kit was originally certified in October 1993 as part of the joint evaluation of beta-lactam test kits by the U.S. Food and Drug Administration and the AOAC Research Institute. The evaluation was completed in 1993 and validated the performance results of the SNAP Beta-Lactam test obtained with visual observations only.

The new evaluation directly compared the results obtained from visual observations to those obtained using a manufacturer supplied "Image Reader." Analyses were performed for all experiments using both the Image Reader and the visual interpretation method, with one technician analyzing and recording data from visual observations, and a second technician analyzing and recording data using the Image Reader. Technicians did not share analytical results, and analyses were recorded separately.

The comparison evaluation consisted of three studies: a dose-response study, a study on the potential interference caused by somatic cells, and a study on the potential interference caused by bacterial cells. The dose-response and the somatic cell interference studies were conducted at the University of New Hampshire, Department of Animal and Nutritional Sciences. The bacterial interference study was performed by the firm using coded samples prepared by the FDA Center for Food Safety and Applied Nutrition, Laboratory Quality Assurance Branch. Bacterial interference results were delivered to the AOAC Research Institute, de-coded, and reviewed by the Research Institute and the FDA Center for Veterinary Medicine.

Table 1. Dose response comparison

Sample	Visual	Image reader
Control	0% (0/30)	0% (0/30)
Penicillin G (1 ppb)	0% (0/6)	0% (0/6)
Penicillin G (2 ppb)	0% (0/6)	0% (0/6)
Penicillin G (3 ppb)	33% (2/6)	67% (4/6)
Penicillin G (4 ppb)	67% (4/6)	100% (6/6)
Penicillin G (5 ppb)	100% (6/6)	100% (6/6)
Penicillin G (6 ppb)	100% (6/6)	100% (6/6)
Cephapirin (2 ppb)	0% (0/6)	0% (0/6)
Cephapirin (4 ppb)	83% (5/6)	83% (5/6)
Cephapirin (6 ppb)	100% (6/6)	100% (6/6)
Cephapirin (8 ppb)	100% (6/6)	100% (6/6)
Cephapirin (10 ppb)	100% (6/6)	100% (6/6)

Table 2. Selectivity and sensitivity

Sample	Visual	Reader
Zero control milk	0% (0/30)	0% (0/30)
Penicillin G (5 ppb)	97% (29/30) 100% (60/60)[1]	100% (30/30)
Cephapirin (8 ppb)	100% (30/30)	100% (30/30)

[1] An additional 60 samples were analyzed according to testing protocol. SNAP™ Beta-Lactam test kit meets sensitivity requirements.

Table 3. Interference from somatic cells

Sample	Visual	Reader
Zero control milk	0% (0/30)	0% (0/30)
Penicillin G (5ppb)	100% (30/30)	100% (30/30)

Dose-Response Comparison

Analytical results from visual observations were compared to those obtained from the Image Reader by devel-

More Publications from AOAC INTERNATIONAL...

Statistical Manual of the AOAC.
93 pages. 1975. Softbound. ISBN 0-935584-15-3.
$34.00* in North America (USA, Canada, Mexico)
$38.00* outside North America
Do-it-yourself statistical techniques for interlaboratory collaborative tests.

Use of Statistics to Develop and Evaluate Analytical Methods.
xvi + 183 pages. 1985. Softbound.
ISBN 0-935584-31-5.
$65.00* in North America (USA, Canada, Mexico)
$68.00* outside North America
A basic reference for evaluating collaborative studies and a natural extension to the Statistical Manual of the AOAC.

FDA Food Additives Analytical Manual (FAAM).
2 volumes. Softbound.
Volume I: 364 pages. 1983. ISBN 0-935584-22-6.
Volume II: xiv + 346 pages. 1987.
ISBN 0-935584-34-X.
$139.00* in North America (USA, Canada, Mexico)
$157.00* outside North America
Provides analytical methodology for determining compliance with food additive regulations.

Classification of Visible Defects Poster.
1984. 24" x 36" chart. In color with photographs.
ISBN 0-935584-27-7.
Single chart: $19.00* in North America (USA, Canada, Mexico), $24.00* outside North America.
Package of 10 charts: $55.00* in North America (USA, Canada, Mexico), $63.00* outside North America.
For rapid identification of can defects.

Classification of Visible Flexible Package Defects Poster.
1989. 24" x 36" chart. Two-sided. In color with photographs. ISBN 0-935584-39-0.
One chart: $29.00* in North America (USA, Canada, Mexico), $34.00* outside North America. Package of 10 charts: $89.00* in North America (USA, Canada, Mexico), $97.00* outside North America.
For rapid identification of flexible package defects.

Principles of Food Analysis for Filth, Decomposition, and Foreign Matter — FDA Technical Bulletin No. 1, Second Edition.
1981. 286 pages. Softbound. ISBN 0-935584-33-1.
$65.00* in North America (USA, Canada, Mexico)
$73.00* outside North America
A comprehensive source of information on food sanitation analysis.

Key for Identification of Mandibles of Stored-Food Insects.
1985. vi + 166 pages. Softbound. ISBN 0-935584-32-3.
$66.00* in North America (USA, Canada, Mexico)
$71.00* outside North America
Enables food sanitation analysts to identify species from all major stored-food insect pest groups.

***Members: subtract 10% discount**

To order your AOAC publications, send your name and address along with your check (US funds on US banks only, please) or credit card information (VISA, MasterCard, American Express, Diners Club, or JCB). When paying by credit card please include: type of credit card, card number, expiration date and your signature.

Mail to:
AOAC INTERNATIONAL-J
1970 Chain Bridge Road
Dept. 0742
McLean, VA 22109-0742, USA

OR

Place credit card orders by phone:
+1-703-552-3032
or FAX:
+1-703-522-5468.

The Scientific Association Dedicated to Analytical Excellence®

AOAC INTERNATIONAL

oping a concentration-response curve for penicillin G and cephapirin (see Table 1).

Six samples of zero control milk fortified with either penicillin G or cephapirin at six concentrations were independently analyzed using the visual interpretation method and the Image Reader. Samples were blinded and randomized with regard to antibiotic concentration. All analyses were performed strictly according to directions provided by the manufacturer, and samples were analyzed over 2 days.

The results of the dose response study were accepted as demonstrating substantial equivalence between the results obtained from visual observations and those obtained using the Image Reader.

Sensitivity and Selectivity

Sensitivity and selectivity attributes were studied as a part of the dose-response study. Thirty samples of milk verified to contain no antibiotics, 30 samples of milk fortified with 5 ppb of penicillin G, and 30 samples of milk fortified with milk fortified with 8 ppb of cephapirin were randomly interspersed with dose response samples.

Beta-lactam test kits are required to demonstrate at least 90% sensitivity with a minimum 95% confidence level for each claimed beta-lactam drug, and 90% selectivity with a minimum 95% confidence to obtain "Performance Tested" certification. Test kits meet this criteria if no failures occur (e.g., false-negative or false-positive results in 30 trials). Test kits fail if three or more failures are obtained from the initial trial of 30 samples. Test kits that yield one or two failures from the initial trial require additional testing. Additional analyses are performed on a new, independent series of 60 samples. Test kits fail if two or more failures occur in the second set of 60 samples.

The independently conducted testing demonstrated that the results of the Image Reader are equivalent to those of the visual interpretation method, and that both methods meet or exceed minimum sensitivity and selectivity requirements (see Table 2).

Interference from Somatic Cells

This experiment was designed to ascertain the affects of high levels of somatic cells (10^6/Ml) in bulk tank milk on the sensitivity and selectivity performance of test kits.

Thirty samples of milk contaminated with 10^6/Ml somatic cells were fortified with 5 ppb of penicillin G and combined with 30 unfortified samples contaminated with 10^6/Ml somatic cells. The samples were combined and randomized with regard to the presence or absence of antibiotic.

Results indicated that high levels of somatic cells did not affect the performance of the SNAP™ Beta-Lactam test kit, and that the results using the Image Reader were equivalent to those of the visual interpretation method (see Table 3).

Interference from Bacterial Cells

The objective of this study was to examine test kit performance in the presence of bacterial contamination that may be encountered by users of test kits. The FDA Center for Food Safety and Applied Nutrition, Laboratory Quality Assurance Branch, prepared penicillin G and cephapirin fortified milk samples contaminated with mixtures of gram positive and gram negative bacterial strains that have recently been isolated from lactating cattle. These samples were coded and shipped to the manufacturer who assayed five portions of each sample.

Data submitted by IDEXX Laboratories, Inc., correctly identified the presence or absence of antibiotic drugs with no discrepancies between the two interpretation methods. Results from the bacterial interference study also supported the removal of a cautionary statement on package inserts that high levels of bacteria may produce false-positive results.

New Packaging Method Tested to Improve Standard Reference Material Availability

The following article is based on information provided by James T. Tanner and William Horwitz of the FDA Center for Food Safety and Applied Nutrition in Washington, DC, and previously distributed by the International Organization for Standardization. It reports on a new packaging method for standard reference materials.

Two years ago, FDA scientists prepared, spray-dried, and packaged an infant formula under nitrogen in individual packets weighing approximately 30 g each. Since then, these packets have been stored and routinely analyzed. Shelf life appears good, but testing is still being carried out.

If successful, this method of packaging could be applied to other organic compounds to ensure that their nutrient contents are stable for a "reasonable" time. These compounds could then be produced and made available as standard reference materials.

Methods Provide Only Half of the Requirement

Standard reference materials are necessary for regulatory agencies because methods "provide only half of the requirement." In its regulatory programs, FDA uses AOAC methods, as well as methods developed by the American Association of Cereal Chemists, the American Oil Chemists Society, the International Organization for Standardization, and others. However, in addition to well-studied methods, some means of determining that the methods were performed correctly is also necessary.

Obtaining acceptable results with validated methodology for a reference material that has a known concentration

of the analyte and is similar in composition to the material being analyzed is presumptive evidence that the method was performed correctly. This combination provides a strong case that the results obtained for the test materials are correct. Reference materials, for which the true values are known, are important for this validation. From a regulator's point of view, the use of appropriate reference materials is desirable for determining compliance with existing regulations.

Reference materials are also necessary to determine the systematic error of new methods. Previously, some AOAC methods had used standard additions for checking the presence of method bias, when material of known concentration was not available. Although this technique is useful under some conditions, it really only measures that analyst's ability to recover what was added at the measurement stage and not their ability to determine what was in the matrix. For this reason, the technique of standard additions may give unreliable information.

The determination of precision is frequently used as a measure of the success of a method because of the ability of a laboratory to obtain the same values, as well as to replicate the results of other laboratories. This is an important part of method evaluation but does not address the accuracy question.

Unfortunately, reference materials are not available for many products and analytes. The main focus has been the major and trace elements and not the various organic compounds that are the major components of food. The main reason for this focus has been due to the fact that organic components change with time and, until now, have not been shelf stable. Therefore, the exact "true" concentration at the time of use could not be assigned.

Shelf Life Is One of Two Problem Areas

The AOAC Task Force On Methods For Nutrient Labeling Analyses was formed by AOAC INTERNATIONAL to determine the availability of methods to enforce the Nutrition Labeling and Education Act. It reported that the useful shelf life of standard reference materials is one of two problem areas that must be resolved before reference materials for organic nutrients content can be made available. The other problem focused on the selection of matrix materials to represent many different foods.

The task force addressed the latter question in a creative way. Food is composed basically of only three essential components: protein, carbohydrate, and fat. Frequently, analysis of a food is not successful because of interference from one or more of these components. If food composition were plotted within a triangle with 100% fat, 100% protein, and 100% carbohydrate at the respective vertices with the concentration of each component decreasing as the side of the triangle opposite the vertex is approached, then the composition of food could be divided into nine different groups, each encompassing a range of concentrations of the three components (protein, carbohydrate, and fat). If a method of analysis were successful for the nine different groups, then it should be applicable to all types of food. Such a method could then be accepted as applicable to food in general rather than to the specific matrix materials for which the method was studied, as is the current AOAC requirement.

The logical extension of this approach would be to have reference materials available for each of the nine groups. An appropriate reference material would be provided for each food type or category. By using the nine different groups as part of the method-performance study and having a reference material for each group, all foods would have a method and a reference material similar to the actual food that could be used for regulatory purposes. This type of verification is part of the infant formula program.

In the case of infant formula, methods of analysis have been developed and collaboratively studied because infant formula is the most highly regulated food in the United States today. It represents the sole source of nutrition for a large segment of the population, namely, infants. As part of the Infant Formula Act of 1980, companies are required to manufacture formula within specified limits, and FDA is required to monitor the formulas to ensure that they are within those limits. Because of differences in methodology, many questions have arisen as to the "true" concentrations of some analytes in the products. Currently, there are methods for infant formulation that both industry and FDA have agreed are to be used for regulatory analyses. These methods are now part of AOAC's Official Methods of Analysis and have been collaboratively studied by FDA, infant formula manufacturers, and several commercial laboratories. However, no reference material is currently available for validating method performance in each laboratory.

Therefore, the first priority for FDA scientists is to produce reference materials for the nine food groups named above and for products in areas where a critical need exists for reliable analyses, such as medical foods. A reference analytical method of known reliability coupled with a standard reference material to monitor analytical performance is the most important requirement for a regulatory agency. With results produced by using this combination, the agency can proceed with appropriate regulatory action that is based on sound analytical science.

New Products

New PathoScreen Medium Detects Waterborne Pathogens

Detect fecal contamination within 24 to 48 h with new PathoScreen medium. PathoScreen is a reliable, inexpensive medium that is well-suited for monitoring drinking water systems in developing tropical countries, in remote field locations, and disaster or emergency situations. You can easily detect hydrogen-sulfide producing bacteria in drinking water, surface water, and recreational water. PathoScreen detects *Salmonella, Proteus, Klebsiella, Citrobacter, Clostridium, Edwardsiella*, and other hydrogen-sulfide producing organisms proven to be associated with fecal contamination and the presence of coliforms. In most tropical climates, indigenous *E. coli* produces positive reactions when traditional coliform tests are used. These positive reactions may not indicate fecal contamination. However, indigenous *E. coli* do not interfere with the PathoScreen test, which makes it an excellent alternative to coliform testing. PathoScreen medium is ready-to-use and packaged in single-dose pillows for either Presence/Absence or Most Probable Number testing. Just pour a PathoScreen medium pillow into the sample, incubate, and read the results. Positive results are easily identified by the formation of a black solution. You do not need an incubator, just keep samples at a constant temperature. Hach Co.
Circle No. 342 on reader service card.

New Autosampler Vials Offered with Five Different Caps

Thermo Separation Products offers a complete new line of autosampler vials with options for every application. The line consists of five different vials types: screw and crimp-cap vials, shell vials, caps with pre-loaded septa, and microvolume inserts. Amber glass is also available for light-sensitive samples. A re-sealable convenience package prevents vial contamination. The vials have undergone an extensive testing process by the company's quality control engineers. They are designed and tested to be fully compatible with all Spectra SYSTEM, SpectraSERIES, and SP88XX autosamplers for guaranteed reliability. Thermo Separation Products.
Circle No. 343 on reader service card.

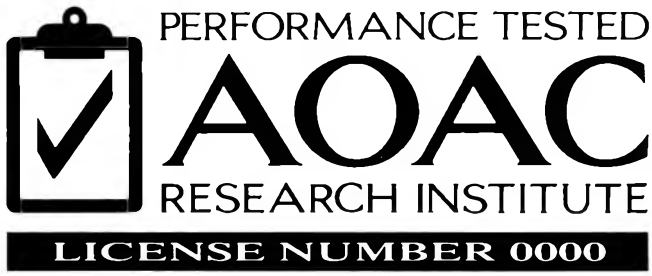
GENESIS Series of Robotic Sample Processors

The new GENESIS series of robotic sample processors (RSPs) has been developed by TECAN as a platform for both current and future laboratory automation projects. TECAN's proven expertise and the application of new engineering technologies provide high throughput, outstanding flexibility, safe operation, and a wide range of new features. Modular development of the GENESIS series provides systems optimized for cost-effective performance in every laboratory. Instruments with four or eight tip pipetting arms are available. Any combination of Teflon-coated stainless steel tips and disposable tips (200 µL and 1000 µL; with or without filters) can be chosen. Both series of disposable tips can be used in the same run without changing the system hardware. The GENESIS series worksurface is designed for unlimited flexibility. Microplates, deep well plates, tube racks, sample tube strips, reagent bottles, disposal tip racks, and a wide range of other carriers can be positioned by the operator anywhere on the worksurface. Reliability is ensured by the GENESIS series' advanced positive identification system. The system reads both horizontal and vertical bar codes to identify and locate sources (e.g., sample tubes) and destinations (e.g., microplates) in any position on the worksurface. Further reliability is provided by liquid level detection that is effective with disposable as well as standard tips. The sensitivity of liquid level detection is adjustable for optimized performance both with conductive liquids and common nonconductive liquids such as DMSO. Integrated sensors check that disposable tips are properly attached and remain attached during pipetting and arm movement. Vertical tip movement is controlled to allow piercing of caps on tubes but prevent injury to operators. Advanced software in a Windows environment makes even the most complex liquid handling procedures easy to control. Day-to-day operation is simple through the graphical user interface. A revolutionary new process implementation and execution method makes programming straightforward. Integration of specialized user functions into the system software is simple and allows full customization of the GENESIS series RSPs to individual applications. TECAN US.
Circle No. 344 on reader service card.

New Membrane Air Dryer from Balston

Balston, Inc. has synergized its superior coalescing technology with a proven, innovative membrane system that will supply oil and particulate free, dry compressed air to dew points as low as −40°F. This new technology offers a reliable, efficient, and economical alternative to pressure swing and refrigerant dryer technologies as there are no moving parts and no electrical requirements. Water vapor from the compressed air supply passes through the membranes' hollow fibers. At the same time, a small portion of the dry air product is redirected along the length of the fibers to sweep out the water vapor, which has permeated the membrane. The moisture-laden sweep gas is then vented to the atmosphere and clean dry air is sup-

When it Comes to Test Kits, Look For This Mark...



To Ensure AOAC Research Institute Testing and Certification

The AOAC Research Institute provides **third party validation of product performance claims** through the Institute's test kit performance testing program.

Why use a *"PERFORMANCE TESTED"* certified test kit?

You can be sure the TEST KIT PERFORMANCE CLAIMS have been independently validated for:

- ✓ sensitivity
- ✓ calibration curve
- ✓ comparison to other methods
- ✓ precision
- ✓ cross reactivity
- ✓ accuracy
- ✓ ruggedness
- ✓ selectivity
- ✓ detection limits

You can be sure that *TEST KIT LABELS & DESCRIPTIVE INSERTS* have also been independently validated

- ✓ Validated instructions for use ensure consistent results
- ✓ Inclusion of important information on labels and inserts enables users to make better informed decisions about appropriate use of a test kit
- ✓ Consistent labeling and insert format enables users to easily compare performance characteristics among "*PERFORMANCE TESTED*" certified test kits
- ✓ Annual validation and review of labels and inserts ensures continued accuracy of product claims

"PERFORMANCE TESTED" certified test kits are recognized

- ✓ FDA's Center for Veterinary Medicine recognizes *"PERFORMANCE TESTED"* certified test kits for official surveillance of milk for antibiotics
- ✓ USDA's Federal Grain Inspection Service and the American Association of Cereal Chemists recognize the AOAC Research Institute's Test Kit Performance Testing Program

How can you use *"PERFORMANCE TESTED"* certified test kits?

- ✓ All routine food processing surveillance and monitoring programs

For more information, contact the AOAC Research Institute:

2200 Wilson Boulevard, Suite 400 • Arlington, VA 22201-3301 USA
Phone: +1-703-522-2529 or Fax: +1-703-522-5468

plied to the application. The lightweight, compact membrane air dryer can be easily mounted on an existing air line. A high efficiency coalescing prefilter is installed directly upstream from the dryer module to protect the membrane from potential contamination caused by pipe scale, liquids, or other solids. The only maintenance required to keep the system operational is changing the prefilter cartridge once each year—a 5 min procedure. Applications include: low dew point instrument air, pneumatic equipment, pressurizing electronic cabinets, analytical instrumentation, prevention of freeze-ups, dry air for hazardous areas, and general laboratory air supply. Balston, Inc.
Circle No. 345 on reader service card.

USEPA—Accepted Phosphorus Method for Wastewater Testing

Hach's new Test 'N Tube phosphorus method is now USEPA-accepted for NPDES compliance monitoring. Operators can use the Test 'N Tube method to report both total phosphorus and orthophosphate results. The method is easier to use than traditional methods and requires less apparatus than any other phosphorus method currently available. Hach's Test 'N Tube phosphorus system enables operators to monitor phosphorus throughout the wastewater treatment process. Operators can detect from 0 to 5.00 mg/L total phosphorus and orthophosphate. For maximum convenience and economy, operators can reuse vials or dispose of them after analysis. Hach offers the space-saving COD Reactor for digesting phosphorus samples. The reactor holds up to twenty-five 16 mm Test 'N Tube vials, and replaces the hot plates and digestion flasks used in traditional methods. Operators prepare, digest, and measure sample concentrations in a single vial. For colorimetric measurement of digested samples, Hach offers a choice of several colorimeters and spectrophotometers preprogrammed with the calibration data for the Test 'N Tube phosphorus method. No calculations or graphs are required. Hach Co.
Circle No. 346 on reader service card.

Columbus Washer

The Columbus washer offers the best combination of wash features in a unique, compact case design. A completely contained liquid system allows the choice of any bottle type or size, and eliminates the need for external vacuum pumps that take additional bench space. Selectable dispense and aspiration rates, and wash head speed, provide the capability for gentle cell washing as well as robust wash procedures. Up to four different wash liquids can be selected automatically to permit the use of four different wash reagents without changing bottles and reconnecting tubing for different wash buffers and rinse solutions. Onboard software features a simple step-by-step menu structure and stores up to 30 programs. Easy to use and maintain, Columbus offers the selection of features you need in a microplate strip washer, at an affordable price. TECAN US.
Circle No. 347 on reader service card.

Azlon Distinction Water Still

Thomas Scientific is now offering a new compact water still with highly desirable features at an economical price. Twin thermostats provide added protection in case of feed water failure or boiler rupture. The heating element is silica sheathed for more even boiling and easier cleaning. Fast output of up to 4 Lph. The Distinction water still also meets the most recent international electrical safety standards. Thomas Scientific.
Circle No. 348 on reader service card.

Ohaus Precision Advanced Balances Assure GLP, Provide Five-Year Warranty

Ohaus Corp.'s precision advanced series of toploading balances has combined greatly enhanced performance capabilities and a new GLP protocol with a new 5-year warranty against defects in materials or workmanship. According to a company spokesman: "Toploading balances that cost a thousand dollars or more don't deliver a better audit trail for verification and none are guaranteed to do it longer." For customers required to comply with external standards or regulations, the selectable GLP protocol provides time and date, calibration data, a unique balance ID number, and more. The precision advanced series also features memory capacity for 255 values used with the statistics function. An additional dozen new features include typical applications such as filling, formulating, and animal weighing, plus auto tare, net/gross, volume determination, and more. All are covered under the 5-year warranty. Ohaus Corp.
Circle No. 349 on reader service card.

New 3M Extraction Disks for Oil and Grease Eliminate Freon

A new method for performing routine monitoring of effluent water for oil and grease contamination, eliminating the use of Freon, has been developed by 3M. The 3M Emporc solid phase extraction disks for oil and grease and total petroleum hydrocarbons (TPHs) meet the proposed new EPA method 1664, which will replace EPA method 413.1 that authorized gravimetric methods using Freon 113. The proposed method approves the use of *n*-hexane as the extraction solvent for both solid-phase extraction (SPE) and liquid-liquid extraction (LLE) and is expected to go into effect January 1, 1995. EPA method 1664 will

be one of the first performance-based methods permitting method modification to overcome interferences or lower the cost of measurements. When compared to LLE, Empore extraction disks for oil and grease are much easier to use both in terms of setup and extraction time. The disk extraction can be run in about 10 min in contrast to a minimum of 35 to 45 min/extraction for LLE. Because emulsions can commonly form between the aqueous and organic layers in an LLE separatory funnel, total sample preparation time can easily extend beyond an hour when you include the procedure to break up the emulsion. Emulsions are eliminated by the Empore extraction disk. Another benefit of the new extraction disk is that it reduces solvent use to approximately 40% of that required for LLE extractions. Only minimal disk preparation is required, with 25 to 30 mL of solvent used during the elution step. Total solvent use for the LLE method can easily exceed 100 mL for the total procedure, including washing and rinsing the apparatus. The 3M Empore extraction disks for oil and grease provide a high rate of flow and excellent recoveries because the thin, 0.5 mm, porous membranes consist of C-18 silica particles enmeshed in an inert, stable, uniform, nonpolar, and very high density fibril matrix that provide a high degree of separating power. During the extraction process, dissolved analytes are adsorbed onto the bonded silica and then eluted for analysis. The disk will extract 5 to 1000 mg of organic material with no loss of recovery. They are available in 47 and 90 mm sizes from J.T. Baker, Inc., Fisher Scientific International. and Varian Sample Preparation Products. 3M.
Circle No. 350 on reader service card.

Fully Automated Procedure for LC Determination of Aldehydes in Drinking Water

A new application note from Thermo Separation Products describes a fully automated procedure for LC determination of trace levels of aldehydes in drinking water. The procedure reduces the total analysis time of EPA method 8315 from 2 h to less than 30 min by completely automating sample cleanup, SPE preconcentration, derivatization, and analysis steps. Additional benefits of the procedure over the manual EPA approach include an improved method detection limit of less than 1 ppb for formaldehyde and acetaldehyde and a significant reduction in the solvent waste generated. Thermo Separation Products.
Circle No. 351 on reader service card.

Misonix Mystaire Ductless Fume Enclosure Video

A new video is available from Misonix on the company's complete line of Mystaire ductless fume enclosures. Designed for use in clinical, biomedical, research, industrial, forensic, and academic laboratories, Mystaire fume enclosures are available in six standard models in widths of 24, 36, and 48 in. Custom configurations are also available. Misonix.
Circle No. 352 on reader service card.

COMING IN THE NEXT ISSUE

DRUGS, COSMETICS, FORENSIC SCIENCES
Reversed-phase Liquid Chromatographic Determination of Cromolyn Sodium in Drug Substance and Select Dosage Forms—Linda L. Ng

FOOD BIOLOGICAL CONTAMINANTS
Development of a New Series of Agricultural/Food Reference Materials for Analytical Quality Control of Elemental Determinations—Milan Ihnat

FOOD CHEMICAL CONTAMINATS
Liquid Chromatographic Determination of Nifursol in Concentrates, Premixes, and Finished Turkey Feeds—Ellen De Vries, Richard C. Bas, and Henny Kuil

FOOD COMPOSITION AND ADDITIVES
Radionuclides in Domestic and Imported Foods in the United States, 1987–1992—William C. Cunningham, David L. Anderson, and Edmond J Baratta

RESIDUES AND TRACE ELEMENTS
Evaluation of Two Field Screening Test Kits for the Detection of PCBs in Soil by Immunoassay—Larry C. Waters, Rob R. Smith, Joe H. Stewart, Roger A. Jenkins, and Richard W. Counts

PESTICIDE PROGRAM

RESIDUE MONITORING

1993

FOOD AND DRUG ADMINISTRATION

This is the seventh annual report summarizing the results of the Food and Drug Administration's (FDA) pesticide residue monitoring program. The 6 previous reports, which were published in the *Journal of the Association of Official Analytical Chemists/Journal of* AOAC INTERNATIONAL, presented results from Fiscal Years (FY) 1987 through 1992. The present report includes findings obtained during FY93 (October 1, 1992 through September 30, 1993) under regulatory and incidence/level monitoring. Selected Total Diet Study results for 1991-1993 are also included. The findings from FY93, as in previous years, continue to indicate that the levels of pesticide residues found in the U.S. food supply are generally well below established limits.

FDA Monitoring Program

Three federal government agencies share responsibility for the regulation of pesticides (1). The Environmental Protection Agency (EPA) registers (i.e., approves) the use of pesticides and sets tolerances (the maximum amount of a residue that is permitted in or on a food) if use of that particular pesticide may result in residues in or on food (2). Except for meat, poultry, and certain egg products, for which the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) is responsible, FDA is charged with enforcing tolerances in imported foods and in domestically produced food shipped in interstate commerce. FDA also acquires incidence/level data on particular commodity/pesticide combinations and carries out its annual market basket survey, the Total Diet Study. In 1990, USDA's Agricultural Marketing Service (AMS) initiated, through contracts with participating states, a residue testing program directed primarily at raw agricultural products (fruits and vegetables). FSIS and AMS pesticide residue data are reported independently by those agencies.

Regulatory Monitoring

One aspect of FDA's monitoring program involves the sampling of individual lots of domestically produced and imported foods and analysis of these foods for pesticide residues. Domestic samples are collected as close as possible to the point of production in the distribution system; import samples are collected at the point of entry into U.S. commerce. Emphasis is on the raw agricultural product, which is analyzed as the unwashed, whole, raw commodity, that is, with the peel or skin intact. Processed foods are also included. If illegal residues (above EPA tolerance or no tolerance for that particular food/pesticide combination) are found in domestic samples, FDA can invoke various sanctions, such as a seizure or injunction. For imports, shipments may be stopped at the port of entry when illegal residues are found. "Automatic detention" may be invoked for imports based on the finding of 1 violative shipment if there is reason to believe that the same situation will exist in future lots during the same shipping season for a specific shipper, grower, geographic area, or country.

The food samples collected are classified as either "surveillance" or "compliance." Most samples that FDA collects are the surveillance type; that is, there is no prior knowledge or evidence that a specific food shipment contains illegal pesticide residues. Compliance samples are taken as follow-up to the finding of an illegal residue or when there is other evidence of a pesticide residue problem.

FDA establishes monitoring priorities through development of an annual National Sampling Plan, which is a compilation of 6 Regional Sampling Plans prepared by FDA personnel throughout the United States. Each annual Plan is designed to provide for sampling and pesticide residue analyses of domestic and imported foods of dietary importance, including products from approximately 80 countries, all 50 states, and Puerto Rico. Some deviations from the Plan may occur depending on circumstances in the individual FDA Districts.

Factors considered in preparing the Sampling Plan include review of recently generated FDA residue data and those produced by the states, regional intelligence on pesticide use, dietary importance of the food, information on the amount of imported food and domestic food that enters interstate commerce, chemical characteristics and toxicity of the pesticide, and production volume/pesticide usage patterns.

ANALYTICAL METHODS

To analyze the large numbers of samples whose pesticide treatment history is usually unknown, analytical methods capable of simultaneously determining a number of pesticide residues are used. These multiresidue methods (MRMs) can determine about half of the approximately 300 pesticides with EPA tolerances, and many others that have no tolerances. The most

commonly used MRMs can also detect many metabolites, impurities, and alteration products of pesticides with and without tolerances (3).

Single residue methods (SRMs) or selective MRMs are used to determine pesticides not covered by an MRM (3). An SRM usually determines 1 pesticide; a selective MRM measures a relatively small number of chemically related pesticides. These types of methods are usually more resource-intensive per residue, and they may require at least as much time to perform as an MRM. They are much less cost efficient than MRMs.

The lower limit of residue measurement in FDA's determination of a specific pesticide is usually well below tolerance levels, which generally range from 0.1 to 50 parts per million (ppm). Residues present at 0.01 ppm and above are usually measurable; however, for individual pesticides, this limit may range from 0.005 to 1 ppm. In this report, the term "trace" is used to indicate residues detected, but at levels below the limit of quantitation.

FDA/STATE COOPERATION

Personnel in FDA field offices interact with their counterparts in most states to carry out more effective pesticide residue monitoring. The extent of these cooperative efforts varies among the states and depends on the size and scope of the pesticide program in the individual states, i.e., states in which agriculture is a major industry tend to have greater resources and more personnel devoted to agriculture-related programs.

FDA also acquires and uses state-generated pesticide residue data to complement its own and other federally sponsored residue programs. For many years, FDA has supported, through a contract with Mississippi State University (MSU), the "Foodcontam" database, which is a compilation of state-collected residue data.

ANIMAL FEEDS

In addition to monitoring foods for human consumption, FDA also samples and analyzes domestic and imported feeds for pesticide residues. This monitoring is carried out under the direction of FDA's Center for Veterinary Medicine (CVM) via its Feed Contaminants Compliance Program.

CVM also reviews pesticide residue data supplied by various states under "Feedcon," a database operated by MSU under the auspices of the Association of American Feed Control Officials. These data are reviewed periodically by CVM so that potential problems stemming from pesticide residues in foods of animal origin may be identified.

INTERNATIONAL ACTIVITIES

FDA has obtained information on foreign pesticide usage via contract for several years. Under the current contract with Landell Mills (Bath, England), FDA receives pesticide usage data each year for about 30 countries that export food to the United States. These data allow FDA to more accurately target particular pesticide/commodity/country combinations for monitoring.

In 1993, FDA continued to work with foreign governments and food producers to promote pesticide usage practices for foreign-grown foods that are consistent with U.S. registrations and tolerances. In some of these activities, FDA worked closely with staffs from EPA, USDA, and the Agency for International Development (AID) to assist foreign producers and governments in developing countries in understanding the U.S. pesticide regulatory system and in establishing their own pesticide regulatory infrastructure.

These programs were focused primarily on Central America. The activities included work with Guatemalan government and industry officials to establish pesticide usage control and oversight for snowpeas and other vegetables to ensure better compliance of Guatemalan exports with U.S. tolerances. In addition, a multiagency effort by FDA, EPA, and AID was continued throughout Central America to establish new analytical laboratories and improve existing ones so that Central American food exports to the United States and elsewhere could be monitored for compliance with residue tolerances.

For several years, FDA and other U.S. agencies have worked with the Organization of American States' Inter-American Institute for Cooperation on Agriculture (IICA), which is headquartered in San Jose, Costa Rica, to promote the development of regulatory infrastructures in the Americas. In 1993, IICA conducted a technical/regulatory seminar series for Andean countries in Quito, Ecuador. Similar training for Caribbean countries is in process.

Canada, Mexico, and the United States continued their longstanding collaboration on pesticide issues. FDA and the other agencies worked extensively on a bilateral basis with both Canada and Mexico to resolve pesticide residue issues, both for food imports into the United States and U.S. exports to these countries. U.S.-Canada pesticide issues were addressed under the auspices of the Canada-United States Trade Agreement's Technical Working Group on Pesticides; U.S.-Mexico issues were covered by FDA, EPA, and USDA in discussions with Mexico's Comision Intersecretarial para el Control del Proceso y Uso de Plaguicidas, Fertilizantes y Substancias Toxicas, known as CICOPLAFEST.

In another area, FDA worked with the government of New Zealand to develop a New Zealand export certification program for selected commodities designed to ensure that only U.S.-registered pesticides are used in their production. This program reflects FDA's recognition of the effectiveness of New Zealand's pesticide usage control procedures, which have resulted in excellent compliance with U.S. tolerances.

FDA also provided technical and regulatory input on pesticide-related activities to Japan and South Korea concerning U.S. exports of rice and wheat to those countries.

Incidence/Level Monitoring

A complementary approach to regulatory monitoring, incidence/level monitoring is used to add to FDA's knowledge about certain pesticide/commodity combinations by analyzing particular types of foods to determine the presence and levels of selected pesticides. In 1993, surveys of important aquaculture products and milk were conducted under incidence/level monitoring. In addition, a statistically based monitoring survey of pears and tomatoes, which had been initiated in 1992, was completed.

FDA data acquired under regulatory monitoring are extensive; however, they are not statistically representative of the overall residue situation for a particular pesticide, commodity, or place of origin. In FDA's surveillance sampling for pesticide residues, sampling bias may be incurred by weighting sampling toward such factors as commodity or place of origin with a history of violations or large volume of production or import shipments. In addition, the total number of samples of a given commodity analyzed for a particular pesticide each year may not be sufficient to draw specific conclusions about the residue situation for the whole volume of that commodity in commerce.

For the statistically based monitoring project, pears and tomatoes were chosen as test commodities because each has a significant domestic and import component, is consumed raw (and often unpeeled), is available year round, and has tolerances for about 90 different pesticide chemicals. The objective of the survey was to determine whether violation rates, frequency of occurrence of residues, and residue levels obtained from such a sampling regimen differed from those obtained through FDA's traditional surveillance approach.

Most FDA Districts participated in collecting the samples. Analyses were performed by the Buffalo (pears) and Minneapolis (tomatoes) District Laboratories. The goal was to collect and analyze about 1600 pear samples and 1600 tomato samples (800 domestic and 800 import of each) during the project.

FIGURE 1. SUMMARY OF RESULTS (DOMESTIC) BY COMMODITY GROUP OF 1993 SAMPLE ANALYSES FOR PESTICIDE RESIDUES *(SURVEILLANCE SAMPLES ONLY)*

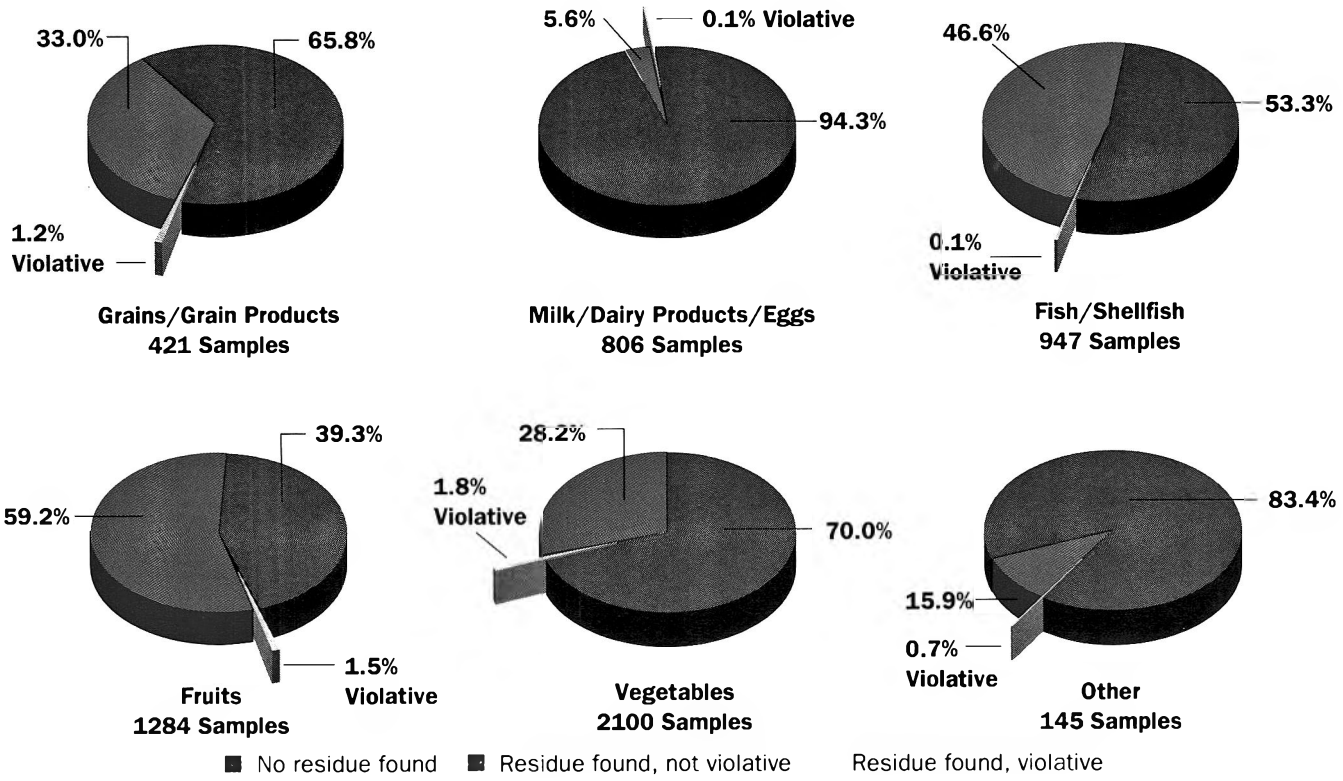
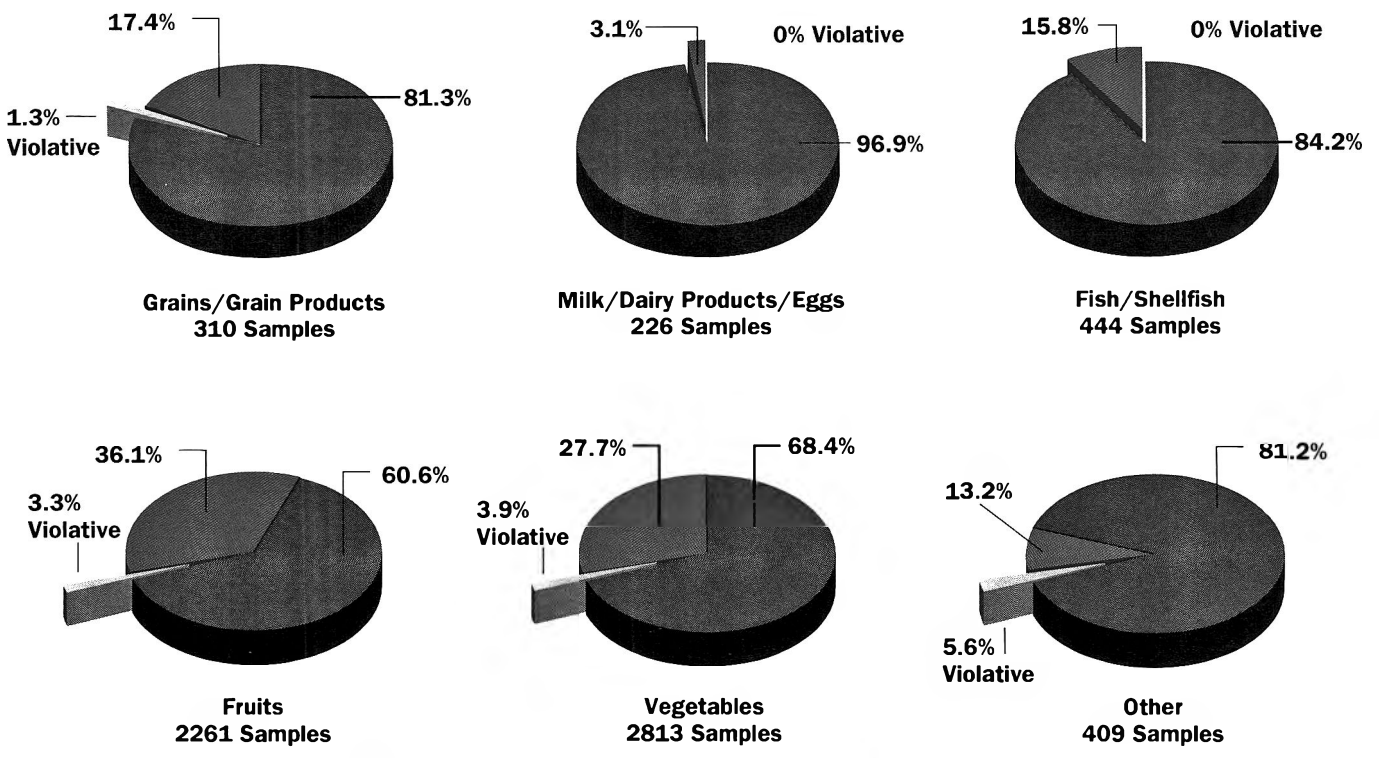


FIGURE 2. SUMMARY OF RESULTS (IMPORT) BY COMMODITY GROUP OF 1993 SAMPLE ANALYSES FOR PESTICIDE RESIDUES *(SURVEILLANCE SAMPLES ONLY)*



Total Diet Study

The Total Diet Study is another major element of FDA's pesticide residue monitoring program (4). In its 6 previous annual pesticide reports (5-10), FDA provided Total Diet Study findings for 1987-1991. In September 1991, FDA implemented revisions to the Total Diet Study that were formulated in 1990 (11). These revisions primarily consisted of collection and analysis of an updated and expanded number (currently 261) of food items, addition of 6 age/sex groups (for a total of 14), and revised analytical coverage. The 1987-88 USDA Nationwide Food Consumption Survey was utilized to revise the food list and diets (11). Additional details of the recent revision are presented elsewhere (12,13).

In carrying out the Total Diet Study, FDA personnel purchase foods from supermarkets or grocery stores 4 times per year, once from each of 4 geographic regions of the country. Between September 1991 and July 1993, 6 market baskets were collected. The 261 foods that comprise each market basket represent over 3500 different foods reported in the USDA food consumption survey; for example, apple pie represents all fruit pies and fruit pastries. Each collection is a composite of like foods purchased in 3 cities in a given region. The foods are prepared table-ready and then analyzed for pesticide residues (as well as radionuclides, industrial chemicals, toxic elements, trace and macro elements, vitamin B_6, and folic acid). The levels of pesticides found, used in conjunction with food consumption data, are used to estimate the dietary intakes of the pesticide residues.

Results and Discussion

Regulatory Monitoring

In 1993, 12,751 samples (12,166 surveillance and 585 compliance) were analyzed under regulatory monitoring. Of these, 5926 were domestic and 6825 were imports.

Figure 1 shows the percentage of the 5703 domestic surveillance samples by commodity group with no residues found, nonviolative residues found, and violative residues found. As in earlier years, fruits and vegetables accounted for the largest proportion of the commodities analyzed in 1993; those 2 commodity groups comprised 59% of the total number of domestic surveillance samples. In 1993, no violative residues were found in 99% of all domestic surveillance samples (the same percentage as in 1991 and 1992).

Appendix A contains more detailed data on domestic surveillance monitoring findings by commodity, including the total number of samples analyzed, the percent samples with no residues found, and the percent violative samples. Of the 5703 domestic surveillance samples, 64% had no detectable residues, less than 1% had over-tolerance residues, and 1% had residues of pesticides for which there was no tolerance for that particular pesticide/commodity combination. In the largest commodity groups, fruits and vegetables, 39 and 70% of the samples, respectively, had no residues detected. About 1% of the fruit samples and about 2% of the vegetable samples contained violative residues (Figure 1). In the milk/dairy products/eggs group, 94% of the samples had no residues detected. In the category Other, which includes a variety of foods, 83% of the samples had no residues detected.

The findings by commodity group for the 6463 import surveillance samples are shown in Figure 2. Fruits and vegetables accounted for 79% of these samples. Overall, no violative residues were found in about 97% of the import surveillance samples (98% in 1991 and 96% in 1992).

Appendix B contains detailed data on the import surveillance samples. Of the 6463 samples analyzed, 69% had no residues detected, less than 1% had over-tolerance residues, and 3% had residues for which there was no tolerance for that particular pesticide/commodity combination. Fruits and vegetables had 61 and 68%, respectively, with no residues detected. Each of these

TABLE 1. FOREIGN COUNTRIES AND NUMBER OF SAMPLES[a] COLLECTED AND ANALYZED IN 1993

Country	No. of Samples
Mexico	1820
Chile	612
Canada	368
Thailand	271
The Netherlands	268
Italy	241
China, People's Rep. of	234
Guatemala	212
Dominican Republic	177
New Zealand	173
Costa Rica	164
Spain	164
Honduras	154
India	126
Colombia	118
Jamaica	103
Argentina	93
Belgium	87
Greece	69
Ecuador	62
Brazil	61
Peru	59
Taiwan	59
Indonesia	58
Turkey	58
Korea, Rep. of	52
Venezuela	49
Germany	48
Japan	47
Australia	46
Israel	44
France	40
United Kingdom	40
Portugal	38
Trinidad & Tobago	38
Philippines	37
Hong Kong	29
Denmark	27
Uruguay	26
Haiti	24
El Salvador	22
Lebanon	22
Pakistan	21
Grenada	19
Panama	18
Hungary	17
Poland	17
Austria	16
Morocco	16
Iceland	15
Singapore	14
Nicaragua	13
Switzerland	13
Egypt	12
Unspecified	17

Country
Ten or fewer samples collected from the following:
Bahamas
Bangladesh
Barbados
Belize
Bolivia
Bosnia-Herzegovina
British Indian Ocean Terr.
British Virgin Islands
Bulgaria
Burma
Cayman Islands
Croatia
Czechoslovakia
Dem. People's Rep. of Korea
Ethiopia
Faeroe Islands
Fiji
Finland
Ghana
Greenland
Guyana
Ireland
Ivory Coast
Jordan
Kenya
Liberia
Liechtenstein
Lithuania
Luxembourg
Macedonia
Madagascar
Malawi
Malaysia
Mozambique
Netherlands Antilles
Nigeria
Norway
Oman
Papua New Guinea
Russia
Saudi Arabia
Slovenia
South Africa
Soviet Union (former)
Sri Lanka
St. Helena
St. Lucia
Surinam
Sweden
Tanzania
Tonga
Yugoslavia (former)

[a] Surveillance plus compliance samples.

groups had less than 1% with over-tolerance residues and 3% with no-tolerance residues. No residues were found in 97% of the milk/dairy products/eggs group and 84% of the fish/shellfish group, and no violative residues were found in either group.

Pesticide monitoring data collected under FDA's regulatory monitoring approach in 1993 are available to the public as a computer database. This database summarizes FDA 1993 regulatory monitoring coverage and findings by country/commodity/pesticide combination. The database also includes the monitoring data by individual sample from which the summary information was compiled. Information on purchase of the database is provided at the end of this report.

GEOGRAPHIC COVERAGE

Domestic. - In 1993, domestic surveillance samples were collected from all 50 states and Puerto Rico. The largest numbers of samples were collected from the states in which agriculture is a major industry. *Import.* - Samples representing food shipments from 107 countries were collected. Table 1 lists the numbers of samples collected and the countries from which they originated. Mexico, as usual, was the source of the largest number of samples, reflecting the volume and diversity of commodities imported from that country, especially during the winter months.

PESTICIDE COVERAGE

Table 2 lists the 325 pesticides that were detectable by the methods used; the 91 pesticides that were actually found are indicated.

FDA conducts ongoing research to expand the pesticide coverage of its monitoring program. This research includes testing the behavior of new or previously untested pesticides through existing analytical methods, and development of new methods to cover pesticides which cannot be determined by existing methods used by FDA. The research encompasses both U.S.-registered pesticides and foreign-use pesticides that are not registered in the United States. The list of pesticides detectable for 1993 (Table 2) reflects the addition of a number of pesticides whose recovery through the analytical methods used was demonstrated as a result of ongoing research.

In 1993, about 19% of the 12,751 samples were analyzed using 1 or more SRMs or selective MRMs. Of those samples, 55% were domestic and 45% were imports. About 9% of the compliance samples and 19% of the surveillance samples were analyzed using SRMs or selective MRMs.

TABLE 2. PESTICIDES DETECTABLE BY THE METHODS USED AND PESTICIDES FOUND (*) IN 1993 REGULATORY MONITORING[a,b]

Pesticide	Pesticide	Pesticide	Pesticide
Acephate*	Benoxacor	Carbaryl*	3-Chloro-5-methyl-4-nitro-1H pyrazole (Release)
Acetochlor	Bensulide	Carbofuran*	Chloroneb
Alachlor	Benzoylprop-ethyl	Carbon tetrachloride	Chloropropylate
Aldicarb*	BHC*	Carbophenothion*	Chlorothalonil*
Aldoxycarb	Bifenox	Carbosulfan	Chloroxuron
Aldrin*	Bifenthrin	Carboxin	Chlorpropham*
Allethrin	Binapacryl	Chloramben	Chlorpyrifos*
Allidochlor	Bromacil	Chlorbenside	Chlorpyrifos-methyl*
Alpha-cypermethrin	Bromophos	Chlorbromuron	Chlorthiophos
Ametryn	Bromophos-ethyl	Chlorbufam	Clomazone
Aminocarb	Bromopropylate	Chlordane*	Coumaphos
Anilazine*	Bromoxynil	Chlordecone	Crotoxyphos
Aramite	Bufencarb	Chlordimeform*	Crufomate
Atrazine	Bupirimate	Chlorfenvinphos*	Cyanazine
Azinphos-ethyl	Butachlor	Chlorflurecol methyl ester	Cyanofenphos
Azinphos-methyl*	Butocarboxim	Chlorimuron ethyl ester	Cyanophos
Bendiocarb	Butralin	Chlornitrofen	Cycloate
Benfluralin	Cadusafos	Chlorobenzilate	Cyfluthrin
Benodanil	Captafol*	Chloroform	Cyhexatin
Benomyl/carbendazim-[c]	Captan*		

TABLE 2. (cont'd) PESTICIDES DETECTABLE BY THE METHODS USED AND PESTICIDES FOUND (*) IN 1993 REGULATORY MONITORING[a,b]

Pesticide	Pesticide	Pesticide	Pesticide
Cypermethrin*	Famphur	Methabenzthiazuron	Pirimicarb
Cyprazine	Fenamiphos	Methamidophos*	Pirimiphos-ethyl
Daminozide	Fenarimol	Methazole	Pirimiphos-methyl*
DCPA*	Fenbuconazole	Methidathion*	Pretilachlor
DDT*	Fenfuram	Methiocarb*	Probenazole
DEF*	Fenitrothion*	Methomyl*	Prochloraz
Deltamethrin	Fenobucarb	Methoprotryne	Procyazine
Deltamethrin, trans	Fenoxaprop ethyl ester	Methoxychlor*	Procymidone*
Demeton*	Fenoxycarb	Methylene chloride	Prodiamine
Dialifor	Fenpropathrin	Metobromuron	Profenofos*
Di-allate	Fenpropimorph	Metolachlor	Profluralin
Diazinon*	Fenson	Metolcarb	Promecarb
Dicamba	Fensulfothion	Metribuzin	Prometryn
Dichlobenil*	Fenthion*	Mevinphos*	Pronamide*
Dichlofenthion	Fenvalerate	Mirex*	Propachlor
Dichlofluanid	Flamprop-M-isopropyl	Monocrotophos*	Propanil
Dichlone	Flamprop-methyl	Monolinuron	Propargite*
Dichlorvos*	Fluazifop butyl ester	Myclobutanil*	Propazine
Diclobutrazol	Fluchloralin	Naled	Propetamphos
Diclofop-methyl	Flucythrinate	Napropamide	Propham
Dicloran*	Flusilazole	Neburon	Propiconazole
Dicofol*	Fluvalinate	Nitralin	Propoxur
Dicrotophos*	Folpet*	Nitrapyrin	Prothiofos
Dieldrin*	Fonofos*	Nitrofen	Prothoate
Diethatyl-ethyl	Formetanate hydrochloride*	Nitrofluorfen	Pyrazon
Dimethachlor	Formothion	Nitrothal-isopropyl	Pyrazophos
Dimethametryn	Gardona	Norflurazon	Pyrethrins
Dimethoate*	Haloxyfop	Nuarimol	Pyridaphenthion
Dinitramine	Heptachlor*	Octhilinone	Quinalphos*
Dinobuton	Heptenophos	Ofurace	Quintozene*
Dinocap	Hexachlorobenzene*	Omethoate*	Quizalofop ethyl ester
Dioxabenzofos	Hexaconazole	Ovex	R25788
Dioxacarb	Hexazinone	Oxadiazon*	Ronnel
Dioxathion	Imazalil*	Oxadixyl	Schradan
Diphenamid	Imazamethabenz methyl	Oxamyl*	Simazine
Diphenylamine*	ester	Oxycarboxin	Simetryn
Disulfoton*	Iprobenfos	Oxydemeton-methyl	Strobane
Diuron	Iprodione*	Oxyfluorfen	Sulfallate
Edifenphos	Isazofos	Oxythioquinox	Sulfotep*
Endosulfan*	Isofenphos	Paclobutrazol	Sulfur dioxide*
Endrin*	Isoprocarb	Parathion*	Sulphenone
EPN*	Isopropalin	Parathion-methyl*	Sulprofos
EPTC	Isoprothiolane	Pebulate	TCMTB
Esfenvalerate*	Lactofen	Penconazole	TDE*
Etaconazole	Lambda-cyhalothrin	Pendimethalin	Tebupirimfose[d]
Ethalfluralin	Leptophos	Permethrin*	Tecnazene
Ethiofencarb	Lindane*	Perthane	TEPP
Ethion*	Linuron*	Phenothrin	Terbacil
Ethofumesate	Malathion*	Phenthoate	Terbufos*
Ethoprop*	MCPA	Phenylphenol, ortho-	Terbumeton
Ethoxyquin	Mecarbam*	Phorate*	Terbuthylazine
Ethylenebisdithiocarbamates[c,d]	Mephosfolan	Phosalone*	Terbutryn
Ethylene dibromide	Merphos	Phosmet*	Tetradifon*
Ethylene dichloride	Metalaxyl	Phosphamidon*	Tetraiodoethylene
Etridiazole	Metasystox thiol	Piperonyl butoxide	Tetrasul
Etrimfos	Metazachlor	Piperophos	Thiabendazole*

TABLE 2. (cont'd) PESTICIDES DETECTABLE BY THE METHODS USED AND PESTICIDES FOUND (*) IN 1993 REGULATORY MONITORING[a,b]

Pesticide	Pesticide	Pesticide	Pesticide
Thiobencarb	Toxaphene*	Trichlorfon	Triflusulfuron methyl ester
Thiodicarb	Tralomethrin	Trichloronat	Triforine
Thiometon	Triadimefon*	Tricyclazole	Trimethacarb
Thionazin	Triadimenol*	Tridiphane	Vernolate
Thiophanate-methyl	Tri-allate*	Triflumizole	Vinclozolin*
Tolylfluanid	Triazophos	Trifluralin	XMC

[a] The list of pesticides detectable is expressed in terms of the parent pesticide. However, monitoring coverage and findings may have included metabolites, impurities, and alteration products.
[b] Some of these pesticides are no longer manufactured or registered for use in the United States.
[c] The analytical methodology determines carbendazim, which may result from use of benomyl or carbendazim.
[d] Such as maneb.
[e] Proposed common name.

TARGETED PESTICIDES

With the rapid growth of world agricultural trade, public concern about the safety of imported foods that could contain pesticide residues with no U.S. tolerance has increased. Over the past several years, an idea has been expounded that pesticides that are not registered for food use in the United States, but which are manufactured in and exported from the United States to other countries, might be used on foods that are then imported into the United States and contain residues of these unregistered pesticides.

FDA regulatory monitoring has routinely covered some of these unregistered pesticides. Others are used on crops that represent relatively low import volumes to the United States and therefore were not identified as appropriate for routine FDA monitoring.

In 1993, FDA targeted some specific U.S.-manufactured unregistered pesticide/commodity/country combinations based on foreign pesticide usage information. A total of 321 samples of imported foods from 25 countries was analyzed for these pesticides. Table 3 shows the pesticides, commodities, and countries that were targeted. No residues of these pesticides were found in the samples analyzed.

Targeted monitoring for U.S.-manufactured pesticides was also directed to domestically produced foods. Table 4 shows the pesticides and commodities that were covered in 1993 under targeted monitoring for domestic foods. A total of 499 samples was analyzed; 25 (5%) of the samples had residues; none were violative. The pesticide/commodity combinations in which below-tolerance residues were found were benomyl in strawberries (2 residues in 6 samples analyzed), formetanate hydrochloride in apples, nectarines, peaches, and strawberries (13 residues in 130 samples analyzed), propargite in apples (7 residues in 20 samples analyzed), and thiabendazole in oranges (3 residues in 51 samples analyzed).

TABLE 3. PESTICIDE/COMMODITY/COUNTRY TARGETED MONITORING OF IMPORTED FOODS CONDUCTED IN 1993

Pesticide	Commodity	Country
Acetochlor	fruit preserves	Hungary
Butachlor	rice	India, Thailand, United Kingdom
Carbosulfan	beans, lentils, peas	various
Flusilazole	kiwi fruit	Chile, New Zealand
Haloxyfop	mung beans, pineapples	China, Thailand
Nuarimol	artichokes, bananas, cantaloupe, fruit preserves, olive oil, olives, pimentos	various
Prothiofos	apples, cassava, chayotes, dasheens, oranges	various
Terbumeton	kiwi fruit	Chile, New Zealand
Terbuthylazine	apples, mango juice, pineapples, wheat, wine	various
Thiometon	beans, corn, endive, grape juice, lentils, peas	various

TABLE 4. PESTICIDE/COMMODITY TARGETED MONITORING OF DOMESTIC FOODS CONDUCTED IN 1993

Pesticide	Commodity	Pesticide	Commodity
Atrazine	cucumbers, corn, grapes, pineapples	Naled	milk, mushrooms
Benomyl	strawberries	Propargite	apples
Bromoxynil	corn, rice	Propiconazole	wheat
Dinocap	apples, grapes, peaches	Propoxur	rice
EPTC	dry beans	Simazine	cucumbers, grapes
Formetanate hydrochloride	apples, nectarines, peaches, strawberries	Thiabendazole	apples, oranges

SURVEILLANCE/COMPLIANCE VIOLATION RATE COMPARISON

In 1993, 223 domestic and 362 import compliance samples were collected and analyzed (Table 5). Because compliance samples are collected when a pesticide residue problem is known or suspected, violation rates are expectedly higher than those for surveillance samples: 17% for domestic (19% in 1992) and 11% for imports (14% in 1992). The corresponding violation rates for surveillance samples were 1.1% for domestic and 3.3% for imports (Figure 3).

Most of the 1993 compliance samples were collected as follow-up on violative surveillance samples. These included follow-up samples from the same shipment as the violative surveillance sample, follow-up samples from additional product from the same grower or shipper, and audit samples from shipments presented for entry into the United States with a certificate of analysis (i.e., shipments subject to automatic detention).

FOODCONTAM DATA

In 1993, 17 states supplied pesticide residue data via the Foodcontam database. A wide variety of commodities was reflected in the 12,816 samples reported. Table 6 lists the 17 states, the number of samples for each, and the number and percentage of samples with positive and "significant" findings. In this instance, a significant finding indicates a residue that exceeds federal or state regulatory limits, a residue for which there is no tolerance for the particular chemical/commodity combination, or reflects some unusual finding(s). For the 12,816 samples reported, 0.9% of the samples analyzed were classified as significant; this is similar to FDA's domestic surveillance violation rate. Fewer Foodcontam samples were reported by the states in 1993 than in 1992 because there was a net loss of 5 cooperating states due to budgetary constraints which precluded pesticide residue monitoring and because several states directed many of their monitoring activities toward the USDA pesticide data program.

FIGURE 3. DOMESTIC AND IMPORT SURVEILLANCE SAMPLE VIOLATION RATES FOR 1993

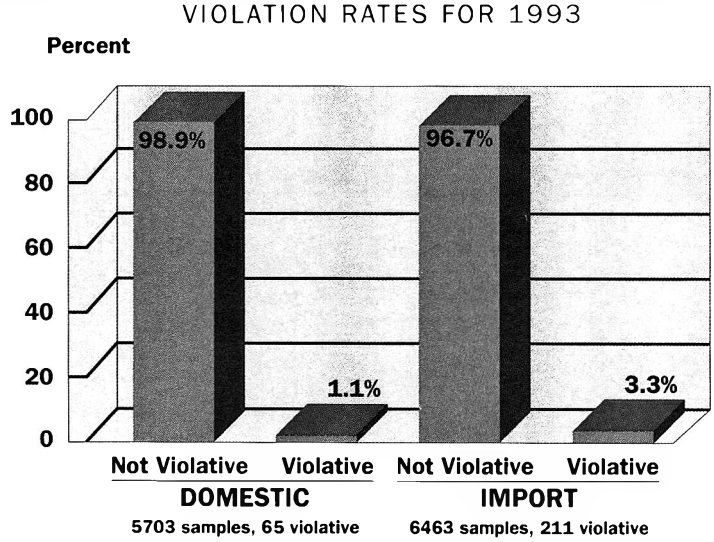


TABLE 5. COMPLIANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %
Domestic			
Grains and grain products	21	62	29
Dairy products	4	100	0
Fish/shellfish	36	97	0
Fruits	28	7	29
Vegetables	81	31	30
Other	53	81	0
Total	**223**	**55**	**17**
Import			
Grains and grain products	20	90	0
Dairy products	9	100	0
Fish	10	90	0
Fruits	96	68	4
Vegetables	199	57	17
Other	28	68	7
Total	**362**	**65**	**11**

TABLE 6. SUMMARY OF FOODCONTAM FINDINGS FOR 1993

State	Total Samples	No. Positive	Positive, %	No. Significant[a]	Significant,[a] %
Arkansas	306	17	5.6	0	0
California	4741	1546	32.6	49	1.0
Delaware	11	1	9.1	0	0
Florida	1296	377	29.1	3	0.2
Georgia	464	90	19.4	4	0.9
Hawaii	184	103	56.0	4	2.2
Indiana	559	469	83.9	0	0
Michigan	217	76	35.0	2	0.9
Minnesota	396	65	16.4	0	0
New York	766	96	12.5	30	3.9
North Carolina	624	178	28.5	3	0.5
Oregon	497	90	18.1	0	0
Pennsylvania	456	223	48.9	10	2.2
Rhode Island	20	4	20.0	4	20.0
Virginia	1413	380	26.9	9	0.6
Washington	87	0	0	0	0
Wisconsin	779	13	1.7	3	0.4
Total	**12,816**	**3728**	**29.1**	**121**	**0.9**

[a] A significant finding denotes a residue that either exceeds federal or state regulatory limits, or a residue for which there is no tolerance for the chemical/food combination, or it reflects an unusual finding.

TABLE 7. SUMMARY OF FINDINGS IN DOMESTIC SURVEILLANCE FEED SAMPLES IN 1993

Type of Feed	Total No. of Samples	Samples with No Residues Found		Samples Violative	
		No.	%	No.	%
Whole/ground grains	173	94	54	1	<1
Mixed feed rations	141	20	14	0	–
Plant by-products	125	62	50	3	2
Animal by-products	87	44	51	0	–
Hay & hay products	43	32	74	4	9
Minerals/supplements	2	2	100	0	–
Total	**571**	**254**	**45**	**8**	**1**

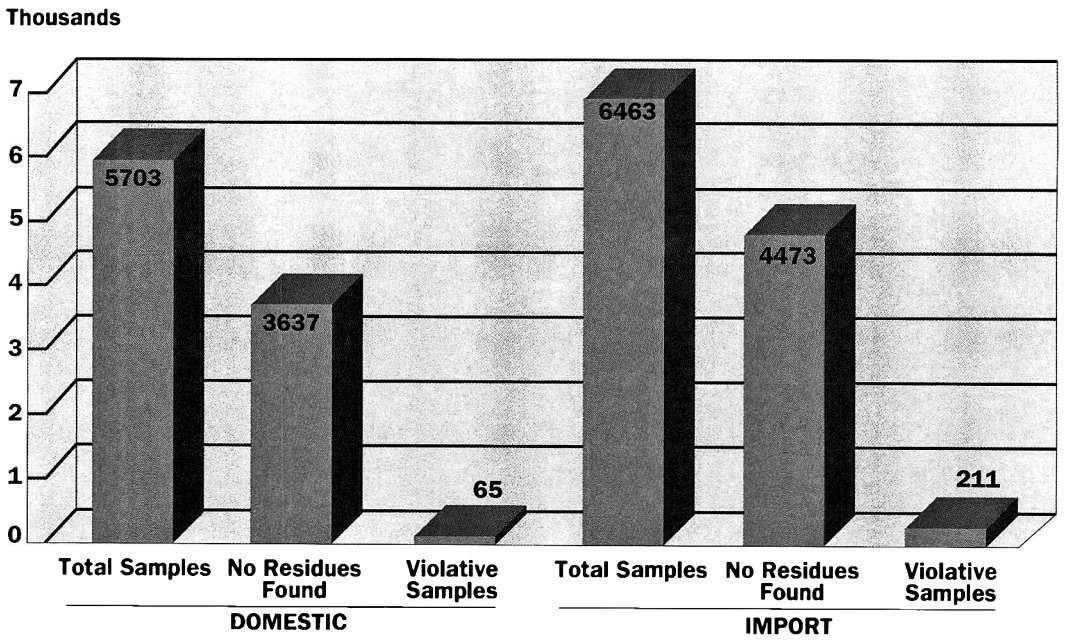
ANIMAL FEEDS

In 1993, 624 domestic feed samples (571 surveillance and 53 compliance) and 75 import feed samples (71 surveillance and 4 compliance) were collected and analyzed by FDA. Of the 571 domestic surveillance samples, 254 (45%) had no pesticide residues detected and 8 (1%) contained violative residues (Table 7). Of the 71 import surveillance samples, 25 (35%) had no pesticide residues detected and 1 (1%) contained violative pesticide residues. The 9 violative domestic and import surveillance samples involved 4 with residues that exceeded a tolerance and 5 with residues for which no tolerance had been established.

In the 317 domestic surveillance feed samples in which 1 or more pesticides were detected, malathion, chlorpyrifos-methyl, diazinon, and chlorpyrifos were most frequently found and accounted for 77% of all residues detected. The findings for these 4 pesticides were as follows:

Pesticide	No. of Samples with Residues	Residue Found, ppm	
		Range	Mean
malathion	232	trace-5.1	0.05
chlorpyrifos-methyl	103	trace-5.1	0.03
diazinon	45	trace-0.38	0.03
chlorpyrifos	32	trace-0.79	0.02

FIGURE 4. COMPARISON OF RESULTS FOR DOMESTIC AND IMPORT SURVEILLANCE SAMPLES IN 1993



Summary: Regulatory Monitoring

In summary, no residues were found in 64% of domestic surveillance samples and 69% of import surveillance samples (Figure 4) analyzed under FDA's regulatory monitoring approach in 1993. Less than 1% of domestic and import surveillance samples had residue levels that were over tolerance and 1% of domestic and 3% of import surveillance samples had residues for which there was no tolerance. The findings for 1993 continue to demonstrate that pesticide residue levels in foods are generally well below EPA tolerances, and they corroborate results presented in earlier reports (5-10).

Incidence/Level Monitoring

In 1993, 3 projects were carried out under this approach to monitoring.

AQUACULTURE SURVEY

Aquaculture is the production and harvest of aquatic and marine species in a water environment other than a "wild" harvest. It has become an important segment of the seafood industry in recent years.

FDA initiated an aquaculture survey in 1990 that has been continued in succeeding years. This survey focuses on persistent halogenated pesticides, which, although no longer registered for use on foods, may be present in the water environment as a result of past agricultural uses on land associated with aquaculture activities.

Thirteen FDA Districts collected a total of 308 samples (this number is included in the count under Fish/Shellfish in Appendix A) from important aquaculture areas of the United States and shipped them to the Buffalo District Laboratory for analysis. The 308 samples included 121 catfish, 48 trout, 34 crayfish and shrimp, 31 oysters, 18 salmon, 16 tilapia, and 40 various other fish/shellfish. These species were emphasized because they represent large segments of the commercial aquaculture harvest.

Of the 308 samples, 137 (44%) had no residues detected. Three catfish samples contained trace-0.02 ppm chlorpyrifos; 2 catfish samples had trace levels of diazinon; and 8 catfish samples contained 0.01-0.09 ppm DCPA (an herbicide, trade name Dacthal). There are no tolerances for these chemicals in fish; therefore, the collecting Districts were notified of these findings for possible regulatory follow-up.

The other findings included DDT (total) found in 170 samples at levels ranging from trace to 1.0 ppm (FDA action level is 5 ppm) and chlordane (total) found in 9 samples at levels of 0.01-0.07 ppm (action level is 0.3 ppm).

MILK SURVEY

This survey takes advantage of an existing federal program that collects milk samples throughout the United States for the purpose of monitoring radionuclides in milk. EPA operates the Environmental Radiation Ambient Monitoring System (ERAMS), which collects and composites samples of pasteurized whole milk from metropolitan areas throughout the country. These composites are also analyzed for pesticide residues through an FDA-supported contract (14).

In 1993, 308 milk samples from 58 metropolitan areas were analyzed. Samples from 21 of the metropolitan areas had no pesticide residues detected. Of the 308 samples, 109 (35%) had detectable residues. The most frequently found residues were *p,p'*-DDE (69 findings) and dieldrin (49 findings). The highest residue level found was 0.01 ppm *p,p'*-DDE (whole milk basis). These results are similar to those found in earlier ERAMS monitoring, and reflect the presence of low levels of these environmentally persistent chemicals in foods of animal origin.

The sample numbers cited above represent about 6 months of collections rather than an entire calendar year since the ERAMS pesticide survey was completed in 1993. The data obtained during the course of the survey have been useful in providing a picture of the pesticide residue situation in milk. FDA will continue to sample and analyze fluid milk through its regulatory monitoring and cooperation with state pesticide authorities.

STATISTICALLY BASED MONITORING SURVEY

Pears. The original goal of the project had been to collect 1600 samples of pears (800 domestic and 800 import). For pears, 710 domestic and 949 import samples (these numbers are not included in the counts under Fruits in Appendixes A and B) were collected from 179 pear establishments and analyzed using both MRMs and SRMs. The number of domestic samples is less than planned because some of the targeted firms were no longer in business or pears were not available from a particular firm at the time of FDA's collection visits. The violation rate for the 710 domestic samples was 0.4%, and for the 949 import samples it was 1.3% (FDA, unpublished data, 1994).

Tomatoes. As with the pears, 1600 samples were to be collected (800 domestic and 800 import). However, for the same reasons as noted above for the pears, the numbers of samples varied from those targeted. A total of 1219 domestic and 144 import samples (these numbers are not included in the counts under Vegetables in Appendixes A and B) were collected from 10 packers and 240 repackers and analyzed using both MRMs and SRMs. The violation rate for the 1219 domestic samples was 2.7% and for the 144 import samples it was 3.5% (FDA, unpublished data, 1994).

The violation rates for these 2 commodities for 1993 surveillance samples were pears, domestic, 4%; imports, 10%; and for tomatoes, domestic, 3%; imports, <1% (Appendixes A and B). In some instances, the violation rates found under statistically based monitoring are considerably lower than those found under regulatory monitoring (surveillance samples) mainly because sampling under the latter approach is somewhat biased. (A detailed report describing the statistically based survey and the results is in preparation.)

Summary: Incidence/Level Monitoring

The findings obtained under this approach, which included the analysis of 308 samples of aquaculture products and 308 samples of milk, were consistent with those obtained under regulatory monitoring. Residues in the aquaculture products and whole milk were, with the exception of no-tolerance residues of 3 pesticides in several catfish samples, within regulatory limits. A statistically based monitoring survey of domestic and imported pears and tomatoes was completed in 1993.

Total Diet Study

The Total Diet Study is unique in that it determines pesticide residues in foods which have been prepared as they would be consumed (4). Of the nearly 300 chemicals that can be determined by the analytical methods used, 99 pesticide and pesticide-related chemicals were found in the foods analyzed in the 6 collections between September 1991 and July 1993. To measure the low levels of pesticides found in the Total Diet Study foods, the analytical methods used are modified to permit measurement at levels 5-10 times lower than those normally used in regulatory monitoring. In general, residues present at or above 1 part per billion can be measured.

Table 8 lists the 19 most frequently found residues, with the total number of findings and the percent occurrence in the 1566 food items analyzed during the 1991-1993 period. Malathion, which is used on a wide variety of crops both pre- and postharvest, was the most frequently found residue. Low levels of DDT residues (principally *p,p'*-DDE) associated with animal-derived foods were the next most prevalent. Table 9 lists the 29 Total Diet Study food items in which no organic pesticide-related residues were found. A large number of these items are beverages. An even larger number of these "no-residue" foods are either strained or junior foods intended for infants and children or foods consumed in significant amounts by infants and children. An extensive review of FDA's monitoring of pesticide residues in infant foods and adult foods

TABLE 8. FREQUENCY OF OCCURRENCE OF PESTICIDE RESIDUES IN TOTAL DIET STUDY (1991-1993)[a]

Pesticide[b]	Total No. of Findings	Occurrence, %
Malathion	332	21
DDE, *p,p'*	298	19
Chlorpyrifos-methyl	251	16
Endosulfan	162	10
Chlorpyrifos	155	10
Dieldrin	142	9
Chlorpropham	90	6
Methamidophos	83	5
Diazinon	72	5
Carbaryl[c]	66	4
Dicloran	61	4
Dimethoate	57	4
Thiabendazole[d]	50	3
Acephate	48	3
Omethoate	40	3
Lindane	36	2
Permethrin	35	2
Propargite[d]	33	2
Ethion	32	2

[a] Based on 6 market baskets collected between September 1991 and July 1993 consisting of 1566 analyzed items.

[b] Isomers, metabolites, and related compounds have not been listed separately; they are covered under the "parent" pesticide from which they arise.

[c] Reflects overall incidence; however, only 95 selected foods per market basket (i.e., 570 items total) were analyzed for *N*-methylcarbamates.

[d] Reflects overall incidence; however, only 50 selected foods per market basket (i.e., 300 items total) were analyzed for these sulfur-containing compounds.

TABLE 9. TOTAL DIET FOOD ITEMS IN WHICH NO ORGANIC PESTICIDE-RELATED RESIDUES WERE DETECTED (1991-1993)[a]

Bananas with tapioca, strained or junior
Beer
Beets, strained or junior
Beverage, fruit-flavored, carbonated
Coffee, decaffeinated, from instant
Coffee, from ground
Cola carbonated beverage
Corn, creamed, strained or junior
Corn flakes
Cream substitute, frozen
Custard pudding, strained or junior
Gelatin dessert
Honey
Infant formula, milk-based, high iron, ready-to-feed
Infant formula, milk-based, low iron, ready-to-feed
Infant formula, soy-based, ready-to-feed
Kidney beans, dry, boiled
Margarine, stick, regular (salted)
Milk, skim
Onions, mature, raw
Peas, strained or junior
Pineapple juice, from frozen concentrate
Pinto beans, dry, boiled
Sugar, white, granulated
Syrup, pancake
Veal cutlet, pan-cooked
Water, tap
Whiskey
Yogurt, plain lowfat

[a] Based on 6 market baskets collected between September 1991 and July 1993 consisting of 1566 analyzed items.

eaten by infants and children has recently been published (15). In that paper, results for 1985-1991 reflecting regulatory, incidence/level, and Total Diet Study monitoring are discussed. Currently, increased monitoring of domestic and imported foods likely to be consumed by children is being implemented by FDA.

Information obtained through the Total Diet Study is used to estimate dietary intakes of pesticides; these intakes are then compared with established standards. Food consumption data to be used in estimating dietary intakes for the revised food list have not been finalized yet. Therefore, dietary intake information for the market baskets collected during this period will be presented in a future report.

Summary: Total Diet Study

In the 1991-1993 period, the types of pesticide residues found in the Total Diet Study and their frequencies of occurrence are consistent with those given in other FDA reports (5-10,15). Pesticide residue levels were generally very low; 29 food items had no pesticide-related residues detected in the 1991-1993 period assessed. The data for 1993 continue to indicate that consumer exposure to pesticide residues from foods is very low.

Summary

A total of 12,751 samples of domestically produced food from all 50 states and Puerto Rico and imported food from 107 countries was analyzed for pesticide residues in 1993. Of these, 12,166 were surveillance samples, which are collected when there is no evidence of a pesticide problem. No residues were found in 64% of the domestic surveillance samples and 69% of the import surveillance samples. Findings in the 585 compliance samples reflect the fact that they are collected and analyzed when a pesticide problem is suspected. Under incidence/level monitoring, 308 samples of aquaculture seafood/shellfish and 308 milk samples were analyzed for pesticide residues. The findings were similar to those from FDA's regulatory monitoring. In addition, a statistically based monitoring survey of pears and tomatoes was completed in 1993. Low violation rates were found for both the domestic and import segments of the 2 commodities. The types of residues found in the Total Diet Study for 1991-1993 were similar to those found in earlier periods.

This report was compiled through the efforts of the following FDA personnel: Norma J. Yess, Marcia G. Houston, Ellis L. Gunderson, Young H. Lee, and Byron O. Bohannon, Office of Plant and Dairy Foods and Beverages, Division of Programs and Enforcement Policy, and Sharon A. Schoen, Office of Management Systems, Division of Information Resources Management, Washington, DC.

Address reprint requests to:
Norma J. Yess
Food and Drug Administration
Office of Plant and Dairy Foods and Beverages
Division of Programs and Enforcement Policy
HFS-308, 200 C Street, SW
Washington, DC 20204

FDA pesticide monitoring data collected under the regulatory monitoring approach in 1993 are available for purchase on personal computer diskettes from the National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161 (telephone 703-487-4650); order number PB94-501681.

REFERENCES

(1) Lombardo, P., & Yess, N.J. (1991) "The FDA Program on Pesticide Residues in Food" *Am. Chem. Soc. Symp. Ser.* **446**, American Chemical Society, Washington, DC, pp. 162-169

(2) *Code of Federal Regulations* (1993) Title 40, U.S. Government Printing Office, Washington, DC, Parts 180, 185, and 186

(3) *Pesticide Analytical Manual* (1968 and revisions) Vols I and II, Food and Drug Administration, Washington, DC (available from National Technical Information Service, Springfield, VA 22161)

(4) Pennington, J.A.T., & Gunderson, E.L. (1987) "History of the Food and Drug Administration's Total Diet Study - 1961 to 1987" J. *Assoc. Off. Anal. Chem.* **70**, 772-782

(5) Food and Drug Administration (1988) "Food and Drug Administration Pesticide Program - Residues in Foods - 1987" J. *Assoc. Off. Anal. Chem.* **71**, 156A-174A

(6) Food and Drug Administration (1989) "Food and Drug Administration Pesticide Program - Residues in Foods - 1988" J. *Assoc. Off. Anal. Chem.* **72**, 133A-152A

(7) Food and Drug Administration (1990) "Food and Drug Administration Pesticide Program - Residues in Foods - 1989" J. *Assoc. Off. Anal. Chem.* **73**, 127A-146A

(8) Food and Drug Administration (1991) "Food and Drug Administration Pesticide Program - Residues in Foods - 1990" J. *Assoc. Off. Anal. Chem.* **74**, 121A-142A

(9) Food and Drug Administration (1992) "Food and Drug Administration Pesticide Program - Residue Monitoring - 1991" J. AOAC *Int.* **75**, 136A-158A

(10) Food and Drug Administration (1993) "Food and Drug Administration Pesticide Program - Residue Monitoring - 1992" J. AOAC *Int.* **76**, 127A-148A

(11) Pennington, J.A.T. (1992) "The 1990 Revision of the FDA Total Diet Study" J. *Nutr. Educ.* **24**, 173-178

(12) Pennington, J.A.T. (1992) "Total Diet Studies: The Identification of Core Foods in the United States Food Supply" *Food Addit. Contam.* **9**, 253-264

(13) Pennington, J.A.T. (1992) "Appendices for the 1990 Revision of the Food and Drug Administration's Total Diet Study" PB92-176239/AS, National Technical Information Service, Springfield, VA

(14) Trotter, W.J., & Dickerson, R. (1993) "Pesticide Residues in Composited Milk Collected Through the U.S. Pasteurized Milk Network" J. AOAC *Int.* **76**, 1220-1225

(15) Yess, N.J., Gunderson, E.L., & Roy, R.R. (1993) "Food and Drug Administration Monitoring of Pesticide Residues in Infant Foods and Adult Foods Eaten by Infants/Children" J. AOAC *Int.* **76**, 492-507

APPENDIX A. ANALYSIS OF DOMESTIC SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
A. Grains and Grain Products				
Barley	15	60	0	0
Corn & corn products	65	63	0	0
Oats	17	59	0	0
Rice & rice products	94	90	0	3
Soybeans	60	85	0	0
Wheat	146	45	<1[a]	<1
Cereal products	13	77	0	0
Other grains & grain products	11	45	0	0
Total	**421**	**66**	**<1[a]**	**<1**
B. Milk/Dairy Products/Eggs				
Cheese/cheese products	124	94	0	0
Eggs	265	96	0	0
Milk & cream/milk products	417	93	0	<1
Total	**806**	**94**	**0**	**<1**
C. Fish/Shellfish				
Total	**947**	**53**	**<1**	**0**
D. Fruits				
Blueberries	28	54	0	4
Grapes	55	80	0	0
Raspberries	19	63	0	11
Strawberries	118	29	0	<1
Other berries	18	44	0	0
Grapefruit	28	36	0	0
Lemons	15	20	0	0
Oranges	122	21	0	0
Other citrus fruits	25	20	0	0
Apples	312	39	0	<1
Pears	26	8	0	4
Apricots	31	6	0	6
Cherries	80	15	0	0
Nectarines	35	14	0	6
Peaches	134	20	4[a]	<1
Other pit fruits	24	71	0	0
Kiwi fruit	20	100	0	0
Papayas	22	91	0	0
Pineapples	13	100	0	0
Other tropical fruits	7	86	0	0

APPENDIX A. (cont'd) ANALYSIS OF DOMESTIC SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
Cantaloupe	53	43	0	0
Watermelon	55	82	0	0
Other vine fruits	15	47	0	13
Fruit jams/jellies/toppings	13	77	0	0
Fruit juices	16	100	0	0
Total	**1284**	**39**	**<1[a]**	**1**
E. Vegetables				
Corn	136	97	0	<1
Green/snow/sugar/sweet peas	61	84	0	0
String beans	90	54	0	0
Other beans & peas	25	96	0	0
Cucumbers	95	67	0	2
Eggplant	38	55	0	5
Peppers	60	43	0	0
Squash	75	63	0	3
Tomatoes	37	65	3	0
Other fruits used as vegetables	8	50	0	0
Asparagus	43	98	0	0
Bok choy	18	83	0	0
Broccoli	60	78	0	2
Cabbage	91	81	0	0
Cauliflower	34	94	0	0
Celery	52	15	0	0
Chinese cabbage	40	90	0	3
Collards	20	75	0	5
Endive/escarole	27	67	0	7
Kale	21	38	5[a]	19
Lettuce	242	57	<1[a]	2
Mustard greens	14	43	0	0
Spinach	41	56	0	2
Other leaf/stem vegetables	48	67	0	10
Mushroom/truffle products	11	55	0	0
Carrots	87	82	0	0
Ginger root	14	100	0	0
Onions/leeks/scallions/shallots	97	89	1	0
Potatoes	213	57	0	0
Radishes	30	90	0	0
Red beets	12	83	0	8
Sugar beets	15	93	0	0
Sweet potatoes	61	69	0	0

APPENDIX A. (cont'd) ANALYSIS OF DOMESTIC SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
Turnips	16	44	0	0
Other root/tuber vegetables	18	44	0	17
Vegetables, dried or paste	127	88	0	<1
Other vegetables/vegetable products	23	83	0	4
Total	**2100**	**70**	**<1[a]**	**2**
F. Other				
Peanuts	34	68	0	0
Other nuts & related products	19	89	0	0
Vegetable oil	19	95	0	0
Honey	28	96	0	0
Baby foods	16	100	0	0
Other food products	29	69	0	3
Total	**145**	**83**	**0**	**<1**
A-F Total	**5703**	**64**	**<1[a]**	**1**

[a] Includes samples that have both residue(s) over tolerance and residue(s) with no tolerance.

APPENDIX B. ANALYSIS OF IMPORT SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
A. Grains and Grain Products				
Rice & rice products	121	93	0	<1
Wheat & wheat products	40	75	0	5
Other grains & grain products	36	81	0	3
Bakery products	24	79	0	0
Macaroni/spaghetti products	60	65	0	0
Noodles/noodle products	29	79	0	0
Total	**310**	**81**	**0**	**1**
B. Milk/Dairy Products/Eggs				
Cheese/cheese products	215	98	0	0
Milk & eggs/egg products	11	73	0	0
Total	**226**	**97**	**0**	**0**
C. Fish/Shellfish				
Total	**444**	**84**	**0**	**0**
D. Fruits				
Blackberries	56	57	0	4
Blueberries	49	73	0	0
Cranberries	11	73	0	0
Grapes	119	28	0	5
Raspberries	102	38	0	<1
Strawberries	123	25	0	11
Other berries	7	71	0	14
Clementines	16	0	0	13
Lemons	22	41	0	5
Limes	50	70	0	0
Oranges	41	71	0	0
Tangelos/tangerines	15	53	0	0
Other citrus fruits	13	100	0	0
Apples	99	38	0	3
Pears	91	46	1[a]	9
Avocados	47	100	0	0
Cherries	30	70	0	0
Nectarines	22	14	0	0
Olives, stuffed	27	89	0	0
Olives, other	56	91	0	4
Peaches	59	29	0	0
Plums	47	57	0	0
Other pit fruits	12	83	0	0
Bananas	187	68	0	0
Kiwi fruit	68	53	0	7

APPENDIX B. (cont'd) ANALYSIS OF IMPORT SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
Mangoes	90	94	0	0
Papayas	113	80	0	10
Pineapples	144	74	3[a]	<1
Plantains	30	93	0	0
Other tropical fruits	69	94	0	6
Cantaloupe	108	35	<1	0
Honeydew	68	22	0	0
Watermelon	52	73	0	4
Other vine fruits	21	62	0	10
Fruit jams & jellies	26	85	0	0
Fruit juices	61	89	0	0
Fruit toppings	23	96	0	0
Fruits, dried or paste	78	83	0	5
Other fruit products	9	78	0	0
Total	**2261**	**61**	**<1[a]**	**3**
E. Vegetables				
Corn	30	97	0	0
Garbanzo beans/chick peas	13	77	0	0
Green/snow/sugar/sweet peas	105	63	0	13
Mung beans	11	82	0	0
String beans	67	51	1[a]	10
Other beans, peas, & corn	51	69	2[a]	2
Cucumbers	101	46	0	0
Eggplant	26	58	0	0
Okra	67	75	0	7
Peppers, hot	329	43	3[a]	5
Peppers, sweet	199	74	<1	0
Pumpkins	18	89	0	0
Squash	155	49	<1[a]	8
Tomatillos	22	41	0	0
Tomatoes	245	61	0	<1
Other fruits used as vegetables	38	79	0	5
Artichokes	45	87	0	4
Asparagus	146	82	3	1
Bamboo shoots	12	100	0	0
Broccoli	81	73	1	0
Broccoli raab	18	72	0	0
Brussels sprouts	13	69	0	0
Cabbage	34	79	0	3
Cauliflower	24	92	0	0
Celery	15	13	0	0
Chicory	32	100	0	0

APPENDIX B. (cont'd) ANALYSIS OF IMPORT SURVEILLANCE SAMPLES BY COMMODITY GROUP IN 1993

Commodity Group	Total No. of Samples	Samples with No Residues Found, %	Samples Violative, %	
			Over Tolerance	No Tolerance
Endive/escarole	61	92	0	0
Kale	11	36	0	0
Lettuce	58	53	2[a]	9
Radicchio	49	88	0	0
Spinach	19	58	0	0
Other leaf/stem vegetables	62	61	0	10
Mushrooms/truffles, whole	61	89	0	0
Mushrooms/truffles, pieces & products	59	90	0	0
Carrots	51	78	0	2
Cassava	12	100	0	0
Garlic, bulb	13	92	0	0
Onions/leeks/scallions	53	89	0	0
Potatoes	49	51	0	0
Radishes	18	56	0	0
Shallots	17	100	0	0
Sweet potatoes	18	94	0	0
Water chestnuts	28	100	0	0
Other root/tuber vegetables	59	93	0	3
Vegetables, dried or paste	182	75	0	4
Vegetables with sauce	26	85	0	0
Other vegetables/vegetable products	10	90	0	0
Total	**2813**	**68**	**<1[a]**	**3**
F. Other				
Spices, whole	31	77	0	10
Other spices, extracts/flavors	9	44	0	11
Cashews	45	67	0	13
Other nuts & nut products	54	87	0	6
Edible seeds	28	86	0	7
Vegetable oils	30	93	0	3
Beverage bases	21	86	0	0
Coffee/tea	28	93	0	4
Water & ice	30	87	0	0
Honey	88	78	0	1
Other food products	45	80	0	11
Total	**409**	**81**	**0**	**3**
A-F Total	**6463**	**69**	**<1[a]**	**3**

[a] Includes samples that have both residue(s) over tolerance and residue(s) with no tolerance.

Cumulative Author Index

Cumulative Author Index

Cumulative Author Index

AGRICULTURAL MATERIALS

Determination of Diclazuril in Avian Feed and Premixes with Gas Chromatography/Mass Spectrometry

W. JOHN BLANCHFLOWER, PETER J. HUGHES, ANDREW CANNAVAN, and D. GLENN KENNEDY
Department of Agriculture, Veterinary Sciences Division, Stormont, Belfast, BT4 3SD, Northern Ireland, UK

A method is presented for the determination of the relatively new anticoccidial drug diclazuril in avian feed and premixes. The drug was extracted from feed with acidified methanol–water, and the extracts were cleaned up by simple liquid–liquid extraction. These were then derivatized with ethereal diazomethane, and the methyl derivatives were analyzed by gas chromatography/mass spectrometry on a bench-top instrument. An internal standard was used to enhance the reproducibility and ruggedness of the assay. The coefficients of variation for a finished feed and premix assayed 5 times each on 3 occasions were 6.6 and 6.0%, respectively. The detection limit of the assay is about 0.02 mg/kg, which is adequate for measuring the normal inclusion rate of 1 mg/kg in finished feed.

Diclazuril (Janssen research compound R64433, Figure 1), which is marketed under the trade name Clinacox, is a relatively new drug used for the prevention and treatment of coccidiosis in chickens (1) and turkeys (2). It is currently licensed for use throughout Europe, Canada, and North America. The recommended inclusion rate is 1 mg/kg in feed. It is more potent than other coccidiostats such as monensin, narasin, and salinomycin, which are normally added to feed at the rate of 60–100 mg/kg (3), and its use is sometimes alternated with these compounds to reduce the possibility of occurrence of anticoccidial drug resistance.

To date, only one method (4), using liquid chromatography, is used to determine diclazuril in feedstuffs. The authors (4) used a ternary gradient system with a run time of about 1 h/sample; however, many peaks other than that from diclazuril showed on the chromatograms from sample extracts. The presence of many peaks is probably due to the high sensitivity demanded from the method because of the low inclusion rate of the analyte in feed. Interference from other compounds likely occurs with some feed samples.

In the method described in this paper, diclazuril is extracted from feed with acidified methanol–water. The extracts are cleaned up by simple liquid–liquid extraction, followed by derivatization with diazomethane. The methyl derivatives are then detected and quantitated by gas chromatography/mass spectrometry (GC/MS), using a bench-top quadrupole GC/MS. An internal standard (IS; Janssen research compound R62646, Figure 1) is used to improve quantitation and ruggedness of the assay.

Received June 9, 1993. Accepted by RN November 4, 1993.

Experimental

Materials

All solvents were GC grade (Rathburn Chemicals, Walkerburn, Scotland, UK). Other chemicals were Analar grade (Merck/BDH Ltd, Poole, Dorset, UK), except for *N*-nitroso-*N*-methylurea, which was obtained from Sigma Chemical Co., Poole, Dorset, UK. Diclazuril and IS (Janssen research compounds R64433 and R62646) were obtained from Janssen Research Products, Lammerdries, Olen, Belgium, and were certified as greater than 99% purity.

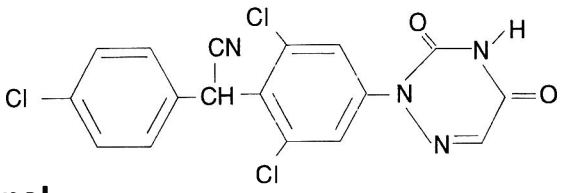
(**a**) *Stock solutions.*—Prepare 500 μg/mL diclazuril stock solution and 500 μg/mL IS stock solution by sonicating in methanol–tetrahydrofuran (50 + 50, v/v). Store in the dark at 4°C for up to 6 months.

(**b**) *Working solutions, 10 μg/mL.*—Prepare by diluting the stock standards in methanol. Store in the dark at 4°C for up to one month.

(**c**) *Diazomethane.*—This reagent must be prepared in an efficient fume cupboard. The supplier's safety precautions for the apparatus and reagents should be followed. Weigh 100–125 mg *N*-nitroso-*N*-methylurea into the inner reaction tube of a millimole-size diazomethane generator (Pierce and Warriner Ltd, Chester, UK) and cap the tube. Pipette 3 mL diethyl ether into the outer tube of the apparatus, fit the inner tube, and place the generator in an ice bath. Using a syringe, slowly add 0.5 mL 4M sodium hydroxide through the septum cap of the reaction tube. Leave on ice for 1 h and then transfer the ethereal diazomethane solution into a glass vial. Add 0.3 mL methanol and store in a freezer at –20°C for up to one week.

(**d**) *Feed samples.*—Premixes (formulated to contain 150 mg diclazuril/kg) and finished poultry meals (formulated to contain 1 mg diclazuril/kg) were obtained from Compton Paddock Laboratories, Newbury, Berkshire, UK. These samples came from various animal feed companies throughout the United Kingdom for quality control purposes. The finished feed samples were milled through a 1 mm sieve before analyses.

Diclazuril



Internal Standard

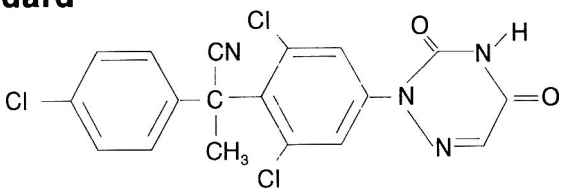


Figure 1. Chemical structures of diclazuril and internal standard.

Apparatus

(a) *GC/MS system.*—HP5995 (Hewlett-Packard Ltd, Stockport, Cheshire, UK) bench-top GC/MS fitted with a direct capillary interface, Chemstation data system, and HP7671A autosampler.

(b) *GC column.*—HP2 (Hewlett-Packard), 25 m × 0.32 mm fused-silica WCOT capillary column coated with a 0.5 μm film of cross-linked 5% phenyl methyl silicone. A small length (ca 0.5 m) was periodically removed from the front of the column if peak broadening became a problem after the analyses of several batches of samples. This could be repeated until the length of the column is reduced to ca 15 m, at which stage it is replaced.

(c) *GC conditions.*—The GC was operated in the split mode with a split ratio of 10:1. The injector port was fitted with a 2 mm id "gooseneck" liner (Restek Corp., Bellefonte, PA), which was packed with a small piece of deactivated silica wool (Restek). The liner was replaced if peak broadening became a problem after several batches of samples had been analyzed. It could be cleaned by boiling in nitric acid, rinsing in distilled water, and drying. It was then deactivated by rinsing with a solution of 5% dimethylchlorosilane in toluene (Alltech Associates, Carnforth, Lancashire, UK) and washed with anhydrous methanol followed by ethyl acetate. The injection port temperature was 330°C, and the column inlet pressure was set to 10 psi. The initial oven temperature was 250°C, and after injection, the temperature was ramped at 15°C/min for a final oven temperature of 325°C. The total run time was 15 min/sample. The derivatized standard and sample extracts (2 μL) were injected with an autosampler.

(d) *MS conditions.*—The temperatures of the transfer line, source, and mass analyzer were 300°, 200°, and 150°C, respectively. The instrument was operated in the electron impact (EI), single-ion-monitoring (SIM) mode and was tuned and calibrated with perfluorotributylamine (Hewlett-Packard). The molecular ions at *m/z* 420 and 419 were monitored for the methyl derivatives of diclazuril and IS, respectively, and peak area data were captured on the Chemstation data system. A dwell time of 100 ms was used for each ion, and the mass window size was set at 0.9 amu.

Sample Extraction

(a) *Finished feeds.*—Weigh aliquots (10 g) of milled feed into 125 mL polyethylene, wide-mouth bottles. Add working IS (1 mL, 10 μg/mL), methanol–water (98 mL, 60 + 40), and hydrochloric acid (1 mL, 11.6N). Shake the bottles for 2 h on a reciprocating shaker and centrifuge for 15 min (2000 × *g*). Transfer aliquots (15 mL) of the supernatants to Quickfit centrifuge tubes and add water (10 mL).

(b) *Premixes.*—Weigh aliquots of premixes (0.5 g) plus aliquots (9.5 g) of a known negative finished feed sample into polyethylene bottles. Add stock IS (150 μL, 500 μg/mL), methanol–water, and hydrochloric acid, as described above, and extract and centrifuge as for finished feeds. Transfer aliquots (2 mL) of the supernatants to centrifuge tubes and add water (23 mL).

Liquid–Liquid Cleanup

Wash the diluted supernatants with hexane (7 mL) by shaking gently for 1 min and centrifuging for 10 min (4°C and 1500 × *g*). Remove and discard the hexane layers and any material that may collect at the interface. Add toluene (4 mL) to all the tubes and shake the tubes gently for 1 min. Centrifuge for 10 min (4°C and 1500 × *g*) and transfer the toluene extracts to clean tubes. Evaporate to dryness under nitrogen at 80°C in a fume cupboard, using a Dri-block heater and needle manifold.

Standards

Pipette aliquots (150 μL) of the working standards (10 μg/mL diclazuril and 10 μg/mL IS) into glass tubes and evaporate to dryness under nitrogen at 60°C.

Derivatization

Add aliquots (0.4 mL) of ethereal diazomethane to the dried standards and sample extracts. Leave at room temperature for 10 min and evaporate to dryness under nitrogen at 40°C. Redissolve the residues in toluene (100 μL) and transfer to autosampler vials.

GC/MS Analyses

Tune and calibrate the GC/MS system and inject aliquots (5 μL) of the derivatized standards and sample extracts. A standard is injected after every 4 sample extracts. Monitor the ions at *m/z* 419 for the internal standard and at *m/z* 420 for diclazuril and collect peak area data.

Calculations

The standards are equivalent to 1 and 150 mg diclazuril/kg in finished feeds and premixes*, respectively, so that the concentrations of diclazuril are calculated by using the following formula:

$$\text{Concentration (mg/kg)} = \frac{A}{B} \times \frac{C}{D}*$$

where A = m/z 419 IS peak area for standard, B = m/z 420 peak area for standard, C = m/z 420 peak area for sample, D = m/z 419 IS peak area for sample, and * = multiply by 150 for premixes.

Results and Discussion

The EI full scan spectra of the methyl derivatives of diclazuril and IS are shown in Figure 2. The molecular ion at m/z 420 was chosen to monitor the derivatized extracts and standards for diclazuril, and the base peak ion at m/z 419 ($M–CH_3$) was chosen to monitor for IS. The chromatograms (Figure 3) for standards and sample extracts were clean and free of interference from other compounds; thus, the assay is very specific for diclazuril. Diclazuril elutes at 7.8 min, and the IS elutes at 8.55 min. The small peaks eluting at 8.55 min on the analyte chromatogram at m/z 420 are due to the isotopic ion of the IS peak at m/z 420 (Figure 2). The specificity of the assay could be further enhanced, if required, by monitoring some of the other prominent ions shown in Figure 2 and calculating the ion ratios.

The reproducibility of the assay was determined by analyzing a meal sample and a premix formulated to contain diclaruzil at 1 and 150 mg/kg, respectively, 5 times on each of 3 days. The results are shown in Table 1. The mean values of diclazuril found in the finished meal sample ranged from 1.04 to 1.13 mg/kg on each of the 3 days, with an overall mean of 1.09 mg/kg. The coefficients of variation (CVs) ranged from 4.5 to 6.6%, with an overall value of 6.6%. The mean values for the premix ranged from 134 to 148 mg/kg over the 3 days, with an overall mean of 142 mg/kg. The CVs ranged from 3.4 to 6.4%, with an overall value of 6.0%. These CVs may be improved by the use of a deuterated diclazuril internal standard for GC/MS instead of compound R62646, but at the present time, deuterated diclaruzil is not available.

The recovery values and linearity of the assay were determined by spiking replicates of a known negative meal sample with diclaruzil at 0.20 to 5 mg/kg and carrying them through the assay. The results are shown in Table 2. Recoveries ranged from 90.7 to 100% when corrected for IS. The absolute recovery without correction for IS was about 50%. This relatively low value reflects the difficulty in extracting diclazuril from aqueous solutions into organic solvents, but with the inclusion of an internal standard, we did not find this to be a major problem. Of the various solvents tried during assay development, toluene was the most efficient. The values for slope, intercept, and linear regression coefficient for diclazuril as calculated from Table 2 were 0.921, 0.006, and 0.999, respectively. The assay is, therefore, linear up to at least 5 mg/kg in feed. The IS concentration used is 1 mg/kg, which is the same as the normal inclusion rate of diclazuril in feed. When the levels of the IS were varied from 0.2 to 5 mg/kg in feed and the diclazuril concentration was kept constant at 1 mg/kg (this was used as internal standard for the purpose of calculations), the standard curve for the IS also was linear, with a regression coefficient of 0.999.

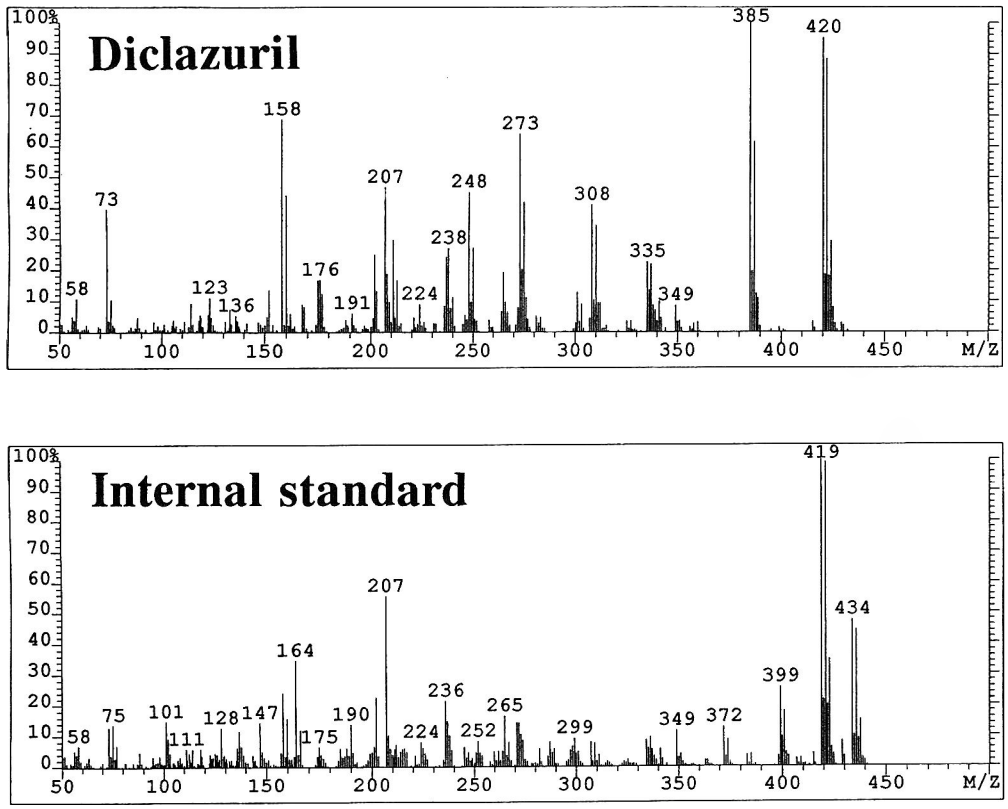


Figure 2. EI full-scan spectra of diclazuril and internal standard.

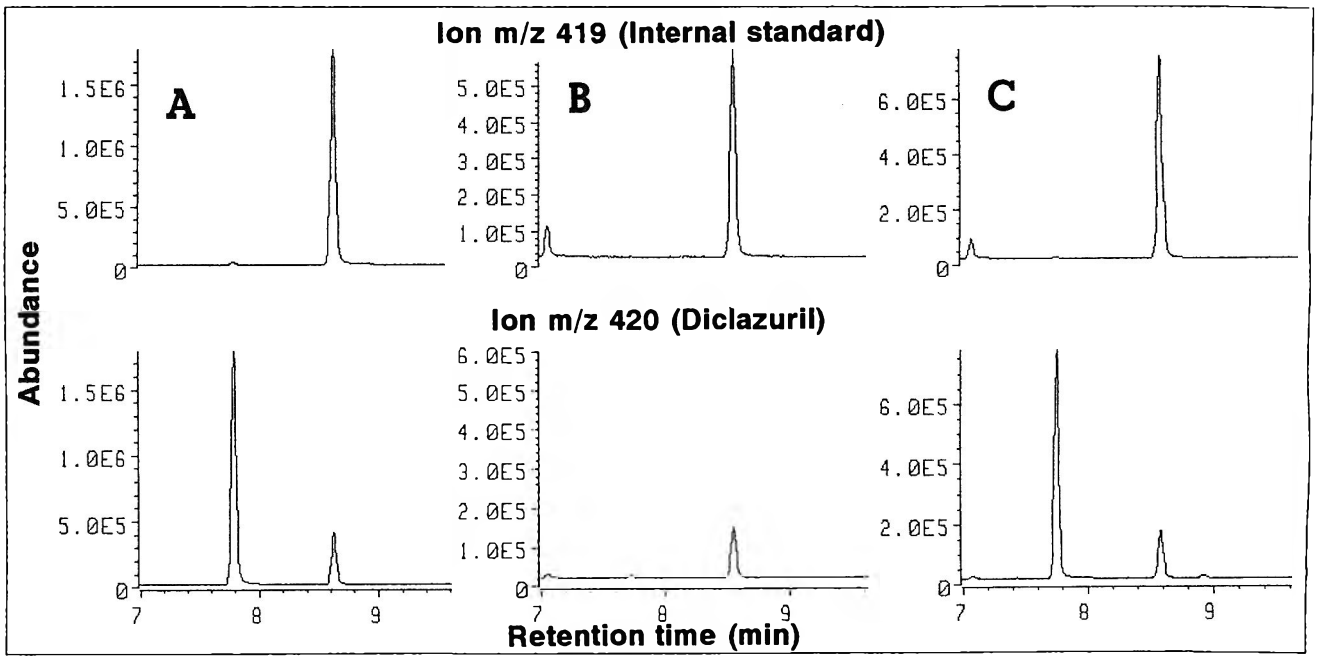


Figure 3. Single-ion chromatograms at *m/z* 420 (diclazuril) and *m/z* 419 (internal standard) for a standard equivalent to 1 mg/kg in feed (A), a negative feed extract (B), and an extract of feed containing diclazuril at 0.9 mg/kg (C). Diclazuril elutes at 7.8 min, and the IS elutes at 8.55 min.

The procedure we adopted for premixes is to add 0.5 g aliquots of the premixes to 9.5 g aliquots of a diclazuril-free finished feed sample and to carry them through the assay as for finished feeds, because some samples of premixes, which are made up in limestone or mineral bases, appear to adsorb some of the IS and diclazuril itself when extracted in the absence of a feed matrix. This results in very low absolute recoveries (typically 10–20%) and, thus, reduced sensitivity of the assay. The presence of a feed matrix during the extraction step eliminates this problem and increases the absolute recovery to values similar to those for finished feeds. This response suggests that components of the feed matrix block active sites in some premix bases.

In the published LC method (4) and in an unpublished LC method (Janssen), samples were extracted overnight. In our GC/MS method, we tried varying the extraction times from 1 h to 18 h and found that 2 h was sufficient for optimum extraction with a reciprocating shaker.

The detection limit of the assay is about 0.02 mg/kg at a signal-to-noise ratio of 3:1, which is adequate for the levels used in medicated feeds. We found, however, that the detection limit for diclazuril and IS can be very dependent on the type of injection port liner fitted to the GC/MS system. We recommend the use of a "gooseneck" splitless liner used in the split mode for best sensitivity and peak shape for diclazuril and IS. Similarly, a cup-type split liner can be used. This liner is packed with a 5 mm depth of deactivated silica beads held in place by a small quantity of deactivated silica wool. We also tried other deactivated injection port liners that work well with other applications, but most of them gave poor sensitivities for diclazuril and IS. Using a normal-type unpacked splitless liner and splitless injection for example, we expected larger peaks

Table 1. Reproducibility of the assay for diclazuril in finished feed and premix

Determination day	Data for finished feed				Data for premix			
	Mean, mg/kg	SD	CV, %	*n*	Mean, mg/kg	SD	CV, %	*n*
Day 1	1.04	0.069	6.6	5	143	5.9	4.1	5
Day 2	1.13	0.068	6.0	5	134	8.6	6.4	5
Day 3	1.10	0.049	4.5	5	148	5.0	3.4	5
Overall	1.09	0.072	6.6	15	142	8.6	6.0	15

Table 2. Recovery and linearity of diclazuril in finished feed

Amount added, mg/kg	Amount found, mg/kg[a]	Recovery, %
0.2	0.200 (0.007)	100.0
0.5	0.476 (0.031)	95.2
1.0	0.932 (0.017)	93.2
2.0	1.813 (0.018)	90.7
5.0	4.624 (0.298)	92.5

[a] Results are the means of triplicate determinations (± SD).

and better sensitivity; instead, the peaks were only just detectable. We have no explanation for this phenomenon and do not know if it depends on the particular make of GC used in the assay. It is, however, an important point of which to be aware when setting up the assay with a different GC/MS system.

Conclusions

The described assay has been used in this laboratory for about 2 years, during which time about 1000 feed samples have been analyzed for diclazuril. The method is relatively fast, specific, and easy to carry out and has not shown any problems of interference by other compounds.

References

(1) Vanparijs, O., Marsboom, R., & Desplenter, L. (1989) *Poultry Sci.* **68**, 489–495

(2) Vanparijs, O., Hermans, L., Van der Flaes, Lapointe, M., & Marsboom, R. (1989) *Avian Dis.* **33**, 422–424

(3) Lapointe, M.R., & Cohen, H. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 480–484

(4) De Cock, J., De Smet, M., & Sneyers, R. (1992) *J. Chromatogr.* **606**, 141–146

AGRICULTURAL MATERIALS

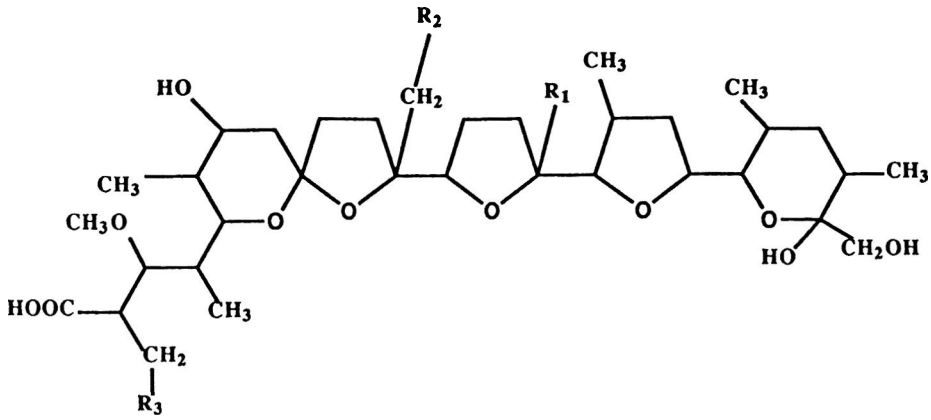
Ruggedness of the Monensin and Narasin Liquid Chromatographic Assays

MARK R. COLEMAN, THOMAS D. MACY, JOHN W. MORAN, and J. MATTHEW RODEWALD
Lilly Research Laboratories, A Division of Eli Lilly & Co., 2001 West Main St, Greenfield, IN 46140-0708

Supplementary validation data were generated for the monensin and narasin liquid chromatographic (LC) assays. Several parameters of the LC system and the sample preparation procedures were evaluated. Feed samples are routinely extracted in methanol–water (9 + 1, v/v). The ratio of methanol to water was varied to evaluate the ruggedness of the extraction procedure. The LC parameters evaluated included flow rates of the mobile phase and vanillin reagent, reactor temperature, and water content of the mobile phase. The resolution of monensin A, monensin B, and narasin A; retention times; tailing factors; peak areas; and peak widths were monitored as LC parameters were varied. The stabilities of monensin and narasin reference standard solutions over time when stored at room temperature and under refrigeration were also monitored. The results show that deviations in the methanol–water ratio of the extraction solution did not significantly affect final assay results. Modification of LC parameters may substantially affect retention time, peak area, and resolution factors.

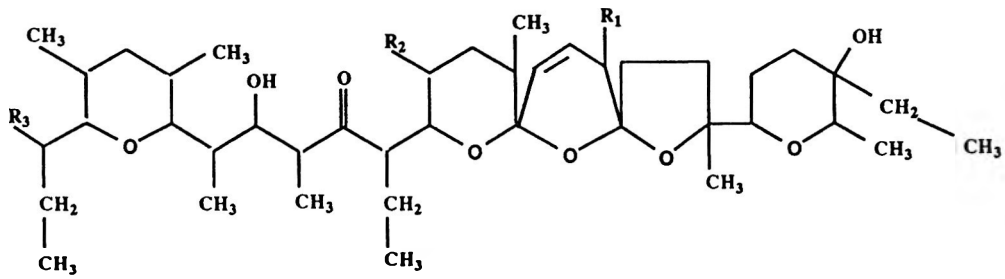
Received July 28, 1993. Accepted by RN November 3, 1993.

The quantitative determination of monensin and narasin (Figures 1 and 2) by liquid chromatography with post-column derivatization (LC–PCD) has been previously described (1, 2). The LC systems used to analyze these compounds, as outlined in Figure 3, are essentially the same, with the only difference being the reference standard used for each assay. These methods are currently being used in a number of laboratories, and the monensin method is currently being evaluated by an AOAC collaborative study as an alternative to the current microbiological methods (3). To prepare for the collaborative study, several additional validation parameters specifically centered on ruggedness were evaluated. The extraction procedure for both methods is a simple extraction of



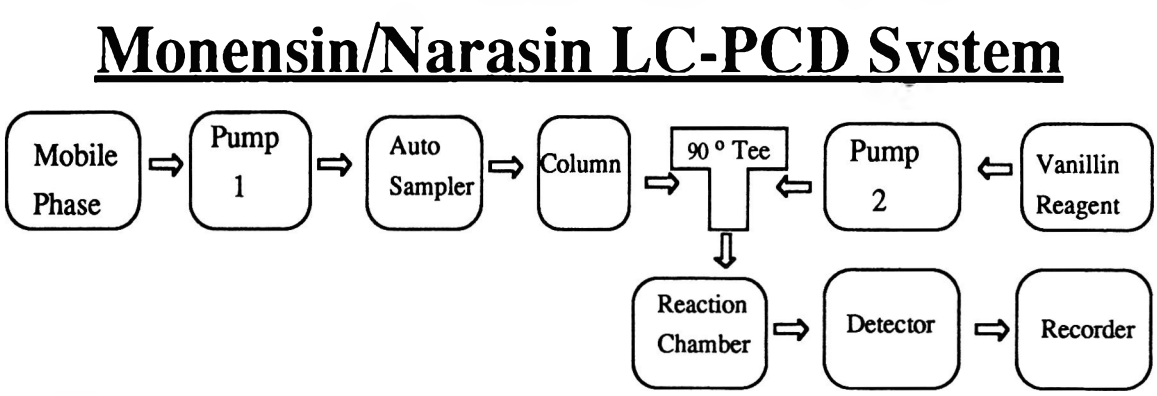
Factor	R_1	R_2	R_3
A	C_2H_5	H	H
B	CH_3	H	H
C	C_2H_5	H	CH_3

Figure 1. Structures of monensin and factors.



Factor	R_1	R_2	R_3
A	OH	CH_3	CO_2H
B	O	CH_3	CO_2H
D	OH	C_2H_5	CO_2H
I	OH	CH_3	CO_2CH_3

Figure 2. Structures of narasin and factors.



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

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

Autosampler = Varian Model 8055, or equivalent, with 200 µL injection loop

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

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

Pump 2 = LDC minipump, or equivalent (0.7 mL/min)

Vanillin reagent = methanol/H_2SO_4/vanillin, 95/2/3 (v/v/w) {Protect from UV light}

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

Reaction chamber = stainless steel reaction chamber (0.02 in x 20 ft) enclosed in a 98°C oven/heater.

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

Recorder = Varian Model 9176, or equivalent

Figure 3. LC-PCD system for analysis of monensin and narasin.

samples with methanol–water (90 + 10, v/v). This ratio was evaluated by extraction of representative poultry and cattle rations and liquid feed supplements. Because there is no cleanup of sample extracts, the only other area to evaluate for ruggedness is the detection system. The parameters considered most critical in the LC system for monensin and narasin were water content of the mobile phase, reactor temperature, acid/vanillin reagent ratio, and flow rates of the mobile phase and vanillin reagent pumps. Each of these parameters was evaluated individually with the resolution mixture to determine how changes in these parameters would affect the established acceptance limits for each method. The information generated will be very useful in evaluating the results of the AOAC INTERNATIONAL collaborative study for the monensin LC assay and for all laboratories that will use this method.

Table 1. Acceptance limits for monensin and narasin LC systems

Parameter	Item measured	Acceptance limit
Resolution	Monensin A and monensin B	>1.25
	Monensin A and narasin A	>3.5
Retention time	Monensin A	600–720 s
	Narasin A	750–1000 s
Tailing factor	Monensin A	<1.4
	Narasin A	<1.4

Table 2. Ruggedness of sample preparation procedure

Feed type	Monensin, g/ton	Methanol/ water ratio, v/v	Mean, g/ton[a]	RSD, %
Poultry	100	85/15	93	10.8
		90/10	97	1.9
		95/5	96	9.3
Cattle	35	85/15	35	12.3
		90/10	35	6.4
		95/5	34	10.9
LFS[b]	200	85/15	211	7.6
		90/10	223	2.5
		95/5	202	2.4

[a] $n = 3$.
[b] LFS = Liquid feed supplement

Table 3. Ruggedness of the LC system for monensin and narasin[a]

Parameter varied from written method	Retention time, s		Peak width		Peak tailing		Peak area		Resolution factors	
	Mon	Nar	Mon	Nar	Mon	Nar	Mon	Nar	Mon A and Mon B	Mon A and Nar
None	637	830	42.9	52.2	1.12	1.15	162841	194565	1.86	4.45
None	619	799	41.9	50.6	1.12	1.14	151656	150827	1.68	4.25
Reaction chamber, 95°C	638	828	43.8	52.7	1.12	1.14	167398	161682	1.76	4.30
Reaction chamber, 90°C	639	829	43.7	52.9	1.12	1.14	150664	132838	1.75	4.29
Mobile phase (96 + 4 + 0.1)	590	738	39.8	46.7	1.12	1.14	179705	230554	1.49	3.72
Mobile phase (90 + 10 + 0.1)	775	1108	54.7	70.8	1.13	1.15	134718	95143	2.36	5.73
MP = 0.7 mL/min, V = 0.5 mL/min	653	845	45.9	54.7	1.12	1.14	158146	133319	1.68	4.16
MP = 0.7 mL/min, V = 0.6 mL/min	653	805	43.1	50.8	1.11	1.12	153145	132651	1.65	4.13
MP = 0.7 mL/min, V = 0.8 mL/min	607	785	40.6	49.0	1.12	1.14	145239	165187	1.76	4.35
MP = 0.6 mL/min, V = 0.7 mL/min	710	919	46.5	55.8	1.12	1.15	172351	195320	1.75	4.44
MP = 0.8 mL/min, V = 0.7 mL/min	544	700	37.9	45.4	1.12	1.14	129377	109599	1.60	4.05
MP = 0.8 mL/min, V = 0.8 mL/min	535	691	36.6	44.1	1.12	1.13	129448	128847	1.66	4.19
MP = 0.6 mL/min, V = 0.6 mL/min	720	928	48.0	57.6	1.03	1.14	180273	188161	1.70	4.31

[a] Mon, monensin A; Nar, narasin A; MP, mobile phase; V, vanillin reagent.

Experimental

Methods and Reagents

The monensin and narasin methods are as described previously (1, 2) with the modifications listed below:

(a) *Mobile phase.*—Methanol–water–acetic acid (940 + 60 + 1). Filter under vacuum through 0.45 μm nylon-66 filter (Cat. No. 38-114, Rainin Instrument Co., Woburn, MA). Degas by stirring for 5–10 min under vacuum or by sparging with helium (recommend 3–5 min). Prepare fresh as required. *(1) Mobile phase A.*—Methanol–water–acetic acid (90 + 10 + 0.1). *(2) Mobile phase B.*—Methanol–water–acetic acid (94 + 6 + 0.1). *(3) Mobile phase C.*—Methanol–water–acetic acid (96 + 4 + 0.1).

(b) *Reaction chamber.*—The reaction chamber temperature is typically 98°C. The reaction chamber temperatures tested were 90°, 95°, and 98°C.

(c) *Extraction solution.*—The extraction solution is typically methanol–water (90 + 10, v/v). The extraction solutions tested were *(1)* methanol–water (85 + 15, v/v), (2) methanol–water (90 + 10, v/v), and *(3)* methanol–water (95 + 5, v/v).

(d) *Flow rates.*—The flow rates of the mobile phase and reagent pumps were modified to evaluate the effect of flow rates. Flow rates of 0.6, 0.7, and 0.8 mL/min were evaluated for the mobile phase pump and 0.5, 0.6, 0.7, and 0.8 mL/min were evaluated for the vanillin reagent pump.

Apparatus

(a) *Liquid chromatograph.*—With postcolumn reactor (Figure 3). A Beckman Model 110B pulse-dampened pump was used to deliver the mobile phase, and a LDC minipump was used to deliver the vanillin reagent. Both pumps were operated at 0.7 mL/min during method development and validation.

LC System Control Parameters

The reproducibility of the LC system to resolve monensin and narasin was evaluated within a day and across days. The parameters evaluated included resolution, tailing, peak width, peak area, and retention time.

(a) *Resolution.*—Prepare resolution mixture as described previously (1, 2) and analyze daily to ensure that the LC system is performing acceptably and that monensin A, monensin B, and narasin A can be separated from other vanillin-positive ionophores. Inject resolution mixture and adjust instrumentation such that the peak height response is 60–90% of full-scale deflection. Calculate the resolution factor R_s for each pair of adjacent peaks as described previously (1, 2). If R_s does not meet the specified requirements, adjust the LC conditions to improve the resolution.

(b) *Retention time.*—The retention time for narasin should be between 750 and 1000 s. The retention time for monensin should be between 600 and 720 s.

Table 4. Stability of monensin reference standard when stored at 5°C and at room temperature

Storage condition	Assay interval, weeks	Monensin theory, μg/mL	Mean, μg/mL	COV, %	Percentage of theory or initial[a]
—[b]	0	10	10.1	1.0	100.6
—	1		10.0	1.1	100.4
—	2		10.2	0.9	102.0
—	3		10.1	0.6	101.3
—	4		10.2	1.1	101.6
—	0	20	20.1	0.3	100.5
—	1		20.1	0.5	100.6
—	2		20.3	0.6	101.4
—	3		20.1	0.6	100.7
—	4		20.4	0.9	101.9
Room temperature	1	10	9.9	0.6	98.0
	2		10.1	1.2	100.0
	3		10.1	1.1	100.3
	4		10.1	1.2	100.8
Room temperature	1	20	19.9	0.2	98.8
	2		20.1	0.5	99.8
	3		20.1	0.4	99.8
	4		20.1	1.7	100.1
5°C	1	10	10.0	0.4	99.1
	2		10.1	0.5	99.9
	3		10.0	1.1	99.4
	4		10.0	0.5	99.3
5°C	1	20	20.0	0.3	99.5
	2		20.1	0.3	99.9
	3		20.0	0.3	99.4
	4		20.1	0.4	99.7

[a] Percentage of theory was calculated for freshly prepared standard. Percentage of initial was calculated for stored standards.
[b] Prepared fresh on the day of assay.

(c) *Tailing factor.*—The tailing factors for monensin A and narasin A, determined at 10% of peak height, must be <1.4. The tailing factor was calculated according to:

$$\text{Tailing} = \frac{10\%\ \text{width}}{2 \times (\text{retention time} - \text{time of front 10\% point})}$$

where 10% width = time of back 10% point – time of front 10% point and retention time = time of the maximum of the fitted Gaussian curve. A 10% point is the point where the response of a side of the peak reaches a height equal to 10% of the difference between the response of the fitted peak maximum and the response of the peak start of finish.

Table 1 lists the established acceptance limits for the monensin and narasin LC assays. These acceptance limits were established based on data from the developing laboratory only and may be modified at the completion of the AOAC INTERNATIONAL collaborative study.

Results and Discussion

Ruggedness of the LC–PCD System

The ruggedness was examined by evaluating several parameters of the chromatographic system and the sample preparation procedure. Samples are routinely extracted in methanol–water (90 + 10). The methanol/water ratio was varied, as shown in Table 2, to evaluate the ruggedness of the extraction solution. The data indicate that similar results are obtained with a methanol/water ratio of 85/15 or 95/5; however, the overall variability is lower when the methanol/water ratio is 90/10. Recovery is also slightly higher with a methanol/water ratio of 90/10. Slight deviations in the methanol/water ratio do not have a significant effect on final results.

Several LC parameters were evaluated to examine method ruggedness (Table 3), including flow rates of the mobile phase and vanillin reagent, reactor temperature, and water content of the mobile phase. Two LC runs were performed with no variations from the methods as written; they served as a reference point. The acceptance limits listed in Table 1 were met when the methods were performed without variation.

When the temperature of the reaction chamber in reduced to either 95° or 90°C, all LC control parameters were still met, but

Table 5. Stability of narasin reference standard when stored at 5°C and at room temperature

Storage condition	Assay interval, weeks	Narasin theory, µg/mL	Mean, µg/mL	COV, %	Percentage of theory or initial[a]
—[b]	0	10	10.2	1.6	100.6
—	1		10.1	0.8	100.8
—	2		10.0	1.4	100.2
—	3		9.8	1.1	98.0
—	4		9.7	2.3	97.4
—	0	20	20.0	2.7	100.5
—	1		20.1	0.7	100.4
—	2		19.7	1.8	98.6
—	3		19.9	0.3	99.4
—	4		19.6	2.2	97.9
Room temperature	1	10	10.0	1.5	98.4
	2		9.8	1.3	95.9
	3		9.7	0.9	95.5
	4		9.6	0.7	94.4
Room temperature	1	20	20.0	0.6	100.3
	2		19.8	0.5	99.2
	3		19.7	0.3	98.7
	4		19.6	0.4	98.3
5°C	1	10	10.0	0.4	98.0
	2		9.7	1.1	95.3
	3		9.6	1.0	94.6
	4		9.4	1.6	92.7
5°C	1	20	19.9	0.8	99.6
	2		19.6	1.1	98.3
	3		19.5	1.1	97.6
	4		19.6	0.8	98.1

[a] Percentage of theory was calculated for freshly prepared standard. Percentage of initial was calculated for stored standards.
[b] Prepared fresh on the day of assay.

there was a decrease in peak area, especially with narasin, which resulted in reduced sensitivity.

An increase in methanol content with a decrease in water content in the mobile phase (methanol–water–acetic acid, 96 + 4 + 0.1) decreased the retention time, increased the peak area, and decreased the resolution factor. A decrease in methanol content with an increase in water content in the mobile phase (methanol–water–acetic acid, 90 + 10 + 0.1) increased the retention time, peak width, and resolution factor but decreased the peak area.

The effect of changing the flow rates of the mobile phase and the vanillin reagent on the LC parameters was evaluated (Table 3). Retention time, peak width, and peak area can be affected by the flow rate; therefore, these parameters must be controlled closely to ensure that the system is functioning within the established control limits.

The data indicate that variations in LC parameters can affect retention time, peak width, peak area, and resolution factors. However, when the method is performed without modification, the established acceptance limits (Table 1) can be met.

Stability of Reference Standards

The stability data for monensin reference standard solutions stored at room temperature and at 5°C for 4 weeks are presented in Table 4. For the standard curve, concentrations of 10 and 20 µg/mL were prepared and assayed at 0, 1, 2, 3, and 4 weeks after storage at room temperature and at 5°C against a freshly prepared standard solution. These data demonstrate that at 4 weeks, the responses for both the 10 and 20 µg/mL standard solutions are above 98% of initial levels and support the stability of standards stored for at least 4 weeks at room temperature.

The stability data for narasin reference standard solutions stored at room temperature and at 5°C for 4 weeks are presented in Table 5. For the standard curve, concentrations of 10 and 20 µg/mL were prepared and assayed at 0, 1, 2, 3, and 4 weeks after storage at room temperature and at 5°C against a freshly prepared standard solution. These data support the stability of standards stored for 2 weeks at room temperature.

Resolution

Resolution was evaluated according to USP XXII, Chapter 621 (4). The reproducibility data (Table 6) were determined by 6 replicate injections of the resolution mixture containing monensin and narasin. The resolution factors for monensin A and monensin B (Mon A–Mon B) and for monensin A and narasin A (Mon A–Nar A) were reproducible, with relative standard deviations (RSDs) of less than 2%. The consistency

Table 6. Retention time, peak tailing, peak area, and resolution factors for monensin A, monensin B, and narasin within a run

	Retention time, s			Peak tailing			Peak area			Resolution factors	
Injection number	Mon A	Mon B	Nar[a]	Mon A	Mon B	Nar	Mon A	Mon B	Nar	Mon A and Mon B	Mon A and Nar
1	642	568	844	1.21	1.07	1.24	175216	6681	134309	1.64	3.83
2	643	571	845	1.22	1.08	1.24	174167	6648	133973	1.63	3.81
3	642	570	844	1.23	1.11	1.25	174830	6597	133852	1.59	3.71
4	640	567	841	1.22	1.08	1.24	173685	6558	134665	1.65	3.82
5	640	568	842	1.22	1.08	1.24	174844	6563	133473	1.64	3.80
6	640	567	841	1.24	1.08	1.26	174136	6503	132178	1.59	3.69
Mean	641	569	843	1.22	1.08	1.25	174480	6592	133742	1.62	3.78
RSD, %	0.21	0.29	0.20	0.84	1.26	0.67	0.33	0.98	0.65	1.64	1.60

[a] Nar = narasin A.

of the retention times of monensin A, monensin B, and narasin A are shown in Table 6. The RSDs for the average retention times of these compounds were less than 0.3%. The individual and average peak areas for monensin A and B and narasin A varied little throughout the 6 injections. The RSDs for the average peak area for these compounds were less than 1.0%. The tailing factors for monensin A, monensin B, and narasin A were consistent between injections, with RSDs of 0.84, 1.26, and 0.67%, respectively. All of these parameters were within the established limits for these methods (Table 1) and were consistent.

The reproducibility of the resolution mixture over time was evaluated (Table 7). These data were obtained from 6 randomly selected resolution determinations over several months. In each case, the resolution mixture was injected and evaluated prior to analyses of samples or reference standards. The individual and average resolution factors for the 6 determinations all passed the acceptance limits required for further analyses, as indicated in Table 1. The individual and average retention times for monensin A, monensin B, and narasin A demonstrated that the retention times were reproducible over time. The individual and average peak areas for monensin A, monensin B, and narasin A are expected to vary to some degree over time, and the degree of variability was acceptable. The individual and average tailing factors for monensin A, monensin B, and narasin A were reproducible over time, with RSDs of 1.1, 2.2, and 1.9%, respectively.

Each chromatographic run included standard solutions (1, 5, 20, and 40 µg/mL) at the beginning and end of each run. The responses from the standard solutions were averaged and used in calculating final results. Standard curves were plotted to evaluate linearity and precision between the beginning and end of each run. The data within a single chromatographic run (Table 6) were highly reproducible; therefore, we recommend running a single resolution mixture on a daily basis and ensuring that the parameters in Table 1 are met prior to analyses of samples.

The data demonstrate that the methods are very rugged and reproducible over time. This information should aid laboratories planning to perform these assays in obtaining acceptable results.

Table 7. Retention time, peak tailing, peak area, and resolution factors for monensin A, monensin B, and narasin over a 3-month period

	Retention time, s			Peak tailing			Peak area			Resolution factors	
Run number	Mon A	Mon B	Nar[a]	Mon A	Mon B	Nar	Mon A	Mon B	Nar	Mon A and Mon B	Mon A and Nar
4538	646	575	838	1.13	1.04	1.18	179595	6411	155796	1.58	3.64
4572	662	582	897	1.14	1.05	1.18	186050	7194	132653	1.55	3.60
4589	697	605	953	1.11	1.06	1.14	155303	5907	106028	1.65	3.85
4619	663	586	879	1.12	1.04	1.13	160983	6364	118247	1.55	3.68
4647	678	594	912	1.11	1.01	1.16	197272	7106	141370	1.44	3.64
4652	696	607	944	1.11	1.08	1.14	200742	7318	142573	1.75	3.95
Mean	674	592	904	1.12	1.05	1.16	179991	6717	132778	1.59	3.73
RSD, %	3.0	2.2	4.7	1.1	2.2	1.9	10.4	8.5	13.6	6.6	3.8

[a] Nar = narasin A.

Acknowledgments

We thank Chester R. Simpson, David A. Dickson, and Amy J. Gilmore for their assistance in generating the data and in preparing the manuscript.

References

(1) Rodewald, J.M., Moran, J.W., Donoho, A.L., & Coleman, M.R. (1992) *J. AOAC Int.* **75**, 272–279

(2) Rodewald, J.M., Moran, J.W., Donoho, A.L., & Coleman, M.R. (1994) *J. AOAC Int.* **77**, 821–828

(3) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA

(4) *The United States Pharmacopeia XXII/The National Formulary XVII* (1990) USP, Rockville, MD

AGRICULTURAL MATERIALS

Determination of Bacitracin-MD in Complete Feed by Microbiological Plate Assay: Collaborative Study

ANIL D. DESAI, ARNOLD HIRSCH, and LARRY A. MUIR
A.L. Laboratories, Inc., 400 State St, Chicago Heights, IL 60411

Collaborators: J. Bell; M. Burkhart; N. Detablan; A. Gardner; M. Hasselberger; A. Knutson; J. Lamb; E. Luigs; D.R. Moore; H. Ragheb; M. Shah

Eleven laboratories evaluated the double extraction procedure for the determination of bacitracin methylene disalicylate in swine and poultry feeds at 22, 44, and 88 g/2000 lb. Bacitracin activity was determined on each of 2 days using blind duplicates in a random order. All collaborators submitted their data for statistical evaluation. No significant difference was found between the assay results and the label claim at 22, 44, and 88 g/2000 lb for swine and poultry feed. For both feed types, reported ranges at 95% confidence limit for 22, 44, and 88 g/2000 lb were 17–30 g/2000 lb, 29–61 g/2000 lb, and 64–112 g/2000 lb, respectively. The microbiological plate assay procedure for determination of BMD in complete feed was adopted first action by AOAC INTERNATIONAL.

[1] Submitted for publication April 20, 1993.

The recommendation was approved by the Committee on Feeds, Fertilizers, and Related Topics, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Board Actions" (1993) *The Referee* **17**, September issue.

Bacitracin is a polypeptide antibiotic produced by *Bacillus licheniformis*. Bacitracin is added to animal feeds for growth promotion, feed efficiency, and disease control and is stabilized with methylene disalicylic acid (MDA). The microbiological assay procedures developed for this study were collaboratively tested by qualified personnel trained to perform microbiological assays. Previous methods developed for measuring zinc bacitracin in feed have also been used for recovery of bacitracin-MD (BMD) from feed (1–4). The new method was developed to improve the accuracy of measuring BMD in feed at levels of 22–88 g/2000 lb.

Collaborative Study

Swine and poultry feeds (400 lb each) were formulated by the Department of Grain Science and Industry, Kansas State University, Manhattan, KS. Fifty pounds of each feed was blended with bacitracin-MD premix (house standard assayed at 52.7 ± 3.4 g/lb activity) at an estimated level of 22, 44, and 88 g BMD activity/2000 lb using a Wenger mixer (50 lb capacity). Swine and poultry feeds at each level were initially assayed prior to shipment of the samples to the collaborators to ascertain the claim potency. Each package was labeled with a sample code in random order without the claim potency. Collaborators were asked to follow the detailed dilution scheme for each sample. Each collaborator was supplied with zinc baci-

tracin working standard (BZn, A.L. Laboratories, Inc.), a slant of *Micrococcus luteus* (ATCC 10240) test culture, a copy of the method, material safety data sheets, homogeneous samples, and data reporting forms. In-house validation of the method was performed at the laboratory of the Office of Indiana State Chemist, West Lafayette, IN.

993.29 Bacitracin-MD (BMD) in Complete Feed—Microbiological Plate Assay Method First Action 1993

(Applicable to determination of 22–88 g/2000 lb [22–88 g/907.18 kg] bacitracin methylene disalicylate in swine and poultry complete feeds.)

Method Performance:

See Table **993.29A** for method performance data.

A. Principle

Bacitracin methylene disalicylate (BMD) in acid-extracted feed samples inhibits growth of *M. luteus*, forming inhibition zones on plates. Results are compared with standard BZn plates and are calculated as standard dose-response lines.

B. Apparatus

See **957.23C(a)–(c)** for cylinders, Petri dishes, and cylinder dispenser.

(**a**) *Shaker.*—Magnetic stirrer or reciprocating shaker is suitable.

(**b**) *Blender.*—With shock-proof motor.

(**c**) *Centrifuge.*—Capable of 5200 rpm (4275 × *g*) (International Equipment Co., 300 2nd Ave, Needham Heights, MA, is suitable source).

(**d**) *Inhibition zone reader.*—Zone reader (Fisher-Lilly Zone Reader, Fisher Scientific, Pittsburgh, PA) or automatic reader.

(**e**) *Incubator.*—Capable of maintaining a 30°–37° temperature range within ± 1°.

Table 993.29A. Method performance for determination of Bacitracin-MD (BMD) in swine and poultry feeds by microbiological plate assay method

BMD, added g/2000 lb	BMD found, g/2000 lb				
	Mean	s_r	s_R	RSD_r, %	RSD_R, %
	Swine feed				
22	22.97	2.02	4.18	8.78	18.22
44	47.62	4.18	9.79	8.78	20.57
88	92.81	9.43	17.20	10.16	18.53
	Poultry feed				
22	22.98	2.13	3.94	9.27	17.15
44	43.95	6.34	7.01	14.42	15.94
88	89.76	7.73	14.08	8.08	15.69

C. Reagents

(**a**) *Methanol.*—(*Note*: Store in tightly closed containers. Keep away from heat, sparks, and open flames.)

(**b**) *Phosphate buffer.*—5%, pH 6.5. Dissolve 22.15 g anhydrous K_2HPO_4 and 27.85 g anhydrous KH_2PO_4 in H_2O and dilute to 1 L.

(**c**) *HCl–methanol solution.*—0.24N. Add 20.4 mL concentrated HCl to 1 L methanol.

(**d**) *Methanol–phosphate buffer.*—5%. Mix 150 mL methanol (**a**) and 2850 mL 5% phosphate buffer, pH 6.5. Adjust pH to 6.5 ± 0.1 with 1.0N HCl.

(**e**) *Test organism.*—*M. luteus* (ATCC 10240, also known as *M. flavus*). Maintain culture and prepare inoculum as in **957.23D(a)**. Before use, determine by trial plates, optimum concentration to add to assay medium to obtain sharp inhibition zones of adequate size (15–18 mm for reference concentration, 0.04 units/mL).

(**f**) *Plate preparation.*—Dissolve 30.5 g agar medium A, **957.23A(a)**, in 1 L H_2O; sterilize at 15 lb steam pressure 20 min, cool to ca 50°, and inoculate with test organism (**e**). Pour ca 12 mL/plate. Store plates inverted at 4°–10°; warm to 22°–25° before dosing.

D. Preparation of Standard Solutions

Accurately weigh an amount of Zinc Bacitracin USP Reference Standard or A.L. Working Standard (BZn) (400 State St, Chicago Heights, IL); dissolve and bring to volume in 50 mL volumetric flask with 0.01N HCl to achieve concentration of 100 units/mL. Stock solution is stable for ca 1 week stored at 4°–10°.

On day of use, prepare intermediate solution (10 units/mL) by diluting 5 mL stock solution to 50 mL with phosphate buffer, **C(b)**.

On day of use, prepare 1 unit/mL solution by diluting 5 mL of intermediate solution (10 units/mL) to 50 mL with phosphate buffer. Use 1 unit/mL solution to prepare the standard working solutions listed in Table **993.29B**, making all dilutions with 5% methanol–phosphate buffer, **C(d)**, in volumetric flasks.

E. Sample Preparation

(1) Grind sample in blender to obtain homogeneous mash of ca 1 mm particle size; avoid fine grinding. Accurately weigh 20–50 g of ground sample to nearest ± 0.1 g into 250 mL centrifuge bottle.

Table 993.29B. Preparation of standard solutions

Volume of 1 unit/mL solution, mL	Diluted volume, mL	Final concentration, units/mL
4	25	0.16
4	50	0.08
4	100	0.04
2	100	0.02
1	100	0.01

Table 993.29C. Dilution scheme for determination of bacitracin-MD (BMD) in swine and poultry feeds by microbiological plate assay method

Feed claim, g BMD/2000 lb	Sample wt, g	Extractions[a]	HCl in methanol, mL[b]	Methanol in pH 6.5 buffer, mL[c]	Total extrations volume, mL	Final dilution
20–25	26	1	45	45	130	5:25
		2	25	25		
40–50	25	1	50	50	150	3:25
		2	25	25		
80–100	21	1	45	45	130	3:50
		2	20	20		

[a] 1 and 2 refer to first, **E(2)**, and second, **E(4)**, sample extractions.
[b] 0.24N HCl in methanol solution, **C(c)**.
[c] 5% Methanol in 5% potassium phosphate buffer, pH 6.5, **C(d)**.

(2) Using volumes in Table **993.29C**, add HCl–methanol solution, **C(c)**, to sample, swirl gently, and add methanol–phosphate buffer, **C(d)**. Place magnetic stir bar in bottle, stopper tightly, and stir vigorously on magnetic stirrer for 15 min.

(3) Centrifuge sample 10 min at 2100 rpm. Decant and save supernatant ("Volume 1") in a stoppered container.

(4) Using appropriate volumes in Table **993.29C**, add HCl–methanol solution to sample, swirl gently, and add methanol–phosphate buffer. Place stir bar in bottle, stopper tightly, and stir vigorously on magnetic stirrer 10 min.

(5) Centrifuge sample 5 min at 2100 rpm. Transfer "Volume 1" back to extraction bottle. (Combined extracts are "Volume 2.") Mix well and centrifuge sample bottle 5 min at 2100 rpm.

(6) On the basis of the expected BMD concentration, dilute an aliquot of supernate to reference concentration (0.04 ± 0.01 units/mL). Final methanol concentration in samples and standards should not vary by >10%.

(7) Store all assay solutions at 4°–10° if plate dosing is delayed more than 1 h.

F. Plating

Bring assay solutions to 20°–25° and shake well before plating.

Dose each cylinder with 250 µL assay solution, following **957.23E**. Incubate plates at constant (± 1°) temperature in 30°–35° range, using same temperature for standards and samples.

G. Calculations

Measure diameter of inhibition zones to the nearest 0.1 mm. Follow **957.23E, F** to calculate standard dose-response line. For manual calculation, draw best fit line. For greater accuracy, use least-square fit linear or polynomial equation calculation from computer or calculator.

Determine BMD in sample by reading concentration from standard response line. Calculate sample potency by converting unit/mL to g/2000 lb of feed as follows:

$$\text{g Bacitracin/2000 lb} = (\text{unit/mL} \times \text{total dilution/sample weight}) \times 21.619$$

Ref.: *J. AOAC Int.* **77**, 1072 (1994).

Table 1. Collaborative study results for determination of bacitracin-MD (BMD) in swine and poultry feeds (day 1), for blind duplicates at 3 levels (claim level)

	Swine samples, g BMD/2000 lb						Poultry samples, g BMD/2000 lb					
Lab.	22		44		88		22		44		88	
1	29.9	27.4	55.3	69.7	98.6	126.9	24.3	25.0	59.7	43.6	90.3	84.1
2	19.0	18.7	39.0	38.7	94.7	68.8	19.0	17.9	46.3	43.6	79.9	69.0
3	14.8	12.1	26.2	22.8	44.6	57.7	13.4	16.3	36.2	22.9	51.3	44.3
4	26.2	29.4	52.2	55.2	102.7	100.5	26.2	33.8	45.3	60.6	109.4	99.9
5	25.9	26.9	44.1	54.6	96.4	95.9	26.2	20.3	48.6	38.1	105.2	118.6
6	27.8	19.1	48.0	45.9	64.9	77.7	27.5	26.4	43.2	49.7	92.2	93.6
7	20.8	19.3	38.3	47.3	84.0	99.4	24.4	20.2	43.3	45.0	89.1	99.5
9	34.6	35.7	60.0	72.0	140.2	158.4	21.6	28.1	64.0	132.9	98.6	57.6
10	20.3	24.9	42.2	47.4	85.5	110.0	21.9	16.9	47.6	44.8	100.8	71.4
11	24.2	24.9	44.8	56.3	77.0	88.5	18.3	19.0	41.8	44.0	86.7	91.5
12	20.5	20.5	42.2	33.8	92.0	109.3	22.7	25.4	37.8	51.2	96.4	93.7
Detn[a]	11	11	11	11	11	11	11	11	11	11	11	11

[a] Number of determinations.
Note: Collaborator 8 withdrew from the study.

Table 2. Collaborative study results for determination of bacitracin-MD (BMD) in swine and poultry feeds (day 2), for blind duplicates at 3 levels (claim level)

	Swine samples, g BMD/2000 lb						Poultry samples, g BMD/2000 lb					
Lab.	22		44		88		22		44		88	
1	26.0	24.3	57.3	50.7	81.0	94.0	18.7	26.8	51.9	34.2	89.4	74.8
2	25.6	14.7	43.6	38.5	72.5	76.3	26.5	27.3	51.2	24.4	100.6	73.8
3	19.0	16.8	23.4	37.8	75.9	79.5	19.8	22.1	35.5	27.7	88.3	73.2
4	27.7	33.0	52.6	67.4	130.5	99.2	34.3	27.8	45.4	49.5	100.6	112.4
5	23.8	25.8	54.1	55.2	96.4	116	25.4	29.8	44.4	57.8	120.5	111.5
6	20.0	22.4	44.7	45.0	89.4	84.1	26.2	21.8	49.3	44.3	88.1	97.6
7	22.3	21.9	47.1	64.5	82.9	104	28.0	17.9	45.3	44.2	106	77.2
9	18.4	18.3	54.4	45.7	95.7	94.2	26.8	22.0	58.0	66.6	103.8	119.7
10	24.3	23.5	49.7	43.0	95.3	98.1	19.7	18.4	40.5	38.1	85.5	72.5
11	18.7	24.1	52.0	50.2	76.0	98.7	18.7	17.4	56.4	41.9	85.2	77.9
12	20.5	16.8	41.1	41.3	85.5	84.8	19.5	20.5	41.1	41.4	84.7	84.8
Detn[a]	11	11	11	11	11	11	11	11	11	11	11	11

[a] Number of determinations.
Note: Collaborator 8 withdrew from the study.

Results and Discussion

Blind duplicates of each material were assayed on each of 2 days. Assay results (Tables 1 and 2) were averaged across days (Table 3) and then statistically analyzed (5). Results were checked for outliers using the Cochran and Grubbs procedure as proposed by the collaborative study guidelines. Statistical evaluation of the results is presented in Table 4. Relative and absolute repeatability standard deviations for the swine feed ranged from 8.8 to 10.2% and from 2.0 to 9.4 g BMD/2000 lb, respectively. Relative and absolute repeatability standard deviations for the poultry feeds ranged from 8.1 to 14.4% and from 2.1 to 7.7 g BMD/2000 lb, respectively. Relative and absolute reproducibility standard deviations for the swine feeds ranged from 18.2 to 20.6% and from 4.2 to 17.2 g BMD/2000 lb, respectively. Relative and absolute reproducibility standard deviations for the poultry feeds ranged from 15.7 to 17.2% and from 3.9 to 14.1 g BMD/2000 lb.

No outliers were detected except for Laboratory 9 for poultry sample at 44 g BMD/2000 lb. Those results (day 2) were excluded from the statistical evaluation.

Collaborators' Comments

Some collaborators indicated that: *(1)* the samples were not homogeneous; *(2)* the final concentration of methanol in the assay solution in 1 of the samples exceeded 10% (ca 14%); *(3)* after the second extraction the container had to be rinsed after transferring "Volume 1" to "Volume 2;" and *(4)* the procedure was lengthy. The last problem could have been solved by using double extraction as an alternative procedure.

Table 3. Collaborative study average results for determination of bacitracin-MD (BMD) in swine and poultry feeds (days 1 and 2)

	Swine samples, g BMD/2000 lb						Poultry samples, g BMD/2000 lb					
Lab.	22		44		88		22		44		88	
1	27.95	25.85	56.30	60.20	89.80	110.45	21.50	25.90	55.80	38.90	88.85	79.45
2	22.30	16.70	41.30	38.60	83.60	72.55	22.75	22.60	48.75	34.00	90.25	71.40
3	16.90	14.45	24.80	30.30	60.25	68.60	16.60	19.20	35.85	25.30	69.80	58.75
4	26.95	31.20	52.40	61.30	116.60	99.85	30.25	30.80	45.35	55.05	105.00	106.15
5	24.85	26.35	49.10	54.90	96.40	105.95	25.80	25.05	46.50	47.95	112.85	115.05
6	23.90	20.75	46.35	45.45	77.15	80.90	26.85	24.10	46.25	47.00	90.15	95.60
7	21.55	20.60	42.70	55.90	83.45	101.70	26.20	19.05	44.30	44.60	97.55	88.35
9	26.50	27.00	57.20	58.85	117.95	126.30	24.20	25.05	61.00[a]	99.75	101.20	88.65
10	22.30	24.20	45.95	45.20	90.40	104.05	20.80	17.65	44.05	41.45	93.20	71.95
11	21.45	24.50	48.40	53.25	76.50	93.60	18.50	18.70	49.10	42.95	85.95	84.70
12	20.50	18.65	41.65	37.55	88.75	97.05	21.10	22.95	39.45	46.30	90.55	89.25

[a] Outlier.
Note: Collaborator 8 withdrew from the study.

Table 4. Statistical evaluation of the collaborative study results for determination of bacitracin (BMD) in swine and poultry feeds (days 1 and 2)

	Swine feed			Poultry feed		
Claim level, g BMD/2000 lb	22	44	88	22	44	88
No. of labs	11	11	11	11	10	11
No. of determinations	22	22	22	22	20	22
Mean, g BMD/2000 lb[a]	22.97	47.62	92.81	22.98	43.95	89.76
s_r	2.02	4.18	9.43	2.13	6.34	7.73
s_R	4.18	9.79	17.20	3.94	7.01	14.08
RSD_r, %	8.78	8.78	10.16	9.27	14.42	8.08
RSD_R, %	18.22	20.57	18.53	17.15	15.94	15.69

[a] Overall mean of the laboratory values = $\bar{X}$.

A few collaborators encountered some practical problems on day 1 or day 2; therefore, the assays were repeated on day 3 and those results were reported.

No major problems with the technique were noted by any collaborator.

Conclusions and Recommendations

On the basis of the statistical evaluation of the test results of this collaborative study, the statistical parameters, e.g., Cochran's test, Grubbs' test, Precision test, *F*-value (95% confidence limit), and % CV calculated without the outliers, were found within U.S. Food and Drug Administration and American Association of Feed Control Officials (AFFCO) assay control limits (± 30% of the claim). The repeatability standard deviations for commercial feeds being assayed routinely at A.L. Laboratories, Inc. are very similar to the statistical data generated by this collaborative study.

On the basis of the results of this study, it is recommended that the method for determination of bacitracin-MD in complete feed by microbiological plate assay procedure be adopted first action.

Acknowledgments

The Associate Referee acknowledges the special efforts of H.S. Ragheb for the in-house validation of the method, helpful suggestions, and guidance; Daniel Mowrey for statistical evaluation; Reidar Sandvik for the technical assistance; Kathy Peterson for her assistance in the preparation of this manuscript; Paul Duquette for reviewing this manuscript; and the following collaborators for their time and cooperation:

James Bell, Barrow-Agee Laboratories, Inc., Memphis, TN

Mike Burkhart, Hazleton Laboratories, Inc., Madison, WI

Nenita Detablan, Iowa Department of Agriculture, Des Moines, IA

Audrey Gardner, Department of Food Science & Technology, Geneva, NY

Mary Lee Hasselberger, Department of Agriculture, Lincoln, NE

Ann Knutson, Colorado Department of Agriculture, Denver, CO

John Lamb, Lilly Corporate Center, Indianapolis, IN

Elizabeth Luigs, A.L. Laboratories, Inc., Production Laboratory, Chicago Heights, IL

Denise Riley Moore, Woodson-Tenent Laboratories, Memphis, TN

Hussein Ragheb, Office of Indiana State Chemist, West Lafayette, IN

Manju Shah, A.L. Laboratories, Inc., Technical Service Laboratory, Chicago Heights, IL

References

(1) Ragheb, H.S., Black, L., & Graham, S. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 526–535

(2) Ragheb, H.S. (1980) *J. Assoc. Off. Anal. Chem.* **62**, 662–670

(3) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **957.24** and **965.48**

(4) Gallagher, J.B., Love, P.W., & Knotts, L.L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1177–1184

(5) Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis (1989) *J. Assoc. Off. Anal. Chem.* **72**, 694–704

AGRICULTURAL MATERIALS

Improved Esterification of Mecoprop for Sensitive Detection on Capillary Gas Chromatography with Mass Selective and Electron Capture Detection

ARTHUR S. LEE, MOO-KI HONG, [1] and ALBERT E. SMITH
University of Georgia Department of Crop and Soil Sciences, Griffin, GA 30223-1797

Analytical methods for determination of the derivatives of the herbicide mecoprop, (±)-2-(4-chloro-2-methylphenoxy)propionic acid (MCPP), by capillary column gas chromatography with mass selective detection (GC–MSD) and electron capture detection (GC–ECD) were studied. A successful procedure was introduced for the ester preparation using H_2SO_4 as the catalyst and the alcohols 2,2,2-trichloroethanol (TCE) or 2,2,2-trifluoroethanol (TFE). The identification and elucidation of MCPP by GC–MSD following the esterification with diazomethane, BF_3–methanol, H_2SO_4–methanol, TCE, TFE, or pentafluorobenzyl bromide (PFB) were carried out. A comparison of the response sensitivities among those MCPP esters was made with GC–ECD. Although the methylation product of MCPP was confirmed with GC–MSD, its low sensitivity to the ECD limited the detection of MCPP. TCE, TFE, and PFB derivatization methods resulted in a high rate of MCPP esterifications and very sensitive ECD molecular responses. On the basis of efficiency, convenience, worker safety, and least sample contamination, TFE esterification was considered as the superior method for MCPP analysis compared with the other methods of derivatization. An accurate method is described for quantifying MCPP in soil leachates by GC–ECD at very low concentrations without the requirement of a complicated cleanup process. As a result, MCPP residues at concentrations of less than 0.1 μg in 100 mL soil leachate were detected.

Mecoprop, (±)-2-(4-chloro-2-methylphenoxy)propionic acid (MCPP), a phenoxy acid herbicide, is used extensively for maintenance of home lawns and golf courses because of its selective weed-control efficiency and low mamalian toxicity. Either high polarity or low volatility of MCPP makes it ineffective for analysis by gas chromatography (GC) without generating a more vaporous derivative. Several methods for the preparation of derivatives of herbicides, to facilitate GC analysis, were reviewed by Cochrane (1). A few methods for esterifying MCPP were studied; most of them involve methylation with diazomethane (2–5), BF_3–methanol mixture (6, 7), and fuming sulfuric acid–ethanol (8). The formation of methyl-MCPP by using diazomethane was confirmed by GC–mass selective detection (MSD) in this study. The U.S. Environmental Protection Agency (EPA) method for MCPP determination (4) uses diazomethane methylation for GC–electron capture detection (ECD) analysis. This method is considered dangerous by many university safety officers, and methyl-MCPP results in a low response when analyzed by GC–ECD. When determining the fate of pesticides in the environment, the use of methods that are very sensitive and accurate is of great importance.

MCPP derivatives, formed to improve the sensitivity and selectivity of subsequent detection by GC–ECD, include the formation of 2-chloroethyl-MCPP (9), 2,2,2-trichloroethyl (TCE)-MCPP (10), and pentafluorobenzyl (PFB)-MCPP (11). Unfortunately, these methods have complicated cleanup procedures that eliminate their utility when processing large numbers of samples. A study (12) using BF_3 as the catalyst indicated that BF_3–2,2,2-trifluoroethanol (TFE) failed to react with MCPP and dicamba (3,6-dichloro-2-methoxybenzoic acid), which elicited our attempt to apply H_2SO_4–TFE for formation of TFE–MCPP.

We report the development of a simple and practicable method for derivatizing MCPP with TFE by using H_2SO_4 as the catalyst. The formation of the resulting ester was confirmed by GC–MSD. The sensitivity of the TFE ester was compared with other esters by GC–ECD. The method was evaluated further with MCPP residue extracted from a soil leachate.

Experimental

Apparatus

The Hewlett-Packard (Sunnyvale, CA) Model 5890 gas chromatograph series II was linked to a HP 3365 series II ChemStation and was equipped with an electron capture detector. The Rtx-1 (RESTEK, Inc., Bellefonte, PA) capillary col-

Received August 3, 1993. Accepted by RN November 4, 1993.

[1] National Institute of Health, Department of Food Chemistry, Seoul 122-020, Republic of Korea

umn, 30 m x 0.53 mm id, had a film thickness of 1 μm and a 5 m guard column connected to the entrance end. The column was connected to the ECD system. Injection port and detector temperatures were 250° and 300°C, respectively. Helium was used to carry analytes at a rate of 13–15 mL/min. The make-up gas was 5% argon in methane. Two microliters of each sample was injected and each sample injection was repeated. Oven conditions were adjusted for each herbicide ester.

The oven temperature conditions for the esters were as follows: (*1*) *TFE esters.*—Initial 130°C (6 min hold), programming rate 30°C/min, final 250°C (5 min hold). (*2*) *Methyl esters.*—Initial 150°C (5 min hold), programming rate 30°C/min, final 250°C (5 min hold). (*3*) *TCE esters.*—Initial 180°C (8 min hold), programming rate 30°C/min, final 250°C (5 min hold). (*4*) *PFB esters.*—Initial 190°C (7 min hold), programming rate 20°C/min, hold 250°C (3 min hold).

The MSD system was a Hewlett-Packard 5890 GC equipped with a 5971A MSD system controlled by an HP G1034B MS ChemStation. We used an HP-5 fused-silica capillary column, 30 m × 0.25 mm id, with a film thickness of 0.25 μm and a splitless injection. The injection port and interface temperatures were 230° and 280°C, respectively. The oven temperature was controlled with an initial temperature of 80°C (3 min hold), a program rate of 20°C/min to a temperature of 200°C (4 min hold), and increased to 250°C (3 min hold). The carrier gas was helium set at a head pressure of 90 kpa. Two microliters of each sample was injected with an automatic sample injector (HP 7673), and each sample injection was repeated. Samples were injected in a sequence program by using an intermittent solvent injection following sets of 3 samples.

Reagents

Pesticide grade hexane, methanol, diethyl ether, and acetone; and analytical grade sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ). TFE, TCE, PFB, BF_3–methanol mixture (14% w/v), and diethylene glycol monoethyl ether were obtained from Sigma Chemical Co. (St. Louis, MO). Diazald, anhydrous sodium sulfate, sodium chloride, and potassium carbonate were obtained from Aldrich Chemical Co. (Milwaukee, WI). MCPP, (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), and dicamba were purchased from Chem Service (West Chester, PA). Diazomethane was made according to the method described in Technical Information Bulletin No. AL-113 (Aldrich Chemical Co.).

The stock solution of MCPP was prepared by dissolving 0.1 g in 100 mL acetone in a 100 mL volumetric flask, and the calibration standards were developed by diluting and derivatizing the stock solution to concentrations ranging from 0.005 to 1.0 mg/L MCPP in hexane.

Diazomethane Methylation

One milliliter of the stock solution of MCPP was placed in a 10 mL Teflon-capped vial and dried slowly under nitrogen. Diazomethane solution (2 mL) was added to the vial, the vial was swirled gently for 1 min, and the reaction was allowed to proceed at room temperature (ca 23°C) for 30 min. The remaining diazomethane was evaporated under a gentle nitrogen stream, and 2 drops of methanol were added to the solution that was completely dried with nitrogen. Two milliliters of hexane was added to the vial, followed by ca 5 mL buffer solution (0.1M NaOH + 0.05M $NaHCO_3$) (ca pH 10), and the mixture was vigorously shaken. The 2 phases were allowed to separate, and a 1 mL aliquot of the hexane layer (top layer) was transfered to a volumetric flask and diluted with hexane to the final volume. All procedures involving diazomethane or BF_3 were conducted under the hood with sufficient air flow.

Methylation-reaction temperatures were controlled in an adjustable water bath (Blue M Co., Blue Island, IL). This same method was used for developing the methyl esters of 2,4,5-T and dicamba.

BF_3–Methanol Methylation

One milliliter of the MCPP stock solution was transferred to a 10 mL Teflon-capped vial and dried slowly under nitrogen. Two milliliters of the BF_3–methanol mixture was added to the vial, and the vial was capped tightly, mixed thoroughly, and allowed to react at 23°, 60°, 80°, and 100°C for 30 min. After the reaction, the remaining BF_3 was evaporated under nitrogen for 30 s. Five milliliters of buffer solution (ca pH 10) and 2 mL hexane were added to the methanol solution, mixed vigorously, and allowed to partition. One milliliter of the hexane layer was pipetted into a volumetric flask, and the solution was diluted to volume with hexane.

H_2SO_4–Methanol Methylation

This procedure was similar to the BF_3–methanol method, except 0.5 mL H_2SO_4 and 1 mL methanol were used as reagents.

H_2SO_4–TFE Esterification

One milliliter of the MCPP stock solution was dried in a Teflon-capped vial under nitrogen, and 0.5 mL H_2SO_4 and 1.0 mL TFE were added to the vial. The vial was capped tightly, the mixture was swirled gently, and the reaction was allowed to occur at room temperature for 30 min. Following the reaction, 1 mL saturated aqueous sodium chloride solution, 4 mL deionized water, and 2 mL hexane were added to the vial. The vial was shaken vigorously. The 2 liquid phases were allowed to separate, and 1 mL of the hexane layer was transferred to a volumetric flask and diluted to volume with hexane. Reaction times of 30, 60, 120, 240, and 480 min were tested to maximize the efficiency of esterification.

H_2SO_4–TCE Esterification

This procedure was similar to the method used for the formation of MCPP–TFE ester, with the addition of a 16 h reaction time and the use of TCE for derivatization of MCPP. The temperature was increased to 60°C for 30, 60, and 120 min to confirm the completion of the reaction.

PFB Esterification

A modification of the method presented by Agemian and Chau (11) was used. One milliliter of the MCPP stock, 0.2 mL PFB (1% v/v), and 2 drops of aqueous potassium carbonate

(30% w/v) solutions were added to a 10 mL vial. The capped vial was vigorously shaken for 1 min, and the reaction occurred at room temperature for 30 min. Following the reaction, 2 mL hexane, 1 mL aqueous saturated NaCl, and 4 mL deionized water were added to the vial. The solution was shaken vigorously for 1 min, then 1 mL of the hexane layer was transferred to a volumetric flask and diluted to volume with hexane.

Several reaction periods were tested to determine the most efficient conditions for forming the PFB derivative.

Extraction of Fortified Soil Leachate Samples

The soil leachate was obtained from lysimeters constructed in the greenhouse (13) and filled with a rooting mix of sand–sphagnum peat moss (85 + 15). The lysimeters subtended growth boxes that contained "Tifdwarf" bermudagrass (*Cynodon dactylon* [L] Pers. X *C. transvaalensis* Burtt-Davy). Soil leachate was fortified using the stock solution of MCPP to give a final concentration of 5 µg/L. The thoroughly mixed solution was filtered using Whatman (Hillsboro, OR) filter paper (GF/A) and homogenized for 30 min before extraction.

A 100 mL aliquot of the fortified leachate was acidified with 2 mL concentrated H_2SO_4 to ca pH 1. The leachate was stirred uniformly, transferred to a 250 mL separatory funnel, and extracted 3 times with 50 mL aliquots of diethyl ether. The diethyl ether extracts were saved. The combined ether extracts were dehydrated over Na_2SO_4 and concentrated to ca 3 mL by using a Kuderna-Danish (K-D) apparatus (SUPELCO, Inc., Bellefonte, PA) in a 60°C water bath. The K-D tube and 3-ball Snyder column were rinsed 3 times with 2 mL diethyl ether, and the rinsate was dried under a stream of nitrogen. The extracted MCPP was esterified according to the methods previously described.

Results and Discussion

The methylation of MCPP by BF_3–methanol (6), diazomethane (5), and H_2SO_4–methanol methods were carried out. There has been no report on the methylation of MCPP with H_2SO_4–methanol. However, the relative response (RR) value, the peak area of analyte per peak area of internal standard, for the product from the H_2SO_4–methanol method was comparable to methyl-MCPP formed according to the BF_3–methanol method. During preparation, the same reaction time (30 min) but different reaction temperatures were used to determine the optimum reaction temperature for each method. The synthesized esters and a certain concentration of methyl-2,4,5-T, the internal standard, were injected into the GC–MSD system to verify the product and quantify the efficiency of the esterification method. The product was identified by comparison of the mass spectra with the respective spectra in a reference library (G1034C MS ChemStation, Hewlett-Packard). Data from the total ion chromatogram (TIC) of MSD are presented in Table 1. The RR value was determined from the ratio of the peak areas of methyl-MCPP to methyl-2,4,5-T. The data indicate that the methylation of MCPP reached an optimum at room temperature (Table 1). Increasing the temperature from room temperature to 60°C during methylation by the BF_3–methanol method improved the methylation efficiency. Further increases in temperature did not influence efficiency of this method, and increasing the temperature above room temperature did not improve the efficiency of esterification by the diazomethane or H_2SO_4–methanol methods. The higher temperatures resulted in the formation of side-products for the H_2SO_4–methanol method. Increasing the temperature above room temperature for the diazomethane method resulted in crystallization in the solution, which could produce an explosion (14). According to the results shown in Table 1, we concluded that the 3 methods can be applied successfully for the methylation of MCPP.

Table 1. Comparison of the RR values for different reagents of the methylations of MCPP[a]

Temperature, °C	BF_3–methanol	Diazomethane	H_2SO_4–methanol
23	3.23 (± 0.19)	4.74 (± 0.07)	4.59 (± 0.09)
60	4.73 (± 0.07)	4.72 (± 0.04)	4.27 (± 0.11)
80	4.65 (± 0.02)	4.77 (± 0.08)	4.21 (± 0.12)
100	4.68 (± 0.08)	4.48 (± 0.10)	4.10 (± 0.18)

[a] RR (from the TIC) = peak area of analyte/peak area of methyl-2,4,5-T (internal standard). RR values are the average of 4 repeat experiments ± standard deviation.

Although the methylation of MCPP could be accomplished, the responses of the respective MCPP derivatives to ECD measurement were not adequately sensitive for our intended use. The low sensitivity of the methyl-MCPP response in GC–ECD is probably due to the low electron withdrawing capacity of the functional groups. The multihalides contained in TFE, TCE, and PFB alcohols could make a major difference in the response of the derivative with MCPP compared to the methyl-MCPP when using GC–ECD determination (10). TFE-MCPP, TCE-MCPP, and PFB-MCPP were successfully developed at room temperature and the derivatives were confirmed by GC–MSD. Figure 1 presents the TICs for the derivatives of MCPP. Identification of the compounds were based on the molecular peak and certain major peaks included in the corresponding mass spectra (Table 2). The internal standard, methyl-2,4,5-T, had a retention time of 11.30 min and was well separated from all MCPP derivatives (Figure 1). All base peaks for the MCPP derivatives were m/z 169, which represented (±)-2(4-chloro-2-methylphenoxy)propane (decarboxylated MCPP). The molecular ions matched the calculated molecular weight for the respective MCPP derivatives. Figure 2 presents the mass spec-

Table 2. Molecular peak and base peak of herbicide esters in mass spectra

Ester	Molecular peak (*m/z*)	Major peaks (*m/z*)
Methyl-MCPP	228	169[a], 142, 107
TFE–MCPP	296	169, 141[a], 107
TCE–MCPP	346	169[a], 142, 107
PFB–MCPP	394	181, 169[a], 142

[a] Base peak in the spectrum.

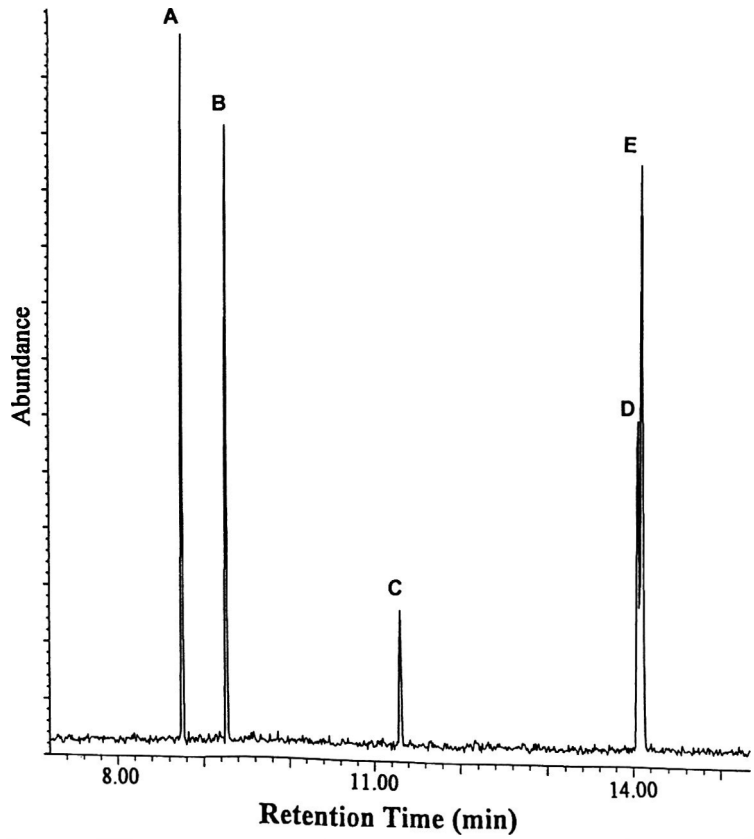


Figure 1. Total ion chromatograms of (A) TFE–MCPP, 2 mg/L; (B) methyl-MCPP, 2 mg/L; (C) methyl-2,4,5,-T, 1 mg/L; (D) TCE–MCPP, 2 mg/L; and (E) PFB–MCPP 2 mg/L.

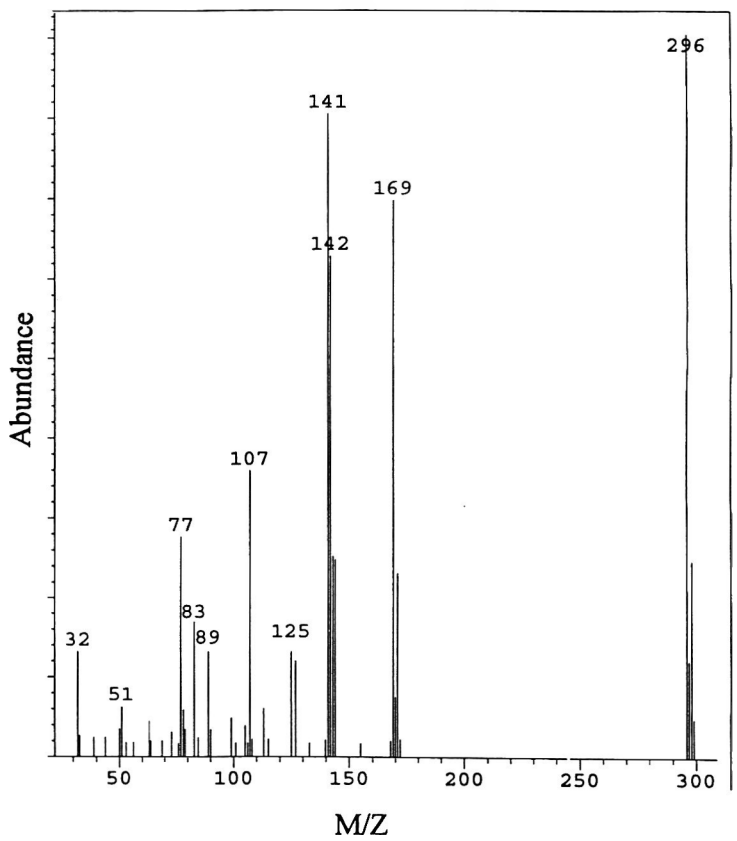


Figure 2. Mass spectrum of TFE–MCPP ester.

Table 3. Comparison of the RR values for different reagents of the esterifications of MCPP[a]

Time, min	TCE	TFE	PFB
30	0.42 (± 0.02)	3.36 (± 0.04)	3.55 (± 0.31)
60	1.55 (± 0.02)	5.61 (± 0.10)	7.10 (± 0.12)
120	2.16 (± 0.04)	7.06 (± 0.16)	9.01 (± 0.11)
240	3.60 (± 0.21)	6.94 (± 0.23)	9.70 (± 0.30)
480	5.31 (± 0.11)	7.07 (± 0.20)	9.60 (± 0.19)
960	5.57 (± 0.05)	—	—

[a] RR (from the TIC) = peak area of analyte/peak area of methyl-2,4,5-T (internal standard). RR values are the average of 3 repeat experiments, ± standard deviation.

trum of TFE–MCPP. The major ions on the spectrum are as follows: *m/z* 296 (molecular ion), *m/z* 169 (MCPP–TFE less TFE–carboxylic ion), *m/z* 141 and 142 (MCPP–TFE less ethyl TFE–carboxylic ion), *m/z* 107 (*m/z* 142 less Cl ion), *m/z* 125 and 83 (TFE ion fragmentation), and *m/z* 77 (MCPP ion fragmentation). According to the above description, the product is MCPP–TFE. The esterification was processed simply and without use of complicated equipment.

Different reaction times were carried out during the preparation to determine an optimum reaction period. Table 3 lists the relative responses of TCE–MCPP, TFE–MCPP, and PFB–MCPP by using methyl-2,4,5-T as the internal standard for determination of the RR values. The TFE–MCPP esterification reaction reached maximum at 2 h. PFB–MCPP required about 4 h to reach a reaction maximum, and TCE–MCPP reaction required more than 8 h to reach a maximum. The saturated state was confirmed by increasing reaction temperature to 60°C during the MCPP–TCE preparation.

The sensitivity of MCPP esters to GC–ECD was investigated (Figure 3). The temperature program described in *Apparatus* for TFE esters was used for this mixture. The separation and retention time of the products were acceptable, except for TCE–MCPP and PFB–MCPP (Figure 3, peak C), and these analyses were conducted individually.

The relative detection limits were obtained by the measurement of each MCPP derivative (100 μg/L) with GC–ECD. The results given in Table 4 were compared by setting the response of methyl-MCPP as 1. As expected, PFB and TCE derivatives resulted in higher detector responses compared with the other esters. TFE–MCPP was not as sensitive as the PFB and TCE derivatives of MCPP. However, it resulted in about 100 times greater response than methyl-MCPP. The estimated limit of detection for MCPP–TFE was 2 pg (2 μL injection of 1 μg/L sample).

These results indicate that the 3 methods for esterification of MCPP (TFE, TCE, and PFB) are superior to diazomethane methylation. We tested these methods for determining MCPP in a soil leachate and for forming products that will interfere during GC–ECD analyses. Concentration response curves were developed for TFE–MCPP, TCE–MCPP, and PFB–MCPP by using methyl-dicamba as the internal standard for

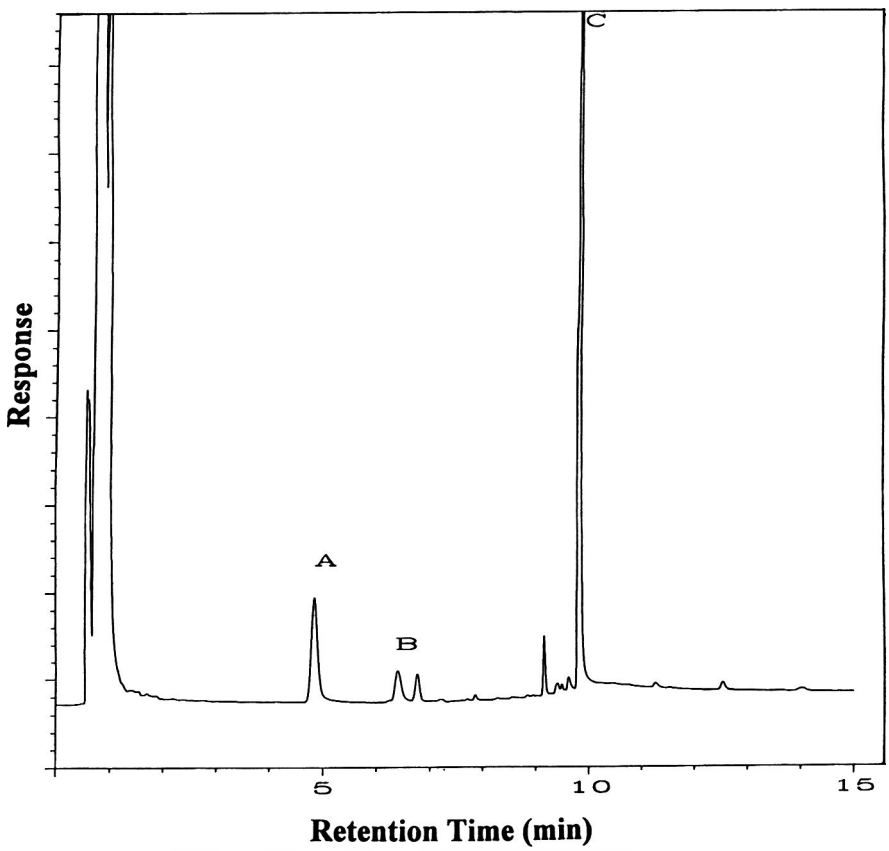


Figure 3. Gas chromatogram of MCPP derivatives (A) 100 μg/L TFE ester; (B) 500 μg/L methyl ester; and (C) 25 μg/L TCE and PFB esters.

Table 4. The sensitivity of MCPP derivatives measured by GC–ECD[a]

Ester	Peak area integration	Peak height integration
Methyl-MCPP	1	1
TFE–MCPP	80	140
TCE–MCPP	660	700
PFB–MCPP	1130	1240

[a] Estimated minimum detection: 1.0 ppb × 2 μL injection for TFE derivatives. Data obtained from the comparison of the responses of 100 ppb samples.

TFE–MCPP and methyl-2,4,5-T for TCE–MCPP and PFB–MCPP. The different internal standards were used for the convenience of measurement. The temperature program conditions during chromatography were selected to obtain product peaks without interference. The RR value was linear over the concentration range of 5.0 to 250 μg/L ($R^2 > 0.99$) for all concentration response curves (curves not included). Fortified (5 μg/L) soil leachate was used for testing the derivatizing methods. Different reaction times were used to ensure reaction optimization. TFE–MCPP and TCE–MCPP esters were developed at a temperature of 60°C for 2 h. PFB–MCPP ester was reacted for 8 h at room temperature. Figure 4 shows the extraction results of 100 mL spiked (Figures 4A and 4C) and nonspiked blank (Figures 4B and 4D) soil leachates. The scale in Figures 4A and 4B is 10 times smaller than their real values. MCPP–PFB (Figure 4A) gave a very strong response (about 120% recovery) to ECD. However, this method resulted in numerous foreign peaks on the chromatogram. This evidence was also shown in Figure 4B, which would require a cleanup procedure before it could be reliably used. The TCE–MCPP method gave similar results (data not shown). Figure 4C shows the results of the TFE–MCPP method for derivatization of MCPP in soil extract. The chromatogram indicates a good peak separation with no interference from peaks of foreign substances in the leachate. The average recovery was above 90% from soil leachate.

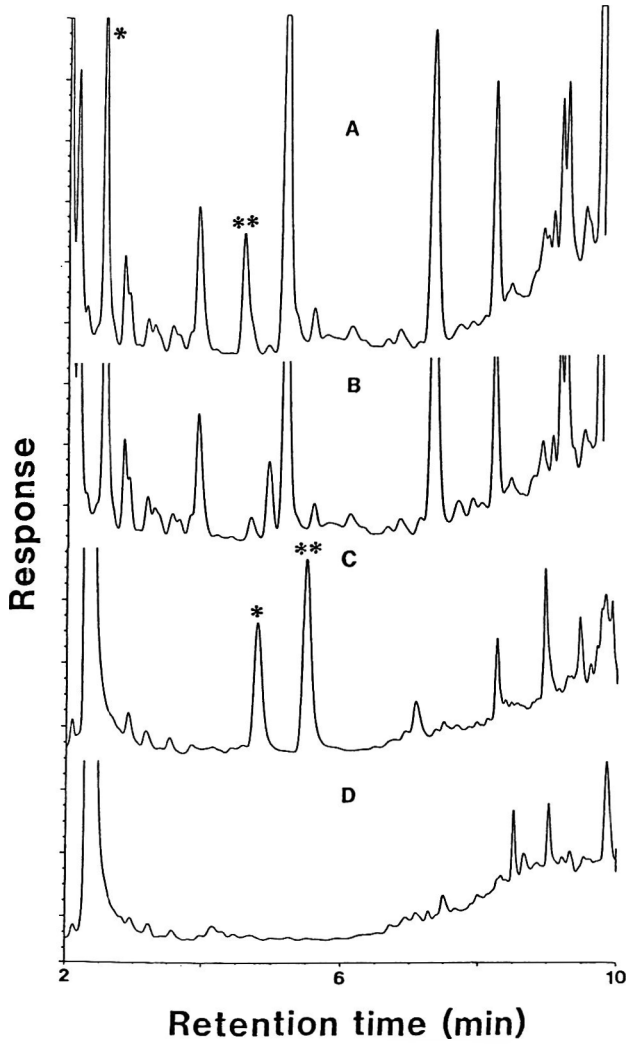


Figure 4. Gas chromatograms of soil leachate samples: (A) 100 μg/L methyl-2,4,5-T, retention time 2.50 min (*); 250 μg/L PFB–MCPP, retention time 4.65 min (**); (B) leachate with PFB only; (C) 250 μg/L TFE–MCPP, retention time 4.81 min (*); 100 μg/L methyl-dicamba, retention time 5.52 min (**); and (D) leachate with TFE only.

Compared with the TCE and PFB methods for esterifying MCPP, the TFE method resulted in slightly less molecular response in the ECD but resulted in a much cleaner chromatogram.

According to the preparation procedure and residue esterification results described above, a method has been developed for analyzing MCPP in soil leachate. The TFE method is simple, safe, economical, and has a low detection limit (≤2 pg by GC–ECD analysis).

Acknowledgments

We wish to thank Nehru Mantripragada for helpful technical assistance. This research was supported by an United States Golf Association research grant.

References

(1) Cochrane, W.P. (1979) *J. Chromatogr. Sci.* **17**, 124–137
(2) Khan, S.U. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 1027–1031
(3) Cessna, A.J., Grover, R., Kerr, L.A., & Aldred, M.L. (1985) *J. Agric. Food Chem.* **33**, 504–507
(4) Gurka, D.F., Shore, F.L., Pan, S.-T., & Amick, E.N. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 970–975
(5) Ngan, F., & Ikesaki, T. (1991) *J. Chromatogr.* **537**, 385–395
(6) Cessna, A.J. (1992) *J. Agric. Food Chem.* **40**, 1154–1157
(7) Gintautas, P.A., Daniel, S.R., & Macalady, D.L. (1992) *Environ. Sci. Technol.* **26**, 517–521
(8) Siltanen, H., & Mutanen, R. (1985) *Chromatographia* **20**, 685–688
(9) Chau, A.S.Y., & Terry, K. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 1294–1301
(10) Mierzwa, S., & Witek, S. (1977) *J. Chromatogr.* **136**, 105–111
(11) Agemian, H., & Chau, A.S.Y. (1976) *Analyst* **101**, 732–737
(12) Tsukioka, T., & Murakami, T. (1989) *J. Chromatogr.* **469**, 351–359
(13) Smith, A.E., Weldon, O., Slaughter, W., Peeler, H., & Mantripragada, N. (1993) *J. Environ. Qual.*, in press
(14) Moore, J.A., & Reed, D.E. (1961) *Org. Synth.* **41**, 16–20

AGRICULTURAL MATERIALS

Particle Concentration Fluorescence Immunoassay for Determination of Tylosin in Premix, Feeds, and Liquid Feed Supplement: Comparison with Turbidimetric Assay

ALAN L. WICKER, DANIEL H. MOWREY, DANIEL J. SWEENEY, and MARK R. COLEMAN
Lilly Research Laboratories, A Division of Eli Lilly & Co., 2001 West Main St, Greenfield, IN 46140-0708
DEBORAH K. MORRIS and CATHERINE L. BROCKUS
International Diagnostic Systems Corp., PO Box 799, St. Joseph, MI 49085

A rapid, competitive particle concentration fluorescence immunoassay (PCFIA) for the determination of tylosin (Tylan) concentrations in extracts of premix, feeds (cattle, chicken, and swine), and liquid feed supplement is described. Tylosin–β-phycoerythrin conjugate, tylosin standard or diluted sample extracts, and rabbit anti-tylosin antibody are incubated for 15 min in PCFIA plates. Rabbit anti-tylosin antibody is then captured, during a 15 min incubation, with goat anti-rabbit antibody that is attached to latex beads. Assay plates are washed to remove unbound tylosin–β-phycoerythrin and unbound, free tylosin. Fluorescence is measured with a fluorimeter. Tylosin concentration is inversely proportional to the β-phycoerythrin fluorescence signal. The method, evaluated and validated with an IDEXX Screen Machine, shows high specificity with regard to compounds expected to be found in the presence of tylosin, and results correlate well with traditional turbidimetric assay results. Hydrolysis of TUA (tylosin–urea adduct), necessary with microbiological methods, and cleanup of extracts beyond filtration are unnecessary with the PCFIA for tylosin.

Received April 9, 1993. Accepted by RN November 3, 1993.

Tylosin is typically assayed by microbiological (1) or turbidimetric methods (2–7). Enzyme immunoassays for detection of compounds used in treatment of animals and compounds of interest in the food industry have been reported (8–16). PCFIA (particle concentration fluorescence immunoassay) has also been used for the detection of gentamicin (17).

The PCFIA for tylosin has 5 major advantages over microbiological methods. (*1*) PCFIA does not require the extensive sample processing necessary with microbiological methods (7). Extraction and filtration are sufficient with PCFIA. Microbiological methods require the use of acidic alumina and, for low-level samples, solid-phase extraction with C_{18} (7). (*2*) Hydrolysis of tylosin–urea adduct (TUA) prior to assay by microbiological methods (18) is not necessary with PCFIA. Tylosin in the presence of urea will form an adduct, which has very little antimicrobial activity (18). Hydrolysis of extracts from samples containing urea (7, 8) is necessary to free tylosin for detection by microbiological assay. PCFIA does not require hydrolysis, because the molecular structure of TUA allows it to react with the anti-tylosin antibody. (*3*) Even with the revised turbidimetric assay (7), the potential exists for compounds and materials to affect microbial growth and the resulting potency determinations. PCFIA specificity, with respect to the compounds and materials evaluated, is quite good. (*4*) Agar plate microbiological assays require overnight incubation (1), and turbidimetric microbiological assays require 3½ to 5 h of incubation (2–7). PCFIA requires approximately 45 min for 1 plate (approximately 1.75 h for 4 plates). (*5*) The PCFIA working range is 1.8 to 180 ng/mL (calibration curve range, 0.018 to 560 ng/mL). The response range for microbiological plate assay is typically 0.25 to 2 μg/mL (1). Microbiological Autoturb assay response range (2–7) is typically 2 to 5 μg/mL.

Existing sample extraction method (6, 7) is utilized. A minimum 1:10 dilution of extracts is required to avoid the effects of methanol and/or matrixes. Specificity evaluation indicates no interference from monensin, narasin, or sulfamethazine concentrations 2 times higher than the highest level expected to be found in the presence of tylosin. However, tilmicosin (Micotil), a macrolide, does interfere, as might be expected.

METHOD

Apparatus

(**a**) *Screen Machine.*—PCFIA instrument (IDEXX Corp.).

(**b**) *Assay plates.*—Cellulose acetate, 0.2 μm (IDEXX Corp.).

(**c**) *Gyratory shaker.*—New Brunswick Scientific, Model G-33, or equivalent.

(**d**) *Filter paper.*—Whatman No. 4, or equivalent; Schleicher and Schuell prepleated filters No. 588, or equivalent.

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

(**f**) *Water baths.*—95° to 99°C for hydrolysis of TUA and 80°C (or steam sterilizer) for inactivation of microbial growth.

(**g**) *Filters, 0.5 μm.*—Cameo (Fisher Scientific), or equivalent.

Safety

Use appropriate protective equipment and techniques to avoid inhalation and direct contact with the reagents, materials, and microorganisms.

Reagents

(**a**) *Solvents.*—Reagent grade methanol, concentrated phosphoric acid, concentrated sodium hydroxide, deionized water, and water from a Milli-Q UF Plus water treatment system.

(**b**) *Phosphate buffers, 0.1M.*—Prepare pH 8 and 7 phosphate buffers according to **42.204(b)** and (**c**), respectively (2).

(**c**) *Autoturb extraction solution.*—Methanol–phosphate buffer (pH 8) (1 + 1, v/v).

(**d**) *PCFIA assay buffer, 0.02M.*—Add 2.88 g potassium phosphate dibasic anhydrous, 0.473 g potassium phosphate monobasic anhydrous, 8.18 g sodium chloride, and 0.5 mL Tween 20 to 1.0 L Milli-Q UF Plus water.

(**e**) *Chromatographic phases.*—Alumina Woelm A, Akt. 1 (acidic alumina) and Sep-Pak C_{18} cartridges (Waters Associates).

(**f**) *Tylosin stock reference standard solution.*—Dry reference standard (tylosin base, Eli Lilly and Co.) for 3 h at 60°C in a vacuum oven. Accurately weigh dried standard to obtain 1000 μg tylosin activity per mL. Dissolve in methanol (1 mL methanol/10 mg standard) and dilute to volume with pH 7 phosphate buffer.

(**g**) *Standard solutions for PCFIA.*—On the day of assay, dilute the stock 1000 μg/mL tylosin standard solution with PCFIA assay buffer to obtain solutions with tylosin concentrations of 560, 180, 56, 18, 5.6, 1.8, 0.56, 0.18, 0.056, and 0.018 ng/mL.

(**h**) *Standard solutions for Autoturb assay of samples containing no urea.*—On the day of assay, pipet 10 mL of the stock 1000 μg/mL tylosin standard solution into a 100 mL volumetric flask. Fill to volume with extraction solution, and mix thoroughly (concentration, 100 μg tylosin activity per mL). Prepare chromatographic column with ca 30 mL acidic alumina, pour contents of volumetric flask over column, and collect the effluent. Dilute with extraction solution to obtain solutions with tylosin concentrations of 2, 3, 4, and 5 μg tylosin activity per mL.

(**i**) *Standard solutions for Autoturb assay of samples containing urea.*—Prepare a solution with 100 μg tylosin activity per mL as indicated in (**h**). Pipet 25 mL column effluent into a 150 mL beaker and add 25 mL pH 8 phosphate buffer. Adjust pH to 5.5 ± 0.2 with concentrated phosphoric acid and place in a water bath at 95°–99°C for 1 h. Let solution equilibrate to room temperature and adjust pH to 8.0 ± 0.1 with sodium hydroxide. Transfer contents of beaker into a volumetric flask (e.g., 25 mL), rinse beaker with extraction solution, and add rinsing to flask. Fill the flask to volume with extraction solution. Dilute with extraction solution to obtain solutions with tylosin concentrations of 2, 3, 4, and 5 μg tylosin activity per mL.

(**j**) *Microorganism and media.*—Maintain *Staphylococcus aureus* ATCC 9144 on Antibiotic Medium 1 slants. Use a loopful from slants to inoculate Difco Antibiotic Medium 3. Incubate for 16–18 h at 37°C on a gyratory shaker to obtain inocu-

lum. On the day of analysis, filter Antibiotic Medium 3 with Schleicher and Schuell prepleated filter paper No. 588 to remove particulates from media. Adjust the pH of medium to 8.0 ± 0.1. Inoculate Antibiotic Medium 3 with ca 20 mL inoculum/L. Dilute samples and standards with 10 mL inoculated Antibiotic Medium 3 by using the Autoturb automated turbidimetric system (19).

(k) *PCFIA reagents.*—Tylosin–β-phycoerythrin conjugate, rabbit anti-tylosin antibody, and latex beads with goat anti-rabbit antibody (International Diagnostic Systems Corp.).

Preparation of Samples and Standard Solutions for PCFIA

Extracts are prepared by placing the specified amount of sample in a suitable container, such as a Mason jar. The specified amounts of extraction solution are added, and the mixtures are agitated on a gyratory shaker for at least 1 h. The extracts are filtered through No. 4 Whatman paper. Following are the sample weight/extraction solution volume ratios for the different matrixes: (*1*) Premix: Extract 10 g sample with 200 mL extraction solution. (*2*) Feed, tylosin levels >100 ppm: Extract 50 g sample with 200 mL extraction solution. (*3*) Feed, tylosin levels <100 ppm: Extract 100 g sample with 400 mL extraction solution. (*4*) Liquid supplements: Extract 20 g sample with 200 mL extraction solution.

Sample extracts and standard solutions are diluted to 1000 ng/mL with PCFIA assay buffer. The 1000 ng/mL solutions are filtered through 0.5 µm Cameo filters. Sample extracts and standard solutions are further diluted to assay concentration (1.8 to 180 ng/mL) with PCFIA assay buffer (Figure 1). Standard solutions include tylosin concentrations at 560, 180, 56, 18, 5.6, 1.8, 0.56, 0.18, 0.056, and 0.018 ng/mL.

Preparation of Samples and Standard Solutions for Autoturb Assay

Samples for Autoturb assay (7, 19) are extracted in the same manner indicated previously for the PCFIA. Additional processing is, however, required for Autoturb assay (Figure 1).

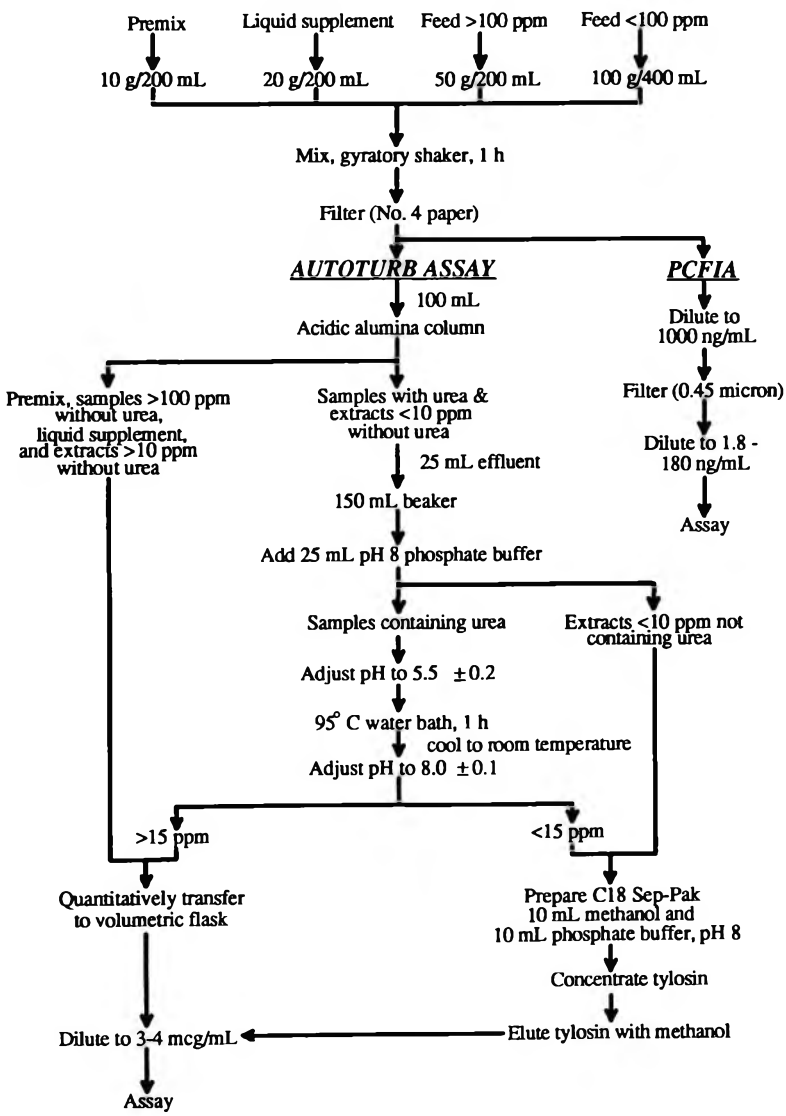


Figure 1. Sample preparation of premix, liquid supplement, and feed samples for tylosin Autoturb assay and PCFIA.

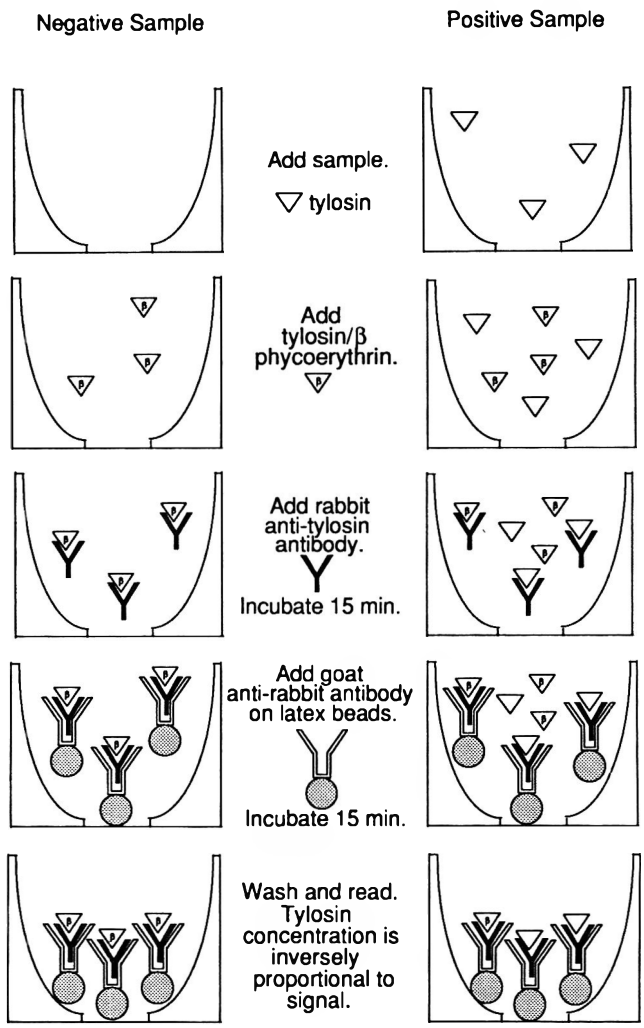


Figure 2. Tylosin PCFIA execution.

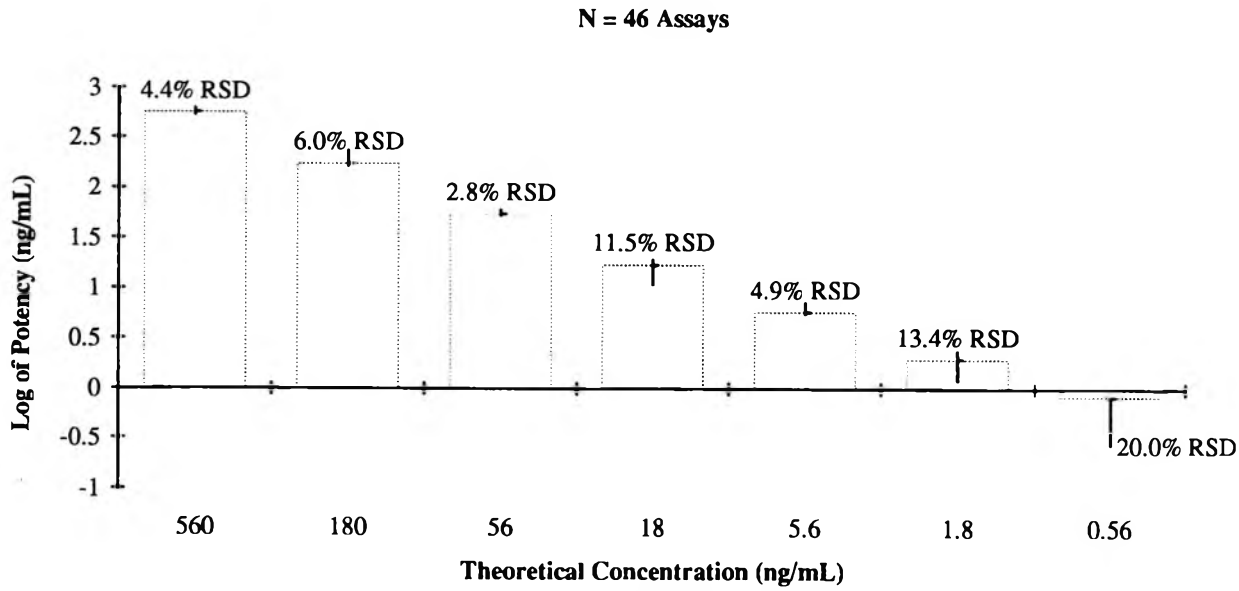


Figure 3. Tylosin PCFIA range. Variability of calibration curve solutions from 0.56 to 560 ng/mL.

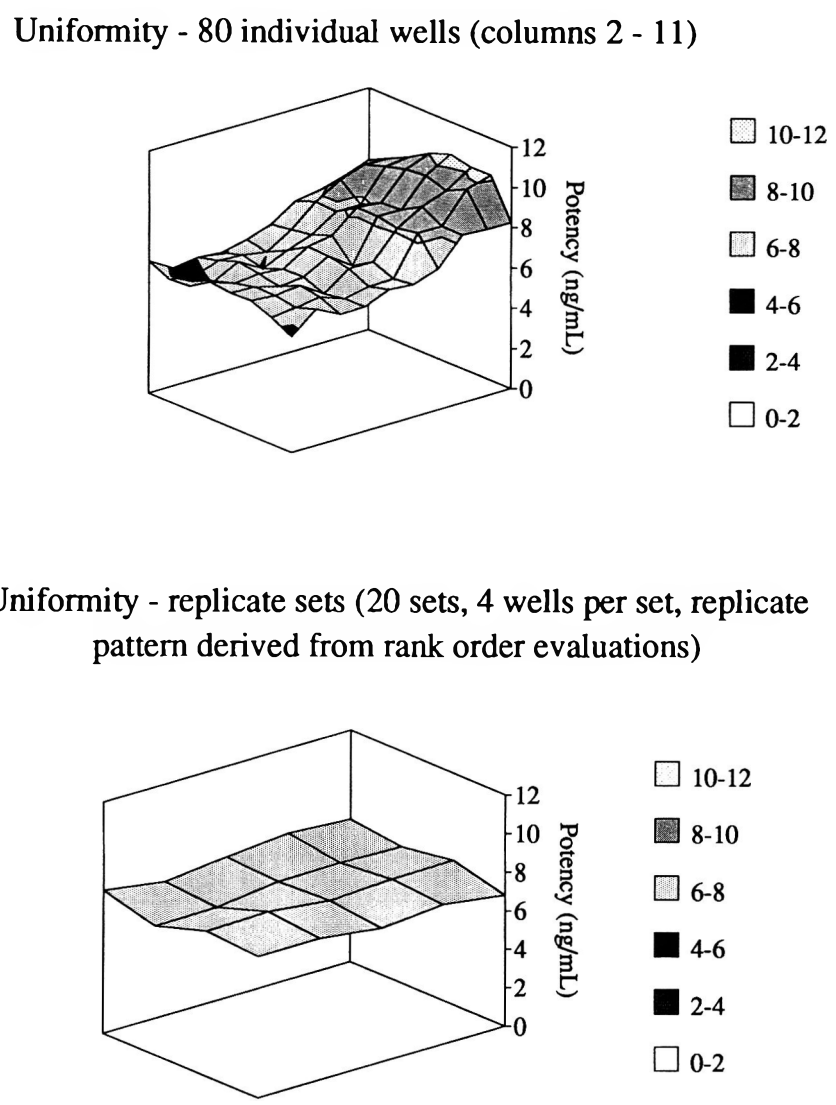


Figure 4. Tylosin PCFIA uniformity (precision) of individual well potencies and replicate set potencies derived from the same data.

For samples containing urea, hydrolysis is performed to free the tylosin so it is microbiologically active (18). Extracts of urea-containing samples are hydrolyzed, after the pH is adjusted to 5.5 ± 0.2 with phosphoric acid, for 1 h at 95°–99°C. After equilibration to room temperature, the pH of the hydrolyzed solutions are adjusted to 8.0 ± 0.1 with sodium hydroxide. Extracts with tylosin concentrations greater than 15 ppm are diluted to 3.0 to 4.0 µg/mL with pH 8 phosphate buffer for assay. Extracts with tylosin concentrations less than 15 ppm are concentrated through C_{18} cartridges. The C_{18} cartridges are conditioned with 10 mL methanol followed by 10 mL pH 8 phosphate buffer. The pH 8 extracts are passed over the conditioned C_{18} cartridges, and the effluent is discarded. Tylosin is eluted from the cartridges with methanol then diluted to 3.0 to 4.0 µg/mL with pH 8 phosphate buffer for assay.

Extracts of samples not containing urea are not hydrolyzed. Premix extracts, extracts of samples containing tylosin at >100 ppm, liquid supplement extracts, and extracts containing tylosin at >10 ppm are diluted to 3.0 to 4.0 µg/mL for assay with pH 8 phosphate buffer. Extracts containing tylosin at <10 ppm are concentrated through C_{18} cartridges. Effluent from the acidic alumina column is diluted with pH 8 phosphate buffer (25 mL + 25 mL). The C_{18} cartridges are conditioned with 10 mL methanol followed by 10 mL pH 8 phosphate buffer. The diluted acidic alumina column effluent is passed over the conditioned C_{18} cartridges, and the effluent is discarded. Tylosin is eluted from the cartridges with methanol and then diluted to 3.0 to 4.0 µg/mL with pH 8 phosphate buffer for assay.

Particle Concentration Fluorescence Immunoassay

PCFIA is performed in special assay plates with 0.22 µm cellulose acetate membranes in the bottom of each well. The plates have a chamber below the wells to facilitate evacuation of liquid from the wells under vacuum. The wells are arranged in standard microtiter plate format (8 rows × 12 columns). The tylosin PCFIA was validated, by comparison with Autoturb assay, by using an IDEXX Screen Machine. After addition of standard and sample solutions to assay plates (20 µL/well), the

Table 1. Tylosin PCFIA specificity: correlation coefficients of potencies from tylosin calibration curve solutions and tylosin calibration curve solutions assayed in the presence of monensin, narasin, or sulfamethazine[a]

	Tylosin	Tylosin and monensin	Tylosin and narasin	Tylosin and sulfamethazine
Tylosin	1.000			
Tylosin and monensin	0.998	1.000		
Tylosin and narasin	1.000	0.999	1.000	
Tylosin and sulfamethazine	1.000	0.999	1.000	1.000

[a] Data are correlation coefficients. Compound concentrations: tylosin, 8 g/ton; monensin, 60 g/ton; narasin, 144 g/ton; sulfamethazine, 200 g/ton.

plates are placed in a Screen Machine for processing. All assay reagents (20 μL each per well) are added by the Screen Machine. Incubation, washing, and reading also are performed by the Screen Machine.

Dosed plates are placed in a Screen Machine where tylosin–β-phycoerythrin conjugate is added to each well, immediately followed by rabbit anti-tylosin antibody. During a 15 min incubation, free tylosin and tylosin–β-phycoerythrin conjugate compete to react with the rabbit anti-tylosin antibody. A mixture of latex beads with goat anti-rabbit antibody attached and reference particles (particles with fluorescent dye attached) is then added to each well. Reference particles are used to compensate for the Screen Machine pipetting and/or reading variability. During a 15 min incubation, rabbit anti-tylosin antibody is captured by the goat anti-rabbit antibody attached to the latex beads. The liquid, containing unbound tylosin and unbound tylosin–β-phycoerythrin conjugate, is then removed through the membranes and lower plate chamber to waste by vacuum. Each well is washed, with the wash solution being likewise removed through the membranes and lower plate chamber to waste by vacuum. The latex beads, with the attached goat anti-rabbit antibody and captured rabbit anti-tylosin antibody, are retained within the wells of the plates The plates are then read, with a fluorometer; the vacuum is sustained during plate reading to maintain the latex beads in a packed state for maximum intensity of the fluorescence signal. The β-phycoerythrin (tylosin–β-phycoerythrin reacted with rabbit anti-tylosin antibody and consequently captured by goat anti-rabbit antibody attached to the latex beads) is excited at 545 nm, and the emission is measured at 575 nm. The reference particle dye is excited at 400 nm, and the emission is measured at 450 nm. Execution of the assay is depicted in Figure 2.

For each well, the ratio of β-phycoerythrin conjugate RFU (relative fluorescence units) to reference particle dye RFU is calculated. Standard and sample data are processed and analyzed to quantify the level of analyte present in test samples based on the dose–response of the standard solutions. Data analyses incorporate many of the ideas discussed by Dudley et al. (20), including use of the 4-parameter logistic model to describe the sigmoidal relationship between analytical response and logarithm of dose. An estimated response at zero concentration, the slope, the EC50 (an estimated concentration at 50% maximum response), and an estimated response at infinite concentration are calculated. A weighted, nonlinear, least-squares procedure similar to that described by Rodbard and Frazier (21) is used to determine the standard calibration curve. The potencies of test samples are then estimated by dose interpolation.

Analysis of the calibration curve parameters and calculated detectable concentrations from the validation study data indicated the parameters that are useful for assessing system suitability: slope, EC50, MDC (minimum detectable concentration or the lowest concentration that results in an expected response significantly different from the expected response at zero concentration), and MMC (maximum measurable concentration or the highest concentration that results in an expected response

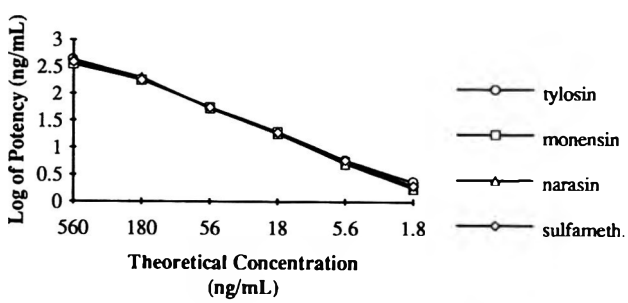


Figure 5. Tylosin PCFIA specificity. Potencies of tylosin calibration curve solutions and tylosin calibration curve solutions assayed in the presence of monensin, narasin, or sulfamethazine.

significantly different from the expected response for infinite concentration).

Autoturb Assay

The Autoturb automated turbidimetric system has been described previously (19). Duplicate tubes of inoculated medium are dosed with 0.10 and 0.15 mL standard or test sample solution (22). After an appropriate period of incubation, turbidities of assay tubes are determined. Point-to-point dose–response curves are constructed from the standard data for each dose size. Test sample potencies are estimated from the appropriate dose–response curve by dose interpolation, and the average potency for each test sample is calculated from the individual determinations.

Results and Discussion

Range

The assay is sensitive and reliable in the range from 1.8 to 560 ng/mL (Figure 3). During validation, each series of standard solutions was used to generate a calibration curve; the data used to generate each calibration curve was run against the curve, and the results were recorded. The recoveries (percentage of theory) and the RSDs (%) were calculated for each concentration. Analysis of the variability of determinations for the solutions indicated, according to Rodbard (23), that the determinations were acceptable in the range from 1.8 to 560 ng/mL.

Assay Limits

The LOD (limit of detection) was determined by using the variability associated with the MDC calculated by the analysis program. The actual LOD was 0.35 ng/mL (the MDC plus 3 standard deviations). In practice, 0.56 ng/mL, the concentration of one of the calibration curve solutions, is considered to be the LOD to facilitate determining the cutoff for unknown samples by comparing the mean unknown sample and LOD signals (β-phycoerythrin RFU divided by reference particle dye RFU). The LOQ (limit of quantification) was determined by using the variability associated with the practical LOD (0.56 ng/mL). The actual LOQ was 0.66 ng/mL (the practical LOD plus 3 standard deviations). Again, the concentration of one of the calibration curve solutions, 1.8 ng/mL, is considered the practical LOQ to facilitate determining the cutoff for unknown samples by comparing the mean unknown sample and LOQ signals (β-phycoerythrin signal divided by reference particle dye signal). Variabilities of the practical LOD (0.56 ng/mL) and LOQ (1.8 ng/mL) potencies are presented in Figure 3.

Table 2. Tylosin PCFIA specificity: monensin, narasin, sulfamethazine, and tilmicosin assayed in the absence of tylosin

Compound	Result, ng/mL
Monensin	< LOD[a]
Narasin	< LOD
Sulfamethazine	< LOD
Tilmicosin	54[b]

[a] LOD = 0.56 ng/mL.
[b] 6.4% of the expected 840 ng/mL.

Precision

The assay was performed with 3 different Screen Machines. Two of the Screen Machines had limited evaluations that resulted in good precision. A notable but reproducible trend was observed when using the Screen Machine for validation. Individual well potencies derived from assay plates dosed in all wells with the same tylosin solution were evaluated (Lilly computer program UNIFORM) to determine overall uniformity (variability) and to obtain variance component estimates. Initially, well-to-well within-plate uniformity was inadequate (median RSD, 22.1%); 80 wells/plate excluding columns 1 and 12; n = 17 plates). We used a rank order approach to analyze the trend (trend analysis) by nonparametric techniques. The or-

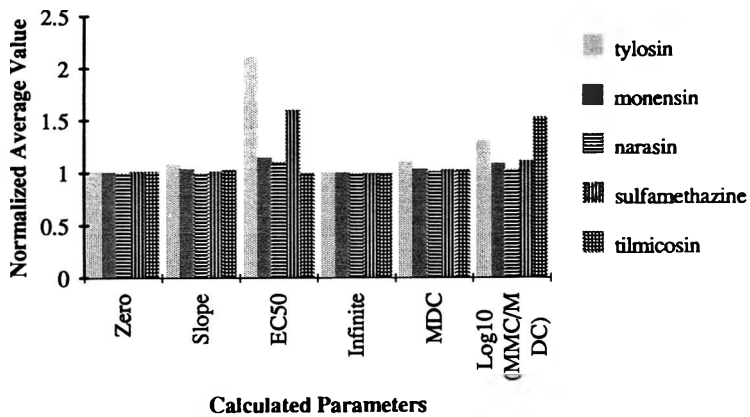
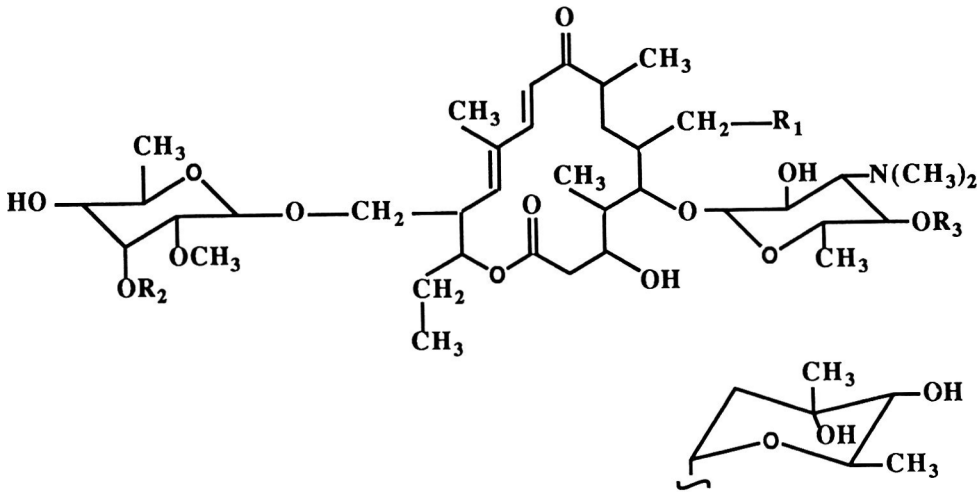


Figure 6. Tylosin PCFIA specificity. Calculated parameters resulting from tylosin calibration curves and tylosin calibration curves assayed in the presence of monensin, narasin, sulfamethazine, or tilmicosin. Data for each parameter were normalized to result in the lowest value being 1 to facilitate charting all parameters on the same scale.



	R_1	R_2	R_3
TYLOSIN (A)	-CHO	$-CH_3$	MYCAROSE
DESMYCOSIN (B)	-CHO	$-CH_3$	-H
MACROSIN (C)	-CHO	-H	MYCAROSE
RELOMYCIN (D)	$-CH_2OH$	$-CH_3$	MYCAROSE

Figure 7. Tylosin factor structures.

dering resulted in a replication pattern using 4 wells per determination to compensate for the trend. The median RSDs (20 replicate sets of 4 wells each per plate) were reduced to 8.5% at 0.56 ng/mL, 6.8% at 5.60 ng/mL, and 5.1% at 56.0 ng/mL after using the rank order replication pattern. Figure 4 graphically presents the trend resulting from the Screen Machine used for validation (80 wells; 5.6 ng/mL tylosin) and also the effect on the same data of the rank order replication pattern (4 wells/determination).

It was determined that 4 plates was the maximum that could be processed at one time in the Screen Machine used for validation. The number of tylosin PCFIA plates that can be processed at the same time in a Screen Machine should be determined by assessing uniformity (precision) as well as calibration curve results. A Screen Machine can accommodate a maximum of 10 plates at the same time. Accuracy and precision were compromised by processing more than 4 plates at a time in the Screen Machine used for validation.

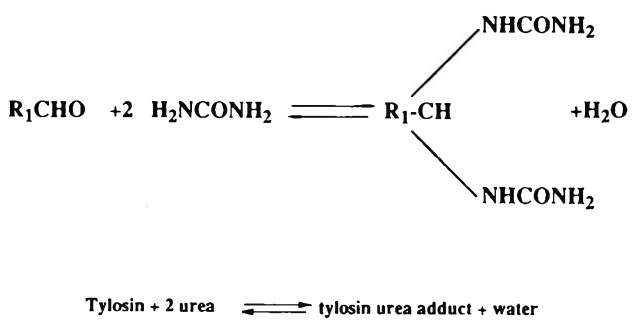


Figure 8. TUA (tylosin–urea adduct) structure.

Specificity

Data were collected from solutions containing tylosin concentrations of 560, 180, 56, 18, 5.6, 1.8, 0.56, 0.18, 0.056, and 0.018 ng/mL. These data were compared to data collected from solutions containing tylosin at the same concentrations and also monensin, narasin, sulfamethazine, or tilmicosin. The compounds used to evaluate for specificity were included at 2 times the highest concentration expected to be found in combination with tylosin, equivalent to the following: monensin at 60 g/ton, narasin at 144 g/ton, sulfamethazine at 200 g/ton, and tilmicosin at 600 g/ton. A total of 2 plates for each were processed. Potencies for each concentration were calculated. Analysis of the data, with tilmicosin excluded, indicated no differences ($p = 0.97$) in tylosin concentration at any dosage level. A chart of the logarithm (ng/mL) of response vs known concentration (ng/mL) for tylosin and for tylosin in the presence of monensin, narasin, or sulfamethazine is presented in Figure 5. Correlation coefficients are presented in Table 1. Tilmicosin, a macrolide, interfered with the assay, as expected, with 6.4% cross-reactivity at a tilmicosin concentration of 840 ng/mL.

In conjunction with the potency analyses, data for tylosin alone and data for tylosin in combination with other compounds were fitted to the 4-parameter logistic model. The 4 curve parameters, A (calculated response at zero concentra-

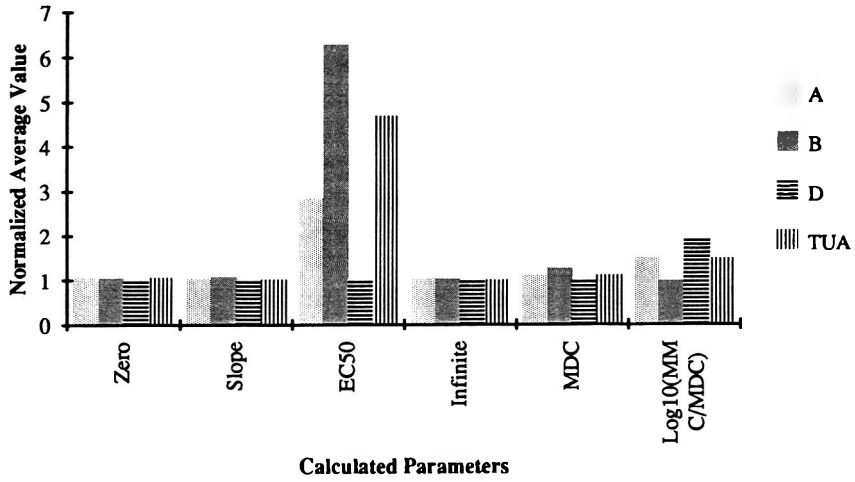


Figure 9. Tylosin PCFIA. Calculated parameters of factors A, B, and D plus TUA.

tion), slope, EC50, and D (calculated response at infinite concentration), as well as MMC, MDC, and $\log_{10}$ (MMC/MDC), were examined for tylosin in combination with each compound and for tylosin alone (Figure 6). Results from tylosin in combination with monensin, narasin, and sulfamethazine showed no differences ($p > 0.05$) from results with tylosin alone. Tilmicosin, a macrolide, resulted in marked differences ($p > 0.01$) in slope, MMC, and MDC. The data indicate that slope, EC50, MMC, and MDC are parameters indicative of assay performance and should be monitored. Slope is logically the most critical. Any atypical value for slope or for more than one of the parameters requires close investigation of the data.

Monensin, narasin, sulfamethazine, and tilmicosin were also assayed in the absence of tylosin at the same levels indicated for the tylosin combination assays at the 5.6 ng/mL tylosin concentration. The actual concentrations of these compounds were monensin, 42.0 ng/mL; narasin, 101 ng/mL; sulfamethazine, 112 ng/mL; and tilmicosin, 840 ng/mL. No interference was indicated, except with tilmicosin (Table 2).

Solutions of tylosin factors B, C, and D and of TUA at 560, 180, 56, 18, 5.6, 1.8, 0.56, 0.18, 0.056, and 0.018 ng/mL were assayed and compared with the reference standard (mostly factor A) to determine their activity. Structures for the factors and for TUA are shown in Figures 7 and 8, respectively. The reference standard was assayed as factor A. The assay results were used to calculate standard curve parameters and measurable concentrations for each solution (Figure 9; factor C excluded

Table 3. Tylosin PCFIA potencies of factors and TUA relative to factor A, indicated as percentage of factor A potency

Factor	Mean potency, %[a]	Potency expressed as a percentage of the potency of factor A, %[a]
A	55.2	100.0
B	16.0	28.9
C	1.3	2.3
D	62.8	113.7
TUA	44.9	81.2

[a] Concentration range analyzed, 1.8 to 180 ng/mL.

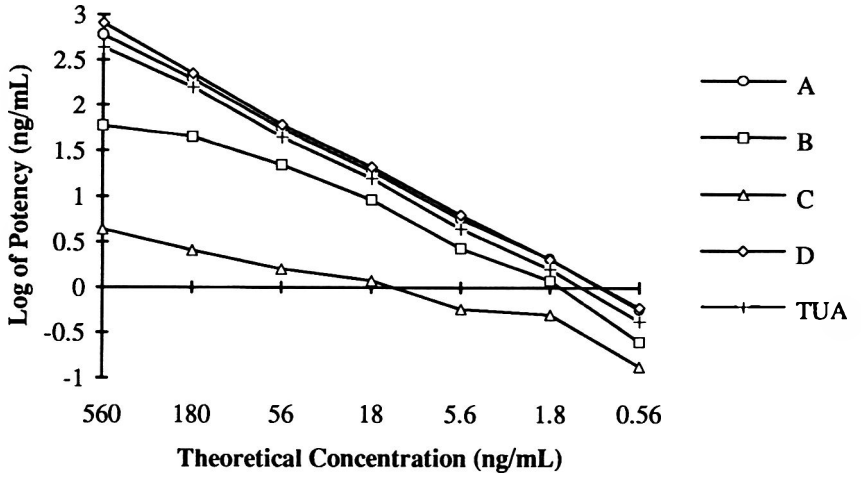


Figure 10. Tylosin PCFIA. Potencies of factors A, B, C, D, and TUA calibration curve solutions.

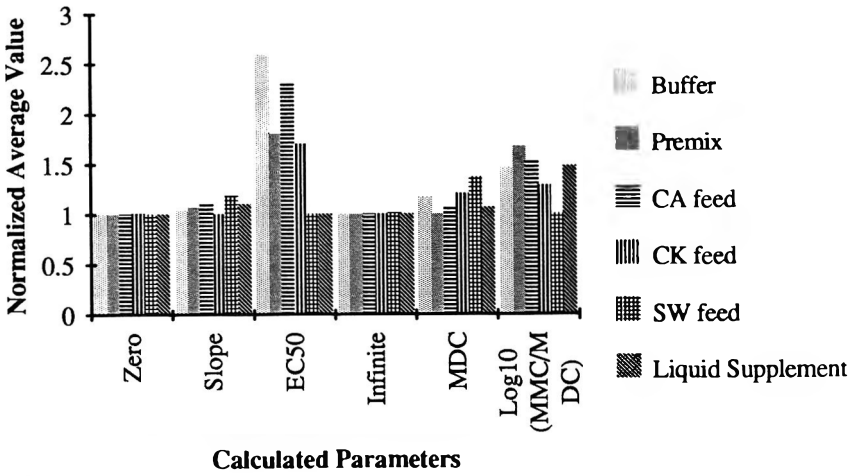


Figure 11. Tylosin PCFIA matrix effects including 560 ng/mL data: calculated parameters from buffer and matrix calibration curves. Data for each parameter were normalized to result in the lowest value being 1 to facilitate charting all parameters on the same scale.

to maintain a reasonable scale; *see* Figure 10), and to determine potencies relative to the reference standard (Figure 10). The potencies relative to factor A (reference standard), expressed as a percentage of the potency of A, were determined from an average of the potencies obtained for the concentrations from 1.8 to 180 ng/mL. The potency data are given in Table 3.

Matrix Effects

Matrix effects were studied with tylosin solutions at 560, 180, 56, 18, 5.6, 1.8, 0.56, 0.18, 0.056, and 0.018 ng/mL. The solutions were prepared by diluting reference standard with assay buffer and extracts of unmedicated, negative-control cattle, chicken, and swine feeds and liquid supplement. The matrixes were evaluated at levels 2 or more times the levels normally encountered. The matrixes simulated a 4 g/ton cattle feed extract, a 4 g/ton chicken feed extract, a 5 g/ton swine feed extract, and a 30 mg/lb liquid supplement extract. In addition, an extract of tylosin premix was diluted with assay buffer. Four plates per matrix were processed for each of 2 periods.

The matrix data were processed, as previously discussed, by using a 4-parameter logistic model to determine the curve parameters A, slope, EC50, and D. Analyses of the parameters provided information on whether the buffer and matrix curves were parallel. Parameters A, slope, and EC50 (Figure 11) were affected ($p < 0.05$), which led to further investigation of the data. The concentration affecting the parameters was 560 ng/mL.

Data from the 560 ng/mL level were removed, and a 4-parameter logistic function was refitted for each matrix. The results (Figure 12) for each parameter for the 6 matrixes were nearly identical ($p > 0.05$). Therefore, these matrixes do not affect assay results. Potencies of the buffer and matrix solutions

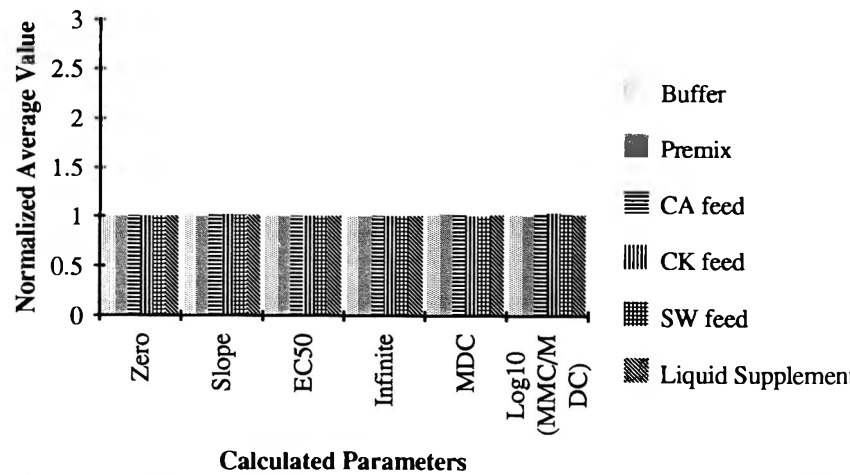


Figure 12. Tylosin PCFIA matrix effects excluding 560 ng/mL data: calculated parameters from buffer and matrix calibration curves. Data for each parameter were normalized to result in the lowest value being 1 to facilitate charting all parameters on the same scale.

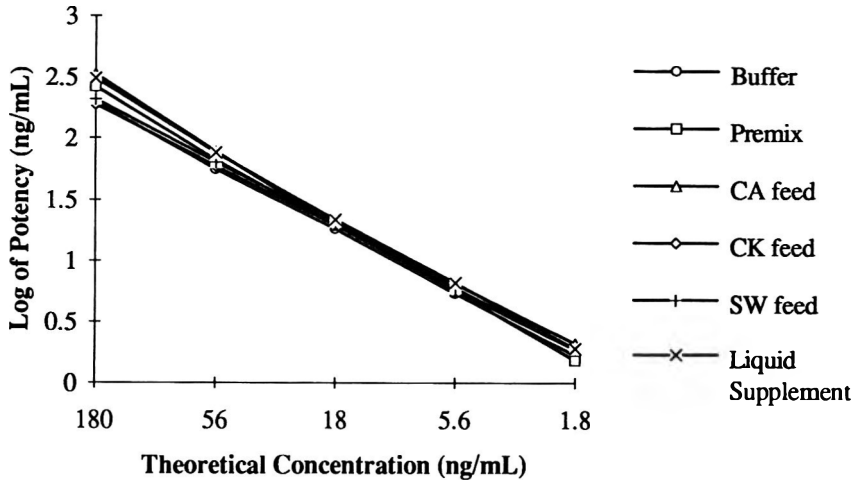


Figure 13. Tylosin PCFIA matrix effects excluding 560 ng/mL data: calibration curve potencies.

from 1.8 to 180 ng/mL are charted in Figure 13. Table 4 contains correlation coefficients for the data.

Accuracy and Comparison of PCFIA and Autoturb

Premix extracts; fortified extracts of cattle, chicken, and swine feeds; and fortified extracts of liquid feed supplement were processed and assayed by PCFIA and Autoturb. The chicken and swine feed extracts were fortified with tylosin reference standard. Cattle feed and liquid feed supplement extracts were fortified with TUA. Average PCFIA and Autoturb recoveries from premixes and feeds containing tylosin at greater than 10 g/ton were 104.0 and 98.6%, respectively. For feeds containing tylosin at ≤10 g/ton, average recoveries were 87.6 and 66.8% for PCFIA and Autoturb, respectively. Average recoveries from liquid supplements were 93.0 and 113.6% for PCFIA and Autoturb, respectively. A graphic comparison of PCFIA and Autoturb results is presented in Figure 14. Correlation coefficients are indicated in Table 5.

Trend Analysis

The following approach was used to analyze the trend associated with the Screen Machine used for validation and for deriving a suitable replicate set pattern.

(*1*) Generate uniformity data (all wells dosed with the same concentration of analyte) on several plates.

(*2*) Rank order the well positions by assay result.

(*3*) Determine coefficients of correlation between rank order vs well position for the data sets. For the data used to derive the pattern in the validation study, the mean correlation coefficient was 0.83 ($n = 61$), with an RSD of 7.6%.

(*4*) If the correlation is reasonable ($r \geq 0.75$), then the reproducibility of the trend is verified.

(*5*) Average the data by well position (mean of all A1 wells, etc.).

(*6*) Rank order the data by mean assay result; take care to maintain the relationship between rank order and well position.

(*7*) Calculate the sum of the rank order (*SO*),

$$SO = N(\frac{N}{2}) + \frac{N}{2}$$

where N = number of observations. Note that this calculation involves rank order, not assay results.

Table 4. Tylosin PCFIA matrix effects excluding 560 ng/mL data: correlation coefficients of calibration curve potencies[a]

	Expected	Buffer	Premix	Cattle feed	Chicken feed	Swine feed	Liquid supplement
Expected	1.000						
Buffer	1.000	1.000					
Premix	1.000	1.000	1.000				
Cattle feed	0.998	0.998	0.998	1.000			
Chicken feed	1.000	1.000	1.000	0.997	1.000		
Swine feed	1.000	1.000	1.000	0.997	1.000	1.000	
Liquid supplement	1.000	1.000	1.000	0.999	0.999	0.999	1.000

[a] Data are correlation coefficients.

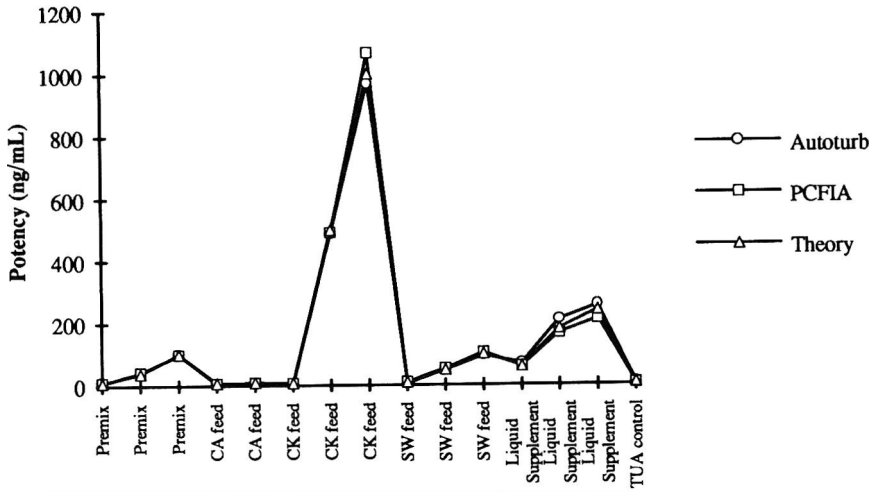


Figure 14. Tylosin PCFIA and Autoturb results from premixes, feeds, liquid supplements, and a TUA control solution.

(*8*) Calculate the number of replicate sets (R) to partition the plate into,

$$R = \frac{N}{W}$$

where N = number of observations and W = number of wells to be averaged per determination (replicate set).

The nature of the trend should be considered in determining the number of wells per replicate set. (Four wells per set were sufficient to compensate for the trend from the Screen Machine used for validation.)

(*9*) Calculate the mean replicate set sum (SR),

$$SR = \frac{SO}{R}$$

where SO = sum of the rank order and R = number of replicate sets.

(*10*) Derive the replication pattern by partitioning the rank order into replicate sets with sums of the individual well rank orders in each set = SR (the mean replicate set sum from step 9). For example, for the data set with rank order 1, 2, 3, 4, 5, 6, 7, 8, the values resulting for steps 7–9 are step 7. SO = 8(8/2) + 8/2 = 36; step 8, R = 8/2 = 4; and step 9, SR = 36/4 = 9. The partition, therefore, is as follows: replicate set 1 = 1 and 8, replicate set 2 = 2 and 7, replicate set 3 = 3 and 6, and replicate set 4 = 4 and 5. If rank order 1 = well A8, rank order 2 = well A7, rank order 3 = well A6, rank order 4 = well A5, rank order 5 = well A4, rank order 6 = well A3, rank order 7 = well A2, and rank order 8 = well A1, then the replication pattern would be replicate set 1 (rank orders 1 and 8) = wells A8 and A1, replicate set 2 (rank orders 2 and 7) = wells A7 and A2, replicate set 3 (rank orders 3 and 6) = wells A6 and A3, and replicate set 4 (rank orders 4 and 5) = wells A5 and A4.

(*11*) Partition the uniformity data, used to derive the replication pattern, into replicate sets according to the derived pattern. Calculate the mean value for each replicate set. Determine if the variability of the values for the replicate sets is within a range acceptable for the intended application of the assay.

Summary and Conclusions

The PCFIA for tylosin produced results comparing favorably with the current Autoturb assay. Sample preparation is considerably less complex and consequently much less time consuming for PCFIA than for microbiological Autoturb analysis (Figure 1). PCFIA requires only filtration (two) and dilution of the extract. Processing of extracts for microbiological assays can include two column steps (acidic alumina and C_{18}) plus hydrolysis (adjust pH, heat, cool, adjust pH), depending on the tylosin concentration and the presence of urea in the sample. PCFIA is also less time consuming than turbidimetric methods. The PCFIA performed as described here requires approximately 1.75 h for 4 plates or 40 samples and controls plus standards. The same 40 samples and controls plus standards would require 3.5 to 5 h of incubation time for a turbidimetric assay plus approximately 38 min to read the 220 assay tubes. Specificity, with respect to the compounds evaluated in the validation study, is also much greater with PCFIA, because these compounds interfere with microbiological assays.

The time and labor savings realized with the PCFIA, as well as the increased specificity, makes the PCFIA a very attractive alternative to turbidimetric and microbiological assays. Laboratories with moderate to high volume sample loads should

Table 5. Correlation coefficients for tylosin PCFIA and Autoturb results from premixes, feeds, liquid supplements, and a TUA control solution[a]

	Expected	Autoturb	PCFIA
Theory	1.000		
Autoturb	0.999	1.000	
PCFIA	0.999	0.996	1.000

[a] Data are correlation coefficients.

consider this method for the determination of tylosin concentrations in premix, liquid supplement, and feed samples.

Acknowledgments

We acknowledge method J426 author and developer J. W. Moran and developer C. Coil for the sample extraction methodology and C. Coil for technical support.

References

(1) Ragheb, H.S., Breunig, H.L., & Scroggs, R.E. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 363–366

(2) *Official Methods of Analysis* (1990) secs **957.23B(b)** and **(c)**

(3) Kavanagh, F.W. (1963) *Analytical Microbiology*, Vol. 1, Academic Press, New York, NY, pp. 369–370

(4) Scroggs, R.E. (1970) *Proceedings of the Symposium on the Determination of Antibiotics in Feed*, Purdue University, pp. 103–114

(5) Kavanagh, F.W. (1972) *Analytical Microbiology*, Vol. 11, Academic Press, New York, NY, pp. 359–364

(6) Coleman, M.R. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 454–456

(7) Coleman, M.R., & Mowrey, D.H. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 927–931

(8) Dixon-Holland, D.E., & Katz, S.E. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 784–789

(9) McCaughey, W.J., Elliott, C.T., & Crooks, S.R.H. (1990) *Food Addit. Contam.* **7**, 259–264

(10) van Egmond, H.P., & Wagstaffe, P.J. (1990) *Food Addit. Contam.* **7**, 239–251

(11) Schmitz, P., Degand, G., Pelzer, G., Gaspar, P., & Maghuin-Togister, G. (1989) *Ann. Med. Vet.* **133**, 427–430

(12) Yao, R.C., & Mahoney, D.F. (1989) *Appl. Environ. Microbiol.* **55**, 1507–1511

(13) Heitzman, R.J. (1988) *Pig Veterinary Society Proceedings* **20**, 90–96

(14) Mount, M.E., Failla, D.L., & Wie, S., (1988) in *Immunoassays for Veterinary and Food Analysis*, M.N. Clifford and R. Jackman (Eds), Elsevier Applied Science Publishers, Ltd, Essex, UK, pp. 301–304

(15) Juptner, C., & Heeschen, W. (1987) in *Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene: Der Tierarzt im Dienst der Lebensmittelhygiene*, September 29 to October 2, 1987, Garmisch-Partenkirchen, pp. 302-307

(16) Arakawa, H., Maeda, M., & Tsuji, A. (1984) *Eisei Kagaku* **30**, 243–246

(17) Varmanelson, P., Nelson, J.E., & Belknap, S.M. (1991) *J. Therapeutic Drug Monitor.* **13**, 260–262

(18) Massey, E.H., & Dennen, D.W. (1973) *J. Agric. Food Chem.* **21**, 112–114

(19) Kuzel, N.R., & Kavanagh, F.W. (1971) *J. Pharm. Sci.* **60**, 767–773

(20) Dudley, R.A., et al. (1985) *Clin. Chem.* **31**, 1264–1271

(21) Rodbard, D., & Frazier, G.R. (1975) *Methods Enzymol.* **37**, 3–22

(22) Kavanagh, F.W. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 6–10

(23) Rodbard, D. (1974) *Clin. Chem.* **20/10**, 1255–1270

CHEMICAL CONTAMINANTS MONITORING

Monitoring Pesticide Residues in Olive Products: Organophosphorus Insecticides in Olives and Oil

CHAIDO LENTZA-RIZOS
Benaki Phytopathological Institute, Pesticide Residue Laboratory, 14561 Kifissia, Greece

A simple and efficient gas–liquid chromatographic method was used to screen and quantitate residues of azinphos ethyl, chlorpyrifos, diazinon, dimethoate, fenthion, fenthion sulfoxide, methidathion, parathion ethyl, and parathion methyl in olive fruit and olive oil. Most olive fruit samples analyzed during 1991–1992 either contained no detectable residues of the insecticides or contained low concentrations of fenthion, dimethoate, and chlorpyrifos. One sample contained fenthion sulfoxide at a level exceeding FAO/WHO Codex Alimentarius maximum residue levels (MRL) for total fenthion. Commercially packed oil samples either contained no detectable residues of the insecticides or contained low concentrations of fenthion, fenthion sulfoxide, and chlorpyrifos. More than half of the virgin oil samples collected from individual growers contained no detectable residues. The others contained mostly fenthion and its sulfoxide metabolite. Of the samples analyzed, 13% exceeded Codex MRL for total fenthion residues. These samples were from a monitoring program targeted at produce most likely to contain residues. Refining procedures seem to act as a decontamination technique for oil containing fenthion residues.

Table olives and olive oil are products of great importance for Greece. They constitute an important part of the national diet and are also exported to foreign countries, including the United States, Japan, Russia, and countries in Northern Europe. Olive trees are attacked by several pests and diseases. Treatments are carried out mostly against insects and scales, namely *Prays oleae, Bactrocera (Dacus) oleae*, and *Saissetia oleae*, which affect both the quantity and quality of the product. Several insecticides are registered for use on olive trees. Among organophosphorus insecticides, those more extensively used are diazinon, dimethoate, parathion ethyl and parathion methyl, chlorpyrifos, azinphos ethyl, methidathion, and fenthion (Ministry of Agriculture, Greece, personal communication).

Received August 16, 1993. Accepted by JM November 16, 1993.

Given the significance of olive products for the country, the concentration of pesticide residues in them is monitored regularly (1) to ensure consumer protection. This paper describes the method used for the simultaneous determination of insecticide residues and gives the results of analyses carried out during 1991–1992.

Literature on insecticide residues in olive products is very scarce and relatively old. Most papers (2–4) refer to residues resulting from supervised field trials. One paper (5) reports monitoring data from 50 olive oil samples collected during 1965–1967 from the Swedish market and analyzed for diazinon, dimethoate, malathion, parathion ethyl, and parathion methyl. The method used involves extraction of pesticides by sweep codistillation and determination by gas–liquid chromatography or cholinesterase inhibition.

METHOD

Apparatus and Reagents

Common pesticide residue analysis equipment was used. Reagents were analytical grade.

Extraction and Cleanup

(a) *Oil.*—A method for the determination of fenthion and its 5 oxidative metabolites in olive oil was developed in our laboratory (6). This method involves (*1*) extraction of residues with *n*-hexane saturated with acetonitrile, (*2*) cleanup by partitioning between *n*-hexane and acetonitrile in the presence of 1% (by volume of acetonitrile) water, and (*3*) oxidation of total fenthion residues to fenthion sulfone and fenthion oxon sulfone.

Only the first 2 steps of this method (extraction and cleanup) were used for diazinon, dimethoate, parathion ethyl, parathion methyl, chlorpyrifos, azinphos ethyl, methidathion, fenthion, and the most important (1, 7) of fenthion's oxidative metabolites, its sulfoxide.

(b) *Olives.*—Stones from a representative sample were removed with a knife, and 50 g flesh was homogenized for 2 min at high speed in an Omni-Mixer pot with 100 g anhydrous sodium sulfate and 75 mL acetone. The pot was cooled, when necessary. The homogenate was filtered under vacuum through a Buchner funnel, paper Whatman sieve, and a layer (0.5–1 cm) of Celite (diatomaceous earth). The cake was collected, the extraction procedure was repeated with a mixture of 25 mL acetone and 50 mL dichloromethane, and the extract was fil-

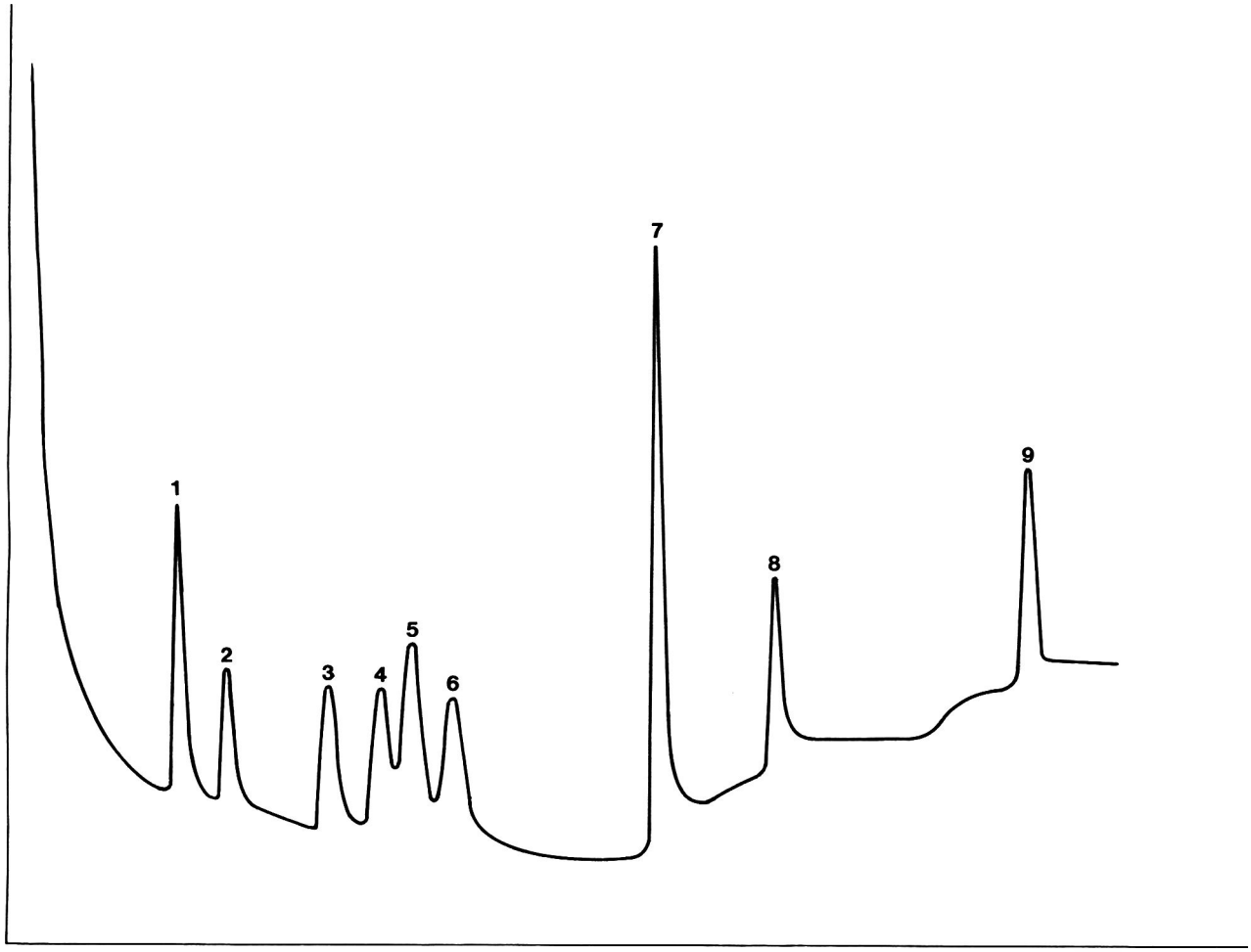


Figure 1. Gas chromatogram of a 3 μL injection of mixed standard made up in acetone: 1, diazinon, 0.3 ng; 2, dimethoate, 0.3 ng; 3, parathion methyl, 0.3 ng; 4, chlorpyrifos, 0.3 ng; 5, parathion ethyl, 0.3 ng; 6, fenthion, 0.3 ng; 7, methidathion, 1.5 ng; 8, fenthion sulfoxide, 1.5 ng; azinphos ethyl, 3 ng. For GC conditions, *see* text.

tered with the same funnel. The pot and funnel were rinsed twice with 12.5 mL each time of an acetone–dichloromethane mixture (1 + 2), and the filtrate was transferred to a 1 L separatory funnel. The walls of the vacuum flask were rinsed with 500 mL of an aqueous solution of 1% NaCl, which was added to the separatory funnel. The funnel was shaken gently and left to equilibrate. The lower (dichloromethane) phase was collected through anhydrous sodium sulfate in a previously weighed round-bottom flask. The cleanup procedure was repeated twice with 50 mL dichloromethane each time. The anhydrous sodium sulfate was rinsed with a few milliliters of dichloromethane, and the extract was evaporated, using a few milliliters of acetone, in a rotary evaporator until all the dichloromethane had been removed. The flask was reweighed, and the quantity of oil remaining was calculated. A 10 g aliquot of this oil, or the whole sample if less than 10 g, was analyzed for pesticide residues by the method for oil analysis, and the results were adjusted where necessary to refer to the actual mass of the sample.

Determination

A gas chromatographic (GC) analysis with packed columns was carried out under temperature-programmed conditions that separated the peaks of the compounds of interest, including that of fenthion sulfoxide, which is the main metabolite of fenthion (1). A Perkin-Elmer Sigma 2 gas chromatograph was used with a nitrogen–phosphorus detector (NPD) and a borosilicate glass column (2 mm × ¼ in. × 6 feet) packed with 5% OV-17 on Gas Chrom. Q., operating under the following conditions: injector temperature, 320°C; detector temperature, 310°C; initial oven temperature, 230°C; initial time, 12 min; initial rate, 16°C/min; final oven temperature, 290°C; final time, 5 min; final rate, 20°C/min; oven post temperature, 310°C; time post, 5 min; bead adjust control, 370; carrier gas (nitrogen) flow rate, 20 mL/min; hydrogen flow rate, 3 mL/min; air flow rate, 100 mL/min; chart speed, 30 cm/h; range, 1; and attenuation, 4.

A mixed standard solution was prepared containing diazinon, dimethoate, parathion ethyl, parathion methyl, and fen-

Table 1. Recoveries of insecticides from fortified olive samples

Compound	Fortification level, mg/kg	Mean rec. of 10 fortified samples, ± SD, %	Limit of detection[a]
Diazinon	0.02	80 ± 20	0.002
Dimethoate	0.02	155 ± 25[b]	0.005
Parathion methyl	0.02	102 ± 9	0.005
Chlorpyrifos	0.02	90 ± 15	0.01
Parathion ethyl	0.02	90 ± 8	0.005
Fenthion	0.02	96 ± 16	0.01
Methidathion	0.1	106 ± 20	0.01
Fenthion sulfoxide	0.1	200[b] ± 22	0.02
Azinphos ethyl	0.2	100 ± 17	0.05

[a] For olives giving ca 10 g of oil from 50 g of analytical sample.
[b] These high values, presumably due to enhancement by crop coextractives, fell to 110% when the peaks of fortified samples were compared with the corresponding peaks of the mixed standard made up in control sample extract.

thion at 0.1 μg/mL; methidathion and fenthion sulfoxide at 0.5 μg/mL; and azinphos ethyl at 1 μg/mL. The same standard solution was used to fortify (spike) control samples and as an external standard for GC. Figure 1 gives a typical chromatogram of this mixed standard.

Method Assessment

Ten control samples (untreated olives) were spiked with the compounds of interest at the following fortification levels: diazinon, dimethoate, parathion methyl, chlorpyifos, parathion ethyl and fenthion at 0.02 mg/kg; methidathion and fenthion sulfoxide at 0.10 mg/kg; and azinphos ethyl at 0.20 mg/kg (compounds of lower sensitivity were spiked at higher levels).

Table 1 gives the mean recoveries and standard deviations for each of the 9 compounds. As noted, dimethoate and fenthion sulfoxide gave high recoveries, presumably due to enhancement by crop coextractives. For this reason, the extracts of the same spiked samples were determined by comparing the peak height of the samples with those of the mixed standard made up in an extract of an unfortified control sample. Recoveries of dimethoate and fenthion sulfoxide fell to 110%. The limits of detection (Table 1)(8) were sufficiently low to enable enforcement of FAO/WHO Codex Alimentarius MRLs (Table 2)(9).

Table 2. FAO/WHO Codex Alimentarius maximum residue limits for olives and oil

Compound	Maximum residue limit, mg/kg			
	Olives		Oil	
	Fresh	Processed	Virgin	Refined
Diazinon	2	—	2	—
Dimethoate	1	0.05[a]	—	0.05[a]
Parathion methyl	—	—	—	—
Chlorpyrifos	—	—	—	—
Parathion ethyl	0.5	—	2	—
Fenthion	1	—	1	—
Methidathion	—	—	—	—
Azinphos ethyl	—	—	—	—

[a] Limit of determination.

Table 3. Concentration ranges of fenthion (parent compound) residues in samples from individual growers

Concentration range, mg/kg	Number of samples within range
ND[a]	62
0.01–0.05	15
0.06–0.10	13
0.11–0.20	9
0.21–0.50	7
0.51–1	2
>1	7

[a] ND, Not detectable (<0.01 mg/kg).

Confirmation of Results

The method was used as a preliminary screening step for all samples. Samples positive for any compound were quantitated more precisely and accurately by using a standard solution of the insecticide initially detected. The results were confirmed on a Varian 370 gas chromatograph with a flame photometric detector and a borosilicate glass column (2 mm × ¼ in. × 1.5 feet) packed with 3% OV-101 on Carbowax 20M–treated Chromosorb 100/120 operating under the following conditions: injector temperature, 250°C; detector temperature, 250°C; initial oven temperature, 180°C; initial time, 5 min; initial rate, 22°C/min; final oven temperature, 200°C; final time, 2 min; carrier gas (helium) flow rate, 30 mL/min; hydrogen flow rate, 140 mL/min; air flow rates, 80 and 170 mL/min; and chart speed, 1 cm/min.

Results and Discussion

Olives

Seven samples of fresh olives (olive fruit without any processing) and 10 samples of processed olives were analyzed. The

Table 4. Concentration ranges of fenthion sulfoxide residues in samples from individual growers

Concentration range, mg/kg	Number of samples within range
ND[a]	86
0.01–0.05	5
0.06–0.10	7
0.11–0.50	4
0.51–1	4
>1	9

[a] ND, Not detectable (<0.01 mg/kg).

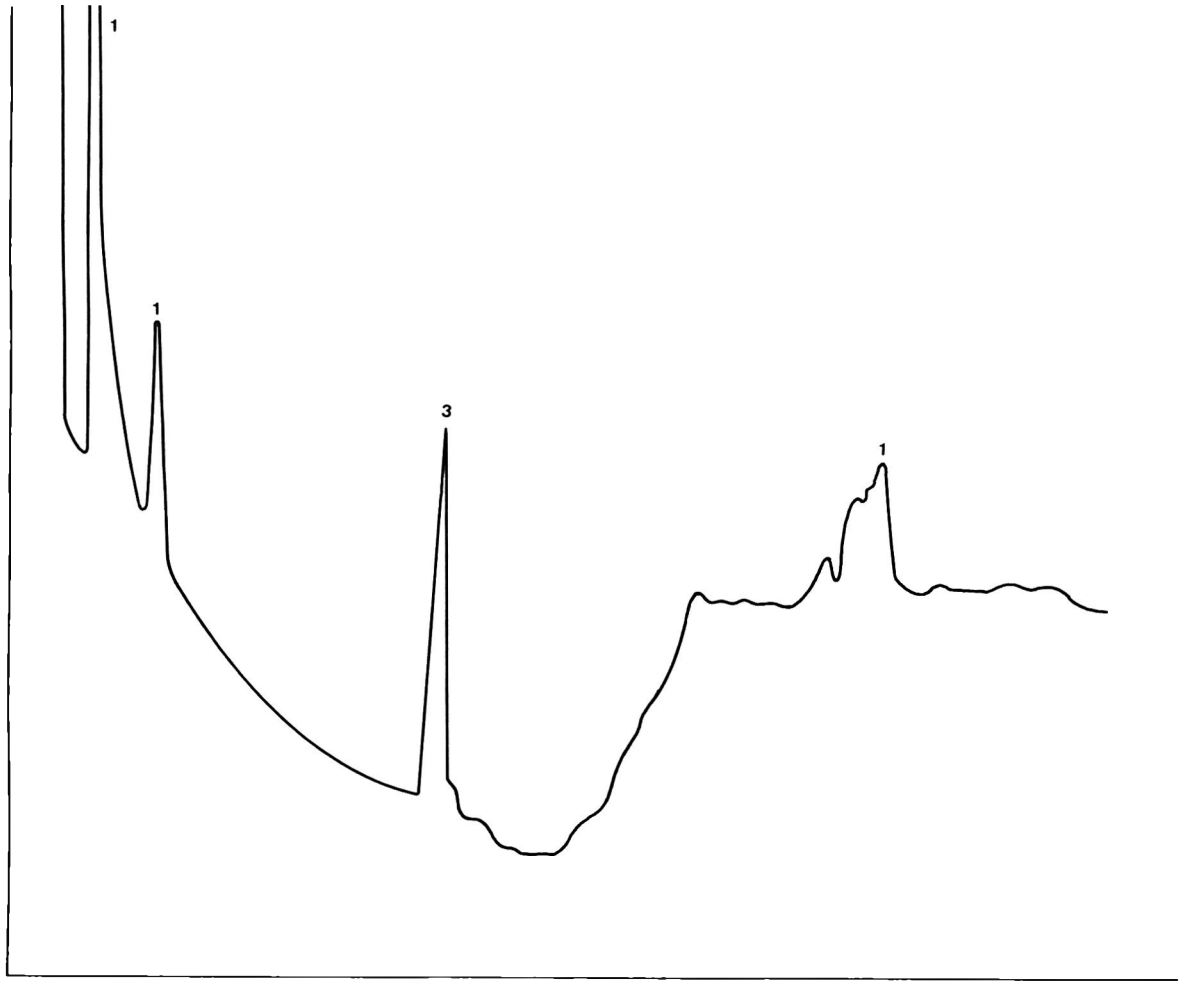


Figure 2. Gas chromatogram of a 3 μL injection of olive fruit extract (5 g/mL) in acetone: 1, peak of crop coextractives; 2, fenthion. GC conditions were the same as those for Figure 1; *see* text.

fresh olive samples were collected from olive groves treated with bait sprays of fenthion or dimethoate close to harvest. The residues detected ranged from 0.01 to 0.15 mg/kg (fenthion) and from 0.005 to 0.07 mg/kg (dimethoate). The processed olive samples were from ready-to-eat packed table olive lots destined for export. Among these samples, 3 contained no detectable residues; 4 contained fenthion or chlorpyrifos at low concentrations ranging from 0.01 to 0.15 mg/kg (chlorpyrifos) and from 0.02 to 0.05 mg/kg (fenthion); one sample contained fenthion and chlorpyrifos at 0.05 and 0.01 mg/kg, respectively; and another contained fenthion and dimethoate both at 0.05 mg/kg. Only one sample (olives prepared by natural fermentation) contained residues of fenthion sulfoxide at a concentration higher than FAO/WHO Codex Alimentarius MRL for total fenthion (9).

Oil

(a) *Commercially packed oil.*—Thirty samples of commercially packed oil were analyzed. Some were virgin oil samples; the categories of other samples (virgin or refined) were not known. Thirteen samples contained no detectable residues. Thirteen samples contained fenthion residues at concentrations ranging from 0.01 to 0.15 mg/kg. Two samples contained both fenthion (0.03 and 0.06 mg/kg) and fenthion sulfoxide (0.02 and 0.08 mg/kg). Finally, 2 samples contained chlorpyrifos residues at 0.03 mg/kg.

(b) *Samples from individual growers.*—A total of 115 samples of virgin oil (oil extracted from olives without any refining) were analyzed. Of these, 59 contained no detectable insecticide residues, one sample contained chlorpyrifos residues at 0.26 mg/kg. one sample contained parathion methyl at 0.01 mg/kg, and another contained parathion ethyl at 0.05 mg/kg. Fenthion residues were detected in 53 samples (Table 3). Fenthion sulfoxide was detected in 29 samples at concentrations ranging from 0.1 to 1.30 mg/kg (Table 4).

These results confirm our previous finding (1) that fenthion, together with its sulfoxide, is the most important residue in oil. They also indicate that the concentration of residues in commercially packed oils is consistently low. This trend is partly attributed to dilution caused by mixing several lots, many of which contain no residues, and partly to refinement procedures. Refinement acts as a decontamination technique, as shown by the following analysis of the deodorization distillate of an olive oil refinery. This distillate, a 3-ton viscous concentrate that remained in the barometric concentrator of the deodorizer was collected over a period of one year after treatment of 5000 tons

of oil. The analysis revealed the following concentrations (mg/kg) of insecticides: diazinon, 0.52 mg/kg; parathion methyl, 0.28; chlorpyrifos, 0.28; methidathion, 2; parathion ethyl, 0.30; and fenthion sulfoxide, 138.

It seems that fenthion and its sulfoxide metabolite are trapped in the concentrator during the procedure. Furthermore, the absence of fenthion parent compound residues indicates that, under the conditions of the deodorization procedure (passage through ultrahot vapor of 190°C under vacuum of 5 mm Hg), it is metabolized to its sulfoxide.

Conclusions

The multiresidue method used to determine 8 insecticides and one important metabolite in olive fruits and olive oil is a simple, time- and cost-effective method that permits handling of large sample loads with simple equipment and materials. This simplicity is particularly important, given that olive trees are grown mainly in countries with limited analytical resources.

Of the total number of samples analyzed during 1991–1992, only one sample of olive fruits exceeded the Codex MRL. None of the commercially packed oil samples contained residues higher than Codex MRLs. Some samples (13% of total) from individual growers contained fenthion and its sulfoxide metabolite at levels exceeding Codex MRLs. However, most of these were surveillance samples: The sampling was designed to focus on a likely problem area, where it was suspected that violative treatments may have been carried out. Thus, the samples were more likely to contain higher levels of residues. The study aimed to confirm the consequences of the misuse of insecticides so that appropriate instructions could be given to government extension agents and olive growers. The oil from which these samples came was not sold commercially nor was it consumed by the producers; instead, it was directed to the soap-making industry.

In relation to other insecticides, the higher incidence of fenthion residues was to be expected because it is the compound mainly used against *Bactrocera (Dacus) oleae* (olive fruit fly), and some treatments are carried out in autumn, that is, close to the harvest period. It is also fat soluble (log of partition coefficient between octanol and water, 4.84).

Acknowledgments

This work was supported by a grant from the Greek Ministry of Agriculture, Plant Protection Service. I am grateful to laboratory assistants C. Bourou and G. Giannopoliti for skillful technical work.

References

(1) Lentza-Rizos, C., & Avramides, E. (1991) *Pest Sci.* **32**, 161–171

(2) Alessandrini, M.E. (1962) *Residue Rev.* **1**, 92–111

(3) Ferreira, J.R., & Tainha, A.M. (1983) *Pest. Sci.* **14**, 167–172

(4) Leone, A.M., Liuzzi, V.A., Gambacorta, G., La Notte, E., Santoro, M., Alviti, F., Laccone, G., Guario, A. (1990) *Riv. Ital. Sost. Grasse* **LXV**, II, 17–28

(5) Renvall, S., & Akerblom, M. (1971) *FAO Plant Prot. Bull.* **3**, 57–61

(6) Lentza-Rizos, C., & Avramides, E. (1990) *Analyst* **115**, 1037–1040

(7) Lentza-Rizos, C., Avramides, E.J., & Roberts, R.A. (1993) *Pest. Sci.* **40**, 63-69

(8) Loftus, M.L., Barray, L.M., & Tomerlin, J.B. (1992) *J. Assoc. Off. Anal. Chem.* **75**, 911–915

(9) FAO/WHO, *Codex Alimentarius Commission*, (1993) CX/PR-2-1993, CCPR

DRUGS, COSMETICS, FORENSIC SCIENCES

Screening of Chloramphenicol Residues in Pork Muscle by Planar Chromatography

J.P. ABJEAN
Centre National d'Etudes Vétérinaires et Alimentaires, Laboratoire des Médicaments Vétérinaires, La Haute Marche, Javené - 35133 Fougeres, France

A rapid planar chromatographic method is described for the qualitative determination of chloramphenicol residues in muscle. The drug is extracted with ethyl acetate and purified by solid-phase extraction. After elution, the collected phase is evaporated to dryness. The residue is dissolved in methanol, spotted on a silica gel plate, and chromatographed. After elution, the chloramphenicol is visualized after reduction with fluorescamine. This method detects chloramphenicol in meat at 10 μg/kg and has a sample throughput of about 25 samples per analyst per day.

Chloramphenicol (CAP) is a very effective broad-spectrum antibiotic. In the European Community, a maximum residue limit (MRL) in foods of animal origin is set at 10 μg/kg. In the United States, the use of CAP in food-producing animals was never authorized. Nevertheless, the monitoring of illegal use is determined also at the 10 μg/kg level (1). For inspection of food of animal origin, a 2-stage strategy may be of interest (2, 3), involving (*1*) screening with a well-suited method and (*2*) confirmation with a method particularly suited for specificity if the drug of interest is of illegal use and suited also for accuracy in case a MRL has been established. In addition to analytical characteristics, such as false-positive and -negative rates, the screening method should have a high sample throughput for the lowest possible cost.

Planar chromatography is particularly well suited for screening purposes. Allen (4) reviewed chromatographic methods for CAP residues in food of animal origin. The only planar chromatographic method that reportedly meets the required sensitivity was difficult to obtain, and we found no other published method consistent with our goal. We, therefore, started our work with a detection method of nitro compounds at nanogram level (5) and with some previously published works describing extraction–purification methods for CAP residues in animal tissues (3, 6, 7).

Received March 19, 1993. Accepted by JW September 8, 1993.

Experimental

Pork muscle was purchased at a local supermarket.

Reagents

(a) *Water.*—Use deionized water (Milli-Q, Millipore).

(b) *Solvents.*—Analytical grade ethyl acetate, hexane, and 1-propanol (Merck); diethyl ether and methanol (Prolabo).

(c) *Sodium carbonate and sodium sulfate.*—Analytical grade (Merck).

(d) *Sep-Pak silica-vac.*—Waters Chromatography Division.

(e) *Si-60 silica gel plates.*—With concentrating zone 10 × 10 or 10 × 20 cm, 0.2 mm (Merck).

(f) *Solution for CAP reduction.*—Dissolve 0.4 g stannous chloride (Merck) in 10 mL 5% acetic acid (Prolabo) in deionized water and add 0.1 mL 5% phenolphthalein (Merck) in dioxane (Merck). The solution is unstable and should be made shortly before use.

(g) *Buffer, pH 8.3.*—Dissolve 19 g boric acid (Sigma Chemical), 19.75 g potassium chloride (Prolabo) in 0.8 L deionized water, adjust pH with concentrated NaOH (Prolabo), and dilute to 1 L.

(h) *Visualizing reagent.*—Dissolve 50 mg fluorescamine (Sigma Chemical) in 500 mL acetone (Merck). The solution is stable for at least 12 months when stored at −18°C.

(i) *Fortification solution.*—Chloramphenicol (Sigma Chemical), 0.1 μg/mL in methanol.

Apparatus

(a) *Chromatographic chamber and sprayer.*—Camag.

(b) *Table-type UV 366 nm UV lamp (6 × 15 W).*—Prolabo.

(c) *Nitrogen evaporator with aluminum block heated to ca 60°C.*—Prolabo.

(d) *Solid-phase extraction system (Prolabo).*—Adaptors, 8 mL reservoirs, and taps (Analytichem).

Extraction

Weigh 1 ± 0.05 g ground muscle in a 12 × 70 mm test tube. Add 2 mL ethyl acetate (disperse the muscle in the organic phase by shaking by hand). Place the tube for 10 min in an ultrasonic bath. Centrifuge for 10 min at 4000 × *g*. Transfer the

Table 1. Raw results of 7 experiments

	Results[a]									
	Fortified sample					Control sample				
Experiment	1	2	3	4	5	6	7	8	9	10
1	+	–	–	+	+	+	–	+	–	–
2	+	+	+	+	+	–	–	–	–	–
3	+	–	?	?	–	–	–	–	–	–
4	+	+	+	+	+	–	–	–	–	–
5	–	+	+	+	+	–	–	–	–	–
6	+	+	+	–[b]	+	–	+[b]	–	–	–
7	+	+	+	?	+	–	–	–	–	–

[a] +, CAP residue present; –, CAP residue absent; ?, doubtful result.
[b] Results to be corrected because inversion was noted.

Table 3. Data for calcuation of sensitivity rate and its standard error

Experiment	m_i[a]	a_i[b]
2	5	5
3	5	3
4	5	5
5	5	4
6	5	5
7	5	5

[a] m_i, number of chloramphenicol-spiked portions.
[b] a_i, number of portions analyzed positive among the known positive test portions.

organic phase to a 15 mL tube containing 0.25 g sodium sulfate. Stir for 1 min and centrifuge for 5 min at 4000 × *g*. Add 5 mL hexane and mix; avoid bringing back the sulfate into suspension.

Purification

Transfer the solution to the reservoir connected to the Sep-Pak and let the solution elute through the silica bed (generally elution proceeds by gravity flow, after initiation by gentle overpressure of the reservoir). Wash the column successively with 2 mL ethyl acetate–hexane (3 + 7) and then 2 mL diethyl ether–hexane (1 + 3). Elute CAP with 5 mL diethyl ether. Evaporate the collected organic phase under a nitrogen flow.

Chromatography

Dissolve the residue in 50 µL methanol. Spot the whole extract on the silica gel plate. At the edges of the plate, spot also 3 and 5 µL CAP solution corresponding to 3 and 5 ng of the drug. Develop with 1-propanol–hexane (1.5 + 8.5) for ca 4 cm.

Visualization

Remove the solvent from the plate solvent with a hair dryer. Then evenly spray the dried plate with stannous chloride solution (spray should moisten but not wet the plate). Let the plate stand for 15 min and then place it under an air flow until it appears dry. Place the plate into a 110°C oven for 15 min. Spray with 2N sodium hydroxide solution; the plate should become slightly pink (carefully avoid an excess of reagent, which would appear as dark background when the plate is observed under 366 nm UV lamps). Evenly spray with buffer solution (*see* remarks for the first reagent). Leave the plate under air flow until it looks dry and place it again in a 110°C oven for 5 min. After the plate has cooled, spray it with fluorescamine solution (ca 10 mL for a 10 × 10 cm plate). Place the plate for 15 min in the dark and examine under 366 nm UV light. CAP appears as a yellow-green spot on a purple background (a somewhat blue background may signify degradation of the

Table 2. Transformed experimental results

	Results[a]											
Experiment	1	2	3	4	5	6	7	8	9	10	Ti[b]	Ti²[c]
1	1	0	0	1	1	0	1	0	1	1	6	36
2	1	1	1	1	1	1	1	1	1	1	10	100
3	1	0	1	1	0	1	1	1	1	1	8	64
4	1	1	1	1	1	1	1	1	1	1	10	100
5	0	1	1	1	1	1	1	1	1	1	9	81
6	1	1	1	1	1	1	1	1	1	1	10	100
7	1	1	1	1	1	1	1	1	1	1	10	100
Si[d]	6	5	6	7	6	6	7	6	7	7	63	581
Si²[e]	36	25	36	49	36	36	49	36	49	49	401	

[a] 0, false result; 1, good result.
[b] Ti, total for the line.
[c] Ti², squared total for the line.
[d] Si, total for the column.
[e] Si², squared total for the column.

Table 4. Data for calculation of specificity rate and its standard error

Experiment	m_i[a]	a_i[b]
2	5	5
3	5	3
4	5	5
5	5	4
6	5	5
7	5	5

[a] m_i, number of negative portions analyzed.
[b] a_i, number of portions analyzed negative among chloramphenicol-free portions.

fluorescamine solution; a yellow background is generally due to an excess of fluorescamine; in both cases, the sensitivity of the method can be dramatically diminished).

Validation

Validation of the method was conducted on the basis of McClure's paper (8). Trials were made on batches of 5 samples spiked at 10 µg/kg and 5 control samples. Samples were fortified by the analyst after they were coded by a second analyst. For analysis of results, 3 rules were followed: (*1*) To insure reproducibility, it was imperative to visualize the standard spot at the 3 ng level. (*2*) Doubtful results were classified as positive. (*3*) Problems noted before the code is discovered may be cause for an authorized result correction. For example, a possible tube inversion was noted in experiment 6 (Table 1). The code revealed that it involved 1 blank and 1 fortified sample. Because the experimental results gave false-positive and false-negative results for these 2 tubes, we assumed that the inversion had been done by the analyst. Consequently, these 2 results were switched and considered correct.

The raw results are summarized in Table 1; the transformed results are given in Table 2. According to McClure (8), statistical results give a χ^2 value of 14.7 for 6 degrees of freedom (df) expressing differences in experiments. Testing the first experiment as the outlier give a χ^2 value of 11.25 ($P = 0.05$; 1 df = 3.412). The test carried out for the other 6 experiments give $\chi^2 = 3.65$ ($P = 0.05$; 5 df = 11.07). Results, therefore, were homogeneous.

Table 5. Data for calculation of false-positive rate and its standard error

Experiment	m_i[a]	a_i[b]	$a_i m_i$	m_i^2
2	5	0	0	25
3	3	0	0	9
4	5	0	0	25
5	4	0	0	16
6	5	0	0	25
7	5	0	0	25

[a] m_i, number of portions analyzed positive among both spiked and chloramphenicol-free portions.
[b] a_i, number of portions analyzed positive among the known negative test portions.

Table 6. Data for calculation of false-negative rate and its standard error

Experiment	m_i[a]	a_i[b]	$a_i m_i$	m_i^2
2	5	0	0	25
3	7	2	14	49
4	5	0	0	25
5	6	1	6	36
6	5	0	0	25
7	5	0	0	25

[a] m_i, number of portions analyzed negative among both spiked and blank test portions.
[b] a_i, number of portions analyzed negative among chloramphenicol-spiked portions.

Method performance was based on the following parameters: (*1*) sensitivity (probability to find positive a sample containing CAP at 10 µg/kg), $P+ = 0.90 \pm 0.068$ (Table 3); (2) specificity (probability to find negative a sample containing no CAP), $P- = 1$ (Table 4); (*3*) the false-positive rate (probability to find positive a sample containing no CAP), $pf+ = 0$ (Table 5); and (*4*) the false-negative rate (probability to find negative a sample containing CAP at 10 µg/kg), $pf- = 0.091 \pm 0.159$ (Table 6).

Discussion

Method validation was conducted, as generally accepted, with fortified samples. The method was also used to analyze pig meat treated with chloramphenicol. The screening method gave a negative result, but the meat contamination was quantified by liquid chromatography to 5 µg/kg. So we assume that the extraction process, assisted with an ultrasonic bath, is effective for incurred residues.

Method performances were calculated for a lot contamination rate of 50%. As it may be expected, the true contamination rate is less than 5%; therefore, the false-negative rate can be calculated (8) as $pf- = 0.0050$. That is, for every 10 000 samples tested as negative, no more than 50 would be expected to be positive (as long as the contamination rate is 5%). Once familiar with the method, one analyst can analyze ca 20–24 samples in one working day. The described method does not require any expensive apparatus, which can be left free for confirmatory purposes. Moreover, the planar chromatographic process does not need time-consuming column equilibration as does liquid chromatography. The analyst, therefore, may, if needed, conduct at the same time the extraction–purification and chromatography of different analytes. For these reasons, planar chromatography may be of interest for regulatory control.

Acknowledgments

I thank A. Bourdais, F. Helleux, and M.H. Moreau for their contribution to the work.

References

(1) Haagsma, N., Schreuder, C., & Rensen, E.R.A. (1986) *J. Chromatogr.*, **363**, 353–359

(2) Stephany, R.W. (1990) in *Proceedings of EuroResidues Conference Noordwijkerhout*, The Netherlands, N. Haagsma, A. Ruiter, & P.B. Czedik-Eysenberg (Eds), Rijksuniversiteit Utrecht, pp. 76–85

(3) Van de Water, C., & Naagsma, N. (1991) *J. Chromatogr.* **566**, 173–185

(4) Allen, E.H. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 990–999

(5) Haefelfinger, P. (1975) *J. Chromatogr.* **111**, 323–329

(6) Arnold, D., & Somogyi, A. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 984–990

(7) Pochard, M.F., Burger, G., Chevalier, M., & Gleizes, E. (1987) *J. Chromatogr.* **409**, 315–323

(8) McClure, F.D. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 953–960

DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Reserpine, Hydralazine HCl, and Hydrochlorothiazide in Tablets by Liquid Chromatography on a Short, Normal-Phase Column

UGO R. CIERI
U.S. Food and Drug Administration, 900 U.S. Customhouse, 2nd and Chestnut Sts, Philadelphia, PA 19106

A procedure is presented for the determination of reserpine, hydralazine HCl, and hydrochlorothiazide in tablets by liquid chromatography. The sample is extracted with methanol, and the extract is filtered through paper. Chromatography is performed in 2 stages, each using a 7.5 cm-long normal-phase column but different mobile phases. In the first stage, intended exclusively for reserpine, the mobile phase consists of methanol containing 2% aqueous 1-pentanesulfonic acid sodium salt at 4 parts/1000. Detection and quantitation of reserpine was by fluorescence at 280 nm (excitation) and 360 nm (emission). In the second stage, the amount of the salt solution in the mobile phase was increased to 5%. Detection and quantitation of hydrochlorothiazide and hydralazine HCl was by UV absorbance at 260 nm. One commercial sample of tablets was analyzed by the proposed method. Two determination of each ingredient were made on a ground composite. Ten individual tablets also were examined.

Received June 18, 1993. Accepted by JW October 4, 1993.

Reserpine, an alkaloid isolated from the root of *Rauwolfia serpentina*, is used primarily to control mild cases of hypertension. Because the drug is rather potent, dosage levels per unit are very low: generally between 0.1 and 0.25 mg. It has been reported (1) that reserpine may easily undergo partial decomposition for a variety of reasons and that the degradation products are therapeutically ineffective.

Some commercial preparations contain, in addition to reserpine, 1 or 2 of the diuretics hydrochlorothiazide, chlorothiazide, hydroflumethiazide, and hydralazine HCl.

One product, official in the U.S. Pharmacopeia (USP), is reserpine–hydralazine HCl–hydrochlorothiazide tablet. In this tablet, dosage levels per unit of the 3 ingredients, in the order listed, are 0.1, 25, and 15 mg (2).

The USP monograph contains 3 assay procedures, one for each ingredient, to be performed on separate portions of a ground composite. Hydrochlorothiazide is determined spectrophotometrically after isolation by ion-exchange chromatography. Hydralazine HCl and reserpine are determined by 2 separate colorimetric procedures.

The advent of liquid chromatography (LC) as an analytical tool has greatly facilitated the analysis of multicomponent drug preparations. Honigberg et al. (3) studied the LC behavior on reversed-phase columns of several drugs that may be present in antihypertensive–diuretic preparations but did not conduct any quantitative determinations.

Different mobile phases were tried, and for the reserpine–hydralazine HCl–hydrochlorothiazide product (2), the authors (3) recommend a 20 + 80 mixture of acetonitrile and 0.1% aqueous ammonium acetate. Hydrochlorothiazide and hydralazine HCl elute quickly and are well-separated, but reserpine elutes extremely slowly and produces a wide peak. Subsequently, a method was published (4) for LC determinations of mixtures of reserpine and hydrochlorothiazide, which used a 30 cm-long normal-phase column, methanol as mobile phase, and 2 detectors arranged in series. Hydrochlorothiazide, which elutes first, is determined by UV absorbance, and reserpine, which follows immediately, is determined by fluorescence. This investigation was initiated to develop a suitable LC procedure for the analysis of the 3-component mixture listed as official in the USP (2).

Experimental

Reagents and Apparatus

(**a**) *Reserpine.*—USP, available from U.S. Pharmacopeial Convention (Rockville, MD), or equivalent.

(**b**) *Hydrochlorothiazide.*—USP, or equivalent.

(**c**) *Hydralazine HCl.*—USP, or equivalent.

(**d**) *Methanol.*—LC grade (Mallinckrodt Co., Paris, KY), or equivalent.

(**e**) *1-Pentanesulfonic acid sodium salt.*—Aldrich Chemical Co. (Milwaukee, WI), or equivalent.

(**f**) *LC column.*—Novapak silica, 7.5 cm × 3.9 mm id (Millipore Corp., Bedford, MA), or equivalent.

(**g**) *Injection valve.*—With 5 µL loop (Rheodyne Corp., Cotati, CA, or Alltech Associates, Deerfield, IL).

(**h**) *Solvent delivery system.*—Model M-45 (Millipore Corp.), or equivalent.

(**i**) *Fluorescence LC monitor.*—Model RF 535, connected to a recorder (Shimadzu Corp., Kyoto, Japan), or equivalent. Set excitation at 280 nm and emission at 360 nm.

(**j**) *UV absorbance LC detector.*—Connected to a recorder (Applied Biosystems, Ramsey, NJ), or equivalent. Set absorbance at 260 nm.

Solutions

(**a**) *Reserpine stock solution.*—Store in the dark; discard after 2 weeks. Weigh accurately ca 50 mg reserpine and transfer to a 100 mL volumetric flask. Add 1.0 mL chloroform, swirl well to dissolve, dilute to volume with methanol, stopper the flask, and mix the contents.

(**b**) *Reserpine intermediate solution.*—Dilute 4.0 mL reserpine stock solution to 200.0 mL with methanol.

(**c**) *Solution of partially degraded reserpine.*—Transfer 0.20 mL reserpine stock solution to a small beaker and evaporate to dryness. Add 1.0 mL chloroform to beaker, swirl to dissolve residue, and irradiate for 10 min under long-wave UV light. Evaporate chloroform to dryness, dissolve residue in warm methanol, dilute to 100 mL with methanol, and mix.

(**d**) *Reference solution.*—Weigh accurately ca 25 mg hydralazine HCl and 15 mg hydrochlorothiazide. Transfer both to a 100 mL volumetric flask, add 40 mL methanol, heat gently to dissolve, cool to room temperature, add 10.0 mL reserpine intermediate solution in methanol to mark, stopper, and mix.

(**e**) *Sample solution.*—(*1*) *Assay of ground composite.*—Weigh at least 20 tablets, calculate average tablet weight, and grind to a uniform powder. Transfer to 100 mL beaker a weighed amount of sample approximately equivalent to 1 tablet, add 40 mL methanol, heat gently on a hot plate, and stir with a glass rod. Remove the beaker from the hot plate as soon as solution begins to simmer, transfer solution to a 100 mL volumetric flask, rinse beaker with several portions of methanol, and add rinsings to the flask. Cool to room temperature, dilute to volume with methanol, stopper, and mix. Filter through paper; discard first 10 mL of filtrate. (*2*) *Assay of single tablet.*—Place a tablet in a 100 mL beaker, add 40 mL methanol, heat gently over a hot plate, carefully crush the tablet with flat-bottom glass rod, and stir. Continue as described in (*1*) beginning with removal as soon as solution begins to simmer.

(**f**) *Salt solution.*—Dissolve 2 g of 1-pentanesulfonic acid sodium salt in 100 mL water.

Determination of Reserpine

To prepare the mobile phase, mix 4 mL salt solution (*f*) with 1000 mL methanol. Connect column to fluorescence detector, and set the flow rate at 0.4 mL/min. Allow system to equilibrate for at least 15 min and then inject an aliquot of a solution of partially degraded reserpine. In addition to reserpine, which elutes after about 3 min, the chromatogram should have 2 smaller peaks, both eluting earlier. The resolution, as defined in ref. 2 (p. 1565), between reserpine and the second degradation peak is not less than 1.5. Inject an aliquot of reference solution. Hydrochlorothiazide elutes after about 1.8 min; the resolution between this peak and that of reserpine is not less than 2.0. The tailing factor of the reserpine peak, as defined in ref. 2 (p. 1567), is not greater than 2.0.

Inject replicate aliquots of the reference solution. The relative standard deviation of reserpine peak response in 4 consecutive chromatograms is not greater than 2.5%. Adjust the composition of the mobile phase if necessary to meet system suitability requirements. Inject reference solution once, sample solution twice, and then reference solution again. Take the average of the responses of reserpine peaks in the sample chromatograms (Ru1) and in the reference chromatograms (Rs1). Determine the amount of reserpine in the sample solution according to the following formula:

$$\text{Reserpine, mg} = (\text{Ru1})\,(W_r)/(\text{Rs1})$$

where W_r is the weight of reserpine (mg) in the reference solution.

If several samples are analyzed successively, as in the case of content uniformity determinations, use the following order of injections: reference, 2 injections of sample 1, reference, 2 injections of sample 2, reference and so on. For each sample, calculate Ru1 as the average response of the reserpine peaks in the chromatograms preceding and following the 2 sample chromatograms. Ru1 also could be calculated as the average response of reserpine peaks in a larger number of reference

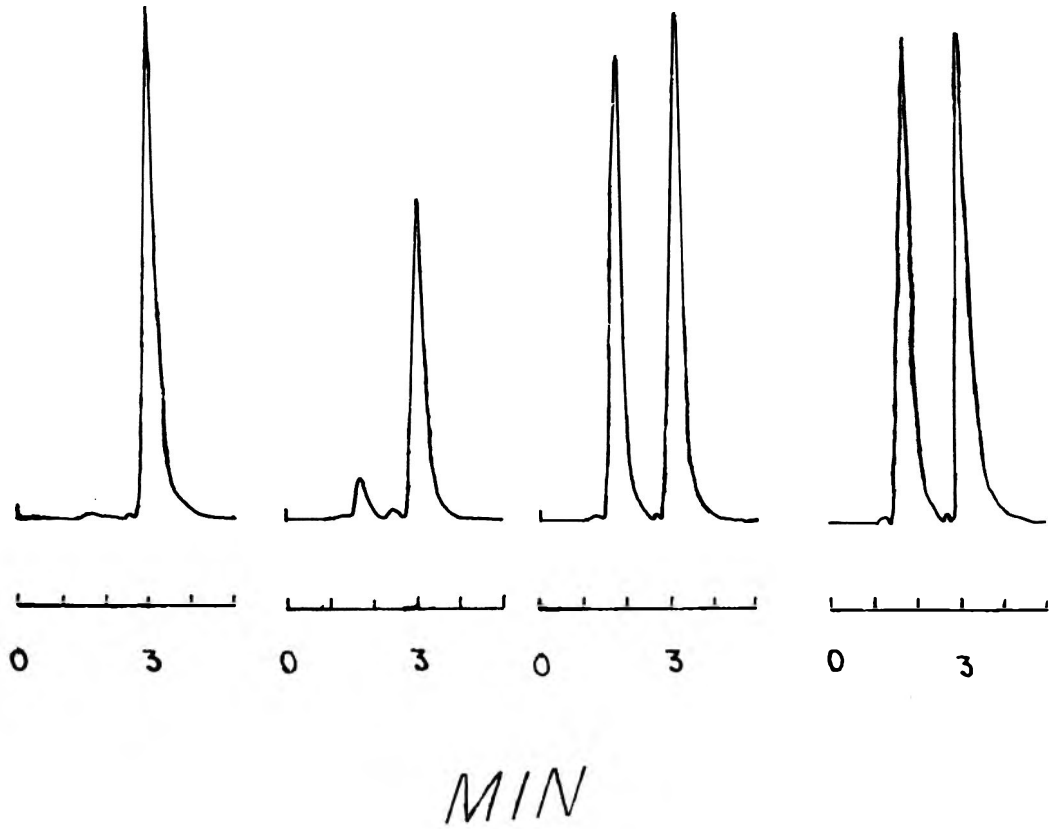


Figure 1. (From left to right) Chromatograms of reserpine, partially degraded reserpine, reference solution, and sample solution. The mobile phase was 2% aqueous 1-pentanesulfonic acid sodium salt–methanol (4 parts/1000). The flow rate was 0.4 mL/min, the volume of injection was 5 μL, and the fluorescence monitor was set at 280 nm (excitation) and 360 nm (emission).

Table. 1. Analysis of commercial tablets

	Hydralazine HCl (Label amount, 25 mg)		Hydrochlorothiazide (Label amount, 15 mg)		Reserpine (Label amount, 0.1 mg)	
Analysis	Amt found, %	% of label	Amt found, mg	% of label	Amt found, mg	% of label
Assay						
Determination 1	23.6	94.4	14.6	97.3	0.0991	99.1
Determination 2	23.7	94.8	14.7	98.0	0.0983	98.3
Content uniformity						
Tablet 1	23.2	92.8	14.8	98.7	0.0958	95.8
Tablet 2	24.1	96.4	15.2	101	0.0972	97.2
Tablet 3	24.6	98.4	15.0	100	0.100	100
Tablet 4	23.3	93.2	14.8	98.7	0.102	102
Tablet 5	24.5	98.0	14.7	98.0	0.0983	98.3
Tablet 6	24.6	98.4	15.1	101	0.0983	98.3
Tablet 7	22.7	90.8	14.6	97.3	0.0959	95.9
Tablet 8	22.9	91.6	14.6	97.3	0.0977	97.7
Tablet 9	23.3	93.2	14.8	98.7	0.0998	99.8
Tablet 10	22.8	91.2	14.0	93.3	0.102	102
Average	23.6	94.4	14.8	98.6	0.0987	98.7
RSD		3.3%		2.3%		2.3%

chromatograms and applied to the determination of several samples, provided that the relative standard deviation of all responses averaged is not greater than 2.5%.

Determination of Hydrochlorothiazide and Hydralazine HCl

To prepare the mobile phase, mix 50 mL salt solution (*f*) with 1000 mL methanol. Connect the column to UV absorbance detector, and set flow rate at 0.6 mL/min. Allow the system to equilibrate for at least 15 min and then inject aliquot of reference solution. Hydrochlorothiazide elutes after about 1 min, and hydralazine HCl elutes after 2 min. The resolution between the 2 peaks is not less than 2.5; the tailing factor for hydrochlorothiazide is not more than 2.0 and that for hydralazine HCl is not more than 3.5. Inject replicate aliquots of reference solution. For both compounds, the relative standard deviation of the peak response in 4 consecutive chromatograms is not greater than 2.0%. Follow the order of injections described in *Determination of Reserpine*.

Determine the amount of hydrochlorothiazide in sample solution according to the following formula:

$$\text{Hydrochlorothiazide (mg)} = (\text{Ru2})(W_t)/(\text{Rs2})$$

where Ru2 is the average peak response of hydrochlorothiazide in 2 sample chromatograms, Rs2 is a similar average in 2 reference chromatograms, and W_t is the weight (mg) of hydrochlorothiazide in the reference solution.

Determine the amount of hydralazine HCl in sample solution according to the following formula:

$$\text{Hydralazine HCl (mg)} = (\text{Ru3})\,(W_z)/(\text{Rs3})$$

where W_z is the weight (mg) of hydralazine HCl in the reference solution and Ru3 and Rs3 are average peak responses of hydralazine HCl in sample and reference chromatograms, respectively.

Results and Discussion

In the LC analysis of multicomponent drug preparations, it is highly desirable to determine all the ingredients in a single system. For this particular product, however, previous information and results of preliminary investigations indicated that it is preferable to use 2 systems: one for determination of reserpine and the other for determination of hydrochlorothiazide and hydralazine HCl.

The procedure for the determination of reserpine is basically the same as the one published earlier for a similar product (4), but minor changes, based on experience and the composition of this mixture, were introduced. A normal-phase column was used, as before, but the length was 7.5 cm rather than 30 cm. The mobile phase, previously pure methanol, was modified to contain 2% aqueous 1-pentanesulfonic acid sodium salt at 4 parts/1000. Detection was done only by fluorescence because the intent at this stage was to determine only reserpine. As seen in Figure 1, chromatograms of reference and sample solutions have 2 peaks, approximately equal in size, due to hydrochlorothiazide and reserpine. Hydralazine HCl elutes extremely late as a wide band, beginning at 30–40 min after injection. Because it is practically nonfluorescent, it is not detected.

If reserpine is partially decomposed, degradation products elute earlier but may not be detected because they are generally small and also because they may overlap with hydrochlorothiazide.

The other 2 ingredients are chromatographed on the same column with a slightly different mobile phase (the percentage of salt solution is increased to about 5%); detection and quantitation is by UV absorbance at 260 nm. Chromatograms of reference and sample solutions have 2 peaks due to hydrochlorothiazide and hydralazine HCl (Figure 2). The quantity of reserpine is too small to produce a detectable peak at the level of detector sensitivity used.

Table 1 shows the results of the analysis of a commercial sample of tablets. Duplicate determinations were made on a ground composite of 20 tablets; 10 tablets were examined for content uniformity.

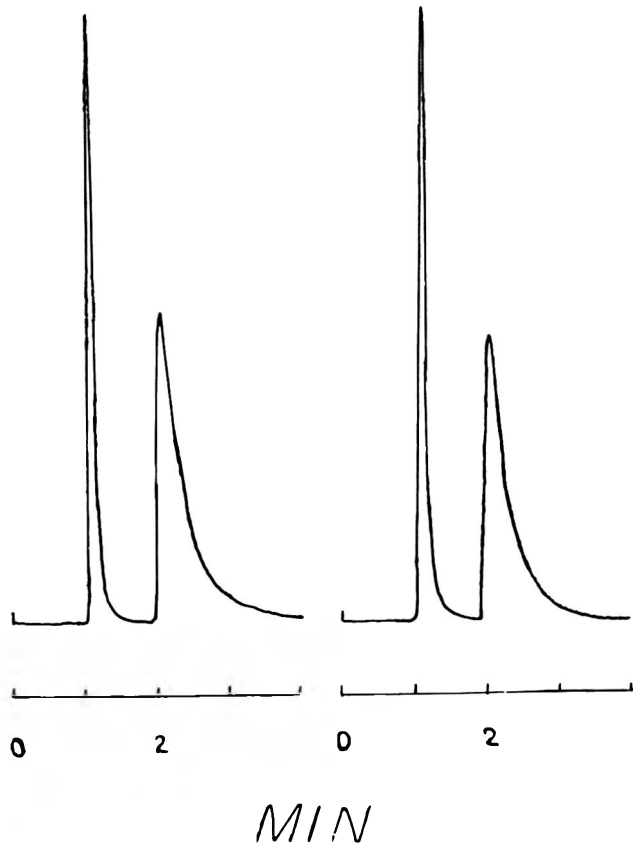


Figure 2. (From left to right) Chromatograms of reference solution and sample solution of hydrochlorothiazide and hydralazine HCl. The mobile phase was 2% aqueous 1-pentanesulfonic acid sodium salt–methanol (50 parts/1000). The flow rate was 0.6 mL/min, the volume of injection was 5 μL, and the UV absorbance detector was set at 260 nm.

References

(1) Wright, G.E., & Tang, T.Y. (1972) *J. Pharm. Sci.* **61**, 299–300

(2) *U.S. Pharmacopeia* (1990) 22nd Revision, U.S. Pharmacopeial Convention, Rockville, MD, pp. 1219–1221

(3) Honigberg, I.L., Stewart, J.T., Smith, A.P., & Hester, D.W. (1980) *J. Pharm. Sci.* **64**, 1201–1204

(4) Cieri, U.R. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 542–544

DRUGS, COSMETICS, FORENSIC SCIENCES

Rapid Ultraviolet Spectrophotometric Assay of Ibuprofen in Liquid Oral Formulations

ASIS K. SANYAL and DINABANDHU LAHA

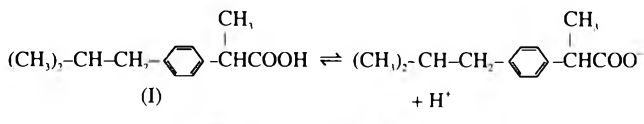
University College of Science, Department of Biochemistry, Microbiology Laboratory, 35 Ballygunge Circular Rd, Calcutta 700 019, India

A selective and sensitive method was developed for rapid estimation of ibuprofen in complex liquid oral formulations. The method is based on spectrophotometric (352 nm) measurement of iodine released by ibuprofen from an iodide–iodate mixture. Absorbance of liberated iodine was linearly related to ibuprofen at concentrations within the range of 10–40 μg/mL. Ibuprofen was made free from interfering excipients and additives through selective extraction with hexane before assay. The results of ibuprofen determination in a syrup and an oral suspension and of recovery experiments performed on various excipients confirmed the applicability of the proposed method to complex formulations.

Ibuprofen, an inhibitor of prostaglandin biosynthesis, has unique anti-inflammatory and analgesic properties. Its combination with paracetamol in particular, has had extensive therapeutic applications in recent years. Most of the methods available for estimation of this drug are based on liquid chromatography (LC).

Official methods (1, 2) for the assay of ibuprofen in pure form or in tablets are based either on acid–base titration or liquid chromatography. The titration method is not suitable for complex liquid formulations like syrups or suspensions. The liquid chromatographic analyses (2–5) described for pharmaceutical and biological samples require expensive instrumental setup not always available for routine analysis particularly in developing countries.

This paper describes a simple, sensitive, and selective spectrophotometric assay of ibuprofen applicable to complex pharmaceuticals. The method is based on the property of the drug (I), which is a propionic acid derivative, to liberate iodine from the iodide and iodate. Hydrogen ion-mediated iodine release from the iodide–iodate mixture is a common property of all acids, the extent and rate of iodine liberation being determined by the strength of the particular acid used.



$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$$

We observed that ibuprofen, by virtue of its free carboxyl group, can liberate iodine quantitatively from the iodide–iodate system.

The iodine liberation reaction by ibuprofen, however, must be performed in an isolated system completely free of any acidic, alkaline, or other compounds that may interfere with the reaction. So it is necessary to have a one-step extraction with a highly nonpolar solvent such as hexane, which can selectively isolate ibuprofen from a complex mixture in a sufficiently pure state necessary for subsequent assay. The applicability of the method for a syrup and an oral suspension, which are the most complex dosage forms of this drug, was demonstrated. There is no officially accepted assay method at present for these formulations.

Received June 19, 1992. Accepted by JW October 4, 1993.

Experimental

Apparatus and Chemicals

A Hitachi Model U-3210 spectrophotometer was used. Authentic samples of ibuprofen were obtained from Boots (United Kingdom). Brufen syrup (Boots Co., Ltd, India) and Ibugesic plus oral suspension (Cipla, Ltd, India) were purchased locally.

Reagents

(a) *Potassium iodate solution.*—2% w/v.
(b) *Potassium iodide solution.*—0.5% w/v.
(c) *Potassium iodide solution.*—0.07% w/v.
(d) *N,N-Dimethylformamide (DMF).*
(e) *Methanol*—MS grade (E. Merck, India, Ltd).

Preparation of Standard

Approximately 100 mg ibuprofen was weighed accurately and dissolved in 30 mL *N,N*-dimethylformamide in a 50 mL volumetric flask. The solution was diluted to volume with the same solvent.

Preparation of Samples (Syrup and Oral Suspension)

The contents of the bottle were shaken vigorously before adding a measured volume (containing ca 100 mg ibuprofen) to 30 mL *N,N*-dimethylformamide in a 50 mL volumetric flask. The contents were mixed well for 1 min to dissolve the drug. The mixture was diluted to volume with the same solvent, and the flask was stoppered and shaken well. The undissolved excipients were allowed to settle and the clear supernatant was used for the subsequent assay.

Determination

To 0.6 mL standard or test solution in a 25 mL glass-stoppered cylinder, 5.4 mL 0.05M HCL was added followed by 5 mL hexane. Ibuprofen was extracted into the organic phase by vigorous shaking of the mixture for 1 min. The mixture was allowed to stand until the layers were separated completely. A portion (2 mL) of the top hexane layer was transferred to a calibrated, glass-stoppered tube (25 mL). The highly volatile solvent was removed easily by evaporation with a gentle stream of nitrogen. The tube may be kept immersed in a thermostatically controlled water bath (50°C) to facilitate evaporation. The residue in the tube was dissolved in 1 mL methanol. Potassium iodate solution (0.5 mL) was then added, and the tube contents were mixed well. The iodine liberation reaction was initiated by the addition of 1 mL 0.5% potassium iodide solution. The tube was then stoppered, and the contents, after thorough mixing, were allowed to stand for 30 min at room temperature (20°–30°C). The reaction mixture was then diluted with 20 mL 0.07% potassium iodide solution, and the absorbance of the solution was measured at 352 nm against an appropriate blank prepared as described above, except that 0.6 mL methanol was taken in place of standard or test solution.

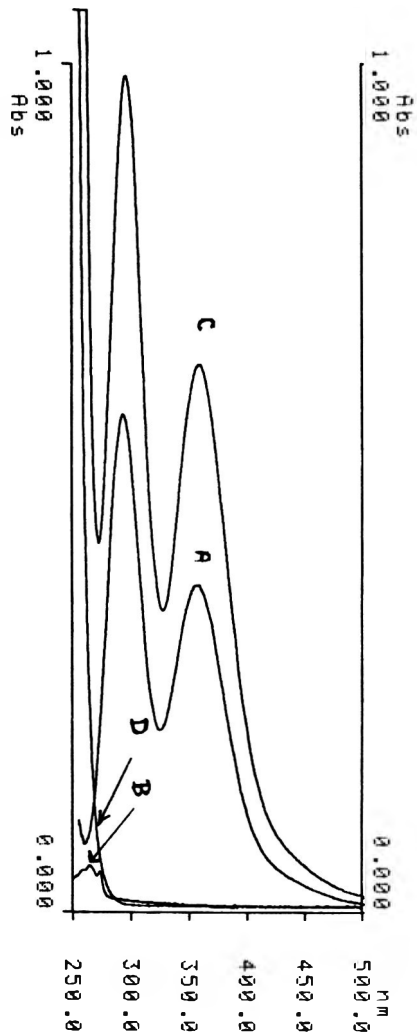


Figure 1. Absorption spectra of (A) iodine in potassium iodide solution (0.08%), (B) ibuprofen, (C) ibuprofen after reaction with iodate and iodide, and (D) reagent blank (iodate and iodide).

Kinetics of Iodine Liberation by Ibuprofen

At suitable time intervals following the addition of 0.5% potassium iodide solution (10 mL) to a mixture of 10 mL ibuprofen solution (prepared in methanol; 600 µg/mL) and 5 mL potassium iodate solution, 2.5 mL aliquots of the reaction mixture was diluted with 20 mL 0.07% potassium iodide solution. The solution was mixed well, and the absorbance at 352 nm was read immediately against a reagent blank prepared by diluting a mixture of 1 mL methanol, 0.5 mL potassium iodate solution, and 1 mL 0.5% potassium iodide solution with 20 mL 0.07% potassium iodide solution.

Calculations

The ibuprofen content of a syrup or an oral suspension was calculated by using the following equation:

$$\text{Ibuprofen (mg/5 mL)} = \frac{U}{S} \times C \times \frac{5}{V} \times D$$

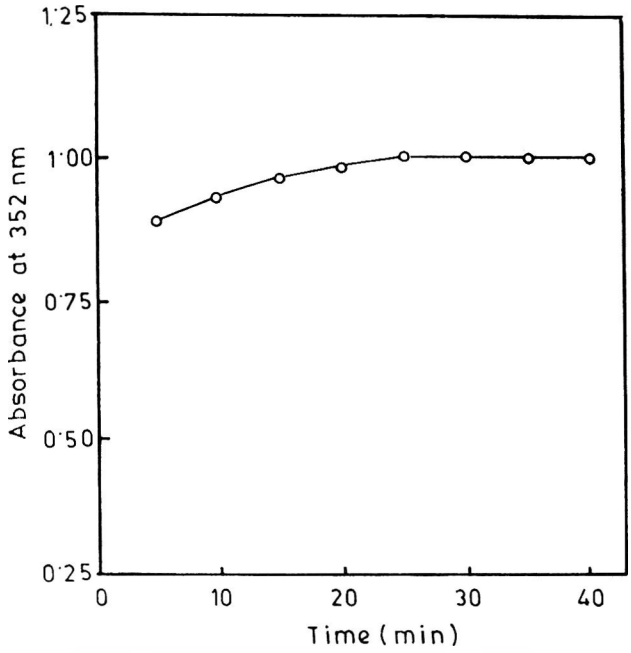


Figure 2. Kinetics of ibuprofen-induced liberation of iodine from iodide–iodate mixture.

where S and U are the absorbance values at 352 nm for standard and sample, respectively; C is the concentration of the standard preparation (mg/mL); V is the volume of sample taken for analysis; and D is the sample dilution.

Results and Discussion

Liberation of iodine by ibuprofen from iodide and iodate can be measured conveniently by ultraviolet spectrophotometry. Iodine shows 2 absorption maxima: at 288 and 352 nm (Figure 1). The maximum at 352 nm was selected for measurement of iodine absorbance because at this longer wavelength, chances of interference by other UV-absorbing substances are less. Moreover, ibuprofen, having negligible absorbance at 352 nm, does not interfere during the measurement of iodine absorbance (Figure 1).

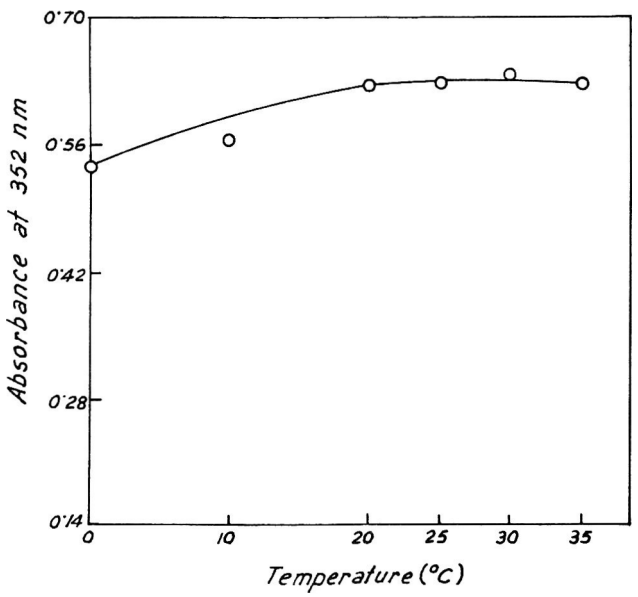


Figure 3. Effect of temperature on iodine liberation by ibuprofen. The reaction was carried out as described in the text at different temperatures.

Table 1. Recovery of ibuprofen from authentic samples[a]

Experiment	Amount added, mg/50 mL	Recovery, %
1	60	99.1 ± 1.3
2	80	99.5 ± 1.5
3	100	100.8 ± 1.1
4	120	99.2 ± 1.8

[a] Authentic samples are standard ibuprofen solutions containing the stated amounts of ibuprofen. Each recovery is the average of 3 determinations ± standard deviation.

The kinetics of iodine liberation by ibuprofen, followed by measuring the absorbance at 352 nm, is shown in Figure 2. The reaction reached steady state within 30 min, and at equilibrium, the absorbance of liberated iodine was linearly related to the concentration of ibuprofen within a range of 10–40 μg/mL (correlation coefficient [r] = 0.9999). This relationship forms the basis of the estimation of ibuprofen by measuring iodine release. Temperature change does not significantly affect iodine liberation by ibuprofen (Figure 3). Considerably high iodine liberation was observed even at 0°C. The reaction attained a steady state at 20°C.

Iodine liberation by ibuprofen can be used to assay the drug in complex samples like syrup and oral suspension. The main sources of interference in such systems are the organic or inorganic acids used as buffering agents. Interference from these substances can be avoided completely by a single extraction with a nonpolar solvent such as hexane. Because of its nonpolar nature, ibuprofen is very soluble in hexane and is easily extracted by this solvent, whereas the other organic acids (e.g., citric, tartaric, lactic, acetic, malic, and fumaric acids) used commonly as additives in liquid oral formulations are hydrophilic and are not soluble in a nonpolar solvent like hexane. For the successful performance of the method, the glass-stoppered

Table 2. Determination of ibuprofen in a syrup and an oral suspension[a]

Sample	Concentration found, mg/5 mL	Percent of label claim
Brufen syrup[b]	99.8 ± 1.4	99.8 ± 1.4
Ibugesic plus oral suspension[c]	100.3 ± 1.6	100.3 ± 1.6

[a] Each result is the mean of 5 independent determinations ± standard deviation.
[b] Label claim, each 5 mL contains 100 mg ibuprofen.
[c] Label claim, each 5 mL contains 100 mg ibuprofen and 162.5 mg paracetamol.

Table 3. Recovery of ibuprofen from commercial samples and some pharmaceutical additives

Commercial sample or additive	Amount added, mg/50 mL of DMF[a]	Recovery, %[b]
Brufen syrup	5[c]	99.5 ± 1.5
Ibugesic plus oral suspension	5[c]	99.7 ± 1.8
Paracetamol	100	100.8 ± 1.2
Citric acid	100	99.2 ± 0.8
Sodium acetate	100	99.6 ± 1.1
Tartaric acid	100	99.2 ± 1.4
Calcium lactate	100	99.9 ± 1.3
Malic acid	100	100.5 ± 1.2
Fumaric acid	100	99.1 ± 1.4
Methyl paraben	20	100.5 ± 1.2
Propyl paraben	20	99.4 ± 0.9
Benzoic acid	8	100.6 ± 0.8
Bronopol	20	99.4 ± 1.5
Povidone	100	100.1 ± 1.3
Vanillin	6	99.8 ± 1.1
Menthol	6	100.2 ± 1.2
Peppermint oil	0.04[c]	100.5 ± 1.3
Saccharin sodium	12	99.8 ± 1.0
Sodium alginate	100	99.9 ± 0.9
Bentonite	100	99.7 ± 1.2
Pineapple	6	99.1 ± 1.2
Tartrazine	2	100.1 ± 1.0
Sunset Yellow	2	99.3 ± 0.9
Carmoisine	2	99.6 ± 1.3

[a] The mixture was processed as described under *Syrup and Oral Suspension* prior to analysis. The commercial samples or additives each contained 100 mg ibuprofen originally.
[b] Each result in the mean of 5 independent determinations ± standard deviation.
[c] Volume added, mL.

tubes used for the iodine liberation reaction should be totally free from any acid, alkali, or grease. It is also important that all the reaction tubes should be allowed to stand undisturbed in a vertical position during the iodine liberation reaction.

The recoveries obtained from authentic samples demonstrate the good accuracy and precision of the proposed method (Table 1). Results of the determination of the ibuprofen content of a syrup and an oral suspension by the proposed method agree well with the declared amount of the drug (Table 2). Quantitation of ibuprofen added to syrup and an oral suspension of known ibuprofen content indicated satisfactory recovery of the drug from these complex mixtures (Table 3). Ibuprofen also was estimated from various pharmaceutic excipients and additives (Table 3). Because the concentrations of various excipients and additives may differ in different commercial formulations, their amount in the binary mixture was selected on the basis of the type of material taken. The results show satisfactory recoveries of the drug from different excipients, indicating lack of interference from these substances. The assay also is not affected by the presence of paracetamol; thus, it can be used to analyze ibuprofen in ibuprofen–paracetamol combinations.

Acknowledgments

We thank A. B. Banerjee, Department of Biochemistry, Calcutta University, for his encouragement during the course of this work.

References

(1) *British Pharmacopeia* (1988) Her Majesty's Stationery Office, London, UK, pp. 307, 956

(2) *United States Pharmacopeia* (1990) 22nd Revision, U.S. Pharmacopeial Convention, Rockville, MD, pp. 682, 683

(3) Asmus, P.A. (1985) *J. Chromatogr.* **331**, 169–179

(4) Albert, K.S., Raabe, A., Garry, M., Antal, E.J., & Gillespie, W.R. (1984) *J. Pharm. Sci.* **73**, 1487–1489

(5) Chai, B., Minkler, P.E., & Hoppel, C.L. (1988) *J. Chromatogr.* **430**, 93–101

Liquid Chromatographic Determination of Multiple Sulfonamide Residues in Bovine Milk: Collaborative Study

MICHAEL D. SMEDLEY
U.S. Food and Drug Administration, Center for Veterinary Medicine, Division of Residue Chemistry, Beltsville, MD 20705

Collaborators: W. Bargo; G. Carignan; J. Jenkins; M. Li; E. Piotrowski; B. Schmid; R. Thompson; C. Thorpe

A collaborative study involving 8 laboratories was conducted on the determination of 8 sulfonamide residues in raw bovine milk using a liquid chromatographic (LC) method. The sulfonamides are extracted with chloroform–acetone, the organic phase is evaporated, the residues are dissolved in an aqueous potassium phosphate solution, and the fatty residues are removed by washing with hexane. The aqueous layer is collected, filtered, and injected onto an LC system, and the analyte is detected by ultraviolet (UV) absorption at 265 nm. To quantitate all 8 sulfonamides isocratically, 2 chromatographic conditions are required: 12% methanol in the mobile phase for 5 sulfonamides, and 30% methanol in the mobile phase for 4 sulfonamides. Sulfamethazine (SMZ), the most widely used sulfonamide, is detected by both systems. Collaborators were instructed to analyze 3 replicates each of control milk and control milk fortified at 3 levels. They were also provided with 20 blind incurred samples (10 samples in duplicate) to analyze. For 10 ppb fortified milk, the average interlaboratory recovery for the 8 sulfonamides ranged from 56.2% for sulfaquinoxaline (SQX) to 82.7% for SMZ in the 12% methanol mobile phase (SMZ12). Also at this level, S_r ranged from 3.2 for SQX to 8.9 for SMZ12, and S_R ranged from 6.9 for sulfadimethoxine to 17.2 for SMZ in the 30% methanol system (SMZ30). At 10 ppb, RSD_r and RSD_R ranged from 5.7% for SQX to 10.8% for SMZ12, and 10.1% for sulfamerazine to 20.9% for SMZ30, respectively. These results demonstrate that the method is suitable for the determination of the 8 sulfonamide residues in milk at 10 ppb. However, the identification of positives by this procedure needs additional confirmation by procedures comparable to the specificity achievable by liquid or gas chromatography combined with mass spectrometry.

Submitted for publication June 8, 1993.
The recommendation was approved by the Committee on Drugs and Related Topics, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Actions" (1993) *The Referee* **17**, September issue.

A liquid chromatographic (LC) multiresidue determinative procedure for 10 sulfonamides in raw milk was developed in the author's laboratory (1). The initial sulfonamides were selected on the basis of informal reports of their use in dairy animals. The present procedure was used in a limited nationwide survey of shelf milk for all 10 residues in 1990 (Smedley et al., unpublished milk survey, U.S. Food and Drug Administration, Washington, DC). The results of that 14-city survey showed the need for a validated method for the determination of sulfonamide residues in milk. Due to questions concerning the quantification of 2 of the drugs, 8 were selected for this study. Therefore, a collaborative study of the procedure was conducted in 4 U.S. Food and Drug Administration laboratories, 2 state laboratories, one U.S. Department of Agriculture laboratory, and a laboratory of Health and Welfare of Canada. This report presents the results of a collaborative study wherein the recovery and precision of the procedure were determined.

Collaborative Study

Prior to the study, each participating laboratory was asked to review the procedure, which was essentially the same as that of Smedley and Weber (1). After the reviews, a few minor clarifications were made to the method and appropriate safety precautions were included. Due to questions concerning the resolution and quantitation of sulfanilamide and sulfamethizole, the method was revised to analyze for 8 sulfonamides instead of the original 10.

The study consisted of 3 phases. Phase I was used to evaluate the participating laboratories' equipment, capabilities, and technical proficiency. It was intended to demonstrate the amount of work a multiresidue method involved and the expertise required by the analyst to interpret the data. If a laboratory determined that it did not have the expertise, adequate instrumentation, or the required time, the laboratory could withdraw at this time before the start of the study. Once a laboratory com-

pleted phase I, it was invited to participate in the evaluation of the method during phases II and III.

Each laboratory was sent ca 1 g of each of 8 sulfonamides, 3 tubes of frozen control milk (50 mL each), and one blind fortified sample. All samples during the study were shipped frozen on dry ice, and the participants were instructed to keep them frozen until analysis.

Phase I involved the construction of standard curves with coefficients of correlation of at least 0.99, and the duplicate analysis of the following: control milk, control milk fortified at 10 ppb of each sulfonamide, and an unknown fortified sample. Participants were told that the unknown sample may contain more than one sulfonamide, necessitating its analysis under both chromatographic conditions. As each laboratory completed phase I, it was instructed to return the results to the sponsor; then the laboratory was sent additional samples and was asked to proceed to phases II and III of the evaluation.

Each participating laboratory was shipped 5 tubes of control milk (50 mL each) for use in phase II and 20 blind samples for use in phase III. During phase II, the laboratories were instructed to analyze the following in triplicate: control milk and fortified control milk, which each laboratory individually fortified at 5, 10, and 20 ppb. The 3 replicates were to be analyzed on 3 different days to provide data of maximum ruggedness. Once the participating laboratory successfully analyzed the 12 samples of phase II, it proceeded to phase III.

During phase III, the participating laboratories analyzed the 20 blind samples. The samples were coded as 20 individually labeled blind tubes of milk, but were actually duplicates of 10 different samples. The 10 blind duplicate samples included a control milk, 6 incurred samples containing one sulfonamide per sample, and 3 incurred samples with 2 sulfonamides each. The incurred residues were in the range of the standard curve, 5–20 ppb.

Participants were instructed to prepare standard curves and analyze one positive and one negative sample daily. A positive control was identified as a control milk sample that the individual laboratories fortified at the 10 ppb level with each sulfonamide. A negative control was identified as a control milk sample. Results from analyzing the positive and negative controls were considered the quality control for the method and instrumentation.

The raw milk used in this study was collected at the author's facility by Herbert F. Righter. Initially 4 cows, with known histories, were obtained from the U.S. Department of Agriculture dairy farm in Beltsville, MD. Raw control milk was collected and analyzed by the author. The milk from these 4 cows was mixed together and used as the control milk for this study. The milk was subdivided into 50 mL tubes and frozen at −80°C until it was shipped. Each cow was dosed with a different sulfonamide drug. Milk at the level of interest was subdivided and frozen. Milk containing multiple sulfonamides was produced by blending the fresh incurred milk from 2 cows dosed with single drugs. This process was repeated with 4 different cows with a similarly documented history, to produce the incurred milk for the remaining 4 sulfonamides.

993.32 Multiple Sulfonamide Residues in Raw Bovine Milk—Liquid Chromatographic Method

First Action 1993

(Applicable to determination of 5–20 ppb of 8 sulfonamides in raw bovine milk: sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfachloropyridazine, sulfadimethoxine, and sulfaquinoxaline.) (*Caution*: *See Appendix: Laboratory Safety* for safe handling of chloroform, acetone, and hexane. Avoid inhalation or contact with eyes or skin. Use only in well-ventilated fume hood. Chloroform is possible carcinogen.)

Method Performance:

See Table **993.32** for method performance data.

Note: In the collaborative study, post-dose milk contained peaks in addition to parent drug, and these are assumed to be metabolites. For confirmation of eluate identity, additional specificity comparable to that provided by liquid or gas chromatography combined with mass spectrometry is required.

A. Principle

Sulfonamides in milk samples are extracted by chloroform–acetone. After solvent evaporation, residues are dissolved in aqueous potassium phosphate solution. Fatty residues are extracted with hexane. The aqueous layer is filtered and analyzed by liquid chromatography (LC). Sulfonamides are detected by ultraviolet (UV) absorption at 265 mm. Eight sulfonamides can be quantitated isocratically: 5 sulfonamides using 12% methanol and 4 using 30% methanol in mobile phase. Sulfamethazine is detected under either condition.

B. Apparatus

(**a**) *LC system.*—Equipped with autoinjector, fixed or variable UV detector, 250 × 4.6 mm reversed-phase C_{18} column with deactivated phases (octadecyldimethylsilyl, 100 Å pore id), and 5 μm particle size; 2 cm guard column of similar packing; and 0.5 μm precolumn filter.

Operating conditions: Oven temperature (C_{18} and guard column), 35 ± 0.2°; flow rate, 1.5 mL/min; standards and sample injection size, 100 μL; run time, 40 min; chart speed, 0.5 cm/min; detector set at 265 nm. (*Note*: If room temperature is not constant, prewarm mobile phase by passing preinjector LC tubing through column heater to attain more reproducible retention times.)

Clean LC system and column by substituting flush solution for mobile phase. Flush column ≥15 min at 1.5 mL/min, or at reduced flow rate overnight. If system will be unused for over 1 week, flush column with methanol–H_2O (60 + 40). Overnight equilibration may be necessary for reproducible sample analysis with 12% methanol system.

(**b**) *Rotary evaporator.*

(**c**) *Polypropylene plastic tubes.*—50 mL volume, plug-sealed.

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

Table 993.32. Method performance for determination of multiple sulfonamides in raw bovine milk by liquid chromatographic method

Sulfonamides[a]	Mean recovery, %	s_r	s_R	RSD_r, %	RSD_R, %	Sulfonamides[a]	Mean recovery, %	s_r	s_R	RSD_r, %	RSD_R, %
	5 ppb Fortified						20 ppb Fortified				
SDZ	74.09	6.00	11.55	8.09	15.58	SDZ	75.01	5.14	6.91	6.85	9.21
STZ	54.64	8.30	14.15	15.20	25.90	STZ	56.14	6.23	9.70	11.10	17.28
SPD	80.46	7.48	12.66	9.29	15.74	SPD	76.23	5.47	7.93	7.18	10.41
SMR	87.06	10.19	12.63	11.70	14.50	SMR	82.44	5.77	8.17	7.00	9.91
SMZ-12	86.82	9.31	9.89	10.72	11.39	SMZ-12	80.73	3.12	5.48	3.87	6.79
SMZ-30	85.38	9.26	27.13	10.85	31.78	SMZ-30	82.87	5.78	11.60	6.98	14.00
SCP	79.08	11.35	12.18	14.35	15.40	SCP	67.88	3.82	5.13	5.56	7.46
SDM	68.03	12.08	13.13	17.76	19.30	SDM	67.05	3.18	5.57	4.74	8.31
SQX	59.11	9.14	14.03	15.46	23.74	SQX	52.55	2.82	6.60	5.37	12.56
	10 ppb Fortified						Incurred Residues, ppb (Blind Samples)				
SDZ	75.36	5.37	7.81	7.13	10.36	SDZ	11.32	0.50	1.25	4.46	11.08
STZ	57.19	4.99	11.56	8.73	20.22	STZ	13.95	0.48	2.50	3.46	17.90
SPD	76.73	5.82	9.54	7.58	12.43	SPD	14.60	0.66	1.53	4.54	10.44
SMR	82.46	6.69	8.36	8.11	10.14	SMR	14.27	0.71	1.14	4.95	8.02
SMZ-12	82.71	8.91	8.91	10.77	10.77	SMZ-12	10.16	1.86	2.25	18.32	22.11
SMZ-30	82.36	6.78	17.20	8.23	20.89	SMZ-30	15.11	1.19	2.57	7.89	16.99
SCP	71.22	6.11	7.81	8.58	10.97	SCP	12.83	2.62	2.72	20.45	21.23
SDM	66.54	4.23	6.85	6.35	10.29	SDM	13.60	1.08	1.82	7.93	13.38
SQX	56.15	3.20	8.94	5.71	15.92	SQX	7.41	1.73	1.84	23.30	24.80

[a] Sulfonamides: SDZ, sulfadiazine; STZ, sulfathiazole; SPD, sulfapyridine; SMR, sulfamerazine; SMZ-12, sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ-30, sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP, sulfachloropyridazine; SDM, sulfadimethoxine; SQX, sulfaquinoxaline.

(e) *Fluted filter paper.*—12.5 cm id, pleated, grade 588, smooth surface, retains coarse precipitates, thickness 0.215 mm, fast speed.

(f) *Nylon filters (N66).*—*(1)* 0.45 μm porosity, 47 mm. *(2)* 2 μm pipet-tip filters.

(g) *Vortex mixer.*

(h) *Freezer.*—Capable of maintaining −80 ± 2° (*See* **E** for use in sample storage).

(i) *Micro weighing funnel.*—To fit 100 mL volumetric flask.

(j) *Volumetric pipets.*—1, 2, 5, and 10 mL; Class A.

(k) *Separatory funnel.*—125 mL, with ground glass stopper and Teflon stopcock.

(l) *Funnel.*—Short stem, 75 mm id.

(m) *Pear-shaped flask.*—100 mL, with 24/40 standard taper neck and stoppers.

(n) *Volumetric flasks.*—50 mL, 100 mL, and 2 L; Class A.

C. Reagents

LC solutions may be stored at room temperature (24 ± 3°) for ≤3 months, unless otherwise noted.

(a) *Water.*—Distilled and deionized, ≤17 megohm-cm.

(b) *Potassium dihydrogen phosphate (PDP) solution.*—0.1M. Dissolve 27.2 g potassium dihydrogen phosphate in H_2O in 2 L volumetric flask, dilute to volume with H_2O, mix, and filter through 0.45 μm nylon filter.

(c) *Solvents.*—Methanol, hexane, acetone (EM Science, Gibbstown, NJ, is suitable source), and chloroform (without ethanol additive) (Burdick & Jackson, Muskegon, MI, is suitable source); all LC grade.

(d) *Mobile phases.—(1) 12% methanol–PDP solution (12 + 88, v/v). (2) 30% methanol–PDP solution (30 + 70, v/v).* Filter methanol through 0.45 μm nylon filter before mixing with 0.1M PDP solution. If LC is equipped with 2 or more reservoirs, pump corresponding percentages of methanol and PDP solution from separate reservoirs for the 2 conditions; otherwise premix mobile phases.

(e) *Flush solutions.—(1) 12% methanol–H_2O (12 + 88, v/v). (2) 30% methanol–H_2O (30 + 70, v/v).* Filter methanol through 0.45 μm nylon filter before mixing with H_2O.

(f) *Extraction solution.*—Chloroform–acetone (2 + 1, v/v). Prepare daily ca 100 mL/sample. Measure chloroform and acetone in separate graduated cylinders, add acetone to chloroform, and mix thoroughly. Let mixture equilibrate (ca 10–15 min) to room temperature before using.

(g) *Sulfonamide standards.—(1) Sulfapyridine (SPD). (2) Sulfathiazole (STZ). (3) Sulfamerazine (SMR). (4) Sulfachloropyridazine (SCP). (5) Sulfamethazine (SMZ). (6) Sulfaqui-*

noxaline (SQX). (7) Sulfadimethoxine (SDM). (8) Sulfadiazine (SDZ). Use Analytical Reference Grade free base for standards preparation (Pfaltz & Bauer, Waterbury, CT, is suitable source for SQX; Sigma Chemical Co., St. Louis, MO, for others).

D. Preparation of Standard Solutions

Store stock solutions at <10° for no longer than 6 months. Use deionized, distilled, and 0.45 μm nylon-filtered H_2O for sulfonamide standard preparation. To minimize cross-contamination, rinse glassware before use first with 1N HCl, then distilled deionized water, and then methanol; remove stopcocks from separatory funnels to ensure thorough rinsing. Use Class A volumetric glassware.

(**a**) *Stock solutions.*—Accurately weigh ca 100 mg, to nearest 0.1 mg, STZ, SMR, SCP, SMZ, SDM, SDZ, and SPD standards and 10 mg SQX standard at room temperature into separate glass weighing boats, transfer to separate 100 mL volumetric flasks. Dissolve in methanol, sonicating if necessary; then allow these solutions to reequilibrate to room temperature, dilute to volume with methanol, and mix thoroughly. SQX stock solution is 100 μg/mL (due to lower solubility in methanol); other stock solutions are 1 mg/mL.

(**b**) *Intermediate solutions (10 μg/mL).*—Using separate pipets, transfer 1.0 mL of each stock solution, except 10.0 mL for SQX, into separate 100 mL volumetric flasks, dilute to volume with H_2O, and mix thoroughly.

(**c**) *Fortification solution (1 μg/mL of each sulfonamide).*—Using separate 10 mL pipets, transfer 10.0 mL of each intermediate standard solution into single 100 mL volumetric flask, dilute to volume with H_2O, and mix thoroughly.

(**d**) *Sulfonamide standards.—(1) 200 ng/mL (20 ppb).*—Dilute 10.0 mL fortification solution to 50 mL with H_2O in 50 mL volumetric flask and mix thoroughly. *(2) 100 ng/mL (10 ppb).*—Dilute 10.0 mL fortification solution to 100 mL with H_2O in 100 mL volumetric flask and mix thoroughly. (*3*) 50 ng/mL (5 ppb).—Dilute 5.0 mL fortification solution to 100 mL with H_2O in 100 mL volumetric flask and mix thoroughly.

E. Sample Storage

Store fresh raw bulk tank milk at <10°. If milk will not be analyzed within 2–3 days, subdivide into polypropylene plastic tubes and store at –80°. If this is not possible, store samples frozen at the lowest temperature possible. Thaw frozen milk in slightly warm tap water on day of analysis and mix by shaking tube before sampling.

Stored at –80°, milk and sulfonamide residues are stable for ca 12 months; stored at –15°, milk is stable only 3–4 months.

F. Sample Extraction

Place fluted filter paper, **B(e)**, in funnel, **B(l)**, and wash filter with 5 mL extraction solution, **C(f)**; discard eluate. Pipet 10 mL milk sample into separatory funnel, **B(k)**.

(When testing for recovery is desired, fortify samples by adding 50, 100, or 200 μL fortification solution **D(c)** to 10 mL milk in separatory funnel [5, 10, or 20 ppb fortified samples].)

Add 50 mL extraction solution to milk in separatory funnel and stopper. Shake milk and extraction solution vigorously 1 min; carefully vent through stopper. (*Note:* Venting separatory funnel through stopper is critical. Do not vent through stopcock. Milk solids clogging stopcock may result in sample loss.)

Shake for 1 additional min, vent, and let phases separate 1 min. Repeat shaking sequence and let phases separate 5 min. Draw off extraction solution, filtering through washed fluted filter and collecting in pear-shaped flask, **B(m)**. (*Note*: Take extreme care to prevent any milk from entering stopcock.)

Add 25 mL extraction solution to separatory funnel and repeat exactly as before. After filtering second extraction into same pear-shaped flask, rinse filter 2× with 5 mL extraction solution, collecting washings in same pear-shaped flask.

G. Sample Preparation

Carefully evaporate extract solution just to dryness on rotary evaporator at 32 ± 2°. During first few minutes, closely regulate vacuum to prevent foaming.

Dissolve residue in 1 mL 0.1M PDP solution, **C(b)**, by agitating vigorously 1 min on vortex mixer.

Immediately add 5 mL hexane and vortex 1 min. Let phases separate 2 min, then immediately vortex 1 more min. Let phases separate ≥15 min before removing aqueous (bottom) layer.

Using 1 mL pipettor, draw off aqueous layer and immediately place nylon pipet-tip filter on pipet tip. (*Note:* Aqueous layer will be homogeneous; therefore it is not necessary to remove 100% of layer; 50–75% of aqueous layer is adequate for 2 injections.) Filter aqueous layer into glass tube or autoinjector vial (or subdivide into 2 vials if 2 LC systems are being used).

Prepared samples, in sealed injection vials, may be stored in autoinjector at room temperature overnight while being injected. Sealed injection vials may be stored at <10° up to 24 h before injection.

H. Chromatographic Systems

Two mobile phase systems are used to resolve 8 sulfonamides: 12% methanol–88% 0.1M PDP solution mobile phase ("12% system") for SDZ, STZ, SPD, SMR, and SMZ; and 30% methanol–70% 0.1M PDP solution mobile phase ("30% system") for SMZ, SCP, SDM, and SQX (*Note*: SMZ is detected in both systems). Set up 2 separate LC systems for the 2 mobile phases, or if only one LC is available, analyze samples using 12% system first, then equilibrate 1 h with 30% system and analyze remaining samples. Reequilibrate to 12% system for several hours or overnight at reduced flow rate. To optimize resolution for different LC columns and pumps, vary methanol concentration in mobile phase between 10 and 30%. (*Note:* Use of 12 and 30% systems minimizes background and provides adequate resolution for all 8 sulfonamides, but does not necessarily reflect best resolution for individual sulfonamides.)

Table 1. Interferences (ppb) in control milk, collaborative study results for determination of sulfonamide residues

		Sulfonamides[a]								
Lab.	Rep.[b]	SDZ	STZ	SPD	SMR	SMZ12	SMZ30	SCP	SDM	SQX
A	1	1.06	ND[c]	2.01	1.33	ND	ND	ND	ND	ND
	2	ND	ND	1.65	2.20	ND	ND	ND	ND	ND
	3	ND	ND	1.31	0.98	ND	ND	ND	ND	ND
B	1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	1	ND	1.00	0.50	ND	ND	0.71	0.27	ND	ND
	2	ND	1.74	ND	ND	ND	ND	1.70	ND	ND
	3	ND	1.75	1.94	ND	ND	1.45	0.08	ND	ND
D	1	ND	ND	ND	ND	ND	2.30	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	0.41[d]	ND	ND	ND	ND	ND	ND	ND	ND
E	1	ND	ND	ND	ND	ND	2.70	1.80	ND	ND
	2	ND	ND	ND	ND	ND	2.20	2.30	ND	ND
	3	ND	ND	ND	ND	ND	2.20	1.80	ND	ND
F	1	ND	0.50	0.90	0.50	ND	1.00	1.20	ND	1.40
	2	ND	0.40	0.40	0.40	ND	0.60	1.00	ND	1.40
	3	ND	0.40	0.40	0.30	ND	0.50	1.30	ND	1.30
G	1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	2.60	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND
H	1	ND	ND	ND	0.17	ND	ND	1.02	ND	ND
	2	ND	ND	ND	ND	ND	ND	1.55	ND	0.62
	3	ND	ND	ND	ND	ND	0.23	ND	ND	ND
Av.		0.06	0.24	0.38	0.35	0.00	0.58	0.58	0.00	0.20

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Rep. = replicate.

[c] ND = none detected.

[d] Value was recalculated using consistent baseline technique. Note that Laboratory D's integrator did not draw baselines.

I. Standard Curve

Check system suitability before sample determination by injecting 100 µL of 5, 10, and 20 ppb sulfonamide standards in duplicate under both LC conditions. Prepare 8 standard curves using peak heights. Correlation coefficients should be ≥0.99.

J. Sample Determination

Inject, in duplicate, 100 µL of redissolved sample extract, **G**, into LC system.

K. Calculations

Calculate linear regression coefficients using least-squares regression for each sulfonamide of interest (concentration vs peak height) to obtain values for:

$$Y = mX + b$$

then solve for concentration of unknown, X, from equation of the line where Y is peak height at corresponding sulfonamide retention time of the samples.

(*Note*: Extracted dried residues from 10 mL milk samples are redissolved in final volume of 1 mL; therefore, an analysis of 100 ng/mL in final extract is equivalent to 10 ppb of that analyte in milk sample.)

L. Notes

(**a**) This method was developed using raw milk. In limited use of the method with retail fluid milk, one interference was observed and was identified by mass spectrometry as theobromine. Sulfadiazine and theobromine coelute under the specified LC conditions; however, they may be fully resolved by using 6% methanol mobile phase and 30° column temperature. Their UV spectra are not sufficiently different to be distinguished using wavelength ratio technique.

(**b**) In the collaborative study, post-dose milk contained peaks in addition to parent drug, and these are assumed to be metabolites. For confirmation of eluate identity, additional specificity comparable to that provided by liquid or gas chromatography combined with mass spectrometry is required. For added confidence in identity, use wavelength ratio comparisons (250/265 nm).

Table 2. Collaborative study results (%) for determination of multiple sulfonamide residues in 5 ppb fortified milk

		Sulfonamides[a]								
Lab.	Rep.[b]	SDZ	STZ	SPD	SMR	SMZ12	SMZ30	SCP	SDM	SQX
A	1	87.00	57.40	100.80	114.20	78.60	64.00	74.40	69.20	50.80
	2	85.40	62.80	90.50	119.50	94.20	65.40	70.60	81.20	56.10
	3	76.00	39.20	88.60	75.80	70.80	90.20	106.80	87.80	59.00
B	1	83.10	74.30	91.40	98.00	89.60	92.70	—[c]	63.30	56.00
	2	82.50	70.70	106.90	101.20	103.60	96.30	—[c]	53.20	61.40
	3	85.30	74.40	98.90	104.00	101.80	103.60	—[c]	85.30	54.00
C	1	58.94	57.43	72.29	77.61	79.25	69.07	84.45	74.38	73.87
	2	59.68	81.86	86.37	76.66	98.13	63.66	68.50	68.63	57.36
	3	50.19	73.56	70.41	85.28	88.07	57.72	100.70	61.24	53.68
D	1	63.00	42.00	71.00	87.00	75.00	—[d]	60.00[e]	64.00	61.00
	2	77.00	48.00	77.00	79.00	91.00	—[d]	70.00[e]	64.00	55.00
	3	83.00[e]	48.00	75.00	74.00	91.00	—[d]	66.00[e]	66.00	55.00
E	1	70.60	42.40	74.80	81.10	85.30	126.20	77.60	73.50	65.60
	2	78.50	52.10	85.00	85.50	95.40	137.80	76.90	79.80	69.00
	3	67.00	40.40	72.10	76.60	92.90	139.70	75.70	90.60	71.60
F	1	85.80	59.00	81.80	89.80	80.60	100.40	89.00	71.20	76.40
	2	82.40	57.20	84.40	84.40	85.20	90.80	88.80	68.00	63.00
	3	91.60	64.60	85.60	82.40	81.00	91.60	88.20	62.00	85.20
G	1	63.50	38.20	57.80	91.90	73.50	44.00	61.60	36.30	22.00
	2	57.90	63.70	77.20	85.90	84.50	66.00	68.80	63.30	36.40
	3	68.00	47.20	60.20	68.70	77.80	66.00	90.00	66.90	45.50
H	1	68.30	36.30	70.50	86.30	85.70	71.50	79.70	79.00	55.60
	2	73.40	39.20	67.00	80.70	74.70	70.40	82.50	39.90	81.20
	3	80.10	41.40	85.50	83.90	106.10	86.00	80.50	63.90	53.90
Av. recovery		74.09	54.64	80.46	87.06	86.82	85.38	79.08	68.03	59.11
Int. CV[f]		15.58	25.90	15.74	14.50	11.39	31.78	15.40	19.30	23.74

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Rep. = replicate.

[c] Laboratory B did not get adequate resolution for SCP. Data have been omitted because in many cases recovery data were calculated from either a peak at the wrong retention time or from a large coeluting interference peak.

[d] Results from Laboratory D could not be recalculated from manual measurements because of off-scale peaks.

[e] Values were recalculated using manual peak height measurements.

[f] Int. CV = interlaboratory coefficient of variation.

(c) Some metabolites can be used as aids in identification of parent compounds, i.e., in 12% system, incurred SMZ has one metabolite peak ca 30 s after STZ peak. Incurred SPD and STZ each have one metabolite peak ca 2 and 4 min, respectively, after SMZ peak. SQX presence in 30% system can be verified by 3 metabolite peaks: 2 peaks eluting within 6 min after SQX and one peak eluting a few minutes after SCP peak. These metabolites do not interfere with analysis of any sulfonamides detected in 30% system.

(d) Incurred SCP and SQX are both quantified using 30% system. If an extract containing SCP is analyzed for other sulfonamides using 12% system, one very broad metabolite peak elutes at ca 65 min (25 min after the 40 min run time). When SCP is detected in 30% system, follow that sample by a flush injection in 12% system, or a 30 min wait before next injection, or equilibrate and reinject the following day. Incurred SCP and incurred SQX both have large metabolite peaks that coelute with SDZ on some LC systems. In this case, incurred SDZ cannot be quantified in samples that also contain incurred SCP or SQX. It is important to evaluate chromatograms from 30% system for presence of SCP or SQX before quantifying a peak at SDZ retention time using 12% system.

(e) SDM also gives one very small metabolite peak near SDZ retention time, only equivalent to 1–2 ppb.

(f) Standard addition may be used to distinguish parent sulfonamide peaks from possible metabolite peaks.

Ref.: *J. AOAC Int.* **77**, 1112 (1994).

Results and Discussion

Initially 13 laboratories agreed to participate in the collaborative study. All were sent the method for review with milk samples and standards to complete phase I. After 5 months, 2 laboratories had not yet started phase I, and one participant

Table 3. Collaborative study results (%) for determination of multiple sulfonamide residues in 10 ppb fortified milk

		Sulfonamides[a]								
Lab.	Rep.[b]	SDZ	STZ	SPD	SMR	SMZ12	SMZ30	SCP	SDM	SQX
A	1	84.00	61.10	91.20	100.80	88.70	72.50	78.70	68.50	54.70
	2	81.50	64.90	83.40	98.00	94.60	71.30	70.10	72.80	57.70
	3	78.10	50.30	73.50	78.90	65.00	86.50	88.10	70.00	55.30
B	1	80.80	70.10	89.20	91.90	92.40	88.60	—[c]	63.70	55.30
	2	80.80	70.00	88.30	93.80	84.00	91.30	—[c]	63.30	61.00
	3	76.40	66.10	83.20	86.50	87.00	87.80	—[c]	66.70	56.40
C	1	68.59	68.01	72.96	78.42	72.08	69.74	62.56	65.73	64.91
	2	62.22	73.76	81.77	72.38	87.37	71.67	80.13	74.41	65.62
	3	70.04	75.05	73.68	78.50	76.47	57.78	79.25	64.71	61.77
D	1	65.00	48.00	72.00	79.00	77.00	—[d]	55.00[e]	65.00	58.00
	2	76.00	52.00	77.00	78.00	88.00	—[d]	69.00[e]	69.00	55.00
	3	88.00	51.00	72.00	78.00	89.00	—[d]	66.00[e]	72.00	60.00
E	1	71.50	41.00	77.00	79.50	80.80	113.40	69.80	80.20	60.00
	2	82.40	52.10	85.00	88.00	89.70	107.90	69.30	76.50	69.00
	3	77.60	47.70	83.80	83.20	89.30	114.40	68.60	74.00	62.10
F	1	84.30	67.30	84.90	82.80	—[f]	96.50	80.20	66.90	64.10
	2	79.60	65.00	81.80	84.60	86.70	85.50	78.00	67.20	60.70
	3	88.80	63.70	79.60	80.40	84.30	88.80	75.50	59.40	65.70
G	1	67.10	47.10	53.80	63.80	66.70	60.00	63.40	54.60	40.50
	2	65.20	61.10	73.10	87.50	85.80	66.60	67.80	64.20	40.90
	3	70.40	50.00	62.40	75.00	80.70	69.40	64.30	65.10	43.20
H	1	70.20	48.00	72.80	81.10	83.40	73.80	66.90	62.70	51.00
	2	67.20	37.20	61.80	74.50	67.40	67.20	68.30	51.70	44.40
	3	72.90	42.10	67.40	84.50	85.90	88.80	74.60	58.50	40.30
Av. recovery		75.36	57.19	76.73	82.46	82.71	82.36	71.22	66.54	56.15
Int. CV[g]		10.36	20.22	12.43	10.14	10.77	20.89	10.97	10.29	15.92

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Rep. = replicate.

[c] Laboratory B did not get adequate resolution for SCP. Data have been omitted because in many cases recovery data were calculated from either a peak at the wrong retention time or from a large coeluting interference peak.

[d] Results from Laboratory D could not be recalculated from manual measurements because of off-scale peaks.

[e] Values were recalculated using manual peak height measurements.

[f] Analyst omitted data because of irregular peak shape.

[g] Int. CV = interlaboratory coefficient of variation.

had withdrawn because of a major instrument failure. Ten laboratories completed phase I and submitted their results. Results from one laboratory were dropped due to inadequate instrumentation. Therefore, samples for phases II and III of the study were sent to 9 laboratories. After 6 months, 8 laboratories had completed the study and submitted their data. The ninth laboratory withdrew from the study due to time constraints.

Tables 1–4 contain the results from analyses of the control milk and recovery data for the 5, 10, and 20 ppb levels fortified milk by the 8 participating laboratories. For 10 ppb fortified milk the average interlaboratory recovery for the 8 sulfonamides ranged from 56.2% for SQX to 82.7% for SMZ12. Also at this level of fortification, S_r ranged from 3.2 for SQX to 8.9 for SMZ12, and S_R ranged from 6.9 for SDM to 17.2 for SMZ30. The RSD_r and RSD_R values at the 10 ppb level ranged from 5.7% for SQX to 10.8% for SMZ12, and 10.1% for SMR to 20.9% for SMZ30, respectively. These values fall on the curve which Hall and Selinger (2) called the Horwitz trumpet.

Table 5 contains the data from all 8 laboratories for the 20 blind incurred samples. Table 6 contains the statistical analyses of these blind incurred samples. The 20 blind samples contained a total of 30 sulfonamide residues. Sulfamethazine was evaluated in both mobile phase systems. Three laboratories correctly identified all 20 blind samples, and one laboratory only misidentified one metabolite as a parent. The other 4 laboratories either did not detect a parent, identified some metabolites as parents, or both. In 3 separate samples, a total of 3 parent sulfonamides were not detected. A total of 240 possibilities existed in the blind incurred samples. Across all laboratories, the procedure correctly identified over 98% of the parent drug residues present. In no instance where an incurred residue was present

Table 4. Collaborative study results (%) for determination of multiple sulfonamide residues in 20 ppb fortified milk

		Sulfonamide[a]								
Lab.	Rep.[b]	SDZ	STZ	SPD	SMR	SMZ12	SMZ30	SCP	SDM	SQX
A	1	80.10	60.10	86.60	90.30	85.70	82.60	75.60	69.10	52.80
	2	77.80	64.30	79.90	84.60	83.50	77.60	68.70	68.50	53.80
	3	76.40	54.70	76.50	82.10	78.60	81.90	75.40	75.10	61.50
B	1	77.90	65.60	83.20	84.30	86.40	87.00	—[c]	65.10	46.70
	2	84.80	70.60	89.40	90.60	87.90	96.00	—[c]	67.20	49.50
	3	80.80	63.90	85.40	86.50	89.80	100.80	—[c]	66.50	45.40
C	1	71.59	64.79	72.76	74.19	76.67	67.61	63.26	63.88	53.18
	2	68.58	58.02	67.02	69.36	75.56	72.24	70.14	66.75	53.94
	3	68.27	60.59	67.53	71.63	71.69	63.73	66.69	60.67	49.80
D	1	64.00	52.00	70.00	74.00	74.00	—[d]	53.00[e]	62.00	52.00
	2	76.00	52.00	75.00	78.00	77.00	—[d]	—[d]	68.00	53.00
	3	81.00	54.00	73.00	77.00	78.00	—[d]	—[d]	70.00	54.00
E	1	76.90	41.70	80.30	82.70	85.30	99.20	69.80	76.00	60.00
	2	86.70	48.10	85.00	88.00	87.40	92.50	67.40	76.50	64.40
	3	74.70	40.40	78.80	82.30	84.80	103.70	63.50	80.10	62.70
F	1	80.50	66.50	80.50	83.00	86.00	88.50	76.00	65.50	58.50
	2	77.50	65.00	80.00	82.50	81.50	83.50	72.00	64.50	57.00
	3	79.50	64.00	79.00	81.00	81.00	79.50	71.50	61.00	58.00
G	1	58.50	41.30	59.90	103.10	72.80	74.00	58.90	64.70	39.90
	2	67.10	57.90	70.30	97.90	82.40	76.80	66.60	63.80	42.60
	3	70.60	54.20	78.90	78.10	82.30	79.60	63.00	65.60	47.70
H	1	77.50	62.00	76.80	85.00	78.30	79.20	73.00	65.40	50.40
	2	66.90	36.10	59.60	72.00	72.60	67.20	63.20	56.80	49.60
	3	76.50	49.60	74.00	80.40	78.30	87.00	72.10	66.50	44.70
Av. recovery		75.01	56.14	76.23	82.44	80.73	82.87	67.88	67.05	52.55
Int. CV[f]		9.21	17.28	10.41	9.91	6.79	14.00	7.46	8.31	12.56

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Rep. = replicate.

[c] Laboratory B did not get adequate resolution for SCP. Data have been omitted because in many cases recovery data were calculated from either a peak at the wrong retention time or from a large coeluting interference peak.

[d] Results from Laboratory D could not be recalculated from manual measurements because of off-scale peaks.

[e] Values were recalculated using manual peak height measurements. This single replicate was omitted during statistical analysis.

[f] Int. CV = interlaboratory coefficient of variation.

was the sample classified as negative. The 3 undetected residues were correctly identified and quantitated in their replicates. One of these was an SMZ12 residue; however, the peak was clearly in the chromatogram and the analyst had correctly identified SMZ in 30% methanol. Another of the undetected parents was intentionally not quantitated because a small interference peak was identified as SCP; therefore, this analyst reported that he could not quantitate SDZ because SCP was present.

Sometimes metabolites were quantitated as parent sulfonamides. This happened 16 times during the study. In all cases where a metabolite was identified and quantitated as a parent sulfonamide, the sample had already been identified as containing a sulfonamide. Of these 16 incorrectly identified peaks, 7 mistakes were made by one participant. Also, in the tables of results of the 20 blind samples, 3 participants reported a total of 29 values as "LF" (less than 5 ppb sulfonamide detected). They did not quantitate these values because they were below the standard curve. Of these 29 low level results reported, 17 were reported by one laboratory. Chromatograms from this laboratory contained very noisy baselines, possibly indicating some instrument problems.

One laboratory did not achieve adequate resolution for SCP. The SCP data from this laboratory have been omitted because in many cases recovery data were calculated from either a peak at the wrong retention time or from a large co-eluting interference peak. Two of the 20 blind samples were control milk. When the random SCP data from this laboratory were deleted, 100% of the blind samples which were control milk were identified correctly as not containing any sulfonamide greater than 5 ppb. Seven laboratories reported "ND" (none detected) for all sulfonamides in both replicates of the control milk; however, the laboratory, which reported a total of 17 LFs, reported one LF in each of its control replicates.

Table 5. Collaborative study results (ppb) for sulfonamide residues determination in blind incurred samples

	Laboratory								
Code[a]	A	B	C	D	E	F	G	H	Average[b]
1.SDZ	12.0	10.95	8.95	12.50	10.70	11.32	10.61	11.8	10.73
2.SCP	11.9	—[c]	12.31	19.8	11.7	14.42	10.08	11.9	12.36
3.SMZ12	12.5	32.58	—[d]	14.7	15.8	14.72	13.40	16.5	13.80
3.SMZ30	15.8	17.11	13.68	17.2	18.2	15.80	13.03	16.6	14.70
3.SDM	16.4	14.75	14.06	12.4	15.0	13.49	10.71	14.2	12.73
4.SQX	7.1	5.82	5.89	3.9	7.0	6.83	7.03	9.91	8.95
5.SMZ12	14.5	15.33	14.83	15.4	14.0	14.72	13.40	14.8	13.80
5.SMZ30	14.7	16.19	15.97	18.1	14.2	15.60	13.19	15.1	14.70
5.SDM	14.4	14.03	16.23	14.2	ND[e]	12.89	10.24	12.4	12.73
7.SPD	14.5	16.98	12.87	15.0	14.9	13.47	13.61	15.3	14.83
8.SPD	14.3	17.18	12.28	15.2	13.3	13.10	14.88	16.8	14.83
10.STZ	13.5	16.65	14.08	14.7	8.8	15.51	13.09	15.8	12.89
10.SMZ12	14.0	15.91	12.82	14.9	13.9	14.57	13.76	17.9	15.45
10.SMZ30	16.8	16.51	10.21	16.4	15.1	15.69	11.16	15.3	13.53
11.SQX	7.5	6.78	6.95	7.3	12.0	9.31	7.03	8.24	8.95
12.SMZ12	13.0	9.88	8.02	10.1	10.1	10.25	8.45	10.4	10.11
12.SMZ30	9.6	11.10	8.60	12.9	9.6	10.41	11.21	8.73	9.29
13.SDZ	12.4	11.17	8.85	11.1	10.9	11.26	10.23	10.9	10.86
13.SMR	15.2	15.09	12.40	14.6	12.7	14.79	13.83	15.0	14.66
14.SDZ	13.3	11.63	8.76	12.1	11.7	11.62	10.60	12.6	10.73
15.SDZ	12.8	11.61	—[f]	11.0	10.3	11.37	10.32	11.5	10.86
15.SMR	14.8	15.54	11.65	14.4	15.3	14.67	13.83	14.5	14.66
16.STZ	13.8	16.94	14.35	15.1	8.4	15.80	12.65	14.1	12.89
16.SMZ12	16.1	16.08	10.99	13.9	14.2	14.92	13.09	15.7	15.45
16.SMZ30	17.0	16.07	10.42	17.1	15.2	15.80	14.04	19.0	13.53
17.SMZ12	9.2	11.18	7.54	10.0	10.4	10.38	7.13	16.5	10.11
17.SMZ30	10.3	12.90	8.69	11.9	10.3	11.03	9.22	10.0	9.29
18.SCP	11.4	—[c]	17.18	11.8	9.7	13.51	11.96	11.9	12.36
19.SDM	8.6	9.42	8.56	9.3	8.9	8.76	6.52	8.69	8.69
20.SDM	8.8	8.94	8.81	9.2	8.8	8.59	6.52	8.69	8.69

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Average of 3 analyses extracted by the author.

[c] Laboratory B did not get adequate resolution for SCP. Data have been omitted because in many cases recovery data were calculated from either a peak at the wrong retention time or from a large coeluting interference peak.

[d] One of the incurred parent sulfonamides was not detected in a multi-incurred sample.

[e] ND = none detected.

[f] A small interference peak was identified as SCP; therefore, this analyst reported that he could not quantitate (NQ) SDZ when SCP was present.

One laboratory did not realize that a large late eluting peak would follow an injection that contained incurred SCP; therefore, this peak appeared in blind sample 2 coeluted with SMZ in blind sample 3. A 32.58 ppb value was reported for the SMZ value in 12% methanol. The Cochran test determined this value to be an outlier. The same participant found only 17.11 ppb for SMZ when this sample was injected in 30% methanol. This value was much closer to the 14.7 ppb reference value.

Collaborators' Comments

Some of the collaborators made comments and helpful suggestions in their reports at the conclusion of the study. The following is an outline of comments with responses:

One laboratory reported that "The interpretation of the chromatograms from the milk samples was difficult...metabolite information will have to be included and discussed in the method to prevent laboratories from identifying false positives." However, the collaborator was able to correctly identify the 20 blind incurred samples with no false positives.

A second laboratory suggested that "The use of relative retention values of certain metabolites with respect to the parent compound can be helpful in verifying the presence of a residue..." This collaborator also wrote that the method was interesting "to work with from an interpretative standpoint." The relative retention values that this participant used as an aid to identification must have been a useful tool because all 20 blind

Table 6. Summary of the collaborative study results from Table 5

Sulfonamide[a]	Sample No.	No. of replicates	$\bar{X}$	S_r	S_R	RSD_r, %	RSD_R, %
SDZ	1, 14	8	11.32	0.50	1.25	4.46	11.08
SCP	2, 18	7	12.83	2.62	2.72	20.45	21.23
SMZ12[b]	3, 5	7	15.88	4.69	4.93	29.54	31.07
SMZ12[c]	3, 5	6	14.54	0.94	1.12	6.47	7.69
SMZ30	3, 5	8	15.65	1.29	1.62	8.21	10.36
SDM	3, 5	7	13.60	1.08	1.82	7.93	13.38
SQX	4, 11	8	7.41	1.73	1.84	23.30	24.80
SPD	7, 8	8	14.60	0.66	1.53	4.54	10.44
STZ	10, 16	8	13.95	0.48	2.50	3.46	17.90
SMZ12	10, 16	8	14.55	0.95	1.65	6.50	11.36
SMZ30	10, 16	8	15.11	1.19	2.57	7.89	16.99
SMZ12	12, 17	8	10.16	1.86	2.25	18.32	22.11
SMZ30	12, 17	8	10.41	0.84	1.40	8.04	13.44
SDZ	13, 15	7	11.20	0.28	0.76	2.51	6.80
SMR	13, 15	8	14.27	0.71	1.14	4.95	8.02
SDM	19, 20	8	8.57	0.15	0.87	1.80	10.11

[a] SDZ = sulfadiazine; STZ = sulfathiazole; SPD = sulfapyridine; SMR = sulfamerazine; SMZ12 = sulfamethazine analyzed with the 12% methanol–88% 0.1M potassium dihydrogen phosphate solution mobile phase; SMZ30 = sulfamethazine analyzed with the 30% methanol–70% 0.1M potassium dihydrogen phosphate solution mobile phase; SCP = sulfachloropyridazine; SDM = sulfadimethoxine; SQX = sulfaquinoxaline.

[b] Cochran's test determined that Laboratory B was an outlier.

[c] Values were recalculated deleting data from Laboratory B.

Note: Blind samples 6 and 9 were control milk.

samples were identified correctly. However, because the author has not confirmed the identity of the metabolite peaks, he did not feel that this technique should become part of the procedure. This technique may be very beneficial after a completed metabolism study on each of the sulfonamides.

Another participating laboratory reported that "Because of the summer heat... [it was] ...impossible to hold the column temperature stable at 35°C." The analyst attempted to use 40°C; however, she experienced abnormal peak shape, co-elution, and absence of metabolite peak. The decision was made to rerun 4 samples on a cooler day. However, it should be noted that the 20 blind samples were all identified correctly by this laboratory.

One laboratory reported that "over 400 hours were required to complete the validation... Considerable expertise is needed for the correct and accurate interpretation of the sample chromatograms which is the most difficult part of the analysis." All sulfonamides were correctly identified in the blind incurred samples and only one metabolite was incorrectly quantitated as being a parent sulfonamide. It is very beneficial to know that this 8 residue method could be evaluated in only 400 h. A single residue method is normally evaluated in 45 days or 360 h. It should be noted that 8 times the information was acquired in only 10% more time.

Another laboratory only reported their results and stated that they were "...interested in the outcome of the study." Another collaborator reported that their LC system was too noisy, so another LC system was used, which gave "...very satisfactory results...." One laboratory reported the availability of only one LC unit with no auto-injection system; thus, the number of samples run each day was limited. The results of this laboratory demonstrate that only one LC is sufficient to analyze both chromatograph conditions.

The last laboratory reported that "...Some sulfa peaks were difficult to identify because of the presence of metabolites... No major difficulty was encountered while performing the extraction procedure... It was found fast and simple."

Conclusions

Using the data from all 8 laboratories, only 3 of a total of 240 incurred residues were undetected; in 16 cases metabolites were quantitated as parent sulfonamides, and 29 traces of sulfonamides were incorrectly identified. Across all laboratories, the procedure correctly identified over 98% of the parent drug residues present. When the data from only one laboratory are omitted, these values change to one sulfonamide residue undetected out of the incurred residues, 9 metabolites quantitated as parent sulfonamides, and 12 traces of sulfonamides incorrectly identified. When the data from this one laboratory are omitted, the procedure correctly identified over 99% of the incurred drug residues present in the blind samples. In no instance where an incurred residue was present was the sample classified as negative.

Horwitz (3, 4) predicted a 32% interlaboratory coefficient of variation (CV) at the 10 ppb concentration level. The values in Table 3 show that the interlaboratory CVs for this study are below 21% at 10 ppb. These results along with adequate recoveries and minimum interferences in the controls demonstrate

that the method is suitable for the determination of the 8 sulfonamide residues in milk at 5–20 ppb.

Recommendation

On the basis of the results of this study, it is recommended that the liquid chromatographic method for determination of multiple sulfonamide residues in bovine milk be adopted first action.

Acknowledgments

The author would like to thank the U.S. Department of Agriculture's dairy farm, Beltsville, MD, for providing 8 cows with a known history to be used in the study, and Herbert F. Righter for providing the control and incurred milk. The author also thanks the following individuals who participated in the collaborative study:

William Bargo, U.S. Food and Drug Administration, Baltimore, MD

Germain Carignan, Health and Welfare Canada, Ottawa, Canada

Jane Jenkins, Georgia Department of Agriculture, Atlanta, GA

Min Li, California Department of Food and Agriculture, Sacramento, CA

Ed Piotrowski, U.S. Department of Agriculture, ARS, Philadelphia, PA

Bob Schmid, U.S. Food and Drug Administration, Denver, CO

Richard D. Thompson, U.S. Food and Drug Administration, Minneapolis, MN

Charles Thorpe, U.S. Food and Drug Administration, Washington, DC

References

(1) Smedley, M.D., & Weber, J.D. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 875–879

(2) Hall, P., & Selinger, B. (1989) *Anal. Chem.* **61**, 1465–1466

(3) Horwitz, W. (1982) *Anal. Chem.* **54**, 67A–76A

(4) Horwitz, W., Kamps, L.R., & Boyer, K.W. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1344–1354

DRUGS, COSMETICS, FORENSIC SCIENCES

Simultaneous Multiresidue Analysis of β-Lactam Antibiotics in Bovine Milk by Liquid Chromatography with Ultraviolet Detection and Confirmation by Electrospray Mass Spectrometry

KRYSTYNA L. TYCZKOWSKA, ROBERT D. VOYKSNER, [1] ROLF F. STRAUB, [1] and ARTHUR L. ARONSON
North Carolina State University, College of Veterinary Medicine, Clinical Pharmacology Unit, Raleigh, NC 27606

A multiresidue analytical method was developed for the simultaneous determination of amoxicillin, cephapirin, procaine penicillin G, ampicillin, cloxacillin, and ceftiofur in bovine milk. The method involved ultrafiltration of milk diluted with an equal volume of 50% acetonitrile through a 10 000 dalton molecular mass cutoff filter. Separation of these β-lactam antibiotics from other milk components was performed by ion-paired (octane- and dodecanesulfonate) liquid chromatography using a phenyl column eluted with acetonitrile–water solution. Ultraviolet absorbance of the column effluent was monitored in the 200–350 nm range of a photodiode-array detector. For quantitation, the chromatograms were acquired at λ210 nm for penicillin G, ampicillin, and cloxacillin; λ230 nm for amoxicillin; and λ290 for cephapirin, procaine, and ceftiofur. The limit of detection for the simultaneous determination of these antibiotics was estimated to be 100 ppb. Liquid chromatography/electrospray mass spectrometry could be used to confirm these antibiotics for quantities down to 100 pg entering the mass spectrometer.

Received December 3, 1992. Accepted by JW September 9, 1993.

A preliminary report on this work was presented at the 106th AOAC INTERNATIONAL Annual Meeting and Exposition, August 31–September 3, 1992, Cincinnati, OH.

[1] Research Triangle Institute, Analytical and Chemical Sciences, PO Box 12194, Research Triangle Park, NC 27709

β-Lactams probably are the most widely used class of antibiotics in veterinary medicine for the treatment of bacterial infections (1). β-Lactam antibiotics consist of 2 types of compounds: penicillins and cephalosporins. The β-lactam antibiotics studied in this report are used to treat bacterial infections in lactating cows: procaine penicillin G, cloxacillin, ampicillin, amoxicillin, cephapirin, and ceftiofur. These antibiotics have either amphoteric or acidic properties. The penicillins have a basic structure consisting of bulky side chains attached to a 6-aminopenicillanic acid nucleus (Figure 1A). A 7-aminocephalosporanic acid nucleus (Figure 1B) is incorporated into the structure of cephalosporins. Few reported methods involve multiple analysis of β-lactam antibiotic residues in milk (2–5) or serum (6–7). Those involving the determination of β-lactam antibiotics in milk at concentrations less than 50 ppb are based on derivatization of analytes and fluorescence detection (3) or capillary gas chromatography with thermionic phosphorus/nitrogen detection (5).

There are no literature reports of the simultaneous determination of these 6 β-lactam antibiotics in bovine milk. This paper describes a liquid chromatographic (LC) procedure for simultaneous determination of penicillin G, cloxacillin, ampicillin, amoxicillin, cephapirin, and ceftiofur in bovine milk. The proposed method includes a simple sample preparation (ultrafiltration), isocratic reversed-phase separation, and UV-VIS detection. The limit of detection for simultaneous determination is 100 ppb of each β-lactam antibiotic in milk. However, we have reported estimated detection limits for penicillin G of 10 ppb, cloxacillin and ceftiofur of 50 ppb, ampicillin of 75 ppb, and amoxicillin of 100 ppb when these β-lactam antibiotics were analyzed individually (8–11). The methodologies described in these papers involved detection and measurement of β-lactam antibiotics directly from milk ultrafiltrates by ion-paired chromatography.

Liquid chromatography/electrospray–mass spectrometry (LC/ES–MS) has the capability of efficiently generating ions from a wide variety of compounds down to the low pg level, and it is a very mild ionization technique (12–15). As we continue our interest in developing new LC/MS methods to confirm drug residues in biological matrixes, we have applied electrospray ionization for the analysis of a mixture of 5 β-lactam antibiotics in milk ultrafiltrate.

Experimental

Materials and Reagents for LC–UV Analysis

The LC solutions were made from highest purity solvent grade acetonitrile (American Burdick & Jackson, Muskegon, MI). LC grade water was obtained from Hydro Services and Supplies (Research Triangle Park, NC). The ion-pairing reagents, octanesulfonate (S8) and dodecanesulfonate (S12) were obtained from Regis (Morton Grove, IL). The microseparation system, Centricon-10, employing a molecular mass cutoff filter of 10 000 Daltons, was supplied by Amicon Division of W.R. Grace (Danvers, MA).

Ceftiofur hydrochloride standard was supplied by the Upjohn Co. (Kalamazoo, MI). Cloxacillin, procaine penicillin G, ampicillin, amoxicillin, and cephapirin standards were obtained from Sigma Chemical Co. (St. Louis, MO). A 1 mg/mL concentration of each β-lactam antibiotic (calculated as a free compound) stock solution was prepared daily in acetonitrile–water (50 + 50, v/v). Working standard solutions of 1 and 10 μg/mL were prepared daily with the same diluent. All standards were protected from light with aluminum foil and amber vials.

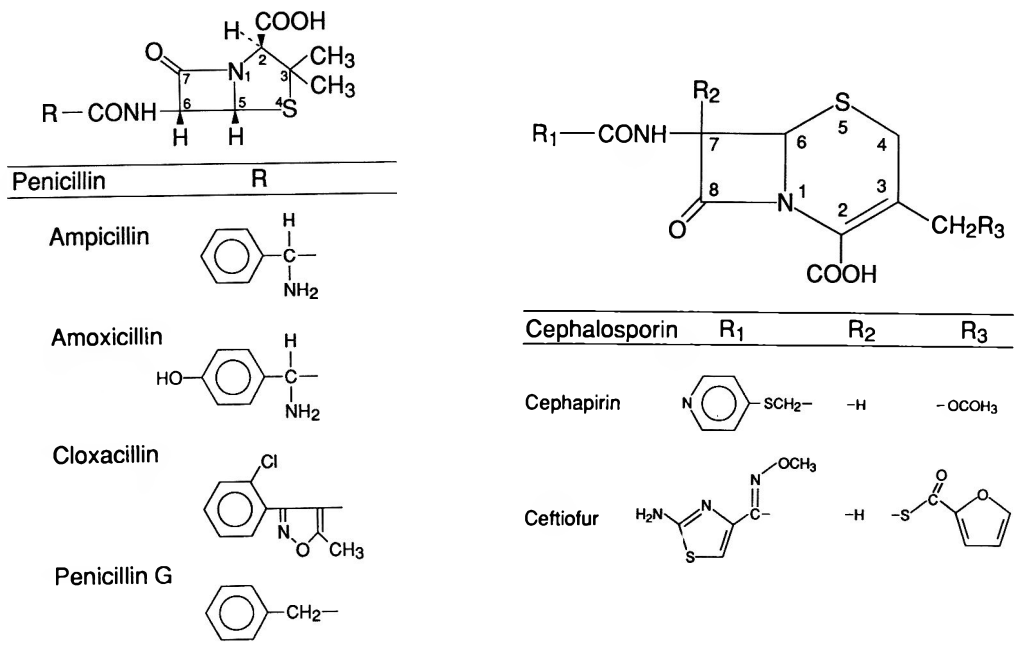


Figure 1. Chemical structures of (A) penicillins and (B) cephalosporins.

Materials and Reagents for LC/ES–MS Analysis

Water was distilled twice and purified with a Milli-Q water system (Millipore Corp., Bedford, MA) prior to use. Methanol (MeOH) and acetonitrile (ACN) were of LC/GC grade quality (Baxter Healthcare Corp., Muskegon, MI). For mobile phase, additive acetic acid (HOAc) from Aldrich Chemicals (Milwaukee, WI) was used. Arginine and gramicidin S from *Bacillus brevis* (Sigma Chemical Co.) were employed as tuning compounds in positive ion ES operation.

Sample Preparation Procedure

Aliquots (500 µL) of milk were diluted with an equal volume of acetonitrile–water (50 + 50, v/v) in the microseparation system equipped with a 10 000 Daltons molecular mass cutoff filter. Samples were vortex-mixed for 10–15 s and centrifuged for approximately 30 min at 4000 × *g* with 45° fixed-angle rotor. A 10–100 µL aliquot of colorless ultrafiltrate was injected into an LC system equipped with a UV-VIS photodiode array (PDA) detector or a 0.5 µL aliquot was injected into the LC/ES–MS system.

Liquid Chromatography with UV–VIS Detection

The LC equipment consisted of a Waters Model 600W multisolvent delivery system with a Waters U6K injector and temperature control accessory set at 40°C. This was coupled to a Model 990 (plus) UV-VIS PDA detector (Waters Chromatography Division, Milford, MA). The LC separations were performed using a mobile phase consisting of 18% acetonitrile in water (v/v), 0.25% phosphoric acid (v/v), 0.30% triethylamine (v/v) in 0.25 mM octanesulfonate, and 4.75 mM dodecanesulfonate aqueous solution. The column was an Ultremex 3 µm, phenyl column, 250 nm × 4.6 mm id (Phenomenex, Torrance, CA). The column effluent was analyzed within 200–350 nm using the PDA detector. The recoveries of β-lactam antibiotics from bovine milk samples were determined by analyzing from 5 to 7 spikes at the 1 ppm level at 210, 230, and 289.6 nm (Table 1).

After the areas of β-lactam antibiotic standards and bovine milk samples were compared, the quantity of β-lactam antibiotics determined by LC–UV detection was calculated as follows:

$$\mu g/mL = \frac{\beta\text{–lactam antibiotic (ng)} \times 2}{\text{injection volume } (\mu L)}$$

Usually, the injection volume was between 10 and 100 µL. The multiplication by 2 in the equation accounts for the dilution of serum (1 + 1) with the solution for releasing protein-bound drug.

Liquid Chromatography with ES—MS Detection

The eluents used to separate the drug mixture were delivered by an ISCO 100D syringe pump/pump controller, Model 174262, with a 5 µL sample loop (ISCO, Inc., Lincoln, NE). The samples were injected with a Valco C14W.5 injector with a 0.5 µL sample loop (ISCO) and separated on a 150 × 2 mm id Ultremex 3 µm C_{18} column (Phenomenex). A Waters Model 484 MS tunable absorbance detector was linked to the higher flow rate port of an AcuRate IC-70 splitter (LC Packings, Inc., San Francisco, CA) and set to 230 nm. A Hewlett-Packard Model HP 3396A integrator (Hewlett-Packard, Avondale, PA) was used to record the UV absorption chromatograms. A computer-controlled Analytica ES–MS interface, Model 101737, with autotune capability (Analytica of Branford, Inc., Branford, CT) was connected to a Hewlett-Packard Model HP 5989A single quadruple mass spectrometer (Hewlett-Packard, Palo Alto, CA). For instrument control and data acquisition, a Hewlett-Packard Model HP Vectra 80486-33 EISA personal computer/MS-DOS Chemstation software version M2.43 was used.

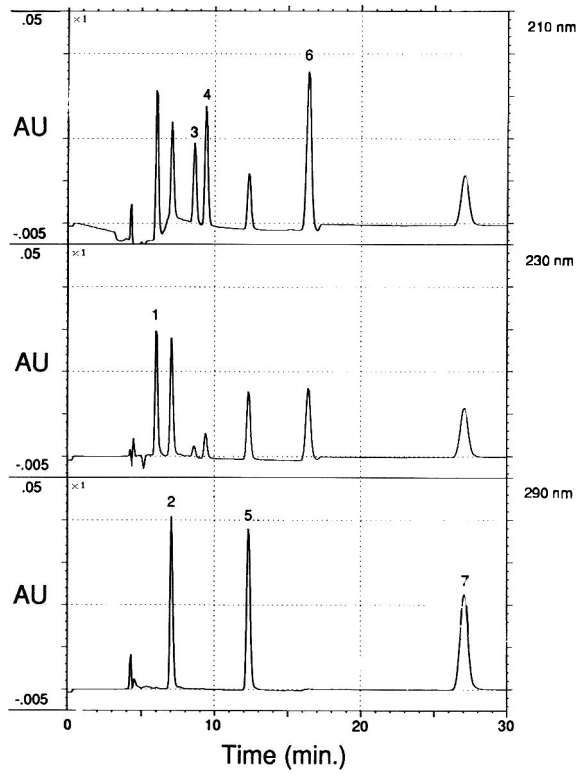
LC separation was performed using a mobile phase consisting of 40% (v/v) acetonitrile and 1% (v/v) acetic acid in water (pH 3.0). The mobile phase flow rate into the 1:70 splitter was 300 µL/min. The splitting reduced the flow rate of the eluent entering the ES interface to 4.3 µL/min.

The Analytica LC/ES–MS interface was autotuned with a synthetic mixture of the 5 β-lactam antibiotics (100 ng/µL each). Best results for all 5 drugs in positive ion mode were achieved by setting the cylindrical electrode (V_1) at ca −3.3 KV, the end plate (V_2) at −3.6 KV, and the capillary (V_3) at −3.8 KV. The capillary exit (collisional activated dissociation [CAD] voltage) potential was 150 V; best results were obtained with skimmer (S) and lens (L) voltages of S_1 = 52 V, L_1 = 54 V, S_2 = 22 V, L_2 = 16 V, and L_3 = −98 V for an overall good sensitivity. The mass spectrometer scanned from *m/z* 40–500 at a step size of 0.1 unit and a rate of 0.33 scan/s in full scan mode. The threshold was 500 and the abundance of each mass was sampled 6 times during a scan. In selected ion monitoring

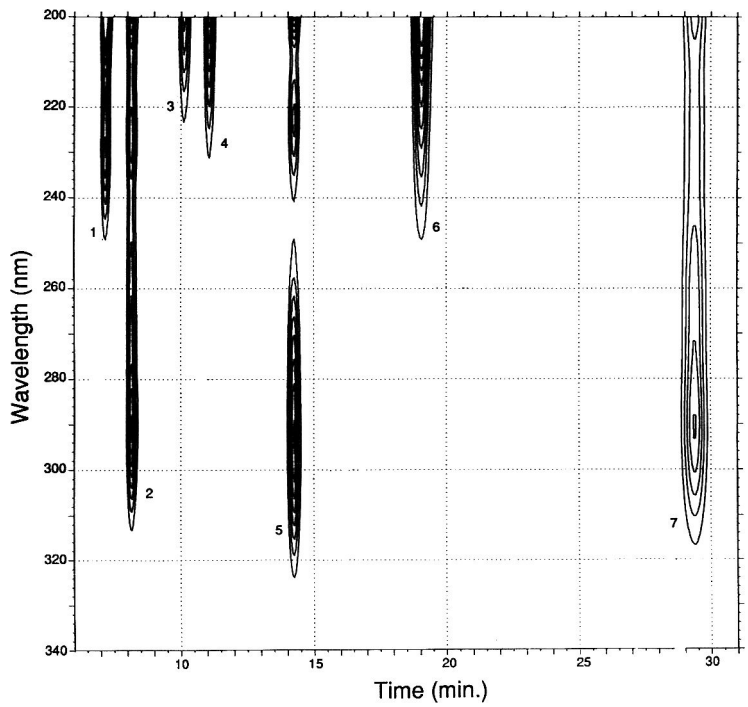
Table 1. Statistical summary of simultaneous analysis of β-lactam antibiotics in bovine milk by LC–UV–VIS

	Penicillin G[a] λ210 nm	Procaine λ290 nm	Amoxicillin λ230 nm	Cloxacillin λ210 nm	Ampicillin λ210 nm	Cephapirin λ290 nm	Ceftiofur λ290 nm
Amount spiked, ppb	1000	700	1000	1000	1000	1000	1000
No. Replications, n	5	7	7	7	7	7	6
Recovery Range	79.6-91.7	92.9-103.6	56.9-85.0	85.1-98.5	70.6-98.6	80-98.5	89-106.3
Mean, ±	86.2	98.0	69.2	90.5	87.4	88.1	95.1
SD	4.98	4.44	3.91	3.91	6.33	6.37	6.52
CV, %	5.78	4.53	15.5	4.32	7.25	7.23	6.86

[a] Penicillin G was determined when the mobile phase contained 18% acetonitrile (v/v), 0.25% phosphoric acid (80%) (v/v), 0.3% triethylamine (v/v) in 0.5 mM octanosulfonate, and 9.5 mM dodecanesulfonate aqueous solution.

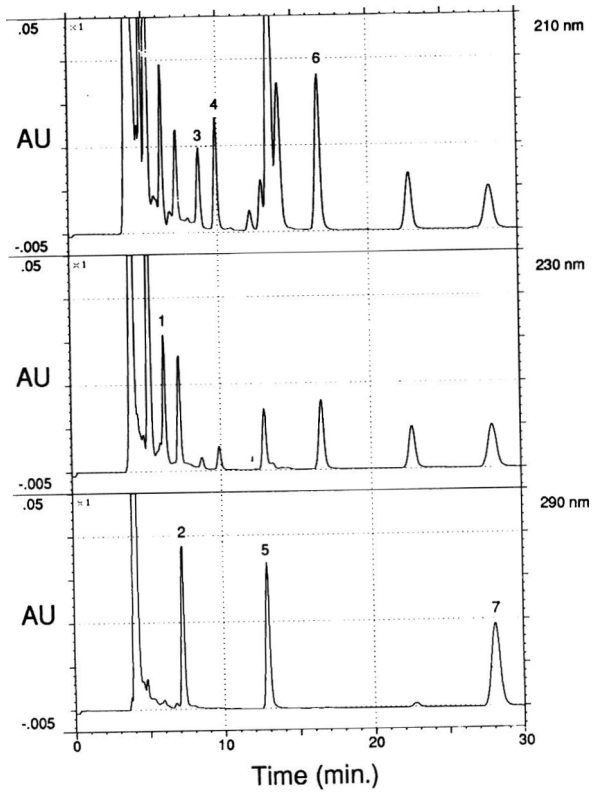


A

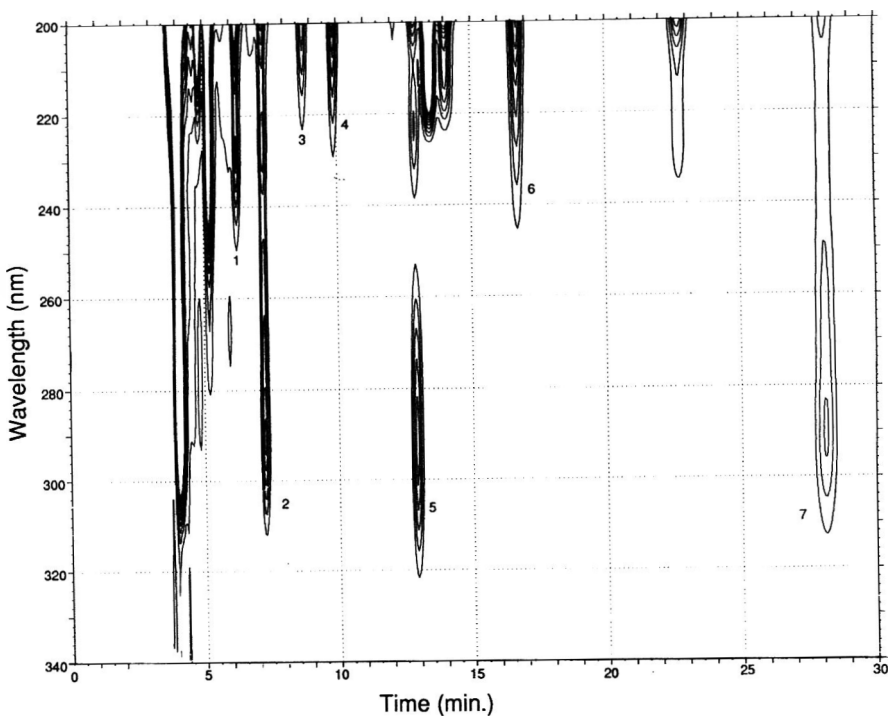


B

Figure 2. (A) LC/UV-PDA chromatograms for 6 β-lactam antibiotics (200 ng) and procaine (140 ng) in aqueous standards: (1) amoxicillin (230 nm), (2) cephapirin (290 nm), (3) penicillin G (210 nm), (4) ampicillin (210 nm), (5) procaine (290 nm), (6) cloxacillin (210 nm), and (7) ceftiofur (290 nm). The mobile phase was 18% acetonitrile (v/v), 0.25% phosphoric acid (80%) (v/v), 0.3% triethylamine (v/v) in 0.25 mM octanesulfonate, and 4.75 mM dodecanesulfonate aqueous solution. (B) UV absorbance contour plot for 7 compounds introduced in Figure 2A and acquired in 200–340 nm range.



A



B

Figure 3. (A) LC/UV-PDA chromatograms for bovine milk sample spiked with 10 ppm of each β-lactam antibiotic: (1) amoxicillin (230 nm), (2) cephapirin (290 nm), (3) penicillin G (210 nm), (4) ampicillin (210 nm), (5) procaine (290 nm), (6) cloxacillin (210 nm), and (7) ceftiofur (290 nm). Injection volume was 40 μL. The mobile phase was the same as that stated in Figure 2A. (B) UV absorbance contour plot for 7 compounds introduced in Figure 3A.

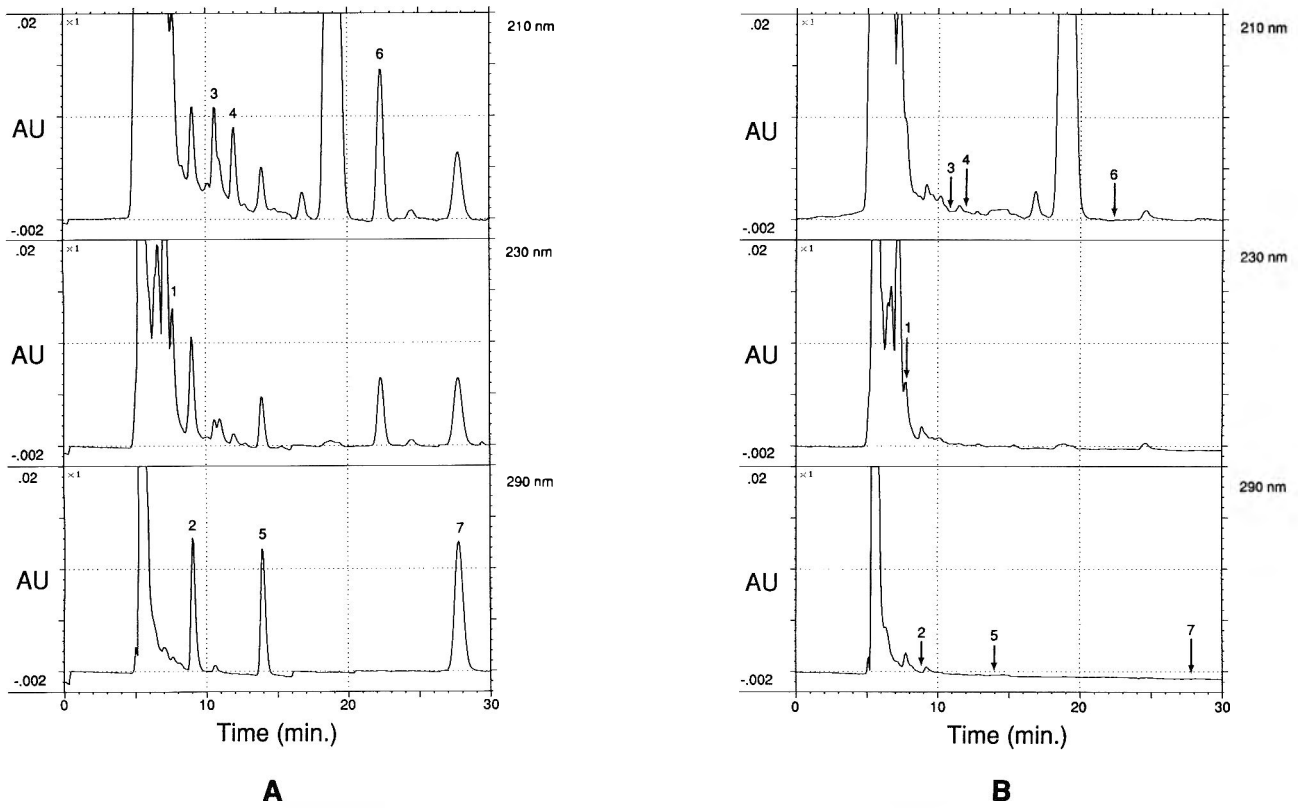


Figure 4. (A) LC/UV-PDA chromatograms for bovine milk sample spiked with 1 ppm of each β-lactam antibiotic: (1) amoxicillin (230 nm), (2) cephapirin (290 nm), (3) penicillin G (210 nm), (4) ampicillin (210 nm), (5) procaine (290 nm), (6) cloxacillin (210 nm), and (7) ceftiofur (290 nm). Injection volume was 80 μL. The mobile phase was the same as that stated in Figure 2A. (B) LC/UV-PDA chromatograms for blank bovine milk sample recorded at 210, 230, and 290 nm. Injection volume was 80 μL. The mobile phase was the same as that stated in Figure 2A.

(SIM), data acquisition dwell time of 200 ms resulted in 4.33 cycles/s.

Results and Discussion

Simultaneous Multiresidue LC–UV Analysis of Six β-Lactam Antibiotics in Bovine Milk

General.—A major goal of this study was to develop an LC procedure that allowed the simultaneous determination of 6 β-lactam antibiotics in bovine milk: procaine penicillin G, ampicillin, amoxicillin, cloxacillin, cephapirin, and ceftiofur. These β-lactam antibiotics were chosen because they are used therapeutically in lactating cows. Although cephapirin is biotransformed to desacetylcephapirin in milk, this metabolite was not included in the multiple analysis of β-lactam antibiotics due to a lack of standard. The methodology concerning the simultaneous determination of cephapirin and desacetylcephapirin in bovine milk was discussed elsewhere (8).

Simultaneous multiresidue analysis for these 6 β-lactam antibiotics is complicated by their variable chemical characteristics. Penicillin G, cloxacillin, and cephapirin have neutral side chains, whereas amoxicillin, ceftiofur, and ampicillin have basic side chains. Protein binding varies widely, for example, 48% for penicillin G and 71% for cloxacillin in cattle (16). It has been suggested that multiresidue analysis that includes compounds with neutral and acidic side chains would be unsatisfactory because of matrix effects (17, 18). However, recent advances in column design (e.g., the Ultremex 3 μm, phenyl column, 250 × 4.6 mm id) that were used in this study allowed for multiresidue analysis with a detection limit estimated to be 100 ppb. Future studies in this laboratory will use a more sensitive detector, a Waters Model 996 UV-VIS photodiode array detector, to achieve a detection limit of 10–20 ppb for the multiresidue analysis of β-lactam antibiotics.

Development of the mobile phase.—To perform the simultaneous LC separation of 6 β-lactam antibiotics in bovine milk, the gradient solvent system was used initially. Because a long (250 mm) phenyl 3 μm column was used as a stationary phase, a long column stabilization time was required between analyses. Also, the separation of amoxicillin from milk components was poor even at the 10 ppm level. The isocratic solvent system was used with the mobile phase consisting of 18–20% acetonitrile and different amounts of ion-pairing reagents: octanesulfonate and dodecanesulfonate in 0.1% phosphoric acid solution. Under these conditions, the β-lactam antibiotics were eluted as follows: amoxicillin, penicillin G, ampicillin, cephapirin, cloxacillin, procaine, and ceftiofur (data not shown). The separation of amoxicillin, penicillin G, and ampicillin from bovine milk components was inadequate. The addition of triethylamine to the mobile phase changed the sequence of elution of β-lactam antibiotics. This time amoxicillin

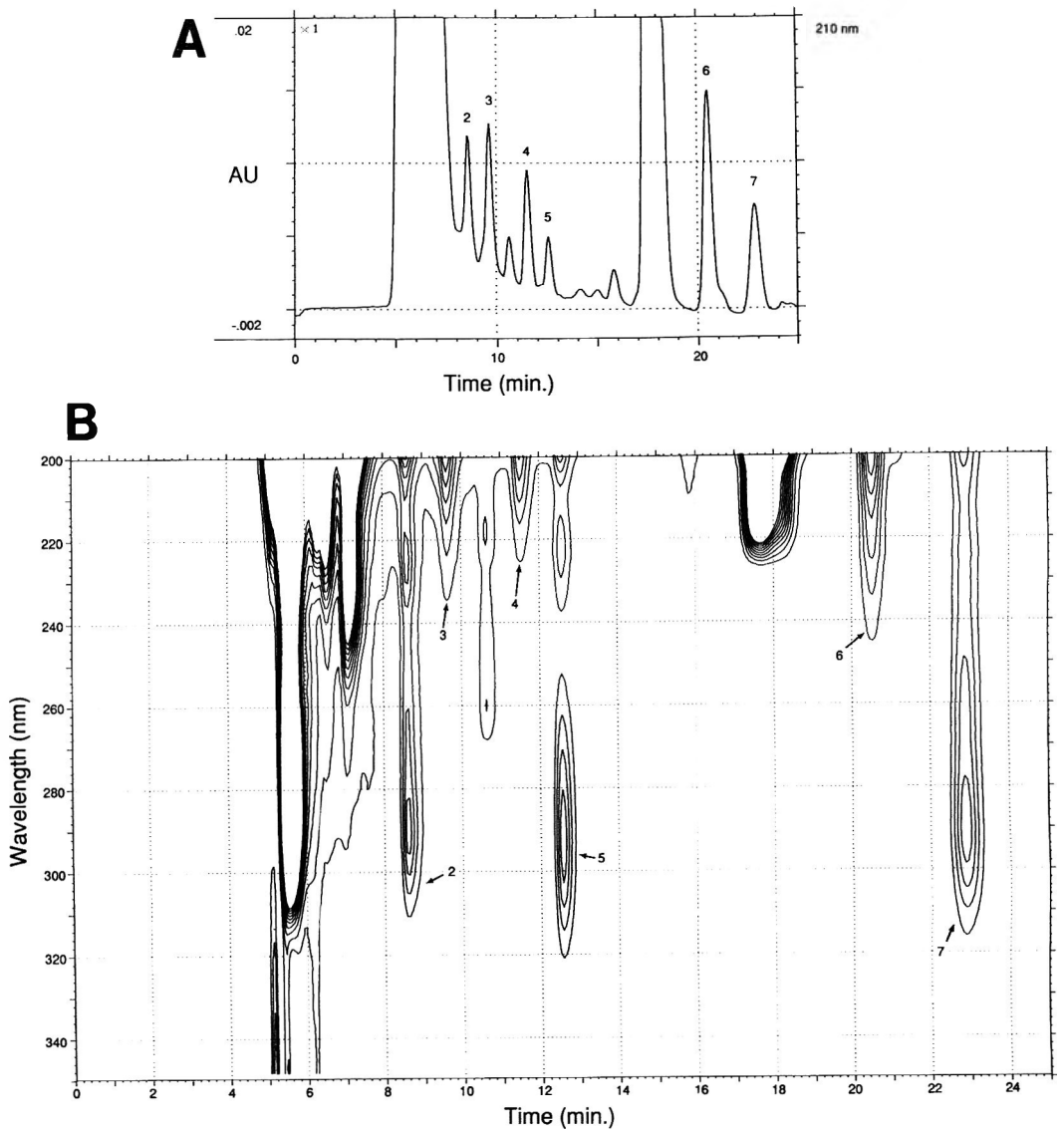


Figure 5. (A) LC/UV-PDA chromatogram for bovine milk sample spiked with 1 ppm of each β-lactam antibiotic and recorded at 210 nm. The mobile phase was 18% acetonitrile (v/v), 0.25% phosphoric acid (v/v), 0.3% triethylamine (v/v) in 0.5 mM octanesulfonate acid, and 4.5 mM dodecanesulfonate aqueous solution. (B) UV asbsorbance contour plot for peaks introduced in Figure 5A. Note that peak No. 3 is penicillin G.

was first; cephapirin (which can be acquired at λ_{max} 290 nm) was second; and penicillin G, ampicillin, procaine, cloxacillin, and ceftiofur were third (Figures 2–5). The final mobile phase was 18% acetonitrile (v/v), 0.25% phosphoric acid (v/v), 0.5% triethylamine (v/v) in 0.25 mM octanesulfonate, and 4.75 mM dodecanesulfonate aqueous solution. Under these conditions, the separation of all 6 β-lactam antibiotics in bovine milk at 10 ppm level was excellent. At 1 ppm concentration, there was no baseline resolution of ampicillin on the bovine milk chromatogram, and recovery of this analyte was approximately 70% (n = 7, at 230 nm).

Chromatographic column repeatability.—Three different phenyl (3 μm) columns (25 × 4.6 nm) were used. Although variabilities in retention times and sensitivity occurred, all columns gave adequate separation of the β-lactam antibiotics in this multiresidue study.

Release of β-lactam antibiotics from milk proteins.—All β-lactam antibiotics exhibited binding to serum, plasma, or milk proteins (16). Several solutions were evaluated for their ability to release these 6 antibiotics from milk proteins: various amounts of acetonitrile with or without 1% phosphoric acid, ethanol, and a mixture of acetonitrile and ethanol. The use of ethanol as a releasing solution was very successful for 5 β-lactam antibiotics, but it caused the rapid ethanolysis of amoxicillin (19). The most effective releasing solution for all 6 β-lactam antibiotics was 50% acetonitrile in water. Recoveries of 69.2–97.6% were achieved when milk samples spiked with 1 ppm of each β-lactam antibiotic (0.7 ppm procaine) were analyzed (Table 1).

LC–UV–VIS.—A chromatogram of 200 ng amoxicillin, cephapirin, penicillin G, ampicillin, cloxacillin, procaine (140 ng), and ceftiofur aqueous standards is shown in Fig-

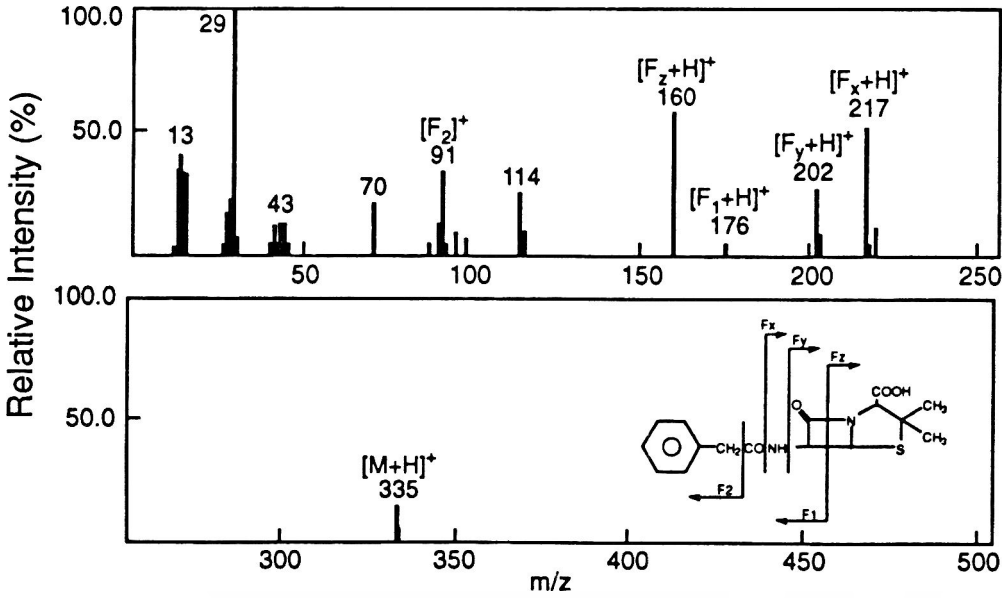


Figure 6. Electrospray mass spectrum of penicillin G at CAD voltage of +320 V in methanol–water (1 + 1).

ure 2A. This chromatogram was acquired at wavelengths of 210, 230, and 290 nm. At 210 nm wavelength, penicillin G, ampicillin, and cloxacillin exhibited good resolution and sensitivity. Although better sensitivity can be achieved at 210 nm, amoxicillin was acquired at 230 nm to reduce the matrix background. However, this β-lactam antibiotic was eluted as a first analyte, just after major matrix peaks on milk chromatograms.

Figure 2B shows a UV-VIS absorbance contour plot for 6 β-lactam antibiotics in standard solutions. The separation of amoxicillin, cephapirin, penicillin G, ampicillin, procaine, cloxacillin, and ceftiofur between 200 and 340 nm was excellent. Excellent separation also occurred when bovine milk was spiked with 10 ppm of each β-lactam antibiotic (Figure 3A). A UV-VIS absorbance contour plot for peaks introduced in Figure 3A is shown in Figure 3B. This revealed clean analytical windows for cephapirin, penicillin G, ampicillin, cloxacillin, and ceftiofur. The contour plot for amoxicillin in bovine milk showed some interferences. The procaine peak was not baseline resolved within the 200–225 nm range, but its LC–UV-VIS detection was not interfered with because of the peak's λ_{max} 290 nm.

Figure 4A exhibits a chromatogram of bovine milk spiked with 1 ppm of each β-lactam antibiotic. The amoxicillin peak (No. 1) was not baseline resolved, and the penicillin G peak (No. 3) showed small interferences on its downslope. Good separation from milk components was achieved with cephapirin, ampicillin, procaine, cloxacillin, and ceftiofur.

Figure 4B shows a blank chromatogram of milk ultrafiltrate acquired at 210, 230, and 290 nm. Although recoveries of this β-lactam antibiotic were 56.9–85.0% (Table 1), the analytical window for amoxicillin contained an interfering peak. Also, the penicillin G peak showed a small interference in its analytical window (Figure 4A, peak No. 3).

Development of the mobile phase for penicillin G.—Procaine penicillin G is the most commonly used β-lactam antibiotic in cattle. Therefore, a different mobile phase was developed to achieve a clean analytical window for this drug. The mobile phase was 18% acetonitrile (v/v), 0.25% phosphoric acid (v/v), 0.3% triethylamine (v/v) in 0.5 mM octanesulfonate, and 4.5 mM dodecanesulfonate aqueous solution. Figure 5A exhibits a chromatogram of bovine milk spiked with 1 ppm of each β-lactam antibiotic. There was no separation of amoxicillin from milk components, but penicillin G and other analytes were separated very well, which is shown on the UV contour plot (Figure 5B).

Linearity.—The study on linearity of the UV-VIS detector response was performed by injecting β-lactam antibiotic standards containing 10 to 1000 ng. Each concentration was analyzed twice. The relationship between peak area and concentration of aqueous β-lactam antibiotic standards was linear within this range with correlation coefficients above 0.99 (n = 5). The UV-VIS detection limit was estimated to be 100 ppb (ng/mL) for each of them, using an injection volume of 100 μL and based on a 3:1 signal-to-noise ratio. This was recorded at 210 nm for penicillin G, ampicillin, and cloxacillin; at 230 nm for amoxicillin; and at 290 nm for cephapirin, ceftiofur, and procaine.

Statistics.—Table 1 summarizes the statistical data obtained from bovine milk spiked with 1 ppm of each β-lactam antibiotic. Recoveries of amoxicillin, ampicillin, cloxacillin, cephapirin, ceftiofur, and procaine were determined by methodology described in *Experimental.* Penicillin G analysis was performed using a different proportion of ion-pairing reagents in the mobile phase (S8:S12 = 1:9). This resulted in recoveries of 79.6–91.7% (n = 5) with a coefficient of variation of 5.78%. Average recoveries of cloxacillin, ampicillin, cephapirin, ceftiofur, and procaine from bovine milk were 90.5, 87.4, 88.1, 95.1, and 98.0% with coefficients of variation of 4.32, 7.25, 7.23, 6.56, and 4.53%, respectively. Recovery of amoxicillin from bovine milk was 69.2% with a coefficient of variation of 15.5% due to poor resolution of this analyte from milk components at the 1 ppm level. When bovine milk samples were

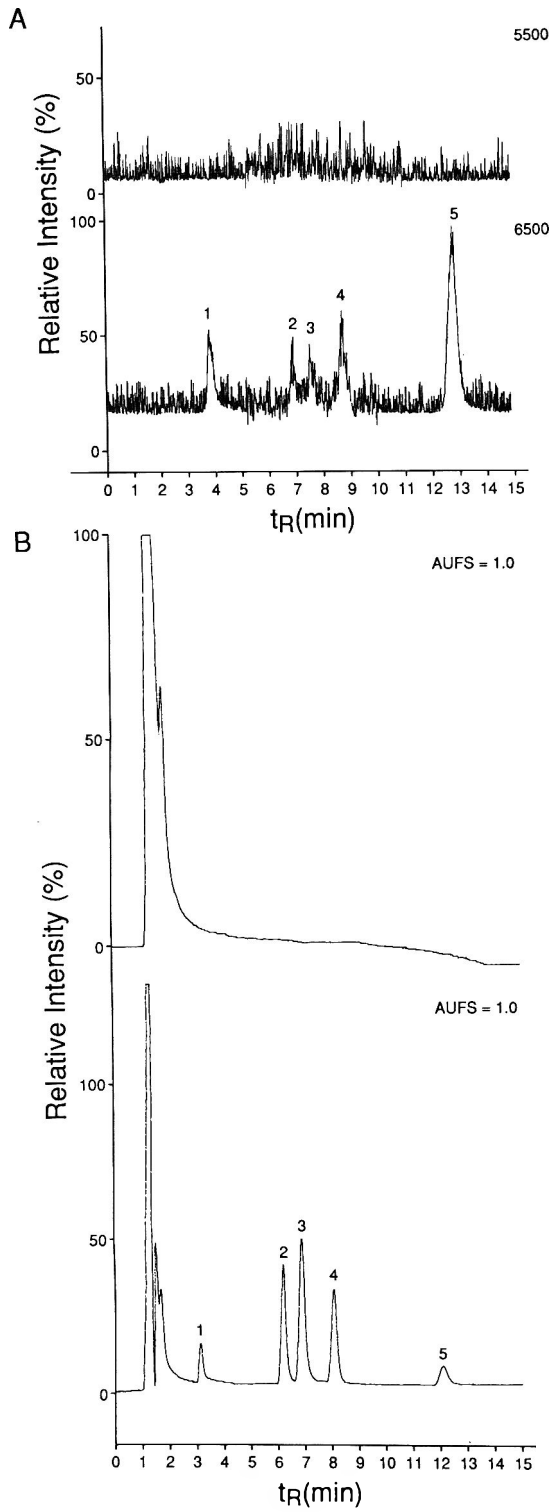


Figure 7. (A) LC/ES–MS reconstructed total ion current of a blank milk ultrafiltrate and a milk ultrafiltrate spiked with 428 pg of each drug in the SIM acquisition mode and the (B) corresponding LC–UV chromatograms recorded at 230 nm. Peaks and ions monitored: 1 = penicillin G (*m/z* 335, 217, 160); 2 = cloxacillin (*m/z* 436, 277, 222); 3 = cephapirin (*m/z* 424, 226, 151); 4 = amoxicillin (*m/z* 366, 348, 160); 5 = ampicillin (*m/z* 350, 192, 174). Injection volume, 0.5 μL; flow rate, 300 μL/min reduced to 4.3 μL/min by a 70:1 split; mobile phase, 40% (v/v) acetonitrile and 1% (v/v) acetic acid in water.

spiked with 10 ppm of each β-lactam antibiotic, the recoveries exceeded 90% ($n = 5$).

Simultaneous Multiresidue LC/ES–MS Analysis of Five β-Lactam Antibiotics in Bovine Milk

Effect of CAD voltage of positive ion MS spectra.—The increase in potential difference between the capillary and the skimmer in the interface resulted in the transfer of more internal energy into the molecule through collisional activating, which formed structurally relevant product ions. Most of the CAD fragment ions detected for each β-lactam antibiotic could be identified based on sample cleavages of the various groups from the molecule. As a representative example of all 5 tested β-lactams, the mass spectrum of penicillin G at CAD voltage of +320 V is presented in Figure 6. Low CAD voltage (e.g., +80 V) generally forms molecular adducts and very few characteristic fragment ions (data not shown). High CAD potentials usually generate a pattern of characteristic cleavage products, among them the $[C_6H_9NSO_2 + H]^+$ ion at *m/z* 160, formed by β-lactam antibiotic ring opening and an ion at *m/z* 114 formed after a further loss of [COOH]. Fragments formed by the cleavage of the amide moiety are more specific for the different penicillins. The peak at *m/z* 91 is characteristic for a loss of the $[C_6H_5CH_2]^+$ ion in penicillin G. The CAD fragmentation of other β-lactam antibiotics in the electrospray transport region has been reported by Straub and Voyksner (20).

The relative intensity of the CAD fragmentation that formed from the β-lactam antibiotics was reproducible in day-to-day operations. Ion ratios varied less than 10% in run-to-run analysis within a day when the $[M + H]^+$ and the 2 most intense fragment ions were monitored. Long-term stability required adjusting the capillary voltage to achieve the same ion ratios. This was a result of contamination in the electrospray transport over a period of several months, which required a slow increase (total of 10–20 V) of capillary exit voltage to achieve the same extent of fragmentation. Usually, the capillary voltage was checked daily for a standard mixture of β-lactam antibiotics to ensure the extent of fragmentation would meet a historical average. No difference in ion ratios was observed between solvent standard mixtures and milk extracts containing the β-lactam antibiotics.

LC/ES–MS.—The combination of LC with ES–MS could specifically detect ampicillin, amoxicillin, cephapirin, cloxacillin, and penicillin G directly from milk ultrafiltrate. To achieve lower detection limits, SIM was incorporated to monitor the $[M + H]^+$ and the 2 most intense fragment ions for each β-lactam antibiotic. Usually, fragment ions were chosen that were specific for each β-lactam antibiotic (charge not on the lactam ring) and were above *m/z* 150 to provide the best specificity. Other ions could also be monitored, such as the common ion fragment for all the β-lactam antibiotics at *m/z* 160, to aid in screening for these antibiotics or other specific ions to improve the specificity of the analysis. The LC/MS total ion current chromatograms (sum of the ion currents for the 3 ions that were monitored for each β-lactam antibiotic) are shown in Figure 7A for 428 pg/β-lactam antibiotic spiked into milk ultrafiltrate. Each β-lactam antibiotic can be clearly detected, free

from common ion interference as demonstrated from the LC/MS chromatogram for analysis of the blank milk ultrafiltrate. The LC–UV chromatogram (230 nm) is also shown for the spiked milk extract and the blank in Figure 7B. The LC/MS method can be used to confirm the presence of these β-lactam antibiotics down to 100 pg/component in the milk ultrafiltrate. Currently, we are evaluating the use of capillary columns (0.32 mm id) and on-column concentration of β-lactam antibiotic in milk ultrafiltrate to improve detection limits to the 1–10 ppb range (21).

Conclusions

A multiresidue analytical method for the simultaneous determination of amoxicillin, cephapirin, procaine penicillin G, ampicillin, cloxacillin, and ceftiofur was relatively specific and sensitive when analyzing these drugs in bovine milk. Confirmation of these β-lactam antibiotics can be accomplished by LC/ES–MS. The electrospray method offers the specificity (at least 3 ions for monitoring) and sensitivity to monitor low ppb concentrations of these antibiotics in bovine milk.

Acknowledgments

This research was supported by the U.S. Food and Drug Administration under Cooperative Agreement No. FD-U-000589.

We thank W. Giddens for typing and preparing the manuscript.

References

(1) Papich, M.G. (1984) *J. Am. Vet. Med. Assoc.* **184**, 344–347

(2) Moats, W.A. (1983) *J. Agric. Food Chem.* **31**, 880–883

(3) Munns, R.K., Shimoda, W., Roybal, J.E., & Vieira, C. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 968–971

(4) Tsuji, A., Niyanmoto, E., & Yamana, T. (1978) *J. Pharmacol.* **30**, 811–813

(5) Meetschen, U., & Petz, M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 373–379

(6) Tyczkowska, K.L., & Aronson, A.L. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 773–775

(7) Rudrik, J.T., & Bawdon, R.E. (1981) *J. Liq. Chromatogr.* **4**, 1525–1545

(8) Tyczkowska, K.L., Voyksner, R.D., & Aronson, A.L. (1991) *J. Vet. Pharmacol. Therap.* **14**, 51–60

(9) Tyczkowska, K.L., Voyksner, R.D., Anderson, K.L., & Aronson, A.L. (1992) *J. Chromatogr.* **614**, 123–134

(10) Tyczkowska, K.L., Voyksner, R.D., & Aronson, A.L. (1989) *J. Chromatogr.* **490**, 101–113

(11) Voyksner, R.D., Tyczkowska, K.L., & Aronson, A.L. (1991) *J. Chromatogr.* **567**, 389–404

(12) Whitehouse, C.M., Dreyer, R.N., Yamashita, M., & Fenn, J.B. (1985) *Anal. Chem.* **57**, 675–679

(13) Smith, R.D., Loo, J.A., Edmonds, C.G., Barinage, C.J., & Udseth, H.R. (1990) *Anal. Chem.* **62**, 882–899

(14) Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., & Whitehouse, C.M. (1990) *Mass Spectrom. Rev.* **9**, 37–70

(15) Voyksner, R.D., & Pack, T. (1991) *Rapid Commun. Mass Spectrom.* **5**, 263–268

(16) Baggot, J.D. (1977) *Principles of Drug Disposition in Domestic Animals*, W.B. Saunders Co., Philadelphia, PA, p. 66

(17) Moats, W.A., & Malisch, R. (1992) *J. AOAC Int.* **75**, 257–260

(18) Moats, W.A. (1992) *J. Chromatogr.* **593**, 15–20

(19) Tyczkowska, K.L., Voyksner, R.D., & Aronson, A.L. (1992) *J. Chromatogr.* **594**, 195–201

(20) Straub, R.F., & Voyksner, R.D. (1993) *J. Chromatogr.* **647**, 167–181

(21) Voyksner, R.D., Linden, M., & Straub, R.F. *Proceedings from the 41st Annual ASMS Conference on Mass Spectrometry and Allied Topics*, San Francisco, CA, May 30–June 4, 1993, paper MP8.

Determination of Bronopol, Bronidox, and Methyldibromo Glutaronitrile in Cosmetics by Liquid Chromatography with Electrochemical Detection

J.W. WEYLAND, A. STERN, and J. ROOSELAAR
Inspectie Gezondheidsbescherming/Keuringsdienst van Waren, POB 777, 7500 AT Enschede, The Netherlands

A method for the simultaneous determination of methyldibromo glutaronitrile, bronopol, and bronidox in cosmetics, based on liquid chromatography with electrochemical detection, is described. The method is suitable for both aqueous products and emulsions. The detection limit is better than 0.002% for all analytes. Recoveries from an emulsion, spiked to concentrations of 0.03% of the analytes, were 100.4% for bronopol [relative standard deviation (RSD), 0.43%], 97.1% for bronidox (RSD, 0.83%), and 98.4% for methyldibromo glutaronitrile (RSD, 1.7%). Repeatabilities were calculated from 10 replicate analyses of commercial samples. The repeatability for bronopol from an emulsion containing 0.047% bronopol was 0.0027% (RSD, 1.99%); for bronidox from a lotion containing 0.027% bronidox, 0.0014% (RSD, 1.86%); and for methyldibromo glutaronitrile from an emulsion containing 0.031% methyldibromo glutaronitrile, 0.0019% (RSD, 2.16%). A ruggedness test showed that sample amount influenced the results for all 3 analytes. The results obtained for bronidox also depended on detection parameters and composition of extraction solvent. The method was applied to 138 cosmetic products and performed trouble free during these analyses. Bronopol was found in 14 samples, and bronidox was found in 4 samples, including a baby hair lotion, in which it is prohibited. Methyldibromo glutaronitrile was present in 27 samples, including creams, lotions, and sun protection cosmetics.

Recently, we reported the determination of methyldibromo glutaronitrile in cosmetics, based on reversed-phase liquid chromatography (RP-LC) in combination with reductive electrochemical detection. The method allowed reliable quantification of methyldibromo glutaronitrile in both aqueous products (like shampoos and bath foams) and emulsions (like vanishing creams and milks) (1). With minor modifications, the method can be applied to the simultaneous determination of 2 additional preservatives: bronopol and bronidox.

Received February 26, 1993. Accepted by JW September 9, 1993.

Methyldibromo glutaronitrile, bronopol, and bronidox are important preservatives in cosmetics.

Methyldibromo glutaronitrile (1,2-dibromo-2,4-dicyanobutane; Tektamer; main ingredient in Euxyl K400) is used as a preservative in both aqueous products (shampoos, bath foams, etc.) and emulsions (vanishing creams, etc.) at concentrations between 0.01 and 0.04%. In the European Community (EC), the maximum level allowed in cosmetics is 0.1%; the use in sun protection cosmetics is restricted to a maximum level of 0.025%. The use of methyldibromo glutaronitrile is rapidly increasing. This study shows that approximately 20% of all cosmetics sold in the Dutch market contain this preservative. Methods for the determination of methyldibromo glutaronitrile in cosmetics include gas chromatography (GC), both with packed columns combined with a phosphorous–nitrogen selective detector and capillary gas chromatography with electron capture detection (2); polarography (3); and liquid chromatography (LC) with UV detection (4). All these methods have certain disadvantages such as complicated execution (GC) or lack of specificity (polarography). LC with UV detection suffers from interferences caused by matrix components that absorb at the low detection wavelength that must be used (220 nm). The combination of LC and electrochemical detection greatly improves specificity (1).

Bronopol (2-bromo-2-nitro-1,3-propanediol) is also used as a general preservative in aqueous products and emulsions. It is used in concentrations between 0.01 and 0.1%; the maximum level allowed by EC directive 76/768 is 0.1%. Bronopol is a simple molecule that hardly shows UV absorption and is, therefore, difficult to quantify reliably by liquid chromatography. It can be determined by its release of formaldehyde under specific conditions, which can be used to advantage in a postcolumn reaction similar to the EC method for the determination of free formaldehyde in cosmetics. Obviously, this procedure (5, 6) is cumbersome. Alternatives that have been described include polarography (7) and gas chromatography (8).

Bronidox (5-bromo-5-nitro-1,3-dioxane) is used as a preservative in aqueous products, as well as raw materials like detergents. Concentrations used vary between 0.005 and 0.1%. In the EC, the use of bronidox as a preservative is restricted to rinse-off cosmetics, with a maximum concentration of 0.1%.

Table 1. Ruggedness test: factors investigated, factor levels, experimental design, and results[a]

Experiment	Factor combination							Bronopol, %	Bronidox, %	MDBGN, %
s	A	B	C	D	E	F	G	0.0328	0.0322	0.0319
t	A	B	c	D	e	f	g	0.0328	0.0297	0.0311
u	A	b	C	d	E	f	g	0.0313	0.0288	0.0294
v	A	b	c	d	e	F	G	0.0327	0.0312	0.0315
w	a	B	C	d	e	F	g	0.0314	0.0290	0.0300
x	a	B	c	d	E	f	G	0.0320	0.0284	0.0296
y	a	b	C	D	e	f	G	0.0311	0.0299	0.0304
z	a	b	c	D	E	F	g	0.0301	0.0296	0.0299
							$\bar{x}$	0.0319	0.0299	0.0305
							$\bar{s}$	0.00117	0.00127	0.00092
							RSD	3.6%	4.2%	3.0%

[a] Ruggedness test according to Youden and Steiner (11). Sample: cleansing milk spiked with 0.03% bronidox, 0.03% bronopol, and 0.03% methyldibromo glutaronitrile (MDBGN). Conditions: as described in the text, but the following factors were varied as indicated in the table:

A/a	Amount of sample (g)	**2**	1.5
B/b	Heating time in water bath during extraction (min)	**5**	10
C/c	Shaking time during extraction (min)	**1**	2
D/d	Composition of extraction solvent (water–methanol, v/v)	**2 + 8**	3 + 7
E/e	Column oven temperature (°C)	**40**	room
F/f	Pulse time of electrochemical detector (ms)	**100**	50
G/g	Working potential (V)	**–0.4**	–0.5

The factor levels prescribed in the method are denoted by **bold** print and are represented by capitals.

Methods for the determination of bronidox are based on liquid chromatography (9) and gas chromatography (10). The LC methods, based on UV detection, are not very suitable for the determination of bronidox in finished products. The sensitivity is poor, and liquid chromatography lacks selectivity, again because of the need to use low detection wavelengths.

Although the gas chromatographic determinations for both bronopol (8) and bronidox (10) are selective and appear to work well, the method described here is an interesting alternative because it allows simultaneous determination of bronopol, bronidox, and methyldibromo glutaronitrile in one procedure. It combines a selectivity and sensitivity comparable with the GLC methods with a very simple sample extraction procedure, which contrasts favorably with the extensive extraction procedures required by gas chromatographic methods.

METHOD

Reagents and Apparatus

(**a**) *Solvents.*—Acetonitrile, methanol, acetone, and water; all LC grade.

(**b**) *Extraction solvent.*—Water–methanol (2 + 8, v/v).

(**c**) *Mobile phase.*—Dissolve 2.84 g sodium sulfate decahydrate (p.a.) and 0.12 g sodium chloride (p.a.) in 600 mL water and 400 mL acetone.

(**d**) *Standard stock solution.*—Accurately weigh 20 mg bronopol, 20 mg bronidox, and 20 mg methyldibromo glutaronitrile in a 50 mL volumetric flask; dissolve in methanol; and dilute to volume with methanol.

(**e**) *LC system.*—Hewlett-Packard 1090 M HPLC and 1049 A electrochemical detector (Hewlett-Packard, Waldbronn, BRD); Lichrospher 100 RP 8 analytical column, 5 µm, 250 × 4 mm (Merck, Darmstadt, Germany). Operating conditions: flow rate, 1 mL/min; injection volume, 10 µL; column oven temperature, 40°C. Detector configuration: gold working electrode, solid-state silver reference electrode; detection mode, reduction pulse mode: +1 V during 10 ms, –1 V during 10 ms, –0.4 V during 100 ms (measuring potential); sensitivity, 500 µA; response time, 2 s; controlled at 40°C.

Sample Preparation and Extraction

Accurately weigh ca 2 g sample into a 50 mL volumetric flask and add ca 30 mL extraction solvent (water–methanol, 2 + 8, v/v). Heat 5 min in a water bath at 60°C, shake 1 min until a homogeneous suspension/emulsion is obtained, cool, and dilute to volume with water–methanol (2 + 8, v/v). Filter with a

paper filter or, when the filtrate is not clear, with a 0.45 μm pore-size membrane filter (test solution).

Chromatography and Quantitation

(**a**) *Chromatography.*—Thoroughly degas the mobile phase by flushing with helium. Avoid the use of plastic tubing because this allows diffusion of oxygen into the mobile phase, which increases the baseline noise. When the mobile phase is free of oxygen, the helium flow may be diminished. Allow the system to condition 1 h at the prescribed mobile phase flow while constantly flushing with a small stream of helium. (In routine analysis, it is advantageous to maintain a low mobile phase and helium flow during the night while continually operating the detector to save stabilizing time the next day.)

(**b**) *Calibration.*—Transfer by pipette 5.00 mL standard stock solution (**d**) into a 50.00 mL volumetric flask and dilute to volume with extraction solvent (**b**) (solution S). Transfer by pipette 5.00 mL solution S into a 10 mL volumetric flask, 6.00 mL into a 25 mL volumetric flask, 3.00 mL into a 25 mL volumetric flask, and 3.00 mL into a 50 mL volumetric flask. Add extraction solvent (**b**) to volume. These calibration standards should be freshly prepared because dilute solutions of bronopol are not stable. Inject 10 μL of each of the calibration standards and measure the peak areas (or heights) for the analytes. For each analyte, construct calibration curves relating the appropriate peak areas (or heights) to the concentrations of the calibration standards.

(**c**) *Quantitation.*—Inject 10 μL of the test solution and record the peak areas (or heights) in the chromatogram obtained. Read the concentration $c_{ts}i$ of each analyte in the test solution from the appropriate calibration curve, and calculate the concentration in the sample from $c_s i$ (%, m/m) = $5 \times c_{ts}i / m$, where m is the amount of sample taken for the analysis in grams and $c_{ts}i$ (mg/mL) is the concentration of the analyte in the test solution as read from the calibration graph.

Results and Discussion

Compared with the conditions in the method previously described for the determination of methyldibromo glutaronitrile (1), the conditions in this method are slightly different. The detection potential was decreased from –0.6 to –0.4 V. At this potential, methyldibromo glutaronitrile, bronopol, and bronidox can be detected reliably. Bronopol and bronidox are at the plateau of clearly defined waves, although the potential is not quite at the plateau for methyldibromo glutaronitrile. Previous investigations have shown, however, that at –0.6 V the assay of methyldibromo glutaronitrile is sensitive to variations in the measuring potential, possibly because of increased background current. At this lower potential, the results for methyldibromo glutaronitrile are no longer sensitive for small changes in the detection potential so that the modification actually improves the ruggedness of the method as far as methyldibromo glutaronitrile is concerned. In addition, minor modifications were made in the preparation of standard solutions and the calibration procedures, and the sample cleanup procedure also was changed.

Average retention times under the conditions described were 3.6 min for bronopol, 8.1 min for bronidox, and 9.3 min for methyldibromo glutaronitrile (Figure 1). When the mobile phase is degassed and recycled over several days, retention times may increase because of evaporation of acetone from the mobile phase. Because identification and integration are based on retention times, recycling the mobile phase over longer periods is not recommended and calibration should be performed regularly. A typical standard curve for bronopol concentrations from 0.25 to 4 mg/100 mL gave a correlation coefficient of 0.9997 based on peak area. For bronidox the correlation coefficient was 1.000, and for methyldibromo glutaronitrile, it was 0.9995.

Recovery experiments were performed with a cleansing milk (previously analyzed to ensure that no analytes were present) spiked to levels of approximately 0.03% for each analyte. Recoveries, measured as the average of 10 independent extraction experiments, were 100.4% [relative standard deviation (RSD), 0.43%] for bronopol, 97.1% (RSD, 0.83) for bronidox, and 98.4% (RSD, 1.7) for methyl glutaronitrile. For all analytes, the detection limit was better than 0.002%.

Estimates of the repeatability were obtained by performing 10 replicate analyses on commercial samples. The repeatability for bronopol measured in an emulsion containing 0.047% was 0.0027%, (RSD, 1.99%). For bronidox, the repeatability was 0.0014% (RSD, 1.86%) when measured from a lotion containing 0.027% bronidox, and for methyldibromo glutaronitrile, the repeatability was 0.0019% (RSD, 2.16%) when measured from an emulsion with 0.031% of the analyte.

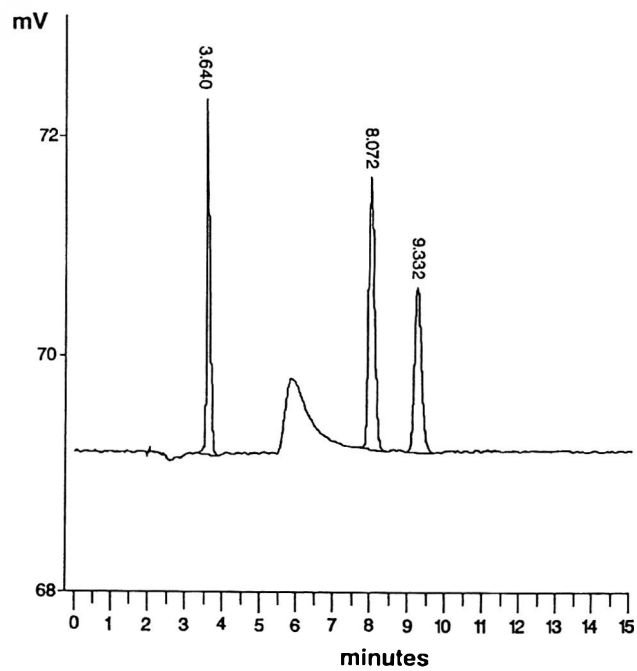


Figure 1. Chromatogram of standard solution of the analytes (9.6 μg/mL of each). Conditions are as described in the text. Retention times: bronopol, 3.6 min; bronidox, 8.1 min; and methyldibromo glutaronitrile, 9.3 min.

Table 2. Results of ruggedness test: differences between results of the experiments at different levels for each factor[a]

Factor		Calculation	Difference between factor levels, δ		
			Results, % ($\times 10^{-3}$)		
			Bronopol	Bronidox	MDBGN
A or a	Amount of sample, g	¼ ((s + t + u + v) − (w + x + y + z))	1.48	1.26	0.97
B or b	Heating time in water bath during extraction, min	¼ ((s + t + w + x) − (u + v + y + z))	1.19	−0.06	0.35
C or c	Shaking time during extraction, min	¼ ((s + u + w + y) − (t + v + x + z))	0.04	0.27	−0.12
D or d	Composition of extraction solvent, v/v (water–methanol)	¼ ((s + t + y + z) − (u + v + w + x))	0.08	0.99	0.70
E or e	Column oven temperature, °C	¼ ((s + u + x + z) − (t + v + w + y))	−0.22	−0.23	−0.55
F or f	Pulse time of electrochemical detector, ms	¼ ((s + v + w + z) − (t + u + x + y))	0.21	1.31	0.71
G or g	Working potential, V	¼ ((s + v + x + y) − (t + u + w + z))	1.02	1.13	0.73

[a] From the results of the 8 determinations listed in Table 1, the influence of each factor is calculated as the difference between the experiments at the 2 factor levels (calculation). A factor has a significant influence on the result if the difference between the results at the 2 factor levels exceeds $\sqrt{2}\sigma$. Critical values are 0.00134 for bronopol, 0.00072 for bronidox, and 0.00094 for methyldibromo glutaronitrile.

The method was subjected to a ruggedness test to assess its sensitivity for small changes in conditions according to the scheme given by Youden and Steiner (11). Variables investigated included sample amount, extraction time and temperature, composition of extraction solvent, LC column oven temperature, and electrochemical detection parameters (Table 1). The latter were included because the determination of methyldibromo glutaronitrile appeared sensitive to variations in the detection potential in a ruggedness test performed on the previous version of the method (1). Experiments for the ruggedness test were performed on the spiked sample described earlier.

Table 1 lists the investigated factors, the factor level settings, and the results of the 8 experiments performed in the ruggedness test. The differences between the factor levels were purposely made large to clearly identify the parameters that influence the results, and they exceed the differences among parameter settings that would be expected to exist among different laboratories. The standard deviations (s) of the 8 individual results found were 0.00117 for bronopol, (RSD, 3.6%), 0.00127 for bronidox (RSD, 4.2%), and 0.00092 for methyldibromo glutaronitrile (RSD, 3.0%) (Table 1).

Table 2 lists the differences (δ) between the results of the experiments performed at the 2 levels for each factor. A large difference for a factor implies that variations in the setting of this factor have a large influence on the results. The effect of a factor can be considered significant ($P < 0.05$) when δ exceeds $\sqrt{2}\sigma$. The critical values are 0.00134 for bronopol, 0.00072 for

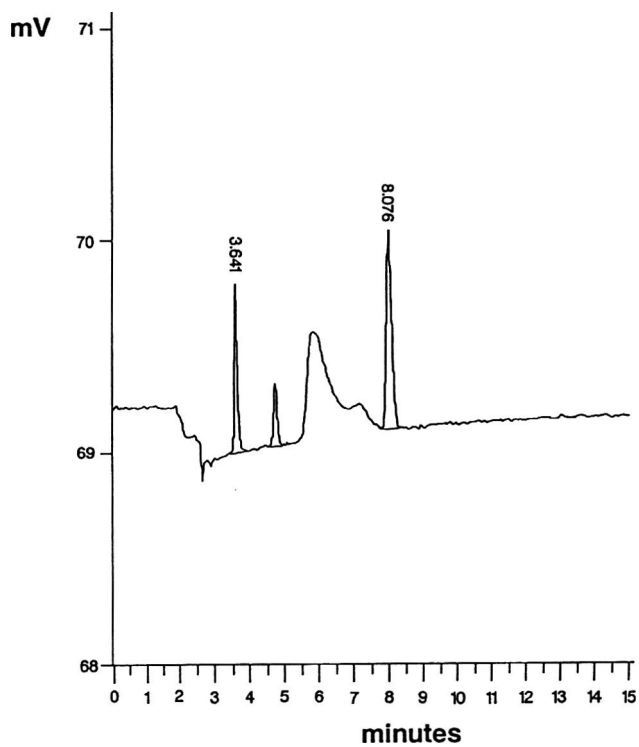


Figure 2. Typical chromatogram of shampoo sample containing 0.013% bronopol (3.6 min) and 0.019% bronidox (8.1 min). Conditions are as described in the text.

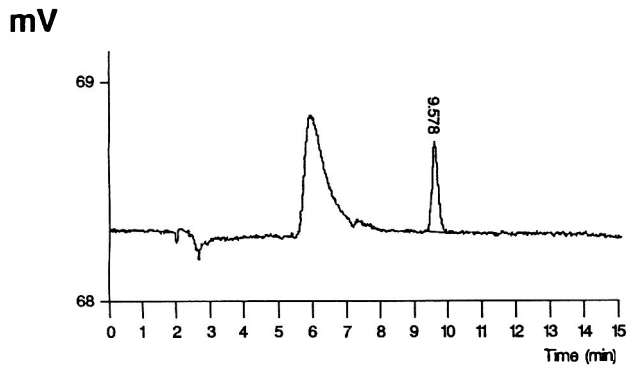


Figure 3. Typical chromatogram of shampoo sample containing 0.014% methyldibromo glutaronitrile (9.6 min). Conditions are as described in the text; mobile phase was recycled.

bronidox, and 0.00094 for methyldibromo glutaronitrile. When these values are compared with the differences (δ) in Table 2, a significant effect is observed for sample amount for all analytes. In addition, results for bronidox are influenced by detection parameters and composition of extraction solvent. These results, however, were obtained with exaggerated variations in factor levels, a situation that is not likely to be observed in practice. With such variations, the RSD values were between 3.0% for methyldibromo glutaronitrile and 4.2% for bronidox (Table 1). These values indicate (and probably overestimate) the reproducibility that would be expected when the method is used by different laboratories under different conditions. Such standard deviations are wholly acceptable at this concentration level.

The method has been in regular use in our laboratory during the previous half year. During this period, 138 samples were analyzed: aqueous products (like shampoos and bath foams), and emulsions (like vanishing creams and milks). The method proved easy to use; interferences have not been observed. Typical chromatograms of samples are shown in Figures 2 and 3. Bronopol was identified in 14 samples (about 10%) in concentrations between 0.006 and 0.07%. Bronidox was found in only 4 samples; 3 samples were shampoos or bath foams, in which bronidox was combined with bronopol. The remaining sample was a baby hair lotion, in which the use of bronidox is prohibited. Methyldibromo glutaronitrile was present in 27 (20%) of the samples in concentrations between 0.005 and 0.033%. Samples containing this preservative included aqueous products, creams, moisturized paper tissues, and sun protection cosmetics.

References

(1) Rooselaar, J., & Weyland, J.W. (1993) *Int. J. Cosmetic Sci.* **15**, 23–32

(2) de Kruijf, N., Rijk, M.A.H., & Schouten, A. (1987) TNO-report A 87.286/261300, CIVO-TNO, Zeist, The Netherlands

(3) Wojciechowsky, M., & Osteryoung, J. (1985) *Anal. Chem.* **57**, 927–933

(4) Product Information, Euxyl K400, Schulke & Mayr GmbH, Norderstedt, Germany

(5) Commission Directive 90/207/EC (1990) Publikatieblad nr L **185**, 92–101

(6) Engelhardt, H., & Klinkner, N. (1985) *Chromatographia* **20**, 559

(7) Croshaw, B., & Holland, V.R. (1984) in *Cosmetic and Drug Preservation*, J.J. Kabara (Ed.), Marcel Dekker, Inc., New York and Basel, pp. 31–62

(8) Liem, D.H. (1975) *Anti-Microbiele Stoffen in Cosmetica*, IGB/KvW Enschede, The Netherlands

(9) Henkel & Cie (1981) *Quantitative Determination of Bronidox in Shampoos by Means of HPLC*, Henkel information sheet, Düsseldorf, Germany

(10) Mattissek, R.Z. (1983) *Lebensmittel. Unters. Forsch.* **177**, 271

(11) Youden, W.J., & Steiner, E.N. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

DRUGS, COSMETICS, FORENSIC SCIENCES

Confirmation of Leucogentian Violet in Chicken Fat by Gas Chromatography/Mass Spectrometry

ROGER T. WILSON
U.S. Department of Agriculture, Food Safety and Inspection Service, Midwestern Laboratory, PO Box 5080, St. Louis, MO 63115
JOHN WONG and JOHN JOHNSTON [1]
U.S. Department of Agriculture, Food Safety and Inspection Service, Western Laboratory, PO Box 4008, Alameda, CA 94501
ROBERT EPSTEIN [2]
U.S. Department of Agriculture, Food and Safety and Inspection Service, Chemistry Division, Washington, DC 20250
DAVID N. HELLER
U.S. Food and Drug Administration, Center for Veterinary Medicine, BARC-East, Bldg 328A, Beltsville, MD 20705

A gas chromatographic/mass spectrometric (GC/MS) procedure for confirming the identity of leucogentian violet (LGV) in chicken fat was developed for regulatory application. The unused portion of the extract remaining from a determinative procedure was back-extracted into an organic phase, concentrated, and analyzed by GC/MS. Confirmation of the identity of LGV was based on matching the retention times and relative abundances of 6 ions in the extract to corresponding values obtained for the LGV standard. The procedure was validated by replicate analyses of negative control, fortified control, and residue-incurred chicken fat. The presence of LGV was confirmed by the GC/MS procedure in all samples found to contain LGV by prior liquid chromatographic analyses. There were no interferences in the control samples.

The U.S. Food and Drug Administration (FDA) recently revoked its interim policy permitting the use of gentian violet (GV) at levels of up to 8 ppm as a mold inhibitor in poultry feed (1). Leucogentian violet (LGV) is a nonpolar metabolite of GV that accumulates in the fat of poultry raised on GV-containing feed. The analysis of LGV by liquid chromatography (LC) with electrochemical (2) or photometric (3) detection has been described. More recently, procedures for the determination of LGV in chicken fat by LC with electrochemical (4) or ultraviolet (5) detection have been developed. The LC procedures were validated for regulatory use at the 10 ppb level in a recent multilaboratory trial (6). Regulatory action is further supported by a procedure that firmly identifies the presence of LGV if a violation is found. Although mass spectrometry has been applied to the analysis of the GV parent drug (7, 8), mass spectral analysis of the LGV metabolite has not been reported to date.

The proposed gas chromatographic/mass spectrometric (GC/MS) procedure uses the extract remaining from the determinative LC procedures (4, 5). The procedure was developed by analysts at the USDA Food Safety and Inspection Service and was validated in the Beltsville laboratory of the FDA Center for Veterinary Medicine. The validation samples included 5 controls, 8 samples containing incurred residues, and 15 fortified samples (5 replicates at 3 levels: 5, 10, and 20 ppb). The residue-incurred samples were found to contain LGV at 5–30 ppb by LC. At least 1 mL of each extract remained after the LC analyses.

Experimental

Leucogentian violet standard was obtained from the Sigma Chemical Co. (Cat. No. L-5760, St. Louis, MO). A stock solution (100 ng/μL) was prepared by accurately weighing 10 mg LGV standard, transferring to a 100 mL volumetric flask, and diluting to volume with methanol. Injection standards were prepared by diluting 10, 25, 50, or 100 μL stock solution to a volume of 1 mL with acetonitrile. The resulting solutions contained LGV at 1, 2.5, 5, or 10 ng/μL and were equivalent to extracts of fat containing LGV at 2, 5, 10, or 20 ng/g (ppb).

The extracts remaining after LC analysis were stored in injection vials at room temperature up to 14 days prior to GC/MS analysis. A 1 mL portion of each extract (dissolved in acetonitrile–water [1 + 1]) was transferred to a 15 mL glass centrifuge tube. Toluene (2 mL) was added. The tube was shaken on a vortex mixer for 1 min and centrifuged at 1000 rpm for 10 min. The upper (organic) phase was carefully transferred with a Pasteur pipet to a glass culture tube. The solution was evaporated to dryness under a nitrogen stream at ambient temperature.

The dried residue was dissolved in 120 μL acetonitrile, and the tube was shaken on a vortex mixer for 15 s. The solution

Received April 22, 1993. Accepted by JW October 4, 1993.

[1] Current address: U.S. Department of Agriculture, Animal and Plant Health Inspection Service, PO Box 25266, Denver, CO 80225.

[2] Current address: U.S. Department of Agriculture, Agricultural Marketing Service, PO Box 94656, Washington, DC 20090-6456.

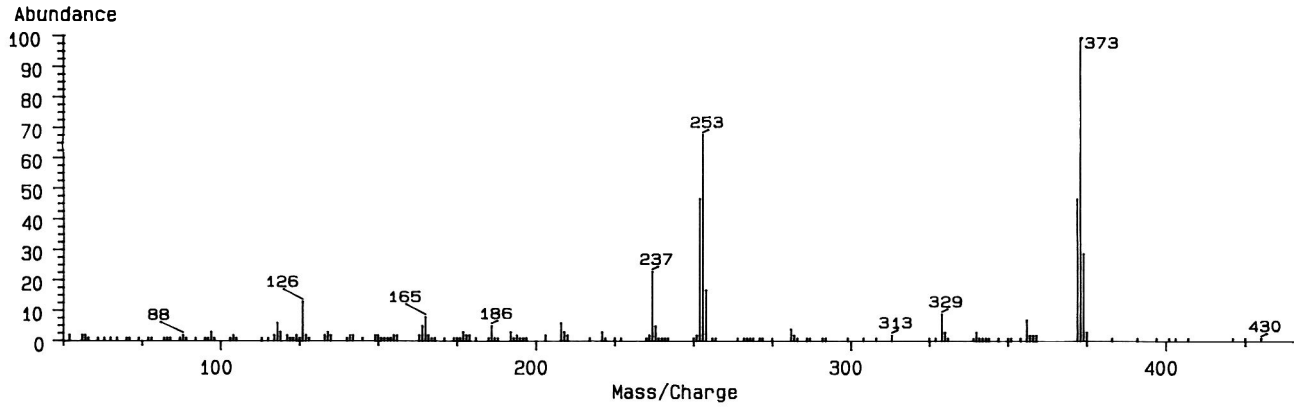


Figure 1. Electron ionization mass spectrum of leucogentian violet.

was transferred with a Pasteur pipet to a conical injection vial, then evaporated to dryness under a gentle nitrogen stream at ambient temperature. Acetonitrile (20 µL) was added, and the vial was capped and shaken on a vortex mixer for 5 s. A 2 µL portion was injected for GC/MS analysis.

Analyses were carried out on a Model 5890 gas chromatograph interfaced to a 5970A mass-selective detector (MSD) and controlled by a UNIX Chemstation data system (Hewlett-Packard, Palo Alto, CA). The splitless injector was used, with a 1 min purge delay. Injector and transfer line temperatures were 270°C. Chromatographic separation was performed on a DB-1 (100% methyl silicone) capillary column, 30 m × 0.25 mm id, with a 0.25 µm film thickness (J&W Scientific, Folsom, CA). The column temperature was held at 150°C for 1 min, ramped at 16°C/min to 300°C, and held for 20 min. The linear velocity of the helium carrier gas at 300°C was measured at 25.5 cm/s. LGV eluted at about 18 min.

The MSD was operated in electron ionization (EI) mode. The ionization energy was 70 eV, and the emission current was 300 µA. The source temperature was nominally 200°C. The MSD was tuned for unit resolution, with a peak width of 0.5 amu at half height. The multiplier gain was 2000–2200 V during this study. The MSD was calibrated with the data system's "autotune" procedure on days 1, 2, and 4 of the validation. The autotune normalizes ion abundances of the tuning compound to target values. On day 3, the autotune normalization was inadvertently not done, although the mass axis was calibrated as on the other days.

Data acquisition consisted of selected-ion monitoring at *m/z* 373.20 (molecular ion), 372.20, 253.15, 252.15, 237.15, and 126.10 for a dwell time of 200 ms each. The scan time was approximately 1.25 s/scan. These *m/z* values represented the major ions in the EI mass spectrum of LGV (Figure 1). The mass defects were taken from measured masses reported in full-scan acquisition. Proposed structures of these and other ions are shown in Figure 2. The ions at *m/z* 372 and 252 were most likely formed by loss of hydrogen from the central carbon atom to form a resonance-stabilized cation. Assignment of the ion at *m/z* 126 as doubly charged was confirmed by observation of an isotopic ion at half mass. Relative abundances were calculated from the peak areas of each selected ion chromatogram, after assigning a value of 100% to the most abundant ion.

Sets of standards at various levels were injected before and after all samples. Standard levels were selected to cover the range of values measured by the LC procedure. An acetonitrile solvent blank was injected prior to every residue-incurred sample to demonstrate that LGV carryover did not occur.

Results and Discussion

All the validation data are presented in Table 1. Each sample is compared individually with the average of all LGV standards run each day. Only the difference between each test sample and the average of standards is shown to simplify comparison. The retention times of LGV in the total-ion chromatograms differed by less than 0.05 min from those of standards run the same day, well within the normally acceptable limit of 2% variation (9). The use of 3 ions or more for selected-ion monitoring conforms to the recommendations of Sphon (10). The reproducibility of relative abundance ratios was acceptable. Relative abundances in test samples were required to match those in standards run the same day within a margin of 10 percentage point units. This criterion was met in every case. The height of the LGV peak in each single-ion chromatogram was at least 3 times higher than

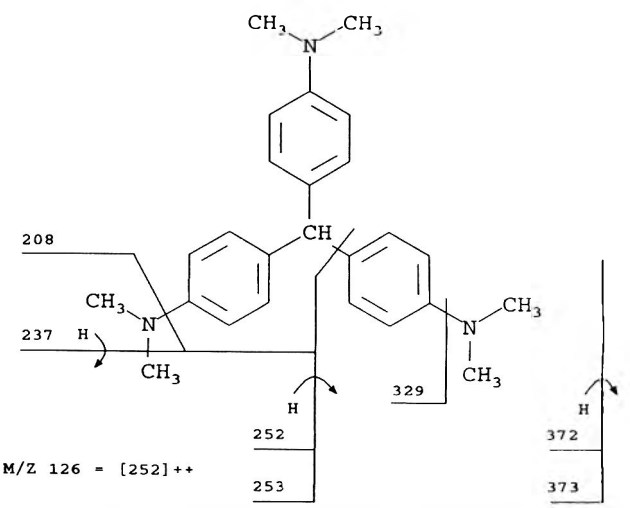


Figure 2. Proposed fragmentation of leucogentian violet in electron ionization.

Table 1. Validation data for GC/MS confirmation of LGV

Validation day	Sample	Retention time, min	Relative abundance (%) of ion at *m/z* or difference from standard (%)[a]					
			126	237	252	253	372	373
Day 1	Standards 5, 10, 20 ppb (av.)	17.862	13.3	26.5	50.6	77.7	41.8	100.0
	Fortified samples							
	5 ppb, Rep 1	−0.013	−2.5	−0.5	−0.4	0.6	−1.6	0.0
	10 ppb, Rep 1	−0.013	0.1	−0.7	−0.2	1.0	−0.4	0.0
	20 ppb, Rep 1	−0.008	0.6	0.5	1.4	1.5	0.8	0.0
	Incurred sample 1	−0.007	0.3	−0.3	−1.3	1.7	−0.1	0.0
Day 2	Standards 5, 10, 20 ppb (av.)	17.842	15.8	27.9	52.7	80.9	42.0	100.0
	Fortified samples							
	5 ppb, Rep 2	−0.028	−0.6	−0.5	−0.5	2.1	−1.2	0.0
	10 ppb, Rep 2	−0.011	−1.9	−3.2	−4.2	−7.8	−2.0	0.0
	20 ppb, Rep 2	−0.034	−0.2	−0.3	−0.7	−2.2	0.0	0.0
	Incurred samples							
	Incurred sample 3	−0.038	0.3	−1.3	0.8	0.4	−0.6	0.0
	Incurred sample 4	−0.040	0.3	0.5	0.0	−0.4	0.4	0.0
Day 3	Standards 2, 10, 20 ppb (av.)	17.755	19.7	35.8	62.8	94.2	41.4	99.4
	Fortified samples							
	5 ppb, Rep 3	−0.009	−3.8	−1.2	1.7	5.8	−2.9	−4.6
	10 ppb, Rep 3	−0.004	−0.7	1.7	2.8	5.8	0.5	−0.1
	20 ppb, Rep 3	−0.001	−0.3	1.0	2.9	5.8	0.4	0.0
	5 ppb, Rep 4	−0.005	−3.7	1.4	1.2	5.8	−4.4	−6.4
	10 ppb, Rep 4	0.017	−2.5	−2.0	−4.1	−4.3	−1.1	0.6
	20 ppb, Rep 4	−0.004	0.3	1.6	2.7	5.5	1.1	0.6
	Incurred sample 5	−0.004	−1.3	0.7	1.1	5.8	−1.5	−3.6
Day 4	Standards 5, 10 ppb (av.)	17.728	12.4	29.7	53.8	82.1	43.1	100.0
	Fortified samples							
	5 ppb, Rep 5	0.003	−2.2	−0.2	−3.6	−1.0	−3.1	0.0
	10 ppb, Rep 5	0.005	0.0	−1.2	0.9	1.8	0.3	0.0
	20 ppb, Rep 5	0.009	0.5	−0.4	−0.5	1.0	0.1	0.0
	Incurred samples							
	Incurred sample 2	−0.002	0.8	0.9	1.6	3.1	0.9	0.0
	Incurred sample 6	0.000	1.1	0.5	1.1	1.5	1.1	0.0
	Incurred sample 7	0.004	1.3	0.7	0.4	1.0	0.7	0.0
	Incurred sample 8	−0.006	1.4	0.7	0.4	1.4	1.4	0.0

[a] Values are relative abundances for standards and differences from standard for samples.

the apparent peak height in blank control samples. Because there were no apparent matrix interferences at the retention time of LGV, the noise level corresponded to instrument background.

The presence of LGV was confirmed in all fortified and residue-incurred samples. None of the control samples produced a false-positive response for LGV. There were no interferences at the retention time of LGV in any control sample or solvent blank (data not shown).

When the autotune normalization was carried out (days 1, 2, and 4), the LGV relative abundances showed little day-to-day variation. Normalization involves adjusting the response in one mass range relative to another range to meet target values for relative abundance. When the normalization was not done (day 3), LGV abundances returned to a slightly different pattern. The ions at *m/z* 253 and 373 were nearly of the same intensity, and in a few cases, *m/z* 253 was the base peak instead of *m/z* 373. For this reason, the average relative abundance of the ion at *m/z* 373 in standards was calculated to be 99.4% instead of 100%.

Selected-ion chromatograms at the retention time of LGV for standard, control tissue, fortified tissue, and residue-incurred tissue are shown in Figures 3–6. An additional metabolite of GV identified as leucomethyl violet (LMV) was observed in the residue-incurred samples. The mass spectrum of LMV from an incurred fat sample is shown in Figure 7. The LMV molecular ion appears at *m/z* 359, corresponding to LGV with hydrogen substituted for one methyl group. LMV fragment ions appear at many of the same *m/z* values as LGV, but not at *m/z* 372 or 373. The retention time of LMV was approximately 0.1 min less than that of LGV under the experimental conditions described. The resolution from LGV was sufficient to enable the peaks to be integrated separately (Figure 6). The LMV peak was less visible in the fortified samples, but not in

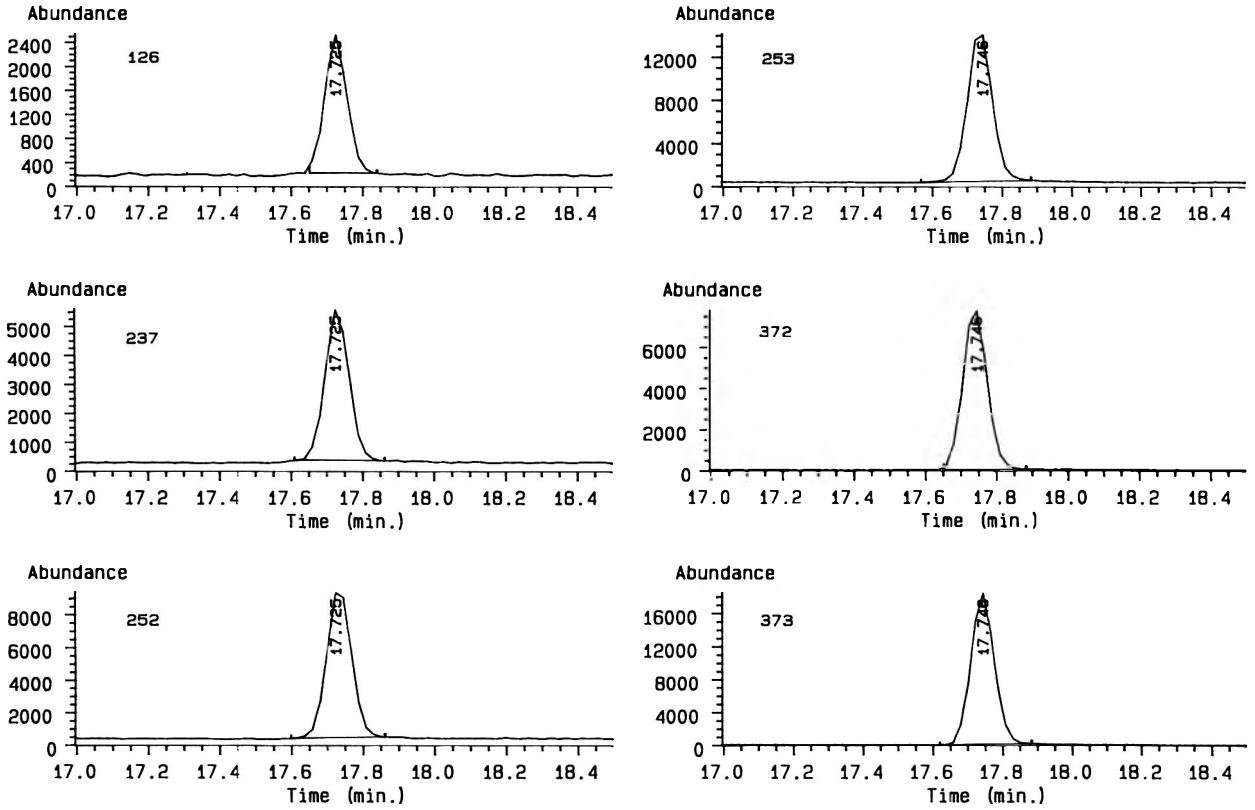


Figure 3. Single-ion chromatograms of 5 ng LGV standard, equivalent to 10 ppb LGV in fat.

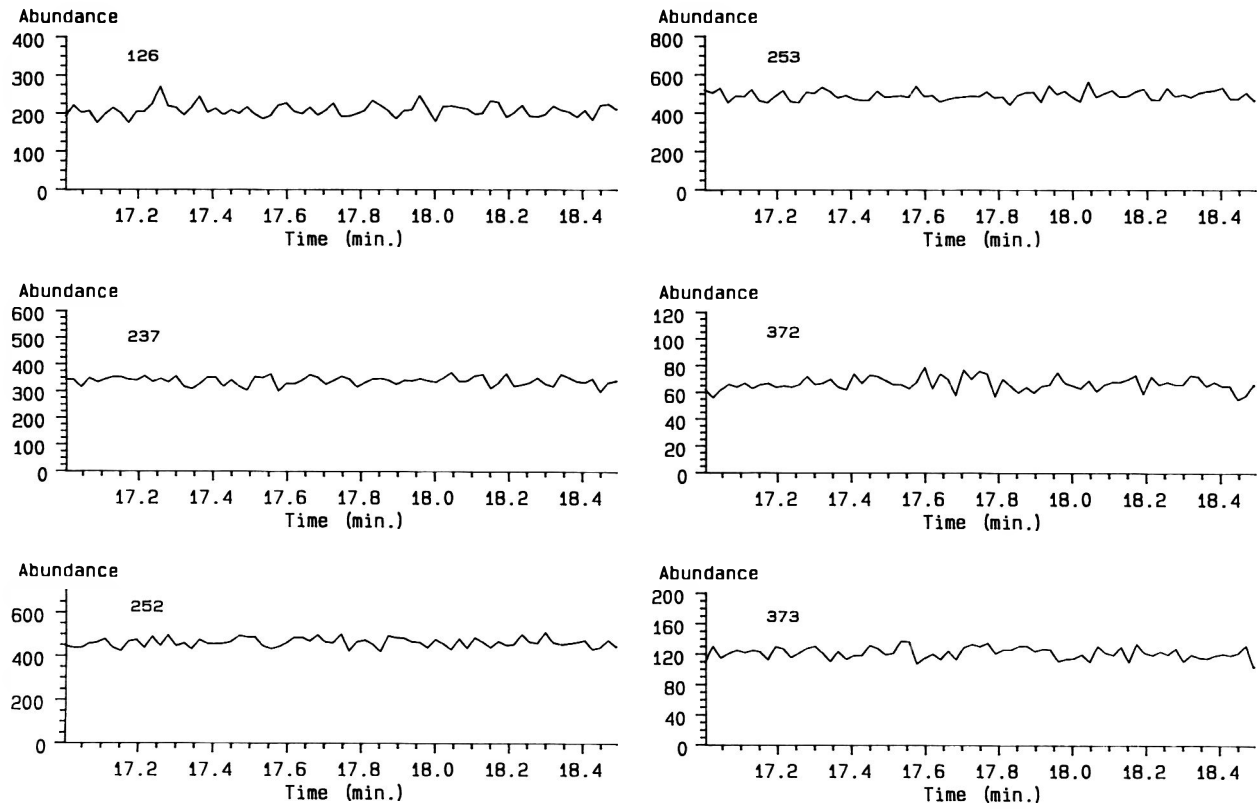


Figure 4. Single-ion chromatograms of control fat extract.

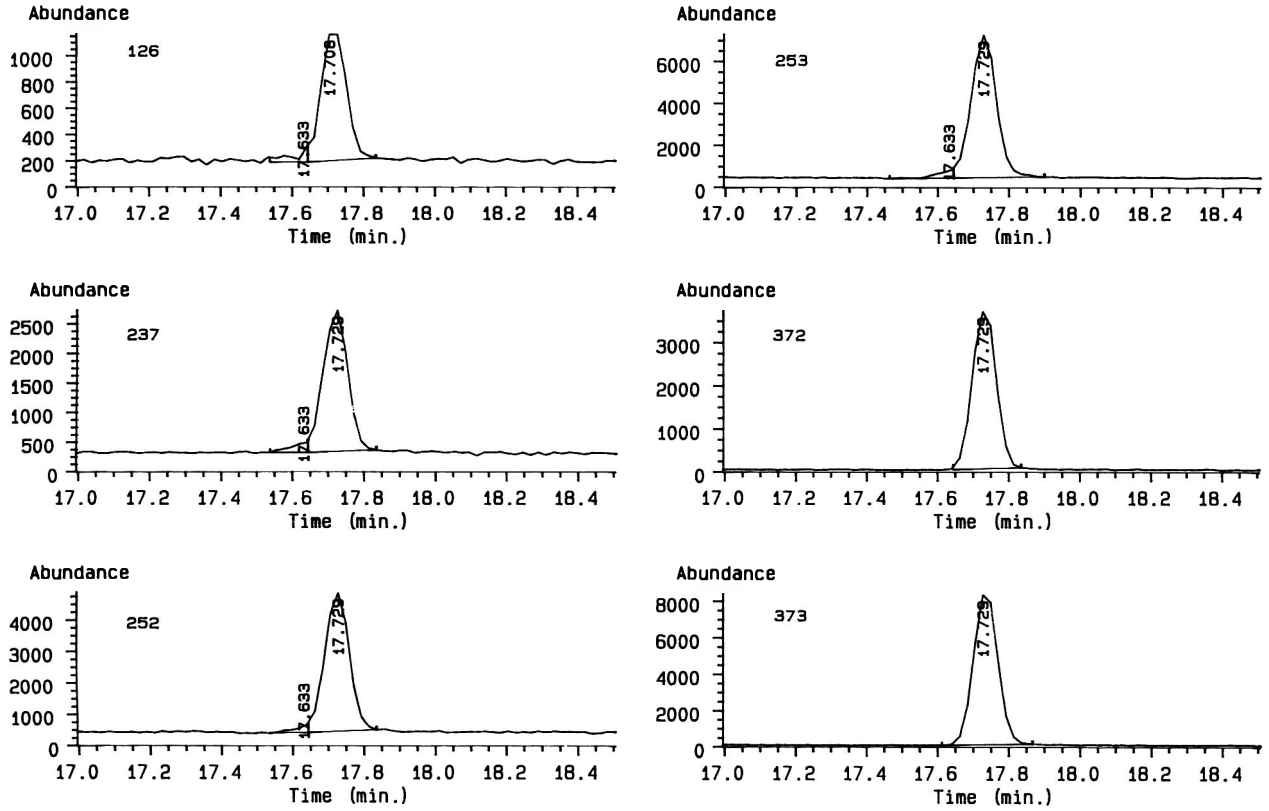


Figure 5. Single-ion chromatograms of extract from fat fortified with LGV at 10 ppb.

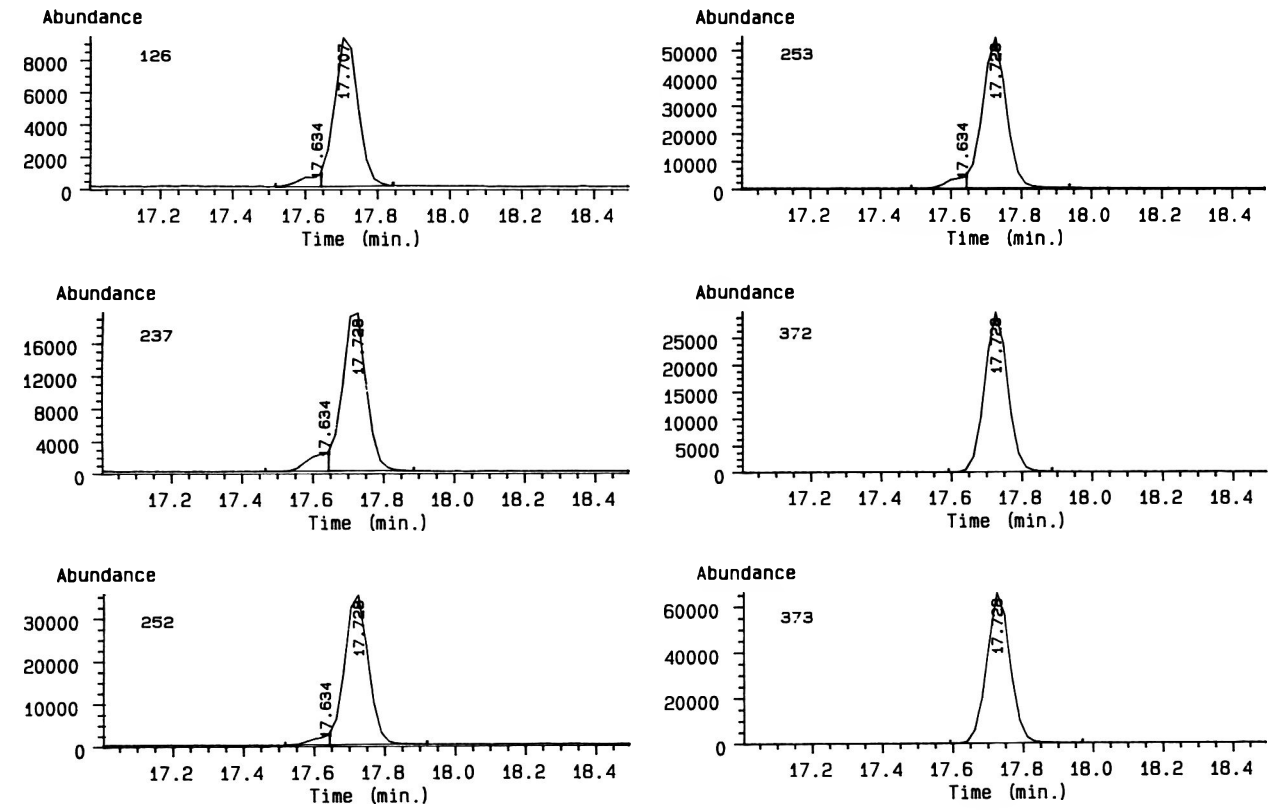


Figure 6. Single-ion chromatograms of extract from residue-incurred fat found to contain LGV at approximately 30 ppb.

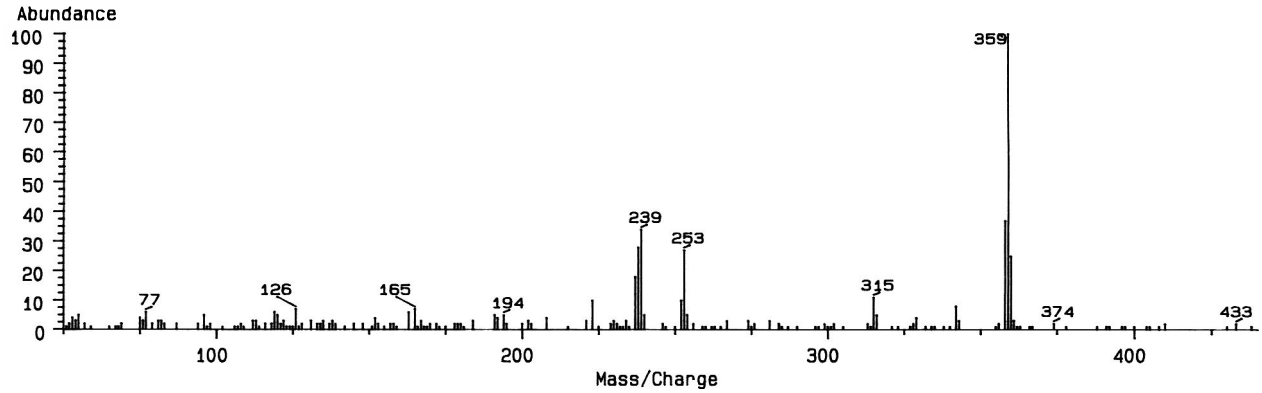


Figure 7. Electron ionization mass spectrum of leucomethyl violet.

the comparison standards. It was detected in standards only if injections of several hundred nanograms were made. Because LMV was detected in extracts of tissues fortified at a low level, some LGV must be converted to LMV during the extraction process.

After the completion of the validation study, it was shown that a final column temperature of 270°C rather than 300°C was sufficient to fully resolve LGV from LMV. The retention time was increased by about 8 min. An example is shown in Figure 8. These conditions are recommended for future application of this procedure.

A DB-5 capillary column (5% phenyl–95% methyl silicone, J&W) was tested with a residue-incurred sample. LGV and LMV were not separated on this column. Although the level of interference was fairly low, use of the DB-5 column is not recommended.

The ions monitored for the validation included the 6 most abundant and structurally significant fragment ions observed with the equipment and conditions used. Other diagnostic ions could have been used as well, including those at *m/z* 208 and 329. The procedure confirmed the identity of LGV in all fat samples fortified at 5 ppb. All ions except that at *m/z* 126 appeared with signal-to-noise levels well above 3:1 in the samples fortified at 5 ppb. The procedure, thus, may be amenable to application below 5 ppb by not using the ion at m/z 126 or by injecting more extract.

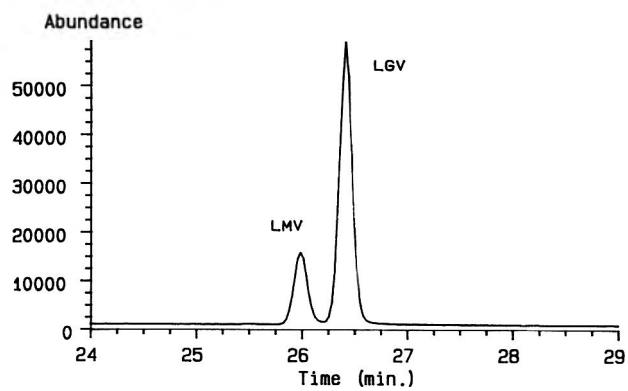


Figure 8. Resolution of leucogentian violet and leucomethyl violet by using a final oven temperature of 270°C: total-ion chromatogram from selected-ion monitoring of a residue-incurred fat sample.

Conclusions

A simple GC/MS procedure for confirming the identity of LGV in chicken fat was developed for regulatory application. Selected-ion monitoring with a GC/MS instrument provided sufficient specificity at the 10 ppb level for regulatory application. The procedure was validated by a second analyst in a different laboratory. There are many positive attributes to the GC/MS procedure. It uses the extract remaining from an LC determinative procedure and can be carried out on a benchtop mass spectrometer.

Acknowledgments

Extracts for the LC procedures were prepared by Patricia G. Schermerhorn of the FDA Center for Veterinary Medicine, Division of Residue Chemistry.

References

(1) *Fed. Regist.* (1991) **56**, 40502
(2) Roybal, J.E., Munns, R.K., Hurlbut, J.A., & Shimoda, W. (1989) *J. Chromatogr.* **467**, 259–266
(3) Allen, J.L., & Meinertz, J.R. (1991) *J. Chromatogr.* **536**, 217–222
(4) Munns, R.K., Roybal, J.E., Hurlbut, J.A., & Shimoda, W. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 705–708
(5) Munns, R.K., Roybal, J.E., Hurlbut, J.A., & Shimoda, W. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 705–708
(6) Schermerhorn, P.G., & Munns, R.K., *J. AOAC Int.*, in press
(7) Talwar, S.K. (1985) *Ind. J. Pharm. Sci.* **47**, 26–27
(8) Chait, B.T., & Field, F.H. (1985) *J. Am. Chem. Soc.* **107**, 6743–6744
(9) Cairns, T., Siegmund, E.G., & Stamp, J.J. (1989) *Mass Spectrom. Rev.* **8**, 93–117
(10) Sphon, J.A. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1247–1252

FOOD BIOLOGICAL CONTAMINANTS

Extraction of Light Filth from Bean Paste: Collaborative Study

JOHN R. BRYCE and LARRY E. GLAZE
U.S. Food and Drug Administration, Division of Microanalytical Evaluations, Washington, DC 20204

Collaborators: D.W. Bakker; C.K. Cook; M.J. Cote; R.R. Galacci; M. Ghobrial; J. Luanprida; R.E. Olson; J.Palau; H.E. Semey; M.L. Silva

Bean paste is a popular Asian food frequently imported to the United States. The main varieties are: hot bean and blackbean, which are used in pastry fillings; and soybean paste, which is usually used as a condiment. A new method was developed for the extraction of light filth from bean pastes containing beans and flour, and from hot bean paste containing red pepper. A 100 g test portion is boiled in tap water containing Igepal DM–710 and CO–730 and washed with hot tap water on a No. 230 sieve. The residue is transferred to a beaker and boiled in isopropanol in a reflux apparatus. The mixture is transferred to a No. 230 sieve. The residue is washed again, transferred to a 2 L trap flask with 40% isopropanol, boiled with magnetic stirring, cooled, and trapped off with flotation liquid [mineral oil–heptane (85 + 15, v/v)]. Ten laboratories participated in a collaborative study validating the extraction method for the detection of light filth. Average recoveries were 94.9 and 82.8% for insect fragments and rat hairs, respectively. The method has been adopted first action by AOAC INTERNATIONAL.

Import of bean paste, which is a traditional food in many parts of Asia, has increased in the United States in recent years. The 3 main varieties of bean paste sold in the United States are: hot bean, blackbean, and soybean. The hot bean and blackbean varieties are primarily used as pastry fillings in Asian cuisine. Soybean paste is usually used as a condiment. These products typically include a bean (i.e., kidney bean, horsebean, or soybean), flour (i.e., rice or wheat), and, in the case of hot bean paste, red pepper.

Submitted for publication April 1, 1993.

The recommendation was approved by the Committee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Board Actions" (1993) *The Referee* **17**, September issue.

This report was presented at the 106th AOAC INTERNATIONAL Annual Meeting, September 2, 1992, Cincinnati, OH.

Collaborative Study

In the study, 2 retail jars each containing 500 g bean paste (soybean with wheat flour variety) and 8 vials containing spike material mixed in granular sucrose were sent to each collaborator. The 2 jars of bean paste provided sufficient amount of test material for 2 practice runs and 6 test portions of 100 g each. Two spike vials were identified as practice samples and the 6 coded vials for the study were duplicates of 3 spike levels. The low spike level consisted of 5 insect fragments (elytral squares of *Tribolium confusum*, ca 0.5 × 0.5 mm) and 5 rat hairs (cut to 2–3 mm); the middle and high spike levels consisted of 15 and 30 insect fragments and 10 and 15 rat hairs, respectively. The collaborators were instructed to use part of the 400 mL hot tap water to wash out the contents of a spike vial into a 1.5 L beaker containing a weighed test portion. Discrepancies between collaborator and Associate Referee counts were resolved by an experienced analyst acting as an impartial third party.

993.28 Light Filth in Bean Paste—Flotation Method

First Action 1993

(Applicable to determination of light filth in bean paste.)

Method Performance:

See Table **993.28** for method performance.

A. Principle

Bean paste sample is treated with surfactants and is wet-sieved with hot water to remove surfactants and some

emulsified fats and oils. Residue is defatted with isopropanol, wet-sieved, boiled in 40% isopropanol, cooled, and trapped 2× with flotation liquid in aqueous 40% isopropanol to recover light filth.

Light filth, such as insect fragments and hairs, is unaffected by emulsification and defatting steps. In contrast to bean paste, light filth is attracted to oil phase in trapping procedure. When oil phase is trapped off, light filth is assessed microscopically.

Table 993.28. Method performance for determination of light filth in bean paste by flotation method[a]

Spike added	$\bar{X}$	s_r	s_R	RSD_r, %	RSD_R, %
			Rat hairs		
5	4.55(4.50)	1.63(1.00)	1.63(1.05)	35.8(22.2)	35.8(23.4)
10	8.85(8.65)	2.04(1.56)	2.09(1.56)	23.0(18.1)	23.6(18.1)
15	12.15(11.70)	2.20(1.67)	2.28(1.85)	18.1(14.3)	18.8(15.8)
			Insect fragments		
5	4.35(4.10)	0.87(1.00)	1.05(1.02)	19.9(24.4)	24.1(24.9)
15	14.20(14.25)	1.22(1.32)	1.45(1.53)	8.6(9.3)	10.2(10.7)
30	28.00(29.11)	1.87(1.05)	2.81(1.05)	6.7(3.6)	10.1(3.6)

[a] Third party counts in parentheses.

B. Apparatus

(**a**) *Sieve.—(1)* No. 230 plain-weave, **945.75B(r)**; and *(2)* sieve handle, **945.75B(t)** (*see* "Changes in Official Methods of Analysis" 3rd Supplement, 1992, p. 128).

(**b**) *Reflux apparatus.*—**975.49A(e)**.

(**c**) *Wildman trap flask.*—2 L, **945.75B(h)**(*4*).

(**d**) *Filter paper.*—Ruled, **945.75B(i)**.

C. Reagents

(**a**) *Surfactant mixture.*—Igepal CO-730, **945.75C(j)**(*1*), and Igepal DM-710, **945.75C(j)**(*2*). Prepare surfactant mixture, DM-710 and CO-730 (1 + 2).

(**b**) *Isopropanol solutions.—(1)* 100%. *(2)* 40% aqueous solution.

(**c**) *Flotation liquid.*—**945.75C(k)**.

D. Isolation

Accurately weigh 100 g bean paste to nearest 0.5 g into 1.5 L beaker. Add ca 400 mL hot tap water and hand-stir 1 min. Bring volume in beaker to 600 mL with hot (55°–70°) tap water. Add 15 mL surfactant mixture, **C(a)**, cover beaker with watch glass, and bring to boil with magnetic stirring, **945.75B(n)**. Remove watchglass and boil for 10 min.

Transfer beaker contents portionwise to sieve, **B(a)**(*1*). Wet-sieve, **970.66B(a)**, between portions. Rinse beaker and transfer washings to sieve. Retain beaker. Wet-sieve, using forceful stream of hot tap water until rinse is clear. Use of sieve handle, **B(a)**(*2*), is recommended. (*Note*: If sieve becomes clogged during sieving, gently tap on side of sieve to drain excess water.) Wet residue on sieve with 100% isopropanol, **C(b)**(*1*), to reduce foam.

Quantitatively transfer residue to retained beaker using 100% isopropanol. Wash sides of beaker with 100% isopropanol from wash bottle; and then fill beaker to 400 mL with 100% isopropanol. Defat contents by boiling 5 min, using reflux apparatus, **B(b)**. Quantitatively transfer contents from reflux apparatus to sieve.

Wet-sieve until rinse is clear. Wash residue on sieve with 40% isopropanol, **C(b)**(*2*), and quantitatively transfer to trap flask, **B(c)**. Wash sides of flask with 40% isopropanol from wash bottle; then fill to 600 mL with 40% isopropanol. Bring to boil and boil gently 10 min with magnetic stirring. Remove from heat and loosen any adhering materials from sides of flask using stopper (wafer) and by washing sides of flask with 40% isopropanol. Cool flask contents to 28°–30° in water bath.

Remove from water bath and add 60 mL flotation liquid, **C(c)**. Stir magnetically 10 min, **970.66B(c)**. Fill flask with 40% isopropanol by pouring slowly down stirring rod. Stir intermittently 10 min and let stand undisturbed 20 min. Gently spin stopper to remove sediment and trap off, **970.66B(b)**, into 400–600 mL beaker, using 40% isopropanol. Add 35 mL flotation liquid to flask and hand-stir with gentle up-and-down motion 30 s. Fill flask to top with 40% isopropanol and let stand undisturbed 20 min. Trap off as before, using 100% isopropanol, and filter entire beaker contents through filter, **B(d)**, using 2 filters if necessary.

Examine microscopically, **945.75B(o)**(2), at ca 30×.

Ref.: *J. AOAC Int.* **77**, 1143 (1994).

Results and Discussion

Results for recoveries of rat hairs are shown in Table 1. Mean recoveries for the 3 spike levels ranged from 78 to 90% with 82.8% overall (third party counts). Collaborator 3 recovered from each of the 2 low level test portions one hair more than the number of hairs used for spiking. This collaborator noted that the sieve used in the study was back-washed after each analysis, and no other explanation could be found for this result.

Results from recoveries of insect fragments are shown in Table 2. Mean recoveries for the 3 spike levels ranged from 82 to 97% with 94.9% overall (third party counts). The data from Collaborator 1 obtained from the analyses of high-level samples were eliminated as outliers, calculated by the single-Grubbs test. Collaborators 4 and 6 detected in medium-level samples more numbers of insect fragments than used for spiking.

The amount of residue on the extraction papers ranged from light (some analyses of one collaborator) to very heavy (one analysis of another collaborator who used 2 extraction pa-

Table 1. Collaborative study results[a] for recovery of rat hair from bean paste (blind duplicates)

	Spike level					
Lab.	5		10		15	
1	5	2(3)	7(5)	9(10)	9(8)	10(9)
2	3(2)	4(5)	5(8)	10(9)	10	12
3	5(6)	9(6)	9(10)	10(9)	15(14)	7(9)
4	6(5)	5	14(10)	8	14(11)	13
5	5	5	12(10)	10	15(14)	15(13)
6	7(5)	3(4)	10	10(9)	10	12
7	5	3	9(10)	9(8)	15(14)	12
8	3(5)	5(4)	5(6)	8(9)	13(12)	14
9	4	3	8	8	13(12)	11(12)
10	5	4(5)	7	9	10	13
$\bar{X}$	4.55(4.50)		8.85(8.65)		12.15(11.70)	
$\bar{X}$, %	91.0(90.0)		88.5(86.5)		81.0(78.0)	
s_r	1.63(1.00)		2.04(1.56)		2.20(1.67)	
s_R	1.63(1.05)		2.09(1.56)		2.28(1.85)	
RSD_r, %	35.8(22.2)		23.0(18.1)		18.1(14.3)	
RSD_R, %	35.8(23.4)		23.6(18.1)		18.8(15.8)	

[a] Third party counts in parentheses.

pers). Six collaborators used 2–3 extraction papers for some analyses.

Eight collaborators analyzed one test portion at a time. Their extraction times ranged from 2.5 to 4 h per test portion (average 3.19 h). Two collaborators, who analyzed all test portions at one time, had extraction times of 5 and 9 h (average 7 h, or 1.16 h per test portion). Some collaborators included set-up time in the reporting of extraction times. The authors' average extraction time was about 2.5 h per test portion. Plate-counting times of one test portion ranged from 8.5 to 45 min (average 22.1 min).

The collaborative study was performed using one type of bean paste. An intralaboratory study was conducted prior to the collaborative study, using 15 commercially available brand products of bean paste. Analytical results for insect fragments ranged from 93 to 100% (av. 98%) recovery. Results for rat hairs ranged from 87 to 100% (av. 94%) recovery.

Collaborators' Comments

Wording of the method was changed to clarify the procedure. One collaborator had to repeat the study following the correct procedure. At the suggestion of another collaborator,

Table 2. Collaborative study results[a] for recovery of insect fragment spikes from bean paste (blind duplicates)

	Spike level					
Lab.	5		15		30	
1	5	4	12	14	25(26)[b]	19(19)[b]
2	5(4)	5(3)	14	11(10)	29	30
3	4	3(4)	12(15)	15	30	28
4	3	5	17	15	27	29
5	4	4	14(13)	15	30	30(28)
6	5(4)	5	16	15	29	30
7	5	5(3)	15	14	28	26(29)
8	4	1	14(13)	15	28	30
9	5	5	13(14)	13	28(29)	24(30)
10	5	5	15	15	30	30
$\bar{X}$	4.35(4.10)		14.20(14.25)		28.00(29.11)	
$\bar{X}$, %	87.0(82.0)		94.7(95.0)		93.3(97.0)	
s_r	0.87(1.00)		1.22(1.32)		1.87(1.05)	
s_R	1.05(1.02)		1.45(1.53)		2.81(1.05)	
RSD_r, %	19.9(24.4)		8.6(9.3)		6.7(3.6)	
RSD_R, %	24.1(24.9)		10.2(10.7)		10.1(3.6)	

[a] Third party count in parentheses.
[b] Outlier calculated by single-Grubbs test; not included in calculations.

the volume of beaker used to receive the trapped material was changed from "400" to "400–600 mL."

Recommendation

On the basis of the results obtained in the study, it is recommended that the method for the extraction of light filth from bean paste be adopted first action.

Acknowledgments

The authors express their appreciation to the following analysts for participating in this study:

Douglas W. Bakker, Minnesota Dept. of Agriculture, St. Paul, MN

Carolyn K. Cook, U.S. Dept. of Agriculture, Athens, GA

Mark J. Cote, U.S. Food and Drug Administration, Philadelphia, PA

Raymond R. Galacci, U.S. Food and Drug Administration, Baltimore, MD

Mary Ghobrial, Silliker Laboratories, Garwood, NJ

Jiraporn Luanprida, Ministry of Agriculture and Cooperatives, Bangkok, Thailand

Richard E. Olson, U.S. Food and Drug Administration, Kansas City, MO

Juaquin Palau, U.S. Food and Drug Administration, San Juan, PR

Howard E. Semey, U.S. Food and Drug Administration, San Francisco, CA

Mary L. Silva, Rhode Island Dept. of Health, Providence, RI

The authors also thank Jack L. Boese, Division of Microbiology, U.S. Food and Drug Administration, for review of the manuscript and Richard H. Albert, Division of Mathematics, U.S. Food and Drug Administration, for statistical analyses.

FOOD BIOLOGICAL CONTAMINANTS

Chemical Test for Mammalian Feces in Ground Black Pepper: Collaborative Study

HARRIET R. GERBER [1]

U.S. Food and Drug Administration, Division of Microanalytical Evaluations, Washington, DC 20204

Collaborators: J.R. Bryce; M.P. Chaput; G.R. Dzidowski; R.L. Heitzman; M. Langridge; R.E. Olson; M.L. Silva; B.A. Spring; W.A. Sumner; D.Y. Tobias

A collaborative study for determining mammalian feces in ground black pepper was conducted, using a modified version of the AOAC Official Method 986.28 for mammalian feces in grain products. With the proposed method, the presence of alkaline phosphatase, an enzyme found in mammalian feces, was determined by using phenolphthalein diphosphate as the enzyme substrate in a test agar medium containing 1% agar in a borate buffer, pH 9.5. Ground black pepper was stirred in water to extract interfering color. The mixture was filtered and the residue was scattered on plates of liquid test agar. The alkaline phosphatase cleaved phosphate radicals from phenolphthalein diphosphate, generating free phenolphthalein, which appeared as pink to red-purple around the fecal particles in the previously colorless medium. For the 10- and 20-particle spike level, collaborators recovered averages of 12.3 and 24.1 particles, respectively. The experimental background was zero. Collaborators reported that the method was clear and easy to perform.

Submitted for publication January 4, 1993.

The recommendation was approved by the Committee on Microbiology and Extraneous Materials. The method was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Board Actions" (1993) *The Referee* **17**, September issue.

[1] Present address: U.S. Food and Drug Administration, Div. of Planning, Evaluation and Management, 5600 Fishers Lane, Rockville, MD 20857.

There is currently no official method for determining the presence of rodent excreta particles in ground black pepper. A newly developed method based on the corn meal method (1) uses the reaction of alkaline phosphatase, an enzyme found in feces, with the substrate phenolphthalein diphosphate to determine the presence of fecal matter. The reaction generates free phenolphthalein, which in the presence of borate buffer, pH 9.5, appears pink to red-purple. A water extraction is used to remove interfering color from the pepper which would otherwise leach into the test medium and mask the color. The enzymatic reaction makes it possible to visualize the presence of particles smaller than 250 μm. The proposed method is relatively rapid and easy to perform, and uses no hazardous chemicals.

Collaborative Study

Black Pepper

The ground black pepper used in this study was obtained commercially. Test portions (4–10 g) gave no positive reactions, and a zero background level of fecal particles was assumed.

Fecal Particles

Mouse feces were ground, sieved, and collected so that the particles passed through a No. 50 sieve and were retained on a No. 60 sieve. The spike particles used in this study were 97–100% positive in this test (2).

Ruggedness Testing

Ruggedness testing was performed using 8 combinations of the following factors analyzed in duplicate: *(1)* temperatures of liquid test agar, 37° and 47°C; *(2)* amounts of agar per Petri dish, 145 and 155 mL; *(3)* extraction times, 5 and 15 min; *(4)* wash times, 20 and 60 s; *(5)* vacuum filtering times, 5 and 15 min; *(6)* test agar gelling times, 20 and 40 min; *(7)* water bath incubation times, 10 and 20 min. The ruggedness testing showed that the method had some latitude within the tested limits. Good recovery of spike material was obtained when the above factors were varied from the optimum as listed above. The ruggedness tests achieved an average recovery of 97% with a range of 75–135% for 20 spike particles.

Collaborative Study Package

Each collaborator was provided with 2 practice and 6 spiked test portions, each containing 10 g of pepper. Each practice test portion was spiked with 20 fecal particles. Other test portions contained 3 levels of fecal particles: 0, 10, and 20, each provided in duplicate. Practice test portions were labeled A and B; other test portions were randomly numbered 1 through 6. Collaborators were also provided with mouse fecal particles to be used as positive controls, a copy of the proposed method, instructions, recording forms, and a sheet for comments. Expected minimum recoveries for the practice test portions were given, and collaborators were requested to contact the Associate Referee if these minimum results were not obtained.

993.27 Mammalian Feces in Ground Black Pepper—Alkaline Phosphatase Detection Method

First Action 1993

(Applicable to determination of mammalian feces in ground black pepper.)

Method Performance:

Black pepper, 10-particle spike/10 g pepper

Mean recovery = 122.5%

$s_r = 3.8$; $s_R = 4.3$; $RSD_r = 30.7\%$; $RSD_R = 34.8\%$

Black pepper, 20-particle spike/10 g pepper

Mean recovery = 120.3%

$s_r = 5.4$; $s_R = 7.2$; $RSD_r = 22.5\%$; $RSD_R = 29.9\%$

A. Principle

Most mammalian intestinal tracts contain alkaline phosphatase (AKP). At test conditions, pH 9.5 and 42°, AKP hydrolyzes phosphate radicals from substrate/pH indicator phenolphthalein diphosphate to produce light pink to red-purple color from free phenolphthalein.

B. Apparatus

(a) *Water bath.*—Capable of maintaining 42 ± 1°.

(b) *Hot plate/stirrer and stir bars.*—*See* **945.75B(n)**; 41 mm ovoid stir bars are recommended.

(c) *Petri plates.*—Disposable plastic, or glass; 150 × 20 or 150 × 15 mm.

(d) *Weighing boats.*—8.1 × 8.1 × 1.9 cm, 100 mL capacity, or similar size.

C. Reagents

(a) *Magnesium chloride solution.*—Dissolve 0.203 g $MgCl_2 \cdot 6H_2O$ and dilute to 500 mL with H_2O. Solution has indefinite shelf life.

(b) *Phenolphthalein diphosphate.*—Tetrasodium salt, ca 95%. Store in desiccator at <0°.

(c) *Stock test reagent.*—Dissolve 19.0 g borax ($Na_2B_4O_7 \cdot 10H_2O$) and 6.28 g anhydrous Na_2CO_3 in 1 L H_2O with stirring. Add 0.94 g phenolphthalein diphosphate, **(b)**, and stir while adding 2 mL $MgCl_2$ solution, **(a)**. Reagent is stable ca 4 months at room temperature. Reagent should be discarded if not colorless and ca pH 9.5 (degraded phenolphthalein diphosphate produces pink color in test reagent).

(d) *Agar.*—Bacto agar (Difco).

(e) *Liquid test agar.*—Measure equal volumes (half total test agar volume needed) of stock test reagent, **(c)**, and H_2O into separate beakers. Beaker for H_2O must be large enough for 2× volume of H_2O measured. Place beaker with H_2O on hot plate/stirrer, add stir bar, and with rapid stirring add enough agar to H_2O to yield 2% agar solution (1.5 g agar/75 mL H_2O). Continue stirring and heat to boil (avoid foam-over). Place cover glass over beaker to prevent heat loss. When agar begins to foam, add measured stock test reagent, pouring reagent

down side of beaker to prevent agar from coming out of solution. Stir rapidly over heat ca 1 min. Prepare fresh daily and maintain at 42°; use 150 mL/plate.

D. Determination

Prepare 4 plates for each 10 g test portion of ground black pepper and one plate for positive control.

Prepare liquid test agar, **C(e)**, and maintain in water bath at 42 ± 1°. Weigh 10 g ground black pepper from each well-mixed test portion. Add pepper, stir bar, and ca 300 mL H_2O to 400 mL beaker. Place beaker on stirrer and stir contents rapidly 10 min. Move beaker around on stirrer to ensure that all pepper particles are suspended. (*Note*: Failure to suspend all pepper particles will result in inadequate color leaching which interferes with detecting positive results.)

Transfer beaker contents to 8 in. No. 50 sieve. Rinse beaker and stir bar into sieve using gentle stream of cool water from water aerator as in **945.75B(a)**. Direct gentle spray of cool water onto pepper until water through sieve is clear of any pepper particles (≥30 s). Quantitatively transfer sieve retainings to 90 mm Hirsch funnel **945.75B(k)** containing 11 cm filter (medium porosity, ca 8–11 μm particle retention, Whatman No. 1 or 2 is suitable) over wire mesh screen. Vacuum-filter, continuing 10 min after most visible liquid has been removed.

Pour ca 150 mL 42° liquid test agar into each plate. Carefully remove filter from funnel, loosen pepper particles using metal spatula, and distribute pepper from each test sample equally on agar surface of 4 plates. Scrape residual pepper particles from filter into plates.

Mix contents of each plate well to distribute particles evenly. Scrape spatula on side of one plate to remove any adhering material. (*Note*: Equal particle distribution among and within dishes is essential; concentrations of pepper particles could physically block visibility of positive color.)

Prepare positive control by thinly scattering some ground, known rodent feces evenly on one plate.

Allow test agar to gel completely (no agar flows as dish is slightly tipped), with lids removed, ca 20 min. Small fan may be used to hasten gelling.

E. Reading Results

When agar is firm, examine agar surface such that line of sight is perpendicular to plate while plate is held at 45° to white background surface, with light source directed onto plate from above. Rotate plate as needed to view agar clearly, checking for pink to red-purple (positive) spots. Positive spots in test sample plates should be same color as spots in positive control plate. Mark positive spots on plate lid, using grease pencil, and mark lid and bottom together, using waterproof marker, so lid can be replaced in same orientation.

Place plates in 42° water bath and incubate 15 min. Remove plates from bath, wipe inside lid to remove fog. Hold lid so bottom edge of lid is 2–3 mm above top edge of plate base while plate is read and marked. Replace lid. Repeat 15 min incubation and marking of positive spots.

Spots that appear and then are not seen on subsequent checks, and spots that appear anywhere in agar are counted. Record number of spots as fecal particles/10 g test portion.

(*Note*: Amount, intensity, and range of color [light pink to red-purple] will vary, depending on fecal particle size, species, and diet of animal. Particles <250 μm can be identified.)

Ref.: *J. AOAC Int.* **77**, 1146 (1994).

Results and Discussion

The results of the collaborative study are presented in Table 1. The average number of positive particles reported by collaborators for the 0, 10, and 20 spike levels was 0, 12.3 (123%), and 24 (120%), respectively. The range of identified positive particles for the 10 and 20 spike levels was 6–24 and 14–44, respectively. One outlier was identified (Collaborator 3, test portion 6) by the tests of Grubbs and Cochran (3, 4) and was not used in the statistical analysis.

Many collaborators reported a higher number of positives than the spike number added. This result could have been caused by breaking up of spike particles, stray background particles, or counting of something other than a positive spot.

Two collaborators who did not have a No. 50 sieve used sieves Nos. 45 and 60. Recoveries obtained with these sieves were within limits of the other collaborators' results.

One collaborator observed a yellow color in the agar. The color could have been the result of incomplete extraction of the black pepper particles. Recoveries reported by this collaborator were good; therefore, the yellow color apparently did not interfere with the identification of positive particles.

Table 1. Collaborative study results for determination of mammalian feces in ground black pepper

	No. of fecal particles					
	Spike level = 0		Spike level = 10		Spike level = 20	
Lab.	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
1	0	0	8	8	20	14
2	0	0	10	24	30	19
3	0	4[a]	10	10	17	24
4	0	0	13	14	44	30
5	0	0	13	11	24	30
6	0	0	16	12	25	34
7	0	0	15	14	21	29
8	0	0	13	19	23	23
9	0	0	8	13	19	18
10	0	0	8	6	19	18
$\bar{X}$			12.3		24.1	
$\bar{X}$, %			122.5		120.3	
s_r			3.9		4.1	
s_R			6.0		7.4	
RSD_r, %			30.7		22.5	
RSD_R, %			34.8		29.9	

[a] Outlier; not used in statistical analysis.

One collaborator prepared 2% agar and stored the aliquots in a refrigerator. The agar was melted by microwave when needed, and added to a 2× test solution before use. The results from these tests were good, indicating that no problems were encountered when the test agar was prepared in this manner.

One collaborator saw no positives in a control and test sample. This result was probably caused by not cooling the test agar to 42°C before adding the ground black pepper. The indicator enzyme, alkaline phosphatase, is deactivated at temperatures above 55°C. The collaborator did find positives in a replacement test portion. This problem points out the need for collaborators to read through the entire method and follow the method as written. Critical points are noted in the method and must be heeded.

Another collaborator noted that the agar in some dishes of gelled agar rotated during handling. Agar movement causes inaccurate counting of positive spots. Allowing the agar to gel completely before placing it in the water bath will prevent most movement. Analysts should observe the gel for movement during counting. In general, collaborators stated that the method was easy to follow and simple to perform.

Recommendation

The proposed method gives high recoveries of fecal particles, eliminates the use of toxic chemicals, and is simple and rapid to perform. On the basis of the results of this study, it is recommended that the alkaline phosphatase method, a chemical test for mammalian feces in ground black pepper, be adopted first action.

Acknowledgments

The author thanks the following collaborators for their work and constructive comments:

John R. Bryce, U.S. Food and Drug Administration, Los Angeles, CA

Maria P. Chaput, U.S. Food and Drug Administration, Winchester Engineering and Analytical Center, Winchester, MA

Gary R. Dzidowski, U.S. Food and Drug Administration, Detroit, MI

Roger L. Heitzman, U.S. Food and Drug Administration, Washington, DC

Maryann Langridge, Ontario Ministry of Health, LSB, Etobicoke, Ontario, Canada

Richard E. Olson, U.S. Food and Drug Administration, Kansas City, KS

Mary Lou Silva, Rhode Island Department of Health, Providence, RI

Barbara A. Spring, U.S. Food and Drug Administration, Dallas, TX

Wil A. Sumner, U.S. Food and Drug Administration, San Francisco, CA

David Y. Tobias, U.S. Food and Drug Administration, New York, NY

The author also thanks Jack Boese, Paris Brickey, and John Gecan, U.S. Food and Drug Administration, Division of Microanalytical Evaluations, Washington, DC, for reviewing the manuscript, and Foster D. McClure, Linda K. Bullock, and Richard H. Albert, U.S. Food and Drug Administration, Division of Mathematics, Washington, DC, for statistical evaluation of the data.

References

(1) Gerber, H.R. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 766–769

(2) Gerber, H.R. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 496–498

(3) Grubbs, S.E. (1950) *Ann. Math. Stat.* **21**, 27–58

(4) Cochran, W.G. (1941) *Ann. Eugene.* (Lond.) **11**, 47–52

Extraction of Light Filth from Whole Wheat Flour, Flotation Method: Collaborative Study

LARRY E. GLAZE and JOHN R. BRYCE
U.S. Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: R.B. Bradicich; M.P. Chaput; G.R. Dzidowski; S. Friedman; M.L. Obert; J.C. Romer; W.T. VanVelzen; E.C. Washbon

An additional extraction/flotation method for the determination of light filth in whole wheat flour was validated through a collaborative study. A 50 g test portion is boiled in a 3% HCl solution. The mixture is washed with hot tap water on a No. 230 sieve. Then the residue is boiled in isopropanol, transferred to a No. 230 sieve, and washed again. The residue is transferred to a Wildman trap flask using 40% isopropanol. The filth is isolated by flotation in mineral oil and a mixture of Tween 80 and Na_4EDTA in 40% isopropanol. Average recoveries by 8 collaborators were 88.8 and 91.7% for insect fragments and rat hairs, respectively. The extraction/flotation method for determination of light filth in whole wheat flour has been adopted first action by AOAC INTERNATIONAL as an additional procedure to the AOAC Official Method 941.16, Filth in Grain Products.

The AOAC Official Method **941.16**, Filth in Grain Products, uses chloroform and carbon tetrachloride, exposing the analyst to possible contact with phosgene gas during drying. Extraction papers are covered with heavy debris, which makes the counting of filth elements difficult.

An additional method has been developed that omits the use of chloroform and carbon tetrachloride, eliminates possible exposure to phosgene gas, and yields acceptable recoveries of filth elements on relatively clean extraction papers. A 50 g test portion is digested by boiling in a 3% HCl solution, and the mixture is sieved. The residue is defatted by boiling in isopropanol and the mixture is sieved again. The filth is trapped with mineral oil in a mixture of Tween 80 and Na_4EDTA in 40% isopropanol.

Submitted for publication April 21, 1993.

The recommendation was approved by the Committee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Board Actions" (1993) *The Referee* **17**, September issue.

This report was presented at the 105th AOAC INTERNATIONAL Annual Meeting, August 12-15, 1991, Phoenix, AZ.

Collaborative Study

Six 50 g test portions of whole wheat flour spiked at 3 levels and 2 spiked practice samples were sent to each of 8 collaborators. The low spike level consisted of 5 insect fragments (elytral squares of *Tribolium confusum*, ca 0.5 mm^2) and 5 rat hairs (2–3 mm long); the middle and high spike levels consisted of 15 and 30 insect fragments, and 10 and 15 rat hairs, respectively. The collaborators were instructed to report results and analytical times, and to return the extraction papers to the Associate Referee, so their results could be checked.

993.26 Light Filth in Whole Wheat Flour—Flotation Method

First Action 1993

(Applicable to determination of light filth in whole wheat flour.)

Method Performance:

See Table **993.26** for method performance.

A. Principle

Whole wheat flour is digested without effect on insect exoskeleton or mammalian hair contaminants. These oleophilic filth elements are separated from nonoleophilic food products by attraction to the oil phase of an oil–aqueous mixture. The oil phase is trapped off, filtered, and examined microscopically for filth elements.

B. Apparatus

(a) *Sieve.—(1)* No. 230 plain-weave, **945.75B(r)**; and *(2)* sieve handle, **945.75B(t)** (*see* "Changes in Official Methods of Analysis," 3rd Supplement, 1992, p. 128).

(b) *Reflux apparatus.*—**975.49A(e)**.

(c) *Wildman trap flask.*—2 L, **945.75B(h)**(*4*).

(d) *Filter paper.*—Ruled, **945.75B(i)**.

Table 993.26. Method performance for determination of light filth in whole wheat flour by flotation method

Spike added/50 g flour	Mean	s_r	s_R	RSD_r, %	RSD_R, %
		Rat hairs			
5	4.8	1.4	1.4	29.8	29.8
	(4.4)[a]	(1.5)	(1.5)	(34.3)	(34.3)
10	9.1	1.2	1.2	13.5	13.5
	(9.0)			(13.0)	(13.0)
15	14.4	1.2	1.3	8.3	9.2
	(14.1)	(1.0)	(1.2)	(6.9)	(8.5)
		Insect fragments			
5	4.7	0.8	0.8	16.0	16.0
15	12.6	1.1	3.0	8.9	23.6
	(13.8)	(1.5)	(1.6)	(10.8)	(11.5)
30	25.9	3.6	4.8	14.0	18.4
		(3.5)	(4.6)	(13.6)	(17.9)

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

C. Reagents

(a) *HCl solution.*—3%. Add 24 mL HCl to 776 mL H_2O.

(b) *Isopropanol solutions.*—*(1)* 100%. *(2)* 40% aqueous solution.

(c) *Mineral oil.*—**945.75C(p)**.

(d) *Tween 80–40% isopropanol solution.*—**945.75C(x)**.

(e) *Na_4EDTA–40% isopropanol solution.*—**945.75C(z)**.

D. Isolation

Add 800 mL 3% HCl solution, **C(a)**, to 2 L beaker. Place on preheated hot plate and magnetically stir so stirring bar is visible in vortex, **945.75B(n)**. Accurately weigh 50 g whole wheat flour to nearest 0.5 g into 250 mL beaker. Transfer flour portionwise to 3% HCl solution. Rinse sides of 250 mL beaker with 3% HCl solution from wash bottle and add washings to 2 L beaker. Cover with watch glass and bring to full boil. Remove watch glass and boil gently 15 min with magnetic stirring.

Wet-sieve, **970.66B(a)**, portionwise on sieve, **B(a)***(1)*, with gentle stream of hot (50°–70°C) tap water until rinse is clear. Use of sieve handle, **B(a)***(2)* or equivalent, is recommended. Retain original beaker. Wash residue to side of sieve with hot tap water, and rinse residue with 100% isopropanol, **C(b)***(1)*.

Quantitatively transfer residue to original beaker, washing with 100% isopropanol. Add 100% isopropanol to 400 mL mark on beaker and boil gently 5 min, using reflux apparatus, **B(b)**, inserted into beaker top. Remove beaker from reflux apparatus and quantitatively transfer beaker contents to sieve.

Wet-sieve with gentle stream of hot tap water until rinse is clear. Wet residue on sieve with 40% isopropanol, **C(b)***(2)*, and quantitatively transfer residue to trap flask, **B(c)**, using 40% isopropanol. Dilute to 600 mL with 40% isopropanol and boil gently 5 min with magnetic stirring. Remove from heat, add 65 mL mineral oil, **C(c)**, and magnetically stir 3 min, **970.66B(c)**. Let stand 1–2 min after stirring.

Add mixture of 5 mL Tween 80–40% isopropanol solution, **C(d)**, and 5 mL Na_4EDTA–40% isopropanol solution, **C(e)**, slowly, down stirring rod. Hand-stir 30 s with gentle rotary motion. Let stand undisturbed 1–2 min. Fill flask with 40% isopropanol, clamp rod, and let stand 30 min. Stir bottom contents every 5 min for first 20 min and leave undisturbed for final 10 min. Spin stopper (wafer) to remove any trapped residue and trap off, **970.66B(b)**, into 400 mL beaker, using 40% isopropanol to rinse neck of flask. Add 40 mL mineral oil to flask and hand-stir 15 s with gentle up-and-down motion. Fill flask with 40% isopropanol and let stand for 20 min. Spin stopper and trap off as before, rinsing neck with 100% isopropanol.

Filter beaker contents through filter, **B(d)**, and examine microscopically at ca 30×.

Ref.: *J. AOAC Int.* **77**, 1143 (1994).

Results and Discussion

The statistical analyses of the results are presented in Tables 1 and 2. The outliers in the study were identified by the single-Grubbs test [*J. Assoc. Off. Anal. Chem.* **72**(1989), No. 4].

Results for recoveries of rat hairs are given in Table 1. Mean recoveries for all 3 spike levels ranged from 87.4 to 94.0% (third party counts). The Petri dishes returned by Collaborator 6 were very wet, with excess glycerine-alcohol running out of the dishes onto the packaging material. This may have caused a loss of hairs in transit and the low recoveries (for 5 of the 6 test portions) found by the Associate Referee compared to those reported by the collaborator. The mean of 87.4% for

Table 1. Collaborative results for recovery of rat hairs (blind duplicates) from whole wheat flour

	Spike level					
Lab.	5		10		15	
1	7 (5)[a]	5	10	8	15	15
2	5 (4)	5	9	9	16 (14)	15
3	2	6	10	10	11	14
4	4	5	9	9	15	15
5	5 (4)	5	10	8	14	16
6	5 (4)	6 (5)	7	10 (9)	15 (14)	15 (14)
7	2 (1)	5	7[b] (11)	6[b] (8)	12 (13)	15 (13)
8	4 (5)	5	10 (9)	8	14	14 (13)
$\bar{X}$	4.75 (4.37)		9.07 (9.0)		14.4 (14.1)	
$\bar{X}$, %	95.0 (87.4)		90.7 (90.0)		96.0 (94.0)	
s_r	1.42 (1.50)		1.22 (1.17)		1.20 (0.97)	
s_R	1.42 (1.50)		1.22 (1.17)		1.33 (1.20)	
RSD_r, %	29.8 (34.3)		13.5 (13.0)		8.3 (6.9)	
RSD_R, %	29.8 (34.3)		13.5 (13.0)		9.2 (8.5)	

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

[b] Outlier by single-Grubbs test; not included in calculations.

Table 2. Collaborative study results for recovery of insect fragments from whole wheat flour (blind duplicates)

	Spike Level					
Lab.	5		15		30	
1	5	5	14	14	28 (27)[a]	28
2	2[b]	1[b]	7 (7[b])	6 (6[b])	27	21 (22)
3	3	3	15	17	0[b]	8[b]
4	5	5	13	15	29	28
5	5	5	11	12 (14)	27 (28)	27
6	5	5	12	13	28	29 (28)
7	3	5	12 (15)	12	12	23
8	5	5	16 (15)	13	30	25
$\bar{X}$	4.7		12.63 (13.79)		25.86	
$\bar{X}$, %	94.0		85.9 (91.9)		86.2	
s_r	0.75		1.12 (1.49)		3.62 (3.52)	
s_R	0.75		2.98 (1.59)		4.76 (4.63)	
RSD_r, %	16.0		8.9 (10.8)		14.0 (13.6)	
RSD_R, %	16.0		23.6 (11.5)		18.4 (17.9)	

[a] Third party counts are in parentheses if different from those of collaborator, or if determined to be an outlier.
[b] Outlier by single-Grubbs test; not included in calculations.

the low spike level reflects the counts of the third party. Results obtained by Collaborator 7 on test samples spiked at the medium level were identified as outliers. No explanation could be found for the low recoveries.

Results for recoveries of insect fragments are given in Table 2. Mean recoveries for all 3 spike levels ranged from 86.2 to 94.0%. Recoveries by Collaborator 2 were very low from all test portions spiked at low and medium levels, and low from one test portion spiked at the high level. A possible explanation for this was the collaborator's decision not to use the reflux (solvent saver) apparatus specified in the method. Instead, a watchglass was used to cover the beakers. Although no significant loss of solvent was noted after 5 min boil, research has shown that the use of the reflux apparatus improves defatting of the product, and consequently improves filth recovery for some products. No other variable seems to explain the very low recoveries found by Collaborator 2. The results from the low level samples and the third party counts for the medium level samples (but not Collaborator's counts) were identified as outliers.

Collaborator 5 reported a small accidental spill during the analysis of one test portion spiked at the medium level, which might have caused the low recovery (11 of 15 insect fragments). No explanation was found for the low recoveries of insect fragments at the high level by Collaborator 3, which were identified as outliers and removed by the single-Grubbs test.

The amount of residue on the extraction papers was subjectively evaluated by the Associate Referee as ranging from very light to very heavy. On average, extraction papers for the proposed method were lighter or cleaner than those for the AOAC Official Method **941.16**. Residue for all 6 test portions was very light to medium for 5 of 8 collaborators, whereas residue for Collaborator 8 was light to medium for 4 of 6 test portions, and heavy for 2. Residue for Collaborator 7 was light to medium for 4 of 6 test portions, heavy for one, and very heavy for another. All plates submitted by Collaborator 5 were very heavy, even though the residue was spread across 2 filter papers per test portion.

The average analytical time required for all 6 test portions was 19.8 h, with 16.9 h used for preparation and extraction and 2.9 h used for reading extraction papers. Individual collaborator times reported for preparation and extraction ranged from 3.5 to 34.5 h. The wide range in times can be explained, in part, by the number of test portions extracted concurrently by each collaborator. Collaborators that reported times near 3.5 h analyzed all test portions concurrently, resulting in a total analytical time slightly longer than that for one portion, which was about 2.5 h in intralaboratory tests. Other collaborators analyzed one or 2 test portions at a time, needed longer preparation times, or were slower, resulting in correspondingly longer analytical times.

Recommendation

The results of this study indicate that the proposed method for the extraction of light filth from whole wheat flour is reliable and safer, and produces cleaner extraction papers than the AOAC Official Method **941.16**. On the basis of the results of this study, it is recommended that this additional extraction/flotation method for determination of light filth in whole wheat flour be adopted first action.

Acknowledgments

The authors express their appreciation to the following U.S. Food and Drug Administration analysts for participating in this study:

Reno B. Bradicich, Dallas, TX

Maria P. Chaput, Winchester Engineering and Analytical Center, Winchester, MA

Gary R. Dzidowski, Detroit, MI

Sharon Friedman, Baltimore, MD

Michele L. Obert, Kansas City, MO

James C. Romer, Denver, CO

W. T. VanVelzen, Seattle, WA

Edwin C. Washbon, Los Angeles, CA

Thanks also to Jack L. Boese, Division of Microbiology, U.S. Food and Drug Administration, for review of the manuscript, and to Foster D. McClure, Division of Mathematics, U.S. Food and Drug Administration, for statistical analyses.

FOOD BIOLOGICAL CONTAMINANTS

Alternative Sieving Method for Extraction of Light Filth from Cheeses: Collaborative Study

MARVIN J. NAKASHIMA
U.S. Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: D.W. Bakker; G.R. Dzidowski; G. Haeberle; L.S. Lawless; T.A. Rammer; M.A. Sogol; R.L. Trauba; G. Tucker; M.D. Wirth

A collaborative study was conducted on an alternative sieving method for the extraction of light filth from cheeses. The alternative method was developed that is applicable to broad variety of cheeses. A 225 g test portion is dispersed in a solution of 5.7% HCl, Igepal CO-730, and Igepal DM-710. Digested cheese is wet-sieved on a No. 230 sieve. The residue is treated with Tergitol Anionic 4, transferred to 1% sodium lauryl sulfate solution, heated, and maintained at 65°–75°C for 10 min. The residue is washed with these 2 surfactants a maximum of 4 times until it is reduced to an amount that is filterable. The residue is filtered and the filter papers are examined microscopically at a magnification of ca 30×. Average recoveries by 9 collaborators for 3 spike levels of rat hairs (5, 10, and 15) were 80, 68, and 81%, respectively; for insect fragments (5, 15, and 30) recoveries were 97, 90, and 92%, respectively. The alternative sieving method for extraction of light filth from cheeses has been adopted first action by AOAC INTERNATIONAL.

The AOAC Official Method **960.49C-E** (1) provides procedures for 3 groups of cheeses: soft and semi-soft; hard; and cheeses containing mold, plant tissue, and spices. The hard cheese method was evaluated collaboratively (2), solely to gather information about residues, residue interference, and filtration speed. None of the above procedures was used in a collaborative study to obtain recovery data.

The alternative sieving method is applicable to all cheeses included in AOAC Official Methods for determination of filth, except bloom rind cheeses (e.g., brie), smoked cheeses, and cheeses containing plant tissue, herbs, and spices. The method expands the coverage to include semi-hard and processed cheeses that contain whey, plant gums, or emulsifiers (e.g., sodium phosphate, sodium citrate).

The following cheeses were tested in-house in advance of the collaborative study: *(1)* hard cheeses: Parmesan, Romano, Sardo Romano, Pecorino Romano, Jarlsberg, Goya, and sbrinz; *(2)* semi-hard cheeses: provolone, Edam, havarti, Alpenzeller, Cantal, Telemark, etorki, opequon gold, arina, colby jack, fontinella, and Swiss; *(3)* semi-soft cheeses: feta (goat and sheep milk), gjetost, muenster, Monterey Jack, ricotta salada, goat log, Port Salut, and mozzarella; *(4)* soft cheeses: cream, cottage, and Bel Paese; *(5)* blue cheeses: blue, Danish blue, Roquefort, Gorgonzola, and Stilton; and *(6)* processed cheeses: cold pack cheese food, pasteurized processed cheese, pasteurized processed cheese food, and pasteurized processed cheese spread.

Results from 97 replicates of hard, semi-hard, semi-soft, soft, and blue cheese, and 16 kinds/brands of processed cheese, gave rat hair and insect fragment recoveries of 91 and 96%, standard deviations of 0.9 and 0.95, and relative standard deviations of 9.9 and 2.9%, respectively.

Collaborative Study

Each collaborator received 6 randomly numbered jars, each containing 225 g test portions of grated parmesan cheese spiked with rat hairs and insect fragments. Duplicates of 3 levels of each analyte were provided: 5 rat fur hairs (2.5–3.5 mm long) and 5 insect fragments (elytral squares of *Tribolium confusum* ca 0.5 mm^2), 10 rat fur hairs and 15 insect fragments, and 15 rat fur hairs and 30 insect fragments. Collaborators were instructed to report their analytical times and to return the filter papers to the author to check their results.

Submitted for publication June 7, 1993.

The recommendation was approved by the Committee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1994) *J.AOAC Int.* 77, 43A, and "Official Methods Board Actions" (1994) *The Referee* 17, February issue.

This report was presented at the 106th AOAC INTERNATIONAL Annual Meeting, September 2, 1992, Cincinnati, OH.

994.05 Light Filth in Cheeses—Alternative Sieving Method

First Action 1994

(Applicable to hard, semi-hard, semi-soft, soft, blue, and processed cheeses containing at least one of following: whey, plant gums, or emulsifiers [e.g., sodium phosphate, sodium citrate].) Not applicable to bloom rind cheeses (e.g., brie), smoked cheeses, and cheeses containing plant tissue, herbs, and spices.

Method Performance:

See Table **994.05** for method performance.

A. Principle

Cheese is digested in acid and 2 surfactants and wet-sieved on No. 230 screen. Residue is dispersed with successive treatments of 2 additional surfactants to attain filterable amounts of residue. Filth (hairs and insect fragments) is unaffected by the treatments and is retained on the sieve. The residue is filtered and the filth is counted microscopically.

B. Reagents

(**a**) *Emulsifiers.*—Igepal CO-730, **945.75C(j)**(*1*), and Igepal DM-710, **945.75C(j)**(2), or equivalent.

(**b**) *Wetting agent.*—Sodium tetradecyl sulfate (Tergitol Anionic 4, Sigma Chemical Co., or equivalent).

(**c**) *Detergent solution.*—Sodium lauryl sulfate, **945.75C(i)**.

C. Preparation of Test Samples

Trim any waxes, paper labels, molds, and other coatings from the cheese surface. Add 225 g of test sample to 2 L beaker as follows: *(1)* Cut cheese in ca 13 mm cubes. Cut to ca 5 mm pieces products that have hardened, products made from whey, and processed products containing ≥1 of following: whey, plant gums, or emulsifiers (e.g., sodium phosphate, sodium citrate). *(2)* Grate product normally grated for use (e.g., Parmesan, Romano). *(3)* Use tablespoon to sample grated or spreadable product.

D. Isolation

Add 1 L hot tap H_20 (>50°C) and 60 mL HCl (37%) to test sample in beaker. Add 5 mL Igepal CO-730, and 10 mL Igepal DM-710, **B(a)**. Cover beaker with watch glass and bring mixture to full boil with magnetic stirring. Remove watch glass and boil mixture 30 min with magnetic stirring, so that top of stirring bar is visible at bottom of vortex. Wet-sieve portionwise, **970.66B(a)**, on No. 230 plain-weave sieve, **945.75B(r)**, with forceful stream of hot tap H_2O until foaming is minimal or none. Add ca 10 mL Tergitol Anionic 4, **B(b)**, and gently wet-sieve until foaming is minimal or none.

Place sieve (submerged sufficiently to cover residue) in pan of 1% sodium lauryl sulfate, **B(c)**, maintained at 65°–75°C (use hot plate to heat solution). Gently swirl residue to disperse, then let pan stand for 10 min. Gently and *thoroughly* wash sides of sieve with hot tap H_2O, and then gently wet-sieve, **970.66B(a)**, residue with hot tap H_2O. Repeat tergitol/sodium lauryl sulfate washes until residue is reduced to filterable amounts. Do not exceed 4 washes.

Quantitatively transfer residue with H_2O to 400 mL beaker. Filter beaker contents with suction through rapid filter paper, **945.75B(i)**, using Hirsch funnel, **945.75B(k)**. Wash beaker with H_2O and filter washings. Examine microscopically at ca 30×, **945.75B(o)**(*2*).

Ref.: *J. AOAC Int.* **77**, 1153 (1994).

Results and Discussion

Of the 9 collaborators participating in the study, Collaborators F and H replaced the collaborators who could not repeat the study. Data from Collaborator G represent repeated analysis. Collaborators were asked to repeat the study if they previously exceeded the specified maximum of 4 tergitol/sodium lauryl sulfate washes (e.g., 18–20), if they did not use the aerator specified in the method, or if they used filter paper without cupped sides.

Collaborators' counts of recoveries of spike rat hairs and insect fragments (Tables 1 and 2, respectively) were checked by the author, and the results were verified by an expert microanalyst (third party) when the author's counts differed from

Table 994.05. Method performance for filth (rat hairs and insect fragments) in Parmesan cheese

Spike added	$\bar{X}$	$\bar{X}$, %	r	R	RSD_r	RSD_R
			Rat hairs			
5	3.9 (4.0)[a]	78.9 (80.0)	0.8 (0.5)	0.9 (1.1)	20.5 (12.5)	23.1 (27.5)
10	6.3 (6.8)	63.3 (68.3)	1.4 (1.5)	2.1 (1.9)	22.2 (22.1)	33.3 (27.9)
15	11.6 (12.1)	77.4 (80.7)	2.7 (2.6)	3.2 (2.7)	23.3 (21.5)	27.6 (22.3)
			Insect fragments			
5	4.8	97.1 (96.7)	0.4 (1.0)	0.5 (1.0)	8.3 (20.8)	10.4 (20.8)
15	12.7 (13.4)	84.8 (89.6)	3.0 (2.0)	3.7 (2.5)	23.6 (14.9)	29.1 (18.6)
30	26.2 (27.4)	87.3 (91.5)	1.4 (2.5)	3.9 (3.2)	5.3 (9.1)	14.9 (11.7)

[a] Third party counts in parentheses.

the collaborator's. Before an analysis of variance of the data was made, Grubbs and Cochran tests were used to find outliers. Table 2 shows that for insect fragments 2 pairs of counts by Collaborator F and one pair of counts by the Collaborator G were excluded.

Average recoveries (third party counts) for 3 spike levels were 80, 68, and 81% for rat hairs (Table 1) and 97, 90, and 92% for insect fragments (Table 2). Variability measures for rat hairs were high (Table 1) and for insect fragments (Table 2) were low to moderately high, but all were in the acceptable range.

Recoveries of rat hairs were lower than expected (intralaboratory recoveries of rat hairs were 91% for 97 replications), considering that no trapping is involved, and transfers are kept to a minimum because the product is reduced to filterable amounts while it is on the sieve.

Comments from collaborators illustrate steps in which hairs could have been lost. Collaborator C, whose recoveries are at or above the average, commented that the use of a forceful stream of water for the initial sieving of the digestate could force hairs through the sieve. That collaborator's water system was also prone to pressure surges, which made it difficult to wash the sieve gently.

Because the data of Collaborator D were on the low side of average recoveries, this collaborator was asked to examine the sieve used in the study for sources of spike losses. The collaborator reported finding gaps in the soldering of the screen to the frame and after closer inspection found 2 spike insect fragments in a crevice. This could partly explain Collaborator D's low recoveries of both analytes.

Collaborator A, whose recoveries were below average, filtered the sodium lauryl sulfate solution used in 3 test portions and found 2 spike hairs. Collaborator D also found a spike rat hair from filtered sodium lauryl sulfate solution. Because the openings on a No. 230 sieve are 0.063 mm (63 μm) and rat fur hair is about 0.020–0.025 mm (20–25 μm) wide, a hair could pass through a sieve opening.

Collaborator G recovered 6 rat hairs (confirmed by the author) from a test portion spiked with 5 rat hairs. From the previous test portion 9 of 10 spike hairs were recovered. This suggests that a hair remaining from a previous test portion contaminated a subsequent test portion.

Collaborator F's recovery of 7 insect fragments from a test portion to which only 5 were added was preceded by a test portion from which only 22 of 30 insect fragments were recovered. Insufficient cleaning (back-washing) of the sieves by the Collaborators F and G is indicated by the results.

The incomplete collection and transfer of sieve residues after the sodium lauryl sulfate soaking is another possible source of rat hair losses, next to incorrect sieving techniques such as splashing or pouring a perpendicular stream of liquid onto the sieve. However, Collaborator A filtered the back-wash water from the No. 230 sieve after the analysis of 3 test portions and found no spike hairs.

The method entails dispersing, up to 4 times, a small quantity of product with spike material that is not visible. When the residue reaches filterable amounts, collecting and transferring the small amount of material is difficult, and gauging whether all areas of the sieve have been completely washed is more difficult. To emphasize the need for completely concentrating residues after the final sodium lauryl sulfate soaking, the instruction for the wet-sieving step has been reworded and a reference for the sieving technique, **970.66B(a)**(1), has been added. The instruction now reads "Gently and **thoroughly** wash sides of sieve ... then gently wet-sieve, **970.66B(a)**...."

Collaborators took an average of 1.9 h (range, 1–3.25 h) to complete the extraction and 0.26 h (range, 0.17–0.4 h) to count the plates. All collaborators used only one filter paper per test

Table 1. Collaborative results for recovery of rat hair spikes (blind duplicates) from Parmesan cheese

	Spike level					
Collaborator	5		10		15	
A	3	2	5	5	5	11
B	4	4	9	10	15	9
C	4	4	9	8	13 (12)[a]	15
D	4 (3)	3	7	5	11	10
E	5	5	2 (3)	6 (7)	13	13
F	3	4	8	5	8 (9)	15
G	3 (6)	5	5 (9)	4 (7)	7 (11)	8 (13)
H	5 (4)	3	6	6	13	14
I	5	5	8	6	15	14
$\bar{X}$	3.9 (4)		6.3 (6.8)		11.6 (12.1)	
$\bar{X}$, %	78.9 (80)		63.3 (68.3)		77.4 (80.7)	
s_r	0.8 (0.5)		1.4 (1.5)		2.7 (2.6)	
s_R	0.9 (1.1)		2.1 (1.9)		3.2 (2.7)	
RSD_r, %	20.5 (12.5)		22.2 (22.1)		23.3 (21.5)	
RSD_R, %	23.1 (27.5)		33.3 (27.9)		27.6 (22.3)	

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

Table 2. Collaborative results for recovery of insect fragment spikes (blind duplicates) from Parmesan cheese

	Spike level					
Collaborator	5		15		30	
A	5	5	14	15	28	28 (30)[a]
B	4 (5)	4	15	15	30	29
C	5	5	15	15	27 (28)	29 (30)
D	5 (4)	5	13	7 (9)	19 (22)	20
E	5	4	8 (9)	10 (11)	28 (30)	26 (27)
F	7[b](7)	4[b](4)	13	15	20[b](22)	30 (30)
G	1[c](3)	3[c](5)	2 (8)	13 (15)	20 (28)	22 (28)
H	5 (6)	6 (5)	15	14 (15)	25 (24)	29
I	5	5	15	15	30	29
$\bar{X}$	4.8		12.7 (13.4)		26.2 (27.4)	
$\bar{X}$, %	97.1 (96.7)		84.8 (89.6)		87.3 (91.5)	
s_r	0.4 (1.0)		3.0 (2.0)		1.4 (2.5)	
s_R	0.5 (1.0)		3.7 (2.5)		3.9 (3.2)	
RSD_r, %	8.3 (20.8)		23.6 (14.9)		5.3 (9.1)	
RSD_R, %	10.4 (20.8)		29.1 (18.6)		14.9 (11.7)	

[a] Third party counts are in parentheses if different from those of collaborator, or if either duplicate value is an outlier.
[b] Outlier by Cochran test.
[c] Outlier by Grubbs test.

portion, but all plates from Collaborator G had sufficient product residue, so the residue could have been spread onto 2 plates.

On average, collaborators used the maximum number (i.e., 4) of tergitol/sodium lauryl sulfate washes allowed in the method. However, 4 collaborators exceeded the number for tergitol treatments and 3 collaborators exceeded the number for sodium lauryl sulfate treatments by 1 and 2 additional washings and soakings, respectively, because of misinterpretation of the instructions. These collaborators understood that more than 4 treatment cycles were allowed. This instruction step has been changed to read, "Do not exceed 4 washes."

Two other clarifications were also made. In the description of the heating step of the sodium lauryl sulfate treatment, 2 collaborators interpreted the text to mean that the temperature did not have to be maintained at 65°–75°C, and allowed their solutions to cool during the 10 min standing period.

The General Referee for Filth and Extraneous Materials in Foods and Drugs interpreted the instruction to mean that the entire sieve (screen and frame) should be submerged in the solution for the heating period. Therefore, this description was changed to read "Place sieve (submerged sufficiently to cover residue) in a pan of 1% sodium lauryl sulfate, **B(c)**, maintained at 65°–75°C. (Use a hot plate to heat solution). Gently swirl residue and then let pan stand for 10 min."

Recommendation

On the basis of the results of this study, it is recommended that the proposed alternative method for extraction of sieved light filth from cheeses be adopted first action.

Acknowledgments

The author thanks the following analysts for participating in this study:

Douglas W. Bakker, Minnesota Department of Agriculture, St. Paul, MN

Gary R. Dzidowski, U.S. Food and Drug Administration, Detroit, MI

Gordon Haeberle, Sunshine Biscuit, Inc., Sayreville, NJ

Lori S. Lawless, U.S. Food and Drug Administration, Baltimore, MD

Terri A. Rammer, Land O'Lakes, Inc., Spencer, WI

Marsha A. Sogol, Kraft General Foods, Inc., Glenview, IL

Richard L. Trauba, U.S. Food and Drug Administration, Minneapolis, MN

Geoffrey Tucker, O.D. Kurtz Associates, Inc., Melbourne, FL

Michael D. Wirth, U.S. Food and Drug Administration, Buffalo, NY

The author also thanks Linda K. Bullock and Richard H. Albert, U.S. Food and Drug Administration, Division of Mathematics, Washington, DC, for their assistance and statistical analyses; and John R. Bryce, U.S. Food and Drug Administration, Division of Microbiology, Washington, DC, for check-counting of collaborative extraction plates.

References

(1) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **960.49C-E** and **970.66B(a)**

(2) Stephens, R.L. (1967) *J. Assoc. Off. Anal. Chem.* **50**, 501–504

FOOD BIOLOGICAL CONTAMINANTS

Effects of Incubation Atmosphere, Novobiocin, and Modified Plating Media on the Efficiency of a DNA Probe for Recovering *Shigella flexneri*

FELICIA B. SATCHELL, VERNEAL R. BRUCE, WALLACE H. ANDREWS, GERALDINE JUNE, PATRICIA S. SHERROD, THOMAS S. HAMMACK, and PETER FENG
U.S. Food and Drug Administration, Division of Microbiological Studies, 200 C St, SW, Washington, DC 20204

An oligonucleotide probe specific for the invasion plasmid of *Shigella* spp. was used to study the effect of several culture method variables described in the *Bacteriological Analytical Manual* for retention of the plasmid in *Shigella flexneri*. Results of colony hybridization analyses showed that, in many instances, a slightly greater number of *Shigella* were detected by the DNA probe on MacConkey agar than on trypticase soy agar. Elevating the incubation temperature from 35° to 42°C slightly reduced the number of *Shigella* detectable by the probe. However, neither aerobic nor anaerobic incubation atmosphere or the inclusion of novobiocin in the media showed any consistent effect on the recovery of *S. flexneri* by the probe. Moreover, the detection efficiency of the probe was not generally affected by the different MacConkey agar formulations tested.

Incidence of foodborne shigellosis in the United States has increased over the past several years. To isolate *Shigella* spp. from foods, the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM) (1) describes a culture method that includes sample enrichment in a novobiocin-supplemented broth under anaerobic conditions and at an elevated temperature, followed by plating on MacConkey agar, a selective medium that contains a bile salts mixture.

Recently, a synthetic oligonucleotide probe was developed that may enable rapid identification of *Shigella* spp. (2). The 18 base DNA probe is specific for the invasion gene on a 140 megadalton plasmid in pathogenic *Shigella* spp., which also encodes for binding of Congo Red dye (3, 4). The application of the plasmid-specific probe is described in BAM (1) and is intended for use in conjunction with the BAM culture method for recovering *Shigella* spp. from foods.

Previous reports, however, suggest that the conditions and media recommended in the BAM culture method may not be suitable for subsequent testing with the plasmid-specific probe. Plasmids of many pathogens are not stable and, therefore, may be easily lost during culture maintenance (5). For instance, inclusion of surfactant in media or incubation at elevated temperatures used for enriching pathogens from foods reportedly causes loss of plasmids in these isolates (6). The antibiotic novobiocin affects the gyrase enzyme of several organisms (7, 8) and may also contribute to plasmid loss in *Shigella*. Furthermore, Tollison and Johnson (6) demonstrated the sensitivity of heat-stressed *S. flexneri* to bile salts. Therefore, the bile salts mixture in MacConkey agar may also cause the loss of plasmids in pathogenic shigellae (6, 9).

In this study, the plasmid-specific oligonucleotide probe was used to examine the effects of several variables of the BAM culture method on the stability of the invasion plasmid in *S. flexneri*. These factors include the effect of 35° vs 42°C incubation temperature, aerobic vs anaerobic atmosphere, the presence or absence of novobiocin, use of a bile salts mixture or Bile Salts No. 3, and the inclusion of Congo Red dye in the plating media to enhance retention of the plasmid.

Received February 4, 1993. Accepted by AP September 17, 1993.

Experimental

Reagents and Media

Trypticase soy broth-yeast extract (TSBYE) and Shigella broth were prepared as recommended in BAM. Three formulations of MacConkey agar (MAC) were prepared as follows (per liter of distilled water): (*1*) MacConkey agar without Bile Salts No. 3 and without bile salts mixture (MNBS)—17 g peptone, 3 g proteose peptone, 10 g lactose, 5 g sodium chloride, 13.5 g agar, 0.03 g neutral red, and 0.001 g crystal violet; (2) MacConkey agar without Bile Salts No. 3 but with bile salts mixture (MBSM)—17 g peptone, 3 g proteose peptone, 10 g lactose, 5 g sodium chloride, 13.5 g agar, 0.03 g neutral red, 0.001 g crystal violet, and 1.5 g bile salts; and (*3*) MacConkey agar with Bile Salts No. 3 and with bile salts mixture (MBBS)—17 g peptone, 3 g proteose peptone, 10 g lactose, 5 g sodium chloride, 13.5 g agar, 0.03 g neutral red, 0.001 g crystal violet, 1.5 g Bile Salts No. 3, and 1.5 g bile salts.

Appropriate amounts of Congo Red dye (Sigma Chemical Co., St. Louis, MO) were added to commercially available MacConkey agar before autoclaving to give final concentra-

Table 1. Effects of plating media and incubation environment on the efficiency of a plasmid-specific probe to recover *Shigella flexneri*

Incubation environment, 35°C	Experiment No.	% of colonies hybridized[a] MAC A[b]	MAC NA[c]	TSA A	TSA NA
Aerobic	1	48	74	54	60
	2	53	72	58	71
	3	77	92	45	ND[d]
	4	94	92	77	85
	5	81	91	60	87
	6	94	33	63	56
	7	77	75	48	35
	8	94	91	78	87
	9	75	78	83	54
	10	77	66	19	64
	Means	77[e]	76[e]	59	67
Anaerobic	1	61	53	59	58
	2	75	77	60	45
	3	82	91	64	76
	4	86	94	54	ND
	5	43	73	32	36
	6	95	ND	90	46
	7	92	82	ND	60
	8	86	74	72	57
	9	67	88	85	90
	10	93	91	72	76
	Means	78[f]	80[f]	65	60

Incubation environment, 42°C	Experiment No.	% of colonies hybridized[a] MAC A[b]	MAC NA[c]	TSA A	TSA NA
Aerobic	1	61	67	54	59
	2	27	42	58	41
	3	80	44	70	46
	4	87	87	70	59
	5	64	67	46	72
	6	78	63	46	ND
	7	40	83	66	61
	8	63	81	72	72
	9	71	88	ND	79
	10	57	58	ND	54
	Means	63	68	60	60
Anaerobic	1	59	46	51	31
	2	52	42	59	42
	3	58	46	42	38
	4	67	47	46	31
	5	36	66	44	66
	6	81	56	67	35
	7	84	62	ND	37
	8	90	54	71	56
	9	86	77	55	41
	10	83	60	65	62
	Means	70[f]	56	56	44

[a] Percentages determined by dividing number of hybrids per filter by CFU per plate.
[b] Antibiotic.
[c] No antibiotic.
[d] Not determined.
[e] Significantly greater than TSA, A.
[f] Significantly greater than TSA, NA.

tions (w/v) of 0.03, 0.01, and 0.003%. All other reagents and media were prepared as recommended in BAM (1).

Cultures

Isolates used in this study were obtained from various sources. Stock cultures of *S. flexneri* were maintained in 50% glycerol and stored at −70°C. All *Shigella* isolates were examined by agarose gel electrophoresis and confirmed to contain the large invasion plasmid (10).

Materials and Methods

A frozen stock culture of *S. flexneri* was inoculated into duplicate tubes containing 10 mL TSBYE and incubated aerobically overnight at 35±2°C. The duplicate cultures were mixed and 2 mL aliquots were subcultured in flasks containing 225 mL *Shigella* broth with or without novobiocin (3 μg/mL). The flasks were incubated aerobically and anaerobically at 35±2° or 42±2°C for 20 h. After incubation, 0.1 mL aliquots from each flask were diluted and plated on commercially available MacConkey agar and on TSBYE with 15 g agar (TSA) for the first phase of the study. For the second phase, 0.1 mL aliquots from each flask were diluted and plated on commercially available MacConkey agar and on MNBS, MBSM, and MBBS agars. For the third phase, 0.1 mL aliquots from each flask were diluted and plated on commercially available MacConkey agar, MacConkey agar with 0.03, 0.01, or 0.003% Congo Red dye, and on TSB with 15 g agar and 0.01% Congo Red dye. The plates were incubated aerobically at 35±2°C for 20 h. The colonies on each plate were counted and then transferred to Whatman No. 541 filter paper for colony hybridization analysis using the plasmid-specific probe. The colonies on the blot were lysed, and the DNA was denatured and neutralized as described previously (1). The plasmid-specific probe was labeled on the 5′ end, using T4 polynucleotide kinase and [τ^{-32}P]ATP (11). The hybridization reagents and conditions used were based on established protocols (12), except that post-hybridization washing of the colony blots was done at 54°C rather than 50°C. The blots were examined by autoradiography.

Table 2. Effect of bile salts on the efficiency of a plasmid-specific probe to recover *Shigella flexneri*

Incubation environment, 35°C	Experiment No.	% of Colonies hybridized[a]				Incubation environment, 42°C	Experiment No.	% of Colonies hybridized[a]			
		−−[b]	−+[c]	+−[d]	++[e]			−−[b]	−+[c]	+−[d]	++[e]
Aerobic, no novobiocin	1	54	46	42	46	Aerobic, no novobiocin	1	49	51	48	57
	2	19	26	29	34		2	22	50	34	32
	3	46	43	44	56		3	41	40	44	60
	4	49	48	44	28		4	49	36	43	49
	Means	42	41	40	41		Means	40	44	42	50
Aerobic, novobiocin	1	50	31	54	34	Aerobic, novobiocin	1	54	35	42	32
	2	25	27	19	45		2	34	30	27	40
	3	36	44	27	61		3	47	48	44	49
	4	50	52	59	57		4	50	ND[f]	43	ND
	Means	40	39	40	49		Means	46	38	39	40
Anaerobic, no novobiocin	1	61	46	50	44	Anaerobic, no novobiocin	1	53	38	46	41
	2	27	45	27	48		2	21	34	24	18
	3	49	43	42	46		3	38	35	35	50
	4	38	66	63	50		4	40	ND	68	46
	Means	44	50	46	47		Means	38	36	43	39
Anaerobic, novobiocin	1	46	51	53	47	Anaerobic, novobiocin	1	51	37	53	62
	2	52	42	53	34		2	18	33	26	27
	3	40	46	41	38		3	49	56	57	46
	4	32	48	42	56		4	45	ND	57	ND
	Means	43	47	47	44		Means	41	42	48	45

[a] Percentages determined by dividing number of hybrids per filter by CFU per plate.
[b] MacConkey agar without bile salts mixture and without Bile Salts No. 3.
[c] Commercially available MacConkey agar without bile salts mixture but with Bile Salts No. 3.
[d] MacConkey agar with bile salts mixture but without Bile Salts No. 3.
[e] MacConkey agar with bile salts mixture and with Bile Salts No. 3.
[f] Not determined.

The results were statistically analyzed using an analysis of variance technique and Duncan's New Multiple Range Test to make pairwise comparisons among means obtained for any particular condition of incubation environment, °C (13). The significance level chosen was $\alpha = 0.05$.

Results and Discussion

Colony hybridization studies showed that, in many instances, the DNA probe identified a higher percentage of *Shigella* isolates plated on MacConkey agar than on TSA (Table 1). This finding was unexpected and suggests that the selectivity of the MacConkey medium may facilitate the retention of plasmid by shigellae. Elevating the incubation temperature of plates from 35±2° to 42±2°C showed only a slight effect in the percentage of colonies that hybridized with the probe (Tables 1 and 2). Although other studies (10) showed that incubation at 42°C may affect plasmid integrity, interfere with replication, and cause plasmid loss, such a temperature effect was not clearly observed in this study.

Inclusion of novobiocin in the enrichment broth did not significantly affect the efficiency of the DNA probe; about equal percentages of *Shigella* isolates were detected from media with or without novobiocin (Tables 2 and 3). Novobiocin affects bacterial DNA gyrase and interferes with DNA supercoiling (7, 8); however, in this study, it had no apparent effect on the stability of the invasion plasmid in *Shigella* spp. (9). Likewise, the DNA probe identified approximately equal percentages of cells in plates incubated under aerobic or anaerobic conditions (Tables 1–3); therefore, the stability of the invasion plasmid in *Shigella* was not affected by incubation atmosphere.

Tollison and Johnson (6) reported that *S. flexneri* became sensitive to bile salts after sublethal heat treatment. In this study, similar percentages of colonies plated on media without bile salts or with a bile salts mixture and/or Bile Salts No. 3 were identified as *Shigella* by the probe (Table 2); hence, bile

Table 3. Effect of Congo Red dye on the efficiency of a plasmid-specific probe to recover *Shigella flexneri*

Incubation environment, °C	Experiment number	% of colonies hybridized[a]				
		MAC[b]	TSA[c]	0.03CR[d]	0.1CR[e]	0.3CRf[f]
Aerobic, no novobiocin, 35°C	1	63	68	57	79	65
	2	59	64	59	50	68
	3	48	51	55	59	87
	4	52	42	47	59	61
	5	54	54	60	47	63
	6	36	48	52	56	83
	Means	52	55	55	58	71[g]
Aerobic, novobiocin, 35°C	1	44	45	48	41	45
	2	57	56	84	45	80
	3	52	47	57	80	68
	4	55	68	54	34	61
	5	64	57	63	88	74
	6	63	61	59	67	59
	Means	56	56	61	59	65
Anaerobic, no novobiocin, 42°C	1	58	54	55	70	63
	2	60	59	60	100	80
	3	67	60	59	65	60
	4	57	59	62	60	33
	5	53	51	55	50	74
	6	49	53	54	67	69
	Means	57	56	58	69	63
Anaerobic, novobiocin, 42°C	1	43	54	60	43	45
	2	72	89	60	78	69
	3	53	51	39	89	85
	4	63	68	53	60	83
	5	41	48	63	55	68
	6	45	55	54	61	77
	Means	53	61	55	64	71[g]

[a] Percentages determined by dividing number of hybrids per filter by CFU per plate.
[b] Commercially available MacConkey agar.
[c] Trypticase soy broth with 15 g/L of agar and 0.1 g/L of Congo Red added.
[d] MacConkey agar with 0.03 g/L of Congo Red added.
[e] MacConkey agar with 0.1 g/L of Congo Red added.
[f] MacConkey agar with 0.3 g/L of Congo Red added.
[g] Significantly greater than MAC.

salts did not affect the detection efficiency of the plasmid-specific probe. The absence of *Shigella* sensitivity to bile salts may be attributed to the use of unheated *Shigella* cells and to the lower concentration of bile salts used for these studies.

The invasion plasmid of *Shigella* also encodes for binding of Congo Red dye (3, 4); therefore, inclusion of Congo Red dye in the plating medium may enhance the retention of plasmid in these isolates. Our study showed that although there was some correlation between the presence of plasmid and the colony uptake of Congo Red dye (data not shown), the presence of 0.01 and 0.003% Congo Red dye did not significantly increase the percentage of colonies detectable by the probe (Table 3). However, at a concentration of 0.03% Congo Red dye in MacConkey medium, a higher percentage of cells was identified as *Shigella* by the DNA probe. Although the effect of Congo Red dye on plasmid stability is inconclusive, this finding suggests that certain levels of Congo Red dye may enable *Shigella* isolates to better retain the large plasmid.

Conclusions

Results of this study showed that most of the variables examined did not significantly affect the stability of the invasion plasmid in *Shigella* spp. Therefore, in accordance with BAM culture method for isolating *Shigella* from foods, samples incubated anaerobically in novobiocin-supplemented *Shigella*

broth at 35±2°C and plated in MacConkey agar are suitable for subsequent DNA probe confirmation with the plasmid-specific synthetic probe. Further studies will determine the effectiveness of this probe for identifying *S. flexneri* in various food types enriched under these conditions.

Acknowledgments

We thank Foster D. McClure, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Division of Mathematics, for statistical analysis of the data.

References

(1) U.S. Food and Drug Administration (1992) *Bacteriological Analytical Manual*, 7th Ed., AOAC, Arlington, VA

(2) Lampel, K., & Jagow, J. (1989) *Abstr. Annu. Meet. Am. Soc. Microbiol.*, **P 14**, p. 321

(3) Sakai, T., Sasakawa, C., Makino, S., Kamata, K., & Yoshikawa, M. (1985) *Infect. Immun.* **51**, 476–482

(4) Maurelli, A., Blackmon, B., & Curtiss, R. (1983) *Infect. Immun.* **43**, 397–401

(5) Lampel, K., Jagow, J., Trucksess, M., & Hill, W. (1990) *Appl. Environ. Microbiol.* **56**, 1536–1540

(6) Tollison, S., & Johnson, M. (1985) *Appl. Environ. Microbiol.* **50**, 337–341

(7) Sanzey, B. (1979) *J. Bacteriol.* **138**, 40–47

(8) Sioud, M., Baldacci, G., de Recondo, A., & Forterre, P. (1988) *Nucleic Acids Res.* **16**, 1379–1390

(9) Mehlman, I., & Wentz, B. (1984) *Abstr. Annu. Meet. Am. Soc. Microbiol.*, **P 3**, p. 200

(10) Satchell, F., Stephenson, P., Andrews, W., Lampel, K., Jagow, J., & Allen, G. (1990) *Abstr. Annu. Meet. Assoc. Off. Anal. Chem.* **345**, p. 213

(11) Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

(12) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA

(13) Kirk, R.E. (1968) *Experimental Design: Procedures for the Behavioral Sciences*, Brooks/Cole Publishing Co., Belmont, CA

FOOD CHEMICAL CONTAMINANTS

Quantitation and Stability of Fumonisins B_1 and B_2 in Milk

CHRIS M. MARAGOS and JOHN L. RICHARD
U.S. Department of Agriculture/Agricultural Research Service, National Center for Agriculture Utilization Research, Mycotoxin Research Unit, 1815 N. University St, Peoria, IL 61604

The well-documented presence of fumonisin mycotoxins B_1 and B_2 (FB_1 and FB_2) in corn raises the possibility that these toxins are carried over into the milk of animals fed with contaminated feed. The presence of FB_1 and FB_2 in milk has not been assessed because of the lack of sensitive analytical techniques for this matrix. Two methods, liquid chromatography (LC) and enzyme-linked immunosorbent assay (ELISA), were adapted for the analysis of milk. The ELISA, produced commercially for screening corn, required no sample preparation and was reproducible but was of low sensitivity [concentration that inhibits color development by 50% (IC_{50}), 1200–1600 ng FB_1/mL]. The more sensitive LC method involves serial extraction of milk with methanol–acetone and strong anion exchange followed by derivatization with naphthalene-2,3-dicarboxaldehyde. Recoveries of 50 ng FB_1 and FB_2/mL from unpasteurized and unhomogenized milk were 84 and 83%, respectively (limit of detection, 5 ng/mL). Recoveries of FB_1 from whole homogenized milk (76%) were slightly lower. Heating milk for 30 min at 62°C, to mimic pasteurization, did not significantly reduce FB_1 or FB_2 recovery, nor did storing milk for 11 days at 4°C. The LC method was applied to 165 samples of milk, only 1 of which was positive. This finding suggests that exposure of humans to FB_1 and FB_2 from milk is low.

Fumonisins are a recently described group of mycotoxins produced by the molds *Fusarium moniliforme* and *F. proliferatum* (1–4). The fumonisins have been associated with several diseases in animals, including leukoencephalomalacia in horses (5), pulmonary edema in pigs (PPE) (6), and hepatocarcinogenesis in rats (7). Although the hazard that fumonisins pose to humans has not been determined, exposure has been linked to esophageal cancer in the Transkei region of southern Africa (8).

Received July 16, 1993. Accepted by GJ September 20, 1993.

Mention of companies or products by name does not imply their endorsement by the U.S. Department of Agriculture over others not cited.

The molds that produce fumonisins are common parasites and saprophytes of corn. Swine feeds suspected of causing PPE contain fumonisin B_1 (FB_1) at 20 to 360 ppm, and problem feeds for horses contain 8 to 117 ppm (9). The widespread use of corn in feed for dairy cattle raises the possibility that human exposure can occur indirectly through edible products (milk and meat) from exposed animals, a progression known to occur with the aflatoxins. Assessment of exposure through milk has been hindered because, heretofore, no analytical methods were available for fumonisins in this matrix.

The objective of this study was to develop methods for the analysis of FB_1 and fumonisin B_2 (FB_2) in milk. Two analytical methods were developed: a liquid chromatographic (LC) method sensitive to 5 ng FB_1 or FB_2/mL milk and an immunoassay, adapted from a commercial enzyme-linked immunosorbent assay (ELISA) sensitive to 250 ng/mL. Results suggest that the LC method is sufficient for the screening of milk for FB_1 and FB_2.

Materials and Methods: Liquid Chromatographic Analysis

Safety note: Fumonisins B_1 and B_2 reportedly promote cancer in rats (7) and should be handled with appropriate caution.

Extraction of Milk for Liquid Chromatographic Analysis

Milk was extracted with methanol–acetone, and then FB_1 and FB_2 were isolated from the organic extract by using solid-phase extraction (SPE) columns with a strong anion exchange (SAX) bonded phase. The fumonisins were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) and separated by LC. The fluorescent products were quantitated.

Reagents

(a) *Water.*—Use deionized water throughout (Nanopure II, Sybron/Barnstead).

(b) *Solvents.*—LC grade methanol, acetone (Fisher Scientific), and acetonitrile (Burdick & Jackson) were used throughout.

(c) *NDA.*—Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Molecular Probes, Inc., Eugene, OR (lot 2411-3), dissolved to a concentration of 0.25 mg/mL in methanol, and stored at −12°C.

(d) *General chemicals.*—Acetic acid (glacial), sodium borate, and sodium cyanide were ACS reagent grade and obtained from major suppliers.

(e) *Standards.*—Standard fumonisins B_1 and B_2, used for recovery and stability experiments, were purchased from Sigma Chemical Co. (lots 121H0066, 102H0850, and 100H05801). Each fumonisin was dissolved to 125 μg/mL in acetonitrile–water (1 + 1), distributed into 11 mm diameter amber vials (National Scientific Company, Cat. No. C4011-2), sealed with crimp tops, and stored at −20°C until use. A working standard (1.00 μg/mL) was prepared daily by diluting the stock solution (warmed to room temperature) with water.

(f) *Mobile phases.*—Methanol–water–acetic acid (75 + 24 + 1, v/v/v) (A) and acetonitrile–water–acetic acid (75 + 24 + 1) (B). Sparge solutions with helium before and during use. Prepare (B) fresh daily to maintain reproducible retention times.

Apparatus

(a) *Shaker.*—Burrell wrist-action shaker Model 75.

(b) *Centrifuge.*—Sorvall Model RC-2 refrigerated centrifuge with Model SS-34 rotor.

(c) *Centrifuge tubes.*—50 mL capacity, Teflon.

(d) *Vacuum evaporation.*—Brinkmann Rotovapor-R, with 50°C water bath and 125 mL Pyrex flasks.

(e) *Storage tubes.*—50 mL capacity disposable centrifuge tubes, polypropylene (Sarstedt).

(f) *Solid-phase extraction columns.*—Bond Elut strong anion exchange (SAX); Varian, 3CC with 500 mg sorbent mass (Cat. No. 1210-2044); lots 182795, 181955, and 182345.

(g) *Syringe hand pump and coupling.*—Vicam (Somerville, MA).

(h) *Screw-cap vials.*—60 × 17 mm amber vials with Teflon-lined caps (Scientific Specialties Service, Inc., Cat. No. B75582).

(i) *Heating block.*—Reacti-Therm heating module, Model 18780 (Pierce Chemical Co.).

(j) *Chromatography.*—Spectra-Physics SP8700 solvent delivery system; 8750 mixer; Rheodyne 7125 injector with 50 μL sample loop; Applied Biosystems, Inc., Spheri-5 column (RP-18, 220 × 4.6 mm, 5 μm) and RP-18 NewGuard column; Spectra-Physics fluorescence detector Model FL2000 and integrator Model SP4270.

Procedure

(a) *Samples.*—Pasteurized, homogenized milk (whole, 2%, or skim) with vitamins A and D added was purchased from a local (Peoria, IL) cafeteria, stored refrigerated, and used prior to the manufacturer's expiration date. Unpasteurized milk was obtained from the Wisconsin Department of Agriculture, Trade, and Consumer Protection as part of a joint survey of mycotoxin contamination (the results of which will be published separately). Unpasteurized milk, stored at 4°C, was used within 4 days of collection.

(b) *Organic extraction.*—For recovery studies, FB_1 and FB_2 were added to milk at 50 ng/mL before extraction. Fifteen milliliters of methanol–acetone (1 + 1, v/v) was added to 5 mL milk, and the mixture was shaken for 10 min. The mixture was cooled in a dry ice–methanol bath for 10 min and then centrifuged at 10 000 rpm for 10 min at −20°C. The supernatant was transferred to a 50 mL Teflon centrifuge tube and held at −70°C. The pellet was mixed with an additional 15 mL methanol–acetone and extracted as described above but without shaking. The supernatant solutions from the first and second extracts were pooled and allowed to cool to −70°C. The combined extract was centrifuged at 12 000 rpm for 3 min at −20°C and stored in polypropylene centrifuge tubes at −20°C.

(c) *SAX column cleanup.*—A modification of the SAX method of Shephard et al. (10) was used to further isolate the fumonisins from this extract. On the day of assay, the organic extract was transferred to a 125 mL flask and concentrated under vacuum at 50°C to 1–2 mL, and 5 mL methanol was added. The SAX column was conditioned with 5 mL methanol and 5 mL methanol–water (3 + 2, v/v). The extract was applied at a flow rate of 1 mL/min, the flask was rinsed with 2.5 mL methanol–water, and the rinse was applied to the column. After the column was washed with 5 mL methanol, the fumonisins were eluted into an amber vial with 7 mL 5% acetic acid in methanol. The acidic extract was dried under a gentle stream of nitrogen at 60°C and then derivatized with NDA.

(d) *Derivatization with NDA.*—Dried SAX extracts of milk were solubilized in 0.5 mL methanol–water (1 + 1, v/v). Fumonisin standards were diluted from the working stock solution to obtain the desired concentration, with a final methanol/water ratio of 1:1. The extract or standard was derivatized with NDA by using a modification of the method of Ware et al. (11, 12). One milliliter of 0.05M borate buffer (pH 9.5) was added to the extract, followed by 0.2 mL sodium cyanide (0.1 mg/mL) and 0.2 mL NDA (0.25 mg/mL). After 15 min at 60°C, the derivatized solution was allowed to cool and then diluted with 3.1 mL acetonitrile–water (3 + 2, v/v).

(e) *Quantitation of FB_1 and FB_2 by liquid chromatography.*—The NDA derivatives of FB_1 and FB_2 were separated and quantitated by reversed-phase LC with fluorescence detection. The column was equilibrated with 70% mobile phase A and 30% mobile phase B at a flow rate of 1 mL/min. The sample (50 μL) was injected, and at 2.6 min, the mobile phase was changed to 55% A + 45% B. At 10.1 min, the mobile phase was changed to 100% B, and at 14.1 min, the mobile phase was returned to the original mixture. Fluorescence was elicited with excitation at 250 nm and detected at 470 nm. The fumonisin contents of unknowns were calculated by comparing peak heights to those of FB_1 and FB_2 standards derivatized concurrently and chromatographed on the same day as the samples.

Materials and Methods: Enzyme-Linked Immunosorbent Assay (ELISA)

A competitive direct ELISA, available commercially for screening corn for fumonisins, was adapted here for use with milk.

Reagents

(a) *ELISA kits.*—Fumonisin Agri-Screen (Neogen Corp., Lansing, MI; Cat. No. 70/8810), serial 18003. Kits contain two 12-well, antibody-coated strips; two 12-well mixing strips; fu-

monisin–peroxidase conjugate solution; enzyme substrate; and stop reagent. The kit format was that of a direct-competitive ELISA, in which free toxin and toxin–peroxidase conjugate compete for a limited number of antibody–binding sites on microtiter wells. After the microtiter wells are washed, the amount of labeled toxin that remains bound is measured colorimetrically after addition of substrate, incubation, and addition of stop reagent.

(b) *Wash solution.*—Phosphate-buffered saline (10 mM, pH 7.4) with 0.02% (v/v) Tween 20 added.

Apparatus

(a) *Strip holder.*—Removawell, Dynatech Laboratories, Inc. (Cat. No. 011-010-6604).

(b) *ELISA microplate washer.*—Bio-Tek Instruments, Model EL403, washes all wells of a 96-well microtiter plate concurrently.

(c) *Incubator.*—Microplate incubator (Scientific Products, Cat. No. J1668-1), adjusted to 37°C.

(d) *Microplate reader.*—Dynatech Laboratories, Inc., Model MR5000, equipped with a 650 nm filter and interfaced with a Gateway 2000 486DX2/50 computer.

(e) *Pipets.*—Multichannel pippetor (Costar 8-channel pipet adjustable over the range 20–200 µL); Gilson Pipetman continuously adjustable pipets with disposable tips P-100, P-200, P-1000, and P-5000.

(f) *Reagent reservoirs.*—For use with multichannel pipets (Biorad, Cat. No. 224-4872).

Quantitation of FB_1 by ELISA

Fumonisin B_1 was added to milk previously verified by LC as having FB_1 and FB_2 at less than 5 ng/mL to achieve final concentrations of 10 to 5000 ng/mL. The milk was analyzed by a modified direct-competitive ELISA that is commercially available for analysis of corn. Modifications include the use of multichannel pipets, an automatic microplate washer, and a competition incubation at 37°C for 30 min rather than at room temperature. Also, microtiter plates were washed 4 times with 0.35 mL wash solution per well rather than with water. Negative controls consisted of water and unspiked milk; positive controls consisted of fumonisins added to water and a spiked corn extract.

Results and Discussion

Analysis of Fumonisins in Milk by ELISA

The ELISA was adapted from a commercially available kit with minor modifications. The advantages of the ELISA are that no sample preparation is required, multiple samples can be analyzed concurrently, and the assay is rapid (total time, about 1 h). However, although the assay was reproducible (Figure 1), substantial amounts of fumonisins must be present for adequate detection with the ELISA. The lowest toxin concentrations beyond 2 standard deviations of the toxin-free control, a parameter often used as an estimate of assay sensitivity, ranged from 100 to 300 ng FB_1/mL. The concentrations required to inhibit color development by 50% (IC_{50}) ranged from 1260 to 1600 ng/mL (Figure 1). Evidently, matrix effects influence estimates of toxin concentration. These likely include the fat and protein contents of the sample. A preliminary study (data not shown) indicated that adding a small amount of protein (1% albumin) slightly enhanced the sensitivity, but increasing the fat content may have an opposite effect (Figure 1). This result suggests that, for unknown samples, the standard curve should be obtained with toxin-free milk of similar composition.

The concentration range over which the ELISA could be used reliably was determined from the standard curve (Figure 1) and a precision profile of coefficient of variation (CV) vs toxin concentration in whole milk (Figure 2) (13). For example, if a CV of 10% is tolerable, the assay can be used to measure fumonisins at concentrations between 250 and 2500 ng/mL whole milk. Before using any immunoassay kit, the user must construct a precision profile to estimate variability within the laboratory.

Although the useful range for the assay was narrow, no sample preparation was required, and the assay was very simple to perform. Substantial improvements in the ELISA sensitivity likely can be obtained by altering the assay format, by using antibodies with higher affinity, and possibly by altering the fumonisin–peroxidase conjugate. The only published ELISA for fumonisins, upon which the current method is based, gave an IC_{50} of 480–790 ng FB_1/mL in buffer (14). The assay has cross-reactivity with FB_2 and fumonisin B_3 (FB_3) but not with the

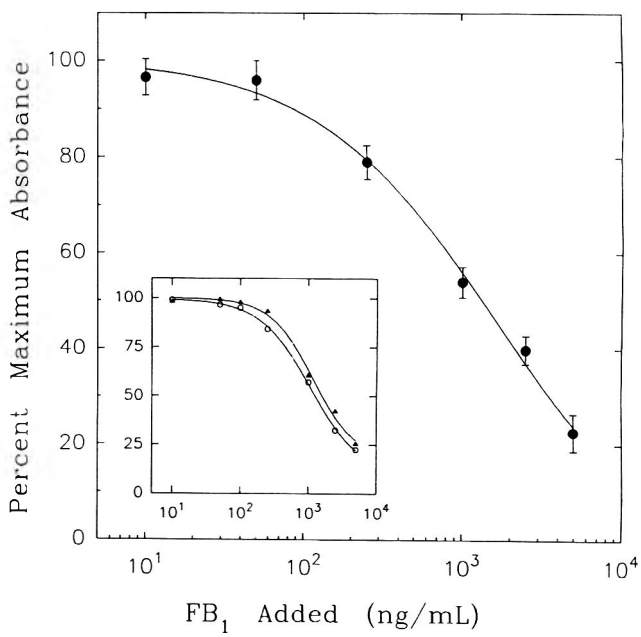


Figure 1. Standard curve of FB_1 added to whole milk (●). Insert: standard curves of FB_1 added to water (▲) and skim milk (○). The concentrations of FB_1 required to inhibit color development by 50% (IC_{50}) were 1340, 1600, and 1260 ng/mL for whole milk, water, and skim milk, respectively. Average absorbances for toxin-free controls with each of these matrixes were 0.988, 0.917, and 0.887.

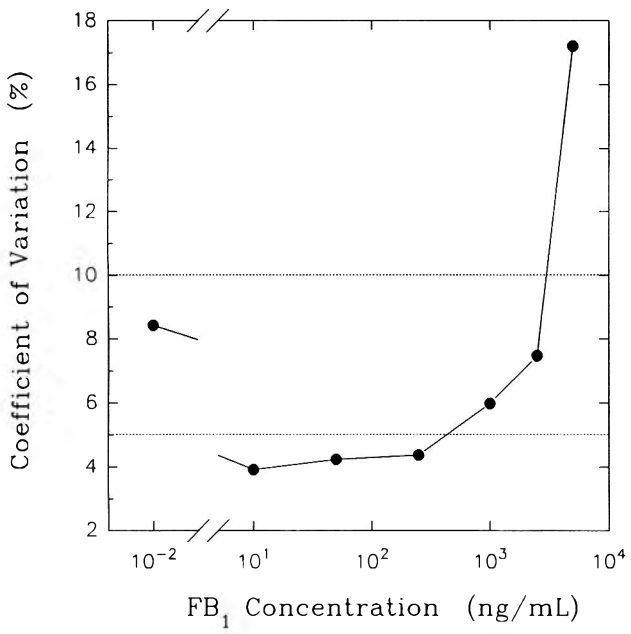


Figure 2. Precision profile of the fumonisin ELISA. Data are from 4 separate experiments with triplicate determinations (n = 12) of FB_1 in whole, homogenized milk.

hydrolysis product of fumonisin B_1 (HFB_1) or tricarballylic acid (14). The approximately 2-fold greater IC_{50} observed in our study most likely results from optimization of the assay for corn (rather than milk) by the manufacturer.

Analysis of Milk by Liquid Chromatography

More sensitive than the ELISA method was an LC method based on organic extraction followed by solid-phase extraction with a SAX column. The analytical recoveries of FB_1 and FB_2 from several varieties of fluid milk are described in Table 1. The chromatograms for unspiked raw milk show a stable baseline near the retention times of FB_1 and FB_2 (Figure 3). When FB_1 and FB_2 were added to this milk at levels of 2 and 1 ng/mL, respectively, they were detected. A level of 5 ng/mL produced peaks with a signal-to-noise ratio of >4. This sensitivity is superior to that reported for FB_1 in plasma and urine (50 ng/mL) with a signal-to-noise ratio of 4 (10). Recoveries of FB_1 ranged from 76.3 ± 7.4% in whole milk to 88.1 ± 7.8% in low-fat milk; recoveries of FB_2 ranged from 81 ± 3.1 to 85.9 ± 13.1% (Table 1). Milk fat content did not affect FB_1 or FB_2 recovery with this method (Table 1).

A number of factors affect the practical application of this method. Milk that was near the expiration date yielded an extract with peaks having retention times near those of FB_1 and FB_2, and care must be taken in their interpretation. In the present study, coinjection of derivatized FB_1 and FB_2 with the suspect extract was sufficient to indicate that the peaks in poor-quality milk were not due to either of these toxins. Interferences were generally not a problem provided the samples were either pasteurized and/or homogenized milk or were raw milk extracted within 7 days of collection. In addition to sample freshness, other conditions improved the results, including use of fresh NDA (prepared within 1 week of use) and fresh mobile phase. In addition, allowing the methanol–acetone extracts to remain at –20°C for several days reduced the background interferences in poor-quality milk.

Stability of Fumonisins in Milk

Fumonisins B_1 and B_2 are stable in corn but not in heated corn products (15). Because milk undergoes heat treatment (pasteurization), the effect of temperature on fumonisin recovery was examined. Recoveries of FB_1 and FB_2 did not change when spiked whole milk was held for up to 11 days at 4°C (Table 2). Similarly, spiked raw milk heated to 62°C for 30 min (a heat treatment equivalent to pasteurization) did not show any loss of FB_1 or FB_2. Subjecting spiked, raw milk to 90°C for 30 min reduced recoveries slightly: by 7% for FB_1 and 13% for FB_2. Thus, heat treatment of milk, except at very high temperatures, would not substantially reduce FB_1 or FB_2. If fumonisins are present in unpasteurized milk, they may be expected to carry over into the pasteurized product.

Natural Occurrence of Fumonisins in Milk

To help determine the exposure of humans to fumonisins through milk, a limited survey was conducted in cooperation with the state of Wisconsin. Over 150 samples of unpasteurized, unhomogenized milk were collected from various sites throughout the state between March 15 and May 3, 1993, by the Wisconsin Department of Agriculture, Trade, and Consumer Protection. The survey was initiated as a result of the severe mold problem reported in the 1992 Wisconsin corn crop (16). Of the 155 samples analyzed, only 1 was found, by the LC procedure, to contain greater than 5 ng FB_1/mL. In addition, 10 samples of whole, homogenized milk purchased in Peoria, IL, all contained less than 5 ng FB_1 and FB_2/mL.

The single positive sample, when cleaned up by using the procedures indicated here and analyzed by gas chromatography/mass spectrometry (GC/MS) contained substantially less FB_1 than expected (1.29 ng/mL; Ron Plattner, personal communication). This result indicates that substantial variability exists, as well as the potential for false-positive responses, when the LC method is used for quantitation near the limit of detection. Despite this limitation, the extent of contamination of milk with fumonisins, above 5 ng/mL, clearly was low even

Table 1. Recoveries of FB_1 and FB_2 from milk by LC method

Matrix	Recovery, %[a]		
	FB_1	FB_2	n
Whole milk	76.3 ± 7.4	85.9 ± 13.1	17
Low-fat milk	88.1 ± 7.8	81.0 ± 3.1	6
Skim milk	78.0 ± 6.1	82.4 ± 3.3	6
Raw milk[b]	84.1 ± 7.5	83.4 ± 5.1	11

[a] Recovery of each fumonisin added at 50 ng/mL.
[b] Unpasteurized and unhomogenized milk.

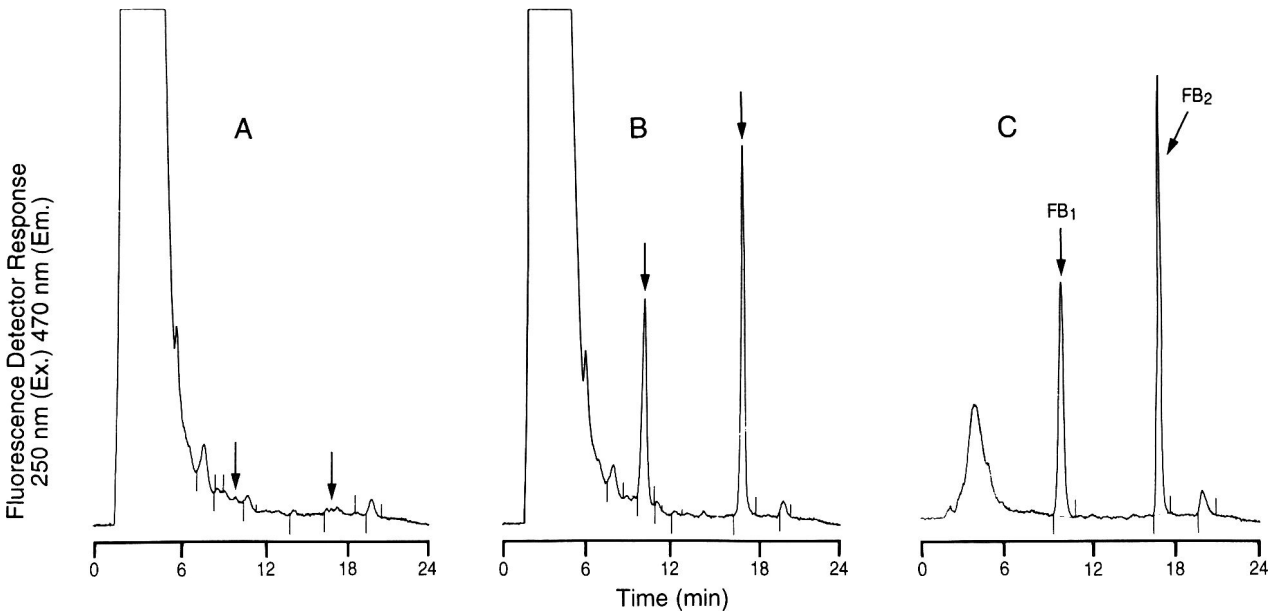


Figure 3. Analysis of fumonisins in raw (unpasteurized, unhomogenized) milk by liquid chromatography. (*A*) toxin-free control; (*B*) the same milk as in (*A*) to which 50 ng each of FB_1 and FB_2 was added per mL; (*C*) standard FB_1 and FB_2 in methanol–water (1 + 1, v/v) at a level equivalent to 50 ng/mL of milk.

under conditions where the potential for *Fusarium* contamination of corn was substantial. The extent that fumonisin in feed is carried over into milk needs to be determined. The levels of FB_1 and FB_2 in milk produced in other years, when there is a problem with fumonisins in corn, also should be investigated.

Conclusions

The analytical methods described for measuring fumonisins B_1 and B_2 in milk differ substantially in sensitivity and ease of use. The ELISA method can be performed rapidly, but the sensitivity is poor. Currently under development are more sensitive immunoassays to replace the present ELISA method. Although more tedious, the LC method is more sensitive (limit of detection, 5 ng/mL) and, in addition, identifies the fumonisin present. The latter method permits the screening of milk for the presence of FB_1 or FB_2 and will allow determination of the extent to which fumonisins are carried over from diet to milk. Sample preparation for the chromatographic method, however, is sufficiently laborious that we are investigating alternatives, such as replacing the organic extraction with an affinity column and using tandem C_{18}–SAX solid-phase extractions (17).

Table 2. Effect of storage time and heat treatment on recovery

Matrix	Temp., °C	Time[b]	Recovery, %[a] FB_1	Recovery, %[a] FB_2	*n*
Whole milk	NT[c]	0[c]	68.3 ± 3.4	75.1 ± 4.16	6
	4	1 day	80.2 ± 3.1	86.0 ± 2.7	3
	4	6 days	76.1 ± 2.6	88.2 ± 3.7	3
	4	11 days	78.1 ± 9.0	81.4 ± 7.2	3
Raw milk	NT[c]	0	82.0 ± 7.8	84.2 ± 5.7	8
	62	30 min	79.0 ± 7.8	80.3 ± 5.1	6
	90	30 min	75.3 ± 5.1	71.5 ± 4.0	6

[a] Recovery of FB_1 or FB_2 added at 50 ng/mL.
[b] Time during which the sample was held at the indicated temperature.
[c] No treatment; control sample was extracted immediately after addition of FB_1 and FB_2.

Acknowledgments

We thank James Selkirk and John Daubert of the Wisconsin Department of Agriculture, Trade, and Consumer Protection and Dr. Fun Sun Chu of the University of Wisconsin-Madison for providing the samples of unpasteurized, unhomogenized milk. We also thank Ron Plattner of the USDA (Peoria, IL) for checking the extract of our positive milk sample by using his GC/MS method.

References

(1) Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.F.O., Spiteller, G., & Vleggaar, R. (1988) *J. Chem. Soc. Chem. Commun.* **11**, 743–745

(2) Ross, P.F., Nelson, P.E., Richard, J.L., Osweiler, G.D., Rice, L.G., Plattner, R.D., & Wilson, T.M. (1990) *Appl. Environ. Microbiol.* **56**, 3225–3226

(3) Thiel, P.G., Marasas, W.F.O., Sydenham, E.W., Shephard, G.S., Gelderblom, W.C.A., & Nieuwenhuis, J.J. (1991) *Appl. Environ. Microbiol.* **57**, 1089–1093

(4) Nelson, P.E., Plattner, R.D., Shackelford, D.D., & Desjardins, A.E. (1992) *Appl. Environ. Microbiol.* **58**, 984–989

(5) Wilson, T.M., Ross, P.F., Owens, D.L., Rice, L.G., Green, S.A., Jenkins, S.J., & Nelson, H.A. (1992) *Mycopathologia* **117**, 115–120

(6) Colvin, B.M., & Harrison, L.R. (1992) *Mycopathologia* **117**, 79–82

(7) Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R., & Kriek, N.P.J. (1988) *Appl. Environ. Microbiol.* **54**, 1806–1811

(8) Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., Van Schalkwyk, D.J., & Koch, K.R. (1990) *J. Agric. Food Chem.* **38**, 1900–1903

(9) Ross, P.F., Rice, L.G., Osweiler, G.D., Nelson, P.E., Richard, J.L., & Wilson, T.M. (1992) *Mycopathologia* **117**, 109–114

(10) Shephard, G.S., Thiel, P.G., & Sydenham, E.W. (1992) *J. Chromatogr.* **574**, 299–304

(11) Ware, G.M. (1990) Abstracts of the Food Drug Officials Southern States Annual Spring Conference, April 8–11, St. Petersburg, FL, p. 15

(12) Bennett, G.A., & Richard, J.L. (1994) *J. AOAC Int.* **2**, 501–506

(13) Feldkamp, C.S. (1992) in *Immunochemical Assays and Biosensor Technology for the 1990s*, R.M. Nakamura, Y. Kasahara, & G.A. Rechnitz (Eds), American Society for Microbiology, Washington, DC, pp. 83–109

(14) Azcona-Olivera, J.I., Abouzied, M.M., Plattner, R.D., & Pestka, J.J. (1992) *J. Agric. Food Chem.* **40**, 531–534

(15) Scott, P.M., & Lawrence, G.A. Abstracts of the 106th AOAC INTERNATIONAL Annual Meeting and Exposition, Abstract 204, AOAC INTERNATIONAL, Arlington, VA, p. 144

(16) Smalley, E.B., & Chu, F.S. (1993) Symposium on Molds, Mycotoxins, and Their Effects on Agricultural Animals, University of Wisconsin, March 24, Madison, WI, p. 7

(17) Holcomb, M., Thompson, H.C., Jr, & Hankins, L.J. (1993) *J. Agric. Food Chem.* **41**, 764–767

FOOD CHEMICAL CONTAMINANTS

Application of Gas Chromatography/Matrix Isolation/Fourier Transform Infrared Spectroscopy to the Identification of Pyrrolizidine Alkaloids from Comfrey Root (*Symphytum officinale* L.)

MAGDI M. MOSSOBA, HUBERT S. LIN, [1] DENIS ANDRZEJEWSKI, and JAMES A. SPHON
U.S. Food and Drug Administration, Division of General Scientific Support, Washington, DC 20204
JOSEPH M. BETZ, LEE J. MILLER, ROBERT M. EPPLEY, MARY W. TRUCKSESS, and SAMUEL W. PAGE
U.S. Food and Drug Administration, Division of Natural Products, Washington, DC 20204

This paper demonstrates that pyrrolizidine alkaloids (PAs) extracted from comfrey root grown in Washington State (USA) can be identified by gas chromatography/matrix isolation/Fourier transform infrared (GC/MI/FTIR) spectroscopy. Infrared spectral bands observed in the fingerprint region were unique even for closely related structures. The identities of the 4 major components, intermedine, lycopsamine, 7-acetylintermedine, and 7-acetyllycopsamine, were confirmed by comparison with standards. Confirmation was also obtained by using the established techniques of electron ionization and positive ion chemical ionization gas chromatography/mass spectrometry. The infrared spectra observed for the components of the root extract were consistent with known structures of specific PAs. The identities of the minor components, symphytine and its isomers symlandine and/or symviridine, in the same extract were not confirmed.

Received August 20, 1993. Accepted by AP November 19, 1993.

[1]Physical Science Aide (summers of 1991 and 1992). Currently a student at the University of Maryland Medical School, Baltimore, MD.

Comfrey (*Symphytum* sp.) is a perennial with green hairy leaves that is grown as animal feed and used by herbalists in salads or as a vegetable, and as a medicinal herb (1, 2). It is also consumed as herbal tea and "green drink" (3), and is available as root or leaf, and in capsule form at health food stores (4).

However, comfrey contains toxic (5–8) and carcinogenic (9–12) unsaturated pyrrolizidine alkaloids (PAs), which are also found in contaminated cereal (13) and other food products. The role of PAs in the etiology of human and animal liver disease is well documented: Liver enzymes convert unsaturated PAs into the corresponding pyrroles, which are potent alkylating agents (14–16). PA-containing plants are known to cause the slow death of livestock (17, 18), and regular ingestion of comfrey root capsules or comfrey leaves was implicated in severe liver damage in humans (19).

Many techniques have been used to determine both the total and individual PA content of plants, including thin-layer chromatography (20–23), liquid chromatography (15, 23–28), gas chromatography (GC) (1, 21, 29–33), and supercritical fluid chromatography (33). In a recent study on the analysis of botanical materials and commercial comfrey products for PAs, a method was developed for the extraction, solid-phase concentration, and capillary GC determination of these alkaloids (34). These procedures were used in the present work.

The structural elucidation of PAs by x-ray diffraction (35, 36), nuclear magnetic resonance spectroscopy (37–39), or infrared spectroscopy (40, 41) requires the isolation of each PA of interest from botanicals. However, the application of tandem techniques, such as GC/mass spectrometry (MS) (30, 31) or GC/Fourier transform infrared (FTIR) spectroscopy (42), makes it possible to confirm the identity of individual PAs in plant extracts containing complex mixtures of PAs as long as standards are available.

In the present work components of a comfrey root (*Symphytum officinale* L.) extract were identified by capillary GC/matrix isolation (MI)/FTIR spectroscopy (43, 44) as well as by commonly used electron ionization (EI) and positive ion chemical ionization (PICI) GC/MS techniques, and the spectra were compared with those of several available PA standards. Distinctive IR spectral bands were found for different isomers. These IR data were consistent with the known functional groups of the corresponding PAs found in this extract.

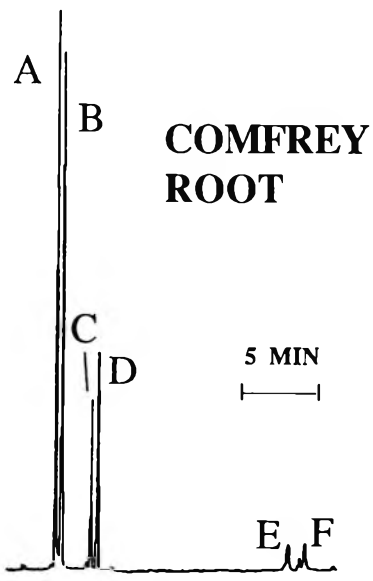
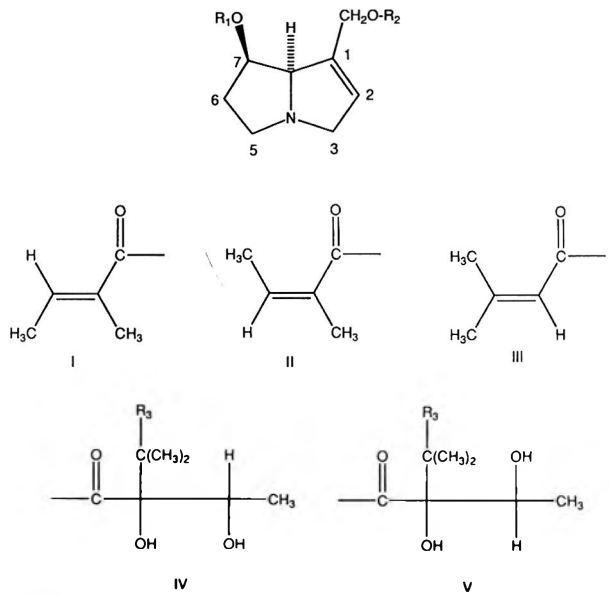


Figure 1. Gas chromatogram for comfrey root (*Symphytum officinale* L.) extract. The elution sequence of the observed FID profile components was A, intermedine; B, lycopsamine; C, 7-acetylintermedine; D, 7-acetyllycopsamine; E and F, see text.

Experimental

Materials

Intermedine, lycopsamine, 7-acetylintermedine, and 7-acetyllycopsamine were a gift from R.J. Molyneux (U.S. Department of Agriculture, Albany, CA). Air-dried, domestic comfrey roots grown in Washington State (USA) were obtained from a bulk botanical dealer. The botanical identity of the ground bulk material was confirmed to be *S. officinale* on the basis of several criteria. The absence of echimidine reported in the present study (*see Results*) is in agreement with the chemotaxonomic work of Huizing et al. (45), which firmly established the absence of echimidine in *S. officinale* L. and confirmed the identity of echimidine in *S. asperum* and *S. xuplandicum*. Identification of the root powder was also based on the allantoin content, alkaloid pattern, and microscopic appearance, which were reported by Huizing et al. (45) and Awang et al. (46) and considered to be sufficient for confirmatory identification of the root powder. Chloroform and methanol were purchased from Baxter/B&J (McGaw, IL). Zinc dust, sulfuric acid, and



NAME	R_1	R_2	R_3
lycopsamine	H	IV	H
intermedine	H	V	H
7-acetyllycopsamine	CH_3CO	IV	H
7-acetylintermedine	CH_3CO	V	H
symphytine	I	IV	H
symlandine	II	IV	H
symviridine	III	IV	H
echimidine	II	IV or V	OH

Figure 2. Structures of pyrrolizidine alkaloids.

ammonium hydroxide were acquired from J.T. Baker Chemical Co. (Phillipsburg, NJ). Celite and Whatman No. 1 filter paper were obtained from Fisher Scientific Co. (Pittsburgh, PA). ChemElut CE-1020 columns were purchased from Varian Sample Preparation Products (Harbor City, CA). Regisil [bis(trimethylsilyl)trifluoroacetamide (BSTFA)] and Derivasil [mixture of pyridine, BSTFA, trimethylchlorosilane, and (trimethylsilyl)imidazole] were obtained from Regis Chemical Co. (Morton Grove, IL).

Test Sample Preparation

Ten grams of ground comfrey root and 5 g Celite were shaken for 1 h with 10 mL 30% ammonium hydroxide and 250 mL chloroform–methanol (85 + 15). The mixture was filtered and the filtrate was evaporated at 45°C on a rotary evaporator. The *N*-oxides of the PAs in the residue were reduced to the corresponding free bases by dissolving the residue in 50 mL 2N sulfuric acid and adding 2.5 g zinc dust to the solution. This slurry was stirred for 2 h and filtered. Concentrated ammonium hydroxide (7.4N, 5 mL) was added, with stirring, to the collected filtrate (15 mL). The resulting basic solution was poured into a ChemElut 1020 column and allowed to equilibrate for 5 min. The PAs were eluted from the column with 100 mL chloroform–ammonium hydroxide (99 + 1), and the eluate was evaporated to dryness at 45°C on a rotary evaporator. The residue was resuspended in 1 mL methanol for GC analysis.

Trimethylsilyl Derivatization

Echimidine and swazine test samples were treated with 50 µL Regisil–Derivasil (1 + 2), and the vials were sealed with screw caps fitted with Teflon-faced silicone septa. The resulting solutions were mixed on a Vortex mixer and heated at 60°C for 30 min for derivatization.

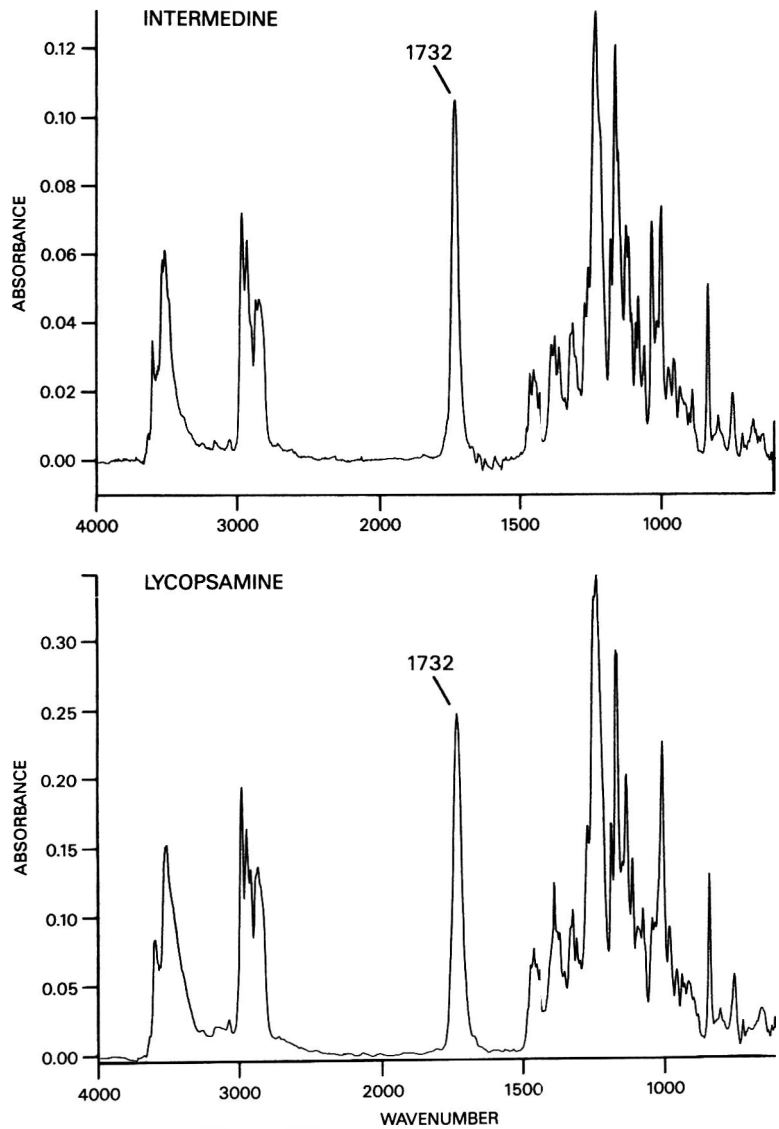


Figure 3. MI/FTIR spectra collected at 4 cm^{-1} resolution for the major comfrey root PA constituents, intermedine and lycopsamine (peaks A and B, Figure 1).

Table 1. Observed MI/FTIR frequencies (cm^{-1}) for PAs in comfrey root extract

Assignment	INT[a]	LYC	ACI	ACL	E	F
OH stretch						
free	3609	3601	3602	3606	3605	3601
associated	3526	3516	3511	3521	3522	3520
=C–H stretch	3071	3070			3077	3077
CH_3 asym str	2980	2977	2983	2982	2982	2985
CH_2 asym str	2944	2943	2943	2943	2942	2948
CH str	2917	2915	2919	2919	2921	2921
CH_3 sym str	2882	2882	2878	2882	2881	2883
CH_2 sym str	2859	2865			2864	
	2826	2830	2818	2816	2812	2813
C=O stretch						
CH_3COOR			1749	1749		
R'COOR	1732	1732	1732	1732	1722	1726
C=C stretch					1660	1658
CH_3 asym bend	1475	1474	1475	1476	1474	
	1462	1461	1454	1460		1454
CH_2 sym bend	1441	1441	1442	1442	1443	1446
CH_3 sym bend	1387	1388	1387	1387	1385	1386
	1372	1369	1376	1376	1368	
OH in-plane bend	1264	1269		1267	1368	
(O)C-O-C str	1235	1235	1244	1245	1273	1234
					1258	
					1247	
C-O str in						
tertiary ROH	1164	1165	1164	1166	1164	1164
secondary ROH	1124	1129	1125	1132	1139	1152
ring OH	999	1001				
ring breathing	891					
CH out-of-plane bend	836	836	825	826	833	
						819
C-C-C def bet 2 rings	750	748			745	

[a] Abbreviations: INT = intermedine; LYC = lycopsamine; ACI = 7-acetylintermedine; ACL = 7-acetyllycopsamine; E = GC peak E; F = GC peak F.

GC/IR Instrumentation

Gas chromatographic separations were performed on a Hewlett-Packard Model 5890 instrument (Avondale, PA) equipped with a flame ionization detector and a Hewlett-Packard Model 3392A integrator. A 50 m × 0.32 mm (id) RSL-200 capillary column (Chrompack, Inc., Bridgewater, NJ) with a 0.19 μm stationary phase film was used. Helium containing 1.5% argon (Matheson Gas Products, Secaucus, NJ) at ca 27 cm/s linear velocity was used as the carrier gas, and helium (99.995%) at 30 mL/min was used as the makeup gas to the detector. The injector and detector temperatures were 250 and 300°C, respectively. The carrier gas mixture was purified by using a Hydro-Purge II filter (Alltech Associates, Deerfield, IL) and a heated gas purifier filter (Supelco, Bellefonte, PA). The injection mode was splitless and a 10 μL Hamilton 701N syringe was used. Injections of ca 1 μL, containing analytes in the nanogram range, were made 6 s after the start of a run, and the total injection time was 15 s. The injector was purged 1 min after the start of the run. The initial column oven temperature was 45°C with a 2 min hold, followed by a 20°C/min increase to 220°C; the oven was held at this temperature for about 15 min until the analysis was complete.

A column effluent split ratio was calculated from the flame ionization detection (FID) response factors (area counts per nanogram injected) obtained with the column directly inserted

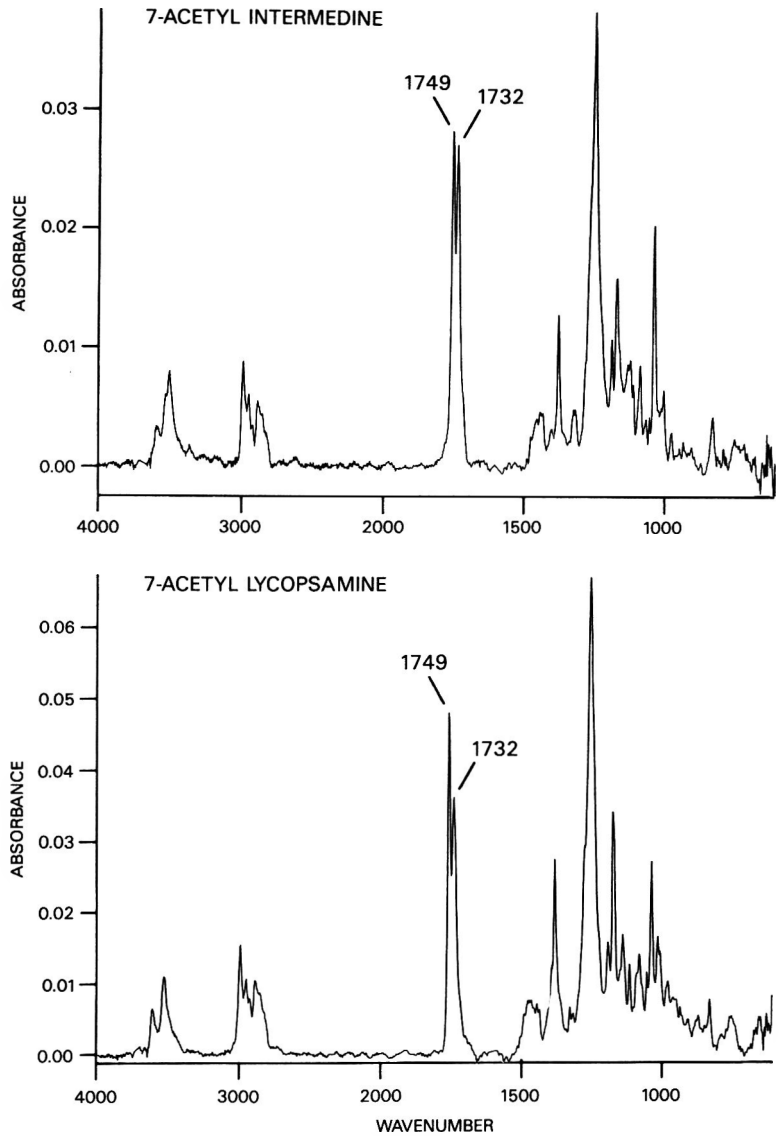


Figure 4. MI/FTIR spectra collected at 4 cm^{-1} resolution for the major PA constituents in comfrey root, 7-acetylintermedine and 7-acetyllycopsamine (peaks C and D, Figure 1).

into the flame ionization detector, and again with the column attached to the GC/MI interface. The split ratio was 4:1, MI/IR:FID.

A Sirius Model 100 FTIR spectrometer (Mattson Instruments, Inc., Madison, WI) equipped with an MI Cryolect interface operating at 12°K under vacuum was used. This system has been described in detail (43, 44). The MI method involved adding argon (1.5% by volume) to the GC carrier gas (helium) and trapping the effluent onto the outer rim of a slowly rotating (at ca 3 mm/min) gold disk held at cryogenic temperatures. During a run, helium was removed by the vacuum pumps, and the analyte molecules surrounded by an excess of argon atoms were frozen into a solid matrix on the gold disk. The IR-transparent argon matrix containing the isolated analytes was subsequently analyzed by IR spectroscopy. The position of each analyte peak on the Cryolect collection disk was indexed by its observed GC retention time. Procedures were previously described in detail (47) for reproducibly locating a peak maximum on the collection disk and for optimizing the performance of the system. These latter procedures, which include optical alignment, can minimize the extent of post-column peak broadening.

GC/MI/FTIR Analysis

Three hundred analyte interferograms were coadded (2 min 43 s at 4 cm^{-1} resolution), and the background (300 scans) was usually collected before or after the analyte peak. The 4 cm^{-1} resolution was chosen because it matched the natural half-widths of the IR bands.

GC/MS Instrumentation

EI mass spectra were obtained on a Finnigan Mat TSQ-46 mass spectrometer interfaced to an Incos 2300 data system with revision D software. The instrument operating parameters were 140°C source temperature, 0.35 mA emission, 70 eV ionization energy, 10^{-8} A/V pre-amplifier, and −5 kV conver-

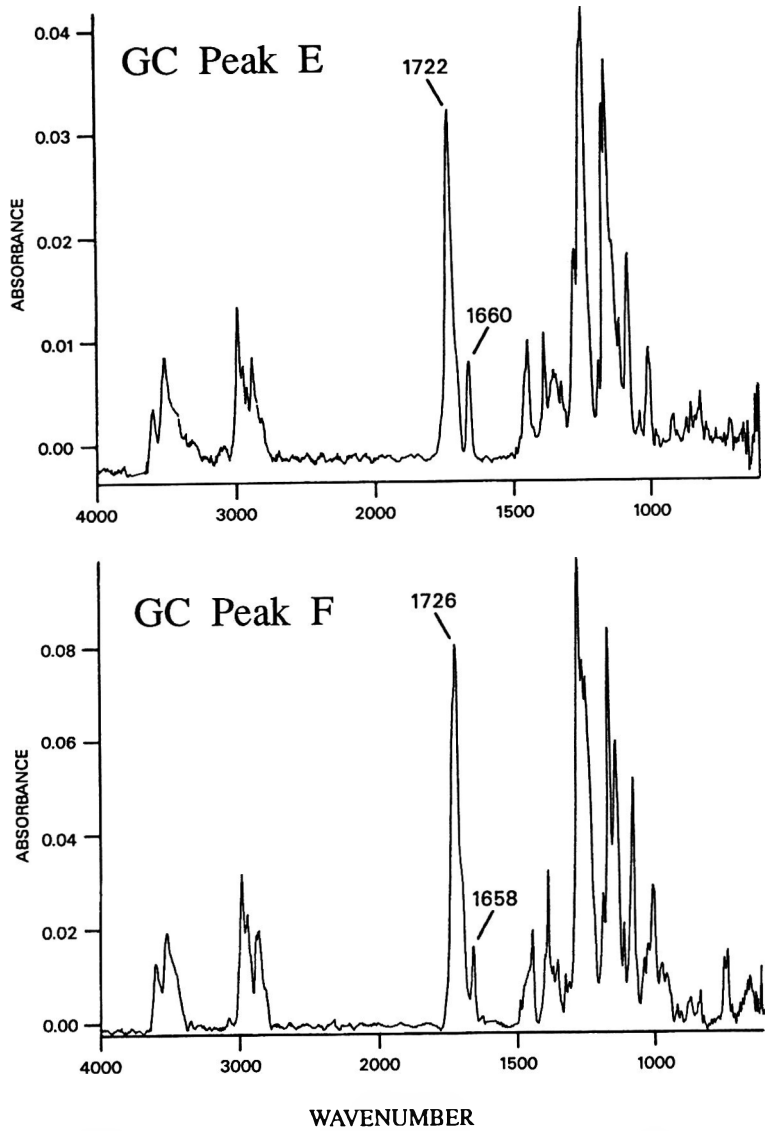


Figure 5. MI/FTIR spectra collected at 4 cm^{-1} resolution for GC peaks E and F (Figure 1). See text.

sion dynode. The instrument was scanned from *m/z* 45 to 665 in 1.0 s. Perfluorotributylamine was used to tune the instrument and calibrate the data system. An Alltech Heliflex AT-5 (formerly RSL-200) 50 m × 0.32 mm (id) fused-silica capillary column with 0.3 μm film thickness was used. Splitless injections were made at 40°C; after 1 min the oven temperature was ramped to 120°C and programmed to 240°C at 4°C/min. Injector temperature was 240°C, transfer line temperature was 250°C, and linear velocity (helium) was 40 cm/s.

PICI mass spectra were obtained on a Finnigan Mat TSQ-46 mass spectrometer with methane as the reagent gas at 0.23 Torr source pressure and 100°C source temperature. The mass spectrometer was scanned from *m/z* 65 to 665 in 1.0 s. The GC column and conditions were as described above.

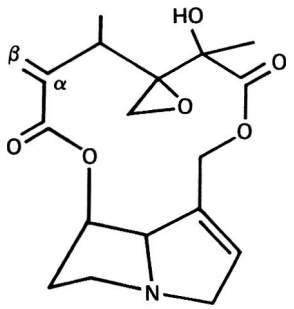
Results and Discussion

Separation of components in comfrey root extract by capillary GC produced a chromatogram (Figure 1) with 4 major and several minor peaks. By comparison with standards, the identities of the major peaks were confirmed by GC/MI/FTIR spectroscopy and by EI and PICI GC/MS as the monoester acyclic diastereomeric PA pairs intermedine and lycopsamine and their corresponding diester acyclic 7-acetyl derivatives (Figure 2).

The IR spectra for intermedine and lycopsamine are shown in Figure 3. The possiblity of *intra*molecular hydrogen bonding was previously reported (36). FTIR band positions for free and associated OH stretching vibrations for the PAs investigated are listed in Table 1, which also includes other band assignments.

Several stretching vibration bands of weak-to-medium intensity were observed for the different hydrocarbon groups (=CH, –CH, CH_2, and CH_3) in the range 3077–2812 cm^{-1} (Table 1). The low frequency bands, between 2830 and 2812 cm^{-1}, are probably due to symmetric stretching of the CH_2 groups adjacent to the nitrogen atom (41, 42).

In PAs, the vibrations of the acetate ester can be easily distinguished from those of the other ester groups. Specifically,



SWAZINE

Figure 6. Molecular structure for the pyrrolizidine alkaloid swazine.

the carbonyl stretching vibration was found at 1749 cm^{-1} for the acetate ester and near or below 1732 cm^{-1} for the other esters (Figure 4). It is noted that the ester carbonyl vibration of lowest frequency was observed at 1722 cm^{-1} (Figure 5).

The molecular ion MS data obtained for the pair of analytes that eluted last (peaks E and F, Figure 1) are consistent with those of symphytine (Figure 2), which is one of the 5 major PAs found in comfrey root (4, 29), and one or both of its geometric isomers, symlandine and symviridine (48; Figure 2). The observed PICI spectrum for the compound corresponding to either peak E or peak F showed a protonated molecular ion at *m/z* 382. It is noted that echimidine was not found in this extract because the PICI spectrum of echimidine showed a molecular ion response at *m/z* 397. Standards of symlandine, symphytine, and symviridine were not available to confirm the identity of these compounds in comfrey root extract.

Acyclic diester PAs, such as symphytine, have a second ester group in which the carbonyl is conjugated to a double bond (C=C stretching vibration around 1660 cm^{-1}; Figure 5). This alpha-beta unsaturation is expected to weaken the ester carbonyl C=O bond and to lower its stretching frequency relative to that of the saturated ester carbonyl at C-9 (40). Each of the IR spectra observed for the analytes corresponding to these 2 minor GC peaks exhibited a carbonyl band with an apparent shoulder. The saturated ester C=O group is probably *intra*molecularly hydrogen-bonded to the OH group on the adjacent carbon (Table 1). As a result, the C=O stretching vibration of this ester group may also be shifted to lower energy. The coincidence of the shifts of the C=O stretching vibrations for the ester containing the *intra*molecularly hydrogen-bonded carbonyl and the ester in which the carbonyl is conjugated to a double bond may explain why only a single C=O stretching band was observed.

One way to verify this coincidence would be to eliminate the hydrogen bonding by converting the hydroxyl groups to trimethylsilyl (TMS) functional groups. Unfortunately, symphytine, symlandine, and symviridine standards are not currently available. However, this approach was used for the related diester acyclic echimidine (Figure 6) and the diester macrocyclic swazine (Figure 6) in an earlier investigation in our laboratory (M.M. Mossoba, 1986, unpublished data). For echimidine-TMS the position of the alpha-beta unsaturated C=O stretching frequency shifted by 27 cm^{-1} to a lower value (1724 cm^{-1}) relative to that of the saturated ester carbonyl (1751 cm^{-1}). Similarly, a doublet separated by 9 cm^{-1} was observed for the 2 carbonyl bands (1739 and 1748 cm^{-1}) in swazine–TMS. Without TMS derivatization, these diester PAs also exhibited a single carbonyl band. The small dihedral angle of 54° between C=C and C=O, found for swazine by x-ray diffraction (35), means that they are *cis*-oriented with little conjugation between them (35), which is consistent with the small shift (9 cm^{-1}) observed for the alpha-beta unsaturated ester carbonyl band.

The intense stretching vibrations for the ester carbon–oxygen single bonds in the various PAs investigated fell in the range 1235–1273 cm^{-1} (Table 1). For alpha-beta unsaturated ester carbonyl groups in PAs, an inverse relationship was found between the C=O and the (O)C-O stretching vibrations: The lower the C=O frequency is, the higher the frequency of the carbonyl–oxygen single bond becomes (41). As shown in Table 1, the PA analyte with the low frequency of 1722 cm^{-1} for the ester carbonyl band exhibited a relatively high (O)C-O stretching vibration (1273 cm^{-1}), which is consistent with the presence of an alpha-beta unsaturated ester group.

Ring deformation modes such as ring breathing (near 890 cm^{-1}) and the C-C-C deformation vibration involving the carbon that is common to both necine rings (near 750 cm^{-1}) were also observed (Table 1).

Conclusions

This work shows the applicability of GC/MI/FTIR spectroscopy to PA isomer identification because subtle structural variations exhibited distinguishable IR spectral differences. The identities of 4 major PA constituents of a comfrey root extract were confirmed by comparison with standards by using GC/MI/FTIR spectroscopy and conventional EI and PICI GC/MS techniques. Observed IR bands were consistent with *intra*molecular hydrogen bonding (near 3520 cm^{-1}) and with the presence of particular functionalities, such as an acetate ester (1749 cm^{-1}), or a double bond on the C-7 side chain (around 1660 cm^{-1}). When standards were unavailable, PICI/MS provided critical molecular ion information.

Acknowledgments

The authors are grateful to C.C.J. Culvenor (Commonwealth of Scientific and Industrial Research Organisation, Division of Animal Health, Parkeville, Victoria, Australia) and R.J. Molyneux (Agricultural Research Service, Western Regional Research Center, Albany, CA) for their generous gifts of pyrrolizidine alkaloid standards.

References

(1) Culvenor, C.C.J., Clarke, M., Edgar, J.A., Frahn, J.L., Jago, M.V., Peterson, J.E., & Smith, L.W. (1980) *Experientia* **36**, 377–389

(2) Meyer, C. (1973) *The Herbalist*, Wehman Brothers Publishers, Hackensack, NJ

(3) Hills, L.D. (1976) *Comfrey, Past, Present and Future*, Faber and Faber, London, UK

(4) Vollmer, J.J., Steiner, N.C., Larsen, G.Y., Muirhead, K.M., & Molyneux, R.J. (1987) *J. Chem. Ed.* **64**, 1027–1030

(5) Fox, D.W., Hart, M.C., Bergeson, P.S., Jarrett, P.B., Stillman, A.E., & Huxtable, R.J. (1978) *J. Pediatr.* **93**, 980–982

(6) Huxtable, R.J. (1980) *Perspect. Biol. Med.* **24**, 1–14

(7) Huxtable, R.J., Luthy, J., & Zweifel, U. (1986) *N. Engl. J. Med.* **315**, 1095

(8) International Programme on Chemical Safety (1988) *Environmental Health Criteria 80: Pyrrolizidine Alkaloids*, World Health Organization, Geneva, Switzerland

(9) Bach, N., Thung, S.N., & Schaffner, F. (1989) *Am. J. Med.* **87**, 97–99

(10) Hirono, I., Mori, H., & Haga, M. (1978) *J. Natl. Cancer Inst.* **61**, 865–869

(11) Hirono, I., Haga, M., Fujii, M., Matsuura, S., Matsubara, N., Nakayama, M., Furuya, T., Hikichi, M., Takanashi, H., Uchida, E., Hosaka, S., & Ueno, I. (1979) *J. Natl. Cancer Inst.* **63**, 469–472

(12) Hirono, I., Mori, H., Haga, M., Fujii, M., Yamada, K., Takanashi, H., Uchida, E., Hosaka, S., Ueno, I., Matsushima, I., Umezava, K., & Shirai, A. (1979) in *Naturally Occurring Carcinogens, Mutagens, and Modulators of Carcinogenesis*, University Park Press, Baltimore, pp. 79–87

(13) Tandon, H.D., Tandon, B.M., & Mattocks, A.R. (1978) *Am. J. Gastroenterol.* **70**, 607–613

(14) Mattocks, A.R. (1981) *Chem. Biol. Interact.* **35**, 301–310

(15) Mattocks, A.R. (1986) *Chemistry and Toxicology of Pyrrolizidine Alkaloids*, Academic Press, Orlando, FL

(16) Mattocks, A.R., & White, I.N.H. (1971) *Chem. Biol. Interact.* **3**, 383–396

(17) Hooper, P.T. (1978) in *Effects of Poisonous Plants on Livestock*, K.R. Van Kampen & I.F. James (Eds), Academic Press, New York, NY, pp. 161–176

(18) Molyneux, R.J., Johnson, A.R., Roitman, J.N., & Benson, M.E. (1979) *J. Agric. Food Chem.* **27**, 494–499

(19) Ridker, P.M., Ohkuma, S., McDermott, W.V., Trey, C., & Huxtable, R.J. (1985) *Gastroenterology* **88**, 1050–1054

(20) Brauchli, J., Luthy, J., Zweifel, U., & Schlatter, C. (1982) *Experientia* **38**, 1085–1087

(21) Chalmers, A.H., Culvenor, C.C.J., & Smith, L.W. (1965) *J. Chromatogr.* **20**, 270–277

(22) Sharma, R.K., Khajuria, G.S., & Atal, C.K. (1965) *J. Chromatogr.* **19**, 433–434

(23) Wagner, H., Neidhardt, U., & Tittel, G. (1981) *Planta Med.* **41**, 232–239

(24) Dimenna, G.P., Krick, T.P., & Segall, H.J. (1980) *J. Chromatogr.* **192**, 474–478

(25) Ramsdell, A.S., & Buhler, D.R. (1981) *J. Chromatogr.* **210**, 154–158

(26) Segall, H.J. (1979) *J. Liq. Chromatogr.* **2**, 429–436

(27) Segall, H.J. (1979) *J. Liq. Chromatogr.* **2**, 1319–1323

(28) Tittel, G., Hinz, H., & Wagner, H. (1979) *Planta Med.* **37**, 1–8

(29) Culvenor, C.C.J., Edgar, J.A., Frahn, J.L., & Smith, L.W. (1980) *Aust. J. Chem.* **33**, 1105–1113

(30) Stelljes, M.E., Kelley, R.B., & Seiber, J.N. (1992) in *Poisonous Plants: Proceedings of the Third International Symposium*, L.F. James (Ed.), Iowa State University Press, Ames, IA

(31) Stelljes, M.E., Kelley, R.B., Molyneux, R.J., & Seiber, J.N. (1991) *J. Nat. Prod.* **54**, 759–773

(32) Wiedenfeld, H., Pastewka, U., Stengl, P., & Roeder, E. (1981) *Planta Med.* **41**, 124–128

(33) Holzer, G., Zalkow, L.H., & Asibal, C.F. (1987) *J. Chromatogr.* **400**, 317–322

(34) Betz, J.M., Eppley, R.M., Taylor, W.C., & Andrzejewski, D. (1994) *J. Pharm. Sci.* **83**, 649–653

(35) Laing, M., & Sommerville, P. (1972) *Tetrahedron Lett.* **51**, 5183–5186

(36) Mackay, M.F., Sadek, M., & Culvenor, C.C.J. (1983) *Acta Cryst.* **C39**, 785–788

(37) Culvenor, C.C.J., Heffernan, M.L., & Woods, W.G. (1965) *Aust. J. Chem.* **18**, 1605–1624

(38) Jones, A.J., Culvenor, C.C.J., & Smith, L.W. (1982) *Aust. J. Chem.* **35**, 1173–1184

(39) Roeder, E. (1990) *Phytochemistry* **29**, 11–29

(40) Culvenor, C.C.J., & Dal Bon, R. (1964) *Aust. J. Chem.* **17**, 1296–1300

(41) Gupta, V.P., Handoo, S.K., & Sawhney, R.S. (1975) *Curr. Sci.* **44**, 451–454

(42) Bicchi, C., Rubiolo, P., & Frattini, C. (1989) *J. Chromatogr.* **473**, 161–170

(43) Bourne, S., Reedy, G., Coffey, P., & Mattson, D. (1984) *Am. Lab. (Fairfield, Conn.)* **16**, 90–101

(44) Reedy, G.T., Ettinger, D.C., Schneider, J.F., & Bourne, S. (1985) *Anal. Chem.* **57**, 1602–1609

(45) Huizing, H.J., Gadella, Th.W.J., & Kliphuis, E. (1982) *Plant Syst. Evol.* **140**, 279–282

(46) Awang, D.V.C., Girard, M., & Kindack, D. (1988) in *International Congress on Natural Products Research*, University of Utah, Salt Lake City, UT, Abstract 0:51

(47) Mossoba, M.M., Niemann, R.A., & Chen, J.-Y.T. (1989) *Anal. Chem.* **61**, 1678–1685

(48) Roeder, E., Bourauel, T., & Neuberger, V. (1992) *Phytochemistry* **31**, 4041–4042

FOOD CHEMICAL CONTAMINANTS

Reversed-Phase Liquid Chromatographic Determination of Hypoglycin A (HG-A) in Canned Ackee Fruit Samples

GHULAM SARWAR and HERBERT G. BOTTING
Health and Welfare Canada, Health Protection Branch, Food Directorate, Banting Building, Tunney's Pasture, Ottawa, ON, K1A 0L2, Canada

A reversed-phase liquid chromatographic (LC) method involving precolumn derivatization with phenylisothiocyanate (PITC) was developed for determining levels of hypoglycin A (HG-A) in canned ackee fruit samples. HG-A was extracted by homogenizing the drained fruit in 80% ethanol. By using a Waters Pico-Tag amino acid analysis 15-cm-long column (which is also used for analyzing protein hydrolysates and biological samples) and an LC system, the baseline separation of HG-A from other amino acids was completed in about 6 min. The total time for analysis and equilibration was 16 min. HG-A levels in the edible portion of fruit in 18 cans varied from 18.27 to 87.50 mg HG-A/can. Recoveries of added standard HG-A averaged 101%. To our knowledge, this is the first report of the use of this method to determine HG-A in ackee fruit.

Hypoglycin A (HG-A) or 2-amino-4,5-methylenehex-5-enoic acid is a water-soluble toxic compound found in unripe fruits and seeds of ackee (*Blighia sapida*) (1). The ackee tree is indigenous to Jamaica but has been introduced into Southern Florida (2).

The unripe ackee aril (fleshy edible material) contains HG-A at 100–111 mg/100 g (3, 4). When the fruit is ripe, the HG-A levels decrease to less than 10 mg/100 g. Cooked ripe ackee fruit is nontoxic and has been a staple part of the Jamaican diet for centuries (5). The ingestion of unripe ackee fruit products, however, is associated with Jamaican "vomiting disease" (3–6). HG-A, the toxic component of ackee fruit, was identified as the causative agent of Jamaican vomiting sickness, characterized by repeated vomiting, severe acidosis, and hypoglycemia accompanied by depletion of liver glycogen (4–6). The sale of imported Jamaican canned ackee fruit products is limited in Canada. The concern, however, does exist that the high levels of HG-A may be present in these products if immature fruit is packed.

Several workers have reported ion-exchange or thin-layer chromatographic methods for the determination of HG-A (7–10). However, only Chase et al. (9, 10) obtained baseline separation between HG-A and leucine or other amino acids. They used ion-exchange chromatography using cation-exchange resin and postcolumn derivatization with ninhydrin, in a liquid chromatographic (LC) system. This improved method was successfully applied in determining the HG-A content of various parts of the ackee fruit at different stages of ripeness (2).

The LC separation of precolumn phenylisothiocyanate (PITC) derivatives, commercially developed as the Pico-Tag method (11), was adapted in our laboratory for rapid analysis of all amino acids in protein hydrolysates (12 min) and nutritionally important amino acids in deproteinized physiological samples (20 min) (12, 13). It was of interest to apply the PITC derivatization methodology to the analysis of HG-A in ackee fruit products.

Experimental

Reagents

The chemicals and solvents used were of analytical and chromatographic grade, respectively.

(a) *Acetonitrile, methanol, glacial acetic acid, and phosphoric acid.*—(J.T. Baker Chemical Co., Phillipsburg, NJ).

(b) *Alcohol, 80%.*—Prepared by diluting 90% anhydrous ethyl alcohol (Commercial Alcohols, Inc., Toronto, ON) with distilled water.

(c) *Disodium hydrogen phosphate.*—(Mallinckrodt, Inc., Science Products Division, St. Louis, MO).

(d) *Sodium acetate, anhydrous.*—(BDH, Ltd, Poole, Dorset, UK).

(e) *PITC.*—(Pierce Chemical Co., Rockford, IL).

(f) *Triethylamine, TEA-99%.*—(Aldrich Chemical Co., Inc.).

(g) *HG-A standard.*—The standard used in this study was kindly donated by Kay Tanaka, Yale University School of Medicine. Tanaka isolated pure HG-A from an ackee fruit seed extract, and its purity was confirmed by other workers (10). Standard HG-A stock solution was prepared by dissolving 2.562 mg in 3 mL 0.1N HCl. A working standard solution of 85.4 μg/mL was prepared by dilution of the stock (1 to 10) with distilled water. The instrument was calibrated at 3 levels by derivatizing 10, 20, and 40 μL of the working standard solution.

(h) *Eluant A.*—0.14M sodium acetate containing 0.05% TEA and 6% acetonitrile, pH 6.1 with glacial acetic acid.

Received April 5, 1993. Accepted by AP September 3, 1993.

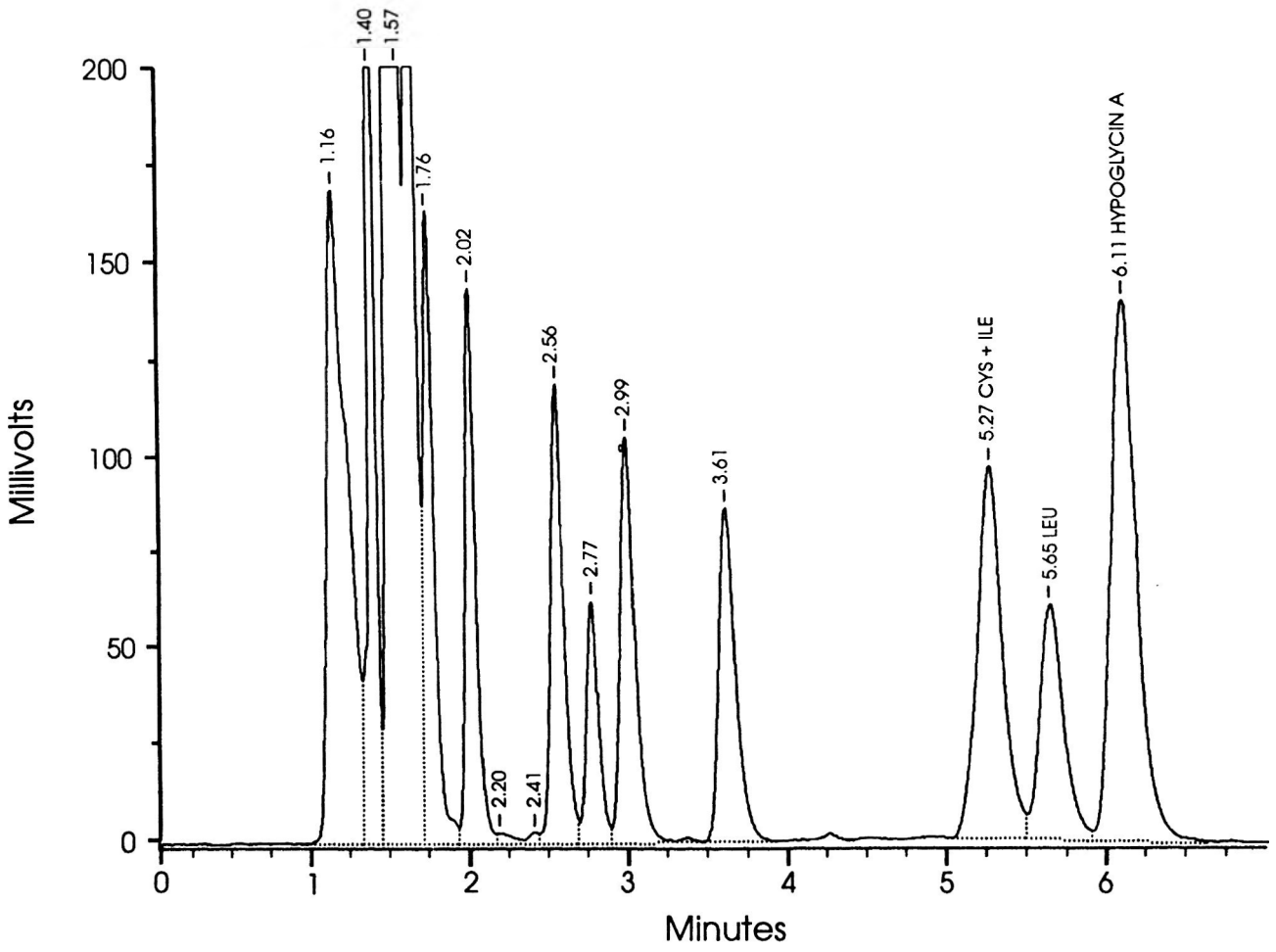


Figure 1. Chromatogram of HG-A plus 17 amino acids standard as PTC-derivatives.

(i) *Eluant B.*—Acetonitrile–water, 60 + 40.

(j) *Sample diluent.*—Prepared by dissolving 710 mg disodium hydrogen phosphate in 1 L water–acetonitrile (19 + 1) and by adjusting the pH to 7.4 with phosphoric acid.

Apparatus

(a) *Liquid chromatographic system.*—Consisting of 2 Model 510 pumps, a 712 Wisp autoinjector, a Model 490 multiwavelength detector, an 810 data system, and a temperature control module. Pico-Tag amino acid analysis column, 150 × 3.9 mm (Cat. No. 88131, Waters Chromatography Division, Millipore Corp., Millford, MA).

(b) *Food processor.*—Cuisinart DLC-10E (Weil Co., Ltd, Downsview, ON).

(c) *Polytron homogenizer.*—Model PT 10-35 (Brinkman Instruments, Inc., Westbury, NY).

(d) *Centrifuge.*—Model IEC CENTRA-7R with an 831a rotor (International Equipment Co., Division of Damon Corp., Needham Heights, MA).

(e) *Analytical evaporator.*—Model Evap (Organomation Assoc., Inc., South Berlin, MA).

(f) *Milli-Q-Water system.*—To generate high-purity distilled water (Millipore Corp., Bedford, MA).

Sample Preparation

Eighteen cans (540 mL each) of commercially packed ackee fruit imported from Jamaica and representing 2 brands (A, 11 cans; B, 7 cans) were selected for analysis. Canned fruit was drained to separate solids (the edible portion) from liquid (brine). For brand A, the weights of solids and liquid averaged 382.4 ± 17.2 and 179.3 ± 23.5 g/can, respectively. For brand B, the weights of solids and liquid averaged 405.1 ± 10.7 and 148.1 ± 14.4 g/can, respectively.

Drained fruit from the entire can was blended in a food processor until smooth. Approximately 2.5 g blended fruit was homogenized in duplicate in 10 mL 80% ethanol for 60 s at a speed setting of 6. The homogenate was centrifuged at 1000 × *g* for 15 min. Approximately 1 mL of the supernatant and the corresponding brine sample were filtered through a 0.22 µm filter (Millex-GS, Millipore Corp., Bedford, MA).

Derivatization

Forty microliters of the filtered fruit supernatant, 20 µL filtered brine, and standards (10, 20, and 40 µL) were derivatized with PITC as described previously (13). PITC derivatization was carried out in 75 × 10 mm culture tubes (American Hospital Supply Canada, Inc., Mississauga, ON). Samples of the fil-

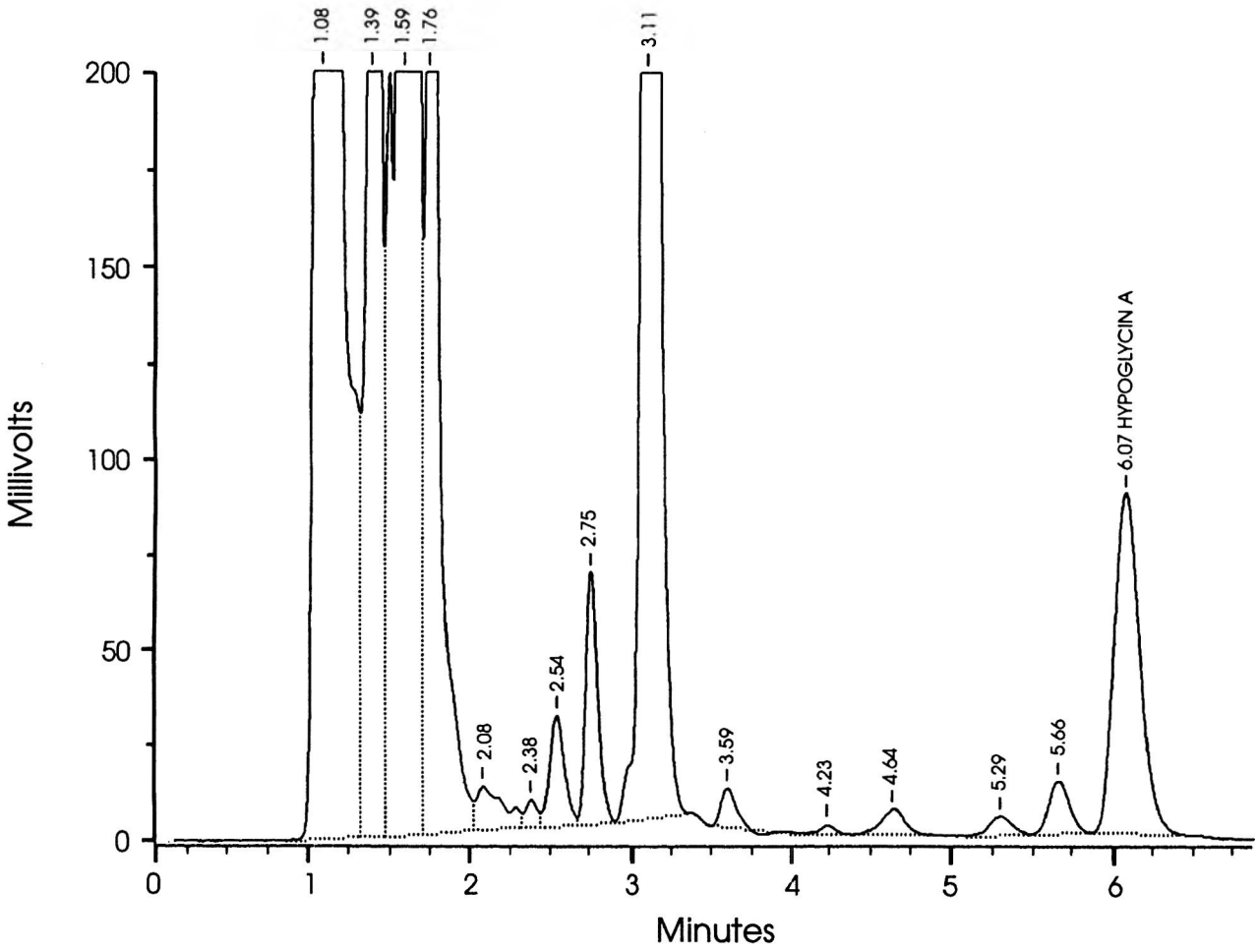


Figure 2. Chromatogram of HG-A (as PTC-derivative) in ethanol extract of drained solids in canned ackee fruit.

tered fruit extract, brine, and standard HG-A were evaporated to dryness at 35°C under nitrogen for 15 min on an analytical evaporator.

The derivatizing solution was prepared by mixing 200 µL methanol, 50 µL water, 50 µL TEA, and 20 µL PITC. HG-A was derivatized by adding 50 µL derivatizing solution to the tubes containing dried samples of standard, fruit extract, or brine. The samples were mixed, and the tubes were covered with Parafilm and allowed to stand for 20 min at room temperature for completion of derivatization. Excess reagent was evaporated under nitrogen for 15 min at 35°C, the derivatized dried samples were redissolved in 200 µL sample diluent, and 20 µL was injected onto the column for chromatography.

Chromatography

Elution was commenced at 1 mL/min with 75% eluant A and 25% eluant B. After 6 min, the column was washed for 3 min at 1.5 mL/min with 100% eluant B and then re-equilibrated at 1.5 mL/min for 6.6 min to initial conditions. The column temperature was maintained at 25°C. The detection range was set at 0–200 millivolts, and the UV absorbance was observed at 254 nm.

Calculations

The amounts of HG-A in fruit and brine were calculated as follows:

$$HG\text{–}A_{fruit} = \left(\frac{ASAM}{ASTD}\right) \times (\mu g/mL\ STD) \times \left(\frac{DF}{W}\right) \times \frac{1}{10}$$

$$HG\text{–}A_{brine} = \left(\frac{ASAM}{ASTD}\right) \times (\mu g/mL\ STD) \times \left(\frac{VSTD}{VSAM}\right) \times \frac{1}{10}$$

where $HG\text{–}A_{fruit}$ = mg HG–A/100 g solid fruit, $HG\text{–}A_{brine}$ = mg HG-A/100 g liquid brine, *ASAM* = HG–A area in sample chromatogram; *ASTD* = HG-A area in standard chromatogram; *DF* = dilution factor, 10 mL; *W* = weight of fruit, about 2.5 g; *VSTD* = volume of standard derivatized; *VSAM* = volume of sample derivatized.

Results and Discussion

The separations of PTC (phenylthiocarbamyl)-HGA in standard, solid fruit, and liquid brine are shown in Figures 1, 2, and 3, respectively. The baseline resolution of HG-A from

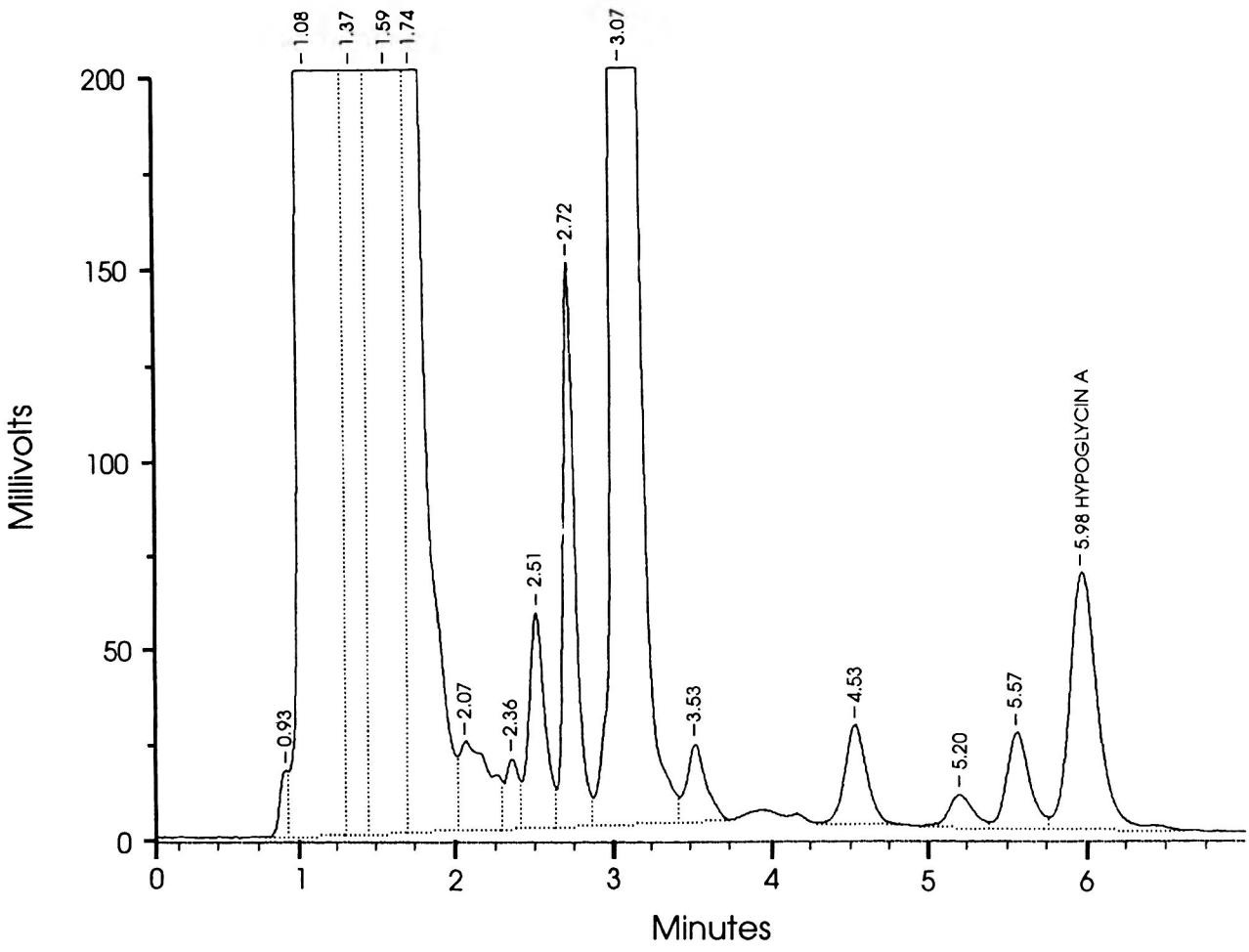


Figure 3. Chromatogram of HG-A (as PTC-derivative) in brine used for packing canned ackee fruit.

leucine and other amino acids was achieved in each case in about 6 min. The standard response was linear from 85 to 340 ng (r = 0.98); the limit of detection of HG-A was 10 ng. System suitability was tested by injecting the standard solution 6 times; a coefficient of variation of 0.4% was obtained. Recovery from the fruit was estimated by adding 156 and 237 μg HG-A to blended fruit aliquots before homogenization. Recoveries were 100 and 102%, respectively. Recovery from brine was determined by adding 20 μL of the working standard to 20 μL brine before derivatization. This recovery was 104%.

The present data on linearity of the standard response, limit of detection, variability, and recoveries were comparable with those reported previously (10) for ion-exchange chromatographic determination of HG-A. The purity of the HG-A peak was established by Chase et al. (10).

The HG-A contents of 18 cans of ackee are shown in Table 1. The liquid was analyzed in duplicate. The solid fruit was extracted with 80% ethanol in duplicate, and each extract was analyzed twice. The canning liquid (brine) contained a significant amount of HG-A (Table 1), which may have leached into the liquid from the fruit. On average, brand A products contained higher levels of HG-A compared with brand B (91.57 vs 59.69 mg HG-A/can). There was, however, considerable can-to-can variability for each brand. Similar variability was reported previously (7, 10). HG-A contents in aril (edible portion), seeds, and husks of ackee fruit at various stages of ripeness have been determined (2). HG-A levels in the unripe ackee fruit (grown in Florida) seed, aril, and husk were 939, 711, and 41.6 mg/100 g, respectively. When the fruit is ripe, the levels of HG-A decreased to 269 mg/100 g seed, remained unchanged in the husk, and decreased to below 1.2 mg/100 g aril. Canned ackee fruit, consisting entirely of Jamaican-grown arils, however, contained HG-A at an average of 9.4 mg/100 g (2). The difference in HG-A contents of the canned aril (Jamaican origin) and the ripe aril from the fresh fruit (grown in Florida) was attributed to processing conditions, fruit cultivar, and/or growing conditions.

The 2 brands analyzed contained an average of 14.18 and 9.72 mg HG-A/100 g solid fruit (Table 1). These values are comparable with those reported previously for similar products (7, 10).

In conclusion, the LC method reported in this paper can be used for accurate, rapid, and reproducible determination of HG-A in canned ackee fruit samples. To our knowledge, this is the first report on the LC analysis of precolumn PITC-derivatives with HG-A. A comparison of the LC method with an established ion-exchange method was not considered necessary in this study because amino acid analysis by LC of precolumn

PITC-derivatives using a reversed-phased Pico-Tag column (the one used in the present investigation) was successfully validated by the ion-exchange chromatographic methods in several research centers (12, 14, 15). Moreover, the HG-A values found in this investigation (Table 1) were comparable with those reported for similar products analyzed by ion-exchange methods (7, 10).

Acknowledgments

We are grateful to R.W. Peace for his valuable suggestions.

References

(1) Hassal, C.H., & Reyle, K. (1955) *Biochem. J.* **60**, 334–339

(2) Chase, G.W., Jr, Landen, W.O., Jr, & Soliman, A.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 318–319

(3) Manchester, K.L. (1974) *FEBS Lett.* **40**, 5133–5139

(4) Ellington, E.V. (1975) in *Pan-American Association of Biochemical Societies Symposium, Hypoglycin*, E. A. Kean (Ed.), Academic Press, New York, NY, pp. 1–2

(5) Tanaka, K., & Ikeda, Y. (1990) in *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*, K. Tanaka & P.M. Coates (Eds), Alan R. Liss, Inc., pp. 167–184

(6) Tanaka, K., Isselbacher, K.J., & Shih, V. (1972) *Science* **175**, 69–71

(7) Scott, P.M., Botting, H.G., Kennedy, B.P.C., & Knipfel, J.E. (1974) *J. Food Sci.* **39**, 1057–1058

(8) Patrick, S.J. (1962) *Can. J. Biochem. Physiol.* **40**, 1195

(9) Chase, G.W., Jr, Landen, W.O., Jr, & Soliman, A.M. (1988) *J. Chromatogr.* **456**, 431–434

(10) Chase, G.W., Jr, Landen, W.O., Jr, Gelbaum, L.T., & Soliman, A.M. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 374–377

(11) Bidlingmeyer, B.A., Cohen, S.A., & Tarvin, T.L. (1984) *J. Chromatogr.* **336**, 93–104

(12) Sarwar, G., Botting, H.G., & Peace, R.W. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 1172–1175

(13) Sarwar, G., & Botting, H.G. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 470–475

(14) Beaver, R.W., Wilson, D.M., Jones, H.M., & Haydon, K.D. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 425–428

(15) White, J.A., Hart, R.J., & Fry, J.C. (1986) *J. Autom. Chem.* **8**, 170–177

FOOD CHEMICAL CONTAMINANTS

Analysis by Liquid Chromatography of Fusarochromanone (TDP-1) Added to Corn and Wheat

YICHUN XU, C. J. MIROCHA, and W. XIE
University of Minnesota, Department of Plant Pathology, St. Paul, MN 55108

An analytical method was developed for the detection of fusarochromanone (TDP-1) in corn and wheat by liquid chromatography. Extraction was done with methanol–water–ammonium hydroxide (90 + 10 + 2), and cleanup was by elution through a silica Sep-Pak cartridge. The limit of sensitivity of TDP-1, detected on the basis of its fluorescence properties, is 200 pg, whereas the detection limit of the method for corn and wheat is 5 ng/g. The average recoveries of TDP-1 added at 5, 25, 100, 500, and 1000 ppb were 86% for corn and 83% for wheat. Confirmation was done by thin-layer chromatography.

Received June 1, 1993. Accepted by GJ September 23, 1993.

Fusarochromanone (TDP-1) is a natural toxin produced by certain isolates of *Fusarium equiseti* that were first discovered in Fairbanks, Alaska. One of the isolates was named Alaska 2-2 (1). The structure of TDP-1 was determined by Pathre et al. (2) as 2,2-dimethyl-5-amino-6-(3′-amino-4′-hydroxylbutyryl)-4-chromone. It causes tibial dyschondroplasia in avian species as well as reduced hatchability of fertilized eggs (3). The molecule is highly conjugated, which is advantageous analytically, because it fluoresces an intense blue when irradiated with long-wave UV light (365 nm).

Fusarium isolates that produce fusarochromanone are rare and have been associated only with northern colder climates such as in Alaska, Denmark, and Germany. An isolate was found naturally occurring in cereal feed associated with tibial dyschondroplasia in Denmark (4). Ulf Thrane (personal communication) sent us 10 isolates of *Fusarium equiseti* found in Denmark, all of which produced the toxin. Isolates such as

these are infrequently found; therefore, this organism must be selectively distributed in soil. However, fusarochromanone-producing isolates can be obtained readily from their original source of isolation in Fairbanks, Alaska.

Numerous derivatives of the parent compound, fusarochromanone, have been isolated from the fungus grown in laboratory culture. Among them are the monoacetate (5), 4 fatty acid derivatives (6), formyl and diacetyl derivatives (7), and a C–3′ hydroxy, C–4′ methoxy derivative (8). The chromatographic behavior of these derivatives are different from that of TDP-1, and they normally are not found in the same extract because their concentration relative to TDP-1 is very low. Moreover, their toxicity is not known.

The objective of this work was to develop an easy and reliable method of analysis based on the intense fluorescence of fusarochromanone when irradiated with long-wave ultraviolet light after separation by LC. Analyses of TDP-1 in corn or wheat naturally infected with *F. equiseti* were not done because such samples were not available.

Experimental

Materials

(a) *Fusarochromanone.*—Produced in our laboratory from an isolate of *Fusarium equiseti* (Alaska 2-2) grown on a solid rice medium. It was extracted and purified as described by Lee et al. (1).

(b) *Corn and wheat samples.*—Obtained from the local market.

Apparatus

(a) *Mill.*—Stein laboratory mill M-2 (Fred Stein Laboratories, Inc., Atchison, KS).

(b) *Cleanup column.*—Sep-Pak silica cartridge (Waters Chromatography, Division of Millipore Co., Milford, MA).

(c) *LC instrument.*—Shimadzu Co., Kyoto, Japan. Column, C_{18} reversed-phase (Waters, μBondapak), 300 mm × 3.9 mm id; detector: Shimadzu RF0530 fluorescence LC monitor detector; wavelength: emission, 450 nm; excitation, 384 nm.

(d) *Centrifuge.*—Sorvall refrigerated automatic.

Reagents

All organic solvents used were reagent grade or better.

(a) *Extraction solvent.*—Methanol–water–29.6% ammonium hydroxide (90 + 10 + 2).

(b) *LC mobile phase solution.*—Mobile phase A, acetonitrile–water–acetic acid (10 + 90 + 1); mobile phase B, acetonitrile–water–acetic acid (40 + 60 + 1). All solvents were LC grade. The acetic acid used was 99.7% pure.

Sample Preparations

(a) *Spiked corn and wheat samples.*—Corn (120 g) was finely ground in a Stein mill and divided into six 20 g portions. Each 20 g portion was spiked with TDP-1 at 20, 10, 2, 0.5, or 0.1 μg to give a final concentration of 1 ppm, 500 ppb, 100 ppb, 25 ppb, or 5 ppb, respectively. TDP-1, dissolved in methanol at the required concentration, was mixed thoroughly with finely divided corn portions; the spiked sample then was dried to less than 15% moisture under nitrogen. Clean corn was used as the control. Wheat samples were spiked in the same manner.

(b) *Standard TDP-1 solutions.*—The preparation of crystalline TDP-1 is described by Pathre et al. (2). TDP-1 (0.86 mg) was dissolved in 8.6 mL methanol and used as the stock standard solution A (0.1 mg/mL). A portion (0.1 mL) of solution A was diluted with 0.9 mL methanol and was called stock solution B (10 μg/mL). Both solutions were stored in a freezer in the dark.

A portion of solution B (0.2 mL) was transferred into a vial and dried under nitrogen; the residue was dissolved with 1 mL methanol containing 1% acetic acid (2000 ng/mL). This solution was diluted with methanol (containing 1% acetic acid) to give final TDP-1 concentrations of 2000, 1000, 100, 50, and 10 ng/mL.

Extraction

Twenty grams of corn or wheat (fresh weight with moisture content of ca 15%) was placed into a 500 mL Erlenmeyer flask, 100 mL of the extraction solvent was added, and the flask was capped with aluminum foil. The flask was placed on a reciprocating shaker for 1 h, and then the contents were filtered through Whatman No. 4 filter paper. A portion of the filtrate (20 mL) was placed in a 250 mL round-bottom flask and dried under vacuum in a rotary evaporator.

Cleanup

A Sep-Pak (Waters) silica gel cartridge was used after it had been washed with 4 mL methanol followed by 4 mL chloroform. The dried sample (in a round-bottom flask) was dissolved in 1.6 mL methanol–acetic acid (3 + 1), after which 18.4 mL chloroform was added. After 10 min, a precipitate was formed, and the mixture was transferred to a centrifuge tube and centrifuged at 3000 rpm for 10 min. A portion of this solution (10 mL) was transferred to the Sep-Pak cartridge. The cartridge was washed with 4 mL chloroform–acetic acid (100 + 2) followed by 8 mL chloroform–methanol–acetic acid (90 + 10 + 2). The cartridge was eluted with 16 mL chloroform–methanol–ammonium hydroxide (85 + 15 + 3); the eluate was collected and dried under nitrogen.

Detection by LC

The dried eluate was dissolved in 1 mL methanol containing 1% acetic acid. The sample (20 μL) was injected into the LC column with a 20 μL injector loop. The column was run as follows: mobile phase A (100%) was run for 3 min at 1.5 mL/min, mobile phase B was added at 1.5 mL/min for 0.5 min, and at the end of this period, solvent phase B (100%) was continued for 7 min. The retention time for TDP-1 was 8.1–8.3 min. The sample size equivalent per injection was 40 mg.

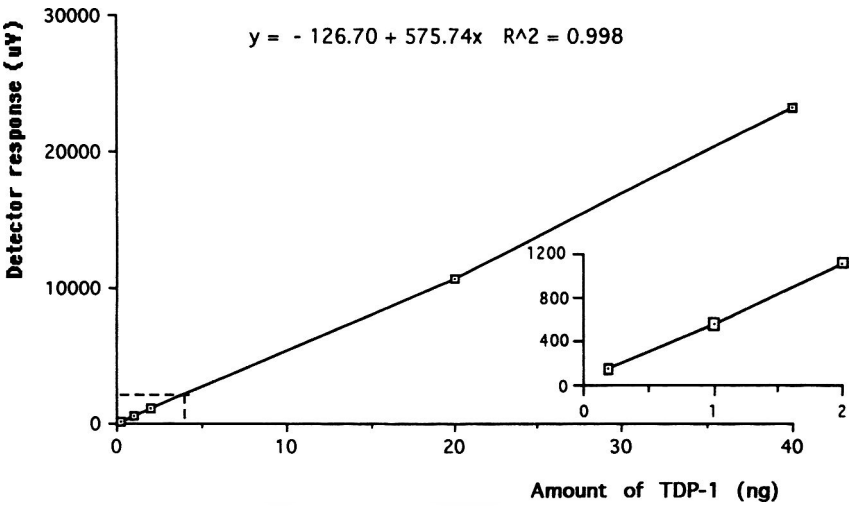


Figure 1. Standard curve based on peak height for the detection of fusarochromanone (TDP-1) by fluorescence LC. The inset shows detection at 0.2, 1, and 2 ng.

Results and Discussion

A standard curve was constructed for fusarochromanone (0.2, 1, 2, 20, and 40 ng injected) to determine the quantitative linear relationship in peak height (Figure 1). The correlation coefficients were 1.0 for peak height and 0.998 for peak area. The inset in Figure 1 shows an amplification of the linear response between 0.2 and 2 ng injected, which demonstrates confidence limits of at least 200 pg in the low-dose-response area. Similar relationships were found with area as the quantitative determinant (data not presented).

The recovery of fusarochromanone was determined by spiking corn samples with 0.1, 0.5, 2, 10, and 20 μg fusarochromanone so as to give concentrations of 5, 25, 100, 500, and 1000 ppb. Three separate extractions and determinations were made for each concentration, as shown in Table 1. The average recoveries were 77, 96, 89, 82, and 87%, respectively, for the 5 concentrations. The average recovery for all concentrations tested was 86%, which is well within the acceptable range for an analytical method.

Similar results were obtained when wheat (see Table 2) was spiked with similar amounts (0.1, 0.5, 2, 10, and 20 μg). The average recoveries were 77, 85, 92, 81, and 79%, respectively, for the 5 concentrations, or an average of 83% for all concentrations tested. For both corn and wheat, the cleanup procedure is efficient enough to eliminate most of the interfering sub-

Table 1. Recovery of fusarochromanone from spiked corn[a]

Amount added		Amount detected				
μg	ppb	μg	ppb	Rec., %	Av., %	SD, %
0	0					
0.1	5.0	0.064	3.2	64.0		
		0.086	4.3	86.0	76.7	9.29
		0.080	4.0	80.0		
0.5	25	0.481	24.1	96.4		
		0.486	24.3	97.2	96.3	0.82
		0.476	23.8	95.2		
2.0	100	1.899	94.9	94.9		
		1.650	82.5	92.5	88.7	8.66
		1.777	88.8	88.8		
10	500	9.392	469.6	93.9		
		7.511	375.5	75.1	81.7	8.66
		7.604	380.2	76.0		
20	1000	18.503	925.2	92.5		
		15.538	776.9	77.7	87.1	6.65
		18.195	909.7	91.0		

[a] Each average is based on 3 separate experiments.

Table 2. Recovery of fusarochromanone from spiked wheat[a]

Amount added		Amount detected				
µg	ppb	µg	ppb	Rec., %	Av., %	SD, %
0	0					
0.1	5	0.084	4.2	84.0		
		0.059	2.9	58.0	76.5	13.19
		0.087	4.4	87.6		
0.5	25	0.376	18.8	75.2		
		0.427	21.3	85.3	84.7	7.48
		0.468	23.4	93.5		
2.0	100	1.853	92.6	92.6		
		1.918	95.9	95.9	92.3	3.11
		1.766	88.3	88.3		
10	500	8.403	420.1	84.0		
		8.276	413.8	82.7	81.0	3.37
		7.628	381.4	76.3		
20	1000	15.543	777.1	77.7		
		15.709	785.4	78.5	79.1	1.45
		16.215	810.8	81.1		

[a] Each average is based on 3 separate experiments.

stances so that a good window is allowed for fusarochromanone detection.

The fluorescence detector response for fusarochromanone extracted from corn spiked at 25 ppb (25 ng/g) is shown in Figure 2. Note the peak at the retention time of 8.16 min. The interfering substance at the retention time of 7.99 min is found in both corn and wheat samples. At higher concentrations (e.g., 50 and 100 ppb), the peak at 7.99 min, although present, is eliminated from the computer plot because of normalization to the dominant peak and is not significant. A similar LC chromatograph is shown for wheat spiked at 25 ppb (Figure 3), which is almost identical to that for corn. The substance with a retention time of 7.9 min is seen at 25 ppb but is masked at higher concentrations. This substance is reduced in quantity by precipitation with chloroform after initial extraction.

Confirmation or verification of the identity of fusarochromanone is an important component of this analytical method. Confirmatory analysis by thin-layer chromatography (TLC), after the same preparation for LC, and normal-phase silica gel chromatography on analytical plates give excellent resolution with chloroform–methanol–ammonium hydroxide (45 + 5 + 1 or 40 + 10 + 1). An R_f value of 0.31 or 0.56, respectively, is obtained for fusarochromanone. The detection limit on TLC plates is 3 ng when irradiated at 366 and 5 ng when irradiated at 254 nm. Fusarochromanone gives a positive (turns pink) ninhydrin reaction; it turns yellow-green when sprayed with

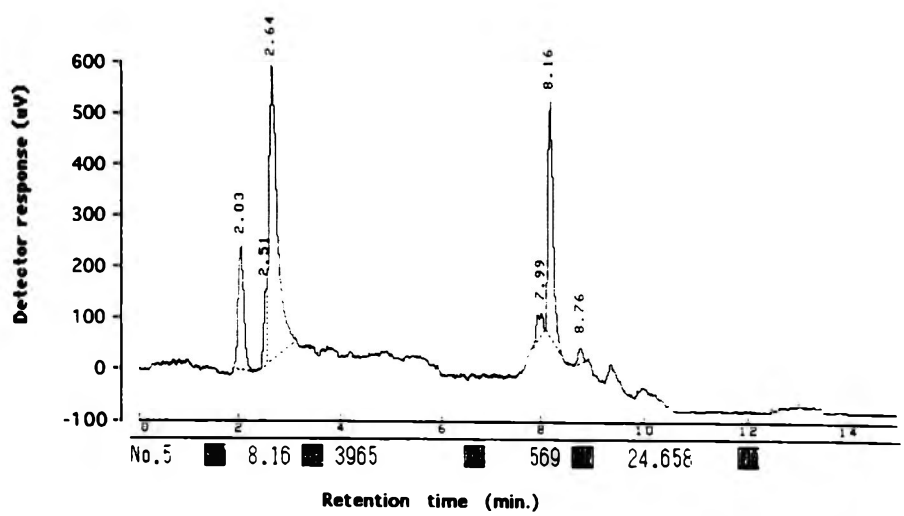


Figure 2. LC detection of fusarochromanone in corn spiked at 25 ppb. The retention time was 8.16 min, with a peak height of 569 and a peak area of 3965. The area of the peak at 8.16 min represents 24.6% of the total area.

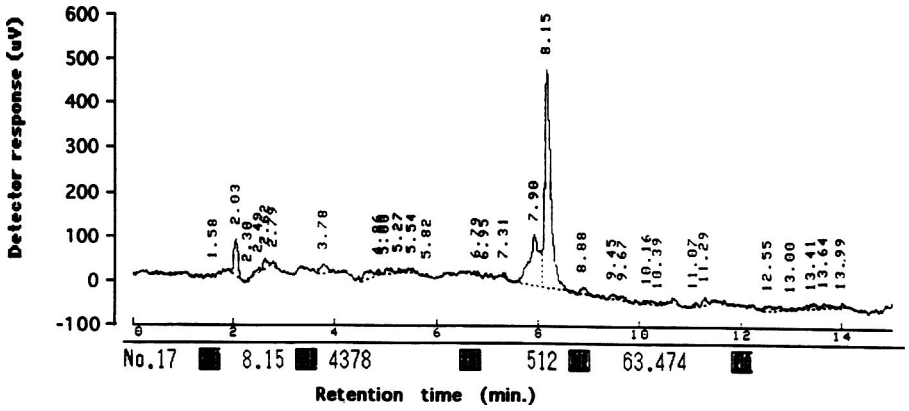


Figure 3. LC detection of fusarochromanone in wheat spiked at 25 ppb. The retention time was 8.15 min, with a peak height of 512 and a peak area of 4378. The area of the peak at 8.15 min represents 63% of the total area.

either 20% sulfuric acid in methanol or *p*-anisaldehyde solution. These 3 colorimetric reagents have limits of detection of 250 ng. Fusarochromanone is relatively unstable when exposed to laboratory fluorescent light; standards should be stored in the dark in a freezer.

Summary

A method was developed for the detection of fusarochromanone in corn and wheat. Detection by LC is based on the intense fluorescence properties of fusarochromanone when irradiated with ultraviolet light. The limit of detection of a standard by LC is 200 pg; the limit of detection of the method for corn and wheat is 5 ppb or 5 ng/g. Confirmation can be made by thin-layer chromatography.

References

(1) Lee, Y.W., Mirocha, C.J., Schroeder, D.J., & Walser, M.M. (1985) *Appl. Environ. Microbiol.* **50**, 102–107

(2) Pathre, S.V., Gleason, W.B., Lee, Y.W., & Mirocha, C.J. (1986) *Can. J. Chem.* **64**, 1308–1311

(3) Lee, Y.W., Mirocha, C.J., Schroeder, D.J., & Hamre, M.L. (1985) *Poultry Sci.* **64**, 1077–1082

(4) Krogh, P., Christensen, D.H., Hald, B., Harlou, B., Larsen, C., Pedersen, E.J., & Thrane, U. (1989) *Appl. Environ. Microbiol.* **55**, 3184–3188

(5) Xie, W., Mirocha, C.J., Pawlosky, R.J., Wen, Y., & Xu, X. (1989) *Appl. Environ. Microbiol.* **55**, 794–797

(6) Xie, W., Mirocha, C.J., Wen, Y., Cheong, W.J., & Pawlosky, R.J. (1991) *J. Agric. Food Chem.* **39**, 1757–1761

(7) Xie, W., Mirocha, C.J., & Wen, Y. (1991) *J. Nat. Products* **54**, 1165–1167

(8) Xie, W., Mirocha, C.J., Wen, Y., & Pawlosky, R.J. (1990) *Appl. Environ. Microbiol.* **56**, 2946–2948

FOOD COMPOSITION AND ADDITIVES

Determination of the Sum of Dimer and Polymer Triglycerides and of Acid Value of Used Frying Fats and Oils by Near Infrared Reflectance Spectroscopy

ANDRIES J. BOOT and ANDRIES J. SPEEK
Food Inspection Service Amsterdam, Regional Inspectorate for Health Protection, Hoogte Kadijk 401, 1018 BK Amsterdam, The Netherlands

Near infrared reflectance spectroscopy (NIRS) in the transflection mode was applied to determine the sum of dimer and polymer triglycerides (DPTG) contents and acid value of used frying fats and oils. A filter instrument and a calibration sample set were used to determine DPTG content and acid value. For each parameter, a 7-wavelength calibration was developed using multiple linear regression analysis. For a validation set comprising 44 samples for the NIRS-DPTG determination in the range of 2.2 to 32.7% m/m, the correlation coefficient between NIRS and liquid chromatography (LC) was 0.976, with a standard error of prediction (SEP) of 1.8% m/m. For a validation set comprising 36 samples for the NIRS-acid value determination in the range of 0.30 to 18.8 mg potassium hydroxide per gram of sample (mg KOH/g), the correlation coefficient between NIRS and titration was 0.996, with a SEP of 0.33 mg KOH/g. Validation after routine operation for 1 year provided SEPs of 2.3% m/m and 0.44 mg KOH/g for DPTG and acid value determination, respectively. NIRS screening of 1400 samples collected during 1992 precluded the need for 1149 DPTG determinations by LC (82.1%) and 1033 acid value determinations by titration (73.8%), which are methods the judicature in The Netherlands accepted, because those samples appeared to comply with legislation.

Toxicity of Dimer and Polymer Triglycerides (DPTG) and Free Fatty Acids and Legislation

Potential formation of carcinogenic substances and compounds with antinutritional properties during use of frying fats and oils was reviewed by Clark and Serbia (1). Oxidation of monomer fat molecules followed by formation of dimeric, trimeric, and higher polymers by using frying temperatures higher than the recommended 180°C represents a health risk to consumers (2). Another reaction during frying is hydrolyzation of fat caused by the regular addition of water through the foods to be fried, resulting in the release of free fatty acids. The latter compounds cause an off-flavor taste, although not a health risk, which the legislature regards as a quality parameter.

The sum of DPTG is expressed as a percentage of total weight of triglycerides (% m/m). Acid value (saponification value), the amount of potassium hydroxide (KOH) required to neutralize diluted fat on phenolphthalein indicator, is expressed as mg KOH/g of fat. To ensure consumer safety, maximum limits for DPTG content and acid value of used frying fats and oils are incorporated in the "Preparation and Preservation of Food Decree," enforced on March 1, 1993, as a part of the Dutch Food and Commodity Act (3), i.e., 16% m/m and 4.5 mg KOH/g, respectively.

Food inspectors of the 13 regional Food Inspection Services in The Netherlands regularly examine restaurants and snack bars in their region. Part of their objective is to collect suspended frying fat and oil samples to determine DPTG content and acid value. Their selection criteria are color observation and comparison of smoking and foaming at a specified temperature. DPTG content and acid value are determined by liquid chromatography (LC) and titration, respectively, the only methods the judicature in The Netherlands accepted. Although these methods have good accuracy and precision, they are laborious, involve use of chemicals, and are time-consuming. Therefore, because food law enforcement practitioners only require knowing whether the DPTG content and/or acid value exceeds the maximum regulatory limits, the development of a rapid screening procedure was desirable.

Applicability of Near Infrared Reflectance Spectroscopy (NIRS)

NIRS was evaluated as a direct, rapid, and nondestructive tool for estimating various major components in solid and semi-solid foods and agricultural commodities. Its main advantage is that the sample only needs to be homogenized or ground before simultaneous measurement of several constituents in less than 1 min per sample. Because no chemicals are used and the instruments are robust, NIRS is a relatively low-cost technique. Its main disadvantage is that small matrix variations

Received June 8, 1993. Accepted by JL on September 28, 1993.

may cause considerable bias. Furthermore, the detection limit is relatively high (approximately 0.1% on a dry matter basis). Because NIRS is a semi-empirical technique, it can never replace specific analytical methods. The latter are necessary to calibrate and validate the NIRS methods.

We studied the applicability of a filter NIRS instrument to estimate DPTG content and acid value of used frying fat and oil samples. However, NIRS data are not accepted by the judicature in The Netherlands. Therefore, we attempted to apply NIRS as a screening method to preclude application of LC and/or titration, methods accepted by the judicature, to those samples in compliance with the Dutch Food and Commodity Act.

Several workers have investigated the possibility of assessing acid value of used frying fats with methods that were less laborious than titration. Levina and Litvinova (4) used infrared (IR) spectroscopy to assess objectionable compounds in reused frying oils. Although they noted differences in certain bands in the IR spectrum that correlated with the acid value, no further IR application was pursued.

Correlations between NIRS data and acid value of fats were studied by Frankel et al. (5). They used NIRS at 2260 nm to assess the free fatty acid concentration of soy beans, which were stored under different moisture conditions. A strong correlation with titration ($r = 0.864$) was reported.

To the best of our knowledge, no earlier work was published concerning the simultaneous determination of the food safety/quality parameters for DPTG content and acid value of used frying fats and oils using NIRS.

Experimental

Selection of Samples for Calibration and Validation

The NIRS equipment was calibrated by collecting spectral data from a sample set that was also analyzed by using reference methods for DPTG and acid value, LC and titration, respectively. Food inspectors collected used frying fats and oil samples from randomly selected restaurants and snack bars in Amsterdam and its region over a 4-month period. To provide a highly robust calibration, product variability was maximized in the sample set by selecting fats and oils used for a large range of snacks. The ratio of fat:oil samples was 70:30 and in accordance with the application ratio in the Amsterdam region. The samples were stored at ambient temperature for duplicate determination of LC-DPTG content and titration-acid value.

After 171 samples were analyzed for LC-DPTG content and titration-acid value, 41 samples were removed from the total sample pool to achieve an approximately homogeneous frequency distribution for LC-DPTG. The remaining 130 samples were split into a calibration set comprising 86 samples and a validation set comprising 44 samples, both with an approximately homogeneous LC-DPTG frequency distribution.

The same set of 171 samples was used to similarly compose acid value calibration and validation sets comprising 107 and 36 samples, respectively.

Determination of DPTG Content by LC

The DPTG content in the samples of the calibration and validation sets was determined using an LC method, collaboratively tested by the 13 regional Food Inspection Services in The Netherlands (6).

After a 1 to 20 volumetric dilution of used frying fat or oil with tetrahydrofuran, a 20 µL aliquot of the resulting solution was injected on a gel-permeation LC column, 300 × 7.8 mm id stainless steel, packed with a spherical gel consisting of styrene–divinylbenzene copolymer with 5–10 µm particle size and pore size 500 Å (e.g., styragel, or equivalent) and eluted with tetrahydrofuran at a flow rate of 0.8 mL/min. The polymer, dimer, and monomer triglycerides were eluted after approximately 10, 10.6, and 11.4 min, respectively, and were detected by measuring the refractive index of the eluate. No external or internal standards were used. The DPTG content was measured as the sum of the detector responses of DPTG expressed as percentage of the total detector response.

Determination of Acid Value by Titration

The acid value of the samples from the calibration and validation sets was determined through a modification of the acid–base titration employed by the International Union of Pure and Applied Chemistry (IUPAC), method number 2.201-1979 (7). Toluene was used instead of diethylether because carbon dioxide is less soluble in toluene. Furthermore, no potentiometric monitoring was used.

After 5 to 10 g of used frying fat or oil was diluted with 50 mL of a mixture of toluene and ethanol (50 + 50, v/v), the resulting solution was titrated with 0.1N KOH in ethanol with phenolphthalein as indicator. The acid value was expressed as weight of potassium hydroxide (in mg) used to neutralize 1 g of fat or oil (mg KOH/g).

NIRS Instrumentation

Transflectance (superposed reflectance and transmittance) data were collected from the fat and oil samples using a Technicon InfraAlyzer-type 450 NIR spectrometer (Technicon Industrial Systems, Tarrytown, NY) in the transflectance mode and equipped with a thermostated flowcell, a liquid sample drawer (Technicon P/N 189-B302502), a standard set of 19 filters in the NIR spectral region, and a lead sulfide detector. Filter locations and wavelengths in nm were 01/1445, 02/1690, 03/1722, 04/1734, 05/1759, 06/1778, 07/1818, 08/1940, 09/1982, 10/2100, 11/2139, 12/2180, 13/2190, 14/2208, 15/2230, 16/2270, 17/2310, 18/2356, and 19/2348. These wavelengths were preselected by the manufacturer because they correspond to known absorption bands of various major food constituents. The NIR spectrometer was connected via an RS-232C interface with a personal computer for data collection.

Preparation of Samples for Spectroscopic Measurement

The samples were placed for 1 h in a water bath at 62°C. The liquified samples were homogenized by manual swirling and

were filtered with a Schleicher and Schuell paper filter No. 604 to remove solid remains of snacks.

NIRS Procedure

After the equipment was equilibrated and the flowcell was thermostated at 62 ± 0.5°C, the liquified samples were brought into the flowcell using a 20 mL syringe. The transflectance data at the 19 selected wavelengths were recorded directly. Each sample was measured in duplicate. The transflectance data were transformed by the NIR spectrometer microprocessor to logarithmic values that were collected by and stored in a personal computer. After the NIRS-DPTG content and NIRS-acid value were calculated through the relevant calibration equation by the instrument microprocessor, the final result was obtained by manually calculating and rounding off the average of the duplicate values.

Calibration

The NIR spectrometer used the following general equation to calculate the concentration of a constituent in a sample from the NIRS data:

$$C = k_0 + \sum_{i=1}^{p} k_i \times \log\left(\frac{1}{R_i}\right)$$

where C is the calculated concentration of the constituent in the sample, k_0 is a constant, k_i is a constant for the corresponding wavelength indicating its importance to compositional information, R_i is the transflection at a given wavelength, and p is the number of wavelengths.

Calibration was achieved by subjecting the logarithmic transflectance data of 19 wavelengths with their corresponding data obtained by LC-DPTG or titration-acid value to multiple regression analysis to determine k_0 and k_i. Next, the calculated standard error of calibration (SEC) was used to check the performance of the 19 wavelength model. The SEC measures how well NIRS data approximated data obtained by LC or titration for the calibration sample set and was calculated using the following equation:

$$SEC = \sqrt{\left(\frac{\sum_{i=1}^{N_c}(Y_{i\,ref} - Y_{i\,nirs})^2}{N_c - p - 1}\right)}$$

where $Y_{i\,ref}$ and $Y_{i\,nirs}$ are DPTG content or acid value analyzed by the relevant reference method and by NIRS, respectively, and N_c is the number of samples of the calibration sample set.

Samples with an absolute residue $(Y_{i\,ref} - Y_{i\,nirs})$ higher than 3 times the SEC were removed from the calibration set. The optimal wavelength set that provided the best predictive equation for relating the NIRS transflectance data to data obtained by LC or titration was achieved through the remaining sample set by using forward stepwise multiple linear regression search, i.e., finding the highest F-ratio that was calculated using the following equation:

$$F - ratio = \frac{r^2}{1 - r^2} \times \frac{N_c - p - 1}{p}$$

where r^2 is the multiple correlation coefficient, N_c is the number of samples, and p is the number of wavelengths. With a given data set of N samples, the highest F-ratio was obtained by selecting the wavelength set with the highest multiple correlation coefficient together with the minimum number of wavelengths.

Wavelength selections and corresponding constants obtained for both parameters were entered into the NIR spectrometer memory; then the calibrated instrument was ready to predict both parameters of the unknown sample by using the relevant calibration constants.

The relationship between sample temperature and NIRS-DPTG and NIRS-acid value was also determined.

Validation

Validation of the calibration implies that the NIRS-predicted DPTG contents and acid values of the validation sample set were compared to their corresponding reference values that were obtained using LC and titration, respectively. A t-test was used to determine whether the value of the intercept significantly differed from 0 and whether the slope significantly differed from 1. After the calibration was validated, the standard error of prediction (SEP), which characterized the deviation of the NIRS values of the validation sample set from their corresponding reference values, was calculated using the following equation:

$$SEP = \sqrt{\left(\frac{\sum_{j=1}^{N_V}[(Y_{j\,ref} - Y_{j\,nirs}) - bias]^2}{N_V - 1}\right)}$$

where N_V is the number of samples used for the validation. The bias of the validation sample set was calculated using the following equation:

$$bias = \frac{\sum_{j=1}^{N_v}(Y_{j\,ref} - Y_{j\,nirs})}{N_v}$$

Finally, the precision of a single NIRS determination, s_r, was calculated for both sample sets from the differences of duplicates using the following equation:

$$s_r = \sqrt{\left(\frac{\sum_{i=1}^{N}(Y_{i\,nir1} - Y_{i\,nir2})^2}{2N}\right)}$$

where $Y_{i\,nir1}$ and $Y_{i\,nir2}$ are a pair of NIRS duplicate values, and N is N_c or N_v.

Results and Discussion

The Near Infrared Transflectance Spectrum

A representative NIRS transflectance spectrum of used frying fat based on the 19 preselected wavelengths is shown in Figure 1. The transflectance spectrum is characterized by strong absorption between 2310 and 2348 nm and less strong absorption between 1722 and 2140 nm.

Calibration and Validation

The stepwise multilinear regression provided a 7-wavelength calibration for each parameter. Table 1 provides the 7 selected wavelengths for NIRS-DPTG content, as well as for NIRS-acid value determination that included the corresponding constants of the calibration equations. The statistical summary of calibration and its validation is given in Table 2. The calibration statistics show a correlation between the LC-DPTG and titration-acid value with their corresponding NIRS transflectance values at the relevant 7 wavelengths with correlation coefficients of 0.976 and 0.996, respectively.

Validation provided intercepts of –0.4% m/m and 0.01 mg KOH/g and slopes of 1.04 and 0.993 for DPTG and acid value calibration graphs, respectively. T-tests showed no significant deviation of the intercepts and slopes from 0 and 1, respectively. The obtained SEPs appeared to be of the same extent as the corresponding SECs.

The SEP is particularly important in applying NIRS as a screening technique because NIRS value criteria must be adopted to decide whether a sample complies with legislation or if it will be submitted to LC or titration, the only methods accepted by the judicature. The extent of the SEP determines the deviation of those criteria from the relevant regulatory limits. The obtained SEPs were sufficiently low to obtain practical criteria (*see Efficiency Obtained by NIRS Screening*).

As shown in Table 1, the calibration constants for calculating NIRS-DPTG content are remarkably higher than those for calculating NIRS-acid value. This indicates that the NIRS transflectance spectrum, based on the 19 preselected wavelengths, contains only minor information about the DPTG content so that instrumental noise influences the NIRS-DPTG determination compared with the NIRS-acid value determination. This results in a relative low precision (s_r, Table 2) of the NIRS-DPTG determination, i.e., 1.3% m/m, as compared to 16% m/m being the maximum regulatory limit (8.1% relative). These precision figures were better for the NIRS-acid value determination, i.e., 0.06 mg KOH/g as compared to 4.5 mg KOH/g as the maximum regulatory limit (1.3% relative). We could not investigate whether the NIRS transflectance spectrum, at other than the 19 preselected wavelengths, contained more compositional information about the DPTG content because a grating instrument was not available.

Table 1. Wavelengths and corresponding calibration coefficients for the simultaneous NIRS determination of DPTG content and acid value of used frying fats and oils with the Technicon InfraAlyzer-type 450 NIR spectrometer

DPTG content		Acid value	
Filter location/wavelength in nm	Calibration constant	Filter location/wavelength in nm	Calibration constant
k_0[a]	88.63	k_0 (*)	–78.14
02/1690	283.2	06/1778	–159.3
03/1722	696.9	09/1982	–569.2
04/1734	–713.3	10/2100	1495
07/1818	2984	11/2139	518.8
11/2139	–6827	13/2190	–1506
12/2180	8195	15/2230	727.1
15/2230	–4610	19/2348	–460.9

[a] This value may vary among instruments and have to be determined for each specific instrument.

The sample temperature dependency of the NIRS-DPTG and NIRS-acid value data (–0.6% m/m and –0.05 mg KOH/g, per °C, respectively, Table 2) appeared to be linear in the range from 60° to 70°C. From these data, we concluded that the thermostating capability of the flowcell (± 0.5°C) was sufficient to minimize the effect of temperature fluctuations.

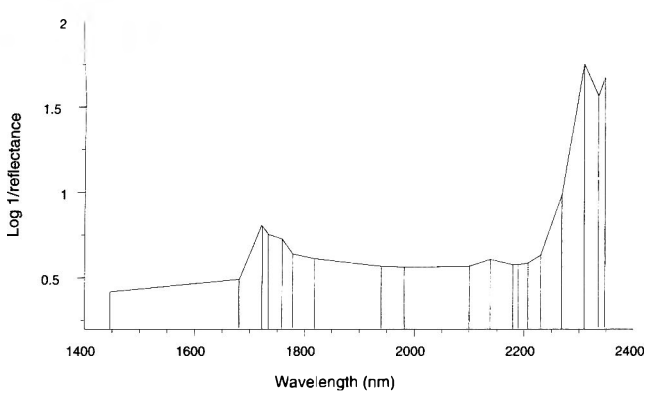


Figure 1. A representative NIRS transflectance spectrum of used frying fat based on the 19 preselected wavelengths.

Validation after Routine Operation for One Year

The calibration was validated again after routine operation for one year by 4 technicians working in shifts during 1992. All NIRS values of samples with an NIRS-DPTG value of 13.0% m/m and higher (N = 251 of total 1400, 17.9%) or with an NIRS-acid value of 4.1 mg KOH/g and higher (N = 367 of total 1400, 26.2%), samples suspected not to comply with legislation, were compared with their reference values obtained using LC or titration, Figures 2 and 3, respectively.

The slope of the DPTG graph (1.002) did not significantly differ from 1, whereas the slope of the acid value graph (0.909) significantly differed from 1. After correction for this deviation, the SEPs were calculated using the formula mentioned (Equation 4). Those "routine" SEPs were 2.3% m/m (1.8) and 0.44 mg KOH/g (0.33) for DPTG content and acid number,

Table 2. Statistical summary of the simultaneous NIRS determination of DPTG content and acid value of used frying fats and oils with the Technicon InfraAlyzer-type 450 NIR spectrometer

	DPTG content, % m/m		Acid value, mg KOH/g	
	Calibration	Validation	Calibration	Validation
Number of samples, N_c / N_v	86	44	107	36
Multiple regression correlation coefficient, r^2	0.968	0.976	0.997	0.996
SEC/SEP	1.9	1.8	0.31	0.33
Precision of a single NIRS analysis, s_r	1.1	1.3	0.06	0.06
Concentration range	0.0–32.7	2.2–31.3	0.30–18.8	0.14–14.5
F-ratio	350		4600	
Sample temperature effect	−0.6% m/m per °C		−0.05 mg KOH/g per °C	
Standard error of chemical methods	0.4 (LC)		0.08 (titration)	

respectively. We regard these increases to be acceptable compared with the original SEPs resulting from validation direct after calibration (Table 2), as given within parentheses.

Efficiency Obtained by NIRS Screening

In practice, used frying fats and oils are sampled on the basis of color observation and comparison of smoking and foaming at a specified temperature. Legal action against users of fat with a DPTG content of 16% m/m and higher and/or with an acid value of 4.5 mg KOH/g and higher can only be based on data obtained by LC or titration. After the NIRS calibration is validated, we decided to submit all samples with an NIRS-DPTG content and/or NIRS-acid value equal or higher than the maximum regulatory limit minus $1.3 \times SEP_v$ to LC and/or titration, i.e., 13.7% m/m for NIRS-DPTG and/or 4.1 mg KOH/g for NIRS-acid value. This meant that NIRS screening for 10% of the samples with NIRS-DPTG content and NIRS-acid value equal to the maximum regulatory limits will lead to the false negative conclusion that these samples comply with the Dutch Food and Commodity Act. We consider this concession to be reasonable because this percentage rapidly decreases for samples with NIRS-DPTG content and/or NIRS-acid value data rising above the relevant maximum regulatory limit. Because we observed some outliers during the calibration procedure, we set our criteria for approval or submission to LC-DPTG analysis of a sample, for the test year 1992, to 13.0% m/m instead of

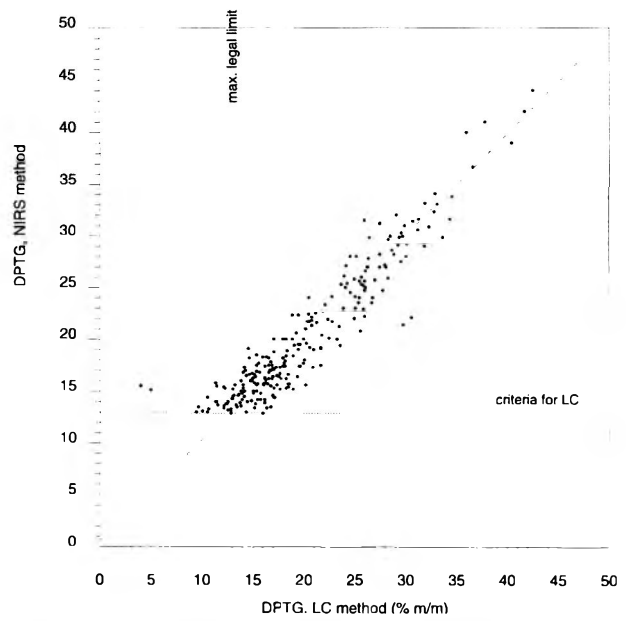


Figure 2. Scatter diagram comparing DPTG contents of 251 samples with an NIRS-DPTG content of 13.0% m/m and higher with their reference values obtained by LC. The SEP is 2.3% m/m.

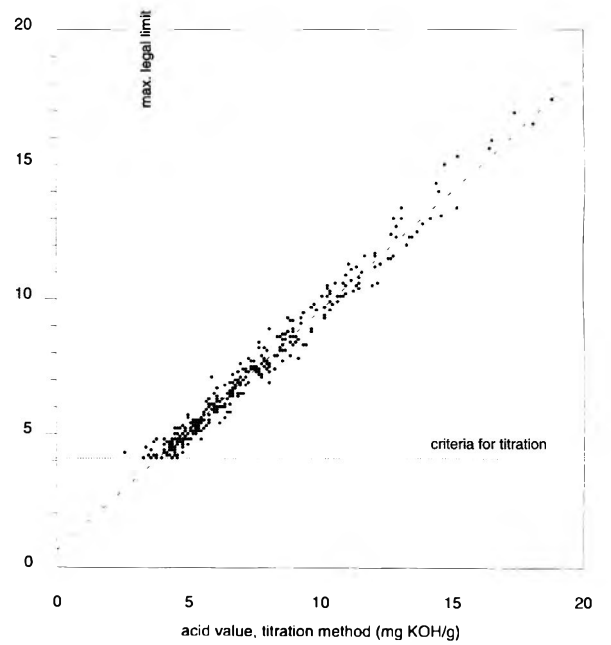


Figure 3. Scatter diagram comparing acid values of 367 samples with an NIRS-acid value of 4.1 mg KOH/g and higher with their reference values obtained by using titration. The SEP is 0.44 mg KOH/g.

13.7% m/m. The 2 DPTG outliers observed (LC-NIRS 4.0–15.5 and 5.1–15.0 % m/m) were later identified as samples that were contaminated with water.

In 1992, food inspectors collected 1400 suspended samples from randomly selected restaurants and snack bars in the Amsterdam region. NIRS screening, considering the chosen criteria, precluded the need for 1149 LC-DPTG analyses (82.1%) and 1033 titration-acid value determinations (73.8%) because those samples appeared to comply with the Dutch Food and Commodity Act. From this, we conclude that NIRS screening is very economical.

Validation after routine operation for 1 year provided SEPs of 2.3% m/m and 0.44 mg KOH/g for NIRS-DPTG content and NIRS-acid value determination, respectively. This implies that we definitively should adopt minimal criteria for submitting samples to LC and/or titration with an NIRS-DPTG content of 13.0% m/m (16–1.3 × 2.3) and/or with an NIRS-acid value of 3.9 mg KOH/g (4.5–1.3 × 0.44).

Quality Control Sample

A ceramic reference disc was used to check overall operation of the NIRS instrument. If the disc reading deviated more than 0.1% relative from day to day and 0.5% relative long term from its average value, equipment service was deemed necessary.

A suitable control sample was obtained by mixing aliquots of 10 used frying fats and 10 used frying oils. The LC-DPTG content and titration-acid value were approximately the relevant maximum regulatory limits. Samples were divided in 100 mL portions that were stored at ambient temperature. An aliquot of such a portion was analyzed in duplicate before each series of samples was analyzed in 1992. After using a portion 10 times, it was discarded.

If the NIRS value of a control sample deviated more than 3 × S_L (intralaboratory standard deviation, double sited) from the average value, or its value deviated more than 2 × S_L (single sited) on 2 successive days of analysis, validation of the calibration, using a validation set comprising at least 20 samples, was necessary. If the calibration appeared to be valid, we concluded that chemical and/or optical properties of the control sample were changed.

NIRS predicted the DPTG content and acid value accurately for at least 1 year and 7 months, respectively. After 7 months, the NIRS-acid value remarkably increased, whereas the titration-acid value was stable for at least 1 year. Unfortunately, because NIR spectra were not stored, we could not explore the wavelength(s) responsible for this deviation.

Conclusions

Based on calibration, validation, and validation after routine operation for one year (particularly the high correlation coefficients and reasonably low SECs and SEPs), we conclude that simultaneous NIRS determination of DPTG content and acid value of used frying fats and oils is sufficiently accurate and precise. The calibration was effective over a wide range for DPTG content (2 to 33% m/m) and acid value (0.2 to 17 mg KOH/g fat). Because NIRS is a low-cost technique that takes about 1 min per sample, large-scale screening of these food safety/quality parameters is possible. We conclude that NIRS screening can be used to assess whether a sample complies with food legislation and that it will save time and chemicals.

References

(1) Clark, W.L., & Serbia, G.G. (1991) *Food Technol.* 84–89

(2) Crampton, E.W., Common, R.H., Farmer, F.A., Well, A.F., & Crawford, D. (1953) *J. Nutr.* **60**, 13

(3) *Dutch Food and Commodity Act—Preparation and Preservation of Food Decree* (1992) Staats Courant Nr. 678, 30 December, The Hague, The Netherlands, ISSN 0169-5037 (Dutch)

(4) Levina, Z.V., & Litvinova, V.D. (1979) *Konservnaya i Ovoshchesushil 'naya Promyshlennost'* **1**, 33–34 (Russian)

(5) Frankel, E.N., Nash, A.M., & Snyder, J.M.T.I. (1987) *J. Am. Oil Chem. Soc.* **64**, 987–992

(6) Beljaars, P.R., van Dijk, R., & Houwen-Claassen, A.A.M. (1994) *J. AOAC Int.* **77**, 667–672

(7) *Standard Methods for the Analysis of Oils, Fats, and Soaps*, International Union of Pure and Applied Chemistry (IUPAC) method number 2.201-1979: Determination of Acid Value and of Acidity

FOOD COMPOSITION AND ADDITIVES

Determination of Cholesterol by *p*-Nitrobenzoate Derivatization and Liquid Chromatography

THOMAS W. HAMILL and ABDEL M. SOLIMAN [1]
U.S. Food and Drug Administration, Atlanta Center for Nutrient Analysis, HFR-SE680, 60 Eighth St, NE, Atlanta, GA 30309

The method involves direct saponification and formation of the sterol *p*-nitrobenzoate (PNB) derivatives or lipid extraction with methylene chloride and isopropyl alcohol, saponification, fatty acid methylation, and formation of the sterol PNB derivatives. The sterol PNB derivatives are separated on a C_8 column with a mobile phase of acetonitrile–hexane–water (250 + 30 + 3) and quantitated by UV detection at 280 nm. The limit of detection for cholesterol is 2 ng. The response was linear over a range of 4 to 250 ng ($r = 0.999$). Assays of NIST SRM 1845 and 1563 by this method gave results that were closer to the certified values than were results obtained by the AOAC gas chromatographic method. Reproducibility was evaluated by using foods containing low, medium, and high levels of cholesterol. The % coefficient of variation ranged from 1.8 to 6.7. Studies on a wide variety of food products (using lipid extraction and saponification–methylation) gave a mean recovery of 87.5 ± 10.1%. Direct saponification of poultry and dairy products gave a mean recovery of 101.3 ± 15.8%. Peak purity was determined by diode array spectrophotometry.

The new regulations for nutritional labeling (1) have increased the need for reliable methods to detect cholesterol in a wide variety of foods. In addition, the definition of "cholesterol free" (2 mg/100 g or referenced amount) challenges the detection limits of current procedures. Many older methods (enzymatic, colorimetric, etc.) are designed for specific food matrixes and lack both specificity and sensitivity. Reviews of available methodology through the early 1980s were written by Naito and David (2), Zak (3) and Sweeny and Weihrauch (4). More recently, Beyer and Jensen (5) reported the overestimation of the cholesterol content of eggs when assayed colorimetrically. They indicated that chromatographic separation was necessary to remove spectral interferences.

Punwar (6) developed a widely used approach for measurement of cholesterol in foods. The method involves lipid extraction, saponification, silyl derivatization, and detection by gas chromatography (GC). Although the lipid extraction and saponification steps are cumbersome, the derivatizing reagents unstable, and the GC column subject to degradation, the method is based on sound scientific principles and is still relied upon. Several improvements have been made. Al-Hasani et al. (7) and Beyer et al. (8) used direct saponification of specific products to speed up the assay. Tsui (9) designed an extraction system using solid-phase isolation and capillary GC to rapidly determine cholesterol in milk. Oles et al. (10) optimized Punwar's (6) lipid extraction and saponification step combined with an NH_2 solid-phase extraction cleanup cartridge and capillary GC to achieve excellent reproducibility in the analysis of sausage, eggs, pretzels, and cookies. Ulbrerth and Reich (11) developed a capillary GC method for the determination of cholesterol in processed foods. They compared direct saponification of the sample with fat extraction followed by saponification. GC results were compared with those of an enzymatic test kit. They concluded that enzymatic detection was not specific, that results of fat extraction are not better than those of direct saponification, that the silylation of cholesterol was not necessary when fused-silica GC columns were used, and that the addition of an antioxidant prevented cholesterol oxidation. Interferences due to α-tocopherol were noted for foods containing both animal and vegetable fats. The study did not include food products containing less than 20 mg cholesterol/100 g.

An earlier qualitative technique (12) detects cholesterol and plant sterols by GC after conversion to the ester with acetic anhydride. Sheppard et al. (13) used anhydride esterification to measure cholesterol and plant sterols in a wide variety of food products. Samples were extracted with chloroform–methanol. The lipid extract was saponified by using an established fatty acid methylation procedure (14). Sterols were esterified with butyric anhydride and detected by GC. The authors achieved 100% recovery of cholesterol through the use of saponification–methylation, and results were reported on foods containing as little as 4 mg cholesterol/100 g.

Temperatures in excess of 200°C are required to volatilize cholesterol for GC. Rees et al. (15) noted that many sterols are thermally decomposed, especially in the presence of heated metal. An internal standard, usually cholestane, is added on the assumption that any chromatographic loss that affects cholesterol also affects cholestane. Liquid-chromatographic proce-

Received May 20, 1993. Accepted by JL September 23, 1993.
[1] Deceased.

dures, which normally do not require elevated temperatures, appear more suitable for cholesterol analysis.

The determination of cholesterol by liquid chromatography (LC) has received less attention than GC approaches. The weak absorbance of cholesterol at low wavelengths presents problems in spectral detection. Several investigators (16–20) assayed underivatized cholesterol by using LC and UV detection in the 203 to 214 nm range. Indyk (21) determined underivatized cholesterol and plant sterols in several food products. Using LC with UV detection at 212 nm, he found 11 mg cholesterol/100 g whole milk, with a detection limit of 40 ng.

Newkirk and Sheppard (22) developed an LC method in which cholesterol was derivatized to its benzoate ester. The lipid extraction, saponification, and methylation steps in the procedure parallel those in the earlier work of Sheppard (13). When the benzoate moiety is added to the sterol molecule, UV detection at 230 nm is possible. The method was applied to a variety of food products that included eggs, butter, and meat products. The procedure gave results similar to those obtained by the GC method of Punwar (6). Novotny et al. (23) measured benzoylated steroid metabolites in blood. Fillion and Zee (24) used benzoylation to measure a mixture of cholesterol oxides. In each case, the benzoylated cholesterol was determined by LC with increased sensitivity compared with results of GC determinations.

The objective of our study was to develop a method for the determination of cholesterol in foods, including those considered to be cholesterol free (i.e., 2 mg/100 g or referenced amount). The present method avoids the problems of extraction, selectivity, sensitivity, and reproducibility usually associated with cholesterol analysis. The method is relatively simple and accommodates a wide variety of foods.

METHOD

Reagents

(**a**) *Acetic acid wash.*—0.02N acetic acid.

(**b**) *Acetonitrile.*—UV grade (Baxter, B&J Brand; Cat. No. 015-4, or equivalent).

(**c**) *Boron trifluoride.*—12.5% in methanol (E.M. Science, Gibbstown, NJ, or equivalent).

(**d**) *Cholesterol calibration standard.*—NIST SRM 911b (Gaithersburg, MD). Prepare as in (**e**).

(**e**) *Cholesterol working standard (50 μg/mL).*—Fluka (Ronkonkoma, NY), or equivalent. Accurately weigh 4–5 mg (± 0.001 mg) and dilute to 100 mL with *n*-hexane.

(**f**) *Magnesium sulfate anhydrous.*—Sigma (Cat. No. M-7506; St. Louis, MO), or equivalent.

(**g**) *KOH solution.*—Dissolve 3 parts of potassium hydroxide pellets by weight (reagent grade) in 2 parts of distilled water by volume. Prepare fresh daily.

(**h**) *Methylene chloride–TEA.*—Add 0.001% triethylamine (TEA) by volume to reagent grade methylene chloride to inhibit acid formation.

(**i**) *Mobile phase.*—Acetonitrile–*n*-hexane–distilled water (250 + 30 + 3). Mix solvents by swirling, and filter through a 0.45 μm nylon filter 3 times to ensure complete solution. The mobile phase may be stirred continually to avoid separation.

(**j**) *4-Nitrobenzoyl chloride (p-nitrobenzoyl chloride) (PNBC).*—Aldrich Chemical Co. (Cat. No. 11,330-8; Milwaukee, WI). Product should be a bright-yellow solid. Blanket with helium and tightly cap immediately after each use.

(**k**) *Pyridine.*—Reagent grade; blanket with helium and tightly cap immediately after each use.

(**l**) *Solvents.*—Benzene, heptane, *n*-hexane (UV grade), isopropyl alcohol, toluene, and isooctane, all reagent grade.

Apparatus

(**a**) *Electric meat and food grinder.*—Oster (Division of Sunbeam Corp., Milwaukee, WI), or equivalent.

(**b**) *Homogenizer.*—Polytron (Brinkmann Instruments, Westbury, NY), or equivalent.

(**c**) *LC system.*—LDC analytical Model 3200 solvent delivery system fitted with a Rheodyne fixed-loop injector and an LDC Model 3200 variable-wavelength detector, or equivalent. Representative conditions for this system are injection volume, 20 μL; mobile-phase flow rate, 1.5 mL/min; and detector wavelength, 280 nm with 0.02 AUFS (absorbance unit full scale).

(**d**) *LC column.*—Zorbax C_8 packed at 6000 psi in a 25 × 0.4 cm id stainless steel column.

(**e**) *LC integrator.*—Hewlett-Packard Model 3390A, or equivalent. Representative settings for this system are zero, 10; attenuation, 2; chart speed, 0.2; peak width, 0.64; threshold, 3 in the peak height mode (*see* integrator manual for setting definitions).

(**f**) *Rotary flash evaporator with condenser.*—Rotovapor EL180 (Buchi Instrument Co.) with a high-vacuum pump (Welch Duo-Seal, Model 1400), a cold trap maintained at <–10°C, and a water bath at 35°C.

Sample Preparations

Mix, grind, or homogenize food products to a homogenous consistency prior to sampling. (*1*) For products where small homogenous portions may be taken directly, saponify the sample and proceed with derivatization and detection. (*2*) For products where lipid extraction is not necessary (oils, etc.), saponify and methylate the sample and then proceed with derivatization and detection. (*3*) For products that cannot be saponified directly (emulsify when extracted, etc.) or when total lipid and fatty acid profile evaluations are desired, extract the lipid, saponify and methylate, and then proceed with derivatization and detection.

Lipid Extraction

Weigh a portion of sample containing 10–500 μg cholesterol and 50–500 mg fat (total lipid) and place it in a 100 mL low-form graduate. The sample taken should be 1–6 mL. Add distilled water to give a total volume of 6–6.5 mL. Add 15 mL isopropyl alcohol and mix with a spatula. Add 6.5–7 g magnesium sulfate and mix. Add 35 mL methylene chloride–TEA and mix. Homogenize the mixture at medium speed for 60 s. Vacuum filter the mixture through fast filter paper. Place the

filtrate in a 250 mL glass stoppered (g/s) round-bottom flask. Wash the graduated cylinder, homogenizer, and filter cake with two 35 mL portions of methylene chloride–TEA. Combine the washes with the filtrate in the 250 mL flask. Add ca 0.5 g magnesium sulfate, and evaporate to dryness in the rotary evaporator. Transfer the residue to a suction filter apparatus fitted with a 50 μm nylon filter using three ca 7 mL portions of isooctane. Collect the filtrates in a 50 mL g/s round-bottom flask and evaporate to dryness in the rotary evaporator. With 2 disposable pipettes (one to add solvent and one to transfer), quantitatively transfer the residue to a volumetric flask and dilute to volume with methylene chloride–TEA. The dilution should contain 1–50 μg cholesterol/mL and 5–50 mg lipid/mL. At this point, a gravimetric evaluation of total lipid can be made by drying an aliquot of the dilution at 95°–100°C for 30 min.

Saponification and Methylation

Take an aliquot of the diluted lipid extract or an oil sample containing 5–200 μg cholesterol and <200 mg lipid. Evaporate the diluted extract to dryness with a stream of helium or dry nitrogen. Saponify and methylate according to the AOAC procedure (14), diluting the esters and sterols to 5.00 mL with heptane. At this point, an aliquot may be taken for fatty acid analysis by GC. Take an aliquot of the heptane dilution and dilute volumetrically with *n*-hexane to a cholesterol concentration of 5–50 μg/mL. Continue with the derivatization.

Direct Saponification

Accurately weigh a portion of sample containing >25 μg cholesterol. The sample should weigh more than 0.05 g but less than 1.0 g. Place the sample in a 125 mL g/s flask. Add 3 mL KOH solution and 15 mL reagent alcohol (95% ethanol–methanol–isopropyl alcohol, 95 + 5 + 5). Fit the flask with an air-cooled condenser and reflux on a steam bath (95°–100°C) for 1 h. Cool and then transfer the digest to a 125 mL separatory funnel with the aid of 15 mL distilled water. Extract the digest 3 times with 30 mL *n*-hexane. Combine the organic phases in a 100 mL volumetric flask (the organic phase may be evaporated in a rotary evaporator and diluted to a smaller volume if necessary) and discard the aqueous phase. Dilute the organic phase to 100 mL with *n*-hexane. Continue with the derivatization step.

Standard Preparation

Prepare as described in *Reagents* (**e**). Label as the standard preparation and use a 2.0 mL aliquot (100 μg) for the derivatization step. Assay working standards by using the calibration standard.

Derivatization

Treat sample and standard preparations separately as follows: Place an aliquot containing 10–200 μg cholesterol in 50 mL g/s round-bottom flask. Evaporate to dryness with a stream of helium or dry nitrogen (dry nitrogen may be substituted for helium throughout the assay). For aliquots larger than 10 mL, evaporate to ca 5 mL in a rotary evaporator and then to dryness with a stream of helium. Add 1.0 mL benzene, blanket with helium, and cap with a glass stopper. Mix carefully; wet and dissolve any visible solid. Uncap and add 0.45 g *p*-nitrobenzoyl chloride (add so that the solids fall directly into the benzene solution). Blanket with helium and cap. Swirl gently for ca 3 s. Uncap and add 5.00 mL pyridine; direct the stream into the benzene mixture. Blanket with helium, cap, and swirl gently for ca 3 s. Uncap and add 1.00 mL 20% aqueous KOH. Blanket, cap, and swirl again. With the cap firmly in place, submerge the reaction flask (ca halfway) in a 70°C water bath for 5 s. Remove and shake vigorously by wrist action for 5 s. Repeat the 5 s heating and 5 s shaking 4 more times. The mixture should be clear and straw-colored at this point. Continue to shake by wrist action for 120 s. Uncap, add 50 mL water, cap, shake vigorously, and transfer to a 250 mL separatory funnel. Complete the transfer with 2 more 50 mL portions of water. Immediately add 30 mL *n*-hexane and shake vigorously. It should take the experienced analyst ca 8 min to complete the reaction (from the addition of benzene to the addition of the 30 mL *n*-hexane). While phases are separating, another derivatization may be started. Drain the aqueous bottom layer into a beaker. Pour only the *n*-hexane layer into a 250 mL g/s round-bottom flask. Repeat the 30 mL *n*-hexane extraction 2 more times; combine the extracts in the 250 mL flask. Neutralize the aqueous phase with acid and discard. Evaporate the combined *n*-hexane extract to dryness in a rotary evaporator. With 2 disposable pipettes, transfer the derivative residue with several 1 to 2 mL portions of *n*-hexane to a volumetric flask. Dilute the sample to a cholesterol concentration of 1–2 μg/mL and the standard to a concentration of 5–6 μg/mL. At this point, prepare 3 standard dilutions of 0.5, 1.0, and 3.0 μg/mL from the standard derivative preparation. Place ca 2 mL of each standard dilution and ca 2 mL derivatized sample dilution in separate 8 mL screw-cap vials. Add ca 2 mL 0.02N acetic acid to each, cap, shake vigorously, and allow phases to separate. Use the upper *n*-hexane phase for the LC determination. The tightly capped solution of *p*-nitrobenzoate (PNB) derivative is stable for several months.

NOTE: Work in a fume hood and wear safety glasses and rubber gloves when preparing the derivative. Avoid contact with or breathing the vapors of *p*-nitrobenzoyl chloride, benzene, and pyridine. Neutralize aqueous waste with 8N acetic acid.

Determination

Inject 20.0 μL of the upper *n*-hexane phase (for fixed-loop injectors, inject 50 μL to ensure complete filling and rinsing) directly into the LC. All peaks should elute in 20 min. Determine the identity of the cholesterol derivative by comparing standard and sample retention times. Plot the linear responses of the standards against their cholesterol concentrations. Determine cholesterol concentration in sample from the standard plot. Calculate the concentration in 100 g of the sample according to the following equation:

$$\text{Cholesterol in sample (mg/100g)} = \frac{\text{Concn in sample (μg/mL)} \times \text{Dilution (mL)} \times 100\text{ g}}{1000(\text{μg/mg}) \times \text{Sample weight (g)}}$$

Results and Discussion

The method is suitable for a wide variety of food matrixes. Depending on their properties, samples may be saponified directly, lipid extracted and then saponified and methylated, or directly saponified and methylated. The derivatization and the detection are the same for each sample.

The National Institute of Standards and Technology (NIST) uses direct saponification (25) to prepare powdered egg samples for cholesterol determination. This approach avoids steps that might lead to cholesterol loss (7, 11). Current work agrees with Indyk (21), who noted underestimation of cholesterol in foods where a significant portion of cholesterol exists in the fat-free phase. NIST SRM 1845 egg powder was assayed for cholesterol by lipid extraction followed by saponification and methylation. The results were lower (16.9 mg/g) than the certified values of 18.8–19.2 mg/g. When the same sample was directly saponified, the certified value was obtained (18.8 mg/g).

Lipid extraction followed by saponification and methylation is useful for products that emulsify when directly saponified. Products such as milk, infant formula, and low-fat cottage cheese are more easily assayed with lipid extraction. The technique is based on the work of Landen (26) for extraction of lipid in milk and infant formula. It involves absorbing the water with anhydrous magnesium sulfate and solubilizing the lipid with isopropyl alcohol–methylene chloride. Modifications were made to Landen's lipid extraction to accommodate foods other than milk and infant formula. Filter paper replaced the sintered glass funnel to avoid cholesterol retention. Two methylene chloride–TEA washes replaced the filter cake extractions. These changes improved cholesterol recovery without lipid loss.

The saponification–methylation step is a well-established method (14) for preparing the fatty acid methyl esters of extracted lipids and oils. Cholesterol is not saponified nor is it methylated by this treatment. Sheppard et al. (13) reported 100% recovery of cholesterol through the method. An attempt to modify this step by omitting the boron trifluoride catalyst resulted in gelatinous emulsions for dairy products. Samples that are mostly fat (vegetable oil, lard, tallow, or the lipid extract for a sample) may be saponified and methylated and then derivatized for cholesterol detection. The advantage of the saponification–methylation step is that an aliquot may be analyzed by GC for fatty acids.

The derivative step is based on the acylation of cholesterol according to the Schotten–Baumann reaction (27). Fitzpatrick and Sigga (28) described the preparation of cholesterol *p*-nitrobenzoate (PNB) and cholesterol benzoate. They indicated that the UV-sensitive derivatives were suitable for analysis by LC. Cholesterol PNB was chosen over cholesterol benzoate because it has a stronger UV absorbance and a maximum at 255 nm. Sugino et al. (29) measured cholesterol oxides in

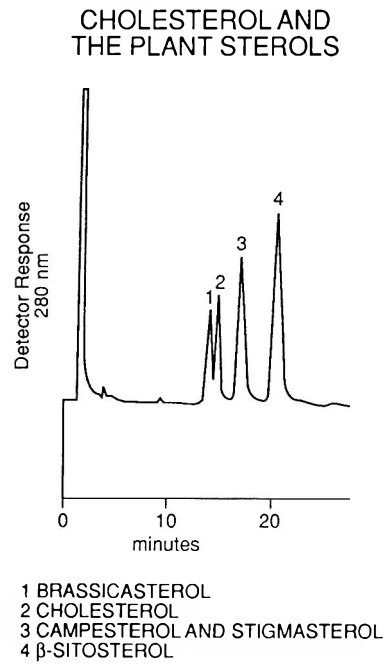


Figure 1. Chromatogram of PNB derivatives of brassicasterol, cholesterol, campesterol, stigmasterol, and β-sitosterol standards, LC parameters: column, Zorbax C_8; mobile phase, acetonitrile–hexane–water (250 + 30 + 3); detection, 280 nm.

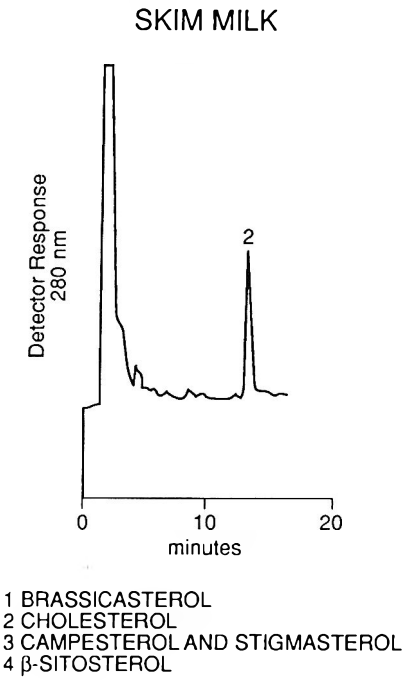


Figure 2. Typical chromatogram of a skim milk sample containing 3.08 mg of cholesterol per 100 g of product. Chromatographic parameters identical to those for Figure 1.

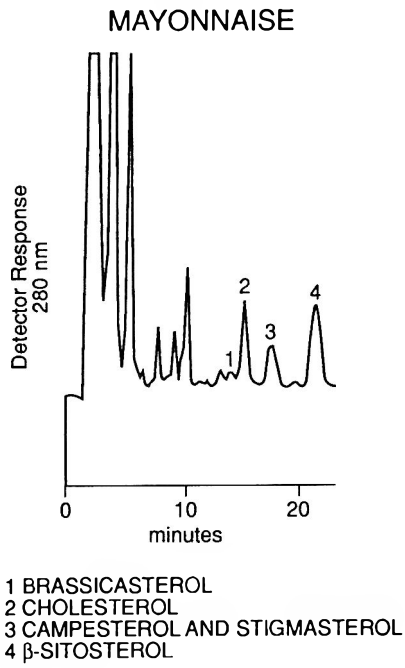


Figure 3. Typical chromatogram of a mayonnaise sample containing 34.7 mg of cholesterol per 100 g of product. Chromatographic parameters indentical to those for Figure 1.

spray-dried eggs by preparing the PNB derivatives. The derivative reaction used by Sugino et al. (29) was designed for microgram quantities for cholesterol oxides in dried egg. Modifications had to be made in the current work to accommodate higher cholesterol concentrations and to eliminate interferences from plant sterols. Benzene was added to solubilize the extracted sterols in a hydrophobic medium because *p*-nitrobenzoyl chloride reacts with water. Recent work has shown that toluene may be substituted for benzene. The direct addition of solid *p*-nitrobenzoyl chloride, pyridine, and 20% KOH in that order gave the most reproducible recoveries of cholesterol PNB. The specified heating and mixing conditions ensure complete reaction. Cholesterol PNB is very hydrophobic; thus, the hexane extraction is almost emulsion-free. Traces of pyridine are removed by washing the hexane extract with an equal volume of 0.02N acetic acid. The acid wash does not affect cholesterol *p*-nitrobenzoate. This precautionary step must be taken because trace levels of pyridine can foul the LC column.

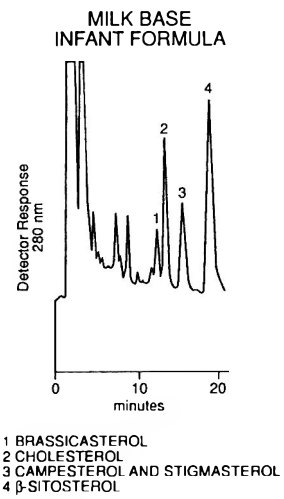


Figure 4. Typical chromatogram of infant formula found to contain 1.28 mg of cholesterol per 100 g of ready-to-feed product. Chromatographic parameters identical to those for Figure 1, except for a 3-fold increase in detector sensitivity.

Table 1. Comparison of assay results of NIST standard reference materials and nonfat dry milk

Product	NIST certified value, mg/g	Value determined by LC method, mg/g	Value determined by AOAC method, mg/g
SRM 1845 Egg powder	19	18.8	13.4
SRM 1563 Coconut oil	0.642	0.659	0.553
Nonfat dry milk	—	0.0254	0.0263

The recommended solvent ratio gave an ideal residence time on the column for cholesterol PNB and the best separation from the plant sterol derivatives. Increasing the hexane volume decreases the residence time, whereas increasing the acetonitrile volume increases the residence time. The mobile phase is at the saturation point for hexane in acetonitrile–water. Filtering 3 times ensures complete miscibility.

Several LC columns were tried, including Zorbax ODS, phenyl, Zorbax C_8, and Zorbax ODS plus Zorbax C_8 in series. Zorbax C_8 gave the best separation of cholesterol PNB from the plant sterol derivatives. Stigmasterol and campesterol derivatives eluted as 1 peak well resolved from the cholesterol derivative. Gordon and Griffith (30) noted the coelution of cholesterol and brassicasterol esters from a C_{18} column. Our approach resolves these 2 components sufficiently to permit cholesterol quantitation. α-Tocopherol interferes with the GC determination of cholesterol (11) in animal fat. The *p*-nitrobenzoyl derivative of *dl*-α-tocopherol was prepared and chroma-

Table 2. Cholesterol content of foods containing low, medium, and high levels of cholesterol

Product	Amount of cholesterol, mg/100 g Mean ± SD	CV, %
Milk-based infant formula[a] (n = 5)	1.31 ± 0.03	2.19
Skim milk (n = 4)	3.08 ± 0.14	4.55
Evaporated whole milk (n = 5)	20.5 ± 1.38	6.73
Cheddar cheese (n = 5)	71.5 ± 1.31	1.83

[a] Reconstituted according to label directions.

Table 3. Determination of cholesterol content and spike recovery of cholesterol from various foods by lipid extraction and saponification

Product	Amt. found,[a] mg/100 g	Amt. added, mg/100 g	Amt. recovered,[b] mg/100 g	Recovery, %
Fresh eggs (edible portion)	342	500	396	79.2
Egg white product (contains dry milk)	2.46	510	410	80.3
Canned egg nog	38.0	62.1	46.2	74.4
		246.8[d]	204.6	82.9
Milk-based infant formula conc. 1-1[c]	1.46	12.1	11.7	96.6
		39.7[d]	35.6	89.7
Milk-based infant formula powder[c]	1.31	1.41	1.01	71.6
Milk-based infant formula conc. 1-1 with whey[c]	2.07	11.9	10.3	86.6
		38.4[d]	35.7	93.0
Milk-based infant formula powder with whey[a]	2.19	5.52	4.37	79.2
Skim milk	3.08	2.17	1.74	80.2
Nonfat dry milk[c]	2.23	1.14	1.02	89.3
Evaporated skim milk[c]	4.13	5.87	5.23	89.1
Cheddar cheese	71.5	109	90.8	83.3
Low-fat cottage cheese	9.49	18.6	17.3	93.0
Vegetable pizza	6.26	9.57	7.18	75.0
		19.8[d]	16.6	84.1
Breaded fish sticks	25.3	15.1	12.3	81.6
Frozen shrimp dinner	44.5	78.2	70.0	89.4
		299.2[d]	306.4	102
Frozen beef dinner	12.9	14.9	16.3	109
		59.3[d]	59.5	100
Fresh pasta	<0.1	2.89	2.92	101

[a] LC data are based on duplicate injection for duplicate or more extractions.
[b] LC data are based on duplicate injections.
[c] Reconstituted according to label directions.
[d] Indicates various spiking levels.

tographed along with cholesterol PNB. Baseline resolution with 3 min between peaks was obtained (results not shown).

Cholesterol PNB has a broad UV absorbance with a maximum at 255 nm. This property permits a variety of wavelength choices for detection. Foods assayed in this study gave the cleanest chromatographs at 280 nm. Quantitative results were obtained for 4 ng cholesterol PNB standard detected at 280 nm.

Figure 1 shows the separation of cholesterol PNB from the PNB derivatives of common plant sterols. Brassicasterol PNB was included to ensure cholesterol PNB resolution and quantitation. Figure 2 shows the chromatogram of cholesterol PNB from a sample of skim milk. The plant sterols are absent, and the saponification step removed triglyceride interferences. Figure 3 (mayonnaise) and Figure 4 (milk-based infant formula) show the ability of this method to determine cholesterol in foods that contain both animal and vegetable fats. The detector sensitivity was increased for the infant formula to accommodate the 1 μg/mL analyte concentration, which represents 20 ng cholesterol PNB on the column.

Table 1 compares results (in duplicate) of this method and the current AOAC procedure (6). NIST certified standards were used for validation. In each case, the PNB method gave values closer to the certified value. NIST uses direct saponification as a modification of the AOAC procedure to ensure complete cholesterol recovery (25). The cholesterol (2 mg/100 g) in non-fat dry milk was barely detectable by the AOAC procedure. By contrast, the PNB method easily handled this analyte level at a 5-fold increase in dilution.

Reproducibility for the method is shown in Table 2. Foods representing low, medium, and high cholesterol contents (2, 20, and 80 mg/100 g, respectively) were assayed by lipid extraction, saponification and methylation, derivatization, and LC detection (n = 5). The method provided reproducible results, even for an infant formula containing a high level of plant sterols.

Assay results (in duplicate) on a wide variety of food products are presented in Table 3. Separate samples of each product were spiked with cholesterol standard. Most products were spiked at levels equal to the expected amount. Several products containing egg, milk, vegetable, cheese, fish, and beef lipids were spiked above the literature values to determine if the method could detect cholesterol in excessive quantities. The difference in spike recoveries was less than 10%, except for the shrimp dinner (89–102%).

Table 4 presents assay results (in duplicate) for poultry and dairy products. Spike recoveries appear better than those shown in Table 3. The mean recovery was 101.3 ± 15.8%, which included the 127% recovery for 2.13 mg/100 g added to evaporated skim milk. Part of the reason for the better results

Table 4. Determination of cholesterol content and spike recoveries from foods by direct saponification

	Label claim, mg/100 g	Amount found, mg/100 g	Amount added, mg/100 g	Amount recovered, mg/100 g	Recovery, %
Reduced-cholesterol liquid eggs	90	113	45.7	43.6	95.4
Chicken eggs (reduced cholesterol)	320	372	320	322	101
Armenian string cheese	60	64.3	57.8	52.2	90.3
Evaporated skim milk	10	5.09	2.13	2.71	127
			8.08	7.08	87.6

may be that direct saponification involves one less analytical step. Other possible reasons have been mentioned in the literature (7).

The PNB method does not overestimate the cholesterol content of fresh eggs and its results agree with recent values (5). Adequate chromatography eliminates the interferences that cause high results. Peak purity of the cholesterol from egg nog was determined by diode array spectrophotometry. Sample and standard peaks matched at all wavelengths. Pure analyte peaks permit determination of trace cholesterol. An egg white product labeled to contain no cholesterol was found to contain 2.46 mg/100 g. Because this product contains dry milk, a small amount of cholesterol is to be expected (1.02 mg cholesterol/100 g was recovered from nonfat dry milk spiked at 1.14 mg/100 g).

In summary, the LC–PNB method permits the determination of cholesterol at various levels in a variety of food products. Reproducible results are obtained for cholesterol in poultry, dairy, vegetable, fish, cereal, and meat products. The Nutrition Labeling and Education Act labeling requirements define "cholesterol free" as less than 2 mg/100 g. This level can now be evaluated quantitatively. The method allows the use of a smaller sample size for ease of handling with a corresponding increase in sensitivity and selectivity.

Acknowledgments

We thank R. R. Eitenmiller of the University of Georgia, Department of Food Science and Technology, for his editorial help with the manuscript; A. J. Sheppard of the U.S. Food and Drug Administration for some valuable suggestions; Edna R. Young for the resource and time management; and Clark Head, Joseph Kramer, Anne Reid, and Dudley Mann, all from the Atlanta Center for Nutrient Analysis of the U.S. Food and Drug Administration, for their analytical help.

References

(1) *Fed. Regist.* (1993) **58**(3), Part IV, 2175–Ç

(2) Naito, H.K., & David, J.A. (1984) in *Laboratory and Research Methods in Biology and Medicine*, Vol. 10, Jon A. Story (Ed.), A.R. Liss, Inc., New York, NY, pp. 1–45

(3) Zak, B. (1980) *Lipids* **15**, 698–704

(4) Sweeney, J.P., & Weihrauch, J.L. (1976) *Crit. Rev. Food Sci. Nutr.* **8**, 131–159

(5) Beyer, R.S., & Jensen, L.S. (1989) *J. Agric. Food Chem.* **37**, 917–920

(6) Punwar, J.K. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 804–810

(7) Al-Hasani, S.M., Shabany, H., & Hlavac, J. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 817–820

(8) Beyer, J.D., Milani, F.X., Dutelle, M.J., & Bradley, R.L., Jr (1989) *J. Assoc. Off. Anal. Chem.* **72**, 746–748

(9) Tsui, I.C. (1988) *J. Assoc. Off. Anal. Chem.* **72**, 421–424

(10) Oles, P., Gates, G., Kensinger, S., Patchell, J., Schumacher, D., Showers, T., & Silcox, A. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 724–728

(11) Ulbreth, F., & Reich, H. (1992) *Food Chem.* **43**, 387–391

(12) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec. **970.51(h)**

(13) Sheppard, A.J., Newkirk, D.R., Hubbard, W.D., & Osgood, T. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1302–1306

(14) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec. **969.33**

(15) Rees, H.H., Donnahey, P.L., & Goodwin, T.W. (1976) *J. Chromatogr.* **116**, 281–291

(16) McCleur, R.H., & Jungalwala, F.B. (1976) *Adv. Exp. Med. Biol.* **76**, 533

(17) Hurst, W.J., Aleo, M.D., & Martin, R.A., Jr (1983) *J. Dairy Sci.* **66**, 2192–2194

(18) Hurst, W.J., Aleo, M.D., & Martin, R.A., Jr (1985) *J. Agric. Food Chem.* **33**, 820–822

(19) Holen, B. (1985) *J. Assoc. Off. Anal. Chem.* **62**, 1344–1346

(20) Brown, H.G. (1987) *J. Assoc. Off. Anal. Chem.* **64**, 106–108

(21) Indyk, H.E. (1990) *Analyst* **115**, 1525–1530

(22) Newkirk, D.R., & Sheppard, A.J. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 54–57

(23) Novotny, M., Alasandro, M., & Koneshi, M. (1983) *Anal. Chem.* **55**, 2375–2377

(24) Fillion, L., & Zee, J.A. (1991) *J. Chromatogr.* **547**, 105–112

(25) Ellerbe, P.M., National Institute of Science and Technology, a private communication on the analysis of SRM 1845

(26) Landen, W.O., Jr (1985) *J. Assoc. Off. Anal. Chem.* **68**, 183–187

(27) *Merck Index* (1993) 10th Ed., Merck & Co., Inc., Rahway, NJ, p. ONR-82

(28) Fitzpatrick, F.A., & Sigga, S. (1973) *Anal. Chem.* **45**, 2310–2314

(29) Sugino, K., Terao, J., Murakami, H., & Matsushita, S. (1986) *J. Agric. Food Chem.* **34**, 36–39

(30) Gordon, M.H., & Griffith, R.E. (1992) *Food Chem.* **43**, 71–78

FOOD COMPOSITION AND ADDITIVES

Comparison and Assessment of the Difference in Total Dietary Fiber in Cooked Dried Legumes as Determined by Five Methods

ROGER MONGEAU and RENÉ BRASSARD
Health Canada, Food Directorate, Banting Research Centre, Nutrition Research Division, Ottawa, ON, K1A 0L2, Canada

Dried beans (kidney and great northern) and dried peas (chick, green, and yellow) were cooked according to package instructions. Total dietary fiber (TDF) was measured by the Mongeau (AOAC 992.16), Prosky (AOAC 985.29), and Lee (AOAC 991.43) methods (A, B, and C, respectively). Non-starch polysaccharides (NSP) were measured by the Englyst gas-chromatographic method that included dimethyl sulfoxide treatment, and the lignin measured separately was added to NSP (method D). TDF was also measured by the Li method (E). TDF values ranged from 7 to 48 g/100 g dry weight. Methods B and C gave similar TDF values, which were higher than those from other methods. For kidney beans I, TDF values by methods B and C were up to 2.5 times higher because of the inclusion of starch. The fiber residues from methods A and C contained the same amounts of arabinose, xylose, mannose, galactose, and uronic acid, but glucose was 4 times higher in residue C than in residue A. When α-amylase from porcine pancreas was incorporated in methods B or C for 5 samples, the discrepancies among methods A, B, and C were reduced by 60–98%.

The Mongeau (AOAC **992.16**), Prosky (AOAC **985.29**), and Lee (AOAC **991.43**) methods, respectively identified as A, B, and C, are gravimetric methods that yield similar total dietary fiber (TDF) values for various foods. Good agreement between methods A and B was reported for the determination of TDF in canned legumes (1). However, large amounts of starch were reported in the residues of cooked dried legumes analyzed by methods B and C (2–4). Similarly, TDF values for dried peas differed by a factor of up to 4 among methods because of inconsistencies in the capacity to digest starch (Mongeau and Brassard, unpublished data). Thus, the determination of dietary fiber is critical for cooked dried legumes. Because of the possibility of significant discrepancies for these foods, this methodological problem must be investigated to achieve agreement among dietary fiber methods and to avoid the accumulation of conflicting data.

Received August 24, 1993. Accepted by JL November 10, 1993.

Methods B and C are similar, and it is not known if their starch-digesting capacities can be improved by using additional digestive enzymes rather than dimethyl sulfoxide (DMSO) or heat treatments. DMSO is known to remove starch, but it is not certain if it removes only glucose from the constituent sugars of the residue (5). A comparison of methods A, B, and C with the Englyst gas-chromatographic method plus lignin (method D) permits monitoring of dietary fiber composition. The Li and Cardozo method (3) is a modification of method B: some enzyme treatments are deleted and a larger protein correction is made to the residue (method E). The Li method provides a base to evaluate the proteolytic activity of enzymes.

The aim of the present work was to compare the TDF values for cooked dried legumes determined by the 5 methods and to see if the variability among methods could be improved by using mammalian digestive enzymes.

Experimental

Materials

Dried red kidney beans I (Goya) and II (Townhouse), great northern beans, and chick peas, bought in the Washington, DC, area, were provided by Li and Cardozo (3). The dried green and yellow peas were bought in Ottawa, Canada.

Sample Preparation

Kidney beans I and II, great northern beans, and chick peas were prepared by U.S. Department of Agriculture (USDA) laboratory as described by Li and Cardozo (3). They were quickly soaked (200 g/L deionized water) and cooked for 1 h, according to package instructions. Kidney beans II were cooked for 2 h. They were then freeze-dried, and a portion of each was sent to the Food Directorate laboratory in Ottawa.

The dried green and yellow peas were brought to boiling and simmered for 3 h according to package instructions. For green peas, the package contents were mixed to ensure homogeneity between 2 cooking batches. Exactly the same time and temperature settings were used for the 2 batches. The green peas listed in Tables 1 and 2 were from the first batch, and those listed in Table 3 were from the second batch. For yellow peas, a single large batch was cooked.

Table 1. TDF in boiled dried green peas: effect of incorporating an APP treatment in methods B and C

Method[a]	TDF,[b] % (dry wt. basis)	Starch in TDF[c]
A with APP at 55°C	9.9 ± 0.1^{w}	2.7
A with APP at 37°C	9.0 ± 0.1^{v}	1.8
B	19.0 ± 0.2^{z}	11.8
B^{+APP}	12.3 ± 0.3^{y}	5.1
C	18.8 ± 0.3^{z}	11.6
C^{+APP}	12.3 ± 0.3^{xy}	5.1
D	7.2 ± 0.2^{u}	0.0
D without DMSO	10.4 ± 0.1^{wx}	3.2

[a] A, rapid method, AOAC **992.16**; B, AOAC **985.29**; C, Tris-Mes method, AOAC **991.43**; C^{APP}, α-amylase from porcine pancreas, for 30 min after heat-stable amylase; D, nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added; E, Li and Cardozo (3). A lignin value of 0.4 was added.

[b] Values are means ± standard deviations for 3 or 4 determinations. Values in this column not sharing the same superscript are significantly ($P < 0.001$) different.

[c] TDF minus TDF by method D.

Dietary Fiber Methods

Total dietary fiber (TDF) was measured by the following methods: A, Mongeau (AOAC **992.16**) (1); B, Prosky (AOAC **985.29**) (6); C, Lee (AOAC **991.43**) (7); D, Englyst GC (8) plus lignin; and E, Li and Cardozo (3). Method E is a simplified Prosky method that includes autoclaving at 130°C and excludes some enzyme treatments. The enzymes used were obtained from the suppliers specified in each method. No substitutes were used.

When α-amylase from porcine pancreas (APP) (Sigma Chemicals Co., Cat. No. A3176) was used with methods B or C, the enzyme was added after treatment of sample with heat-stable amylase. The APP treatment consisted of adding a 5.0% solution of APP in phosphate buffer (0.1M at pH 7.0) for 30 min at 55°C. A volume of 5 mL of the solution was the best compromise between efficiency and economy for methods B and C. Method A includes 2 APP treatments.

Table 2. Effect of APP and protease on amounts of residual protein of methods B and C for boiled dried green peas

Method modifications[a]	Protein,[b] % dry sample
B	5.0 ± 0.0^{v}
B^{+APP}	3.3 ± 0.2^{u}
B^{+APP} protease deleted	5.5
C	6.9 ± 0.1^{w}
C^{+APP}	2.9 ± 0.3^{uv}
C^{+APP} protease deleted	4.9
E without protease	18.7
$E^{120°C}$ without protease	20.0 ± 0.1^{x}

[a] A, rapid method, AOAC **992.16**; B, AOAC **985.29**; C, Tris-Mes method, AOAC **991.43**; C^{APP}, α-amylase from porcine pancreas, for 30 min after heat-stable amylase; D, nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added; E, Li and Cardozo (3). $E^{120°C}$ is the method of Li and Andrews (16).

[b] Values are means ± standard deviations for 2–4 determinations; $n = 1$ when no standard deviation is shown. Values in this column not sharing the same superscript are significantly ($P < 0.001$) different.

Table 3. Constituent sugars of residues of fiber methods C, C^{+APP}, and D for boiled dried green peas

	Amount of sugar, % (dry wt. basis)							
Method[a]	RHA[b]	FUCO	ARA	XYL	MAN	GAL	GLU	URON
C	0.13	0.10	2.66	0.32	0.16	0.62	20.41	1.46
	0.13	0.10	2.46	0.27	0.13	0.56	21.86	1.18
C^{+APP}	0.13	0.09	2.50	0.29	0.16	0.60	6.87	1.35
	0.12	0.09	2.36	0.26	0.16	0.55	7.16	1.25
D[c]	0.09	0.08	2.59	0.28	0.09	0.74	1.75	1.14
	0.12	0.09	2.62	0.28	0.09	0.72	1.55	1.08

[a] Method: nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added

[b] RHA, rhamnose; FUCO, fucose; ARA, arabinose; XYL, xylose; MAN, mannose; GAL, galactose; GLU, glucose; URON, uronic acid.

[c] Green peas used with method D were from the first cooking batch, whereas those used with methods C and C^{+APP} were from the second cooking batch.

Determination of Neutral Sugars and Uronic Acids

The polysaccharide components of fiber residues were analyzed according to the Englyst method (8). After hydrolysis by acid treatments, neutral sugars were measured by gas–liquid chromatography as alditol acetates and uronic acid was measured colorimetrically.

Determination of Lignin

Lignin was measured by 2 methods. Permanganate lignin was measured according to the Robertson and Van Soest procedure as described by Mongeau and Brassard (9): the sample was treated with a neutral detergent, incubated with APP for 65 min, treated with an acid detergent, and then treated mildly with permanganate. These sequential treatments removed most of the nonfiber materials. Permanganate lignin values were added to Englyst nonstarch polysaccharides (NSP) to obtain TDF values.

Klason lignin was the material left after all the enzyme and chemical treatments of the Englyst DMSO method.

Statistical Method

The significance of the effect of APP on TDF analysis of dried green and yellow peas was evaluated by 2-tail unpaired t-test (StatView II, Abacus Concepts, Inc., Berkeley, CA).

Results

Table 4 shows TDF values for beans and chick peas measured by 5 methods. The close agreement of most duplicates indicates good repeatability. The number of analyses was re-

stricted by the limited amounts of samples available from the USDA laboratory. TDF values ranged from 11% for chick peas to 48% (g/100 g, dry weight) for kidney beans I. The unmodified Prosky (B) and Lee (C) methods yielded similar values, which were 1.5 to 2.3 times higher than those from the Mongeau rapid method (A), the Englyst GC method plus lignin (D) or the Li and Cardozo method (E). Analysis by methods A, D, and E of the kidney and great northern beans gave similar dietary fiber contents (18–22%), which were higher than those of chick peas (11–13%). By contrast, with methods B and C, TDF values for kidney beans I (48%) were higher than those for other samples, including chick peas (24–32%). When APP treatment was incorporated in method C (C^{+AAP}), the TDF value decreased to 32% but remained at least 50% higher than those from methods A and D. For kidney beans II and great northern beans, a closer agreement was seen among methods A, C^{+APP}, D, and E. Not enough chick pea sample remained to test method C^{+APP}. Methods A and E gave slightly higher values than method D (Table 4).

Permanganate and Klason lignin contents represented 10% of nonstarch polysaccharides (NSP) or less. They were respectively 1.77 and 1.65% for kidney beans I, 1.41 and 1.36% for kidney beans II, 0.76 and 0.42% for great northern beans, and 0.88 and 0.56% for chick peas. Klason lignin (y) was slightly lower than permanganate lignin (x), but values were highly correlated ($y = 1.27x - 0.53$, $r = 0.993$). The permanganate lignin value was added to the Englyst NSP to obtain the TDF values by method D in Table 4.

Table 5 compares the constituent sugars of residues from methods A, C, and D for kidney beans I. The compositions were similar for uronic acid and neutral sugars, including arabinose/xylose ratios (2.7–2.9). An exception were values of residual glucose, which were 4.4, 7.6, and 30% for methods D, A, and C, respectively. Method D included a DMSO treatment that dissolved starch, and the 4.4% glucose obtained was part of NSP. This result implies that the residual starch contents for methods A and C were 3.2 and 25.6%, respectively. These figures correspond to the difference between their respective TDFs and the TDF by method D (2 and 29%; Table 4).

For kidney beans II, great northern beans, and chick peas, values for total sugars agreed within 0.2%, but glucose values were higher (7.8–11.3%) by method C than by method D (Table 6). This difference accounted for 70–88% of the difference in the TDF values (11.3–14.1%; Table 4).

Because of the lack of availability of the four samples listed in Tables 4–6, green and yellow peas were used to complement the method comparison for boiled legumes. The TDF contents of boiled green peas ranged from 7.2 to 19% depending on method (Table 1). The starch contents of the respective fiber residues, calculated as the difference in TDF from method D, varied from 2.7% (method A) to 11.8% (method B). The starch content was 1.8% when APP was used at 37°C for 18 h in method A and 3.2% when method D was used without DMSO. When methods B and C were used with an APP treatment, most of the residual starch was digested ($P < 0.001$) and 5.1% remained in the fiber residues (Table 1).

Table 4. Total dietary fiber values for beans and chick peas measured by different methods[a]

Sample[b]	Total dietary fiber, % (dry wt. basis)					
	A	B	C	C^{+APP}	D	E
Kidney beans I	20.7	47.1	47.9	31.1	18.9	
	21.3	44.5	47.3	32.1	18.8	
Kidney beans II	21.3	29.0	31.7	22.1	17.7	21.2
	21.9	30.4	31.5	21.4	17.3	21.9
Great northern beans	20.7	29.3	30.6	23.0	19.4	22.3
	20.7	28.9	31.1	23.4	19.6	22.4
Chick peas	12.7	24.9	23.8		10.7	13.2
	12.8	24.3	23.5		10.7	12.8

[a] A, rapid method, AOAC **992.16**; B, AOAC **985.29**; C, Tris-Mes method, AOAC **991.43**; C^{+APP}, α-amylase from porcine pancreas, for 30 min after heat-stable amylase; D, nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added; E, Li and Cardozo (3).

[b] Samples were soaked and boiled for 1 h except for kidney beans II, which were boiled for 2 h.

Because starch digestion increased after incorporation of an APP treatment between the heat-stable amylase and amyloglucosidase treatments, the relative importance of these amylolytic enzymes was tested. When the heat-stable amylase or the amyloglucosidase treatment was deleted in methods B or C (data not shown), the amounts of starch in the residue exceeded the 5.1% given in Table 1 obtained by methods (B^{+APP} and C^{+APP}) using the 3 amylolytic enzymes. Pancreatin was tested in place of APP, but with the concentrations used, less starch was digested because of the lower amylase activity of the enzyme mixture (data not shown).

In contrast to methods B and C, method E does not include a protease treatment. Consequently, the amounts of protein (N × 6.3) to be subtracted from the residues were higher for method E (>18%) than for methods B and C (<7%) (Table 2). The protein content was expressed in percent sample because the amylolytic activity of APP also decreased residue weights. APP and protease treatments together (method B^{+APP} or C^{+APP}) further decreased ($P < 0.001$) residual protein from 5.0–6.9% to about 3% because of the additional proteolytic activity of APP (Table 2). Removal of the protease treatment from method B^{+APP} or C^{+APP} revealed that the proteolytic activity of APP was equivalent to that of the protease alone. Deletion of protease had no effect on starch contents and TDF values.

A second batch of green peas was cooked for analysis of constituent sugars in residues of methods C and C^{+APP} (Table 3). Although cooking conditions were the same as for the first batch (Tables 1 and 2), the total sugar content reached 26.3% (method C, Table 3) compared with 18.8% TDF (method C, Table 1). This result indicated the poor repeatability of method C for cooked green beans. When incorporated in method C, APP did not change the nonglucose sugars but removed 14.1 g/100 g glucose (starch) and consequently decreased the total sugar content of the residue to 12.0% (Ta-

Table 5. Constituent sugars of fiber residues of methods A, C, and D for boiled dried kidney beans I[a]

	Amount of sugar, % (dry wt. basis)							
Method	RHA[b]	FUCO	ARA	XYL	MAN	GAL	GLU	URON
A	0.24	0.37	5.55	2.06	0.31	1.37	7.71	3.00
	0.24	0.39	5.63	2.09	0.32	1.40	7.57	3.07
C	0.22	0.33	4.99	1.63	0.49	1.20	31.6	3.23
	0.26	0.34	5.18	1.98	0.48	1.23	28.7	3.77
D	0.21	0.31	5.39	1.86	0.53	1.27	4.35	3.17
	0.20	0.34	5.28	1.82	0.53	1.29	4.38	3.10

[a] Method: nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added.
[b] Abbreviations: RHA, rhamnose; FUCO, fucose; ARA, arabinose; XYL, xylose; MAN, mannose; GAL, galactose; GLU, glucose; URON, uronic acid.

ble 3). The latter value is similar to the TDF value of 12.3%, which includes about 0.4% lignin (Table 1) and indicates that method C^{+APP} is more independent from variations between cooking batches.

A large batch of yellow peas was cooked to permit various analyses on identical material (Tables 7 and 8). TDF values by methods B and C were at least 40% higher than by methods A and D (Table 7). When the APP treatment was performed at 37°C instead of 55°C in method A, TDF did not decrease significantly (–0.8%, $P > 0.01$). When APP was incorporated in methods B and C, the TDF values were 25% lower, and residual starch decreased from 9.8% or more to 3.8% or less. TDF values by methods A, B^{+APP}, and C^{+APP} were within the 16.7–18.0% range. Deleting the DMSO treatment in method D increased the TDF value to 18.5% (Table 7).

The total amount of polysaccharide components in the hydrolyzed residue of method C reached 25.4%, the majority being glucose (17.4%, Table 8). The APP treatment reduced the glucose content to 9.6%. This accounted for >95% of the reduction in total sugars. The remaining glucose content (9.6%) exceeded the nonstarch glucose content in the residue of method D by 4.1% (Table 8), which is close to the 3.8% obtained gravimetrically (Table 7).

Discussion

Dietary fiber is made of complex and heterogeneous polymeric materials that are not easy to separate from other food components, particularly starch (10–16). Methods for dietary fiber have been improved over the past decade, and a good agreement for TDF analyzed by methods A, B, and D was reported for various foods including canned legumes (1). However, it was reported that a substantial amount of starch remains in the insoluble fiber residue of methods B or C for cooked dried legumes (2–4). The present results confirmed the incomplete removal of starch by methods B or C for 6 legumes. Chick peas were not tested with APP but the difference in the glucose contents of the residues of methods C and D (Table 6) accounted for 88% of the gap in the respective TDF values (Table 4). For kidney beans I, APP reduced the discrepancy between methods A and C from 26.6 to 10.6% (Table 4). For kidney beans II, great northern beans, green peas, and yellow peas, APP reduced the mean discrepancy among methods A, B, and C from 9.9 to 1.8% (Tables 1, 3, 4 and 7–8). Overall for the 5 samples tested, APP reduced the discrepancies among methods A, B, and C by 60–98%.

The decreases in the TDF values observed when using APP in methods B or C were attributable only to removal of starch from their residues. This effect could have been demonstrated by treating the residues of methods B and C with starch-digesting enzymes and by measuring the glucose released in the supernatant. However, the release of glucose depends on the enzymatic conditions of the assay. If the enzymatic conditions do

Table 6. Constituent sugars of fiber residues of methods C and D for 3 legumes

		Amount of sugar, % (dry wt. basis)							
Sample	Method[a]	RHA[b]	FUCO	ARA	XYL	MAN	GAL	GLU	URON
Kidney beans II	C	0.20	0.28	4.54	1.76	0.34	1.07	15.4	2.83
		0.23	0.27	4.50	1.78	0.34	1.11	14.6	3.08
	D	0.20	0.28	4.73	1.73	0.57	1.14	4.88	2.73
		0.21	0.27	4.51	1.70	0.54	1.10	4.81	2.71
Great northern beans	C	0.26	0.33	5.68	2.36	0.36	1.05	12.9	3.81
		0.23	0.30	5.35	2.38	0.34	1.00	12.4	3.93
	D	0.23	0.34	5.87	2.21	0.53	1.05	4.81	3.53
		0.20	0.35	5.78	2.24	0.54	1.06	5.03	3.55
Chick peas	C	0.21	0.10	3.44	0.48	0.27	0.58	13.5	2.28
		0.19	0.10	3.45	0.43	0.25	0.54	14.3	1.95
	D	0.20	0.11	3.83	0.46	0.17	0.56	2.56	1.91
		0.21	0.09	3.92	0.45	0.17	0.58	2.54	1.87

[a] Residues of method C were treated as those of method D (nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added).
[b] Abbreviations: RHA, rhamnose; FUCO, fucose; ARA, arabinose; XYL, xylose; MAN, mannose; GAL, galactose; GLU, glucose; URON, uronic acid.

Table 7. TDF in boiled yellow peas determined by unmodified or modified methods A, B, C, and D

Method[a]	TDF,[b] % (dry wt. basis)	Starch in TDF[c]
A with APP at 55°C	16.7 ± 0.1^{vw}	2.5
A with APP at 37°C	15.9 ± 0.2^{uv}	1.7
B	24.0 ± 0.3^{y}	9.8
B^{+APP}	17.8 ± 0.0^{x}	3.6
C	24.1 ± 0.6^{y}	9.9
C^{+APP}	18.0 ± 0.1^{wx}	3.8
D	14.2 ± 0.1^{u}	0.0
D without DMSO	18.5 ± 1.4^{uvwxy}	4.3

[a] A, rapid method, AOAC **992.16**; B, AOAC **985.29**; C, Tris-Mes method, AOAC **991.43**; C^{APP}, α-amylase from porcine pancreas, for 30 min after heat-stable amylase; D, nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added; E, Li and Cardozo (3). A lignin value of 1.2 was added.

[b] Values are means ± standard deviations for 3 determinations, except for methods B^{+APP} and C^{+APP} ($n = 2$). Values in this column not sharing the same superscript are significantly ($P \leq 0.01$) different.

[c] TDF minus TDF by method D.

not remove all starch, the amount of glucose released in the supernatant will lead to an underestimation of residual starch. On the other hand, if fiber-degrading enzymes are present in the assay, they may release nonstarch glucose, which will lead to overestimation.

In the present work, a different approach was used to estimate the starch in the residue of method C. The residue was hydrolyzed in acidic conditions according to the Englyst GC method. These conditions favor complete depolymerization of fiber polysaccharides into their constituent sugars and minimize sugar degradation. Measuring the sugars by gas chromatography as alditol acetates enables a comparison with NSP constituents determined by the integral Englyst method (8). The enzymes used in the latter method are devoid of fiber-degrading activities, and the DMSO treatment ensures that no starch remains in the residue before acid hydrolysis. This nonstarch glucose value can be subtracted from that found in the residue of the fiber method tested for residual starch. The values for the other constituent sugars of NSP may also permit detection of fiber-degrading enzymes.

Table 8. Constituent sugars of residues of fiber methods C, C^{+APP}, and D for boiled dried yellow peas

Method[a]	Amount of sugar, % (dry wt. basis)							
	RHA[b]	FUCO	ARA	XYL	MAN	GAL	GLU	URON
C	0.23	0.13	3.01	1.29	0.20	0.65	17.35	2.48
	0.22	0.13	2.83	1.39	0.20	0.62	17.45	2.56
C^{+APP}	0.20	0.10	2.99	1.05	0.19	0.63	9.52	2.60
	0.19	0.10	3.00	1.06	0.19	0.63	9.68	2.28
D	0.19	0.10	2.97	1.01	0.12	0.75	5.51	2.22
	0.18	0.11	3.09	1.09	0.11	0.72	5.52	2.25

[a] Residues were treated as those of method D (nonstarch polysaccharides (NSP) according to Englyst GC method using DMSO, with $KMnO_4$ lignin values added).

[b] RHA, rhamnose; FUCO, fucose; ARA, arabinose; XYL, xylose; MAN, mannose; GAL, galactose; GLU, glucose; URON, uronic acid.

APP did not degrade the fiber. For green and yellow peas, APP in method C did not decrease the nonglucose fiber constituents and it did not decrease glucose under the level observed by method D (Tables 3 and 8). For other foods (green beans, carrot, barley, oat bran, high-fiber cereal, and soybran), TDF values obtained gravimetrically by method C with or without APP were similar:

$$C^{+APP} = 0.156 + 0.995(C),\ r^2 = 0.999 \text{ (unpublished data).}$$

APP was incubated at 55°C when used in methods A, B, and C. At 37° or 55°C, the types of enzyme activities of APP are the same but the rate of starch hydrolysis is higher at 55°C. Tables 1 and 7 indicate a slightly better starch digestion at 37°C than at 55°C for method A because a longer incubation was used at 37°C. APP is devoid of fiber-degrading enzyme activities at 37° or 55°C.

Lee and Prosky (5) suggested that DMSO could solubilize hemicelluloses, on the basis of published data including those of Honda et al. (2). However, the present results do not support this hypothesis because the fiber constituent sugars, except glucose, were similar for methods excluding (A and C) and including DMSO (D). There were no definite trends for the 6 legume samples examined (Tables 3, 5, 6, and 8).

APP is an unpurified α-amylase preparation, and it provides a proteolytic activity comparable with that of the protease used in methods B and C (Table 2). This indicates that method B would not take longer if APP replaces the protease, and the capacity to digest starch would be improved. Methods B and C are similar, but the latter uses a Tris-Mes buffer with a temperature-dependent pH, and only one pH adjustment is needed. Because APP works very efficiently at 55°C, the resulting pH would not be compatible with its activity. Thus, if APP replaces the protease in method C, the number of pH adjustments would be the same as in method B.

Unmodified method C included more starch in the fiber residue of the second cooking batch (19.5%, calculated from Table 3) than in the first cooking batch (11.6%, Table 1). However, this inconsistency did not appear when APP was incorporated in method C. The amounts of residual starch were decreased to around 5% for both batches, and the dietary fiber values excluding lignin were close (11.9%, Table 1; 12.0%, Table 3).

Methods A, C, and D showed remarkable agreement in the polysaccharide constituents of dietary fiber, glucose excepted (Tables 3, 5, 6, and 8). Therefore, solving the problem of starch digestion should decrease most of the discrepancy among methods.

References

(1) Mongeau, R., & Brassard, R. (1989) *J. Food Compos. Anal.* **2**, 189–199

(2) Honda, C., Nanba, T., Asaoka, O., Yumoto, K., Hayashi, T., Ikegami, S., & Takai, Y. (1992) *J. Food Hyg. Soc. Jpn.* **33,** 46–51

(3) Li, B.W., & Cardozo, M.S. (1993) *J. Food Sci.* **58,** 929–932

(4) Villanueva, M.J., Redondo, A., Rodriguez, M.D., & Garcia B. (1993) *Fresenius' J. Anal. Chem.* **345,** 247–249

(5) Lee, S.C., & Prosky, L. (1992) *Cereal Foods World* **37,** 765–771

(6) Prosky, L., Asp, N.-G., Furda, I., DeVries, J.W., Schweizer, T.F., & Harland, B.F. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 677–679

(7) Lee, S.C., Prosky, L., & DeVries, J.W. (1992) *J. AOAC Int.* **75,** 395–399

(8) Englyst, H.N., & Cummings, J.H. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 808–814

(9) Mongeau, R., & Brassard, R. (1982) *J. Food Sci.* **47,** 550–555

(10) Marlett, J.A., & Lee, S. (1980) *J. Food Sci.* **45,** 1688–1693

(11) Mongeau, R., & Brassard, R. (1980) *Getreide Mehl und Brot* **34,** 125–127

(12) Rasper, V.F. (1981) in *The Analysis of Dietary Fiber in Food,* W.P.T. James & O. Theander (Eds), Dekker, New York, pp. 29–36

(13) Schaller, D. (1978) *Am. J. Clin. Nutr.* **31,** S99–S102

(14) Southgate, D.A.T. (1981) in *The Analysis of Dietary Fiber in Food,* W.P.T. James & O. Theander (Eds), Dekker, New York, pp. 37–50

(15) Van Soest, P.J. (1981) in *The Analysis of Dietary Fiber in Food,* W.P.T. James & O. Theander (Eds), Dekker, New York, pp. 241–262

(16) Li, B.W., & Andrews, K.W. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 1063–1064

FOOD COMPOSITION AND ADDITIVES

Evaluation of a Gas Chromatographic Method to Identify Irradiated Chicken, Pork, and Beef by Detection of Volatile Hydrocarbons

GEORG A. SCHREIBER, GRIT SCHULZKI, ALMUTH SPIEGELBERG, NORBERT HELLE, and KLAUS W. BÖGL
Federal Institute for Health Protection of Consumers and Veterinary Medicine, General-Pape-Str. 62, D-12101 Berlin, Germany

A method suitable for routine application was used in an interlaboratory study to detect irradiation treatment of chicken carcass, pork, and beef. By using gas chromatographic analysis, 17 participating laboratories determined the quantity of 4 different radiation-induced volatile hydrocarbons (tetradecene, pentadecane, hexadecadiene, and heptadecene) in the fat fraction of coded specimens approximately 3 and 6 months after irradiation. The specimens of each type of meat were supplied by 2 different producers. The dose range tested (0.6–7.5 kGy) included levels commercially used to reduce the number of contaminating microorganisms (1–5 kGy). The method employed permitted a correct identification of irradiated or nonirradiated in 98.3% of the 864 specimens.

Received May 12, 1993. Accepted by JL September 27, 1993.

Treatment of certain types of food by ionizing radiation to increase the shelf life and to reduce health hazards that might be caused by contaminating microorganisms is permissible in a number of countries; in other countries, ionizing radiation is banned. To meet the different national regulations in international trade and to ensure the consumer's free choice to buy irradiated or nonirradiated products, labelling control has become necessary. Various interlaboratory studies have already been performed to evaluate methods that identify irradiation treatment of foods (1–6). The present interlaboratory study describes the gas chromatographic (GC) detection of volatile hydrocarbons (HC) in the fat fraction of raw chicken carcass, pork, and beef. The method was used to identify an irradiation of these products in the standard commercial dose range (1–5 kGy). The method was used for a qualitative judgment only, not for dose estimations.

At the end of the 1960s, details about the detection of irradiated foodstuffs on the basis of changes in the fat fraction were published (7–11). In recent years, interest in the identification of irradiated fatty foodstuffs has grown steadily (12–29). The

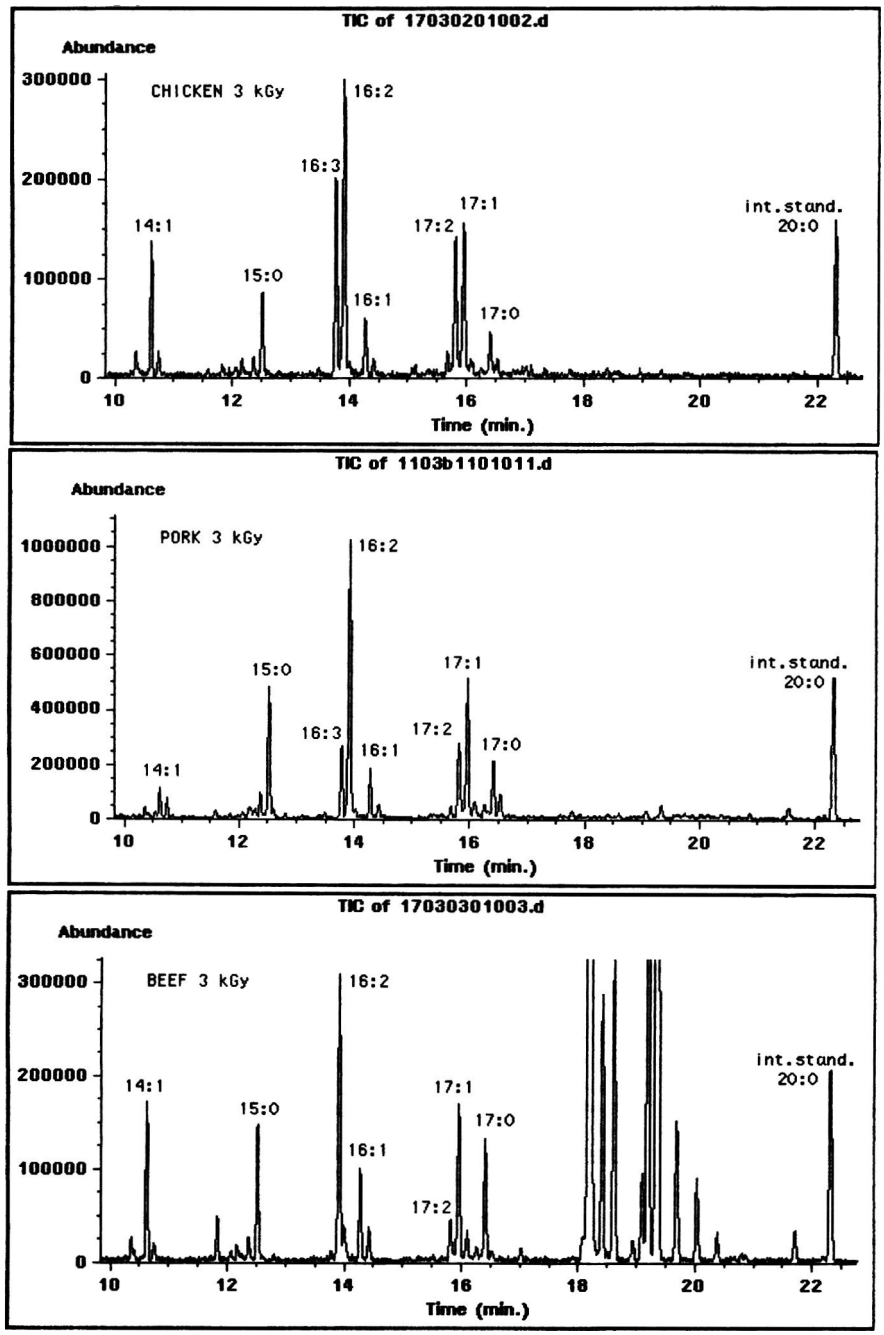


Figure 1. Radiation-induced volatile hydrocarbons from the fat fraction of irradiated chicken carcass, pork, and beef. Hydrocarbons were separated on an HP Ultra 2 capillary column (5% diphenyl–95% dimethyl polysiloxane, 12 m × 0.20 mm id, 0.33 μm film thickness) and registered as total ion chromatograms in a mass spectrometer.

treatment of fatty foodstuffs by ionizing radiation induces a series of changes in the fat fraction from primary and secondary reactions. These changes cannot be classified as radiation-specific because they may also be produced by oxidation processes (27). However, preferential cleavage of certain chemical bonds occurs when triglycerides are treated with ionizing radiation. Among others, 2 types of volatile HCs can be detected in fairly large quantities: one has 1 carbon atom less than the original fatty acid (C_{n-1}), and another has 2 carbon atoms less and an additional double bond in the C-1 position ($C_{n-2:1}$). A cleavage scheme was proposed, based on the identification of the predominant radiolytic products, which makes it possible to predict the main products if the fatty acid composition of the irradiated product is known (30).

The detailed description of the present method drew on proposals (Nawar, unpublished data), experience of the participants (14), and the results of optimization studies for routine application of this method performed by the organizing laboratory (20, 25). For analysis, the fat was extracted to isolate the nonpolar fraction by Florisil column chromatography. This

fraction was separated by GC to quantify 1-tetradecene (14:1), pentadecane (15:0), 1,7-hexadecadiene (16:2), and 8-heptadecene (17:1), which are referred to as markers (Figure 1).

This paper presents a summary of the interlaboratory study. A detailed report including all single data was also published (31).

Experimental

Participants

Fifteen colleagues from food control laboratories, one colleague from a research institute, and the organizing laboratory took part in this study. Each of the participating laboratories was given a letter code.

Test Materials

(**a**) *Chicken carcass homogenates.*—Whole chicken carcasses were irradiated with ca 2 and 6 kGy. After irradiation, the meat (including the skin) was homogenized with a mixer. Each laboratory received 3 aliquots of the 2 kGy homogenate and 3 aliquots of the 6 kGy homogenate. The markers in each aliquot were to be quantitated by parallel analysis. During homogenization, contamination of the homogenates occurred that interfered considerably with the GC detection of 14:1. Therefore, no results are given for 14:1 yields.

(**b**) *Coded specimens.*—The specimens of chicken carcass, pork, and beef were acquired from 2 different producers (referred to as X and Y). The specimens were delivered frozen (in air) in open plastic bags and were stored in air at −20°C. Specimens were only exposed to other temperatures during irradiation and distribution. However, they remained in a frozen state.

From each of the 6 products, some specimens were tested at random by the organizing laboratory to detect possible radiation treatment. In no instance were radiation-induced markers detected in the respective test portions.

Fatty Acid Composition of the Products

Fatty acid analysis was carried out on test portions of 4 different specimens per product. Significant differences in the contents of the main fatty acids could not be revealed between test portions of producer X and of producer Y for the 3 different types of meat. Therefore, the mean contents of palmitic, stearic, oleic, and linoleic acids in X and Y specimens of each type of meat are given (Table 1).

Table 1. Relative concentrations of main fatty acids in the total fat of products[a]

Type of meat	Palmitic acid, FA 16:0, %	Stearic acid, FA 18:0, %	Oleic acid, FA 18:1, %	Linoleic acid, FA 18:2, %
Chicken	20.8 ± 1.2	6.0 ± 0.7	32.0 ± 1.8	25.4 ± 3.2
Pork	25.4 ± 1.3	10.9 ± 1.5	34.7 ± 2.0	10.4 ± 1.3
Beef	23.5 ± 2.0	9.5 ± 2.0	43.0 ± 4.6	1.5 ± 0.9

[a] Fatty acid (FA) analysis was performed by L. Matter, CLUA Duisburg, Germany, by using the method L06.0012 of the collection of Official Methods according to article 35 of the German Foods Act. Values are mean ± standard deviation.

Irradiation of Coded Specimens

Two months after purchase, specimens were packed between 2 layers of ice in polystyrene–cardboard boxes and irradiated with ^{60}Co γ-rays at 3 different dose levels. Each box contained only one type of meat from one producer. The layers of meat measured ca 15 cm. Dosimeters (Gammachrome YR for level 1; Amber Perspex 3042 D for levels 2 and 3 [AEA Technology, Harwell, UK]) were applied at both ends and in the middle of the meat layer. Irradiation temperature was between −15 and −10°C. After the evaluation of the dosimeters, the average doses of each product were determined (31). The following mean dose levels were achieved: level 1, 0.6–0.8 kGy (standard deviations, 12–28%); level 2, 2.5–3.0 kGy (standard deviations, 5–12%); and level 3, 6.7–7.5 kGy (standard deviations, 3–8%). The dose rate of dose level 1 was 0.167 Gy/s, and for the second and third dose levels, it was 1.95 Gy/s. The dose rate error was ca ±10%.

Coding of Specimens

Each laboratory received 54 specimens. The code consisted of a letter identifying the laboratory and a serial number from 1 to 54. Specimens 1–27 were to be examined ca 3 months after irradiation, and specimens 28–54 were to be examined ca 6 months after irradiation. Specimens of chicken carcass (1–9 and 28–36), pork (10–18 and 37–45), and beef (19–27 and 46–54) were to be examined during each study period. Of the 9 specimens per type of meat and study period (9-specimen sets), 5 were provided by producer X and 4 by producer Y.

Dose Distribution

One specimen of each of the 9-specimen sets (No. 2, 11, 20, 31, 40, and 49) was not irradiated. The remaining 8 specimens were either nonirradiated or irradiated within 1 of the 3 dose levels. The distribution was at random.

Times of Analysis of the Coded Specimens

Fat extractions and GC analyses were done by the individual laboratories between 1 and 4 months (specimens 1–27) and between 3 and 8 months after irradiation (specimens 28–54). Mean analysis times were in the third and sixth month.

Fat Extraction

The participants were free to choose between 2 methods of fat extraction. For both methods, test portions of the specimens had to be homogenized with an electric mixer. The extracted fat had to be preserved at 4°C in sealed-glass vials under nitrogen gas until the isolation of the nonpolar fraction. We recommended the use of all the fatty parts of the specimens (the skin in the case of chicken carcass) for extraction. Method A was recommended for chicken carcass and pork and method B for the less-fatty beef. For each analysis, 0.5–1 g fat had to be obtained.

Procedure A.—Heat the test portion to 50°C in a glass centrifuge tube for about 30 min with occasional stirring until melting of the fatty phase has become apparent. Addition of small quantities of water to the homogenate may ease separa-

tion. Centrifuge 10 min at 900 × *g*. Remove the oil phase with a Pasteur pipet. Repeat extraction, if necessary.

Procedure B.—Mix equal parts (w/v) of the homogenized test portion and pentane–isopropanol (3 + 2, v/v) in a stirring blender. Centrifuge 10 min at 900 × *g*. Take the upper oil phase. The residue can be re-extracted with one third of the amount of solvent. Concentrate the unified oil phases by vacuum rotary evaporation at 40–50°C as far as possible. Add 20 mL pentane. Dry the extract over anhydrous sodium sulfate (AR) (heated to 650°C prior to use) for at least 1 h with occasional shaking. Remove the sodium sulfate by filtration. Evaporate the solvent as far as possible. Hexane can be used instead of pentane.

Separation of Hydrocarbons by Florisil Column Chromatography

HCs were to be extracted from the fat fraction by Florisil column chromatography. The Florisil could be used either in activated (by heating to 550°C) or partly deactivated form. Deactivation was to be performed in accordance with the following S 9 method for the analysis of pesticides as used by the German Research Association (32):

Heat Florisil (60–100 mesh) overnight to 550°C. Keep it sealed after cooling (heating removes volatile contaminants). Before use, heat Florisil for at least 5 h to 130°C. After cooling in a desiccator, add 3% water (w/w). Shake for at least 20 min. Keep this mixture sealed for 10–12 h. Use Florisil deactivated in this way only in the course of the next 3 days. Otherwise, heat Florisil again to 130°C and repeat deactivation.

Approximately 20 g of Florisil and a column height of 10 cm (20 mm id) were recommended per test portion. Anhydrous sodium sulfate AR (heated for decontamination to 650°C) could be added on top of Florisil in a 1 cm layer.

For extraction of the nonpolar fraction, mix 1 g liquid fat with 1 mL internal standard (4 μg/mL *n*-eicosane) in pentane or hexane and put it on the column. Elute the HC with 60 mL eluent at a flow rate of 3 mL/min. Remove pentane to a volume of ca 3 mL by rotary evaporation without vacuum at a temperature of 45°C. Use vacuum in case of hexane. About 0.5 mL isooctane may be added to exclude any accidental evaporation to dryness. Concentrate the test portion to ca 1 mL by means of a nitrogen gas flow.

Laboratory W did not follow the given description and used a liquid chromatographic apparatus directly linked to a GC apparatus instead of Florisil chromatography (16).

Gas Chromatography and Detection

The participants could choose their own GC conditions (31). The use of a mass spectrometric (MS) system was recommended for detection because 2 out of 4 markers were not available as standard substances. Seven of the participating laboratories used a flame ionization detection (FID) system instead.

Calculation of Quantities of Hydrocarbons and Determination of Correction Factors for Recovery

The participants had to determine the recovery rates for 1-tetradecene, pentadecane, and 1-heptadecene to correct the results for the radiation-induced HCs 1-tetradecene, pentadecane, and 8-heptadecene in the test portions. 1-Heptadecene and 8-heptadecene were assumed to behave in a virtually identical manner. The results for 16:2 were not corrected because no standard was available.

To determine the recovery rates, mix 1 g fat from nonirradiated chicken carcass with 1 mL of a standard solution containing eicosane (ca 4 μg/mL) as internal standard along with the 3 HCs in similar concentrations. Process the fat under normal conditions. Perform 10 parallel analyses and calculate the correction factors.

Yields for 14:1, 15:0, and 17:1 were corrected for recovery.

Particularities

No results were reported for 10 specimens from the first study period and for 33 specimens from the second. Thirteen specimens were confused during analysis or distribution and were withdrawn from data analysis. Because of detachment of labels during distribution, 3 specimens of one laboratory were considered in the identification part only (all 3 specimens were irradiated but with different doses).

Because of contaminations of solvent or isooctane, one laboratory did not report on the concentrations of 14:1 and 15:0, and another could not determine 15:0 yields in the first study period specimens. For the second study period specimens, no 14:1 values were provided by one laboratory because 14:1 was added to the internal standard solution by mistake.

Two laboratories did not determine 16:2 yields because hexadecadiene standard was not available. However, in those cases in which no peaks were detected in the retention range of 16:2, the result 0 μg/g fat was given by one of these laboratories.

Identification of Coded Specimens as Irradiated

By threshold values (specimens of the first and second study period).—Consider a specimen as irradiated if the calculated quantities for 3 of the 4 markers exceed the following thresholds: for 14:0, 0.15 μg/g fat; for 15:0, 0.15 μg/g fat; for 16:2, 0.25 μg/g fat; and for 17:1, 0.20 μg/g fat.

Without threshold values (specimens of the second study period).—In addition to the identification procedure described above, perform a *second* identification of specimens 28–54 without giving regard to the threshold values. Take the following considerations into account for a decision (2 laboratories did not undertake this additional evaluation): Because 15:0 is a frequent contaminant (solvent, filter paper, etc.), this marker might be found in nonirradiated specimens; the C_{n-1} and $C_{n-2:1}$ markers, which are formed from palmitic and oleic acid (Table 2), must be clearly detectable in test portions of irradiated specimens; irradiation produces the largest quantities of 16:2 and more 17:1 than 14:1 and 15:0 in the 3 types of meat; and unusually small amounts of 14:1 will be found in irradiated-pork specimens.

Data Analysis

For the qualitative analysis (identification), 864 specimens could be considered; for the quantitative data analysis,

Table 2. Radiolytic C_{n-1} and $C_{n-2:1}$ hydrocarbons for main fatty acids

Marker	Palmitic acid, FA 16:0	Stearic acid, FA 18:0	Oleic acid, FA 18:1	Linoleic acid, FA 18:2
C_{n-1}	15:0	17:0	8–17:1	6,9–17:2
$C_{n-2:1}$	1–14:1	1–16:1	1,7–16:2	1,7,10–16:3

861 specimens could be considered. Laboratory O measured unusually high marker yields. Therefore, these values were excluded from the summarizing data analysis, and this exclusion is indicated in captions or footnotes of figures and tables.

Results

Determination of Markers in Chicken Carcass Homogenates

The marker yields in chicken carcass homogenates irradiated with 2 different doses are given in Table 3. The mean values of all laboratories for the markers 15:0, 16:2, and 17:1 showed relative standard deviations between 20 and 38%.

Determination of Markers in the Coded Specimens

Nonirradiated specimens.—In none of the test portions of nonirradiated chicken carcass specimens was 16:2 detected, and only in 3 specimens could 17:1 be detected (laboratory X; first study period). Both 16:2 and 17:1 were found in test portions of 6 nonirradiated beef specimens, and either 16:2 or 17:1 were found in additional 3 and 2 beef specimens, respectively. However, in nearly all cases, the concentrations were extremely low. Marker 16:2 was detected in test portions of 3 nonirradiated pork specimens, 17:1 was detected in 16, and both markers were detected in 6 cases. Marker 15:0 was found frequently in test portions of nonirradiated specimens, and in particular in pork specimens.

Comparison of the marker yields in test portions of X and Y specimens.—No significant differences in marker yields could be detected between test portions of the X and Y specimens for all 3 types of meat. Also, regression slopes coincided perfectly for the 3 and 6 month values (Figure 2). For this reason, the data of the X and Y specimens were combined in the following data analyses.

Mean marker yields 3 and 6 months after irradiation.—Mean values and standard deviations of the marker yields in test portions of chicken carcass, pork, and beef specimens are listed in Table 4 (for the first and second study period). Significant differences could not be shown between yields of first and second period specimens for the 3 types of meat. On the contrary, data coincided very well.

Correction of Data for Recovery

Although yields of 14:1, 15:0, and 17:1 were corrected for recovery, large interlaboratory variations in marker yields were obtained, in particular for pork test portions. Interlaboratory variations in marker yields were not diminished by corrections for recovery (31).

Table 3. Hydrocarbon quantities in 3 aliquots of chicken carcass homogenates irradiated with doses of about 2 and 6 kGy[a]

Laboratory	15:0	16:2	17:1
	2 kGy dose		
A	1.35 ± 19	1.43 ± 11	1.49 ± 16
B	1.33 ± 8	1.99 ± 6	2.07 ± 7
F	1.09 ± 5	1.62 ± 6	1.28 ± 4
H	1.29 ± 9	1.74 ± 6	1.79 ± 7
I	0.77 ± 13	1.27 ± 9	1.38 ± 4
J	2.01 ± 15	—	1.20 ± 37
K	1.16 ± 14	—	1.84 ± 9
L	1.06 ± 7	2.01 ± 6	1.59 ± 8
O	0.81 ± 11	2.98 ± 9	1.62 ± 6
P[b]	0.84 ± 16	1.68 ± 7	1.55 ± 8
Q	1.08 ± 5	2.67 ± 6	1.47 ± 12
S	0.97 ± 9	1.56 ± 7	1.18 ± 9
T[b]	0.59 ± 11	0.80 ± 24	1.05 ± 29
U	1.40 ± 13	2.42 ± 10	1.65 ± 7
V	0.33 ± 55	1.93 ± 12	2.08 ± 9
W	1.18 ± 4	2.10 ± 4	1.79 ± 9
X[b]	1.95 ± 4	1.93 ± 3	1.23 ± 2
Mean	1.13 ± 38	1.88 ± 29	1.54 ± 20
	6 kGy dose		
A	2.64 ± 10	2.80 ± 5	2.43 ± 11
B	2.97 ± 6	5.46 ± 2	6.24 ± 5
F	2.34 ± 34	3.83 ± 14	3.46 ± 9
H	3.41 ± 10	5.95 ± 10	6.19 ± 8
I	1.74 ± 9	4.34 ± 12	4.85 ± 7
J	3.52 ± 29	—	2.91 ± 23
K	2.11 ± 25	—	4.30 ± 11
L	2.38 ± 4	5.55 ± 1	4.27 ± 1
O	1.69 ± 6	7.37 ± 5	4.65 ± 6
P[b]	1.69 ± 4	4.33 ± 6	4.14 ± 5
Q[c]	2.62 ± 8	7.25 ± 6	4.39 ± 5
T[b]	1.41 ± 7	3.00 ± 9	2.88 ± 10
U	3.04 ± 4	6.36 ± 3	4.54 ± 2
V	0.87 ± 27	4.33 ± 11	4.46 ± 10
W	2.57 ± 6	5.50 ± 5	4.47 ± 6
X[b]	4.53 ± 9	5.71 ± 9	3.84 ± 4
Mean	2.47 ± 37	5.13 ± 28	4.25 ± 25

[a] Quantities were determined in 2 parallel analyses as mean (μg/g fat) ± standard deviation (%) σ_n of 6 values.
[b] Mean and σ_{n-1} of 3 determinations.
[c] Mean and σ_{n-1} of 4 determinations.

Dose Response

For assessment of the dose dependencies of marker yields, *R* values of the linear model:

$$Y = c + \alpha \times D$$

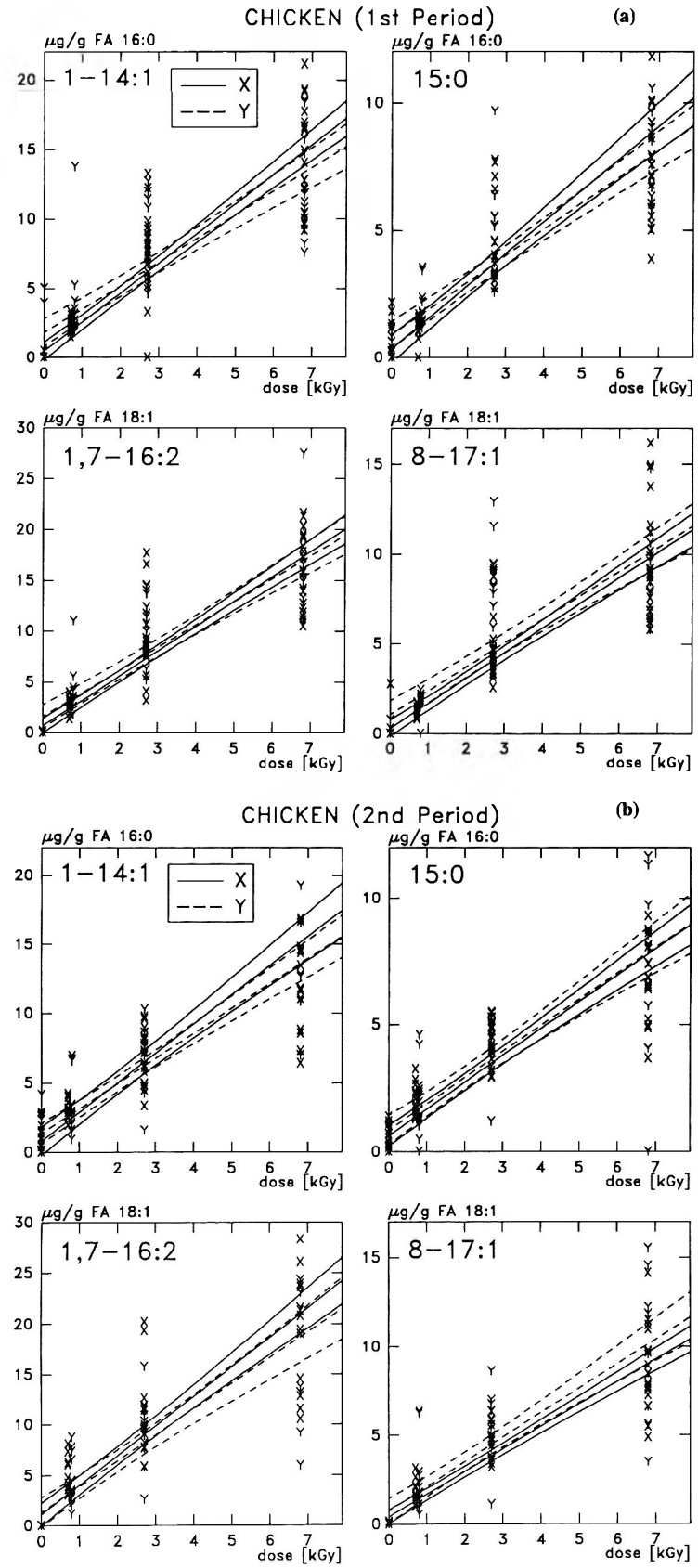


Figure 2. Marker concentrations per precursor fatty acid in fat of first and second period chicken carcass, pork, and beef specimens. 14:1 and 15:0 yields are per gram of palmitic acid; 16:2 and 17:1 yields are per gram of oleic acid. Linear regressions and 95% confidence intervals are given for X and Y specimens, respectively. Data of first and second period specimens were derived from 16 and 15 laboratories, respectively (laboratory O was excluded).

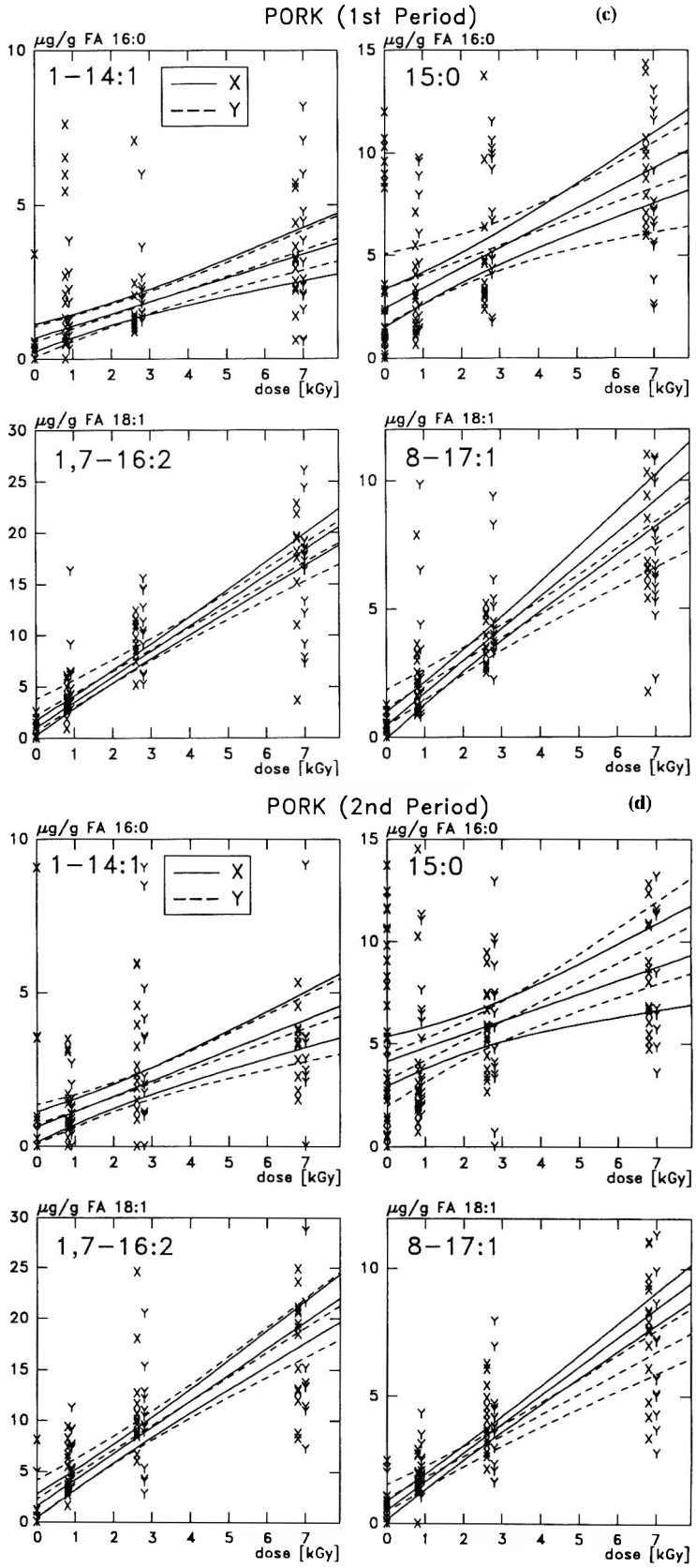


Figure 2. *(continued)*

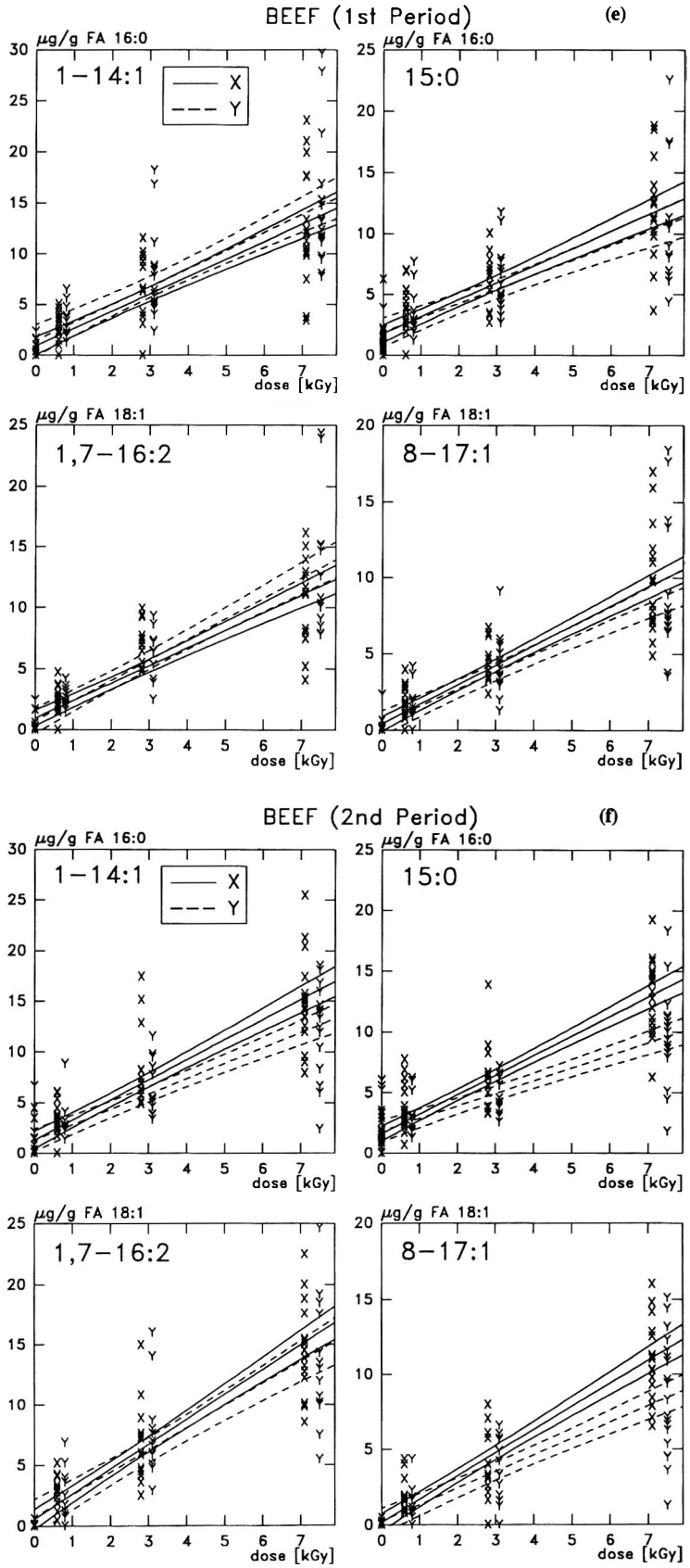


Figure 2. *(continued)*

Table 4. Mean and standard deviations (μg/g) of marker yields obtained on test portions of the first and second period specimens[a]

Mean dose, kGy	Study period	14:1	15:0	16:2	17:1
		Chicken carcass			
0	1st	0.04 ± 0.19	0.02 ± 0.06	0.001 ± 0.007	0.02 ± 0.13
	2nd	0.11 ± 0.21	0.05 ± 0.08	0 ± 0	0.002 ± 0.008
0.75	1st	0.63 ± 0.48	0.30 ± 0.13	1.16 ± 0.58	0.47 ± 0.17
	2nd	0.63 ± 0.29	0.40 ± 0.19	1.32 ± 0.66	0.64 ± 0.39
2.7	1st	1.61 ± 0.60	0.90 ± 0.32	3.24 ± 1.19	1.87 ± 0.86
	2nd	1.41 ± 0.42	0.88 ± 0.20	3.22 ± 1.23	1.57 ± 0.47
6.8	1st	2.80 ± 0.80	1.66 ± 0.62	5.35 ± 1.31	2.94 ± 0.87
	2nd	3.01 ± 1.31	1.58 ± 0.63	6.15 ± 2.31	2.91 ± 0.97
		Pork			
0	1st	0.03 ± 0.13	0.53 ± 0.90	0.07 ± 0.19	0.05 ± 0.11
	2nd	0.02 ± 0.06	1.00 ± 1.12	0.01 ± 0.06	0.07 ± 0.15
0.85	1st	0.46 ± 0.52	0.84 ± 0.66	1.64 ± 0.97	0.81 ± 0.72
	2nd	0.26 ± 0.22	0.98 ± 0.80	1.85 ± 0.79	0.64 ± 0.26
2.7	1st	0.48 ± 0.35	1.42 ± 0.83	3.42 ± 0.99	1.36 ± 0.44
	2nd	0.68 ± 0.60	1.66 ± 1.28	3.60 ± 1.69	1.34 ± 0.51
6.9	1st	0.79 ± 0.46	1.99 ± 0.73	5.90 ± 1.85	2.47 ± 0.80
	2nd	0.91 ± 0.59	2.28 ± 0.94	6.34 ± 2.73	2.53 ± 0.92
		Beef			
0	1st	0.06 ± 0.13	0.26 ± 0.29	0.05 ± 0.20	0.03 ± 0.16
	2nd	0.11 ± 0.31	0.31 ± 0.35	0.01 ± 0.04	0.01 ± 0.03
0.7	1st	0.67 ± 0.34	0.78 ± 0.44	1.04 ± 0.47	0.68 ± 0.42
	2nd	0.77 ± 0.41	0.80 ± 0.43	1.17 ± 0.71	0.70 ± 0.45
2.9	1st	1.62 ± 0.80	1.35 ± 0.52	3.00 ± 0.91	1.72 ± 0.53
	2nd	1.77 ± 0.81	1.27 ± 0.52	3.04 ± 1.45	1.61 ± 0.85
7.3	1st	3.14 ± 1.40	2.54 ± 1.04	5.31 ± 1.89	3.82 ± 1.54
	2nd	3.21 ± 1.04	2.64 ± 0.86	6.28 ± 2.33	4.20 ± 1.73

[a] Mean and standard deviations were calculated from all single data that were reported by the laboratories (except Laboratory O).

where D = dose and c and α = coefficients, were calculated for each marker in first and second study period specimens of each laboratory and for each type of meat. The mean R values for all 4 markers per laboratory reveal that in most cases similar dose dependencies were achieved on first and second study period specimens within one laboratory. For chicken carcass and beef specimens, very high R values were achieved within nearly all laboratories. For pork specimens, some laboratories could hardly establish a dose dependency of marker yields; nevertheless, high R values were obtained by one laboratory in both study periods and by 3 laboratories in either the first or the second period (31). Mean R values of all laboratories are given for first and second study period specimens of all 3 types of meat in Table 5.

Comparison of the Variations of Marker Concentrations

Relative standard deviations of the mean values obtained for chicken carcass homogenates and irradiated, coded chicken carcass specimens of all laboratories were compared (Table 6). For 15:0, 16:2, and 17:1 yields, the standard deviations of homogenates and coded specimens were very similar.

Table 5. Mean of R values for linear fitting of marker yields to dose for first and second study period specimens[a]

	R values, mean $\pm\sigma_n$	
Meat	First period	Second period
Chicken carcass	0.95 ± 0.05	0.93 ± 0.10
Pork	0.79 ± 0.17	0.77 ± 0.13
Beef	0.93 ± 0.05	0.87 ± 0.18

[a] Yields of each marker and laboratory were linear-fitted dependent of dose to calculate respective R values. Mean R values of the 4 markers were calculated for each laboratory and study period. Mean R values of all laboratories are given.

Table 6. Comparison of relative standard deviations obtained for mean yields of fatty acids 15:0, 16:2, and 17:1 in test portions of irradiated, coded, chicken carcass specimens of the first period and of chicken carcass homogenates[a]

Dose, kGy	Coded/ homogenized	Relative standard deviations, % 15:0	16:2	17:1
0.75	Coded	46	52	35
2.7	Coded	36	36	46
~2	Homogenized	38	26	20
6.8	Coded	37	23	30
~ 6	Homogenized	36	26	25

[a] Relative standard deviations were calculated from mean and standard deviations of all respective data reported by the laboratories (except Laboratory O).

Also, on the individual laboratory level, the variations for homogenized chicken carcass were not shown to be lower than those for coded ones (31).

Comparison of the Marker Concentrations per Fatty Acid Concentration

For the yield of a certain marker per concentration of the precursor fatty acid, nearly the same dose dependencies could be established for the C_{n-1} HCs (15:0 and 17:1) in each type of meat (Figure 3). The high 15:0 yields in test portions of nonirradiated specimens are discussed in *Markers in Nonirradiated Specimens—Contamination*. The 16:2 yields ($C_{n-2:1}$) per oleic acid concentrations were the same in both chicken carcass and pork specimens, but they were somewhat lower in beef specimens. In contrast, the 14:1 concentrations ($C_{n-2:1}$) in chicken carcass and beef specimens were very similar, and the dose dependency of 14:1 yields in pork specimens was much less pronounced.

Ratios of C_{n-1} and $C_{n-2:1}$ Hydrocarbon Yields and Comparison to the Fatty Acid Ratios

Ratios of marker yields were calculated for specimens irradiated within the dose levels 2 and 3 and contrasted with expected values (Table 7).

The ratios of 14:1 to 15:0 yields and of 16:2 to 17:1 yields ($C_{n-2:1}/C_{n-1}$ from palmitic and oleic acids, respectively) were nearly identical in chicken carcass and beef specimens. However, there were extreme differences in both ratios for pork specimens.

The ratios of 14:1 to 16:2 ($C_{n-2:1}$ from palmitic and oleic acids, respectively) as well as of 15:0 to 17:1 (C_{n-1} from palmitic and oleic acids, respectively) were very similar to the fatty acid ratio of 16:0 to 18:1 in the chicken carcass specimens

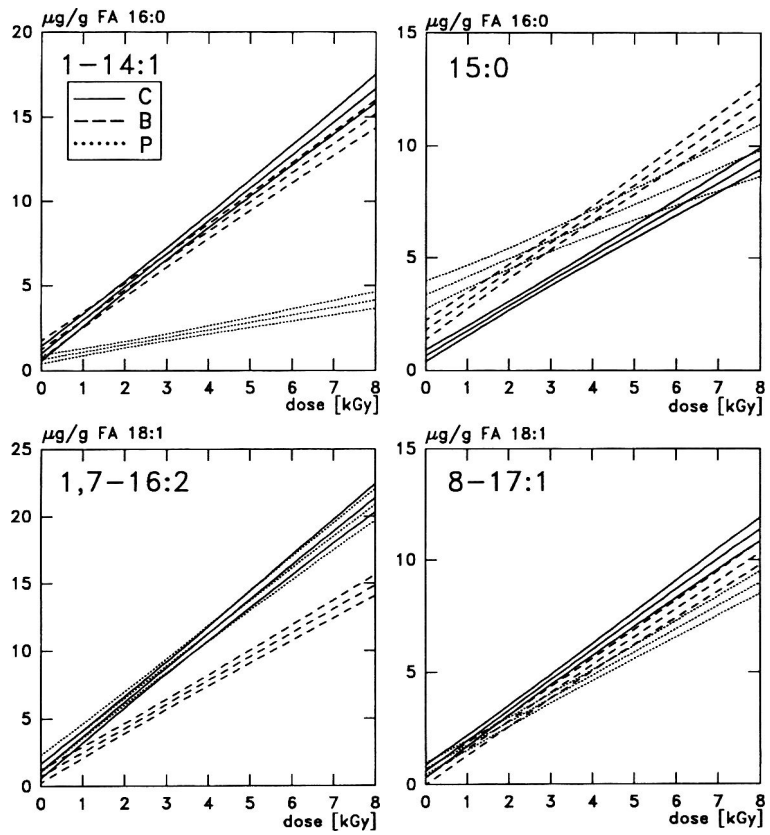


Figure 3. Dose dependencies of marker yields in test portions of chicken carcass (C), pork (P), and beef (B) specimens per precursor fatty acid concentration of palmitic acid (14:1 and 15:0) and oleic acid (16:2 and 17:1). Linear regressions and 95% confidence intervals were calculated for all specimens of both study periods and depicted per type of meat and marker (without Laboratory O values).

Table 7. Comparison of yield and expected ratios for the C_{n-1} and $C_{n-2:1}$ hydrocarbons in test portions of first and second period chicken carcass, pork, and beef specimens irradiated within dose levels 2 and 3[a]

Ratio	Experimental	Expected
	Chicken carcass[b]	
FA 16:0 $C_{n-2:1}/C_{n-1}$	1.8 ± 0.6	R16:0 = R18:1
FA 18:1 $C_{n-2:1}/C_{n-1}$	1.9 ± 0.5	R16:0 = R18:1
FA 16:0 $C_{n-2:1}$/FA 18:1 $C_{n-2:1}$	0.5 ± 0.2	0.65
FA 16:0 C_{n-1}/FA 18:1 C_{n-1}	0.6 ± 0.2	0.65
	Pork[c]	
FA 16:0 $C_{n-2:1}/C_{n-1}$	0.4 ± 0.4	R16:0 = R18:1
FA 18:1 $C_{n-2:1}/C_{n-1}$	2.6 ± 0.7	R16:0 = R18:1
FA 16:0 $C_{n-2:1}$/FA 18:1 $C_{n-2:1}$	0.2 ± 0.2	0.73
FA 16:0 C_{n-1}/FA 18:1 C_{n-1}	1.1 ± 0.6	0.73
	Beef[d]	
FA 16:0 $C_{n-2:1}/C_{n-1}$	1.3 ± 0.4	R16:0 = R18:1
FA 18:1 $C_{n-2:1}/C_{n-1}$	1.5 ± 0.4	R16:0 = R18:1
FA 16:0 $C_{n-2:1}$/FA 18:1 $C_{n-2:1}$	0.6 ± 0.2	0.55
FA 16:0 C_{n-1}/FA 18:1 C_{n-1}	0.7 ± 0.3	0.55

[a] For each specimen, the respective ratios were calculated (except specimens of Laboratory O). Mean and standard deviations of these ratios are given.
[b] FA ratio 16:0/18:1 = 0.65.
[c] FA ratio 16:0/18:1 = 0.73.
[d] FA ratio 16:0/18:1 = 0.55.

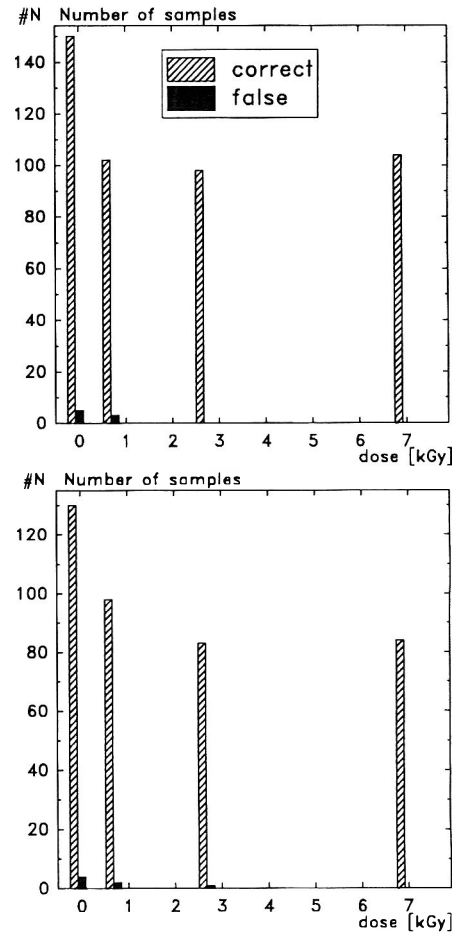


Figure 4. Number of specimens in the first (top; 17 laboratories) and second study periods (bottom; 16 laboratories) that were correctly or falsely identified by means of given threshold values in dependency on the dose. Specimens of the 3 types of meat were combined and depicted by using the mean doses.

(0.65). The HC ratios for beef (fatty acid ratio, 0.55) were also in the expected range. For pork specimens, a ratio of 0.73 was calculated for the 2 fatty acids. The range achieved for 15:0 to 17:1 (1.1 ± 0.6) included the expected value. However, a ratio of 0.2 ± 0.2 was obtained for 14:1 to 16:2.

Identification Rates

Identification by threshold values.—Results that could be considered in the qualitative evaluation were available for 462 specimens from the first part of the interlaboratory test and for 402 specimens from the second part. Five of the 462 specimens in the first part were identified as false positive and 3 as false negative. In the second study period, 4 specimens were wrongly judged as irradiated and 3 specimens were wrongly judged as nonirradiated (Figure 4). This means that in both parts, 98.3% of the specimens had been identified correctly. It is striking that, in particular, pork specimens were incorrectly identified as irradiated (Table 8).

Identification of the second study period specimens without threshold values.—In this additional evaluation of the second study period specimens, which was based on the conclusive detection of markers, 96.6% of the specimens were correctly identified. One specimen was identified as false negative and 11 specimens as false positive. However, Table 9 shows that none of the false positive specimens fulfilled the criteria described in the method section for a clear identification of a specimen as irradiated.

Discussion

The aim of the interlaboratory test—to demonstrate the suitability of the method to detect irradiated chicken carcass, pork, and beef—was clearly achieved. The method was, therefore, accepted as a method for qualitative detection by the LMBG (German Foods Act) Commission for inclusion in the *Official Collection of Methods* according to Article 35 of the German Foods Act. Although the yield of radiation-induced HC is dose-dependent, the method cannot be used for dose estimations in routine control applications because the marker yields depend on irradiation conditions that are normally not known (25, 30).

Table 8. Specimens identified as false negative or false positive using threshold values

Code	Meat	Dose, kGy	Fatty acid, μg/g fat			
			14:1	15:0	16:2	17:1
False negative						
a23	Beef X	0.6	0	0	0	0
k10	Pork X	0.8	0.12	0.30	—	0.30
q33	Chicken Y	0.8	0.57	0.10	0.36	0.17
q51	Beef Y	0.8	0.63	0.30	0	0
t22	Beef X	0.6	0	0	0	1.23
x44	Pork Y	2.8	0	0	0.96	0.55
False positive						
a43	Pork Y	0	0.90	0.90	1.70	0.69
h19	Beef X	0	0	0.23	0.71	0.26
h24	Beef Y	0	0.55	0.90	1.03	0.99
j41	Pork X	0	0.24	3.16	—	0.25
q38	Pork X	0	0.89	0.64	2.80	0.85
q40	Pork X	0	2.30	2.10	0.40	0.31
w10	Pork X	0	0.07	0.31	0.39	0.34
w13	Pork X	0	0.09	0.60	0.64	0.43
w17	Pork Y	0	0.06	0.24	0.32	0.25
Thresholds			0.15	0.15	0.25	0.20

AOAC INTERNATIONAL has been asked to evaluate the method on the basis of the presented results.

To allow a proper rating of the results, it should be stressed that prior to the study, most of the participants were not familiar with the method used. The majority of the laboratories were only given an extremely brief introductory phase (each laboratory received 3 irradiated chicken carcass and pork specimens) before they had to analyze the coded specimens and homogenates.

Table 9. Specimens identified as false negative or false positive not using threshold values

Code	Meat	Dose, kGy	Fatty acid, μg/g fat			
			14:1	15:0	16:2	17:1
False negative						
q51	Beef Y	0.8	0.63	0.30	0	0
False positive						
a43	Pork Y	0	0.90	0.90	1.70	0.69
j40	Pork X	0	0.06	1.99	—	0.21
j41	Pork X	0	0.24	3.16	—	0.25
k40	Pork X	0	0	1.35	—	0.36
q38	Pork X	0	0.89	0.64	2.80	0.85
q40	Pork X	0	2.30	2.10	0.40	0.31
s40	Pork X	0	≤0.14	2.95	≤0.13	0.30
t37	Pork X	0	0	0	0	0
t38	Pork X	0	trace	trace	trace	trace
t52	Beef Y	0	0	0	0	0
t54	Beef Y	0	0	0	0	0

As can be seen from Figure 1 and Table 2, a number of HCs are produced by irradiation. In the interlaboratory test, only tetradecene, pentadecane, hexadecadiene, and heptadecene had to be detected. These HCs were chosen because their parent substances, palmitic and oleic acids, are present in sufficient quantities in the triglycerides of all 3 types of meat. We decided to dispense with the detection of other radiation-induced HCs for the interlaboratory test because a limit had to be placed on the amount of work involved for the participants. Another important reason was that many unsaturated HCs were not available as standard substances. Therefore, identification was difficult, especially if no mass spectrometer was available. Although this also applied to 1,7-hexadecadiene and 8-heptadecene during the interlaboratory test, these 2 substances were nevertheless used as markers for irradiation because they occur in the largest quantities after irradiation (13).

Determination of Markers in Coded Specimens

A summary of the marker concentrations in coded specimens revealed considerable variation among the laboratories. This variation was to be expected for raw data. Therefore, the participants were requested to establish correction factors for the recovery of the individual markers. However, the variation was not reduced by means of correction. Also, the 2 different fat extraction procedures and the use of activated (2 laboratories) and deactivated Florisil could not be correlated with higher- or lower-marker yields. A possible explanation could be the addition of the standards to nonirradiated fat to determine recovery rates, whereas in the case of irradiation, the markers are produced directly in the fat itself. Different molecular arrangements that might influence elution behavior seem to be possible. However, recovery rates are probably too variable among independent analyses, which means that for every analysis, recovery rates have to be determined. Even the establishment of meat-specific recovery rates might be necessary for a proper quantitation of markers. Neither of these options was done in this study.

Despite these problems, the mean dose dependencies of the markers calculated per precursor fatty acid agree with the concentrations that were found by Nawar et al. (13) in the 3 types of meat examined. Only the 14:1 mean concentrations in pork specimens were slightly lower, whereas the 16:2 mean concentrations in the chicken carcass specimens were somewhat higher.

Markers in Nonirradiated Specimens—Contamination

One or several markers were found in some nonirradiated specimens, particularly in pork specimens. Although the markers cannot be regarded as radiation-specific, such results can be attributed to contamination according to our experience. Contamination was indicated because no markers could be deter-

mined in most of the nonirradiated specimens. The respective specimen pools are regarded as homogenous because no quantitative differences could be shown between either the X and Y specimens or the 3 and 6 month specimens of a particular type of meat. Saturated HC and unsaturated HC with the double bond in the C-1 position are substances that occur frequently. They can even be found occasionally in 'high-purity' solvents. The detection of the homologous series of saturated HCs together with radiation-induced markers can be regarded as a conclusive sign of contamination. Florisil, sodium sulfate, filter paper, extraction thimbles, and dirty glassware are frequent sources of contamination. Any contact between the extracted fat and plastics must be avoided as well.

Comparison of the 3 and 6 Month Values

It is a particularly interesting feature of the results that no differences could be established among the marker concentrations in the first and second study period specimens. This shows that the possible production of markers by autoxidation is only of minor significance in quantitative terms and that it does not affect identifiability. The irradiation of the specimens 2 months after purchase meant an extension of the study period to approximately 8 months, during which time no markers were detected in the overwhelming majority of nonirradiated specimens nor could production of markers by autoxidation be established. Moreover, the conformity among the results of the 3 and 6 month measurements shows the stability of markers. This means that an irradiated product can be identified even after prolonged storage.

Determination of HCs in Irradiated Chicken Carcass Homogenates

Homogenates were included in this study to reveal nonoptimized GC conditions and processing problems. Whereas generally high standard deviations should be attributable to both problems, very different relative standard deviations for the 2 homogenates should result from processing problems.

Although the standard deviations of the mean marker concentrations in the homogenates varied greatly, which indicated described problems in some laboratories, *R* values of homogenates and irradiated, coded chicken carcass specimens could not be correlated. Most laboratories that observed a high variation in homogenates (low R values) recorded higher *R* values in coded specimens and vice versa.

A comparison between the *R* values of homogenates and irradiated, coded specimens also showed that, contrary to expectations, the *R* values of homogenates were not noticeably higher. Therefore, the conclusion must be drawn that the errors of various analyses were greater than the variation of the marker concentrations in irradiated, coded chicken carcass specimens of a respective dose level.

Marker Yield per Precursor Fatty Acid and Ratios of Radiation-Induced HCs

According to the cleavage scheme of triglycerides, it was to be expected that the yield of a certain marker per concentration of the precursor fatty acid was the same for all 3 types of meat (30). This was nearly achieved for C_{n-1} HCs. The lower slope of the 15:0 regression line for pork specimens was attributed to contaminations. In comparison to chicken carcass, somewhat higher 15:0 mean yields were achieved for beef. However, for $C_{n-2:1}$ HCs, very low 14:1 yields were obtained in pork specimens. Also, the 16:2 mean yields were somewhat lower for beef compared with chicken carcass or pork specimens. Different efficiencies of ionizing radiation for HC yields might result from different molecular arrangements of fatty acids in the triglycerides of the respective meat. However, this possibility is speculative and needs clarification.

We also assumed that the ratios of radiation-induced $C_{n-2:1}$ and C_{n-1} HCs for both precursor fatty acids would be the same within a single type of meat, i.e., the ratios of yields of 14:1 to 15:0 and 16:2 to 17:1 per type of meat should be the same or at least similar. This proved to be the case for chicken carcass and beef specimens. However, for pork specimens, mean ratios of 0.4 and 2.6 were calculated.

Furthermore, the radiation-induced C_{n-1} (15:0 to 17:1) and $C_{n-2:1}$ HCs (14:1 to 16:2) should be detectable in a ratio that corresponds to the ratio of the fatty acid concentrations (palmitic acid to oleic acid) in this type of meat. Again, this could be shown for chicken carcass and beef specimens, but for pork specimens the $C_{n-2:1}$ ratio was far outside the expected range.

Identification of Specimens

The qualitative part of the interlaboratory test—the identification of the specimens as irradiated or nonirradiated—was the most important aspect. However, the quantitative and qualitative parts cannot be viewed in isolation from one another because the initial fixing of threshold values required a quantitation of the markers. On the basis of given threshold values in both study periods, 98.3% of the specimens were correctly assessed. Even the specimens irradiated with doses of level 1 (approximately 0.6–0.8 kGy) were correctly identified in approximately 97% of the cases. The large number of correctly identified specimens was a result of the requirement that a specimen should only be classified as irradiated if the threshold values of at least 3 of the 4 analyzed markers were exceeded.

False identifications often occurred in laboratories that also reported mixups of specimens during analysis (h24, q33, q38, q40, and q41). Other samples were identified as false positives on the basis of marker yields very close to the threshold values (h19, w10, w13, and w17); for one specimen, 16:2 was not determined (j41).

Despite these high identification rates, the quantitative variations in marker yields made a review of the threshold value identification necessary. By the additional identification in the second study period, which was based on the conclusive detection of all the markers rather than on threshold values, more specimens were identified as false positive and only one irradiated specimen was not detected. However, the data listed in Table 9 show that none of the false positive specimens would have been identified as irradiated if the criteria described in the method section would have been strictly applied. At least, the analysis should have been repeated in cases of suspicion (a43, q38, q40, and s40), which was not done. Thus, the result of this

evaluation appears only at first glance to be worse than the identifications made with the help of threshold values. According to the data and to our experience, false positive identification can be excluded if a specimen is not identified as irradiated until the described conditions have been fulfilled. Furthermore, there is no need to use only the 4 markers that had to be measured in this interlaboratory test. All the anticipated radiation-induced markers produced from the main fatty acids should be determined, at least if a mass spectrometer is available. Of particular importance for a rating is the detection of unsaturated HCs with double bonds in the middle of the chain (see Table 2) because these substances are not known to occur naturally. The proposed method submitted to the German LMBG Commission and the AOAC Commission, therefore, envisages the clear detection of these HCs as a prerequisite for the identification of an irradiated specimen. On the basis of the broad database that was achieved as a result of this interlaboratory test, the ratios of the marker concentrations can be used to confirm an assessment because, in fats of different types of meat, a particular HC pattern is induced (Figure 1). The ratios obtained from the interlaboratory test data match those that can be calculated from the data provided by Nawar et al. (13).

Notes on Methodology

On the basis of the experience gained during the interlaboratory test and the results of other optimization studies performed in parallel (25), the following advice can be given: An optimization of the GC system by means of standards is urgently recommended (temperature program, column, solvent, and injection mode). The organizing laboratory recommends the use of capillary columns with methyl silicon phases (5% diphenyl–95% dimethylpolysiloxane) for HCs separation. The HCs elute separately in groups of the same chain length and in the order of their boiling points (Figure 1).

The use of both activated and deactivated Florisil was permitted for the interlaboratory test. In the meantime, however, the use of activated Florisil is not advisable because polyunsaturated HCs can be retained depending on the level of activation (which, in turn, is dependent on the storage period of Florisil after heating) (20, 25). Moreover, a stable level of activation can be achieved for at least 3 days as a result of partial deactivation by an addition of 3% water. Also, different Florisil batches may have a different retention capacity, which means that the complete elution of the HCs should be tested by fractionating the eluate when the batches are changed. Both hexane and pentane can be used as elution agents. We recommend the addition of isooctane before concentrating the eluate. Sodium sulfate must be heated to remove possible contaminants.

Prospects

The method was tested on 3 types of meat: chicken carcass, beef, and pork. Even though no comprehensive studies were conducted so far that involved other types of fat-containing foods, the method should be able to clearly reveal irradiation in other fat-containing products provided the main fatty acids and, thus, the most important radiation-induced HCs are known. The organizing laboratory conducted promising studies on fish, fruit seeds, and Camembert, for instance.

The reason for low concentrations of 14:1 in pork specimens remains to be revealed. This result has shown that the marker concentration of irradiated products is not determined solely by the fatty acid contents but also by another unknown factor. In contrast to chicken carcass and beef, there is also no straight forward relationship between the ratio of certain alkylcyclobutanones—other radiation-induced substances that are suitable for detection—and the fatty acid content of pork (33). We assume that the reasons are the same. Clarification of this factor is important for the extrapolation of the results to other fat-containing foods.

Conclusions

By means of an interlaboratory test, a comprehensive database was established that shows that irradiated raw chicken carcass, pork, and beef can be clearly identified during the normal storage period. Therefore, the method can be applied in routine control.

Acknowledgments

We are very grateful to the following participants of the interlaboratory study: S.T. Adam and H. Delincée, Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany; U. Bänziger, Kantonales Laboratorium, Solothurn, Switzerland; P. Baumann, Chemisches und Lebensmitteluntersuchungsamt, Dortmund, Germany; Ch. Droz, Kantonales Laboratorium, St. Gallen, Switzerland; S. Estendorfer, Landesuntersuchungsamt für das Gesundheitswesen Südbayern, Oberschleißheim, Germany; A. Känzig and C. Gemperle, Kantonales Laboratorium, Aarau, Switzerland; W. Krölls, Institut für Lebensmittelund Umweltuntersuchungen, Köln, Germany; M. Metschies, Chemische Landesuntersuchungsanstalt, Freiburg, Germany; G. Mildau, W. Ruge, and J. Ammon, Chemische Landesuntersuchungsanstalt, Karlsruhe, Germany; J. Pfordt and H.-U. von Grabowski, Staatliches Chemisches Untersuchungsamt, Oldenburg, Germany; A. Plaga-Lodde and R. Brockmann, Chemisches Untersuchungsamt, Bielefeld, Germany; M. Punkert, Staatliches Chemisches Untersuchungsamt, Lüneburg, Germany; B. Rönnefahrt, Chemisches Untersuchungsamt, Hamm, Germany; H. Stemmer, Gemeinschaftliches Chemisches Untersuchungsinstitut der Städte Wuppertal und Solingen, Wuppertal, Germany; N. Vater, Staatliches Medizinal-Lebensmittelund Veterinäruntersuchungsamt Nordhessen, Kassel, Germany; K. Wilmers, Chemisches Landesuntersuchungsamt Nordrhein Westfalen, Münster, Germany.

We thank Wolfgang Schichtel and Reiner Eidenberger of GAMMASTER MÜNCHEN, Allershausen, who were kind enough to irradiate the specimens. We are grateful as well to the many colleagues in other countries who co-operated with the organizing laboratory. The discussions we had with them considerably influenced the planning of the interlaboratory test. We are particularly grateful to Wassef W. Nawar, Amherst, MA, USA, Hilary M. Stevenson, Belfast, UK, and to Werner

Meier, Zürich, Switzerland. International contacts were made possible by the research program of the European Community Bureau of Reference, Brussels, Belgium, (Contract No. 5348/1/5/340/90/4/BCR-F-10) and by the ADMIT program of the Joint Division of FAO/IAEA, Vienna, Austria.

References

(1) Desrosiers, M.F., McLaughlin, W.L., Sheahen, L.A., Dodd, N.J.F., Lea, J.S., Eveans, J.C., Rowlands, C.C., Raffi, J.J., & Agnel, J.-P.L. (1990) *Int. J. Food Sc. Techn.* **25**, 682–691

(2) Meier W., & Stevenson, M.H. (1993) in *Recent Advances on the Detection of Irradiated Food*, EUR/14315/EN, M. Leonardi, J.J. Raffi, & J.J. Belliardo (Eds), Commission of the European Communities, Brussels, Belgium, pp. 211–218

(3) Raffi, J.J. (1992) *Electron Spin Resonance Intercomparison Studies on Irradiated Foodstuffs*, EUR/13630/EN, Commission of the European Communities, Brussels, Belgium

(4) Schreiber, G.A., Wagner, U., Leffke, A., Helle, N., Ammon, J., Buchholtz, H.-V., Delincée, H., Estendorfer, S., Fuchs, K., von Grabowski, H.-U., Kruspe, W., Mainczyk, K., Münz, H., Nootenboom, H., Schleich, C., Vreden, N., Wiezorek, C., & Bögl, K.W. (1993) *Thermoluminescence Analysis to Detect Irradiated Spices, Herbs and Spice- and Herb Mixtures—an Intercomparison Study*, SozEp-Heft 2/1993, Bundesgesundheitsamt, Berlin, Germany

(5) Schreiber, G.A., Wagner, U., Leffke, A., Helle, N., Ammon, J., Buchholtz, H.-V., Delincée, H., Estendorfer, S., von Grabowski, H.-U., Kruspe, W., Mainczyk, K., Münz, H., Schleich, C., Vreden, N., Wiezorek, C., & Bögl, K.W. (1993) *Thermoluminescence Analysis to Detect Irradiated Fruit and Vegetables—an Intercomparison Study*, SozEp-Heft 3/1993, Bundesgesundheitsamt, Berlin, Germany

(6) Sanderson, D.C.W., Schreiber, G.A., & Carmichael, L.A. (1994) *An European Interlaboratory Trial of TL Detection of Irradiated Herbs and Spices. Phase 1: Preliminary Study*, Commission of the European Communities, Brussels, Belgium, in press

(7) Balboni, J.J., & Nawar, W.W. (1970) *J. Agric. Food Chem.* **18**, 746

(8) Champagne, J.R., & Nawar, W.W. (1969) *Food Sci.* **34**, 335–339

(9) Dubravcic, M.F., & Nawar, W.W. (1969) *J. Agric. Food Chem.* **17**, 639–644

(10) Kavalam, J.R., & Nawar, W.W. (1969) *J. Am. Oil Chem. Soc.* **46**, 387–390

(11) LeTellier, P.R., & Nawar, W.W. (1972) *J. Agric. Food Chem.* **20**, 129

(12) Nawar, W.W. (1988) in *Health Impact, Identification and Dosimetry of Irradiated Foods. Report of a WHO Working Group*, ISH-Heft 125, K.W. Bögl, D.F. Regulla, & M.J. Suess (Eds), Bundesgesundheitsamt, Berlin, Germany, pp. 287–296

(13) Nawar, W.W., Zhu, Z.R., & Yoo, Y.J. (1990) in *Food Irradiation and the Chemist*, D.E. Johnston & M.H. Stevenson (Eds), The Royal Society of Chemistry, London, UK, pp. 13–24

(14) Ammon, J., Mildau, G., Ruge, W., & Delincée, H. (1992) *Deut. Lebensm. Runds.* **88**, 35–37

(15) Lesgard, G., Giamarchi, P., Raffi, J., & Prost, M. (1993) in *Recent Advances on the Detection of Irradiated Food*, EUR/14315/EN, M. Leonardi, J.J. Raffi, & J.J. Belliardo (Eds), Commission of the European Communities, Brussels, Belgium, pp. 240–249

(16) Biedermann, M., Grob, K., & Meier, W. (1990) HRC & CC *J. High Resolut. Chromatogr. & Chromatogr. Comm. 12*, 591–598

(17) Morehouse, K.M., & Ku, Y. (1990) *Radiat. Phys. Chem.* **35**, 337–341

(18) Morehouse, K.M., Ku, Y., Albrecht, H.L., & Yang, G.C. (1991) *Radiat. Phys. Chem.* **38**, 61–68

(19) Morehouse, K.M., & Ku, Y. (1992) *J. Agric. Food Chem.* **40**, 1963–1971

(20) Schulzki, G., Spiegelberg, A., Helle, N., Bögl, K.W., & Schreiber, G.A. (1993) in *Recent Advances on the Detection of Irradiated Food*, EUR/14315/EN, M. Leonardi, J.J. Raffi, & J.J. Belliardo (Eds), Commission of the European Communities, Brussels, Belgium, pp. 250-263

(21) Sjöberg, A.M., Tuominen, J.P., Kiutamo, T., & Luukkonen, S.M. (1992) *J. Sci. Food Agric.* **59**, 65–75

(22) Spiegelberg, A., Heide, L., & Bögl, K.W. (1990) *Bundesgesundhbl.* **8**, 328–332

(23) Spiegelberg, A., Heide, L., & Bögl, K.W. (1991) in *Potential New Methods of Detection of Irradiated Food*, EUR/13331/EN, J.J. Raffi & J.J. Belliardo (Eds), Commission of the European Communities, Brussels, Belgium, pp. 177–186

(24) Spiegelberg, A., Helle, N., Bögl, K.W., & Schreiber, G.A. (1991) in *Strahleninduzierte Fettveränderungen unter Besonderer Berücksichtigung von Geflügel - Grundlagen, Anwendung, Kontrolle*, SozEp-Heft 2/1991, K.W. Bögl, R. Döllstädt, G. Hübner, J.-Th. Mörsel, G.A. Schreiber, & M. Tülsner (Eds), Bundesgesundheitsamt, Berlin, Germany, pp. 261–285

(25) Spiegelberg, A., Schulzki, G., Helle, N., Bögl, K.W., & Schreiber, G.A. (1993) *Radiat. Phys. Chem.*, **43**, 433–444

(26) Delincée, H. (1991) *Analytical Detection Methods for Irradiated Foods. A review of the current literature*, IAEA-TECDOC-587, IAEA, Vienna, Austria

(27) Nawar, W.W. (1986) *Food Rev. Int.*, **21**, 45–78

(28) Schreiber, G.A., Spiegelberg, A., Helle, N., & Bögl, K.W. (1991) in *Strahleninduzierte Fettveränderungen unter Besonderer Berücksichtigung von Geflügel - Grundlagen, Anwendung, Kontrolle*, SozEp-Heft 2/1991, K.W. Bögl, R. Döllstädt, G. Hübner, J.-Th. Mörsel, G.A. Schreiber, & M. Tülsner (Eds), Bundesgesundheitsamt, Berlin, Germany, pp. 235–260

(29) Schreiber, G.A., Helle, N., & Bögl, K.W. (1993) *Int. J. Radiat. Biol.* **63**, 105–130

(30) Nawar, W.W. (1973) in *Proceedings International Colloquium. Identification of Irradiated Food*, EUR 5126 d/e/f/i/n, Commission of the European Communities, Brussels, Belgium, pp. 117–125

(31) Schreiber, G.A., Schulzki, G., Spiegelberg, A., Helle, N., Adam, S., Ammon, J., Baumann, P., Brockmann, R., Bänziger, U., Delincée, H., Droz, Ch., Estendorfer, S., Gemperle, C., von Grabowski, H.-U., Känzig, A., Krölls, W., Matter, L., Metschies, M., Mildau, G., Pfordt, J., Plaga-Lodde, A., Punkert, M., Rönnefahrt, B., Ruge, W., Stemmer, H., Vater, N., Wilmers, K., & Bögl, K.W. (1993) *Gas Chro-*

matographic Analysis of Volatile Hydrocarbons to Detect Irradiated Chicken, Pork, and Beef—an Intercomparison Study, SozEp-Heft 1/1993, Bundesgesundheitsamt, Berlin, Germany

(32) Deutsche Forschungsgemeinschaft (1991) in *Mitteilung VI der Senatskommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel*, VCH Verlagsgesellschaft, Weinheim, Germany, S 9

(33) Stevenson, M.H., Crone, A.V.J., & Hamilton, J.T.G. (1993) in *Recent Advances on the Detection of Irradiated Food*, EUR/14315/EN, M. Leonardi, J.J. Raffi, & J.J. Belliardo (Eds), Commission of the European Communities, Brussels, Belgium, pp. 319–328

FOOD COMPOSITION AND ADDITIVES

Effect of Infrared Analyzer Homogenization Efficiency on Repeatability of Uncorrected Fat A and Fat B Signals

ERIKA B. SMITH, DAVID M. BARBANO, and JOANNA M. LYNCH
Cornell University, Northeast Dairy Foods Research Center, Ithaca, NY 14853
J. RICHARD FLEMING
U.S. Department of Agriculture, Agricultural Marketing Service, Texas Milk Market, Carrollton, TX 75006

Poor repeatability by infrared milk analyzers may be caused by inefficient homogenization as a result of light scattering and the Christiansen effect. The objectives of this study were to identify instruments with good and poor homogenization efficiency and to determine if a difference exists in repeatability performance between instruments with good vs poor homogenization efficiency. Unhomogenized and homogenized portions of the same milk were tested 20 times consecutively on 22 instruments. An instrument was considered to have poor homogenization efficiency if the mean difference in the uncorrected signal between unhomogenized and homogenized portions of the same milk was ≥1.43% of the fat test (i.e., ≥0.05% at 3.5% fat). Instruments were evaluated for repeatability by calculating the sample standard deviation and the range of the latter 19 uncorrected readings for unhomogenized and homogenized milks. When repeatability was evaluated as a function of homogenization efficiency, there was a significant ($p = 0.001$) correlation between poor homogenization efficiency and poor repeatability when testing unhomogenized milk but not when testing homogenized milk. Improved homogenizer performance within infrared milk analyzers is needed to improve the repeatability of raw milk testing.

Received June 7, 1993. Accepted by GL November 17, 1993.

Infrared milk analysis is a rapid and cost-effective method for measuring fat, protein, and lactose concentrations in milk. Infrared milk analyzers use 4 specific sample wavelengths to measure light absorbance by the major structural groups present in milk fat, protein, and lactose in combination with 4 specific reference wavelengths (1). The fat A channel measures the absorbance at 5.73 μm caused by the stretching of carbonyl groups, whereas the absorbance at 3.46 μm caused by the carbon–hydrogen stretch is measured by the fat B channel. The amide groups of milk proteins absorb energy at 6.465 μm, and the hydroxyl groups of lactose absorb energy at 9.610 μm (1).

Accuracy of results obtained from infrared milk analysis is dependent upon adequate mechanical and electrical performance (i.e., performance characteristics) of the instrument in addition to proper instrument calibration by the operator (2). Homogenization efficiency and repeatability are 2 important performance characteristics that the instrument operator must monitor periodically. Inefficient homogenization of raw (unhomogenized) milk within the instrument results in a nonuniform distribution of fat globule size within a milk sample that can cause light scattering and the Christiansen effect (i.e., shift in wavelength of maximum light absorption) (3, 4). A recent survey of 22 infrared analyzers (5) found that more instruments than expected failed the homogenization efficiency criteria as stated in official methods (6, 7).

Table 1. Methods to determine repeatability for the infrared milk analyzer

Method	No. samples[a]	Number of determinations	Repeatability criteria
IDF	1	2	Absolute difference <0.040% (i.e., s_r<0.014); absolute difference <0.060% (i.e., s_r<0.021) for instruments of the first generation
AOAC	8	2/sample	Mean difference between duplicate instrument estimates ≤0.02%; s_r of the difference between duplicate instrument estimates ≤0.02%[b]

[a] Neither the composition nor the type of milk to be used (i.e., raw or homogenized) is specified.
[b] Corresponds to range <0.057% (i.e., range = 2.83 * s_r).

Repeatability is the closeness of agreement between results (i.e., precision) obtained by repeated measurements on identical material by a standard method (8). AOAC considers a fat channel to have acceptable precision if the channel can maintain a standard deviation of the mean difference between duplicate analyses ≤0.02% and a mean difference between duplicate instrument estimates ≤0.02% based on the analysis of 8 different samples (6). The International Dairy Federation states that an infrared milk analyzer has adequate precision if the absolute difference between single determinations on one sample is <0.04% for each channel (7). A summary of these different repeatability criteria is given in Table 1.

The manufacturer of the Foss Milkoscan 104 lists the following technical specifications for the instrument: standard deviation ≤0.02% for samples containing 2–6% fat and relative standard deviation (i.e., coefficient of variation) ≤0.5% for samples containing 6–15% fat (9). Differences among the various methods and criteria for determining instrument repeatability should be reviewed against the manufacturer's claims and from actual data for instrument repeatability from laboratories. Official methods groups should develop a harmonized criterion for repeatability.

A previous study suggests that inefficient homogenization by the infrared analyzer may be responsible for poor repeatability observed when testing unhomogenized milk (10). Because this particular study involved only one instrument, the poorer repeatability observed when testing unhomogenized milk was later attributed to a specific (or manufacturer) instrumental failure (11). However, poor repeatability when testing unhomogenized milk might be correlated with inefficient homogenization by the instrument's homogenizer. When testing raw milk, the fat globule size distribution produced by the homogenizer may be different from one measurement cycle to the next in an instrument with poor homogenization efficiency. This variability may be manifested as poor instrument repeatability. Fat B channel repeatability may be affected more by the instrument's inefficient sample homogenization because of its shorter wavelength than the fat A channel. This occurs because light scattering becomes more pronounced as the diameter of the fat globule approaches the wavelength of light at which a measurement is being made (4, 12, 13).

The objectives of this study were (*1*) to identify instruments with poor and good homogenization efficiency and (*2*) to determine if there is a difference in repeatability performance of the instruments with poor and good homogenizer efficiency.

Table 2. Model and number of instruments in the survey

Instrument model	Abbreviation	Number of instruments
Milkoscan 104[a]	MS 104	7
Milkoscan 134[a]	MS 134	3
Milkoscan 255[a]	MS 255	1
Multispec Dairy Lab 1[b]	DL-1	1
Multispec Dairy Lab 2[b]	DL-2	1
Multispec M1[b]	M-1	3
Multispec M1: Bentley[c]	M-1 B	2
Multispec M2[b]	M-2	4
Total		22

[a] Manufactured by Foss Electric, Hillerod, Denmark.
[b] Manufactured by Multispec, Ltd, York, UK.
[c] The Bentley modification of the Multispec M-1 uses the optical system of the original instrument and then transfers total control of the signal to an external computer utilizing software developed by Bentley Instruments (Chaska, MN).

Experimental

Preparation of Samples

Raw milk (ca 3.3% fat) was pasteurized at 76.6°C, and a portion of the pasteurized, unhomogenized milk was collected. Another portion of the same milk was homogenized using a 2-stage homogenizer (Creamery Package homogenizer, NFG Co., Chicago, IL) at 12 411 kPa (i.e., 1800 psi). Separately, a pasteurized (82.2°C) portion of Half & Half (ca 10% fat) was collected before homogenization and another portion was collected after homogenization using a Gaulin 2-stage homogenizer (APV Gaulin, Inc., Everett, MA) at 12 411 kPa. The un-

Table 3. Fat content of unhomogenized and homogenized portions of the same milk at 2 different fat levels

Sample	Homogenization pressure, kPa	% Fat[a] Low fat level	% Fat[a] High fat level
Unhomogenized	0	3.282	5.990
Homogenized	12411	3.282	5.998

[a] Ether extraction fat values represent the mean of duplicate analyses by 1 analyst.

Table 4. Calculated primary slopes of the uncorrected signals for the fat A and B channels of the instruments participating in the survey

Instrument number	Model	Primary slope	
		Fat A	Fat B
1	DL-1	1.35	0.81
2	DL-2	1.36	1.30
3	MS 104	0.94	0.98
4	MS 104	0.92	0.93
5	MS 104	1.01	1.04
6	MS 104	0.95	1.19
7	MS 104	0.89	0.99
8	MS 104	0.95	0.99
9	MS 104	0.93	1.00
10	MS 134	0.91	1.01
11	MS 134	0.92	0.90
12	MS 134	1.02	1.01
13	MS 255	1.01	0.98
14	M-1	1.05	1.06
15	M-1	1.08	1.02
16	M-1	0.95	0.67
17	M-1 B	0.84	0.62
18	M-1 B	0.98	0.67
19	M-2	1.08	1.05
20	M-2	1.02	1.00
21	M-2	0.84	0.73
22	M-2	0.77	0.62

homogenized and homogenized Half & Half were diluted to 6% fat with pasteurized skim milk. This yielded a pair of pasteurized unhomogenized and homogenized milks at 3.3 and 6% fat for use in evaluating homogenization efficiency and repeatability.

Each test material was mixed, split uniformly into 80 mL Whirl-Pak bags (Nasco, Fort Atkinson, WI), and cooled in crushed ice immediately after processing. After the bags were subdivided into 22 sets of test samples, the test samples were shipped by overnight delivery to 16 laboratories that operated a total of 22 instruments. The model and number of instruments that participated in the survey are shown in Table 2. Each instrument received 5 test portions of the unhomogenized and homogenized milks at 3.3 and 6% fat. All analysts were provided with detailed instructions describing sample handling and testing procedures.

Chemical and Instrumental Analysis

The fat concentrations of the unhomogenized and homogenized milk samples were determined by ether extraction (14) to verify that the fat content of the unhomogenized and homogenized milk in each pair was as close as possible to the same fat content (Table 3). Any differences in fat content between unhomogenized and homogenized milk within each pair were less than 0.01% fat. All 22 instruments included in the survey were used routinely for raw milk testing, and no special adjustments were made to the instruments before conducting the survey.

Table 5. Homogenization efficiency test values: homogenized minus unhomogenized primary slope adjusted uncorrected readings for the fat A and fat B channels for each instrument[a]

Instrument Number	Model	3.3% Fat		6% Fat	
		Fat A	Fat B	Fat A	Fat B
1	DL-1	−0.033	−0.046	−0.104[b]	0.088[b]
2	DL-2	−0.022	−0.022	−0.064	−0.062
3	MS 104	—	0.099[b]	—	0.209[b]
4	MS 104	−0.027	0.008	−0.122[b]	−0.010
5	MS 104	0.008	0.084[b]	0.037	0.257[b]
6	MS 104	−0.010	0.021	−0.012	0.102[b]
7	MS 104	−0.091[b]	0.084[b]	−0.280[b]	0.206[b]
8	MS 104	0.000	−0.011	−0.103[b]	0.043
9	MS 104	−0.005	−0.002	−0.033	−0.004
10	MS 134	−0.059[b]	0.115[b]	−0.296[b]	0.216[b]
11	MS 134	0.004	0.086[b]	−0.078	0.139[b]
12	MS 134	−0.021	0.068[b]	−0.147[b]	0.157[b]
13	MS 255	−0.014	0.005	0.005	0.027
14	M-1	−0.008	0.023	0.050	0.165[b]
15	M-1	0.012	0.032	0.038	0.083
16	M-1	—	—	—	—
17	M-1 B	0.014	—	0.055	—
18	M-1 B	0.015	0.038	0.051	0.098[b]
19	M-2	−0.012	−0.007	−0.065	−0.012
20	M-2	−0.012	0.010	−0.070	0.040
21	M-2	−0.026	0.130[b]	−0.044	0.187[b]
22	M-2	0.003	—	−0.010	—
Number of instruments evaluated		20	19	20	19
Number of failures		2	7	6	11

[a] Data discarded from the fat A channels of instruments 3 and 16 and from the fat B channels of instruments 16, 17, 22 for failing a repeatability evaluation using water.

[b] Difference between uncorrected fat signals for homogenized and unhomogenized milk ≥1.43% of the fat content of the milk pair used for the test.

Evaluation of Homogenization Efficiency and Repeatability

Instrument operators were instructed to zero their instruments before testing and between test materials. The fat A and B channels of each instrument were evaluated first for repeatability on water by collecting 20 consecutive readings on water before the repeatability check was performed on the 4 different types of milk samples. Instruments were considered to exhibit acceptable repeatability on water if the uncorrected signal range of the latter 19 water readings was <0.04%. Only data from those channels that displayed acceptable water repeatability were included in the survey results. If an instrument could not repeat on water, probably another source of the repeatability problem existed separate from the homogenizer.

Table 6. Slope-adjusted repeatability ranges for the fat A and fat B channels

Instrument number	Model	Fat A				Fat B			
		3.3% Fat		6% Fat		3.3% Fat		6% Fat	
		Unhomo.[a]	Homo.	Unhomo.	Homo.	Unhomo.	Homo.	Unhomo.	Homo.
1	DL-1	0.022	0.022	0.037	0.030	0.025	0.012	0.025	0.037
2	DL-2	0.015	0.007	0.022	0.007	0.008	0.015	0.023	0.015
3	MS 104	—	—	—	—	0.041	0.041	0.092	0.031
4	MS 104	0.011	0.000	0.043	0.022	0.032	0.021	0.032	0.021
5	MS 104	0.030	0.020	0.040	0.020	0.048	0.029	0.096	0.019
6	MS 104	0.011	0.011	0.032	0.021	0.034	0.025	0.109	0.034
7	MS 104	0.056	0.034	0.134	0.022	0.030	0.030	0.112	0.020
8	MS 104	0.032	0.021	0.053	0.021	0.020	0.030	0.080	0.040
9	MS 104	0.021	0.021	0.021	0.011	0.020	0.020	0.010	0.040
10	MS 134	0.011	0.011	0.044	0.022	0.020	0.020	0.060	0.020
11	MS 134	0.022	0.011	0.033	0.022	0.067	0.011	0.067	0.011
12	MS 134	0.029	0.010	0.049	0.049	0.049	0.010	0.040	0.040
13	MS 255	0.030	0.030	0.059	0.020	0.031	0.041	0.061	0.041
14	M-1	0.029	0.029	0.076	0.010	0.066	0.019	0.057	0.028
15	M-1	0.009	0.028	0.046	0.019	0.020	0.029	0.059	0.039
16	M-1	—	—	—	—	—	—	—	—
17	M-1 B	0.036	0.024	0.059	0.012	—	—	—	—
18	M-1 B	0.020	0.020	0.041	0.020	0.030	0.059	0.104	0.059
19	M-2	0.018	0.028	0.037	0.055	0.029	0.019	0.057	0.048
20	M-2	0.020	0.010	0.030	0.020	0.020	0.020	0.030	0.020
21	M-2	0.024	0.012	0.059	0.024	0.074	0.045	0.117	0.079
22	M-2	0.026	0.026	0.065	0.026	—	—	—	—

[a] Unhomo. = unhomogenized, homo. = homogenized.

The order of sample testing was as follows: water to set zero, unhomogenized milk at 3.3% fat, homogenized milk at 3.3% fat, unhomogenized milk at 6% fat, homogenized milk at 6% fat, and water for a final zero check. Water was used to check and reset zeros if needed between different types of milk samples. Homogenization efficiency and repeatability were evaluated on each test sample by collecting 20 consecutive uncorrected instrument readings on each type of milk. Because there were 5 Whirl-Pak bags of milk per test sample, this was achieved by taking 4 readings consecutively from each of the 5 Whirl-Pak bags. The milk in each Whirl-Pak bag was mixed by inversion between readings.

The first reading on milk out of 20 was discarded to avoid any carryover effect when changing from water to milk. The primary slope of the uncorrected signal for the fat A and B channels was calculated for each instrument by dividing the mean difference between the uncorrected signal for the homogenized milks containing 3.3 and 6% fat by the mean difference in fat content between the homogenized milks containing 3.3 and 6% fat determined by ether extraction (Table 3). Primary slopes of the uncorrected signal for each fat channel of each instrument that participated in the survey are displayed in Table 4.

When comparing data between several instruments, misleading homogenization efficiency and repeatability comparisons could occur if the primary slope of the uncorrected signal for a channel is significantly larger or smaller than 1.00 from one instrument to the next. Therefore, all uncorrected signals of each instrument were adjusted mathematically to a slope of 1.00 for each channel before homogenization and repeatability values were calculated in this study.

Mean slope-adjusted uncorrected signals for the fat channels were calculated for each instrument by dividing mean uncorrected signals by the primary slope. Official methods state that instruments with a difference between unhomogenized and homogenized portions of the same milk ≥0.05% fail homogenization efficiency (6, 7). A revised homogenization efficiency criterion that can be adjusted for different fat concentrations was applied to the pairs of unhomogenized and homogenized portions of the same milk both at 3.3 and 6% fat, as reported previously (5). An instrument failed the homogenization efficiency test if the difference between unhomogenized and homogenized portions of the same milk was ≥1.43% of the actual fat content of the pair of samples used to evaluate homogenization efficiency. Repeatability was determined by calculating the standard deviation (s_r) and the range of 19 primary slope-adjusted uncorrected instruments' readings for each test sample.

Results and Discussion

On the fat A channel, 2 instruments (numbers 3 and 16) had poor repeatability on water, so only 20 out of 22 instruments were evaluated for homogenization efficiency and repeatabil-

Table 7. Slope-adjusted standard deviation (s_r) data for the fat A and fat B channels

Instrument number	Model	Fat A				Fat B			
		3.3% Fat		6% Fat		3.3% Fat		6% Fat	
		Unhomo.[a]	Homo.	Unhomo.	Homo.	Unhomo.	Homo.	Unhomo.	Homo.
1	DL-1	0.007	0.006	0.012	0.007	0.009	0.006	0.008	0.009
2	DL-2	0.004	0.003	0.006	0.004	0.003	0.005	0.006	0.005
3	MS 104	—	—	—	—	0.012	0.009	0.022	0.008
4	MS 104	0.006	0.000	0.009	0.007	0.008	0.007	0.008	0.006
5	MS 104	0.008	0.006	0.011	0.006	0.012	0.007	0.025	0.006
6	MS 104	0.004	0.004	0.011	0.006	0.010	0.008	0.027	0.008
7	MS 104	0.016	0.007	0.033	0.007	0.009	0.008	0.029	0.006
8	MS 104	0.010	0.009	0.016	0.007	0.006	0.008	0.027	0.010
9	MS 104	0.006	0.006	0.007	0.005	0.005	0.007	0.005	0.012
10	MS 134	0.005	0.005	0.011	0.007	0.005	0.006	0.016	0.005
11	MS 134	0.008	0.005	0.009	0.004	0.020	0.006	0.018	0.006
12	MS 134	0.008	0.005	0.016	0.011	0.013	0.005	0.011	0.012
13	MS 255	0.009	0.012	0.017	0.006	0.009	0.010	0.017	0.011
14	M-1	0.008	0.007	0.019	0.004	0.015	0.005	0.017	0.009
15	M-1	0.005	0.007	0.014	0.007	0.008	0.010	0.021	0.011
16	M-1	—	—	—	—	—	—	—	—
17	M-1 B	0.010	0.006	0.012	0.006	—	—	—	—
18	M-1 B	0.006	0.007	0.011	0.006	0.010	0.020	0.024	0.012
19	M-2	0.005	0.006	0.009	0.011	0.008	0.007	0.013	0.012
20	M-2	0.006	0.004	0.008	0.006	0.005	0.007	0.010	0.007
21	M-2	0.005	0.006	0.018	0.006	0.020	0.013	0.036	0.027
22	M-2	0.010	0.007	0.016	0.007	—	—	—	—

[a] Unhomo. = unhomogenized, homo. = homogenized.

ity on milk for this channel. Three instruments (numbers 16, 17, and 22) displayed poor repeatability on water for the fat B channel. Therefore, only 19 out of 22 instruments were evaluated for homogenization efficiency and repeatability on milk for this channel. Results from the homogenization efficiency evaluation are shown in Table 5. Many instruments were unable to satisfy the revised homogenization efficiency criteria, and more instruments failed those criteria at 6% than at 3.3% fat on both channels.

Tables 6 and 7 contain slope-adjusted uncorrected signal repeatability ranges and slope-adjusted standard deviation (s_r) data from the survey, respectively. Because different groups have used different criteria to evaluate repeatability, we believed that expressing the results in both forms would be beneficial. Many more instruments exceeded the range criterion of <0.04 over 19 readings than exceeded the standard deviation (s_r) criterion of ≤0.02%. Thus, the 2 criteria are not equivalent and assessment of channel repeatability using a range of <0.04 over 19 samples was a more restrictive criterion for evaluating repeatability status.

Results in Tables 5–7 indicate that a correlation may exist between inefficient homogenization and poor repeatability when testing raw milk samples. If this is true, then there should be a statistically significant increase in repeatability range and standard deviation with decreasing homogenization efficiency by the instrument homogenizer when testing unhomogenized milk. However, a statistically significant increase in repeatability range and standard deviation would not be expected for milks that have been efficiently homogenized externally to the infrared milk analyzer. To test this hypothesis, a series of first order regression analyses of variance (ANOVAs) was calculated for each fat channel.

First, the absolute homogenization efficiency test values (Table 5) for the fat A and B channels were adjusted for fat content by dividing each value by the fat content of the milk used (i.e., 3.3 or 6%) to assess homogenization efficiency. This allowed the homogenization efficiency data from milks containing different fat contents to be combined as one data set from each channel. First-order regression analyses were calculated separately of repeatability standard deviation data for unhomogenized and homogenized milks as a function of fat-adjusted absolute homogenization efficiency values and are shown for the fat A (Figures 1 and 2) and the fat B channels (Figures 3 and 4). As the value for fat-adjusted absolute homogenization efficiency increases, the efficiency of homogenization decreases.

An ANOVA was calculated to determine if the slope of each regression line was significantly different from zero (Table 8). The slope of the regression line for the relationship between repeatability standard deviation data for *unhomogenized* milk and homogenization efficiency values for each fat channel was significantly ($p = 0.001$) different from zero (Figures 1 and 3).

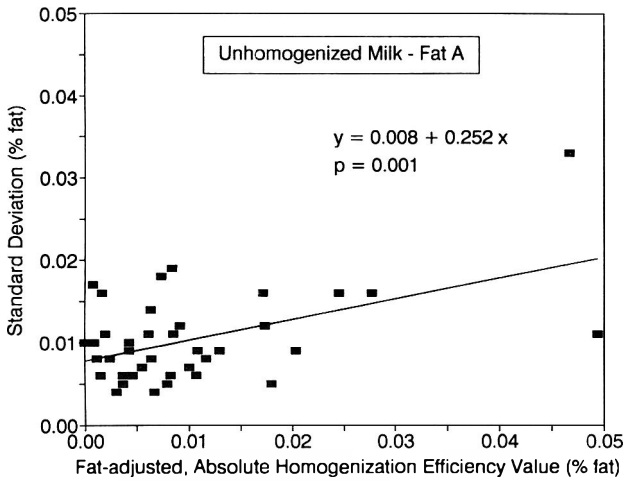


Figure 1. Standard deviation data vs homogenization efficiency values for unhomogenized milk, fat A channel.

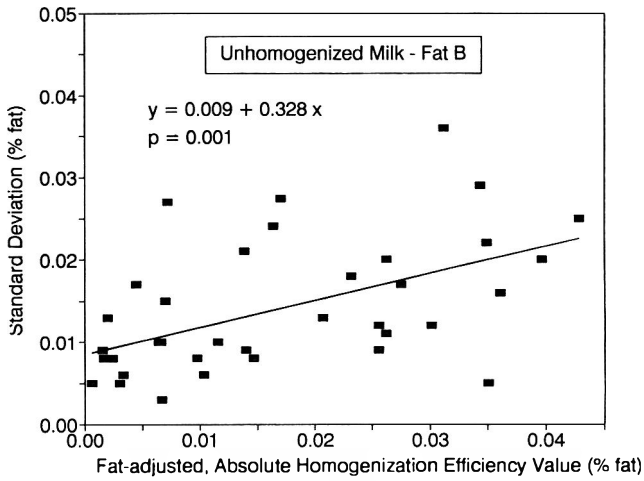


Figure 3. Standard deviation data vs homogenization efficiency values for unhomogenized milk, fat B channel.

On the same instruments, none of the regressions of the repeatability standard deviation data for *homogenized* milk against the homogenization efficiency values were significant (i.e., slope not significantly different from zero) for either the fat A or B channels (Figures 2 and 4). Thus, instruments with inefficient homogenizers exhibited poorer repeatability when testing unhomogenized milk than when testing homogenized milk.

Examination of the repeatability behavior of the fat A and B measurements for homogenized milk (Figures 2 and 4 respectively) indicates the repeatability that the instruments are capable of achieving if the homogenizer within the instrument could perform as efficiently as the dairy plant homogenizer used in this study. For the fat A channel, only 3 out of 40 repeatability standard deviation values were >0.01%, and for the fat B channel only 2 out of 38 were >0.02% (Table 2). This is in contrast to Figures 1 and 3 where repeatability standard deviations on the same instruments are much larger when the homogenizer within the instrument has to homogenize a portion of the same unhomogenized milk. It is also interesting to note that the Y-intercepts for Figures 1–4 are almost identical, which indicates that an instrument with a very efficient homogenizer should be able to achieve similar repeatability standard deviations on unhomogenized and homogenized portions of the same milk. The basic electronic and optical components of these instruments are capable of better repeatability performance than can be achieved currently with the average existing instrument homogenizer.

How does inefficient homogenization within an instrument affect the accuracy of raw milk testing if the instrument is calibrated using raw milk? Calibration adjustment should be minimally affected as long as 3 or more replicates of each calibration milk are averaged. However, the actual samples used for producer payment are generally tested only once. The poor repeatability that occurs because of inefficient homogenization within the instrument will affect producer payment testing by increasing the standard deviation of the difference between chemistry and single infrared tests. In addition, variation in sample temperature within and between days may cause hour-

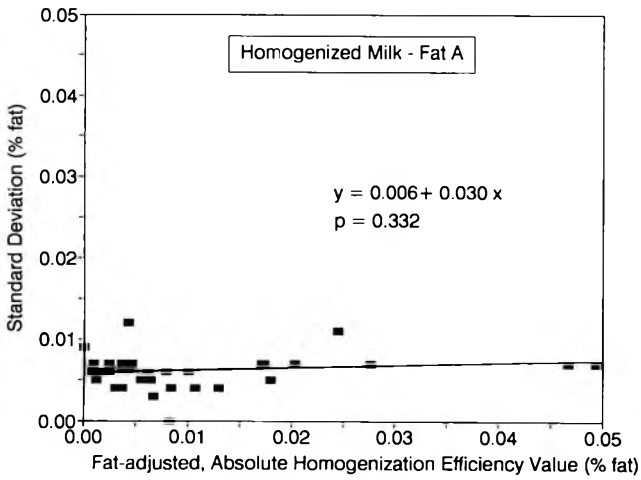


Figure 2. Standard deviation data vs homogenization efficiency values for homogenized milk, fat A channel.

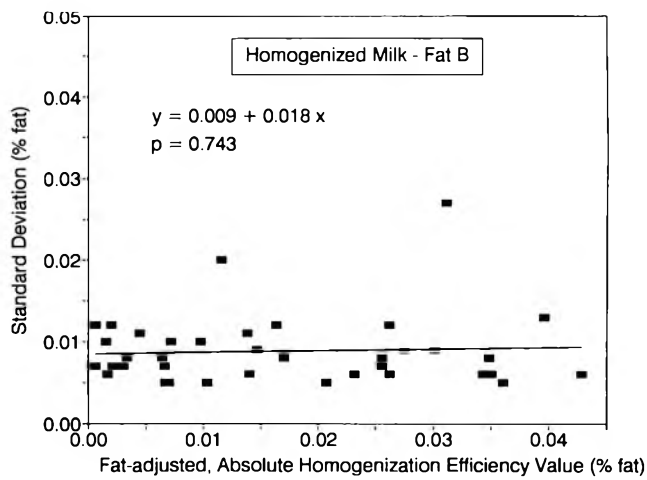


Figure 4. Standard deviation data vs homogenization efficiency values for homogenized milk, fat B channel.

Table 8. Summary of p values from first order regression ANOVA: repeatability range data vs fat adjusted, absolute homogenization efficiency values and repeatability standard deviation (s_r) data vs fat adjusted, absolute homogenization efficiency values

	Range		Standard deviation	
Channel	Unhomo.	Homo.	Unhomo.	Homo.
Fat A	<0.001	0.220	0.001	0.332
Fat B	<0.001	0.793	0.001	0.743

to-hour and day-to-day bias in infrared results when an inefficient homogenizer is used within the instrument. Further work needs to be done on this issue.

Conclusions

Many instruments had poor homogenization efficiency, which is consistent with a previous report (5). Instruments with poor homogenizer performance are more likely to exhibit poor repeatability on both fat channels when testing unhomogenized milk than instruments with good homogenizers. Variation in fat globule size distribution, produced by an inefficient homogenizer, that may occur from one measurement cycle to the next, may account for the poor repeatability. Poor repeatability on raw milk is a concern for producer payment testing because samples are not tested in duplicate. Because of the short wavelength of light used for the fat B channel, fat B channel readings are affected more by homogenization inefficiency and poor repeatability than the fat A channel readings. Improved homogenizer performance is needed within infrared milk analyzers to improve the repeatability of testing raw milk. The different repeatability criteria that are used to judge instrument homogenizer performance need to be reviewed and standardized.

Acknowledgments

We acknowledge the technical assistance of the staff of the Federal Milk Market Laboratories, Northeast Dairy Herd Improvement Association, and the Dairy Quality Control Institute. We are grateful for the assistance of Maureen Chapman, Pat Nelson, and Edna Hu. Assistance from the staff of Foss Electric and Foss Food Technology was very helpful.

References

(1) Biggs, D.A., Johnsson, G., & Sjaunja, L.O. (1987) Analysis of Fat, Protein, and Lactose & Total Solids by Infrared Absorption in *Rapid Indirect Methods for Measuring Milk Components IDF Bulletin 208*, Brussels, Belgium

(2) Barbano, D.M., & Clark, J.L. (1989) *J. Dairy Sci.* **72**, 1627–1636

(3) Shields, J. (1975) The Analysis of Milk by Infrared Absorption, B.Ph. Thesis, University of York, York, UK

(4) Goulden, J.D. (1964) *J. Dairy Research* **31**, 272–284

(5) Smith, E.B., & Barbano, D.M. (1993) *J. Assoc. Off. Anal. Chem.* **76**, 1033–1041

(6) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec. **972.16C**

(7) International Dairy Federation (1990) Determination of Milkfat, Protein, and Lactose—Guide for the Operation of Mid-infrared Instruments, *IDF Standard 208*, Brussels, Belgium

(8) International Dairy Federation (1985) Milk: Definition and Evaluation of the Overall Accuracy of Indirect Methods of Milk Analysis—Application to Calibration Procedure and Quality Control in Dairy Laboratory, *IDF Standard 128*, Brussels, Belgium

(9) Instruction Manual, Milkoscan 100 series, Foss Food Technology, 1985

(10) Sjaunja, L.O. (1984) *Acta. Agric. Scand.* **34**, 249–259

(11) Sjaunja, L.O. (1985) *Acta. Agric. Scand.* **35**, 345–352

(12) Alpert, N.N., Keiser, W.E., & Szymanski, H.A. (1964) *IR: Theory and Practice on Infrared Spectroscopy*, Plenum Press, New York, NY, pp. 329–341

(13) Osbourne, B.G., & Fearn, T. (1986) *Near Infrared Spectroscopy in Food Analysis*, Longman Scientific & Technical, New York, NY, pp. 51–55

(14) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec. **989.05**

RESIDUES AND TRACE ELEMENTS

Determination of Sulfite in Rainwater by Solid-Phase Spectrophotometry

JUAN M. BOSQUE-SENDRA, FRANCISCA MOLINA, and MOUNIR NECHAR
University of Granada, Department of Analytical Chemistry, 18071 Granada, Spain

A simple and specific method was developed for the determination of sulfite in rainwater based on solid-phase spectrophotometry (SPS). Rainwater was collected on morpholine, sulfite was stabilized by forming an adduct that reacted with pararosaniline and formaldehyde, and the reaction product was fixed on Dowex 1-X8 resin. The absorbance of the solid phase was measured directly at 565 and 700 nm. The calibration curve was linear in the concentration range of 1–50 μg/L in solution (relative standard deviation = 1.7%); the International Union of Pure and Applied Chemistry detection limit was 0.8 μg/L. In simulated rainwater, the values of the calibration curve slopes were similar to that of the standard calibration curve slope, and recoveries for 40–497 μg/L sulfite ranged from 97 to 105%. Linearity, analytical sensitivity, and precision were better for the SPS method than the solution method, and the detection and quantitation limit values were lower for the SPS method than the solution method based on the similar reaction. The SPS method was subsequently used to determine sulfite in real rainwater.

Sulfur dioxide, the sulfur oxide released into the atmosphere largely by combustion of sulfur in fuels and coals, dissolves in water and forms bisulfite or sulfite. These compounds can gradually oxidize to sulfuric acid or sulfate, and fall to earth dissolved in rainwater. They acidify the environment and diminish the neutralizing capacity of soil and water. Consequently, the development of analytical methods to determine sulfite at the microgram per liter level independent from sulfate in rain is of great interest.

One method reported for the spectrophotometric analysis of sulfite in water (1, 2) is based on the formation of intensely colored pararosanilinemethylsulfonic acid from the reaction between sulfite, pararosaniline, and formaldehyde in an acid medium (3). This method is simple and highly sensitive.

Solid-phase spectrophotometry (SPS) (4–6) is a technique that includes the preconcentration of the analyte on a solid, with a previous or simultaneous step of reaction to produce a chromogenic compound, and the subsequent measurement of the absorbance in the solid phase. This method provides an increase in selectivity and sensitivity over conventional spectrophotometric methods.

We previously reported on a simple and extremely sensitive spectrophotometric method based on SPS and the reaction previously noted. This method can determine atmospheric sulfur dioxide at the microgram per liter level (6).

In the present paper, SPS is used to determine sulfite in rainwater. We propose a new 250 mL sample system, which presents higher sensitivity and lower detection and quantitation limits than those of the previously used 100 mL system (6). In regard to the experimental parameters, special attention was given to the presence of foreign ions; the influence of rainwater (the matrix) on analyte stability; and on the calibration curve, and the recovery of sulfite.

Received June 18, 1993. Accepted by JS October 18, 1993.

Experimental

Reagents

All chemicals used were analytical grade unless stated otherwise; reverse osmosis-type quality water was used for dilution of reagents and samples, and all experiments were carried out at room temperature.

(**a**) *Solid ion exchangers.*—Dowex 1-X8 (100–200 and 200–400 mesh) anion-exchange resins (Sigma Chemical Co., St. Louis, MO) were used in the chloride form. Each was properly conditioned, air-dried, and stored in polyethylene containers.

(**b**) *Standard sulfite solution, 0.5 g/L.*—Prepared from anhydrous sodium sulfite and dissolved in reverse osmosis water. It was standardized iodometrically.

(**c**) *Working sulfite solutions, 5 mg/L.*—Prepared by appropriate dilution of the fresh stock solution with morpholine solution (0.02% v/v) as stabilizer. This solution was stored under refrigeration (7°–8°C). Under these conditions, the solution is stable for 4 months.

(**d**) *Pararosaniline hydrochloride solution.*—Pararosaniline hydrochloride (0.2 g) (C.I. 42500 Basic Red 9) (Aldrich Chemical Co., Inc., Milwaukee, WI) was dissolved in 1M HCl and purified (7). Six milliliters of this solution was diluted to 100 mL for the working reagent.

(**e**) *Formaldehyde solution, 0.28% (v/v).*—Probus (PQ grade; purity 97%).

(**f**) *Morpholine solutions.*—Solutions of various concentrations, prepared by dilution of the necessary volume of morpholine solution (Panreac, PRS grade; purity 97%) with reverse osmosis water.

(**g**) *Buffer solution.*—Solution of pH 3.0 was prepared from 0.1M potassium hydroxide solution (E. Merck, Darmstadt, Germany) and 0.1M monochloroacetic acid (Fluka AG, Buchs, Switzerland).

Apparatus

(**a**) *Spectrophotometer.*—Spectronic 2000 spectrophotometer equipped with 1 and 10 mm cells (Bausch & Lomb, Rochester, NY).

(**b**) *Agitator.*—Agitaser Model 2000 rotating-bottle agitator (77 rpm) (Agitaser, Barcelona, Spain).

(**c**) *pH meter.*—Crison Digit-501 pH meter with a combined glass-calomel electrode (Crison Instruments, S.A., Alella [Barcelona], Spain).

Absorbance Measurements

Measure the absorbance of the pararosanilinemethylsulfonic acid fixed on the resin in a 1 mm cell at 565 nm (absorption maximum) (6) and at 700 nm (in the range where resin only caused attenuation of light) against a 1 mm cell well-packed with resin equilibrated with water. Measure the absorbance of the blank (a 1 mm cell packed with resin equilibrated with blank solution) at the same wavelengths. The blank absorbance results from the pararosaniline fixed on the exchanger (6).

Obtain the net absorbance, A_{RP}, for the reaction product as follows (5):

$$A_{RP} = A_S - A_B$$

where $A_S = A_{565} - A_{700}$ (for the sample) and $A_B = A_{565} - A_{700}$ (for the blank).

Analytical Procedure

In a 250 mL calibrated flask, add in this order: 5 mL 0.012% purified pararosaniline solution, 5 mL 0.28% formaldehyde solution, an aliquot of the sample (volume depending on analyte concentration) containing either 1–50 μg/L or 2–65 μg/L sulfite stabilized with morpholine (0.02%), and 5 mL 0.1M buffer solution (pH 3.0). Raise the volume to the mark with water. Transfer the solution to a 1000 mL polyethylene bottle and add 0.15 g Dowex 1-X8 (200–400 mesh) or Dowex 1-X8 (100–200 mesh) resin, respectively. Shake the mixture mechanically for 40 or 70 min, respectively. Collect the colored resin beads by filtration under suction and, with a pipette, pack them into a 1 mm cell together with a small portion of the filtrate. Centrifuge the cell for 1 min at 25 × *g*. Measure the absorbances at 565 and at 700 nm for the sample against a 1 mm cell reference similarly packed with resin equilibrated with water. Prepare a blank solution containing the reagents, except the analyte, and treat it as described for the sample. Measure the net absorbance, A_{RP}, for the reaction product as described under *Absorbance Measurements*.

Preparation of Simulated Rainwater

Carry out the procedure proposed by Koch et al. (10) for the preparation of their Standard Reference Material, SRM 2694, Simulated Rainwater. This solution, which is available in 2 concentration levels, contains the cations and anions commonly found in acid rain. We chose SRM 2694-I, a medium ionic level, as the most suitable for our purposes, and the pH of this solution was set at 4.5, to avoid problems with stability of the solution (10).

Collection and Treatment of Rainwater Samples

Use glass funnels of 150–190 mm id to collect rainwater in glass flasks protected from sunlight. The funnels were narrow-stemmed and fitted into the flasks, avoiding direct entry. The collectors were placed in different sites very close to each other, on the flat roof of the university. For each sample, at least 3 collectors were prepared: the first collector, with morpholine to stabilize sulfite, measured the sulfite of the rainwater; the second collector, without any additive, measured the pH of the rainwater; and the third collector, with 2 mL concentrated HNO_3, measured the metal content of the rainwater. For all samples, except Ca(II), the latter determination was carried out by atomic absorption spectrometry (AAS) following chelation of pH 3.6 with sodium diethyl dithiocarbamate (DDC) and extraction into methylisobutyl ketone. Ca(II) was determined by measuring the rainwater sample directly, without extraction. The collectors were exposed for no more than 2 h during rain periods. The samples were stored under refrigeration (7°–8°C) and analyzed within the period of stability of sulfite under our conditions.

Results and Discussion

Optimization of Conditions

The influence of pararosaniline concentration on the absorbance of the reaction product was studied (for a final volume of 250 mL). Net absorbance at 565 nm increased with a corresponding increase in pararosaniline concentration. Absorbance was found to be highest in the range of 3–7 mL 0.012% pararosaniline. In the standard procedure, 5 mL pararosaniline was selected as optimum. This concentration was also used in the standard procedure with a 100 mL sample system for the determination of atmospheric SO_2 (6).

The net absorbance at 565 nm increased with a corresponding increase in formaldehyde concentration, and the optimal values were obtained for 0.28% (v/v) formaldehyde at between 3.5 and 7 mL. In the standard procedure, 5 mL formaldehyde was selected as optimum. This concentration is higher than that used with a 100 mL sample system (6).

With Dowex 1-X8 (100–200 mesh) resin, 70 min of stirring at 77 rpm was found to be optimum, decreasing to 40 min of stirring with Dowex 1-X8 (200–400 mesh) resin. The velocity of stirring (as great as 97 rpm) did not affect the stirring time required. This finding suggests that diffusion through the stationary layer may not be the limiting step in the fixation process.

The centrifugation time was 1 min at 2500 rpm ($25 \times g$). For all measurements, 0.15 g resin was used as a compromise between maximum sensitivity and operativity.

The increase in the ionic strength due to the buffer solution (monochloroacetic acid–potassium monochloroacetate) did not affect the net absorbance in the range of 1×10^{-4}M–1.1×10^{-3}M. Higher concentrations produced a reduction in the net absorbance due to an increase in the absorbance of the blank. This absorbance may be caused by the charge of the anion, whereby pararosaniline is neutralized and fixed on the exchanger by an adsorption process. This behavior of pararosaniline can be confirmed in other types of solid phases. Thus, the fixation of pararosaniline is higher on a cation-exchanger (Dowex 50W in form H^+, 100–200 mesh) than on an anion-exchanger due to the positive charge of pararosaniline. Furthermore, in the presence of a dextran type gel without ion-exchanger groups (Sephadex G-15, 40–120 μ), the fixation of pararosaniline is higher on a cation-exchanger than on an anion-exchanger. This finding confirms the large contribution of the adsorption process in fixation of the pararosaniline.

Stabilization of Working Sulfite Solutions

Problems with the preparation of standard solutions result from the insufficient stability of the sulfite, a consequence of its oxidation into sulfate.

Previously, we established that morpholine (0.02%) is the most suitable stabilizer. Thus, the working sulfite solution (5 mg/L) was stable at room temperature (20°–25°C) for at least 12 days (6). New experiments were carried out on the stability of sulfite solutions with temperature. The results proved that working sulfite solutions in 0.02% morpholine are stable for 4 months if the solutions were stored under refrigeration (7°–8°C). On the other hand, the heating of a working sulfite solution in 0.02% morpholine in the 30°–60°C temperature range for 45 min had no effect on the sulfite content of the solution.

Analytical Data

The most important analytical data are shown in Table 1. The proposed method may be used with resin of 2 bead-sizes. Thus, the 100–200 mesh resin presented lower values of the blank (A = 0.053 as mean), but more stirring (70 min) was necessary. The 200–400 mesh resin, with higher values of the blank (A = 0.158 as mean), needed less stirring (40 min) and provided a greater sensitivity.

The fluctuations in the background absorbance measured for the blank, calculated as the average of 10 determinations and expressed as SD units, for 100–200 mesh resin and 200–400 mesh resin were 0.006 and 0.008 A, respectively. The IUPAC detection limit ($K = 3$) and the quantitation limit ($K = 10$) (8, 9) were calculated for each mesh resin.

The precision of the proposed method, expressed as the RSD for 10 within-day independent determinations (repeatability) and 10 between-day independent determinations (reproducibility), is shown in Table 1.

Table 1. Analytical data

	Dowex 1-X8 anion-exchanger resin	
	100–200 mesh	200–400 mesh
Intercept[a]	0.015 ± 0.005	0.014 ± 0.003
Slope, L/μg[a]	0.016 ± 0.001	0.031 ± 0.001
Lineal dynamic range, μg/L	2–65	1–50
Correlation coefficient	0.997	0.999
Detection limit, μg/L	1.1	0.8
Quantitation limit, μg/L	3.8	2.5
Reproducibility, RSD%[b]	4.6	4.4
Repeatability, RSD%[c]	3.6	1.7

[a] Mean ± standard deviation ($n = 3$).
[b] 17 μg/L SO_3^{2-} (100–200 mesh) and 19 μg/L SO_3^{2-} (200–400 mesh), ($n = 10$).
[c] 41 μg/L SO_3^{2-} (100–200 mesh) and 10 μg/L SO_3^{2-} (200–400 mesh), ($n = 10$).

Effects of Foreign Ions

Interference by foreign ions, which are commonly found in water, was studied. These foreign ions may cause oxidation of the analyte in the determination of sulfite at the 16 μg/L level. Tolerance is defined as the concentration of foreign ion that produces an error of ± 5% in the determination of the analyte. The results are summarized in Table 2. These tolerances can be increased up to 50 mg/L for Cl^-; 100 mg/L for NO_3^-, and 10 mg/L for Ca(II) by using a blank with the foreign ion.

Interference was negative for most of the ions and positive for Cl^-, NO_3^-, SO_4^{2-}, SiO_3^{2-}, Ca(II), Mg(II), and NH_4^+.

The absorbance of the blank was not affected by the presence of most ions tested. However, the absorbance of the blank increased linearly with concentration of some anions. Table 3 summarizes these dependencies and also the false-positive levels, i.e., the maximum concentration of these anions that produce an absorbance lower than that of the IUPAC quantitation limit of the standard procedure, without analyte. Greater concentrations would produce, in the absence of the sulfite, the false positive results.

Table 2. Effect of foreign ions on the determination of 16 μg/L sulfite

Foreign ion	Tolerance level, μg/L
SO_4^{2-}	250000
PO_4^{3-}, SiO_3^{2-}	20000
NO_3^-, Cl^-	5000
F^-	3000
NH_4^+	2000
Ca(II), Mg(II)	1000
NO_2^-, Al(III)	100
Mn(II)	50
Cu(II)	30
Cr(III)	20
Fe(III), Pb(II)	10

Table 3. Effect of foreign anions on the blank absorbance

Anion	Blank absorbance vs concentration of foreign ions	False-positive level, μg/L[a]
SiO_3^{2-}	A = 0.057 + 0.0057 C(mg/L), r = 0.991	2600
NO_3^-	A = 0.043 + 0.0015 C(mg/L), r = 0.996	19000
Cl^-	A = 0.057 + 0.0007 C(mg/L), r = 0.980	21000
SO_4^{2-}	A = 0.052 + 0.0007 C(mg/L), r = 0.999	29000

[a] Maximum concentration of foreign anion that produces an absorbance lower than that of the IUPAC quantitation limit of the present method, in the absence of analyte.

On the other hand, the lineal dependence observed between the absorbance and the concentration of nitrate in the $Ca(NO_3)_2$ or $Mg(NO_3)_2$ solutions, both with and without analyte, is similar to that observed between the absorbance and the concentration of nitrate in the $NaNO_3$ solutions. These findings show that the positive interference observed in the tests for Ca(II) and Mg(II) might be explained by the presence of NO_3^- as the anion in the cation solutions.

Analysis of Sulfite in Simulated Rainwater

Because established and constant conditions are required for some experiments to verify aspects of the application of the proposed method, we prepared a matrix of rainwater by dissolution of salts and acids in water. This simulated rainwater required careful preparation; therefore, we selected the procedure proposed by Koch et al. (10).

Calibration Curves for Simulated Rainwater

We obtained different calibration curves for simulated rainwater. Aliquots of 10, 25, 50, and 100 mL simulated rainwater SRM 2694-I containing concentrations of sulfite in the range of 4–30 μg/L were analyzed as described under *Analytical Procedure*. The slopes of the calibration curves for small volumes had a similar value, and they were equal to the slope of the standard calibration curve of the proposed method. Only sample volumes equal to or higher than 50 mL produced a decrease in the slope of calibration curves.

High pararosaniline concentrations and low pH values are necessary for the formation of pararosanilinemethylsulfonic acid (11). The decrease of the slope is justified when large volumes of sample are used. Thus, for volumes equal to or higher than 50 mL, the relative concentration of the reagents during mixing with the sulfite is lower than the optimum values, and low quantities of reaction product are formed. However, we have no definitive proof to support this hypothesis.

Sampling and Stability of Sulfite

The stability of sulfite in simulated rainwater during and after sampling was studied. Solutions with 0.1 and 0.7 mg/L sulfite on a matrix such as an SRM 2694-I solution and in the presence of 2.4×10^{-3}% and 3.0×10^{-3}% morpholine, respectively, were used. The results of the experiments proved that both solutions were stable for at least 10 days if the solutions were stored under refrigeration (7°–8°C). The stability experiments carried out for similar solutions, but in sampling conditions as described under *Collection and Treatment of Rainwater Samples*, proved that the solution with a low concentration of sulfite was stable for 5 h when the concentration of morpholine was 2.4×10^{-3}%, while the solution with a high concentration of sulfite was stable for 1.5 h if the concentration of morpholine was 3×10^{-3}%. Nevertheless, stability can be increased to 2.5 h if the concentration of morpholine is 7×10^{-3}%.

The presence of certain metals is a problem for the stability of sulfite in solution, due to their catalyzing action in the reaction of oxidation of sulfite. A study of the effect of metals on the stability of sulfite in rainwater after sampling was carried out. The metals selected were Cu(II), Fe(III), Mn(II), and Pb(II) because they are more frequently found in the environment or present a relatively larger interference in the proposed method. For this study, 2 solutions were prepared as follows: on a matrix such as a SRM 2694-I solution, containing 2.4×10^{-3}% or 5.6×10^{-3}% morpholine and 0.09 mg/L or 0.60 mg/L sulfite, respectively, the selected metals were added in equal concentrations of 20 and 50 μg/L, respectively. The results proved that both concentrations of sulfite were stable at least for 14 h if the solutions are stored under refrigeration (7°–8°C).

These studies show that the sampling time of real rainwater should not be longer than 2 h to avoid loss of analyte. On the other hand, if the samples are stored under refrigeration (7°–8°C), the analysis may be delayed between 14 h and 10 days depending on metal concentrations in rainwater.

To confirm these facts, a rainwater sample was collected in 2.5×10^{-3}% morpholine, and the sulfite in 25 mL aliquots of the rainwater sample was analyzed, in duplicate, by the standard procedure with a calibration curve for 3 h. The metal content of the sample was also analyzed, and the results are as follow: Ca(II), 1 mg/L; Fe(III), 51 μg/L; Cu(II), 38 μg/L; Mn(II), 4 μg/L; and Pb(II), 10 μg/L. In this period, the sulfite content in the sample was stable (31 ± 1 μg/L).

Comparison of SPS and Solution Methods

A comparative study of the SPS and the solution method based on the similar reaction (3) with the modifications of Scaringelli et al. (7) was developed. As the best procedure for the study, we chose an application of the statistical model of single lineal regression on the calibration curves (12). It allows a comparative study under similar experimental conditions with a relatively small number of experiments. In addition, this model has the advantage in that the errors inherent in the calibration curves are included.

The study was developed using an SRM 2694-I solution. We analyzed 5 samples with a quantity of sulfite ranging from 0.0 to 4.75 μg, in quintuplicate, by both the SPS and the solution method (final volume 13 mL for the latter). The results obtained are shown in Table 4. Table 5 shows the quality parameters calculated from these results. Examination of the data in Table 5 shows that the SPS method presents better linearity and lower RSD values of the concentration, RSD(C)%, (13) than does the solution method in all ranges of concentrations.

If we compare the analytical sensitivity (14), the SPS method lets us discern between lower differences of concentrations than does the solution method. Furthermore, Table 5 also shows that the quantitation limits for the solution method and the SPS method are about 110 and 4 µg/L, respectively.

On the other hand, Table 4 shows the correlation between the SPS and the solution method. The confidence intervals of slope and intercept do not differ significantly from 1 and 0, respectively, indicating that the accuracy of both methods is similar.

Recovery in Simulated Rainwater

To check the accuracy of the proposed method, a recovery study was carried out in triplicate on a matrix such as an SRM 2694-I solution. Different amounts of sulfite in the range of 10–125 µg were added to 250 mL SRM 2694-I solution, and the percentage of sulfite recovered was determined. Table 6 shows the results obtained for each sample volume analyzed. The percentage of sulfite recovery is between 97 and 105%.

Determination of Sulfite in Rainwater Samples

The proposed method was applied to the determination of sulfite in real rainwater samples by the standard additions method. The rainwater samples were collected as described under *Collection and Treatment of Rainwater Samples* throughout February 1993. The sample volumes collected ranged from 20–150 mL, and the morpholine concentration was between 2.5×10^{-3}% and 7.2×10^{-3}%. The pH of each collection, which ranged between 6.8 and 7.0, was measured in a container without additives. Ca(II), Cu(II), Mn(II), Pb(II), and Fe(III) were measured by AAS in the container with HNO_3. The results were in the range of 10–17 mg/L, 19–57 µg/L, 14–32 µg/L, 22–53 µg/L, and 167–245 µg/L, respectively.

The results of the rainwater analysis are presented in Table 7. The levels of sulfite found ranged from 150 to 480 µg/L. The concentration of sulfite in each sample agreed well when known amounts of sulfite were added before the analyses. The recoveries obtained were 95–105%. These results confirm the validity of the proposed method for the determination of sulfite in rainwater.

Table 4. Comparative study of the SPS and the solution method[a]

Sulfite added, µg	Sulfite found, µg[b]	
	SPS method	Solution method
0.00	–0.05	–0.07
0.95	0.97	1.16
1.90	2.10	2.07
3.33	3.55	3.45
4.75	4.84	4.85

[a] In simulated rainwater.
[b] Each value is an average of 5 determinations.
Linear regression: y(SPS) = –0.045 + 1.016 x(Solution); r= 0.999
For $t_{(P=0.05;\ DF=23)} = 2.069$:
$a \pm ts_a = -0.045 \pm 0.112$
$b \pm ts_b = 1.016 \pm 0.039$

Table 5. Quality parameters of the SPS and the solution method[a]

	SPS method	Solution method
Linearity, %	97.7	96.8
Analytical sensitivity, µg/L	0.8	21
Calibration sensitivity, signal per µg/L	34.9	0.5
Detection limit, µg/L	1.3	34
Quantitation limit, µg/L	4.2	113
	Precision	
µg	RSD(C)%	
0.95	10.7	21.6
1.90	4.6	7.9
3.33	2.9	4.2
4.75	2.3	3.3

[a] The statistical model proposed in ref. (12) was used.

Determination of Sulfite in Mineral Water

The method was applied to the determination of sulfite in water by the standard additions method. A commercially available mineral water, which was not pretreated with chlorine before bottling, was selected as a representative sample. For all measurements, 50 mL water was used. The average value (3 determinations) of the sulfite content was lower than the IUPAC quantitation limit. To check the accuracy of the proposed method, a recovery study was carried out in which various amounts of sulfite were added to 1 L samples of the above mineral water, and aliquots were analyzed as described under *Analytical Procedure*. Recoveries of 97–107% indicate that the proposed method can be applied satisfactorily to the determination of sulfite in mineral water.

Acknowledgments

This research was funded by the Dirección General de Investigación Científica y Técnica (DGICYT) del Ministerio de

Table 6. Recovery study for sulfite in simulated rainwater samples

Sample	Sample taken, mL	Sulfite found, µg[a]	Sulfite in sample, µg/L	Sulfite added, µg/L	Recovery, %
A	5	2.6 ± 0.1	520	497	105
B	10	3.12 ± 0.07	312	298	105
C	25	2.4 ± 0.2	96	99	97
D	50	2.09 ± 0.04	42	40	105

[a] Mean ± standard deviation (n = 3).

Table 7. Determination of sulfite in rainwater samples

Sample	Sample taken, mL	Amount added, µg	Amount found, µg[a]	Recovery, %	Sulfite in sample, µg/L
10/2/93	5	—	2.4	—	480
		1.9	4.2	94.7	—
		4.7	7.2	102.1	—
11/2/93	2	—	0.3 ± 0.1[b]	—	150
A		1.9	2.2 ± 0.1	100.0	—
		4.7	5.0 ± 0.3	100.0	—
11/2/93	4	—	0.7 ± 0.1	—	175
B		0.9	1.6 ± 0.1	100.0	—
		2.4	3.1 ± 0.2	100.0	—
		4.7	5.3 ± 0.2	97.9	—
12/2/93	4	—	0.9 ± 0.2	—	225
		1.9	2.8 ± 0.1	100.0	—
		4.7	5.4 ± 0.3	95.7	—
27/2/93	10	—	1.7 ± 0.3	—	170
		0.9	2.6 ± 0.1	100.0	—
		1.9	3.7 ± 0.1	105.3	—
		2.8	4.4 ± 0.1	96.4	—

[a] Mean ± standard deviation ($n = 3$).
[b] Value lower than IUPAC quantitation limit.

Educación y Ciencia (Spain) (Project No. PS88-0101). M. Nechar received financial support from the Instituto de Cooperación con el Mundo Arabe del Ministerio de Asuntos Exteriores (Spain).

References

(1) Methods for the Examination of Waters and Associated Materials (1987) *Determinations of Sulfite, Sulfur Dioxide, Thiosulphate and Thiocyanate, with Notes on the Determination of Total Sulfur and Other Sulfur Compounds*, HMSO Books, London, UK, pp. 12–18
(2) Davies, T.P. (1984) *Atmos. Environ.* **18**, 2499–2502
(3) West, P.W., & Gaeke, G.C. (1956) *Anal. Chem.* **28**, 1816–1819
(4) Yoshimura, K., & Waki, H. (1985) *Talanta* **32**, 345–352
(5) Capitán, F., Capitán-Vallvey, L.F., Valencia, M.C., Bosque-Sendra, J.M., Molina, F., & de Orbe, I. (1991) *Analusis* **19**, 177–179
(6) Bosque-Sendra, J.M., Molina, F., & López, E. (1991) *Analyst* **116**, 871–874
(7) Scaringelli, F.P., Saltzman, B.E., & Frey, S.A. (1967) *Anal. Chem.* **39**, 1709–1719
(8) IUPAC (1978) *Spectrochim. Acta Part B At. Spectrosc.* **33**, 242–245
(9) Analytical Methods Committee (1987) *Analyst* **112**, 199–204
(10) Koch, W.F., Marinenko, G., & Paule, R.C. (1986) *J. Res. Natl. Bur. Stand.* **91**, 33–41
(11) Dasgupta, P.K., DeCesare, K., & Ullrey, J.C. (1980) *Anal. Chem.* **52**, 1912–1922
(12) Cuadros Rodríguez, L., García Campaña, A.M., Jiménez Linares, C., & Román Ceba, M. (1993) *Anal. Lett.* **26**, 1243–1258
(13) Miller, J.C., & Miller, J.N. (1988) *Statistics for Analytical Chemistry*, Ellis Horwood, Ltd, Chichester, UK, pp. 112–115
(14) Ingle, J.D., Jr (1971) *J. Chem. Ed.* **51**, 100–105

RESIDUES AND TRACE ELEMENTS

Determination of Phthalates in Water and Soil by Tandem Mass Spectrometry Under Chemical Ionization Conditions with Isobutane as Reagent Gas

WILLIAM C. BRUMLEY, ELIZABETH M. SHAFTER, and PAUL E. TILLANDER [1]
U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, PO Box 93478, Las Vegas, NV 89193-3478

Eleven phthalate esters spiked in water and soil were determined by tandem mass spectrometry (MS) under positive chemical ionization mass spectrometry (CIMS) conditions with isobutane as reagent gas. Emphasis was placed on the determination step because tandem MS and CIMS are not widely adopted in current methods of the U.S. Environmental Protection Agency. Extraction by sonication and cleanup by use of a solid-phase extraction cartridge were adopted. The relative response factors gave relative standard deviations (RSDs) of 12–30% when 2 unlabeled internal standards were used. The relative abundances of monitored ions gave relative abundance deviations of less than ± 8%. The method confirms identity, including molecular weight, and quantitates with high specificity. Results obtained with 2 unlabeled internal standards were compared with results obtained with a stable-isotope-labeled internal standard for dioctyl phthalate.

Phthalates are diesters of 1,2-benzenedicarboxylic acid (phthalic acid) and are of almost ubiquitous occurrence because of their widespread use as plasticizers. They are used also as solvents, insect repellants, and additives to plastic explosives.

Phthalates are target analytes described in the U.S. Environmental Protection Agency (EPA) Solid Waste 846 Methods Manual (Methods 8060 and 8270) (1) and in U.S. EPA Method 606 (2). The toxicity, mutagenicity, and carcinogenicity of bis(2-ethylhexyl)phthalate was reviewed recently (3). This compound is listed as "reasonably anticipated to be a carcinogen" (4). Many phthalate esters may be irritating to mucous membranes (5).

Regardless of the health effects in humans, phthalates need to be determined because they are often present as overwhelming interferences and nuisances in samples used for the trace determinations of other pollutants. They are, therefore, of continuing interest to the Environmental Monitoring Systems Laboratory in Las Vegas. It is necessary to identify the troublesome phthalate compounds and to design appropriate steps to eliminate or reduce their effects on analyses.

Contamination by phthalates is also of concern in other areas. In the biomedical field, for example, the presence of phthalates in parenterally introduced fluids and feeding tubes was studied (6, 7). Various analytical approaches to determination of phthalates in foods were reviewed (8).

Some gas chromatographic (GC) methods of determining phthalates involve solid-phase extraction (SPE) in sample preparation (9, 10). Other GC methods involve cleanup with sulfuric acid (11). These methods have been used to determine phthalate contamination of blood plasma (12), the marine environment (13), various plastic tubes (6), infusion products (7), food (8), the aquatic environment (14–16), and solvents (17).

Phthalate determinations are based also on liquid-chromatographic separations (18–20) with UV detection. Polarography was used as a detection technique to determine phthalates in water (21). The use of micellar electrokinetic chromatography to separate phthalates has been reported (22).

Thin-layer chromatography (TLC) also has been used to separate phthalates. SPE of phthalates from water followed by TLC separation was used by Sherma et al. (23). Further TLC work emphasized the general chromatographic behavior of phthalates (24–25).

The mass spectrometry and fragmentation of phthalates by electron impact (EI) ionization were studied by several workers (26–29). Chemical ionization mass spectrometry (CIMS) also has been used to determine phthalates (30, 31). Yinon (32) studied the collision-induced dissociation spectra of phthalates. He found that the base peak of EI mass spectra at *m/z* 149 was formed by 4 alternative pathways. He also observed the presence of fewer fragment ions in mass spectra when only the decompositions of M^+ ions were considered.

Tandem mass spectrometry (MS) is not widely adopted in U.S. EPA methodology, especially as GC/tandem MS applications. Hunt and co-workers (31) presented a scheme for using tandem MS in environmental analysis. In view of the high specificity of tandem MS, we use it when very low levels of analyte are to be determined or when excessive cleanup opera-

Received July 15, 1993. Accepted by JS October 1993.
[1] Deceased.

tions are required. CIMS also is a reasonable choice when molecular weight information is lacking in EI mass spectra or when more selective ionization is sought. In this paper, we present a procedure for determining phthalate esters that is based on tandem MS under positive CIMS with isobutane as reagent gas. Performance data are reported for 11 phthalates, and the specificity of the method is discussed.

Experimental

Chemicals

The phthalate esters were obtained from Chem Service, Inc., West Chester, PA. A standard solution containing compounds **1–11** (defined below) in hexane and a separate internal standard solution containing **12** and **13** were made up with the following concentrations (ng/μL) of phthalate:

1, dimethyl phthalate (12.3); **2**, diethyl phthalate (21.0); **3**, dibutyl phthalate (15.5); **4**, bis(methoxyethyl) phthalate (38.3); **5**, bis(ethoxyethyl) phthalate (58.5); **6**, dicyclohexyl phthalate (81.0); **7**, bis(2-ethylhexyl) phthalate (50.4); **8**, di-*n*-octyl phthalate (70.4); **9**, butylbenzyl phthalate (143.0); **10**, butyloctyl phthalate (65.4); **11**, diamyl phthalate (124.0); **12**, dimethyl isophthalate (20.5); **13**, hexyl-2-ethylhexyl phthalate (74.0).

Sample Preparation

Aqueous samples were subjected to liquid–liquid extraction in a separatory funnel as described in SW-846 Method 3510 (1) with slight modifications. Briefly, 0.25 mL standard was added to 1.5 mL methanol; this solution was spiked into 250 mL deionized (ASTM Type II) water (Barnstead/Thermolyne, Dubuque, IA). Methanol was used to help disperse the phthalates in water. The spiked solution was then extracted successively with 12, 6, and 6 mL methylene chloride, and the 3 extracts were combined. The combined extract was concentrated under a gentle stream of nitrogen while warming with a hot-air gun. A final volume of 0.1–1.0 mL was reached after addition of 0.25 mL internal standard containing **12** and **13**. All spikings were done by syringe (Hamilton Co., Reno, NV).

Soil samples obtained locally in Las Vegas and soil B (L. A. Clark Superfund site, Spotsylvania, VA) were extracted according to SW-846 Method 3550 (1) with slight modifications. Briefly, a 10 g sample of soil was extracted by sonication. Soils were spiked with 100 μL of the standard before extraction. Methylene chloride–acetone (50 + 50, v/v) (30 mL) was used with a sonicator (model W-375, Heat Systems-Ultrasonics, Inc., Farmingdale, NY) for extractions.

Extracts were filtered through a 5.5-cm-diameter filter (Schleicher and Schuell, lot no. 0-891) by using a Buchner funnel connected to a vacuum pump (Edwards Model E2M-1, Crawley Sussex, England). The filtrate was dried by blowing a gentle stream of nitrogen while warming with a hot-air gun. The filtrate residue was dissolved in 1 mL hexane.

The solid-phase extraction (SPE) cleanup was carried out with either a Supelco (Bellefonte, PA) LC-Florisil (LC-Fl) 6 mL cartridge or a Varian (Sunnyvale, CA) LC-Fl 1 mL cartridge. The 6 mL cartridge was used on the heavily contaminated soil B to increase adsorbent capacity. The 1 mL cartridge was used with the local soil. Both sizes of cartridges, although obtained from different manufacturers, performed similarly in experiments with standards. The Supelco LC-Fl cartridge was first washed with 6 mL hexane. The sample was applied and then washed with 6 mL hexane. The phthalates were then eluted with 8 mL hexane–acetone (90 + 10, v/v). The same procedure was followed for the Varian cartridge. The Varian 1 mL cartridge was first washed with 4 mL hexane. The sample was added and then washed with 4 mL hexane. The phthalates were then eluted with 6 mL hexane–acetone (90 + 10, v/v). The eluate in both cases was then spiked with 100 μL internal standard solution containing **12** and **13** or the internal standard containing d_4-dioctyl phthalate and concentrated to 0.1–1.0 mL under a nitrogen stream.

GC/Tandem MS

A Finnigan MAT TSQ-45 instrument was used to analyze the final extracts. The instrument was interfaced to an INCOS 2300 (Nova 4X) data system with TSQ software (Rev 6.6). The collision-activated decompositions (CAD) were monitored under experimental control of the data system. A multiexperiment descriptor (terminology used by INCOS) was created that contained 5 experiment descriptors. Each experiment descriptor controlled the CAD of 3 $(M + H)^+$ ions, with 3 ions monitored per decomposition. This procedure is known in the literature as multiple-reaction monitoring. Some redundancy was incorporated in the descriptors to keep all experiments equivalent [i.e., 12 unique $(M + H)^+$ ions were used among 15 total $(M + H)^+$ ions whose CADs were followed]. The dwell time per ion was 0.18 s, resulting in a total cycle time of 0.54 s per experiment. The number of cycles for each experiment was adjusted to divide the entire run into 5 retention-time windows. Automated procedures were created to process the data.

Samples were introduced to the standard 9610 gas chromatograph (GC) by on-column injections using a modified injector (33). The retention gap consisted of a 3 m × 0.53 mm id, deactivated, fused-silica column. The capillary column was a 30 m × 0.25 mm id SPB-5 (Supelco, Inc., Bellefonte, PA) column with 0.25 μm film thickness. The temperature was programmed from 50° to 300°C at 20°C/min; the flow rate (He) was 30 cm/s at 50°C. The analytical column was connected to the mass spectrometer via an uncoated, deactivated transfer line (1 m × 0.25 mm id) held at 260°C. The isobutane (Aldrich Chemical Co., Milwaukee, WI) reagent gas was introduced from the makeup (GC) side at a source pressure of 0.35–0.40 torr, as read from a standard Finnigan-MAT ionizer pressure gauge. The source temperature was 110°C (dial setting), the emission current was 0.27 mA, the electron energy was 70 eV, the preamplifier was set at 10^{-8} A/V, and the electron multiplier and conversion dynode were set at −1300 V and −3 kV, respectively. The collision gas was Ar (Scott Specialty Gases, Inc., Plumsteadville, PA), maintained at 1.6–2.0 mtorr (Hastings Gauge) so that the analyzer pressure reading was 4.2×10^{-5} torr.

Table 1. Response factor performance data for tandem MS of phthalates

Compound	Reference compound	Av. RF[a]	RSD, %	*m/z* of $(M + H)^+$	*m/z* of other monitored ions[b]
1	12	3.76	12.5	195	163,151
2	12	2.47	15.0	223	177,149
3	13	7.21	25.0	279	205,149
4	13	1.09	23.1	283	207,149
5	13	0.80	23.2	311	221,073
6	13	1.82	24.4	331	249,149
7	13	2.11	23.4	391	261,149
8	13	1.90	30.7	391	261,149
9	13	1.21	25.9	313	205,149
10	13	7.24	23.7	307	219,149
11	13	2.74	19.6	335	205,149
12	—[c]	—	—	195	163,151
13	—	—	—	363	233,149

[a] The RF is calculated by the INCOS data system according to the following equation:
RF = (area of analyte ion × ng of internal standard)/(area of internal standard ion × ng of analyte).
[b] The underlined ions were used for quantitation.
[c] NA, not applicable.

Results and Discussion

Tandem MS

The determination involved a multiexperiment that followed the decompositions of 13 $(M + H)^+$ ions representing the 11 analytes and 2 internal standards. For each decomposition, 3 ions were monitored: the $(M + H)^+$ ion and 2 product ions. (With **1**, only 2 ions were observed in the CAD spectrum.) One of these 3 ions was used for quantitation (the *m/z* values of these ions are underlined in Table 1). The multiexperiment, which consisted of 5 experiments, divided the entire run into 5 retention-time windows. Thus, the total acquisition cycle times (0.54 s) were capable of reproducing the chromatography.

Isobutane was chosen as the reagent gas to maximize the relative abundances of the $(M + H)^+$ ions of the phthalates and to offer some selectivity in terms of compounds that would be ionized on the basis of the gas-phase acidity scale. Table 1 lists response factors (RFs), their relative standard deviations (RSDs), and the ions monitored (the ion used for quantitation is underlined). The data represent a 5-point calibration curve that spanned about a factor of 10 in concentration. Each of the 5 points was determined in triplicate. All quantitations were made within the working range of the calibration curve.

The RSDs for response factors ranged from about 12 to 30%. These RSDs are not untypical for multianalyte determinations with internal standards that are not isotope-labeled analogues of individual analytes (1). These data support the use of this approach to determine phthalates.

It was of interest to compare RSDs obtained with 2 internal standards to those obtained with a stable isotope-labeled internal standard. The RF data for **8**, obtained with a ring-labeled d_4 analogue of **8**, gave an RSD of 4% (data not shown). As expected, the data obtained with isotope-labeled standard showed better precision than the data obtained without isotope-labeled internal standards.

Another parameter of interest in tandem MS determinations is reproducibility of the relative abundances of the ions monitored. This reproducibility affects our ability to confirm the identity of each compound. Table 2 lists the relative abundances of ions monitored and the RSDs for relative abundances. A more common concern to mass spectrometrists is the percent relative abundance error (expressed as ±). These values were below ± 8% and therefore were within customary expectations (for examples, SW-846 Method 8290 specifies a ± 15% relative abundance error for dioxin determinations). Therefore, we concluded that the identity of phthalates could be confirmed by monitoring 3 ions per compound (for compound **1**, only 2 ions were monitored) and observing the responses at the proper retention times.

The CADs observed in this study generally consisted of the loss of ROH (i.e., the alcohol moiety) and the production of *m/z* 149. Figure 1 illustrates a typical product ion spectrum from *m/z* 279 of **3**. Product ions were few compared with the

Table 2. Reproducibility of relative abundances (RA) of ions monitored for phthalates[a]

Compound	RA (first ion), %	RSD, %	RA error, %	RA (second ion), %	RSD, %	RA error, %
1	(195) 17.9	10.9	2.0	NO	NO	NO
2	(223) 25.1	10.0	3.0	(149) 74.2	11.3	8.0
3	(279) 12.6	20.8	3.0	(205) 33.1	6.9	2.0
4	(283) 18.4	6.4	1.0	(149) 0.31	95.0	0.3
5	(311) 15.0	7.9	1.0	(221) 92.6	6.9	6.0
6	(331) 23.4	25.9	6.0	(231) 14.8	16.8	2.0
7	(391) 8.9	13.0	1.0	(261) 4.6	11.5	0.5
8	(391) 5.7	5.3	0.3	(261) 15.3	7.9	1.0
9	(313) 25.3	17.4	4.0	(205) 57.5	12.0	7.0
10	(307) 9.0	12.6	1.0	(219) 23.7	6.4	2.0
11	(335) 9.2	15.7	1.0	(205) 18.7	14.3	3.0

[a] Values in parentheses are *m/z* values of the observed ions. NO, not observed.

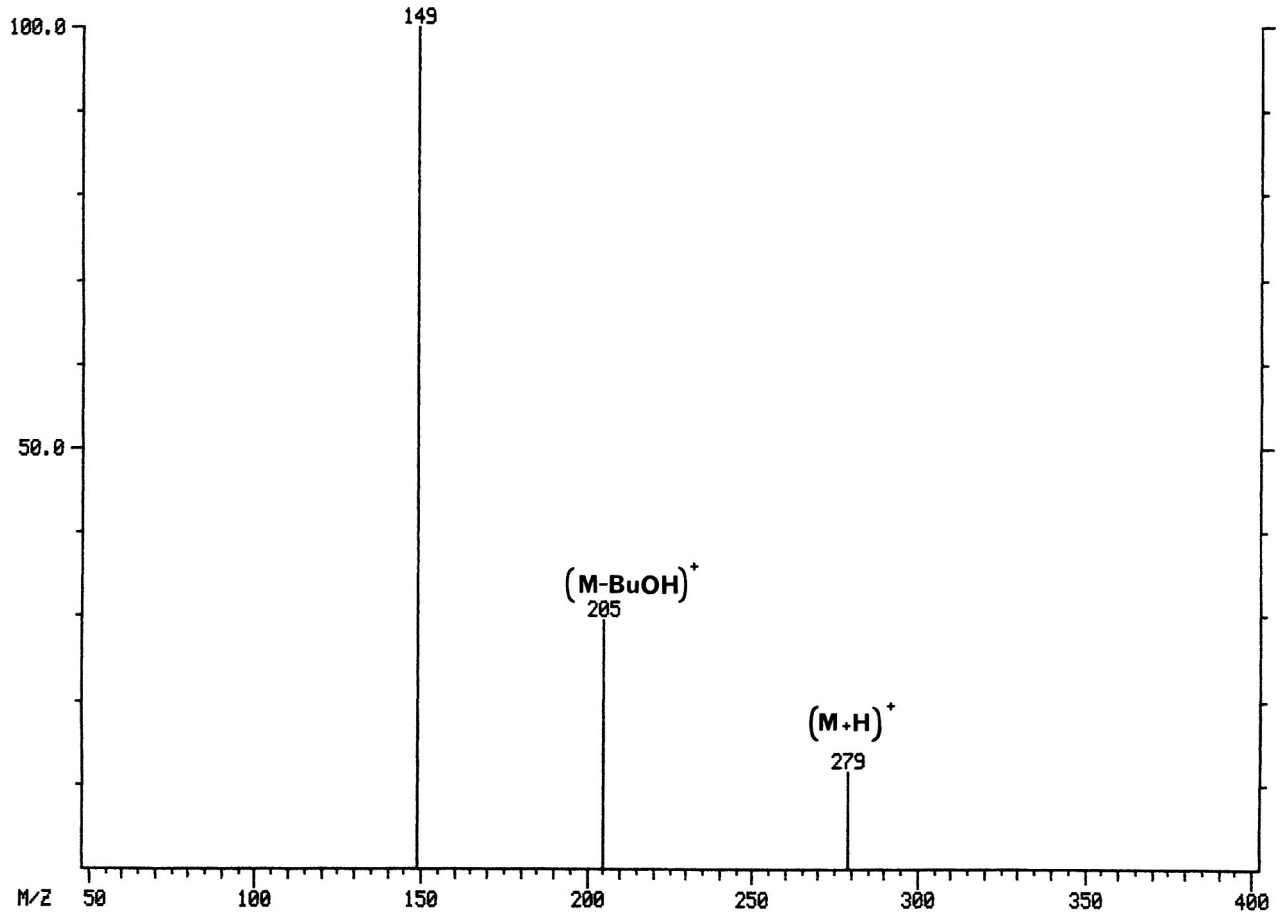


Figure 1. Product ion mass spectrum of *m/z* 279, $(M + H)^+$ of dibutyl phthalate.

numerous fragment ions observed in EI mass spectra. Compound **1** could not readily produce a product ion at *m/z* 149. Compounds **4** and **5** also were exceptions in that a low relative abundance of *m/z* 149 was produced. These compounds (**4** and **5**) showed charge retention on the alcohol moiety of the phthalate ester.

Precision of Determination and Recoveries of Phthalates from Water and Soil

Recoveries determined by tandem MS for spiked water samples are tabulated in Table 3 along with RSDs based on 3 determinations. The overall method precision (from spiking to determination of phthalates in the final extract) was acceptable; RSD values ranged from 2 to 7%.

Results of triplicate determinations of soil spiked with phthalates at 0.1–1.0 μg/g are given in Table 4. The average recoveries ranged from 22 to 68% for compounds not found in the method blank (i.e., all compounds except **3** and **7**). The precision of determination by tandem MS for a single sample extract injected 3 times gave RSDs of 2.5 to 25% for all compounds not in the method blank (i.e., except **3** and **7**). These values parallel the RSDs for the relative response factors of the compounds (*see* Table 1 for comparison). To place this variation in perspective, we studied the precision of determination by tandem MS of a single extract with d_4-dioctyl phthalate as

Table 3. Precision of recovery of phthalates from spiked water[a]

Compound	Rec. 1, %	Rec. 2, %	Rec. 3, %	Av. rec., %	RSD, %
1	92	105	100	99	6.6
2	118	129	124	124	4.5
3[b]	93	194	76	121	53
4	56	65	51	57	7.1
5	73	79	66	73	6.5
6	72	78	81	77	4.5
7	95	98	93	95	2.5
8	91	86	82	86	4.5
9	64	68	71	68	3.5
10	69	77	78	75	4.9
11	72	74	82	76	5.3

[a] The spiking levels for compounds 1 to 11 were, respectively (ng/g): 12.3, 21.0, 15.5, 38.3, 58.5, 81.0, 50.4, 70.4, 143., 65.4, and 124. The recovery was calculated as follows:
ng of analyte = (area of analyte ion × ng of internal standard)/(area of internal standard ion × RF); recovery of analyte (%) = ng of analyte/ng analyte spike × 100%.

[b] Present as a contaminant in syringe blanks.

Table 4. Precision of determination of phthalates in the final extract of a single spiked soil[a]

Compound	Rec. 1, %	Rec. 2, %	Rec. 3, %	Average recovery, %	RSD, %
1	18	22	25	22	16.
2	42	40	47	43	8.4
3[b]	49	161	78	96	61.
4	54	49	51	51	4.9
5	47	64	40	50	25.
6	60	63	61	61	2.5
7[b]	408	411	420	413	1.5
8	66	70	69	68	3.0
9	51	71	48	57	22.
10	54	58	51	54	6.5
11	63	67	62	64	4.1

[a] The spiking level for compounds 1 to 11 were, respectively (μg/g): 0.123, 0.210, 0.155, 0.383, 0.585, 0.810, 0.504, 0.704, 1.43, 0.654, and 1.24.

[b] Present in method blanks.

internal standard. The RSD in this case was 2.2%. This result again pointed to improved precision when the deuterium-labeled internal standard was used.

Figures 2 and 3 show total-ion current responses for a standard and a spiked soil. The 5 retention-time windows are indicated in Figure 2, along with typical retention times. The observed patterns in the total-ion and the individual-ion chromatograms indicated no interferences from the matrix that prevented confirmation of phthalates. Method blanks did indicate that the laboratory was phthalate-contaminated with compounds **3** and **7** and, in later work, compound **2**.

Because the cleanup step used SPE-Fl cartridges, recovery from Florisil affected overall method performance. The recovery precision for Florisil and the observed average recoveries and RSDs were similar to those reported earlier (9).

Recoveries from spiked soils as determined by tandem MS are given in Table 5. The first 3 determinations (soil 1, soil 2, and soil 3) were for 3 portions of soil of local origin; for these, RSDs were 7–44%. Overall method precision (from spiking to final extract) was comparable with that of the multianalyte

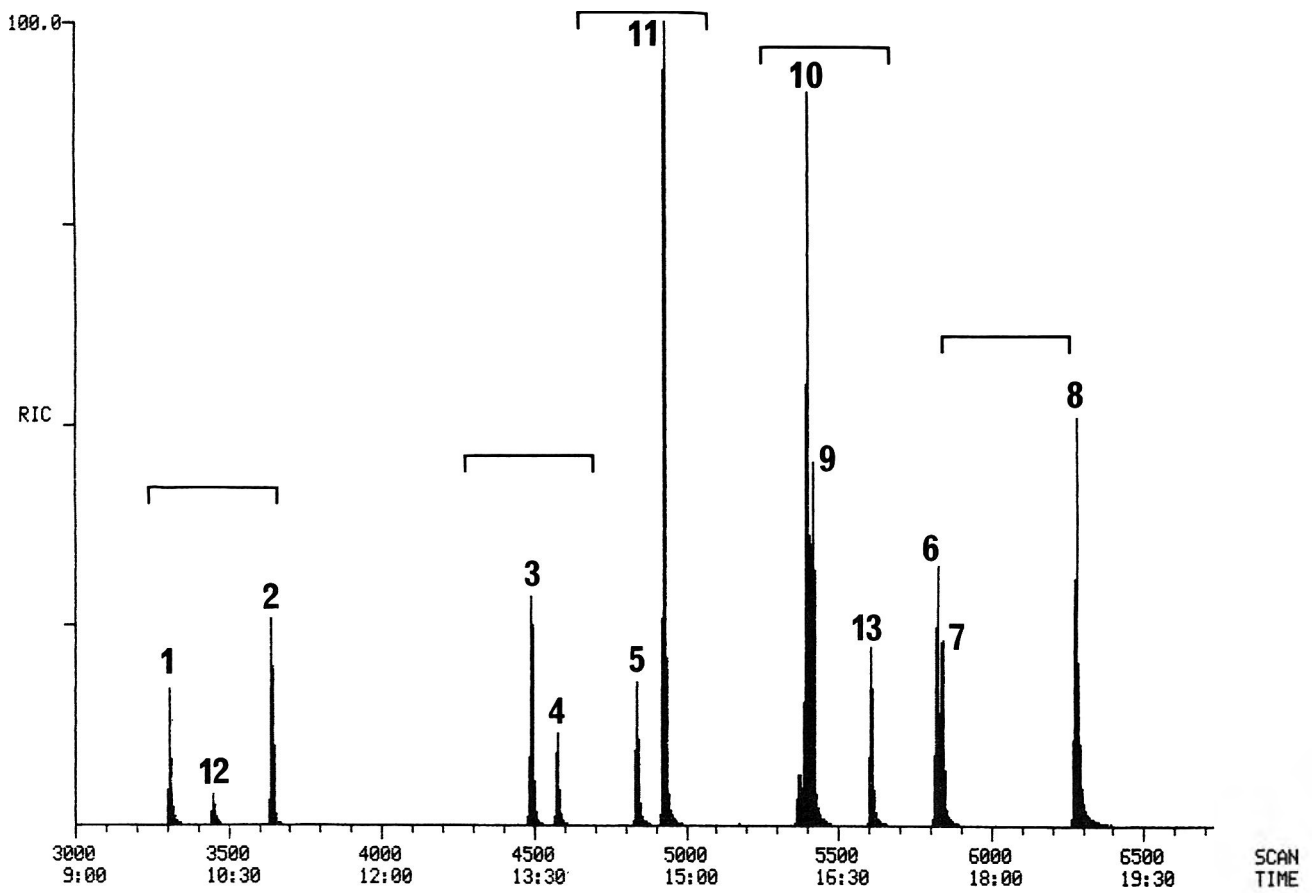


Figure 2. Total-ion current for multiexperiment with standards. Compound numbers are according to the text: 1, dimethyl phthalate, retention time (RT) = 9:56; 2, diethyl phthalate, RT = 10:56; 3, dibutyl phthalate, RT = 13:29; 4, bis(methoxyethyl) phthalate, RT = 13:44; 5, bis(ethoxyethyl) phthalate, RT = 14:32; 6, dicyclohexyl phthalate, RT = 17:29; 7, bis(2-ethylhexyl) phthalate, RT = 17:32; 8, di-*n*-octyl phthalate, RT = 18:51; 9, butylbenzyl phthalate, RT = 16:16; 10, butyoctyl phthalate, RT = 14:48; 11, diamyl phthalate, RT = 16:12; 12, dimethyl isophthalate, RT = 10:22; 13, hexyl-2-ethylhexyl phthalate, RT = 16:50. Brackets indicate the 5 groupings (experiments) making up the multiexperiment.

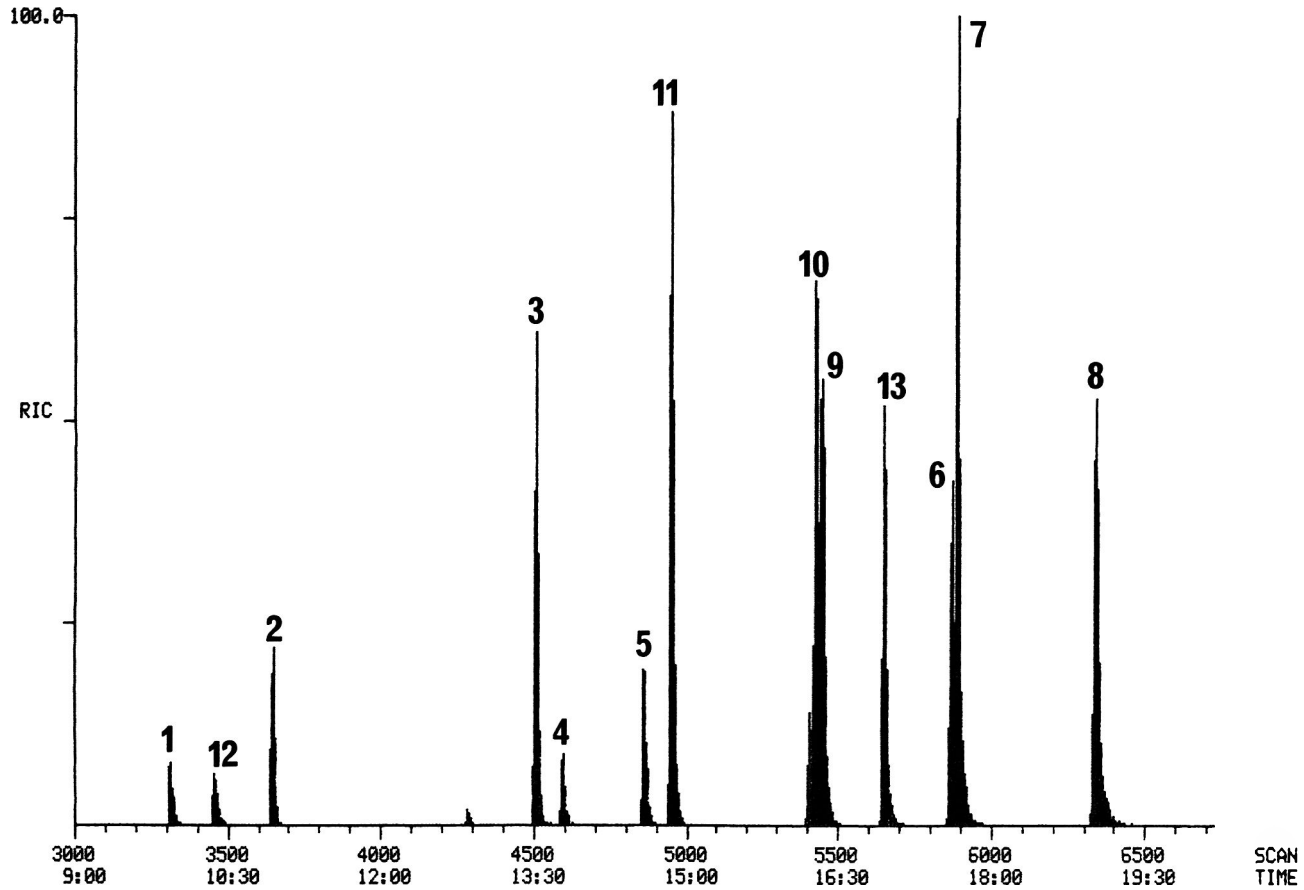


Figure 3. Total ion current for multiexperiment with spiked soil. Compound numbers are as given in Figure 2.

method. The RSDs for overall method precision reflect variances contributed by the extraction step, cleanup step, and determination step.

The fourth example (soil B) given in the table was heavily contaminated with creosote. The recovery of phthalates from this sample actually was better than from the local soil. The results of phthalate determination in an unspiked sample of soil B (given in parentheses relative to the spiking level used in spiked soils) indicated environmental contamination above method contamination blanks for compounds **3**, **7**, **8**, **9**, and **11**. These data represent the confirmation and quantitation of phthalates in a Superfund site soil sample at levels of <1 to >1.0 μg/g.

We also found additional laboratory contamination by compound **2** in subsequent work; this explains the high recovery of

Table 5. Recoveries of phthalates from spiked soils[a]

Compound	Rec. from Soil 1, %	Rec. from Soil 2, %	Rec. from Soil 3, %	Av rec., %	RSD, %	Rec. from Soil B
1	25	43	54	41	36	95
2	47	44	56	46	13	213[b]
3[c]	78	67	95	ND	ND	98
4	51	32	43	42	23	16
5	40	37	35	37	7	59
6	61	41	40	47	25	85
7[c]	420	144	140	ND	ND	752
8[d]	69	76	67	71	4	113 (1)
9[d]	48	38	41	42	12	75 (3)
10	51	24	27	34	44	78
11[d]	62	30	37	43	39	83 (8)

[a] The spiking levels are the same as given in footnote [a] in Table 4. ND, not determined.
[b] Present as a contaminant in the method for this soil.
[c] Present in method blanks.
[d] Present in unspiked Soil B; values in parentheses are percentages of the spiking level.

2 from this soil matrix. We believe that the overall higher recovery was due to improved recovery from SPE-Fl (compare with Table 4). There was no evidence of matrix interference (i.e., responses that were not phthalates). Enhanced recovery due to coextractives has been observed before in SPE (34, 35).

Conclusion

We applied tandem MS under CIMS conditions to the determination of phthalates as environmental contaminants. The precision, specificity, reproducibility of spectra, and calibration statistics were within reasonable expectations for a GC/MS multianalyte method. The use of stable-isotope-labeled internal standards improves method performance in terms of precision and is the technique of choice, but nonlabeled internal standards also produce data comparable with those from traditional MS approaches for multianalyte analyses.

Acknowledgments

This research was funded by the U.S. Environmental Protection Agency through its Office of Research and Development (ORD) and was conducted by the Environmental Monitoring Systems Laboratory in Las Vegas (EMSL-LV) to support ORD's Hazardous Waste Issue. It was subjected to ORD's peer and administrative reviews and was approved as an EPA publication.

References

(1) U.S. EPA (1986) *Test Methods for Evaluating Solid Waste; Physical/Chemical Methods*, Vol 1B, 3rd Ed., U.S. EPA, Washington, DC

(2) *Federal Register*, Pt. 136, App. A, Method 606, 40 CFR CH 1 (July 1, 1992 Edition)

(3) *Registry of Toxic Effects of Chemical Substances*, D. V. Sweet, (Ed.), DHHS (NIOSH): Cincinnati, OH, Publication No. 93-101-2, January 1993

(4) *Sixth Annual Report on Carcinogens*, (1991) DHHS, prepared for NIEHS, Research Triangle Park, NC, p. 172

(5) *The Merck Index*, 11th Ed., Merck & Co., Rahway, NJ, 1989

(6) Khaliq, M.A., Alam, M.S., & Srivastava, S.P. (1992) *Bull. Environ. Contam. Toxicol.* **48**, 572–578

(7) Halkiewicz, J., & Halkiewicz, A. (1988) *Farm. Pollut.* **44**, 16–21

(8) Page, B.D. (1988) *Food and Packaging Interactions*, J.H. Hotchkiss (Ed.), ACS Symposium Series 365, American Chemical Society, pp. 118–135

(9) Lopez-Avila, V., Milanes, J., Dodhiwala, N.S., & Beckert, W.F. (1989) *J. Chromatogr. Sci.* **27**, 209–215

(10) Lopez-Avila, V., Benedicto, J., & Milanes, J. (1989) *Evaluation of Sample Extract Cleanup Using Solid-Phase Extraction Cartridges*. Report EPA/600/4-89/059

(11) Thuren, A., & Soedergren, A. (1987) *Int. J. Environ. Anal. Chem.* **28**, 309–315

(12) Teirlynck, O.A., & Rosseel, M.T. (1985) *J. Chromatogr.* **342**, 399–405

(13) Waldock, M.J. (1983) *Chem. Ecol.* **1**, 261–277

(14) Millar, J.D., Thomas, R.E., & Shattenberg, H.J. (1984) *Method Study 16, Method 606 Phthalate Esters*, Report EPA-600/4-84-056

(15) Kohli, J., Ryan, J.F., & Afghan, B.K. (1989) in *Analysis of Trace Organics in Aquatic Environment*, B.K. Afghan & A.S.Y. Chau (Eds), CRC Press, Boca Raton, FL, pp 243–281

(16) Peterson, J.C., & Freeman, D.H. (1982) *Int. J. Environ. Anal. Chem.* **12**, 277–291

(17) Lopez-Avila, V., Milanes, J., Constantine, F., & Beckert, W.F. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 709–720

(18) Russell, D.J., & McDuffie, B. (1983) *Int. J. Environ. Anal. Chem.* **15**, 165–183

(19) Dong, M.W., & DiCesare, J.L. (1982) *J. Chromatogr. Sci.* **20**, 517–522

(20) Wu, J.C.G. (1991) *J. Environ. Sci. Health, Part A* **A26**, 1363–1385

(21) Tanaka, K., & Takeshita, M. (1984) *Anal. Chim. Acta* **166**, 153–161

(22) Ong, C.P., Lee, H.K., & Li, S.F.Y. (1991) *J. Chromatogr.* **542**, 473–481

(23) Sherma, J., Dryer, J., & Bouvard, J.J. (1986) *Am. Lab.* **18**, 28, 30–32

(24) Albert, L., Aldana, P., & Carbajal, C. (1986) *Rev. Soc. Quim. Mex.* **30**, 143–146

(25) Brinkman, U.A.T., & De Vries, G. (1983) *J. Chromatogr.* **258**, 43–55

(26) Ende, M., & Spiteller, G. (1982) *Mass Spectrom. Rev.* **1**, 29–62

(27) McLafferty, F.W., & Gohlke, R.S. (1959) *Anal. Chem.* **31**, 2076–2082

(28) Emery, E.M. (1960) *Anal. Chem.* **32**, 1495–1506

(29) Djerassi, C., & Fenselau, C. (1965) *J. Am. Chem. Soc.* **87**, 5756–5762

(30) Hunt, D.F., Shabanowitz, J., Harvey, T.M., & Coates, M.L. (1983) *J. Chromatogr.* **271**, 93–105

(31) Hunt, D.F., Shabanowitz, J., Harvey, T.M., & Coates, M.L. (1985) *Anal. Chem.* **57**, 525–537

(32) Yinon, J. (1988) *Org. Mass Spectrom.* **23**, 755–759

(33) Brumley, W.C., Brownrigg, C.M., & Grange, A.H. (1993) *J. Chromatogr.* **633**, 177–183

(34) Loconto, P.R. (1991) *LC-GC* **9**, 460–465

(35) Loconto, P.R. (1991) *LC-GC* **9**, 752–760

RESIDUES AND TRACE ELEMENTS

Determination of Methyl 2-Benzimidazolecarbamate in Bulk Fruit Juice Concentrates by Competitive-Inhibition Enzyme Immunoassay

RODNEY J. BUSHWAY, BARBARA E.S. YOUNG, [1] LANCE R. PARADIS, LEWIS B. PERKINS, SUSAN K. MARTIN, [2] and MYRNA P. BROWN [2]
University of Maine, Department of Food Science, 5736 Holmes Hall, Orono, ME 04469-5736

A polyclonal enzyme immunoassay (EIA) was used to quantitate methyl 2-benzimidazolecarbamate (MBC or carbendazim), a degradation product of benomyl, in bulk fruit juice concentrates. These concentrates are used by industrial producers to prepare juice or juice concentrates sold in supermarkets. Total sample analysis time was less than 18 min without cleanup or 35 min with cleanup. As many as 8 samples can be analyzed simultaneously, with a limit of quantitation of 10 ppb. The assay's dynamic range ran from 0.5 to 20 ppb MBC but was best from 0.5 to 10 ppb. Intra-assay coefficients of variation (CVs) varied from 4.0 to 13% for standards and from 4.1 to 26% for samples. Inter-assay CVs varied from 4.5 to 47% for standards and 5.6 to 22% for samples. Average recovery of several juice concentrates spiked at 10 to 290 ppb was 97%. A total of 140 juice concentrates comprising 20 different kinds of juice were analyzed by 2 EIA methods and one liquid chromatographic (LC) procedure. MBC-positive samples gave the following correlation coefficients: 0.954 for EIA without cleanup vs LC, 0.956 for EIA with cleanup vs LC, and 0.978 for EIA with cleanup vs EIA without cleanup. MBC concentrations in MBC-positive juice samples ranged from 5 to 2960 ppb.

Benomyl, a systemic benzimidazole fungicide, is used for preharvest treatment of fruits and vegetables in the United States. Benomyl degrades rapidly to methyl 2-benzimidazolecarbamate (MBC or carbendazim) in fruits and vegetables and in organic solvents. Carbendazim is used in Europe as a fungicide and thus has basically the same fungal activity as benomyl. With the recent U.S. Ninth Circuit Court of Appeals decision on the regulation of potentially carcinogenic pesticides (1) and the release of the National Academy of Sciences report concerning pesticides and children (2), there is an increased need to monitor MBC in the food supply.

Previous methods including chromatographic and immunoassay techniques have focused on fresh fruits and vegetables (3–7). One enzyme immunoassay (EIA) was used to determine residue levels of MBC in commercial fruit juices and their concentrates (8), but it does not work on bulk juice concentrates because these are several times more concentrated than the consumer or commercial concentrates. The increased strength causes pH and matrix problems with the EIA (8) developed for commercial concentrates.

This paper describes a rapid, inexpensive, and sensitive EIA for the quantitation of MBC in bulk concentrates. Such a method should be very useful to the food industry for monitoring of incoming bulk concentrates for MBC residues.

METHOD

Reagents and Standards

(a) *Reagents.*—All reagents for the preparation of immunogens for raising antisera to MBC were previously described (3, 8). All solvents were obtained from EM Science (Gibbstown, NJ). Phosphate salts, ammonium chloride, and ammonium hydroxide were purschased from VWR (Boston, MA).

(b) *Analytical standard.*—MBC pesticide standard was obtained from the U.S. Environmental Protection Agency (Research Triangle Park, NC).

(c) *Pesticide standard stock solution.*—Weigh 17 mg MBC into a 50 mL volumetric flask, dissolve, and dilute to volume with methanol–acetonitrile (90 + 10). Store at 4°C. It is stable for 2 months.

(d) *Diluting solvent for tube immunoassay with cleanup.*—Place 25 mL mobile phase (h) into a 250 mL volumetric flask and dilute to volume with skim or nonfat dry milk (prepare according to label directions). Prepare fresh.

(e) *Diluting solvents for tube immunoassay without cleanup.*—(*1*) Ammonium chloride (0.5M) containing 0.025 mL ammonium hydroxide per 1 mL ammonium chloride solution. (*2*) Dibasic sodium phosphate (0.2M).

(f) *Pesticide standards for tube immunoassay.*—(*1*) *Intermediate standard.*—Remove 91 µL from corresponding stock

Received August 24, 1993. Accepted by AP November 2, 1993.
[1] Millipore Corp., 80 Ashby Rd, Bedford, MA 01730
[2] Coca-Cola Foods, PO Box 247, Auburndale, FL 33823

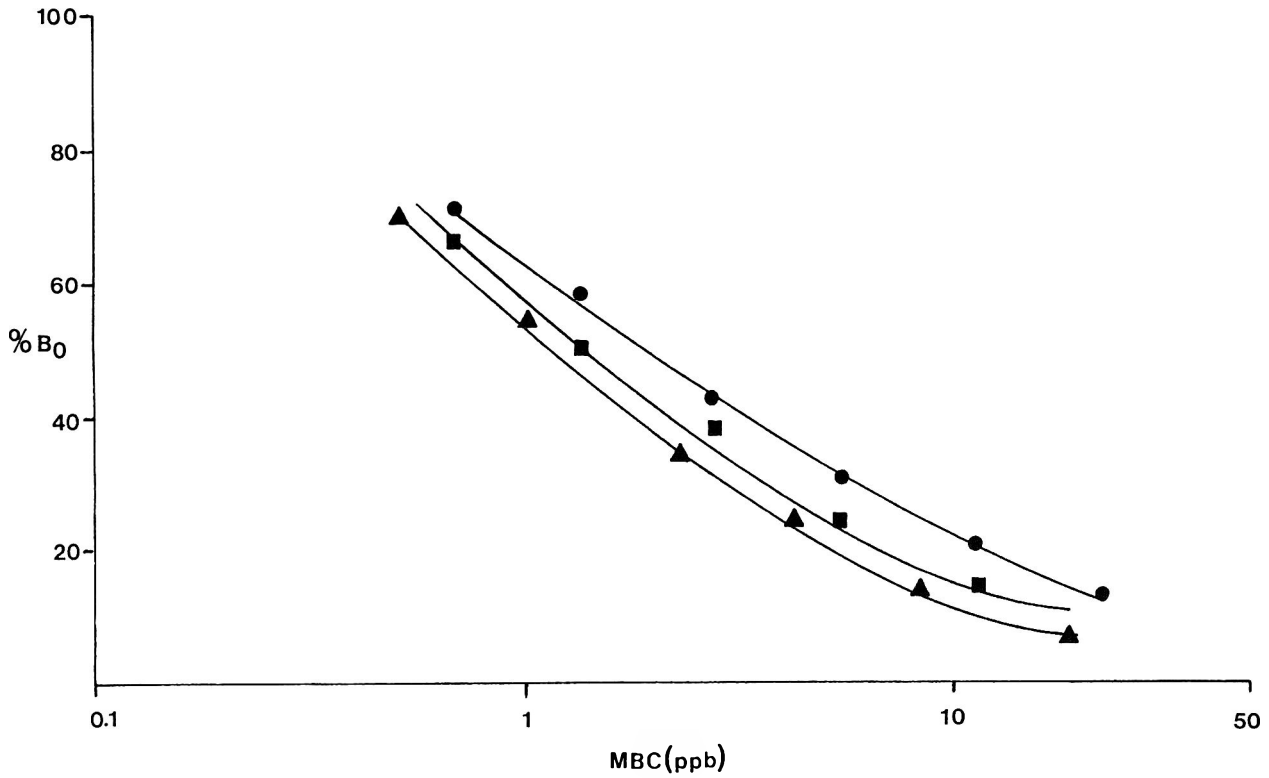


Figure 1. Typical standard curve for MBC in 90% skim milk and 10% LC mobile phase (●); MBC in juice concentrate free of MBC (■); and MBC in buffer (▲).

solution (**c**), place into a 50 mL volumetric flask, and dilute to volume with 50 mL of a solution made up of 1 part of (**e**) (*1*) and 2 parts of (**e**) (*2*). (*2*) *Working standard.*—Remove aliquots (8.75, 17, 40, 70, 140, and 320 µL) for standard curve determination from intermediate standard (*1*) and place each into separate 20 mL scintillation vials. Bring each vial to a total volume of 10 mL with a solution containing 1 part of (**e**) (*1*) and 2 parts of (e) (*2*); sonicate to mix.

(**g**) *MBC standards for LC.*—(*1*) *Intermediate standard.*—Take 1.0 mL from MBC stock and place into a 50 mL volumetric flask. Dilute to volume with LC mobile phase (**h**). (*2*) *Working standards.*—Remove aliquots (1.0, 2.0, 4.0, and 8.0 mL) for standard curve determination from MBC intermediate standard solution and place into separate 50 mL volumetric flasks. Dilute to volume with LC mobile phase (**h**).

(**h**) *Mobile phase.*—Acetonitrile–methanol–water–monoethanolamine (300 + 70 + 440 + 0.1).

(**i**) *Sample extraction solvent.*—Use 5 mL ethanol with 15 mL 2M ammonium chloride adjusted to pH 9.5 with 14.5N ammonium hydroxide. Partition into methylene chloride.

(**j**) *Conjugate synthesis and antisera.*—Follow methods described previously (8). Reagents are provided in the kit (*see* section (**c**) under *Apparatus*).

Apparatus

(**a**) *Liquid chromatograph.*—Equipped with a 1050 isocratic pump (Hewlett-Packard, Avondale, PA), a Valco EQ-60 pneumatic injector (VICI Instruments, Houston, TX), a Waters 470 fluorescence detector (Milford, MA), and a Hewlett-Packard 3396A integrator. Operating conditions: injection volume, 10 µL; flow rate, 1.0 mL/min; fluorescence, excitation at 286 nm and emission at 305 nm; attenuation, 8; gain, 100; filter, 1.5 s.

(**b**) *Chromatographic column.*—Ultracarb 30 ODS, stainless steel, 15 cm × 4.6 id (Phenomenex, Torrance, CA).

(**c**) *EnviroGard EIA benomyl tube kit.*—Contains polystyrene test tubes coated with benomyl–MBC antibodies, enzyme conjugate (horseradish peroxidase bound to MBC), hydrogen peroxide as substrate, and tetramethylbenzidine as chromogen (Millipore Corp., Bedford, MA).

Tube Immunoassay of MBC without Cleanup

Prepare MBC standards as described in (**f**)(*2*). Weigh 1 g bulk fruit juice concentrate into a 20 mL glass scintillation vial. Add 9 mL diluting solvent (e) (*1*). Sonicate for 1 min. Transfer a 1 mL aliquot into a 7 mL glass scintillation vial. Add 2 mL 0.2M dibasic sodium phosphate and sonicate for 1 min. This is the working sample.

Analyze standards and samples by adding 160 µL of each to coated tubes from the tube kit, followed by 160 µL enzyme conjugate (up to 8 samples with 2 controls can be prepared simultaneously). Incubate the tubes for 10 min at room temperature. Rinse the tubes with water to remove unreacted sample and enzyme conjugate. Add 320 µL substrate–chromogen

Table 1. Reproducibility of the MBC tube immunoassay for standards prepared in ammonium chloride–ammonium hydroxide and 0.2M sodium phosphate dibasic

MBC standards, ppb	CV, %	
	Intra-assay[a]	Interassay[b]
0.52	6.8	6.0
1.04	4.1	6.0
2.36	4.0	7.7
4.12	7.3	9.4
8.24	10	22
18.8	11	47

[a] Based on 5 determinations in 1 day.
[b] Based on 10 determinations performed on 10 different days over a period of 2 months.

mixture (1 + 1) or follow the tube kit instructions, which use drops instead of microliters. Incubate the tubes for 5 min; add 1 drop of 2.5N sulfuric acid (stop solution) to stop the reaction. The color changes from blue to yellow.

Measure the intensity of the yellow solution by reading the tubes at 450 nm with a tube reader (Millipore Corp.); alternatively, use a conventional spectrophotometer set at 450 nm. Run 2 control tubes (one at beginning and another at end) with each set of 8 samples to calculate B_0 values (%) of standards and samples [100 × absorbance at 450 nm of standard or sample ÷ average absorbance at 450 nm of negative control made up of 1 part of (**e**) (*1*) and 2 parts of (**e**) (2)]. Use an average value for the negative control. Analyze standard solutions before samples and again after the last sample. (The time difference between the first and the last sample may range from 10 h down to 1 h. Although running only 2 standard sets per day is not standard practice, we have shown that it is effective and saves time and money). Use an average of both standard curves to quantitate MBC in samples. Prepare the standard curve and use it to calculate unknowns. Use semilog paper (3-cycle) to plot the curve; the y axis is B_0 (%) and the x axis is MBC concentration on a log scale.

Table 2. Reproducibility of the MBC tube immunoassay for standards prepared in 90% skim milk and 10% mobile phase

MBC standards, ppb	CV, %	
	Intra-assay[a]	Interassay[b]
0.52	2.4	4.5
1.04	5.7	5.2
2.36	7.1	11
4.12	7.6	14
8.24	11	21
18.8	13	42

[a] Based on 6 determinations in 1 day.
[b] Based on 17 determinations performed on 17 different days over a period of 2 months.

Table 3. Reproducibility of the MBC tube immunoassay for bulk juice concentrates without cleanup

Juice concentrate	MBC, ppb	CV, %	
		Intra-assay[a]	Interassay[b]
Strawberry	340	11	15
Loganberry	1055	13	15
Blueberry	2070	8.9	14
Peach	690	8.9	14
Pineapple	ND[c]	—	—
Apple	31	11	22
Red Grape	39	16	19
Raspberry	248	11	13
Raspberry	91	10	15
Lemon	36	4.1	17
Cherry	59	7.9	17
Pineapple	96	16	22

[a] Based on 6 determinations in 1 day.
[b] Based on 8 determinations performed on 8 different days.
[c] ND, not detected.

Tube Immunoassay of MBC with Cleanup

Extract 5 g bulk juice concentrate by placing it into a 50 mL conical polypropylene centrifuge tube and adding 20 mL extraction solvent (**i**) and 20 mL methylene chloride. Polytron the mixture for 2 min at medium speed. Centrifuge for 3 min at 5000 × *g*. Transfer the bottom layer (methylene chloride) to a 20 mL scintillation vial containing 0.5 g sodium sulfate. Transfer a 10 mL aliquot to another scintillation vial and evaporate the methylene chloride to dryness under air. Add 1 mL mobile

Table 4. Reproducibility of the MBC tube immunoassay for bulk juice concentrates with cleanup

Juice concentrate	MBC, ppb	CV, %	
		Intra-assay[a]	Interassay[b]
Strawberry	303	26	5.6
Loganberry	640	14	10
Blueberry	1840	8.4	14
Peach	857	8.5	6.5
Pineapple	ND[c]	—	—
Apple	29	11	14
Red Grape	32	5.8	7.7
Raspberry	213	5.5	18
Raspberry	65	14	9.9
Lemon	21	22	14
Cherry	37	9.5	20
Pineapple	100	8.9	11

[a] Based on 5 determinations in 1 day.
[b] Based on 5 determinations performed on 5 different days.
[c] ND, not detected.

Table 5. Reproducibility of the MBC LC method for bulk juice concentrates

Juice concentrate	MBC, ppb	CV, % Intra-assay[a]	CV, % Interassay[b]
Strawberry	317	6.9	9.3
Loganberry	692	8.9	7.8
Blueberry	1523	6.1	17
Peach	743	2.0	9.2
Pineapple	ND[c]	—	—
Apple	27	9.6	13
Red Grape	35	5.6	15
Raspberry	229	2.7	13
Raspberry	64	7.6	6.0
Lemon	27	7.6	19
Cherry	92	—	15
Pineapple	84	8.4	20

[a] Based on 6 determinations in 1 day, except for strawberry and pineapple for which there were 5 determinations.
[b] Based on 6 determinations performed on 6 different days, except for loganberry, apple, red grape, both raspberries, lemon, and pineapple (5 determinations) and cherry (4 determinations).
[c] ND, not detected.

phase (**h**) to the residue, sonicate, and transfer to a 1.5 mL polypropylene centrifuge tube. Centrifuge for 5 min at 10 000 $\times g$.

Prepare MBC standards as described in (**f**) (2). For samples, remove 0.1 mL aliquots from the centrifuged samples above. Add 0.9 mL skim milk or nonfat dry milk to each aliquot and sonicate. Any other dilutions will be made with the diluting solvent (**d**). Analyze standards and samples by immunoassay according to the procedure described in the previous section.

LC Determination of MBC

Inject 10 µL from the original 1 mL sample that was used for the immunoassay with cleanup. Use the LC conditions described earlier.

Results and Discussion

The first immunoassay for MBC (8), developed for store-bought juices and concentrates, does not work on the bulk (industrial-strength) concentrates because of pH and matrix effects. Therefore, stronger pH buffer was used to alleviate these problems. With the new buffer system, lower limits of detection and quantitation, compared with those of the previous method (8) for commercial concentrates, were obtained.

Table 6. Accuracy of MBC tube immunoassay for bulk juice concentrates without cleanup[a]

MBC added, ppb	MBC found, ppb	Mean rec., %[b]	CV, %[b]
10	11	111	5.7
40	36	90	6.7
160	135	84	12

[a] Fruit juice concentrates used were orange, grapefruit, lime, lemon and apple. All were shown to be free of MBC by LC at a detection limit of 5 ppb.
[b] Means and CVs are based on 9 determinations.

Table 7. Accuracy of MBC tube immunoassay for bulk juice concentrates with cleanup[a]

MBC added, ppb	MBC found, ppb	Mean rec., %[b]	CV, %[b]
40	43	108	20
290	272	94	5.9

[a] Fruit juice concentrates were pineapple, orange, lemon, grape, apple, grapefruit, and passion fruit. All were shown to be free of MBC by LC at a detection limit of 5 ppb.
[b] Values for the 40 ppb spike were based on 14 determinations; those for the 290 ppb spike were based on 6 determinations.

Typical standard curves for MBC are given in Figure 1. MBC concentrations may be determined from 0.5 to 20 ppb, but concentrations from 0.5 to 10 ppb yield the best results, because the CVs are lower in this range. Samples with concentrations greater than 10 ng/mL (indicated by B_0 <18%) are diluted to bring them within assay range. Figure 1 shows that standard curves and samples will have different B_0 values depending upon the final diluent. Therefore, for immunoassays used for quantitation, the standards and samples must be in the same matrix. Sometimes, however, an artificial diluent can be designed to substitute for the exact matrix, as shown also in Figure 1. For example, the spiked concentrate standards were made from MBC-free bulk juice concentrates, whereas the buffer standards were prepared from a prepared mixture to closely simulate the juice concentrate. The results agree quite well. There is an approximately 6% error in quantitation using the buffer system compared with using the pesticide-free concentrates. This difference may not warrant the trouble of trying to find pesticide-free concentrates.

The limit of quantitation for all methods was 10 ppb, based on the procedure developed in 1980 by a committee of environmental chemists (9). This limit is the smallest amount of MBC that can be determined with good reproducibility. However, the lower limits of detection (which should not be confused with quantitation limits) were 10 ppb for EIA without cleanup and 5 ppb for EIA with cleanup and LC. The limit of detection is the least amount the methods can detect, but these values are not as reproducible, on the basis of statistics, as the values for the lower limits of quantitation (9).

As with any analytical technique, precisions within and between days are very important. Reproducibility results for the MBC tube immunoassay for standards and samples are presented in Tables 1–4. Tables 1 and 2 show the consistency data obtained from MBC standards. Table 1 depicts results of standards prepared in a buffer solution used for analyzing juice concentrates without cleanup. Intra- and interassay CVs ranged from 4.0 to 47%, with most CVs at 10% or less. For the standards made up in mobile phase and milk (used for the cleanup

Table 8. Comparison of the tube immunoassay and LC for the determination of MBC in bulk fruit juice concentrates

Concentrate	MBC, ppb[a]			Concentrate	MBC, ppb[a]		
	EIA with cleanup	EIA without cleanup	LC		EIA with cleanup	EIA without cleanup	LC
Orange	ND	ND	ND	Lemon	44	25	19
Apple	14	15	17	Grapefruit	ND	ND	ND
Apple	ND	ND	ND	Pineapple	ND	ND	ND
Apple	ND	ND	ND	Red Grape	17	13	18
Strawberry	ND	ND	ND	Tangerine	ND	ND	ND
Concord grape	ND	ND	ND	Apple	ND	ND	ND
Apple	ND	ND	ND	Pineapple	186	120	121
Apple	123	—	70	Pineapple	ND	ND	ND
Strawberry	405	376	324	Pineapple	ND	ND	ND
Raspberry	330	272	190	White grape	ND	ND	ND
Con. grape	ND	ND	ND	Pineapple	ND	ND	ND
Con. grape	ND	ND	ND	Pineapple	ND	ND	ND
Con. grape	ND	ND	ND	Pineapple	ND	ND	ND
Red grape	15	6	20	Pineapple	ND	ND	ND
Red grape	15	17	20	Pineapple	ND	ND	ND
Strawberry	900	580	760	Pineapple	ND	ND	ND
Orange	ND	ND	ND	Pineapple	240	340	295
Orange	ND	ND	ND	Pineapple	ND	ND	ND
Orange	ND	ND	ND	Orange	ND	ND	ND
Orange	ND	ND	ND	Apple	123	120	82
Orange	ND	ND	ND	Apple	200	160	113
Orange	ND	ND	ND	Apple	44	60	46
Apple	ND	ND	ND	Apple	69	66	32
Apple	ND	ND	ND	Apple	135	184	170
Apple	—	1060	1095	Red grape	32	51	32
White grape	ND	ND	ND	Pineapple	306	216	187
Apple	ND	ND	ND	Cranberry	ND	ND	ND
Apple	13	10	11	Apple	ND	ND	ND
Apple	ND	ND	ND	Apple	360	512	389
Apple	ND	ND	ND	Apple	26	—	26
Apple	ND	ND	ND	Apple	ND	ND	ND
Apple	ND	ND	ND	Apple	69	72	50
Apple	24	38	38	Apple	14	—	9
Apple	306	72	177	Apple	330	460	337
Apple	ND	7	11	Apple	12	10	—
Apple	14	9	11	Apple	ND	ND	ND
Apple	ND	ND	ND	Apple	ND	ND	ND
Peach	660	720	636	Apple	14	15	18
Peach	750	1040	898	Apple	ND	ND	ND
Peach	330	440	360	Apple	220	220	121
Peach	360	460	393	Raspberry	1410	1640	1210
Peach	—	392	265	Raspberry	60	44	—
Peach	—	48	49	Raspberry	315	328	279
Grapefruit	ND	ND	ND	Raspberry	38	28	35
Grapefruit	ND	ND	ND	Raspberry	1890	1910	2681
Grapefruit	12	10	5	Raspberry	264	172	221
Grapefruit	23	9	5	Raspberry	1740	1680	1266
Grapefruit	ND	ND	ND	Raspberry	360	480	376
Grapefruit	ND	ND	ND	Raspberry	450	512	376
Pineapple	ND	ND	ND	Raspberry	204	188	210
Pineapple	ND	ND	ND	Raspberry	1170	1280	1310
				Raspberry	36	38	37

Table 8. ***(continued)***

Concentrate	MBC, ppb[a]		
	EIA with cleanup	EIA without cleanup	LC
Raspberry	75	148	—
Raspberry	34	—	68
Raspberry	2700	2960	2009
Raspberry	38	32	95
Raspberry	1110	1600	1361
Raspberry	41	38	60
Raspberry	1380	—	1392
Raspberry	810	—	795
Raspberry	1080	—	696
Cherry	16	11	50
Cherry	43	44	67
Cherry	52	64	66
Peach	102	204	185
Peach	870	660	627
Peach	25	44	47
Peach	108	150	185
Raspberry	32	92	74
Cranberry	ND	ND	ND
Orange	ND	ND	ND
Apricot	ND	ND	ND
Cranberry	ND	ND	ND
Orange	ND	ND	ND
Passion fruit	ND	ND	ND
Cranberry	ND	ND	ND
Orange	ND	ND	ND
Passion fruit	ND	ND	ND
Orange	ND	ND	ND
Boisenberry	24	32	39
Lemon	ND	ND	ND
Loganberry	420	980	752
Cranberry	ND	ND	ND
Apple	11	11	15
Black currant	90	156	78
Blueberry	2070	2480	2011
Cranberry	ND	ND	ND
Grapefruit	12	18	24
Cranberry	ND	ND	ND
Cranberry	ND	ND	ND
Cranberry	ND	ND	ND
Cranberry	ND	ND	ND
Concord grape	ND	ND	ND

[a] ND for EIA without cleanup = none detected at a limit of 10 ppb. ND for EIA with cleanup = none detected at a limit of 5 ppb. ND for LC = none detected at a detection limit of 5 ppb. — = missing data. Correlation coefficients: EIA without cleanup vs LC, 0.954 ($y = 0.978x + 22.7$); EIA with cleanup vs LC, 0.956 ($y = 1.08x + 16.9$); EIA with cleanup vs EIA without cleanup, 0.978 ($y = 0.876x + 10.4$).

method) the intra- and interassay CVs varied from 2.4 to 42% (Table 2), with most CVs at 11% or less. Both standard sets (Tables 1 and 2) demonstrated approximately the same reproducibility, except for interassay CVs for 18.8 ppb standard, which were about 40%. For this reason, we recommend that the standard curve be used only to the 10 ppb level to maintain good day-to-day consistency.

The assay reproducibility for some juice concentrates is shown in Tables 3 and 4. Intra- and interassay CVs for the procedure without cleanup ranged from 4.1 to 22%, with most below 17% (Table 3). For the procedure with cleanup, CVs varied from 5.5 to 26% with most values below 15% (Table 4).

A consistency study was performed with the LC method (Table 5). The intra- and interassay CVs ranged from 2.0 to 19%, with most values lower than 10%. The data in Tables 3–5 show that the immunoassay compares well with the more expensive LC procedure in terms of reproducibility.

The accuracy of the immunoassay was tested by analyzing spiked bulk juice concentrates for the procedure with and without cleanup (Tables 6 and 7). Recoveries averaged 95% for the procedure without cleanup and 101% for the procedure with cleanup. Juice concentrates were spiked from 10 to 290 ppb. The consistencies of both EIAs for the accuracy test were also in the range of the previous studies, with CVs ranging from 5.7 to 20%.

Cross-reactivity of the benomyl antibody was discussed in a previous paper (8). The antibody shows varying levels of cross-reactivity toward other benzimidazole and thiophanate fungicides. Except for MBC, which has the same reactivity as benomyl, only one other fungicide in this group, thiabendazole (TBZ), shows sufficient cross-reactivity to cause problems in the quantitation process at the residue level. However, because TBZ is used as a postharvest treatment for fruits, most TBZ is found on the peel. Juice concentrates do not contain substances from the peel; therefore very little if any TBZ is expected to be in concentrates. Using LC, we have not found any TBZ levels in fruit juice concentrates that would interfere with the EIA quantitation of MBC.

An extensive study of the correlation between the 2 immunoassays and LC was carried out on 144 bulk juice concentrates comprising 20 different fruits (Table 8). Of these, 69 contained no detectable levels of MBC by all 3 procedures. For one sample, EIA without cleanup detected no MBC, whereas EIA with cleanup and LC indicated MBC at 7 and 11 ppb, respectively. However, this is not a false-negative result, because the EIA without cleanup has a detection limit of 10 ppb.

A total of 74 concentrates (51%) contained MBC at concentrations ranging from 5 to 2960 ppb, with most concentrations greater than 300 ppb and none near the tolerance for the raw fruit, which varies from 7 to 10 ppm. Several correlations were made with the data for MBC-positive samples. For example the correlation between EIA without cleanup and LC was 0.954 ($y = 0.978x + 22.7$), for EIA with cleanup and LC, it was 0.956 ($y = 1.08x + 16.9$), and for EIA with cleanup and EIA without cleanup, it was 0.978 ($y = 0.876x + 10.4$). The correlation coefficients for all 3 methods were similar (~1), indicating that the 3 methods were in good agreement. Thus matrix effects are nonexistent with the method with no cleanup. The use of EIA without cleanup will save time compared with the other techniques.

The EIA without cleanup for bulk juice concentrate should be very useful both in saving time and money for food processors checking their concentrates for benomyl and/or MBC. Futhermore, such a procedure will help in gathering the pesticide data needed for processed foods so that real risk–benefit analyses can be made.

Acknowledgments

This paper is manuscript 2301 of the University of Maine Agricultural Experiment Station.

References

(1) Winter, C.K. (1993) *Food Technol.* **47**, 81–86

(2) Klis, J.B. (1993) *Food Technol.* **47**, 120–122

(3) Bushway, R.J., Kugabalasooriar, J., Perkins, L.B, Harrison, R.O, Young, B.E.S., & Ferguson, B.S. (1992) *J. AOAC Int.* **75**, 323–327

(4) Gilvydis, D.M., & Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753–761

(5) Newsome, W.H., & Collins, P.G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 1025–1027

(6) Newsome, W.H., & Shields, J.B. (1981) *J. Agric. Food Chem.* **29**, 220–229

(7) Bushway, R.J., Hurst, H.L., Kugabalasooriar, J., & Perkins, L.B. (1991) *J. Chromatogr.* **587**, 321–324

(8) Bushway, R.J., Savage, S.A., & Ferguson, B.S. (1990) *Food Chem.* **35**, 51–58

(9) Anon. (1980) *Anal. Chem.* **52**, 2242–2249

RESIDUES AND TRACE ELEMENTS

Determination of Thiabendazole in Fruits and Vegetables by Competitive-Inhibition Enzyme Immunoassay

RODNEY J. BUSHWAY, BARBARA E.S. YOUNG,[1] LANCE R. PARADIS, and LEWIS B. PERKINS
University of Maine, Department of Food Science, 5736 Holmes Hall, Orono, ME 04469-5736

An enzyme immunoassay (EIA) was developed for analysis of thiabendazole (TBZ) in fruits and vegetables. A commercial kit using a polyclonal antibody for benomyl–carbendazim was used. Total analysis time, including sample preparation, was 35 min. As many as 8 samples can be analyzed simultaneously, with a limit of quantitation of 9 ppb. The assay's dynamic range ran from 9 to 304 ppb TBZ. Intra-assay coefficients of variation (CVs) ranged from 5.0 to 9.6% for standards and 11 to 30% for samples. Interassay CVs varied from 4.4 to 15% for standards and from 10 to 24% for samples. Average recovery from 29 samples spiked at 50–50 000 ppb was 116%. A total of 107 food products comprising 12 different fruits and vegetables and their processed products were analyzed for their TBZ content by EIA and liquid chromatography (LC). Of these samples, 84 were positive for TBZ by both methods, with a correlation coefficient (r) of 0.989. Eight samples had none detected by either technique, and 15 were positive for carbendazim. Concentrations of TBZ ranged from 11 to 94 000 ppb. The immunoassay with methanol sonication shows promise as a rapid screening method for TBZ in foods.

Received August 24, 1993. Accepted by AP November 9, 1993.
[1] Millipore Corp., 80 Ashby Rd, Bedford, MA 01730

Thiabendazole (TBZ) is a systemic fungicide belonging to the benzimidazole and thiophanate class of fungicides. Its primary use is for postharvest treatment of fruits and vegetables to protect produce from the ascomycetes, basidiomycetes, and some deuteromycetes. With the recent publication (1) of the National Academy of Sciences report "Pesticides in Diets of Infants and Children" and because of TBZ's frequent use and amount applied (2), there is a need to measure levels of this fungicide in food products.

Previous methods for analysis of TBZ in foods focused on chromatographic methods using reversed-phase liquid chromatography (LC) (3–9), normal-phase LC (4, 6–7) and gas chromatography (GC) (5). Two EIAs recently were applied to the analysis TBZ (4, 5). Both were plate assays. The first method (4) used a polyclonal antibody to determine TBZ in pear, apple, lemon, grapefruit, and potato at a detection limit of 30 ppb with cleanup. The second method (5) used a mono-

clonal antibody to analyze TBZ in apple and potato with a detection limit of 0.2 ppm without cleanup.

This paper describes an EIA polyclonal tube method for the determination of TBZ in 12 different raw fruits and vegetables and some of their processed products at a detection limit of 9 ppb with cleanup. Preliminary data are given on a procedure without cleanup. Such methods may be very useful for screening foods for TBZ.

METHOD

Reagents and Standards

(**a**) *Reagents.*—All reagents pertaining to the preparation of immunogens for raising antisera to carbendazim (MBC), which also cross-reacts with TBZ, were previously described (4). All solvents were LC grade (VWR, Boston, MA).

(**b**) *Analytical standard.*—TBZ pesticide standard was obtained from the U.S. Environmental Protection Agency. Research Triangle Park, NC.

(c) *Pesticide standard stock solution.*—Weigh 25 mg TBZ into 50 mL volumetric flask, dissolve, and dilute to volume with methanol. Store at 4°C. The standard should be stable for 6 months.

(**d**) *Diluting solvent for tube immunoassay.*—Place 25 mL mobile phase (**g**) into 250 mL volumetric flask and dilute to volume with skim or nonfat dry milk (prepare according to label directions). Prepare fresh.

(**e**) *Pesticide standards for tube immunoassay.*—(*1*) *Intermediate standard.*—Remove 0.1 mL aliquot from corresponding stock solution (**c**), place into 50 mL volumetric flask. and dilute to volume with 50 mL diluting solvent (**d**). (*2*) *Working standard.*— Remove aliquots (95, 190, 380, 760, 1520, and 3040 µL) for standard curve determination from intermediate standard (**1**), and place each into separate 20 mL scintillation vials. Dilute each vial to 10 mL total volume with diluting solvent (**d**), and then sonicate to dissolve residue.

(**f**) *TBZ standards for LC.*—(*1*) *Intermediate standard.*—Take 100 µL aliquot from TBZ stock (**c**), and place into 50 mL volumetric flask. Dilute to volume with LC mobile phase (**g**). (*2*) *Working standards.*—Remove aliquots (0.25, 0.50, 1.0. and 2.0 mL) for standard curve determination from TBZ intermediate standard solution, and place into separate 10 mL volumetric flasks. Dilute to volume with LC mobile phase.

(**g**) *Mobile phase.*—Acetonitrile–methanol–water–monoethanolamine (260 + 70 + 500 + 0.1).

(**h**) *Sample extraction solvent.*—Use 5 mL ethanol with 15 mL 2M ammonium chloride adjusted to pH 9.5 with 14.5N ammonium hydroxide. Partition into methylene chloride.

(**i**) *Conjugate synthesis and antisera.*—Follow methods described previously (4). Reagents are provided in the kit (*see* section (**c**) under *Apparatus*).

Apparatus

(**a**) *Liquid chromatograph.*—510 pump (Waters Associates, Milford, MA); Valco pneumatic injector (VICI Instruments, Houston, TX); Waters 470 fluorescence detector and a Hewlett-Packard (Avondale, PA) 3396A integrator. Operating conditions: injection volume, 10 µL; flow rate, 1.0 mL/min; fluorescence, excitation at 305 nm and emission at 345 nm; attenuation, 8; gain, 100; filter, 1.5 s.

(**b**) *Chromatographic column.*—Ultracarb 30 ODS, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Torrance, CA).

(**c**) *EnviroGard EIA benomyl tube kits.*—Contain polystyrene test tubes coated with antibodies to MBC, enzyme conjugate (horseradish peroxidase bound to MBC), hydrogen peroxide as substrate, and tetramethylbenzidine as chromogen (Millipore Corp., Bedford, MA).

Tube Immunoassay of TBZ with Cleanup

First extract 5 g of a homogenous fresh sample or 0.5 g of a dried sample by placing it into 50 mL conical polypropylene centrifuge tube followed by 20 mL extraction solvent (**h**) and 20 mL methylene chloride. Polytron (Kinematica Model PT-10-35, Kriens-Luzern, Switzerland) the mixture for 2 min at medium speed. Centrifuge 3 min at 5000 × *g*. Transfer the bottom layer (methylene chloride) into 20 mL scintillation vial containing 0.5 g sodium sulfate. Dry 10 mL aliquot of methylene chloride layer under nitrogen. Dissolve residue in 1 mL mobile phase (**g**) and transfer to 1.5 mL polypropylene centrifuge tube. Centrifuge 5 min at 10 000 × *g*.

Prepare TBZ standards as described in (**e**)(*2*). For samples, remove 0.1 mL aliquots from the centifuged samples. Add 0.9 mL skim milk or nonfat dry milk to each aliquot and sonicate. Any other dilutions will be made with the diluting solvent (**d**).

Analyze standards and samples by adding 160 µL of each to coated tubes from the tube kit followed by 160 µL enzyme conjugate (up to 8 samples with 2 controls can be prepared simultaneously). Incubate tubes for 10 min at room temperature. Rinse tubes 4 times by filling with water to remove unreacted sample and enzyme conjugate and allow to drain for 1 min on a paper towel. Add 320 µL substrate–chromogen mixture (1 + 1). If using the kit, follow the instructions because drops of reagents are used. Incubate tubes for 10 min and then add 1 drop 2.5N sulfuric acid (stop solution) to stop the reaction. The color changes from blue to yellow.

Measure intensity of the yellow solution by reading tubes at 450 nm with a tube reader (Millipore Corp.), or use conventional spectrophotometer set at 450 nm. Run 2 control tubes (one at the beginning and the other at the end) with each set of 8 samples to calculate B_0(%) values of standards and samples (100 × absorbance at 450 nm of standard or sample/average absorbance at 450 nm of negative control of diluting solvent (**d**)). Use an average value for negative control. Run a standard curve before samples and again after last samples. (The time difference from first to last samples may range from 10 h down to 1 h. Although running only 2 standard sets per day is not standard practice, we have shown that it is effective and saves time and money). Use an average of both standard curve runs to quantitate TBZ in samples. Prepare the standard curve and use it to calculate unknowns. Use semilog paper (3-cycle) to plot the standard curve, where the y axis is B_0 and the x axis is TBZ concentration on the log scale (Figure 1).

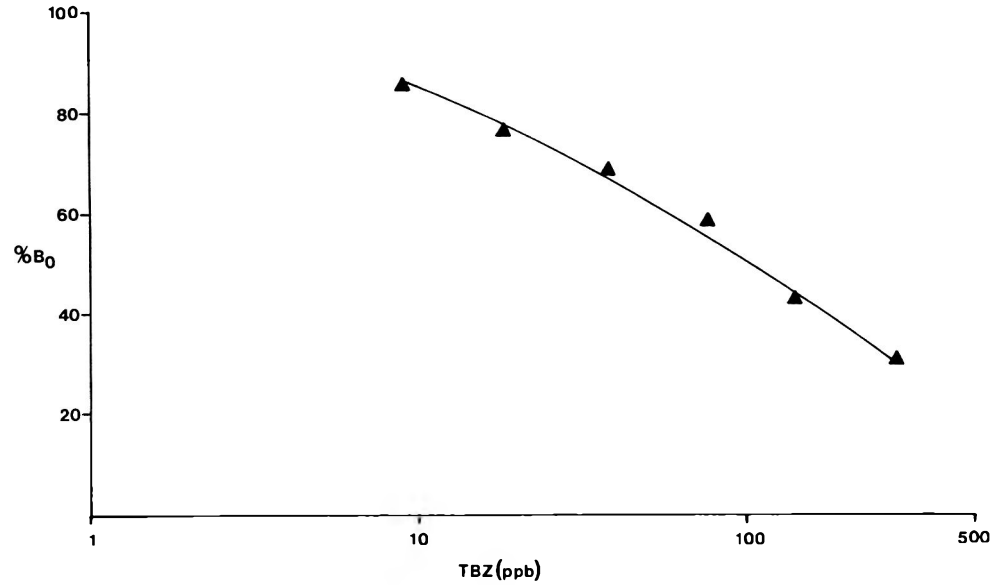


Figure 1. Typical immunoassay standard curve for TBZ.

Tube Immunoassay of TBZ without Cleanup

Weigh the whole fruit or vegetable and place it into 400 mL beaker. Add 50 mL methanol and sonicate 2 min; make sure that the food is rotated during sonication so that the entire peel region contacts the methanol. Make a 1:10 dilution of the sonicate with nonfat dry milk. Follow the immunoassay procedure described above.

LC Determination of TBZ

Inject 10 µL from the original 1 mL sample that was used for immunoassay. Use LC conditions described in *Apparatus* (**a**) and (**b**) and *Reagents* (**f**) and (**g**).

Results and Discussion

A typical standard curve for TBZ is shown in Figure 1. The working concentration of TBZ is 9 to 304 ppb. Samples with a concentration >304 ng/mL (indicated by $B_0 < 25\%$) are simply diluted. (Samples are the first 1:10 dilution of the original cleaned up extract.)

As with any analytical technique, precision within and between days is crucial. Reproducibility results for the TBZ tube immunoassay for standards and samples are presented in Tables 1 and 2. Table 1 shows the consistency data obtained from analyzing TBZ standards. Intra- and interassay CVs ranged from 4.4 to 15%, with most CVs < 10%.

Ten different foods containing TBZ at various concentrations (273 to 100 334 ppb) were used to ascertain the reproducibility of the assay for samples. The results of this intra- and interassay study are given in Table 2. Most CVs were <21% (range, 10–30%). Although these CVs are higher than those of

Table 1. Reproducibility of thiabendazole immunoassay for standards prepared in 10% mobile phase–90% nonfat dry milk

	CV, %	
Thiabendazole, ppb	Intra-assay[a]	Interassay[b]
4.6	5.0	4.4
9.2	5.1	6.8
18	5.2	7.1
37	8.7	8.5
74	9.1	9.7
147	7.5	11
304	9.6	15

[a] Based on 6 determinations in 1 day.
[b] Based on 38 determinations over 18 days.

Table 2. Reproducibility of thiabendazole immunoassay for fruits, vegetables, and their products

	Thiabendazole,	CV,%	
Sample	ppb	Intra-assay[a]	Interassay[b]
Banana peel	1009	15	20
Potato	273	30	15
Orange peel	6225	11	10
Orange flesh	277	21	24
Grapefruit peel	9172	21	10
Grapefruit flesh	470	15	21
Apple	3180	15	16
Pear	958	18	19
Extruded potato peel	100334	26	15
Potato cookie	2964	20	21

[a] Based on 6 determinations in 1 day.
[b] Based on 6 determinations over 6 days.

Table 3. Accuracy of thiabendazole immunoassay for fruits and vegetables[a]

Sample	TBZ added, ppb	TBZ found, ppb	Mean recovery, %	CV, %
Grapefruit peel	50	54	108	20
Grapefruit flesh	50	54	108	4
Apple	50	66	132	13
Orange flesh	50	63	126	18
Banana flesh	50	58	116	12
Banana peel	50	83	166	8
Potato peel	50	71	142	17
Potato flesh	50	59	118	10
Pear	50	61	122	16
Banana peel	200	233	117	8
Banana flesh	200	229	115	16
Potato	200	235	118	5
Pear	200	220	110	20
Apple	200	247	124	16
Orange peel	200	217	109	18
Orange flesh	200	227	114	7
Grapefruit flesh	1000	1115	112	9
Grapefruit peel	1000	1113	112	12
Apple	1000	1143	114	7
Potato	1000	990	99	8
Pear	1000	975	98	10
Banana peel	1000	990	99	8
Banana flesh	1000	1110	111	18
Grapefruit peel	10000	13070	131	17
Grapefruit flesh	10000	11963	120	8
Apple	10000	9720	97	16
Grapefruit flesh	50000	46500	93	9
Grapefruit flesh	50000	48800	98	19
Apple	50000	60200	120	10

[a] Means and CVs are based on 4 determinations.

Table 4. Cross-reactivity of the benomyl/carbendazim antibody

Pesticide	I_{50}, ppb[a]	LLD, ppb[b]
Benomyl	4.0	0.5
MBC	4.0	0.5
Thiabendazole (TBZ)	80.0	5.0
Thiophanate	200.0	43.0
Thiophanate methyl	200.0	43.0
Procymidone	500.0	—
5-Hydroxy-TBZ	800.0	—
Vinchlozolin	1500.0	—

[a] Concentration giving 50% inhibition.

[b] Least detectable dose estimated at B_0 of 80%.

The following pesticides showed no cross-reactivity at a concentration of 1.5 ppm: 2-aminobenzimidazole, iprodione, carbofuran, folpet, salithion, phosalone, alachlor, amitrole, chlormephos, bayleton, pronamide, guthion, tetradifon, metholachlor, dinoseb, acifluorfen, atrazine, benefin, diphenylamine, chlorothalonil, aldicarb, diazinon, metalaxyl, asulam, propachlor, thidiazuron, pyrolan, anilazine, bentazon, butylate, fluchloralin, butachlor, diphenamid, oxythioquinox, secbumeton, pyrazophos, metrazole, rubigan, basalin, morestan, pyrene, carbaryl, chlorpropham, acephate, mercarbam, nitralin, iodofenphos, bendiocarb, oryzalin, fluazifop butyl, linuron, molinate, triforine, dodine, fenthiuron, terbutryn, diuron, phosmet, propoxur, mefluidide, and carbanolate.

the standards, they should be acceptable for a screening procedure.

Recovery studies were also performed on 29 spiked fruit and vegetable samples comprising 6 different vegetables and fruits including separate flesh and peel samples. Results are given in Table 3. Spiking levels varied from 50 to 50 000 ppb, and recoveries ranged from 93 to 166% (mean recovery was 116%, with most recoveries between 93 and 120%). Thus, the accuracy was acceptable. Reproducibility was quite good in this study, with CVs ranging from 4 to 20%. These higher recoveries could be due to either matrix effects or the presence of other pesticides that cross-react with the antibody.

The antisera used in this analysis was raised against benomyl–carbendazim (MBC), but the antibody demonstrates sufficient cross-reactivity toward TBZ to allow detection of TBZ in foods (Table 4). A cross-reactivity between 2 widely used fungicides could lead to a major problem, but, in this case, it does not reduce the effectiveness of the screening procedure. In most instances, TBZ quantitation by EIA will not be affected by MBC. Because benomyl is no longer used in postharvest applications, TBZ is usually not found with benomyl.

The correlation between tube EIA and LC was studied with 107 fruits, vegetables, and their process products. The results support the statements made above concerning potential MBC interferences. A total of 84 samples were positive for TBZ (Table 5), 15 were positive for MBC, and 8 contained no detectable levels of TBZ or MBC. For the 84 TBZ-positive samples, a correlation coefficient of 0.989 was obtained with an equation of $y = 0.80x + 67.6$. This equation indicates a high bias of the EIA method for TBZ concentrations (none of the 84 samples showed any MBC by liquid chromatography (LC) so it was not the cause of the high bias), but the EIA method is still excellent for a screening procedure. The 15 MBC-positive samples were potatoes grown in Wisconsin, where benomyl is widely used on potatoes and some apples.

The data presented in Table 5 gives useful information about the distribution of TBZ in fruits. For example, the peel of treated fruit contains from 83 to 98% of the total amount of TBZ present. Also, processed products, such as potato chips, extruded potato peel, and baked and fried potatoes, contain high levels of TBZ.

Table 5. Comparison of immunoassay and LC for the determination of TBZ in fruits, vegetables, and their products

Sample	TBZ, ppb		Sample	TBZ, ppb	
	Immunoassay	LC		Immunoassay	LC
Extruded potato peel	84000	63635	Red delicious apple	2120	1484
Extruded potato peel	94000	61855	Potato chips	53	46
Dried potato peel	76000	63190	Potato chips	154	130
Grapefruit peel	10000	8633	Potato chips	540	310
Grapefruit flesh	360	233	Potato chips	600	317
Grapefruit peel	8400	4895	Cracker	ND	ND
MacIntosh apple	43	43	Cracker	ND	ND
MacIntosh apple	23	24	Baked potato	53	20
Red delicious apple	6400	3916	Baked potato	1480	1277
Red delicious apple	4200	3827	Fried potato skin	500	356
Pear	580	475	Fried potato skin	352	267
Pear	1192	507	Steak fries	15	8
Apple	43	10	Steak fries	23	13
Apple	31	12	Potato cookie raw	4500	1780
Potato	43	18	Potato cookie extruded	3200	1691
Potato	49	15	Banana peel	466	490
Potato	157	112	Banana flesh	82	102
Potato	173	109	Potato	13	2
Potato	19	11	Potato	11	2
Potato	31	11	Potato chips	ND	ND
Russet potato	114	81	Potato chips	ND	ND
Russet potato	109	88	Round white potato	56	63
Potato chips	17	33	Round white potato	85	60
Potato chips	12	16	Katahdin potato	760	470
Potato chips	1480	892	Extruded potato peel	816	638
Potato chips	1760	1270	Extruded potato peel	26400	28226
Potato chips	760	482	Extruded potato peel	46000	51493
Potato chips	680	534	Extruded potato peel	84000	72471
Lime peel	47	11	Extruded potato peel	74000	61029
Lime flesh	13	4	Raspberry	43	27
Orange peel	3000	2634	Raspberry	13	2
Orange flesh	448	235	Banana flesh	59	57
Lemon peel	319	164	Banana peel	412	282
Lemon flesh	49	21	Potato	340	259
Golden delicious apple	5880	3631	Potato	288	252
Golden delicious apple	5400	4058	Potato	ND	ND
Red delicious apple	900	470	Pear	1320	725
Red delicious apple	900	484	Pear	920	610
MacIntosh apple	19	6	Lime peel	100	10
MacIntosh apple	11	4	Lime flesh	ND	ND
Pear	1000	534	Lemon peel	ND	ND
Pear	1000	579	Lemon flesh	ND	ND
Grapefruit peel	6200	5207	Orange peel	4000	3433
Grapefruit flesh	170	107	Orange flesh	360	328
Banana flesh	30	25	Golden delicious apple	740	412
Golden delicious apple	560	354			
Red delicious apple	2240	1983			

[a] ND, none detected at a detection limit of 9 ppb for EIA and 2 ppb for LC.

Table 6. Comparison of immunoassay with methanol wash and LC for determination of thiabendazole in potatoes

	TBZ, ppm	
Sample	Immunoassay	LC
Potato 1	69.3	57.5
Potato 2	52.1	44.0
Potato 3	96.6	57.5
Potato 4	40.0	29.0
Potato 5	40.5	36.0
Potato 6	16.4	19.0
Potato 7	66.9	57.5
Potato 8	38.5	47.0
Potato 9	12.1	9.0
Potato 10	56.1	55.0

Food matrix effects are commonly encountered in immunoassay methods (10–12). To help prevent such effects, samples were partitioned into methylene chloride after extraction. However, preliminary results indicated that a methanol sonication of the outside of the fruits and vegetables works well as a rapid screening technique. We have tried this procedure on several types of fruits and vegetables and observed that, each time, EIA results that indicated the presence of TBZ were confirmed by LC results. A correlation was determined with this method with 10 potato samples (Table 6). The correlation coefficient was 0.886 and the equation was $y = 1.28x - 3.97$.

Because this antibody does cross-react with both MBC and TBZ at low levels, the EIA method can be used only to screen for both fungicides in fruits and vegetables and their products. The data indicate that this procedure would be a good screening method especially because there are LC methods that can distinguish between MBC and TBZ in a sample. However, if one knows the pesticide history of the food, this immunoassay would be very effective in the quantitation of TBZ, as supported by the data presented.

Acknowledgments

This manuscript is No. 2300 of the University of Maine Agricultural Experiment Station.

References

(1) Klis, J.B. (1993) *Food Technol.* **47**, 120–122

(2) Papadopoulou-Mourkidou, E. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 745–765

(3) Gilvydis, D.M., & Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753–761

(4) Newsome, W.H., & Collins, P.G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 1025–1027

(5) Brandon, D.L., Binder, R.G., Wilson, R.E., & Montague, W.C., Jr (1993) *J. Agric. Food Chem.* **41**, 996–999

(6) Luchtefeld, R.G. (1992) in *Emerging Strategies for Pesticide Analysis*, T. Cairns & J. Sherma (Eds), CRC Press, Ann Arbor, MI, pp. 151–174

(7) Bushway, R.J. (1992) in *Food Analysis by HPLC*, L.M. Nollet (Ed.), Marcel Dekker, New York, NY, pp. 507–578

(8) Wang, Y. (1992) *Sepu* **10**, 354–356

(9) Tsumura-Hasegawa, Y., Tonogai, Y., Nakamura, Y., & Ito, Y. (1992) *Shokuhin Eiseigaku Zasshi* **33**, 258–266

(10) Bushway, R.J., Perkins, B., Savage, S.A., Lekousi, S.L., & Ferguson, B.S. (1989) *Bull. Environ. Contam. Toxicol.* **42**, 899–904

(11) Bushway, R.J., Kugabalasooriar, J., Perkins, L.B., Harrison, R.O., Young, B.E.S., & Ferguson, B.S. (1992) *J. AOAC Int.* **75**, 323–327

(12) Jourdan, S.W., Scutellaro, A.M., Hayes, M.C., & Herzog, D.P. (1992) Paper 263, presented at the 106th Meeting of the Association of Official Analytical Chemists, Cincinnati, OH, August 31–September 2.

RESIDUES AND TRACE ELEMENTS

Determination of Levels of Lead Contamination in Food and Feed Crops

CARMEN CABRERA, CARMEN GALLEGO, MARIA C. LOPEZ, and MARIA L. LORENZO
University of Granada, School of Pharmacy, Department of Nutrition and Bromatology, E-18012 Granada, Spain
ENRIQUE LILLO
Laboratorio Agroalimentario de Granada, Junta de Andalucía, E-18014 Atarfe, Granada, Spain

A rapid, precise procedure is described for the determination of lead in food and feed products with electrothermal atomic absorption spectrophotometry. Samples were mineralized in a microwave acid digestion bomb in the presence of nitric acid and vanadium pentoxide. Lead concentrations were determined directly from digested samples. The detection limit was 0.04 ng/mL. Accuracy and precision were checked against National Institute of Standards and Technology standard reference material. The analytical method was tested with 51 food and feed crops from Mediterranean zones in Spain and found to be suitable for these products. Lead concentrations in samples ranged from not detectable to 2.695 μg/g (fresh weight).

Human activity is leading to increasing levels of lead contamination in the environment. Agricultural products can be markedly affected by urban and industrial wastes allowed to reach farmlands, which occurs when waste water is used for irrigation or when phytosanitary products are repeatedly applied (1). However, the most important source of contamination is atmospheric pollution from industrial or motor vehicle emissions, which represent from 73 to 95% of the total lead load in plants (2). Plant susceptibility to Pb accumulation is also affected by the duration of exposure, climate, physicochemical characteristics of the soil, the plant species in question, or the anatomical part of the plant considered (2–4).

Elevated Pb levels cause a series of metabolic changes in plants, including decreased growth; delayed flowering; lowered chlorophyll content; and reductions in the number and quality of shoots, leaves, and fruits (4). If animals consume contaminated plants, agricultural production may be reduced considerably. Moreover, this process can bring Pb into the human food chain (5). Approximately 80% of the total human Pb intake is supplied by the diet (6, 7). According to FDA data (8), food and feed crops provide approximately 34 to 44% of the dietary intake. There is obviously a need to accurately detect even very low Pb concentrations in foods and possible increases in contamination resulting from environmental pollution.

Received August 6, 1993. Accepted by JS November 17, 1993.

The most frequently recommended techniques for Pb determination in foods include polarography, inductively coupled plasma atomic emission spectrometry (ICP-AES), and electrothermal atomization atomic absorption spectrophotometry (ETA-AAS) (9–12). The latter, when used with an L'vov platform, yields the most reproducible results (13–15). Acid decomposition is the most widely used method for sample digestion. Several combinations of acids and oxidants have been proposed with different time and temperature programs (10–12). One of the best techniques is based on the use of both sealed polytetrafluoroethylene flasks and on microwaves to reduce digestion time (16–19).

We have perfected an analytical method suitable to determine Pb in food and feed crops, based on ETA-AAS with an L'vov platform, following optimization of the temperature-time program for the graphite furnace. The samples are mineralized in a microwave acid digestion bomb. To optimize the procedure, we did a series of assays at different power settings of a microwave oven, combining the settings with different durations of digestion and varying the amounts of sample and nitric acid. The method can be used in quality control assays of food and feed crops grown in Mediterranean coastal regions of Granada (southern Spain), a zone characterized by intensive cropping and the widespread use of fertilizers (including sewage sludge) and pesticides. The farmlands from which we obtained the plant samples are also characterized by their proximity to potential sources of pollution such as industries, heavily traveled highways, and urban communities.

Experimental

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer Model 2380 double-beam atomic absorption spectrophotometer equipped with a deuterium arc background corrector and hollow cathode lamp (Perkin-Elmer Corp., Norwalk, CT); Bausch & Lomb D-5000 chart recorder (Bausch & Lomb, Rochester, NY).

(b) *Graphite furnace.*—Perkin-Elmer HGA-400 (Perkin-Elmer Corp.). Pyrolytic graphite tubes with an L'vov platform.

(c) *Microwave oven.*—Moulinex Model FM-460 with 15–100% full power (600 W) capability in 25% increments.

(d) *Microwave acid digestion bomb.*—Parr Model 4782 (Parr Instrument Co., Moline, IL).

Reagents

(a) *Nitric acid.*—Reagent grade, 65% (E. Merck, Darmstadt, Germany).

(b) *Vanadium pentoxide.*—Analytical reagent grade (E. Merck).

(c) *Lead standard solution.*—Titrisol lead solution (1.00 ± 0.002 g) (E. Merck).

(d) *Ammonium molybdate.*—Reagent grade (E. Merck).

(e) *Water.*—All solutions were prepared from deionized water with a specific resistivity of 18 MΩ.cm, which was obtained by filtering distilled water through a Millipore Milli-Q Model RO15 purifier immediately before use.

Material

To eliminate absorbance due to detergents and samples, all glassware and polyethylene sample containers were washed with tap water after each use, soaked in a 6N HNO_3 solution (at least overnight), and rinsed several times in ultrapure water.

Samples

Samples were collected from farm plots in the coastal region of Granada. We obtained samples from plots located near potential sources of pollution and used random sampling in the rest of the plots.

In the study area, vegetables, fruits, and cereal (*Zea mays*) were grown, although the predominant irrigation crop was sugarcane (*Saccharum officinarum*).

The number of samples collected of each species depended on the relative abundance of the crops. To optimize the amount of plant material, previous assays were done to ensure that samples were homogeneous and representative of the crop investigated (20).

Depending on their morphological features, the samples were peeled or washed with potable water, then rinsed several times in deionized water. Fractions (250 g) of the edible portion of each species were dried at low setting in a microwave oven. The dried samples were ground into a fine powder, homogenized as appropriate, and stored at 4°C in sealed polyethylene jars. Lyophilized samples were prepared of each species and subjected to the same analytical technique. No significant differences were found between data obtained with the two procedures.

Sample Digestion

Dried and homogenized samples (0.200 g) were treated with 2.5 mL 65% HNO_3 and 35 mg V_2O_5 as a catalyst (21), and then digested in a microwave acid digestion bomb. Mineralization was complete in 90 s with the oven at its highest setting. The digestion bomb was cooled by freezing at -18°C for 30–40 min, then the solution was diluted to a total volume of 25 mL in a glass volumetric flask with ultrapure water.

Lead Determination

To avoid the formation of refractory carbides, the tube was pretreated by injecting 10 µL saturated ammonium molybdate solution and by heating according to the time-temperature program optimized for samples (Table 1). A sample aliquot of 10 µL was analyzed under the optimized conditions at a wavelength of 283.3 nm and a slit width of 0.7 nm. The conditions were optimized on the basis of time-temperature studies using National Institute of Standards and Technology (NIST) standard reference material *Citrus leaves* (SRM 1572). Mineralization of the matrix was complete after heating at 500°C for 30 s. The atomization temperature that yielded maximum signals was 2100°C for 6 s, with an integration time of 6 s. Argon was used as the internal gas. Flow was stopped during the atomization stage to increase sensitivity; this did not alter the usable life of the tube. The furnace was cleaned by raising the temperature to 2650°C; the graphite tube was cooled to 20°C between analyses. For the instrumental conditions used, the analytical detection limit was 0.04 ng/mL (22).

The calibration plot was obtained from a working solution of 1 µg/mL and successive dilutions with 10% HNO_3 (65%). The working solution, along with the blank (2.5 mL 65% HNO_3, 35 mg V_2O_5, and ultrapure water to a total volume of 25 mL), was subjected to the same acid digestion bomb treatment as the samples (although this treatment was shown to be unnecessary). The equation for the calibration plot is as follows:

$$\text{Absorbance} = -2.16 \times 10^{-3} + 2.29 \times 10^{-3}\ [\text{Pb, ng/mL}]$$

where $r = 0.9965$ and $\alpha = 1\%$ (α, level of significance). The results were linear over a range of up to 1 µg/mL.

The standard additions method (23) were used to detect possible interferences; standards for digestion and dilution were processed in the same way as samples. The slopes of the calibration plot for spiked samples were similar to the slope of the calibration plot for the standards in acid medium; thus, matrix effects were considered negligible.

Table 1. Instrumental conditions for the determination of lead in food and feed crops

Step	Temp., °C	Ramp time, s	Hold time, s	Gas flow rate, mL Ar/min
Dry	100	20	10	100
	150	15	25	100
Char	500	15	15	100
Atomize	2100	1	5	stop
Clean	2650	1	2	100
	20	1	2	100

Table 2. Accuracy and precision of measured concentrations of lead in NIST standard reference material

Reference material	Pb concn, μg/g dry weight		Recovery, %	Precision RSD, %[b]
	Measured[a]	Certified[a]		
SRM 1572 Citrus leaves	13.1 ± 0.86	13.3 ± 2.4	98.50	9.2

[a] Mean ± standard deviation, at 95% confidence internal about the mean (n = 10).
[b] Relative standard deviation.

Results and Discussion

Accuracy and Precision

The accuracy of this analytical method was checked with recovery assays (20). Known amounts of analyte were added to 5 different randomly chosen samples and the mixtures were processed for acid digestion and dilution as described above for experimental samples. Percentage recoveries ranged from 97.37 to 100.50%.

Accuracy and precision were also checked in 10 determinations of NIST standard reference material (Table 2). In addition, reproducibility of the spectrophotometric determinations and absorbance values from 10 successive measurements in 2 different samples were statistically analyzed (24). The results of the precision tests are shown in Table 3.

Sample Analysis

The procedure described above was used to determine Pb concentrations in 51 samples of 7 different plant species. The mean values and range of concentrations in each species are given in Table 4, in which all data are referred to fresh weight of the edible fraction.

The developed digestion procedure provides rapid, complete mineralization of the sample with minimal losses and avoids external contamination by the reaction vessel or reagents because of the small amounts involved of these materials. Mineralization with the microwave acid digestion bomb technique was complete within 90 s, which represents a significant savings in time when compared with other methods that require hours or even days. The use of a small volume of acid and the simplicity of the entire procedure reduce the risk of contamination, an important factor in the determining trace elements. More than one element at a time can be analyzed in a single analytical solution, and the method is suitable for a wide variety of samples (25–27). The digestion procedure increases the accuracy and precision of the analytical technique and is appropriate for the range of Pb concentrations found in the experimental samples discussed here (28).

Lead was quantified by ETA-AAS with an optimized time-temperature program for the drying, charing, and atomization phases in a graphite furnace. Factors with the potential to cause interferences were eliminated, and the final method was found to be highly accurate and precise. The optimized assay conditions obviate most matrix interferences and other sources of unspecific absorption. Omission of the standard additions method considerably simplifies the analysis. The detection limit and sensitivity are suitable for the range of Pb concentrations encountered and are compatible with estimates given by other authors. Moreover, the analytical accuracy and precision of our method are acceptable (28).

The advantages of this method make it useful for routine analysis, not only for quality control of food and feed crops, but also as a measure of environmental contamination and as a tool in assessing medium- and long-term health risks to humans.

The mean concentration of Pb in the plant samples analyzed in this study was 0.304 ± 0.490 μg/g (mean ± standard deviation). The variability of the data emphasizes the influence of species-specific characteristics on the accumulation of heavy metals by plants and reflects variations in contamination between different sampling points. The highest Pb levels were found in corn (*Zea mays*) and green bean (*Phaseolus vulgaris*). These 2 species are able to absorb large amounts of heavy metals from the soil; in *Zea mays*, absorption is further facilitated by the extensive superficial root system (5). No statistically significant correlation was found between Pb concentration and the proximity of the sampling point to potential sources of contamination (industries, heavily traveled highways, and urban communities). Thus, Pb concentrations in the crops analyzed here may be influenced by as yet undetermined interactions

Table 3. Statistical analysis of results of determinations of lead in food and feed crops by electrothermal atomic absorption spectrometry

Sample	x[a]	S_{n-1}[b]	S_m[c]	$x \pm S_m \cdot t$[d]	$\frac{S_m \cdot t}{x}$, %
Lettuce-1 (*L. sativa*)	0.031	1.767×10^{-3}	5.588×10^{-4}	$0.031 \pm 1.264 \times 10^{-3}$	4.07
Green bean-1 (*P. vulgaris*)	0.080	1.229×10^{-3}	3.887×10^{-4}	$0.080 \pm 8.793 \times 10^{-4}$	1.09

[a] x, mean value of 10 determinations.
[b] S_{n-1}, standard deviation for 10-replicate determinations.
[c] S_m, standard error of the mean.
[d] The value of Student's t was 2.262 for both samples.

Table 4. Concentrations of lead (μg/g fresh weight) in food and feed crops grown on the coast of Granada (Spain)

Sample	No. of samples	Mean	Range
Sugarcane (*S. officinarum*)	24	0.131	0.030–0.265
Sweet corn (*Z. mays*)	11	0.983	0.124–2.695
Potato (*Solanum tuberosum*)	6	0.048	0.025–0.087
Green bean (*P. vulgaris*)	4	0.209	0.119–0.422
Banana (*Musa paradisiaca*)	2	ND[a]	—
Lettuce (*L. sativa*)	2	0.179	0.108–0.250
Tomato (*Lycopersicum esculentum*)	2	0.034	0.034–0.035

[a] Not detectable.

between different types of contamination, in combination with Pb supplied through irrigation water, fertilizers, and pesticides.

Although the values of Pb found in these food and feed crops do not represent an imminent toxicological risk, periodic determinations are advisable because of the large amounts of these products that are consumed as part of the Mediterranean diet.

Acknowledgments

This study was supported by the Control Analítico y Toxicológico de Alimentos y Bebidas de Consumo en Andalucía, program sponsored by the Andalusian Regional Government. Appreciation is expresed to the Laboratorio Agroalimentario de Granada for assistance and to Karen Shashok for translating the original manuscript into English.

References

(1) Martin, M.H., & Coughtrey, P.J. (1982) in *Biological Monitoring of Heavy Metal Pollution*, Applied Science Publishers, New York, NY, pp. 10–80

(2) Dalenberg, J.W., & Van Driel, W. (1990) *Neth. J. Agric. Sci.* **38**, 369–379

(3) Jentschke, G., Fritz, E., & Godbold, D. (1991) *Physiol. Plant.* **81**, 417–422

(4) Setia, N., Kaur, D., & Setia, R.C. (1989) *J. Plant Sci. Res.* **5**, 127–132

(5) Reilly, C. (1980) in *Metal Contamination of Food*, Applied Science Publishers, London, UK, pp. 97–103

(6) WHO (1987) in *Toxicological Evaluation of Certain Food Additives and Contaminants*, the 30th Meeting of the Joint FAO-WHO Expert Committee on Food Additives, Cambridge University Press, Cambridge, UK, pp. 223–230

(7) Goyer, R.A. (1988) in *Handbook of Toxicity of Inorganic Compounds*, H.G. Seiler, & H. Sigel (Eds), Marcel Dekker, New York, NY, pp. 362–363

(8) Mahaffey, K.R., Corneliussen, P.E., Jelinek, C.F., & Fiorino, J.A. (1975) *Environ. Health Perspect.* **12**, 63–69

(9) Dabeka, R.W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1005–1008

(10) Dabeka, R.W., & Makenzie, A.D. (1986) *J. Spectrosc.* **31**, 44–52

(11) Skurikhin, I.M. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 286–293

(12) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, sec. **972.25**

(13) Slavin, W., & Manning, D.C. (1980) *Spectroch. Acta* **35B**, 701–714

(14) Rains, T., Rush, T., & Butler, T. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 995–998

(15) Koirtyohann, S., Kaiser, M., & Hinderberger, E. (1982) *J. Assoc. Off. Chem.* **65**, 999–1004

(16) Friel, J.K., Skinner, C.S., Jackson, S.E., & Longerich, H.P. (1990) *Analyst* **115**, 269–273

(17) Okamoto, K., & Fuwa, K. (1984) *Anal. Chem.* **56**, 1758–1760

(18) Kingston, H.M., & Jassie, L.B. (1986) *Anal. Chem.* **58**, 2534–2541

(19) Stripp, R., & Bogen, D. (1989) *J. Anal. Toxicol.* **13**, 57–59

(20) Horwitz, W. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 455–466

(21) Bonilla, M., Rodriguez, L., & Camara, C. (1987) *J. Anal. At. Spectrom.* **3**, 157–161

(22) Long, G.L., & Winefordner, J.D. (1983) *Anal. Chem.* **55**, 713A–715A

(23) Kalivas, J.H. (1983) *Anal. Chem.* **55**, 565–567

(24) Stiel, R.G. (1982) in *Principles and Procedures of Statistics*, McGraw-Hill Book Co., London, UK

(25) Cabrera, C., Lorenzo, M.L., Gallego, C., López, M.C., & Lillo, E. (1991) *Anal. Chim. Acta* **246**, 375–378

(26) Cabrera, C., Lorenzo, M.L., Gallego, C., López, M.C., & Lillo, E. (1992) *J. Agric. Food Chem.* **40**, 1631–1633

(27) Navarro, M., López, M.C., López, H., & Sanchez, M. (1992) *J. AOAC Int.* **75**, 1029–1031

(28) Horwitz, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 525–530

RESIDUES AND TRACE ELEMENTS

Determination of Inorganic Anions in Water by Ion Chromatography: Collaborative Study

KENNETH W. EDGELL
The Bionetics Corp., 16 Triangle Park Dr, Cincinnati, OH 45246
JAMES E. LONGBOTTOM
Quality Assurance Research Division, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, 26 West Martin Luther King Dr, Cincinnati, OH 45268
JOHN D. PFAFF
Chemistry Research Division, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, 26 West Martin Luther King Dr, Cincinnati, OH 45268

Collaborators: R. Altman; E. Arar; M. Baker; T. Bennett; M. Bolyard; R. Cassat; D. Head; R. Joyce; D. Kersey; R. Latsh; R. Lawrence; J. Meyer; H. Paterson; M. Peden; R. Potts; J. Raia; H. Rhodes; L. Rodriquez; E. Sorbet; J. Vickers; P. Walton; B. Warden

The U.S. Environmental Protection Agency (U.S. EPA) and the American Society for Testing and Materials (ASTM) conducted a joint collaborative study validating an ion chromatographic method for determination of inorganic anions (U.S. EPA method 300.0A and the equivalent proposed revision to ASTM method D4327). This study was conducted to determine the mean recovery and precision of analyses for bromide, chloride, fluoride, nitrate, nitrite, orthophosphate, and sulfate in reagent water, drinking water, and wastewater. The study design was based on Youden's nonreplicate plan for collaborative tests of analytical methods. The test waters were spiked with the anions at 6 concentration levels, prepared as 3 Youden pairs. The 22 volunteer laboratories were instructed to dilute 10 mL sample concentrate to 100 mL test water. A measured volume of sample (20–200 μL) was injected into an ion chromatograph equipped with a guard column, anion exchange column, and a chemical micromembrane suppression device. The anions were then separated using 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate, and measured by a conductivity detector. Submitted data were evaluated using U.S. EPA's IMVS computer program, which follows ASTM D2777-86 statistical guidance. U.S. EPA method 300.0A and ASTM method D4327 were judged acceptable for measurement of the above anions (except sulfate) at concentrations ranging from 0.3 to 25 mg/L and sulfate concentrations from 2.9 to 95 mg/L. Mean recoveries for the 7 anions from all matrixes, as estimated from the linear regression equations, ranged from 95 to 104%. At concentrations above 2–6 mg/L for bromide, fluoride, nitrate, nitrite, and orthophosphate, and above 24 mg/L for sulfate, the overall and single-analyst relative standard deviations were less than 10 and 6%, respectively. As concentrations decreased, precision became more variable. The relative standard deviations of results for chloride were slightly higher than the other anions, especially in matrixes with high chloride background. Analysis of Variance (ANOVA) tests at the 95% confidence interval indicated a statistically significant matrix effect for chloride, nitrite, and nitrate analyses in drinking water compared to analyses in reagent water. Because these matrix effects were caused by the spiking process and not the drinking water itself, the ANOVA determination was not considered to be of practical significance.

Submitted for Publication April 22, 1993.
The recommendation was approved by the Committee on Environmental Quality, and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1993) *J. AOAC Int.* **76**, 125A, and "Official Methods Board Actions" (1993) *The Referee* **17**, September issue.

Ion chromatography using U.S. Environmental Protection Agency (U.S. EPA) method 300 (1) was first approved for determination of nitrates in drinking water by the U.S. EPA in 1984. The method was upgraded as U.S. EPA method 300.0A (2) to incorporate current equipment specifications and in January 1991, ion chromatography was approved for determination of the regulated anions, nitrate and nitrite (3).

The U.S. EPA Environmental Monitoring Systems Laboratory in Cincinnati, OH (EMSL-Cincinnati), develops or selects analytical methods and provides quality assurance (QA) sup-

port for Agency programs that involve water and wastewater regulations. Within EMSL-Cincinnati, the responsibility for providing QA support is assigned to the Quality Assurance Research Division (QARD). One of QARD's activities is to conduct interlaboratory method validation studies to evaluate analytical methods selected for the Agency's operating programs. EMSL-Cincinnati (for U.S. EPA) and American Society for Testing and Materials (ASTM) Committee D-19 have agreed to conduct joint interlaboratory method validation studies on methods of common interest. ASTM, through its consensus standard process, provided a forum for review of analytical methods, study designs, selection of appropriate test materials, and solicitation of volunteer laboratories for the study. U.S. EPA conducted the study and evaluated the data using statistical procedures acceptable to both organizations.

The objectives of this study were 3-fold: *(1)* to characterize multilaboratory performance of U.S. EPA method 300.0A and the equivalent ASTM Method D4327 in terms of recovery, overall precision, and single-analyst precision; *(2)* to compare the recovery and precision for 7 anions in reagent water, drinking water, and wastewater; and *(3)* to develop appropriate QC acceptance limits for use in assessing overall data quality.

The joint study for U.S. EPA method 300.0A was conducted under the direction of QARD, EMSL-Cincinnati. As primary contractor to QARD, The Bionetics Corp. was responsible for preparing and distributing sample concentrates, user instructions, and report forms, reviewing data, and drafting a final report. The raw data were statistically evaluated by QARD using its computer program, IMVS (4), designed for these studies.

Volunteer laboratories were solicited by EMSL-Cincinnati and ASTM Committee D-19, and 25 laboratories who volunteered to participate in the study were sent sample concentrates in October 1990; 22 laboratories returned data by the closure date in December 1990.

Collaborative Study

The study design was based on Youden's nonreplicate design for collaborative evaluation of overall precision, single-analyst precision, and mean recovery for analytical methods (5). Two samples, differing in analyte concentration by 5–20% of the mean of the pair, were analyzed as a Youden pair to provide data for estimating single-analyst precision. In this study, 3 concentration pairs encompassed the relatively low concentration ranges of 0.3–25 mg/L for all anions except sulfate and 2.9–95 mg/L for sulfate. The study design called for each participant to spike the 7 anions into reagent water, drinking water, and a wastewater of interest to them. In addition to the spiked matrixes, each participant was asked to analyze a single unspiked sample (blank) of reagent water, drinking water, and wastewater as background measures. Evaluation of results obtained for spiked reagent water demonstrated method proficiency in recovering the inorganic anions on a sample free of interferences. By difference, recoveries of spikes into drinking water and wastewater tested method suitability for these water types and gave a measure of interference by comparison with results from reagent water. The drinking water and wastewater matrixes used in this study were selected by the participants.

Quality control (QC) requirements consistent with the method were incorporated into this study to monitor various stages of the analytical process. Each participant was sent a concentrate to prepare a QC sample containing the 7 anions at concentrations between 5–25 mg/L. Participants were instructed to analyze the QC sample before analyzing each set of spiked matrix samples. If the QC sample results fell outside of the acceptance limits of ± 10% of spike concentration, the laboratory was directed to reanalyze the QC sample and, if the second analysis also failed, recalibrate its instrument. Because the fluoride anion elutes quickly in the region of the negative baseline excursion caused by water, it was not held to QC acceptance limits required for the other 6 analytes. All QC results, including fluoride results, were requested as part of the study data.

Each participating laboratory received 18 spike solutions (6 unknown solutions for spiking into 3 water matrixes), one known calibration standard concentrate, one QC sample concentrate with true values and acceptance limits, report forms, a questionnaire, an instruction manual, and a formatted 5¼ in. disk for reporting results. Copies of the method were distributed to the participants prior to the study. The sample spiking concentrates, calibration concentrates, and QC sample concentrates were prepared using filter-sterilized (0.22 μm), deionized water, and packaged in flame-sealed 20 mL ampules (12 mL/ampule). The ampules were previously washed with tap water and double rinsed with deionized water. Collaborating laboratories were instructed to calibrate their analytical instruments using the provided calibration standard concentrate, analyze the spiked samples in strict accord with the written method (2), and complete the analyses within 30 days after receipt of the samples. Prior to distribution, the concentrations of ampule solutions were confirmed using U.S. EPA method 300.0A vs standards freshly prepared from neat materials.

Study data were recorded on 5¼ in. data entry disks using EMSL-Cincinnati's software program, RETRIEVE. This MS-DOS based software structures a data file for a collaborative study, produces personalized data entry disks, and then reads the data on the returned disks into a consolidated data file for further computer processing.

993.30 Inorganic Anions in Water—Ion Chromatographic Method

U.S. EPA-ASTM-AOAC Method

First Action 1993

(Applicable to determination of bromide, chloride, fluoride, nitrate-N, nitrite-N, orthophosphate, and sulfate in drinking water and wastewater in the range shown in Table **993.30A**.)

Method performance:

See Table **993.30A** for method performance data.

Table 993.30A. Method performance for determination of inorganic anions in drinking water (DW) and wastewater (WW) by ion chromatographic method

Anion	Water type	Av. Youden pair spikes, mg/L	Mean, mg/L	r^a	R^b	RSD_r, %	RSD_R, %	Mean rec. of spike, %
Bromide	DW	0.74	0.72	0.04	0.12	6.22	16.45	97.96
	DW	5.77	5.70	0.13	0.32	2.32	5.65	98.87
	DW	18.90	18.91	0.57	0.59	2.99	3.10	100.05
	WW	0.74	0.67	0.31	0.31	46.42	46.42	91.16
	WW	5.77	5.73	0.12	0.43	2.05	7.53	99.39
	WW	18.90	18.80	0.66	1.13	3.52	6.03	99.47
Chloride	DW	0.91	1.38	0.38	0.67	27.93	48.31	151.65
	DW	7.15	7.72	0.55	1.08	7.06	14.04	107.97
	DW	23.40	23.93	1.45	2.60	6.05	10.85	102.26
	WW	0.91	1.01	0.33	0.63	33.01	62.86	110.99
	WW	7.15	7.06	0.89	0.92	12.58	13.09	98.74
	WW	23.40	23.02	0.75	2.16	3.27	9.40	98.38
Fluoride	DW	0.30	0.32	0.05	0.08	15.62	26.42	106.67
	DW	2.34	2.30	0.06	0.21	2.48	9.19	98.50
	DW	7.64	7.56	0.24	0.69	3.12	9.15	98.95
	WW	0.30	0.28	0.06	0.12	22.71	43.44	93.33
	WW	2.34	2.37	0.08	0.22	3.23	9.43	101.50
	WW	7.64	7.54	0.22	0.40	2.94	5.36	98.69
Nitrate-N	DW	0.49	0.55	0.03	0.10	5.07	18.53	112.24
	DW	3.86	3.93	0.15	0.49	3.72	12.51	101.81
	DW	12.60	12.71	0.44	0.68	3.49	5.35	100.87
	WW	0.49	0.49	0.05	0.11	10.71	22.29	100.00
	WW	3.86	3.69	0.04	0.24	1.19	6.49	95.60
	WW	12.60	12.42	0.47	0.52	3.78	4.16	98.57
Nitrite-N	DW	0.42	0.36	0.03	0.12	7.31	34.24	85.71
	DW	3.30	3.34	0.07	0.23	2.16	6.81	101.21
	DW	10.80	10.64	0.28	0.53	2.63	4.95	98.52
	WW	0.42	0.39	0.05	0.09	12.40	23.75	92.86
	WW	3.30	3.52	0.07	0.16	2.05	4.55	106.67
	WW	10.80	10.74	0.36	0.83	3.38	7.69	99.44
o-Phosphate	DW	0.81	0.70	0.22	0.35	31.54	50.90	86.96
	DW	6.35	5.98	0.15	0.65	2.53	10.79	94.25
	DW	20.75	20.32	0.59	0.89	2.93	4.40	97.93
	WW	0.81	0.81	0.08	0.18	10.18	21.92	100.62
	WW	6.35	5.94	0.34	0.63	5.74	10.54	93.62
	WW	20.75	20.12	1.22	1.82	6.07	9.06	96.96

Table 993.30A. (*continued*)

Anion	Water type	Av. Youden pair spikes, mg/L	Mean, mg/L	r[a]	R[b]	RSD_r, %	RSD_R, %	Mean rec. of spike, %
Sulfate	DW	3.33	3.26	0.23	0.59	7.16	18.19	98.05
	DW	26.15	26.20	0.28	0.98	1.09	3.73	100.19
	DW	85.50	85.49	2.84	7.77	3.32	9.08	99.99
	WW	3.33	3.42	0.48	0.96	14.16	28.03	102.86
	WW	26.15	26.16	0.68	2.75	2.58	10.52	100.04
	WW	85.50	84.21	3.30	4.87	3.92	5.78	98.49

[a] Repeatability.
[b] Reproducibility.

A. Principle

Anions in test sample are separated by an ion chromatographic system containing guard column, separator column, and suppressor device and are measured using conductivity detector.

B. Apparatus

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

(**b**) *Ion chromatographic system.*—Equipped with guard column, anion separator column (Dionex AS4A, Dionex Corp., Sunnyvale, CA, is suitable), anion suppressor device, syringes, pumps to maintain flow rate of 2 mL/min, 50 µL sample loop, compressed gases, and conductivity detector with ca 1.25 µL internal volume. Data system is recommended for measuring peak areas.

(**c**) *Sample bottles.*—60 mL high-density polyethylene bottles with polypropylene (20 mm thickness) screw cap.

C. Reagents

(**a**) *Reagent water.*—Distilled or deionized water, free of the anions of interest, as determined by this method.

(**b**) *Eluent solution.*—1.7 mM sodium bicarbonate ($NaHCO_3$), 1.8 mM sodium carbonate (Na_2CO_3). Dissolve 0.2856 g $NaHCO_3$ and 0.3816 g Na_2CO_3 and dilute to 2 L in reagent water, (**a**).

(**c**) *Regeneration solution (micromembrane suppressor).*—0.025N sulfuric acid (H_2SO_4). Dilute 2.8 mL concentrated H_2SO_4 to 4 L with reagent water, (**a**).

(**d**) *Stock standard solutions.*—1000 mg/L (1 mg/mL): Stock standard solutions may be purchased as certified solutions or prepared from ACS reagent grade materials (dried at 105°C for 30 min). To prepare 1000 mg/L stock standard solutions, dissolve and dilute to 1 L in reagent water, (**a**), the following: *(1) Bromide (Br^-).*—1.2876 g sodium bromide (NaBr); *(2) Chloride (Cl^-).*—1.6485 g sodium chloride (NaCl); *(3) Fluoride (F^-).*—2.2124 g sodium fluoride (NaF); *(4) Nitrate (NO_3^-–N).*—6.0679 g sodium nitrate ($NaNO_3$); *(5) Nitrite (NO_2^-–N).*—4.9257 g sodium nitrite ($NaNO_2$); *(6) Phosphate (PO_4^{3-}–P).*—4.3937 g potassium phosphate (KH_2PO_4); and *(7) Sulfate (SO_4^{2-}).*—1.8141 g potassium sulfate (K_2SO_4).

Stock standard solutions are stable at least 1 month stored at 4°C. Prepare working standards fresh weekly, except prepare nitrite and phosphate working standards fresh daily.

D. Sample Collection, Preservation, and Storage

Collect samples in scrupulously clean sample bottles, **B(c)**. Sample preservation and holding times for fluoride, bromide, and chloride are "none required" and 28 days; for nitrate-N, nitrite-N, and orthophosphate-P are "cool to 4°C" and 48 h; and for sulfate are "cool to 4°C" and 28 days, respectively.

Method of preservation and holding time for samples are determined by anions of interest that require most preservation and shortest holding time. It is recommended that all samples be cooled to 4°C and held <48 h.

E. Calibration

Use ion chromatographic operating parameters recommended in instrument operation manual and in **B(b).**

Prepare calibration standards at 3 concentration levels minimum and a blank that brackets anticipated sample concentration range. Dilute stock standards, **C(d)**, with reagent water, **C(a)**, in volumetric flasks. If sample analyte concentration exceeds calibration range, dilute sample to fall within range. Calibrate each attenuation range of instrument individually.

Collaborative study linear ranges are ca 0.3–25 mg/L (all anions), except 2–100 mg/L range for sulfate. *See* Table **993.30A** for anion specific ranges.

Inject 0.1–1.0 mL (determined by injection loop volume) of each calibration standard and plot peak height or area responses against concentration.

Nonlinear response can result when separator column capacity is exceeded (overloading). Compare response of sample diluted 1:1 to response of undiluted sample. If calculated responses are equal, samples need not be diluted.

F. Procedure

Load and inject fixed amount of well-mixed sample. Flush injection loop thoroughly with each new sample. Use same size loop for standards and samples. Record resulting peak size in area or peak height units. Automated constant volume injection system may also be used.

Dilute sample with reagent water, **C(a)**, and reanalyze if response for peak exceeds working range of system.

If resulting chromatogram fails to produce adequate resolution, or if identification of specific anions is questionable, fortify sample with an appropriate amount of standard, **C(d)**, and reanalyze.

Note: Retention time is inversely proportional to concentration. Nitrate and sulfate exhibit greatest amount of change, although all anions are affected to some degree. In some cases, this peak migration may produce poor resolution or identification. Small organic acids or carbonate ion coelute with fluoride ion which can affect fluoride quantitation.

G. Calculations

Determine peak height or area counts of anions in samples. Compare with calibration curve to determine concentration.

H. Quality Control

Minimum QC requirements:

(**a**) *Initial demonstration of laboratory capability.*—Prepare and analyze 4 laboratory fortified blanks (LFB) using aliquots of stock standards, **C(d)**, in 100 mL volumetric flasks; dilute to volume with reagent water, **C(a)**, at concentration levels indicated in Table **993.30B**. Calculate percent recovery in each sample. Repeat test if measurements are not in range shown in Table **993.30B**; check instrument calibration.

(**b**) *Assess laboratory reagent blank (LRB).*—Before processing samples, demonstrate that glassware and reagent interferences are under control. Analyze LRB, prepared using reagent water, **C(a)**, with each set of samples or when reagents are changed. If LRB produces a peak within retention time window of any analyte, determine and eliminate source of contamination before processing samples.

(**c**) *Assess fortified matrix recovery.*—Prepare fortified matrix sample using spike level indicated in Table **993.30B** in 100 mL volumetric flask; dilute to volume using appropriate matrix. Analyze one fortified matrix every 10% of routine samples, or one fortified matrix per set, whichever is greater. Fortified concentration should be no less than background concentration of sample selected for fortification, and ideally equal to that used for LRB. Calculate percent recovery for each analyte, corrected for background concentrations measured in unfortified sample, and compare recoveries to control limits in Table **993.30B**. If recovery of any analyte falls outside acceptance limits and laboratory performance for that analyte is in control, recovery problem is judged matrix related, not system related.

(**d**) *Assess field and laboratory precision.*—Collect one field duplicate for each set of samples or every 20 samples to monitor sampling precision. Analyze one laboratory duplicate for each set of samples or every 20 samples to monitor analytical precision.

(**e**) *Assess laboratory performance.*—Verify calibration curve each working day, or whenever anion eluent is changed, and after every 20 samples by analyzing one LFB containing each analyte of interest at concentration levels indicated in Table **993.30B**. If retention time for any analyte varies from expected values by $> \pm 10\%$, repeat test, using fresh calibration standards. If recovery for any analyte varies more than acceptance limits in Table **993.30B**, prepare a new calibration curve for that analyte.

(**f**) At least quarterly, analyze one U.S. EPA (U.S. Environmental Protection Agency) or AALA (American Association for Laboratory Accreditation) Certified Reference Material, or equivalent, as quality control sample to verify that standard solutions and calibration curves have been correctly prepared. Prepare fresh calibration standards and recalibrate if results fall outside acceptance limits in Table **993.30B**.

(**g**) Annual participation in external performance evaluation study is recommended.

Ref.: *J. AOAC Int.* **77**, 1253(1994).

CAS 7647-15-6 Sodium bromide
CAS 7647-14-5 Sodium chloride

Table 993.30B. Quality control acceptance limits for analysis of control sample, as percent of spike value, in determination of inorganic anions in water by ion chromatographic method

Anion	Recommended conc., mg/L	Predicted recovery, mg/L[a]	Predicted std. dev., mg/L[a]	Acceptance limits[b]	
				mg/L	%
Bromide	10.0	9.99	0.44	8.67–11.31	87–113
Chloride	10.0	9.94	0.36	8.86–11.02	89–110
Fluoride	5.0	4.94	0.18	4.40–5.48	88–110
Nitrite-N	5.0	5.18	0.17	4.67–5.69	93–114
Nitrate-N	5.0	4.92	0.25	4.17–5.67	83–113
Phosphate-P	10.0	10.02	0.64	8.10–11.94	81–119
Sulfate	25.0	24.99	1.58	20.25–29.73	81–119

[a] Predicted from linear regression equations derived for collaborative study.
[b] Acceptance limits are defined as $X \pm 3\ s_R$, where X = predicted recovery (mg/L), and s_R = predicted standard deviation (mg/L).

CAS 7681-49-4	Sodium fluoride
CAS 7631-99-4	Sodium nitrate
CAS 7632-00-0	Sodium nitrite
CAS 7778-77-0	Potassium phosphate
CAS 7778-80-5	Potassium sulfate

Treatment of Data

Each data set was initially screened for calculation and transcription errors by looking for extreme data points which were less than one-fifth or greater than 5 times the true value. If found, the responsible laboratory was contacted and asked to review its calculations and transcription of the extreme data points. If an error was found, the laboratory was permitted to substitute corrected results. If an error was not found, extreme data were submitted with other study data for statistical processing.

The QC sample data and narrative submitted by the participants were evaluated for compliance with the acceptance limits of 90–110% of the spike value and for evidence of corrective actions, if appropriate. A pattern of chronic QC sample failures, with no documented corrective action, was considered grounds for removing the laboratory data set from the study.

The participating laboratories were instructed to report background concentrations of the anions in the matrix blanks separately and not to submit "background-corrected" data. Because background corrections were performed by EMSL-Cincinnati, the effect of background concentration on the spiked concentration could be evaluated.

The background-corrected data were grouped by water type, arranged into 6 subsets as defined by the 6-concentration levels, and evaluated using U.S. EPA's IMVS program. First, missing data points from the 6-concentration set were replaced by values estimated from the other concentrations and "less than" and "nondetect" values were converted to zero. Then, outlier tests suggested in the ASTM Standard Practice D2777-86 (6) were applied. The first outlier test was Youden's laboratory ranking procedure (5), which rejects a laboratory that had a consistently higher or lower bias in its submitted data for a given analyte compared to the other laboratories. If a bias was determined, all 6 analyte values for that laboratory were rejected. This procedure was applied to each analyte data set, for each water type, at the 5% level of significance. Next, zeroes, interpolated values, and negative numbers were removed before further analyses. As a final outlier test, Thompson's test for individual outliers (7) was applied to the retained data for a specific water type and sample at the 5% significance level. If an individual data point was rejected based on this test, it was removed from the subset and the test was repeated once more on the remaining data for that water type and sample.

Summary statistics were calculated for the mean recovery and overall precision for each of the 6-concentration levels. Single-analyst precision was calculated for each of the 3 Youden concentration pairs. The IMVS computer program used the summary statistics for the 6-concentration samples to calculate relationships between mean recovery and true concentration and between precision and mean recovery in the form of linear regression equations, using the weighted least squares technique (8, 9). Coefficients of determination of the weighted regression equations (COD_w) were also calculated to evaluate the fit of the regressions to the retained data sets. For these same data, IMVS also determined statistically significant matrix effects between the reagent water and the other matrixes.

Results and Discussion

Initial Data Screen

Of the 22 laboratories that submitted data during this study, 3 laboratory data sets were completely removed and 2 data sets were partially removed. Data from 2 laboratories were excluded because they used instrument configurations outside the scope of the method. Laboratory 22 used an electronically suppressed ion chromatograph, and Laboratory 23 used a chemically suppressed system other than the micromembrane system specified in the method. Data from Laboratory 8 were rejected because this collaborator did not return any data for orthophosphate or sulfate, reported out-of-control nitrate QC results for all 3 QC samples, and reported hydrochloric acid contamination in its reagent water source. Data for bromide, nitrate, and orthophosphate from Laboratory 6 were removed because the laboratory mislabeled 3 of the 7 anions during analysis and quantification. Sulfate and chloride wastewater data from Laboratory 7 were removed because several of its wastewater samples were diluted to different levels than the wastewater blank, making background correction impossible to apply. Data for remaining anions were not affected because no background concentrations were present in the wastewater.

Quality Control Sample Results

Of the 22 laboratories, 11 successfully passed all 18 QC checks (6 anions × 3 analyses). Of the 11 laboratories that failed one or more of the 18 QC checks, only 3 performed corrective action as instructed. Failures of QC sample checks by Laboratory 6 resulted from mislabeled peaks; reanalysis of the QC samples did not improve its results. Laboratories 10 and 17 recalibrated after several unacceptable QC sample results, and both submitted acceptable data after recalibration.

The results reported for fluoride in the QC sample were very encouraging. Although the participating laboratories were not required to achieve the 90–110% acceptance criterion for the fluoride QC sample results, they had no problem in doing so. Only Laboratory 2 showed a low systematic bias for all 3 of its fluoride results. Since recalibration was not required as a result of low fluoride QC recovery, the Laboratory 2 drinking water and wastewater fluoride data sets were rejected as outliers by the ranking test which detected the low systematic bias. Of the 48 submitted fluoride QC results, 3 (all from Laboratory 2) were biased low and only 2 values (random laboratories) were biased high. These results indicate that fluoride can be used effectively in a QC sample to monitor "in control" status of fluoride analyses.

Rejection of Outliers

For the entire study, the IMVS computer program rejected 311 (14.3%) of the 2169 data points submitted to the computer. Across matrix types as outliers were rejected: 9.0% of the reagent water data, 15.0% of the drinking water data, and 19.2% of the wastewater data. The higher percentage of outlier data in the drinking water and wastewater data sets was primarily the result of high chloride, nitrate, and sulfate background concentrations in the samples selected for spiking by Laboratories 1, 2, 9, and 13.

The percentage of data rejected for each analyte is presented in Figure 1. The fluoride analytical results had the largest average percentage (20.6%) of rejected data points, across water types. The majority of these outlier data was removed by the laboratory ranking test, which identified systematic bias in Laboratories 1, 2, 9, and 12 data sets.

The percentage of data rejected for each laboratory is presented in Figure 2. The laboratory ranking test accounted for 62.5% of the total number of outlier data. Laboratories 1, 2, and 15 were judged to have a low systematic bias in some of their submitted data, and Laboratories 9, 10, 17, and 20 were judged to have a high systematic bias in some of their submitted data.

Method Performance for Bromide, Nitrate, Nitrite, Orthophosphate, and Sulfate

The summary statistics, calculated after removal of outlier data, are presented in Table 1. The weighted least squares linear regression equations calculated using the summary statistics are presented in Table 2. The mean recoveries for bromide, nitrate, nitrite, orthophosphate, and sulfate, as estimated from the slopes of the mean recovery regression equations, ranged from 98 to 104% in reagent water, from 95 to 101% in drinking water, and from 93 to 104% in wastewater.

In all water types tested, the overall precision, RSD_R, was less than 10% at concentrations above 2–6 mg/L for bromide, nitrate, nitrite, and orthophosphate, and above 24 mg/L for sulfate. As concentrations decreased to 0.5 mg/L (2.8 mg/L for sulfate), the RSD_R became more variable, 10–30%. The single-analyst precisions were less than 6% at concentration levels above 2–6 mg/L for bromide, nitrate, nitrite, and orthophosphate and above 24 mg/L for sulfate in all water types. As concentrations decreased to 0.5 mg/L (2.8 mg/L for sulfate), the precisions varied 6–20%.

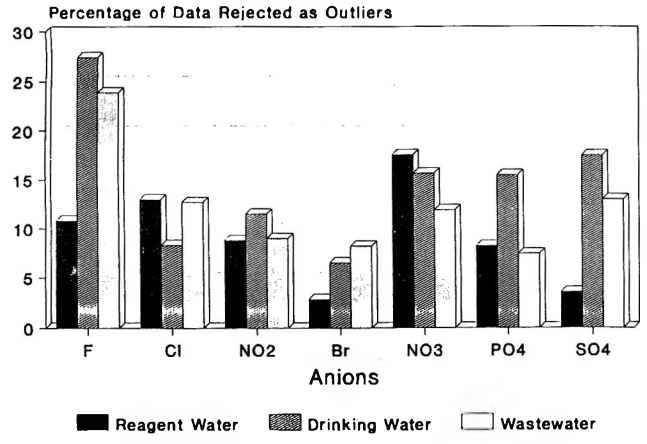


Figure 1. Outlier removal by anion.

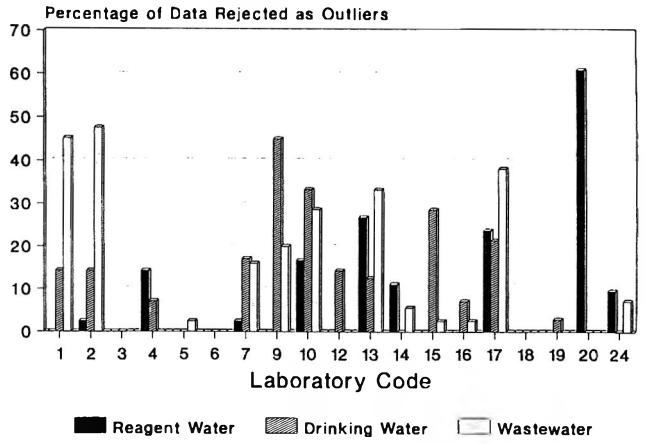


Figure 2. Outlier removal by laboratory.

Method Performance for Chloride Ion

Mean chloride ion recoveries at the 4 highest concentrations were comparable to recoveries obtained from other method analytes ranging from 96 to 104%. However, high biased recoveries were obtained at the lowest concentrations in drinking waters. Recoveries of chloride ion at 1.04 and 0.78 mg/L were 126 and 148%, respectively, from the drinking waters. These higher chloride recoveries may have resulted from reduction of residual chlorine to chloride by nitrite anion present in the spike solution:

$$HNO_2 + Cl_2 + H_2O \rightarrow HNO_3 + 2H^+ + 2Cl^-$$

If so, in addition to higher than expected recoveries of chloride at the lower concentration levels, we would expect to find higher than expected recoveries of nitrate and lower than expected recoveries of nitrite, which is exactly what is seen in Table 1. Additional support for this theory is found in the reagent water results, where the absence of residual chlorine resulted in the nearly 100% recovery of chloride, nitrite, and nitrate.

For reagent water, the overall relative standard deviations (RSD_R) for chloride results were comparable to those obtained for other anions, but RSD_R for drinking water and wastewater matrixes were more variable. At concentrations above 6.5 mg/L, the RSD_R ranged from 11 to 40%. As the concentrations approached 1.0 mg/L, the RSD_R increased to 70%. Mathematical manipulation of a high background chloride ion concentration to determine recovery of a small chloride spike concentration was responsible for the increased variability observed, particularly at the lower concentration levels. The average background concentration of chloride was 18 mg/L in the drinking waters and 29 mg/L in the wastewaters. The single-analyst RSR_r for chloride analyses ranged from 2 to 12% above concentration 6.5 mg/L in all matrixes. As the concentration

Table 1. Summary statistics of the collaborative study results for determination of inorganic anions in water

		Reagent water				Finished drinking water				Wastewater			
Analyte	Concn[a]	N[b]	$\bar{X}$[c]	s_R[d]	s_r[e]	N	$\bar{X}$	s_R	s_r	N	$\bar{X}$	s_R	s_r
Fluoride	0.26	13	0.25	0.08	0.11	10	0.30	0.07	0.04	9	0.28	0.15	0.06
	0.34	14	0.29	0.11		10	0.33	0.03		12	0.35	0.07	
	2.12	14	2.12	0.07	0.12	11	2.10	0.17	0.07	12	2.17	0.21	0.14
	2.55	14	2.49	0.14		11	2.53	0.12		12	2.51	0.16	
	6.79	14	6.76	0.20	0.19	12	6.80	0.51	0.27	11	6.67	0.41	0.22
	8.49	14	8.46	0.30		12	8.33	0.75		11	8.33	0.37	
Chloride	0.78	16	0.80	0.17	0.29	16	1.16	0.51	0.35	13	0.84	0.36	0.59
	1.04	17	1.12	0.46		16	1.32	0.46		15	1.24	0.89	
	6.50	15	6.31	0.27	0.14	16	6.81	0.89	0.91	16	6.45	1.04	0.89
	7.80	16	7.77	0.39		17	7.91	1.53		16	7.68	0.80	
	20.8	15	20.7	0.54	0.62	17	22.9	2.34	1.47	14	20.7	1.21	1.53
	26.0	15	25.9	0.58		17	25.9	2.96		15	24.9	2.04	
Nitrite	0.36	18	0.37	0.04	0.04	16	0.30	0.13	0.03	15	0.37	0.03	0.04
	0.48	18	0.48	0.06		16	0.38	0.13		16	0.46	0.10	
	3.00	16	3.18	0.12	0.06	17	3.01	0.22	0.12	17	3.19	0.14	0.11
	3.60	17	3.83	0.12		17	3.62	0.22		18	3.81	0.20	
	9.60	18	9.84	0.36	0.26	17	9.54	0.38	0.29	17	9.68	0.75	0.33
	12.0	17	12.1	0.27		17	11.6	0.53		18	12.1	0.64	
Bromide	0.63	17	0.69	0.11	0.05	17	0.63	0.12	0.04	15	0.65	0.14	0.09
	0.84	18	0.85	0.12		16	0.81	0.13		14	0.86	0.14	
	5.24	17	5.21	0.22	0.21	17	5.12	0.23	0.13	16	5.24	0.37	0.11
	6.29	18	6.17	0.35		17	6.20	0.30		16	6.29	0.47	
	16.8	17	17.1	0.70	0.36	17	16.9	0.55	0.57	14	16.7	0.70	0.43
	21.0	18	21.3	0.93		16	20.9	0.65		15	21.0	0.64	
Nitrate	0.42	14	0.42	0.04	0.02	15	0.49	0.05	0.03	15	0.43	0.10	0.05
	0.56	15	0.56	0.06		14	0.61	0.08		15	0.53	0.11	
	3.51	13	3.34	0.15	0.08	14	3.47	0.26	0.10	15	3.34	0.23	0.07
	4.21	15	4.05	0.28		15	4.23	0.40		16	4.03	0.26	
	11.2	13	11.1	0.47	0.34	14	11.5	0.63	0.46	14	10.9	0.33	0.47
	14.0	14	14.4	0.61		14	14.2	0.61		14	13.9	0.65	
Phosphate	0.69	12	0.69	0.06	0.06	12	0.67	0.11	0.17	14	0.70	0.09	0.09
	0.92	15	0.98	0.15		12	0.95	0.21		13	0.86	0.13	
	5.77	16	5.72	0.36	0.18	15	5.34	0.41	0.40	16	5.41	0.59	0.34
	6.92	15	6.78	0.42		15	6.29	0.72		16	6.46	0.66	
	18.4	17	18.8	1.04	0.63	14	18.0	0.70	0.59	15	17.9	2.11	1.27
	23.1	14	23.2	0.35		14	22.6	1.07		14	22.7	0.98	
Sulfate	2.85	17	2.83	0.32	0.52	19	2.80	0.52	0.34	15	2.80	0.50	0.41
	3.80	19	3.83	0.92		19	3.79	0.67		16	3.90	0.74	
	23.8	19	24.0	1.67	0.68	17	24.1	1.22	0.53	16	23.4	1.76	0.57
	28.5	19	28.5	1.56		19	28.6	1.74		17	27.9	2.39	
	76.0	18	76.8	3.42	2.33	19	76.6	6.59	2.84	15	74.0	3.25	3.39
	95.0	17	95.7	3.59		19	94.4	8.78		15	93.9	6.16	

[a] C = spike concentration, mg/L.
[b] N = number of retained data points in each set.
[c] $\bar{X}$ = mean recovery, mg/L.
[d] s_R = overall standard deviation, mg/L.
[e] s_r = single-analyst standard deviation, mg/L.

level approached 1.0 mg/L, the RSD_r became more variable (25–50%).

Method Performance for Fluoride Ion

Fluoride was included in this collaborative study even though U.S. EPA method 300.0A and ASTM method D4327 are not recommended for fluoride analysis. Anions that are not retained or only slightly retained by the analytical column elute in the area of the fluoride anion. Also, small organic acids and the carbonate anion coelute with fluoride. Although the matrix waters used in this study were not characterized for the presence or absence of carbonate ion or small organic acids, results

Table 2. Regression equations based on the summary statistics of the collaborative study results for determination of inorganic anions in water

Analyte, mg/L	Reagent water	Drinking water	Wastewater
Fluoride	$\bar{X}$[a] = 0.993C[b] 0.022	$\bar{X}$ = 0.978C + 0.029	$\bar{X}$ = 0.984C + 0.022
(0.26–8.49)	s_R[c] = 0.019$\bar{X}$ + 0.085	s_R = 0.058$\bar{X}$ + 0.039	s_R = 0.027$\bar{X}$ + 0.111
	s_r[d] = 0.009$\bar{X}$ + 0.109	s_r = 0.024$\bar{X}$ + 0.029	s_r = 0.031$\bar{X}$ + 0.050
Chloride	$\bar{X}$ = 0.989C + 0.049	$\bar{X}$ = 0.992C + 0.347	$\bar{X}$ = 0.976C + 0.134
(0.78–26.0)	s_R = 0.021$\bar{X}$ + 0.154	s_R = 0.100$\bar{X}$ + 0.368	s_R = 0.077$\bar{X}$ + 0.476[f]
	s_r = 0.35[e]	s_r = 0.068$\bar{X}$ + 0.265	s_r = 0.046$\bar{X}$ + 0.543
Nitrite	$\bar{X}$ = 1.037C 0.009	$\bar{X}$ = 1.002C 0.076	$\bar{X}$ = 1.032C 0.011
(0.36–12.0)	s_R = 0.026$\bar{X}$ + 0.039	s_R = 0.032$\bar{X}$ + 0.117	s_R = 0.056$\bar{X}$ + 0.033
	s_r = 0.015$\bar{X}$ + 0.035	s_r = 0.028$\bar{X}$ + 0.018	s_r = 0.026$\bar{X}$ + 0.029
Bromide	$\bar{X}$ = 0.995C + 0.040	$\bar{X}$ = 0.991C 0.004	$\bar{X}$ = 0.996C + 0.024
(0.63–21.0)	s_R = 0.035$\bar{X}$ + 0.088	s_R = 0.027$\bar{X}$ + 0.106	s_R = 0.040$\bar{X}$ + 0.112
	s_r = 0.024$\bar{X}$ + 0.030	s_r = 0.024$\bar{X}$ + 0.026	s_r = 0.012$\bar{X}$ + 0.079
Nitrate	$\bar{X}$ = 0.982C + 0.009	$\bar{X}$ = 0.995C + 0.064	$\bar{X}$ = 0.965C + 0.013
(0.42–14.0)	s_R = 0.045$\bar{X}$ + 0.030	s_R = 0.055$\bar{X}$ + 0.069	s_R = 0.038$\bar{X}$ + 0.088
	s_r = 0.023$\bar{X}$ + 0.010	s_r = 0.029$\bar{X}$ + 0.012	s_r = 0.022$\bar{X}$ + 0.040
Phosphate	$\bar{X}$ = 1.000C + 0.021	$\bar{X}$ = 0.948C + 0.038	$\bar{X}$ = 0.952C + 0.019
(0.69–23.1)	s_R = 0.060$\bar{X}$ + 0.042	s_R = 0.060$\bar{X}$ + 0.104[f]	s_R = 0.08961$\bar{X}$ + 0.042[f]
	s_r = 0.026$\bar{X}$ + 0.034	s_r = 0.032$\bar{X}$ + 0.146[f]	s_r = 0.056$\bar{X}$ + 0.041
Sulfate	$\bar{X}$ = 1.008C 0.030	$\bar{X}$ = 1.006C 0.053	$\bar{X}$ = 0.981C + 0.067
(2.85–95.0)	s_R = 0.048$\bar{X}$ + 0.378	s_R = 0.066$\bar{X}$ + 0.358	s_R = 0.059$\bar{X}$ + 0.394
	s_r = 0.015$\bar{X}$ + 0.464	s_r = 0.020$\bar{X}$ + 0.270	s_r = 0.024$\bar{X}$ + 0.322

[a] $\bar{X}$ = mean recovery, mg/L.
[b] C = spike concentration, mg/L.
[c] s_R = overall standard deviation, mg/L.
[d] s_r = single-analyst standard deviation, mg/L.
[e] Regression equation contained negative slope. Average precision is reported.
[f] COD_w <0.50. Regression equation should not be used outside of study concentration range.

obtained for recovery of fluoride spiked at background concentrations in drinking water and wastewater were very encouraging. Of 19 laboratories, 18 reported background concentrations of fluoride in drinking water ranging from 0.050 to 3.28 mg/L with an average of 0.66 mg/L. Only 10 of 18 laboratories reported background concentrations of fluoride in wastewater ranging from 0.060 to 12.8 mg/L. The average fluoride background concentration in wastewater, excluding the 12.8 mg/L value, was 0.47 mg/L.

At all concentrations in all matrixes, mean fluoride ion recoveries were comparable to those for bromide, nitrate, nitrite, orthophosphate, and sulfate. The overall RSD_R across all water types were less than 9% at concentrations above 2.0 mg/L and became more variable (20–50%) as the concentration approached 0.3 mg/L. The precision of results for fluoride anion was comparable to that of the other anions, even with the diverse matrixes analyzed and the potential for interferences from coeluting compounds. The RSD_r for fluoride results ranged from 3 to 10% for concentrations above 2.0 mg/L in all matrixes. As the concentration approached 0.3 mg/L, the RSD_r became more variable, 20–38%.

Effect of Water Type

The reagent water, drinking water, and wastewater data were subjected to an ANOVA test at the 95% confidence interval to determine the effect of water type. The ANOVA test detected a statistically significant matrix effect of drinking water on determinations of chloride, nitrite, and nitrate anions. Residual chlorine appeared to be responsible for matrix effects associated with determinations of chloride, nitrite and nitrate in drinking water. When the spiking solution containing nitrite was added to samples containing residual chlorine, a measurable concentration was oxidized to nitrate anion, producing additional chloride ion as a byproduct. The summary statistics presented in Table 1 show nitrite recoveries to be slightly lower than the spike level while the nitrate and chloride recoveries are slightly higher than the spike level. The biases were particularly noticeable at the lower concentration levels. The ANOVA test identified these biases as matrix related, when compared to results in reagent water. Because these biases were caused by materials introduced by the spiking process in the drinking waters and not by the measurement process, the ANOVA determination was not considered to be of practical significance.

Quality Control Acceptance Limits

One principal use of collaborative study results is to provide a basis for establishing QC acceptance limits for method users. For this study, 90–110% limits were placed on recovery of analytes from the QC sample. This was found to be a reasonable indicator of bias in participating laboratories. Generally, a laboratory with biased QC data also had some submitted data removed as outlier data for showing the same bias. On the basis of study results, a requirement was added to the method for analysis of a fortified reagent water blank with every 20 samples, with acceptance limits set at the predicted mean recovery $\pm 3 \times s_R$. Periodically, laboratories should test their performance and calibration using an independent, certified reference material.

Evaluation of Data from Laboratories Not Using U.S. EPA Method 300.0A

Laboratory 22 used an electronically suppressed ion chromatographic system with 2% lithium borate gluconate as the eluent. Laboratory 23 used chemically suppressed ion chromatography, but not the micromembrane suppression specified in the method. Because U.S. EPA method 300.0A and ASTM method D4327 specify micromembrane chemically suppressed ion chromatography, these laboratory data sets were evaluated independent of the main study data. Laboratory 22 data were comparable to study results submitted by the best laboratories that used chemically suppressed instruments. Laboratory 23 apparently had calibration problems because its 4 highest concentration samples were comparable to the main study data while the 2 lowest concentration levels had a consistently high bias not observed in the main study data.

Insufficient data were obtained to conclude that either of these 2 method deviations would be an acceptable alternative to U.S. EPA method 300.0A or ASTM method D4327.

Conclusions and Recommendations

U.S. EPA method 300.0A and the equivalent ASTM method D4327 were shown to be sufficiently accurate and precise for determination of bromide, chloride, nitrate, nitrite, orthophosphate, and sulfate in drinking water and wastewater in a collaborative study involving 22 laboratories. Equations (Table 2) for method recovery, overall standard deviation, and single-analyst standard deviation can be used to estimate method performance at any concentration within the study range.

Although U.S. EPA method 300.0A and ASTM method D4327 are not currently approved for fluoride analyses performed under the U.S. Safe Drinking Water Act or the Clean Water Act, the methods produced accurate and precise fluoride data in drinking water matrixes and various types of wastewaters. However, caution must be used during fluoride determinations because of possible coeluting interferences.

Performance-based QC acceptance limits using the collaborative study mean recovery and precision estimates were found to be comparable to the ± 10% acceptance limits for QC samples used in this study. Method users should routinely test a laboratory QC sample prepared in reagent water at the concentrations presented in Table 2 and compare results with the performance based acceptance limits.

Most of the data submitted by Laboratory 22 (electronically suppressed instrument) and Laboratory 23 (chemically suppressed but not using a micromembrane) were comparable to the rest of the study. Further research should be conducted to determine if these method variations are equivalent to the U.S. EPA method 300.0A.

On the basis of study results, it is recommended that the ion chromatographic method for determination of inorganic anions in water be adopted first action. This method, along with its U.S. EPA and ASTM counterparts, is also being recommended for inclusion in the National Pollutant Discharge Elimination System (NPDES) table of approved methods for all 7 anions.

Acknowledgments

The following collaborators participated in this study:

Ronald Altman, U.S. Environmental Protection Agency, Region III Laboratory, Annapolis, MD

Elizabeth Arar, Technology Applications, Inc., Cincinnati, OH

Malcom Baker, Dallas Water Utilities Laboratory, Dallas, TX

Thomas Bennett, Jr, U.S. Environmental Protection Agency, Region IV Laboratory, Athens, GA

Mikki Bolyard, U.S. Environmental Protection Agency, Technical Service Division, Cincinnati, OH

Richard Cassat, Arkansas Department PC&E, Little Rock, AR

Dan Head, Kentucky Utilities Co., Ghent, KY

Robert Joyce, Dionex Corp., Sunnyvale, CA

David Kersey, Water Quality Laboratory, Albuquerque, NM

Richard Latsch, North States Power Co./Scherburne, Becher, MN

Robert Lawrence, Waters Chromatography Division, Milford, MA

John Meyer, Scientific Laboratory Division, Albuquerque, NM

Harry Paterson, Los Alamos National Laboratory, Los Alamos, NM

Mark Peden, Illinois State Water Survey, Champaign, IL

Robert Potts, Virginia State Laboratory, Richmond, VA

Joseph Raia, Shell Development Co., Houston, TX

Hal Rhodes, Texaco, Inc., Port Arthur, TX

Leo Rodriguez, AFOEHL/ASA, Brooks AFB, TX

Elaine Sorbet, Louisiana Department of Environmental Quality, Baton Rouge, LA

Johnny Vickers, West Paine Laboratories, Baton Rouge, LA

Paul Walton, GPU Nuclear Power Co., Reading, PA

Bruce Warden, Waste Management, Inc., Geneva, IL

We thank Ed Katz, Environmental Monitoring Systems Laboratory, Cincinnati, OH, for analytical support; John Ortman, AARP at Environmental Monitoring Systems Laboratory, Cincinnati, OH, for computer support; and Elinor Hazel, Bionetics, Corp., Cincinnati, OH, for word processing.

References

(1) O'Dell, J.W., Pfaff, J.D., Gales, M.E., & McKee, G.D. (1984) EPA/600/4-84/017, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH

(2) Pfaff, J.D., Brockhoff, C.A., & O'Dell, J.W. (1989) U.S. EPA method 300.0, Determination of Inorganic Anions in Water by Ion Chromatography, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH

(3) *Fed. Regist.* (1991) **56**, 3526-3597

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

(5) Youden, W.Y., & Steiner, E.M. (1975) *Statistical Manual of the Association of Official Analytical Chemists*, AOAC, Arlington, VA, p. 88

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

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

(8) Edgell, K.W., & Jenkins, E.L. (1989) EPA/600/4-88/034, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH

(9) Draper, N., & Smith, H. (1968) *Applied Regression Analysis*, John Wiley & Sons, New York, NY

RESIDUES AND TRACE ELEMENTS

A Rapid Multiresidue Screen for Organophosphorus, Organochlorine, and *N*-Methyl Carbamate Insecticides in Plant and Animal Tissues

DIRK M. HOLSTEGE, DAVID L. SCHARBERG, ELIZABETH R. TOR, LAURA C. HART, and FRANCIS D. GALEY
University of California, Toxicology Laboratory, California Veterinary Diagnostic Laboratory System, Davis, CA 95616

A multiresidue screen for the quantitative determination of 43 organophosphorus, 17 organochlorine, and 11 *N*-methyl carbamate insecticides in 10 g of plant or animal tissues is described. The insecticides are extracted with 5% ethanol in ethyl acetate (v/v). Samples with high lipid content are cleaned up by automated gel permeation chromatography with a 30% ethyl acetate in hexane (v/v) eluant and in-line silica gel minicolumns. Highly pigmented samples are cleaned up with class-specific solid-phase extraction columns. The concentrated extracts are analyzed by selective detection with gas chromatography or liquid chromatography. Recovery of 71 insecticides ranged from 77 to 113%. Analysis of fortified bovine liver (n = 5) resulted in an average recovery of 96 ± 4% at the 0.5 to 0.05 μg/g level. Analysis of fortified alfalfa hay (n = 5) resulted in a mean recovery of 94 ± 4% at the 0.06 to 0.5 μg/g level, and analysis of fortified fresh tomatoes (n = 5) resulted in an average recovery of 97 ± 3% at the 0.06 to 0.5 μg/g level. Method detection limits ranged from 0.02 to 0.5 μg/g for the compounds studied with a nominal 10 g sample.

Received September 8, 1993. Accepted by JS November 5, 1993.

A multiresidue method (MRM) screening for insecticides for use in a veterinary diagnostic laboratory must be rapid and must test a wide variety of toxic insecticides at relevant toxicological concentrations. The 3 classes of insecticides of common interest are organophosphorus (OP), organochlorine (OC), and *N*-methyl carbamate (MC) insecticides. Samples tested include animal tissue, plant tissue, and ingesta. Thus, the MRM must give good precision and accuracy on samples with varying amounts of pigmentation, moisture content, and lipid content.

There are numerous MRMs testing for a single class of compounds such as OP (1–6), OC (7, 8) or MC (9, 10) insecticides, but few screen for these insecticides with a single sample extraction (11). Even fewer test the diversity of sample types encountered in a veterinary diagnostic laboratory. The MRMs developed by Luke et al. (12, 13) and Specht and Tillkes (14) can be applied to foods of vegetable and animal origin. The

MRM of Lee et al. (15), which was evaluated only on fruits and vegetables, has a cleanup to remove lipids. These MRMs use water-miscible solvents such as acetone or acetonitrile, followed by a water-removal step. This approach has the advantage of extracting a wide variety of pesticides but also extracts a large amount of polar matrix coextractives. Labor-intensive steps, such as liquid–liquid partitioning, column chromatography, or salting out, are required to remove coextracted water.

Previous work (1) has demonstrated the ability of a polar, water-immiscible organic solvent to extract organophosphorus insecticides from plant and animal tissue. The extraction solvent, 10% methanol in methylene chloride (v/v), was sufficiently polar to extract the organophosphorus insecticides, with a minimum of polar coextractives. Trace amounts of coextracted water were removed with sodium sulfate. An automated gel permeation–silica gel solid phase extraction (SPE) cleanup gave satisfactory cleanup of extracts for gas chromatography with flame photometric detection (GC–FPD). The method provides a rapid organophosphorus insecticide screen. One inconvenience of the method was the use of a halogenated solvent, with the associated hazards and higher disposal costs.

The present paper describes an MRM for OP, OC, and MC insecticides in plant and animal tissue and complex matrixes such as ingesta. The insecticides are extracted with 5% ethanol in ethyl acetate (v/v). Samples of high lipid content are cleaned up by gel permeation chromatography with in-line silica gel SPE minicolumns. Samples containing low amounts of lipids are cleaned up as needed with class-specific SPE protocols. Extracts for analysis of OC insecticides are cleaned up on a 1000 mg Florisil SPE column, whereas those for analysis of MC insecticides are cleaned up on a 500 mg aminopropyl SPE column. Sample extracts with low lipid content for OP insecticides are not cleaned up. Samples are analyzed by class-selective instrumentation. OP insecticides are analyzed by GC–FPD; OC insecticides are analyzed by gas chromatography with electron capture detection (GC–ECD). MC insecticides are analyzed by liquid chromatography (LC) followed by postcolumn hydrolysis and derivatization, with fluorometric detection. The MRM was tested for 43 OP, 17 OC, and 11 MC insecticides by means of a recovery study from bovine liver, alfalfa hay, and fresh tomatoes. The variables tested were high lipid, pigment, and water contents.

METHOD

Equipment and Apparatus

(**a**) *Gas chromatographs.*—(*1*) Model 5890, Hewlett-Packard equipped with flame photometric detector, phosphorus filter (525 nm); autosampler Model 7673, Hewlett-Packard, 30 m × 0.53 mm × 1.0 μm DB-17 capillary column (J&W Scientific), and glass insert with 0.5 cm loosely packed silanized glass wool; total helium flow, 70 mL/min; helium carrier gas flow, 15 psi (12 mL/min); septum purge flow, 3 mL/min; detector flows: H_2 at 75 mL/min, air at 100 mL/min; temperature program: 60°C for 0.5 min, 30°C/min to 160°C, 5°C/min to 280°C, 280°C for 4.2 min: run time, 32 min; splitless injection, valve off at 0.5 min; injector temperature, 240°C; detector temperature, 280°C; volume of injection, 2.0 μL. (*2*) Model Sigma 2000, Perkin-Elmer equipped with electron capture detector (ECD), autosampler (Model AS-2000, Perkin-Elmer), 30 m × 0.53 mm × 0.83 μm DB-608 capillary column (J&W Scientific), and glass insert with 0.5 cm loosely packed silanized glass wool; carrier gas flow, helium at 10 psi (4.5 mL/min); detector gas flow, argon–methane (95 + 5) at 6 psi (100 mL/min); temperature program: 60°C for 0 min, 30°C/min to 180°C, 5°C/min to 280°C, hold for 5 min; run time, 32 min; splitless injection; valve off at 0.5 min; injector temperature, 240°C; detector temperature, 320°C; volume of injection, 2.0 μL.

(**b**) *Liquid chromatograph.*—Hewlett-Packard 1090 LC with dual postcolumn reaction system and fluorescence detector (Hitachi 1050), autosampler (HP-1090 LC autosampler), 150 mm × 3.9 mm, 4 μ C8 Nova Pak (Waters); linear solvent gradient: methanol–H_2O–acetonitrile, 4 min at 0 + 85 + 15, 10 min to 40 + 20 + 40 for 1 min at 1 mL/min; post time, 10 min; oven temperature, 50°C; fluorescence detection at 345 nm excitation and 455 nm emission; volume of injection, 10 μL. Dual postcolumn reaction: 2-piston pump (LDC Analytical) pumping 0.05N NaOH at 0.25 mL/min for *N*-methyl carbamate hydrolysis and 0.05% OPA reagent at 0.25 mL/min for derivatization; 100°C heated 0.5 mL reaction coil for hydrolysis (Model CRX 390, Pickering Laboratories, Inc.).

(**c**) *Centrifuge.*—IEC Centra-7R refrigerated centrifuge (International Equipment Co., Needham Heights, MA).

(**d**) *Data output and processing.*—Chromatography data system (Turbochrom data system with 900 series interface, Perkin-Elmer Nelson).

(**e**) *Gel permeation chromatograph.*—Model 1002A, Autoprep GPC, ABC Laboratories) with 5 mL sample loop. Additional fittings included a 4-way switching valve to remove column from solvent path (Rainin Instrument Co.), a 0.5 cm Luer lock to solid-phase extraction column fitting (Bio-Rad Laboratories), low-pressure ¼–28 tube couplings, flangeless PTFE fittings for ⅛ in. and 1/16 in. tubing, and 1/16 in. od × 0.01 mm id PFTE tubing (Rainin Instrument Co.). GPC column: 25 mm id × 180 mm packed with 38 g S-X3 Biobeads, 200–400 mesh (Bio-Rad Laboratories); mobile phase, 30% ethyl acetate in hexane (v/v); flow rate, 5 mL/min; dump cycle, 15 min; collect cycle, 26 min; wash cycle, 5 min. Determine dump, collect, and wash times experimentally by fractionating 1 g corn oil fortified with propetamphos and azinphos methyl. Dump and collect times should allow collection of the fractions between and including the fractions containing propetamphos (first eluted compound) and azinphos methyl (last eluted compound).

(**f**) *Temperature-controlled nitrogen gas evaporator.*—N-Evap Analytical Evaporator (Organomation Assoc., Inc.).

(**g**) *Screw-cap test tubes with PTFE-lined caps.*—10 and 50 mL (Fisher Scientific).

(**h**) *Square French homogenization vessels with Teflon-lined caps.*—250 mL (Fisher Scientific).

(**i**) *Silica gel solid-phase extraction columns.*—6 mL, 500 mg, disposable (Bakerbond SPE, J.T. Baker, Inc.).

(j) *Florisil solid-phase extraction columns.*—6 mL, 1000 mg, disposable (Bakerbond SPE, J.T. Baker, Inc.).

(k) *Aminopropyl solid-phase extraction columns.*—6 mL, 500 mg (Burdick & Jackson).

(l) *Tissue homogenizer.*—Polytron Model PT 10/35 (Brinkman Instruments, Inc.).

(m) *Acrodisc CR filter.*—0.45 μm, chemical resistant (Fisher Scientific).

(n) *Nylaflo nylon membrane filters.*—0.45 μm, 47-mm diameter (Gelman Sciences, Ann Arbor, MI).

Reagents

(a) *Hexane, methanol, ethanol, acetonitrile, ethyl acetate, acetone, and toluene.*—Pesticide grade (Fisher Scientific).

(b) *Sodium sulfate.*—ACS reagent grade (Fisher Scientific) washed with ethyl acetate and oven-dried at 120°C.

(c) *Decanol keeper.*—Add 5 mL decanol to 95 mL acetone.

(d) *Gel permeation chromatography solvent.*—30% ethyl acetate in hexane (v/v).

(e) *0.05N NaOH.*—Filtered through a Nylaflo 0.45 μm filter.

(f) *OPA reagent.*—25 mL filtered (Nylaflo 0.45 μm filter) 1M potassium borate, pH 10.4, + 0.25 g *o*-phthalaldehyde (Sigma Chemical Co., St. Louis, MO) + 0.5 mL mercaptoethanol (LKB Instruments, Inc., Gaithersburg, MD) in 500 mL H_2O.

Standards

Dissolve 25 mg neat insecticide in 25 mL methanol to make a 1000 μg/mL standard solution. Subsequent dilutions are made with toluene (OPs and OCs) or methanol (MCs). The OP compounds are divided into 4 mixtures (Table 1, A–D), the MCs are divided into 3 mixtures (Table 2, A–C), and the OCs are in one standard mixture (Table 3).

Extraction

Thoroughly mix sample to obtain a representative subsample. Prepare plant samples as follows. Place 10 to 25 g sample in a Stein mill sample cup. Add sufficient liquid nitrogen to freeze the sample. Immediately fracture the plant sample by milling for 1 min. Prepare at least 100 g fractured plant material prior to subsampling. Mix tissue samples in Waring blender or chop finely if sample is insufficient for blending.

Weigh 10.0 g frozen sample into homogenizing vessel. Add 50 g sodium sulfate and 100 mL 5% ethanol in ethyl acetate (v/v). Homogenize for 1 min at high speed. Centrifuge extracts at 1200 rpm for 4 min. Pipette a 40 mL aliquot of extract into a 50 mL test tube and cap the tube. Proceed with cleanup procedure on the basis of sample lipid content.

Cleanup of Samples with High Lipid Content (Liver): Gel Permeation Chromatographic (GPC) Cleanup

Add 3 drops of decanol keeper solution to a 20 mL aliquot of sample extract. Carefully evaporate to dryness by using a nitrogen evaporator at 40°C. Add 10 mL GPC solvent and sonicate for 2 min to redissolve the residue. Filter samples through 0.45 μm PTFE filters as they are loaded onto the GPC.

The sample extracts are cleaned up by GPC and silica gel SPE minicolumns attached to the GPC outlet (Figure 1). Prer-

Table 1. Organophosphorus insecticides: CAS registry numbers, standard mixture, typical retention times (RT) under standard conditions (30 M DB-17 megabore), and GPC elution volumes

Compound	CAS registry number	Standard mix	RT, min	GPC volume, mL
Dichlorvos	62-73-7	C	4.5	90–120
Methamidophos	10265-92-6	D	5.1	110–130
Mevinphos E	7786-34-7	A	6.3	100–130
Mevinphos Z	7786-34-7	A	6.6	100–130
Acephate	30560-19-1	D	7.3	110–140
Demeton-O	298-03-3	C	8.2	80–100
Ethoprop	13194-48-4	D	8.7	90–110
Phorate	298-02-2	A	9.6	90–110
Naled	300-76-5	B	9.9	90–130
Terbufos	13071-79-9	A	10.5	70–100
Demeton-S	126-75-0	C	10.5	90–120
Dicrotophos	141-66-2	B	10.8	130–160
Propetamphos	31218-83-4	D	11.0	70–90
Diazinon	333-41-5	A	11.0	70–100
Monocrotophos	919-44-8	D	11.5	110–140
Fonofos	944-22-9	A	11.5	100–120
Disulfoton	298-04-4	B	11.6	80–110
Dioxathion	78-34-2	C	12.0	100–130
Dimethoate	60-51-5	B	12.3	130–150
Phosphamidon E	13171-21-6	C	12.5	110–140
Ronnel	299-84-3	D	13.6	90–120
Phosphamidon Z	13171-21-6	C	13.9	110–140
Methyl Parathion	298-00-0	A	14.0	110–140
Merphos	150-50-5	B	14.4	70–100
Chlorpyrifos	2921-88-2	D	14.8	80–110
Parathion	56-38-2	A	15.0	100–120
Malathion	121-75-5	B	15.1	90–120
Fenthion	55-38-9	C	16.0	120–150
Crufomate	299-86-5	D	16.2	80–110
Isofenphos	25311-71-1	B	16.3	70–100
Chlorfenvinphos	470-90-6	C	16.8	90–120
DEF	78-48-8	A	17.6	70–100
Methidathion	950-37-8	A	18.9	140–170
Crotoxyphos	7700-17-6	D	17.8	100–130
Tetrachlorvinphos	961-11-5	B	18.2	110–130
Profenophos	41198-08-7	D	18.5	90–110
Fenamiphos	22224-92-6	C	18.6	90–120
Ethion	563-12-2	A	20.5	90–110
Carbophenothion	786-19-6	B	21.3	100–120
Fensulfothion	115-90-2	D	21.5	130–160
Triazophos	24017-47-8	D	22.9	110–140
EPN	2104-64-5	C	24.2	120–150
Phosalone	2310-17-0	A	25.3	100–130
Phosmet	732-11-6	B	25.7	160–200
Azinphos methyl	86-50-0	C	27.5	170–210
Coumaphos	56-72-4	A	28.2	120–150

Table 2. Methyl carbamate insecticides: CAS registry numbers, standard mix, typical retention times (RT) under standard conditions (C8 Nova Pak) and GPC elution volumes

Compound	CAS registry number	Standard mix	RT, min	GPC volume, mL
Aldicarb sulfone	1646-88-4	B	3.1	120–150
Oxamyl	23135-22-0	C	3.3	160–200
Methomyl	16752-77-5	B	3.7	150–180
3-OH carbofuran	16655-82-6	B	6.3	100–120
Aldicarb	116-06-3	C	9.6	110–150
Bendiocarb	22781-23-3	C	11.2	80–110
Propoxur	114-26-1	B	11.2	90–120
Carbofuran	1563-66-2	A	11.4	100–130
Carbaryl	63-25-2	C	11.7	110–160
Methiocarb	2032-65-7	A	13.5	100–130
Mexacarbate	315-18-4	C	14.7	100–150

inse silica gel SPE columns with 5 mL GPC solvent and attach to GPC output. Load 5 mL extract into the GPC. Run the GPC program; collect in 125 mL flasks. Remove the GPC column from the solvent path by using a 4-way switching valve. Set the GPC to "load." Change the GPC solvent to 20% acetone in ethyl acetate. Elute SPE columns with 15 mL 20% acetone in ethyl acetate (v/v) at 5 mL/min while running the GPC on dump, 0; collect, 3; and wash, 0. Combine eluate with GPC eluate. Add 3 drops of decanol solution. Evaporate to 10 mL with stream of nitrogen at 40°C. Transfer the residue to a 15 mL Kuderna-Danish receiving flask with 5 mL ethyl acetate. Continue concentration to 0.5 mL. Take a 200 µL aliquot for OP analysis. Take a 50 µL aliquot and dilute to 200 µL with toluene for OC analysis. Carefully evaporate the remaining 250 µL just to dryness with a stream of nitrogen at room temperature. Immediately add 0.5 mL methanol for MC analysis.

Table 3. Organochlorine insecticides: CAS registry numbers, concentration, typical retention times (RT) under standard conditions (DB-608 Megabore) and GPC elution volumes

Compound	CAS registry number	Concn, µg/mL	RT, min	GPC volume, mL
Hexachlorobenzene	118-74-1	0.1	11.0	90–110
α-BHC	319-84-6	0.07	11.5	80–110
Lindane	58-89-9	0.1	12.9	90–110
Heptachlor	76-44-8	0.1	14.1	80–100
Aldrin	309-00-2	0.1	15.3	80–100
Heptachlor epoxide	1024-57-3	0.1	17.3	80–110
γ-Chlordane	5103-74-2	0.1	18.0	70–100
Endosulfan I	959-98-8	0.2	18.8	80–110
p,p'-DDE	72-55-9	0.1	19.6	90–130
Dieldrin	60-57-1	0.1	20.1	90–120
Endrin	72-20-8	0.1	21.8	90–140
p,p'-DDD	72-54-8	0.1	22.1	80–120
Endosulfan II	33213-65-9	0.2	22.6	80–120
p,p'-DDT	50-29-3	0.1	23.6	90–120
Dicofol	115-32-2	0.5	27.2	80–110
p,p'-Methoxychlor	72-43-5	0.2	28.0	110–150
Mirex	2385-85-5	0.1	28.7	80–120

Cleanup of Samples With Low Lipid Content (Alfalfa and Tomato): SPE Cleanups

(a) *Organochlorine insecticides.*—Prerinse 1000 mg Florisil SPE column with 5 mL ethyl acetate followed by 5 mL hexane. Add 2 drops of decanol solution to a 2 mL aliquot of the extract and evaporate to dryness with a stream of nitrogen at 40°C. Immediately add 1 mL 10% ethyl acetate in hexane (v/v). Pipette the sample onto the SPE column, and elute the OCs with 15 mL 10% ethyl acetate in hexane (v/v) at a rate of 1 drop/s. Add 2 drops of decanol solution and 1 mL toluene to the eluate and evaporate to 1.0 mL with a stream of nitrogen at 40°C. Proceed to OC analysis.

(b) *N-Methyl carbamate insecticides.*—Prerinse 500 mg aminopropyl SPE column with 5 mL ethyl acetate followed by 5 mL hexane. Add 2 drops of decanol solution to a 5 mL aliquot of extract and evaporate to dryness with a stream of nitrogen at 40°C. Immediately add 1 mL hexane. Pipette the sample

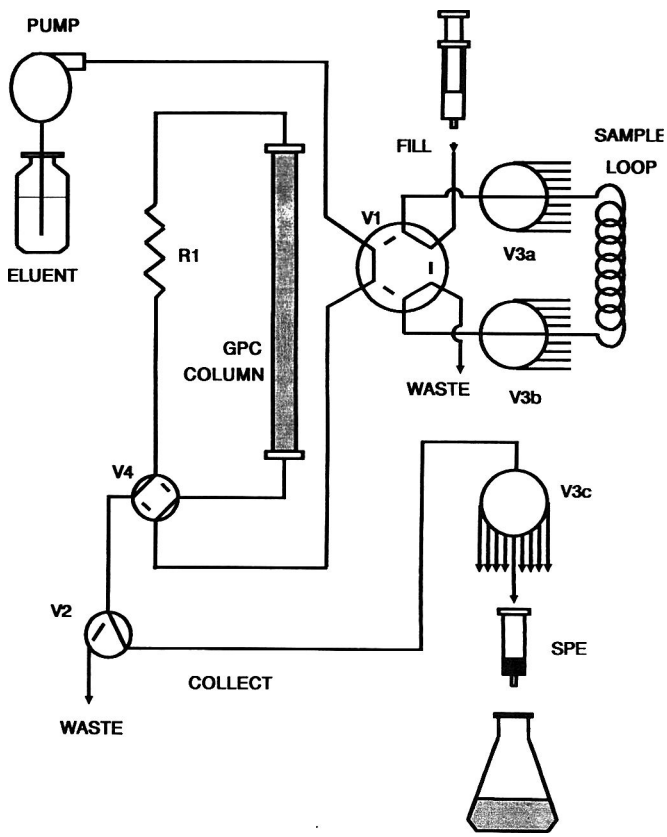


Figure 1. Schematic of GPC–SPE automatic cleanup system. V1 = 6-way manual load–run valve. V2 = 3-way solenoid dump–collect valve. V3 = 24-way, 3-gang solenoid sample selector valve. V4 = 4-way manual eluant bypass valve. R1 = 0.010 in. id capillary restrictor. SPE = silica gel solid phase column.

Table 4. Summary of validation study results

Compound	Fortification level, ppm	Average rec., %	CV, %	Method detection limit, ppm
Alfalfa hay				
3-OH carbofuran	0.5	109	2.0	0.1
Aldicarb	0.5	83	1.8	0.1
Aldicarb sulfone	0.5	83	5.1	0.1
Bendiocarb	0.5	92	4.4	0.1
Carbaryl	0.5	85	2.6	0.1
Carbofuran	0.5	90	4.9	0.1
Methiocarb	0.5	110	4.4	0.1
Methomyl	0.5	89	3.6	0.1
Mexacarbate	0.5	87	7.0	0.1
Oxamyl	0.5	86	6.3	0.1
Propoxur	0.5	88	2.3	0.1
Aldrin	0.125	94	4.6	0.1
α-BHC	0.09	86	3.0	0.05
γ-Chlordane	0.125	103	3.8	0.1
p,p'-DDD	0.125	100	3.4	0.1
p,p'-DDE	0.125	95	4.9	0.1
p,p'-DDT	0.125	98	4.6	0.1
Dicofol	0.6	100	5.9	0.5
Dieldrin	0.25	90	4.9	0.2
Endosulfan I	0.125	93	7.6	0.1
Endosulfan II	0.125	100	4.6	0.1
Endrin	0.125	81	2.6	0.1
HCB	0.06	100	4.0	0.05
Heptachlor	0.125	90	4.2	0.1
Heptachlor epoxide	0.125	93	4.5	0.1
Lindane	0.06	88	3.5	0.05
p,p'-Methoxychlor	0.25	101	3.0	0.2
Mirex	0.25	82	2.6	0.2
Acephate	0.5	84	6.1	0.1
Azinphos methyl	0.5	108	1.7	0.1
Carbophenothion	0.5	99	2.7	0.1
Chlorfenvinphos	0.5	99	1.9	0.08
Chlorpyriphos	0.5	97	7.0	0.04
Coumaphos	0.5	98	2.2	0.1
Crufomate	0.5	95	6.9	0.1
Crotoxyphos	0.5	99	6.7	0.1
DDVP	0.5	83	2.4	0.1
DEF	0.5	96	2.6	0.04
Demeton	0.5	82	3.2	0.12
Diazinon	0.5	96	3.0	0.04
Dicrotophos	0.5	96	1.4	0.1
Dimethoate	0.5	81	3.8	0.08
Dioxathion	0.5	106	0.7	0.2
Disulfoton	0.5	87	2.0	0.08
EPN	0.5	97	1.9	0.1
Ethion	0.5	96	3.2	0.04
Ethoprop	0.5	93	6.7	0.1
Fenamiphos	0.5	92	2.5	0.1
Fensulfothion	0.5	97	6.5	0.1
Fenthion	0.5	96	1.1	0.1
Fonofos	0.5	96	2.4	0.08
Isofenphos	0.5	99	2.6	0.1
Malathion	0.5	99	3.0	0.04
Merphos	0.5	99	3.4	0.08
Methamidophos	0.5	77	5.7	0.1
Methidathion	0.5	97	2.1	0.08
Methyl parathion	0.5	97	2.2	0.04
Mevinphos E	0.5	96	2.8	0.04
Mevinphos Z	0.5	98	2.9	0.04
Monocrotophos	0.5	93	6.6	0.1
Naled	0.5	85	5.9	0.15
Parathion	0.5	95	2.7	0.04
Phorate	0.5	93	1.4	0.04
Phosalone	0.5	99	2.8	0.1
Phosmet	0.5	104	3.9	0.2
Phosphamidon	0.5	88	4.2	0.2
Profenophos	0.5	97	6.4	0.1
Propetamphos	0.5	93	7.3	0.1
Ronnel	0.5	96	7.2	0.1
Terbufos	0.5	94	1.7	0.1
Tetrachlorvinphos	0.5	99	3.1	0.1
Triazophos	0.5	98	6.4	0.1
Bovine liver, pooled results				
3-OH carbofuran	0.5	98	2.0	0.05
Aldicarb	0.5	77	7.3	0.05
Aldicarb sulfone	0.5	97	1.5	0.05
Bendiocarb	0.5	92	2.7	0.05
Carbaryl	0.5	94	1.6	0.05
Carbofuran	0.5	92	1.7	0.05
Methiocarb	0.5	88	4.0	0.05
Methomyl	0.5	91	1.6	0.05
Mexacarbate	0.5	89	3.6	0.05
Oxamyl	0.5	92	1.1	0.05
Propoxur	0.5	93	1.4	0.05
Aldrin	0.1	88	5.4	0.02
α-BHC	0.07	106	3.2	0.02
γ-Chlordane	0.1	102	1.8	0.02
p,p'-DDD	0.1	98	4.1	0.02
p,p'-DDE	0.1	104	3.7	0.02
p,p'-DDT	0.1	101	5.4	0.02
Dicofol	0.5	101	6.5	0.1
Dieldrin	0.2	113	4.9	0.04
Endosulfan I	0.1	102	3.3	0.02
Endosulfan II	0.1	95	5.5	0.02
Endrin	0.1	102	4.2	0.02
HCB	0.05	112	1.8	0.01
Heptachlor	0.1	90	5.1	0.02
Heptachlor epoxide	0.1	87	5.3	0.02
Lindane	0.05	109	1.9	0.01

Table 4. ***(continued)***

Compound	Fortification level, ppm	Average rec., %	CV, %	Method detection limit, ppm
Bovine liver, pooled results *(continued)*				
p,p'-Methoxychlor	0.2	94	5.1	0.04
Mirex	0.2	105	6.3	0.04
Acephate	0.125	89	3.9	0.025
Azinphos methyl	0.125	109	3.6	0.025
Carbophenothion	0.125	98	3.1	0.025
Chlorfenvinphos	0.125	101	2.0	0.02
Chlorpyriphos	0.125	94	1.8	0.01
Coumaphos	0.125	102	6.4	0.025
Crufomate	0.125	96	1.9	0.025
Crotoxyphos	0.125	106	2.0	0.025
DDVP	0.125	100	4.0	0.025
DEF	0.125	95	7.1	0.01
Demeton	0.125	88	7.0	0.03
Diazinon	0.125	91	5.5	0.01
Dicrotophos	0.125	98	5.2	0.025
Dimethoate	0.125	100	4.1	0.02
Dioxathion	0.125	99	1.7	0.05
Disulfoton	0.125	92	4.2	0.02
EPN	0.125	99	2.4	0.025
Ethion	0.125	104	2.9	0.01
Ethoprop	0.125	82	5.1	0.025
Fenamiphos	0.125	78	5.3	0.025
Fensulfothion	0.125	100	2.1	0.025
Fenthion	0.125	85	6.1	0.025
Fonofos	0.125	89	4.6	0.02
Isofenphos	0.125	96	3.5	0.025
Malathion	0.125	97	3.1	0.01
Merphos	0.125	104	6.3	0.02
Methamidophos	0.125	91	6.4	0.025
Methidathion	0.125	103	6.9	0.02
Methyl parathion	0.125	96	6.1	0.01
Mevinphos E	0.125	82	5.0	0.01
Mevinphos Z	0.125	89	5.3	0.01
Monocrotophos	0.125	95	5.2	0.025
Naled	0.125	84	9.2	0.0375
Parathion	0.125	94	6.2	0.01
Phorate	0.125	84	6.1	0.01
Phosalone	0.125	105	7.6	0.025
Phosmet	0.125	104	4.0	0.05
Phosphamidon	0.125	91	2.8	0.05
Profenophos	0.125	100	1.4	0.025
Propetamphos	0.125	85	4.7	0.025
Ronnel	0.125	92	1.9	0.025
Terbufos	0.125	86	4.0	0.025
Tetrachlorvinphos	0.125	98	3.3	0.025
Triazophos	0.125	107	4.4	0.025
Tomato				
3-OH carbofuran	0.5	89	1.4	0.1
Aldicarb	0.5	87	3.2	0.1

Compound	Fortification level, ppm	Average rec., %	CV, %	Method detection limit, ppm
Tomato *(continued)*				
Aldicarb sulfone	0.5	91	1.6	0.1
Bendiocarb	0.5	91	2.6	0.1
Carbaryl	0.5	96	3.0	0.1
Carbofuran	0.5	93	4.3	0.1
Methiocarb	0.5	98	3.3	0.1
Methomyl	0.5	94	1.6	0.1
Mexacarbate	0.5	94	2.9	0.1
Oxamyl	0.5	87	2.3	0.1
Propoxur	0.5	94	2.0	0.1
Aldrin	0.125	100	7.5	0.1
α-BHC	0.09	95	3.2	0.07
γ-Chlordane	0.125	96	2.8	0.1
p,p'-DDD	0.125	96	1.7	0.1
p,p'-DDE	0.125	94	3.4	0.1
p,p'-DDT	0.125	98	2.0	0.1
Dicofol	0.6	104	1.7	0.5
Dieldrin	0.25	93	3.4	0.2
Endosulfan I	0.125	95	2.9	0.1
Endosulfan II	0.125	96	3.8	0.1
Endrin	0.125	96	2.6	0.1
HCB	0.06	88	2.5	0.05
Heptachlor	0.125	87	3.7	0.1
Heptachlor epoxide	0.125	93	3.4	0.1
Lindane	0.06	91	3.1	0.05
p,p'-Methoxychlor	0.25	98	2.0	0.2
Mirex	0.25	92	2.5	0.2
Acephate	0.5	103	1.7	0.1
Azinphos methyl	0.5	110	1.3	0.1
Carbophenothion	0.5	98	3.1	0.1
Chlorfenvinphos	0.5	103	2.0	0.08
Chlorpyriphos	0.5	95	2.0	0.04
Coumaphos	0.5	103	0.6	0.1
Crufomate	0.5	95	2.8	0.1
Crotoxyphos	0.5	98	2.5	0.1
DDVP	0.5	85	9.3	0.1
DEF	0.5	97	2.4	0.04
Demeton	0.5	94	2.8	0.12
Diazinon	0.5	96	1.8	0.04
Dicrotophos	0.5	98	5.2	0.1
Dimethoate	0.5	100	4.1	0.08
Dioxathion	0.5	98	7.3	0.2
Disulfoton	0.5	92	4.2	0.08
EPN	0.5	104	2.0	0.1
Ethion	0.5	98	2.7	0.04
Ethoprop	0.5	92	1.6	0.1
Fenamiphos	0.5	103	1.8	0.1
Fensulfothion	0.5	98	3.0	0.1
Fenthion	0.5	103	2.0	0.1
Fonofos	0.5	95	1.9	0.08
Isofenphos	0.5	96	3.5	0.1

Table 4. ***(continued)***

Compound	Fortification level, ppm	Average rec., %	CV, %	Method detection limit, ppm
		Tomato *(continued)*		
Malathion	0.5	97	3.1	0.04
Merphos	0.5	105	4.6	0.08
Methamidophos	0.5	113	2.0	0.1
Methidathion	0.5	100	1.8	0.08
Methyl parathion	0.5	98	1.8	0.04
Mevinphos E	0.5	93	1.1	0.04
Mevinphos Z	0.5	94	1.6	0.04
Monocrotophos	0.5	100	2.1	0.1
Naled	0.5	109	5.0	0.15
Parathion	0.5	97	2.2	0.04
Phorate	0.5	93	1.6	0.04
Phosalone	0.5	101	1.7	0.1
Phosmet	0.5	104	4.0	0.2
Phosphamidon	0.5	107	1.5	0.2
Profenophos	0.5	96	2.3	0.1
Propetamphos	0.5	95	1.6	0.1
Ronnel	0.5	94	2.0	0.1
Terbufos	0.5	94	1.9	0.1
Tetrachlorvinphos	0.5	98	3.3	0.1
Triazophos	0.5	99	2.2	0.1

onto the SPE column, and wash the column with 9 mL hexane at a rate of 1 drop/s. Discard this wash. Add 15 mL ethyl acetate to the test tube used to evaporate the extract, and use this ethyl acetate to elute the MCs from the SPE column. Add 2 drops of decanol solution to the eluate, and evaporate to dryness with a stream of nitrogen at 40°C. Immediately add 0.5 mL methanol. Proceed to MC analysis.

(**c**) *Organophosphorus insecticides.*—Add 2 drops of decanol solution to a 5 mL aliquot of extract and evaporate to dryness with a stream of nitrogen at 40°C. Immediately add 1 mL toluene. Proceed to OP analysis.

Analysis

(**a**) *Organochlorine insecticides.*—Determine residues by GC–ECD with a DB-608 megabore column. Inject 2 μL analytical standard (0.1 to 0.5 μg/mL, Table 3) and sample extracts at 0.5 g/mL (animal tissue) or 0.2 g/mL (plant material). Quantitate by using external calibration based on injections of OC insecticides.

(**b**) *N-Methyl carbamate insecticides.*—Determine residues by LC with dual postcolumn reaction system and fluorescence detection. Inject 10 μL analytical standard (0.5 μg/mL) and sample extracts at 1.0 g/mL. Quantitate by using external calibration based on injections of 5 ng MC insecticides.

(**c**) *Organophosphorus insecticides.*—Determine residues by GC–FPD with a DB-17 megabore column. Inject 2 μL analytical standard (0.5 μg/mL) and sample extracts at 2 g/mL (animal tissue) or 0.5 g/mL (plant material). Quantitate by using external calibration based on injections of 1 ng OP insecticides.

Validation Study

Pooled alfalfa hay, fresh tomato, and bovine liver were fortified with each insecticide standard at a level that was 2 to 10 times the detection limit reported by this laboratory (Table 4). Five replicate fortifications for each matrix type were prepared.

Results and Discussion

The recoveries of 71 insecticides are presented in Table 4. The method quantitatively extracted the polar insecticides. Matrix coextractives were removed without loss of the more volatile or the polar insecticides. Extraction with 5% ethanol in ethyl acetate (v/v) resulted in a multiresidue extraction. After appropriate cleanup steps, good precision and accuracy for high-lipid-content (bovine liver), highly pigmented (alfalfa hay), and high-water-content (tomato) samples were obtained.

The use of 5% ethanol in ethyl acetate remedied the problems associated with the use of a polar extraction solvent such as acetone. This solvent mixture had sufficient polarity to extract highly polar compounds such as acephate, methomyl, and methamidophos, and yet was immiscible with water. Trace amounts of water in the solvent extract were removed by sodium sulfate.

Development of Solvent

Development of the solvent system was based on several criteria. No solvent was used that was hazardous or was expensive to dispose of. A volatile solvent system must be used because rapid evaporation of a large solvent volume would be required without loss of volatile compounds. The solvent system must be sufficiently polar to extract the most polar insecticides and yet be immiscible with water. The extract must be clear so that aliquots could be removed without time-consuming filtrations. The final sample extract should have a minimum of matrix coextractives, but extraction of the pesticides of interest must be quantitative.

Halogenated solvents, such as methylene chloride and acetonitrile, were eliminated from consideration because of higher hazard and disposal cost. Solvent mixtures with large percentages of water-miscible solvents such as methanol, ethanol, and acetone were not considered because of water miscibility. Toluene, propanol, larger alcohols, isooctane, and cyclohexane were eliminated because of insufficient volatility.

Ethyl acetate, modified by addition of acetone, methanol, or ethanol for increased polarity was chosen as a starting point for investigation. First, the ability of various percentages of acetone, methanol, and ethanol in ethyl acetate were compared for extraction of matrix coextractives from hydrated alfalfa hay. Alfalfa hay (5 g) was wetted with 10 mL H_2O and extracted with 100 mL of 5, 10, and 20% of methanol or ethanol in ethyl acetate and 20% acetone in ethyl acetate. Methanol (10%) in methylene chloride was also tested as a reference to earlier work (1). A 10 mL aliquot of the extract was taken and evaporated to dryness, and the residue was redissolved in 1 mL ethyl

Table 5. Comparison of volatile matrix coextractives from 5 g alfalfa hay extracted with 100 mL solvent mixture

Solvent mixture	Total area (×1000)
5% Methanol in ethyl acetate	451
10% Methanol in ethyl acetate	463
5% Ethanol in ethyl acetate	646
20% Methanol in ethyl acetate	938
10% Methanol in methylene chloride	1080
10% Ethanol in ethyl acetate	1104
20% Acetone in ethyl acetate	1140
20% Ethanol in ethyl acetate	1304

acetate. A volume of 2 µL was injected on a PE Sigma 2000 GC with flame ionization detection (FID) and a 30 m × 0.53 mm × 1 µm DB-17 column and the temperature programmed from 60° to 280°C at 10°C/min. The areas for all peaks after the solvent front were summed to estimate volatile coextractive content. The mixtures containing 5% methanol, 10% methanol, and 5% ethanol in ethyl acetate gave the least total areas and thus the lowest overall volatile matrix coextractives (Table 5).

The ability of 100 mL of these solvent mixtures to extract the polar compounds acephate and methamidophos from 10 mL H_2O was examined next. Each solvent mixture (10 mL) examined for matrix coextractives was added to 10 mL H_2O with 50 g Na_2SO_4, and the mixture was homogenized for 1 min. Of these solvents, only 5% and 10% methanol in ethyl acetate, 5% ethanol in ethyl acetate, and 20% acetone in ethyl acetate gave clear extracts, indicating a lower concentration of water. Because 5% ethanol or 5% methanol in ethyl acetate and 10% methanol in ethyl acetate both gave clear extracts and low matrix coextractives, only these solvent mixtures were examined further. Water (10 mL) was fortified at 5 µg/g with acephate and methamidophos, and the mixture was homogenized with 100 mL solvent and 50 g Na_2SO_4 for 1 min. Aliquots were evaporated to dryness with 3 drops of 5% decanol in acetone at 40°C, and residues were analyzed via GC–FPD. All spike recoveries were less than 70%. The trial was repeated with 5 mL H_2O, with 5% ethanol in ethyl acetate giving approximately 10% higher recoveries than the other two solvents (Table 6).

Table 6. Recoveries from 5 mL H_2O fortified with acephate and methamidophos at 5 µg/g (n = 2)

	Acephate		Methamidophos	
Solvent	Rec., %	CV, %	Rec., %	CV, %
5% Ethanol in ethyl acetate	98	4.0	105	1.8
5% Methanol in ethyl acetate	90	3.9	95	1.8
10% Methanol in ethyl acetate	90	0.3	92	3.7

The ability of solvents to extract various OP insecticides from liver was examined. Liver (10 g) was fortified at 1 µg/g with OP standard mixture D (Table 1). This mixture contains the most polar OPs (methamidophos, acephate, and monocrotophos) and the least polar (on the basis of retention on silica gel SPE columns), ronnel and chlorpyriphos. Duplicate fortified livers were extracted with 100 mL solvent, cleaned up by GPC and in-line silica gel SPE columns, and analyzed by GC–FPD as in the cleanup section of the method. The solvent 5% ethanol in ethyl acetate yielded an average recovery of 99% (CV, 2.3%) for all OPs in the mixture, whereas 5% methanol in ethyl acetate gave a recovery of 92% (CV, 4.4%), and 10% methanol in ethyl acetate yielded a recovery of 86% (CV, 8.0%).

Ethyl acetate containing 5% ethanol gave the best overall performance as an extraction solvent. It resulted in a minimum of volatile coextractives, the best extractability of polar insecticides from H_2O, and the highest recovery of insecticides with a wide range of polarities from liver.

Development of Cleanup Procedure

The GPC cleanup conditions are similar to those of previous work (1), except that GPC solvent was used. Hexane containing 40% ethyl acetate (v/v) was replaced with 30% ethyl acetate in hexane. This solvent mixture allowed for the use of a shorter column and thus smaller collection volumes. Hexane and ethyl acetate were used because of their ease of removal by evaporation compared with other solvent systems (14) and the desire to avoid halogenated solvents (16). All insecticides were eluted from the GPC under the listed conditions in a volume of 130 mL (Tables 1–3).

Cleanup steps for samples with low lipid content were selected to provide a rapid and selective cleanup based on the analysis being performed. Analysis of OP insecticides in alfalfa hay required no cleanup because of the selective nature of the FPD. MC and OC insecticides were adequately cleaned up with the SPE procedures (Figure 2).

Development of Chromatographic Procedure

The chromatographic conditions are similar to those of other authors (1, 15, 17). Both short analysis times and reduced numbers of false-positive results were considered when selecting these parameters. The use of a DB-608 megabore column was especially useful because it separated all the major OC insecticides (Figure 2). GPC/silica gel cleanup enabled analysis of tissue extracts at 2 g/mL (OP insecticides) and 0.5 g/mL (MC insecticides) without chromatographic problems (Figures 3 and 4). The use of autosamplers enabled unattended overnight analyses. Longer chromatographic runs and thus better resolution between pesticides and interfering peaks were therefore possible with only minimal personnel time.

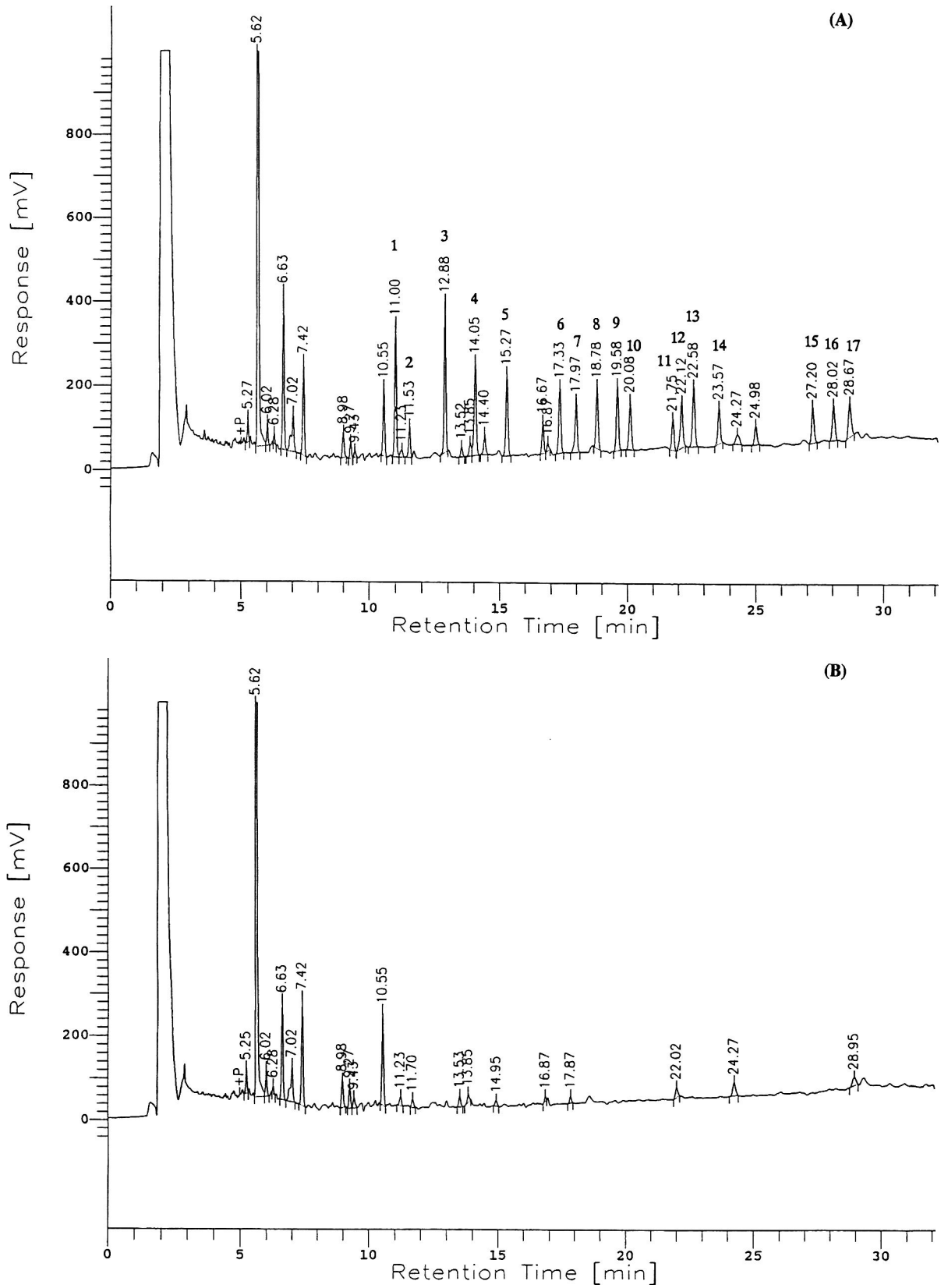


Figure 2. (A) GC/ECD chromatogram of alfalfa hay extract fortified at 0.0625 to 0.25 ppm with OC insecticides (see Table 4). A 2 μL volume of a 0.2 g/mL sample extract was injected under standard GC conditions. Peaks: (1) HCB, (2) α-BHC, (3) lindane, (4) heptachlor, (5) aldrin, (6) heptachlor epoxide, (7) γ-chlordane, (8) endosulfan I, (9) *p,p'*-DDE, (10) dieldrin, (11) endrin, (12) *p,p'*-DDD, (13) endosulfan II, (14) *p,p'*-DDT, (15) dicofol, (16) *p,p'*-methoxychlor and (17) mirex. (B) GC/ECD chromatogram of control alfalfa hay extract. A 2 μL volume of a 0.2 g/mL sample extract was injected under standard GC conditions.

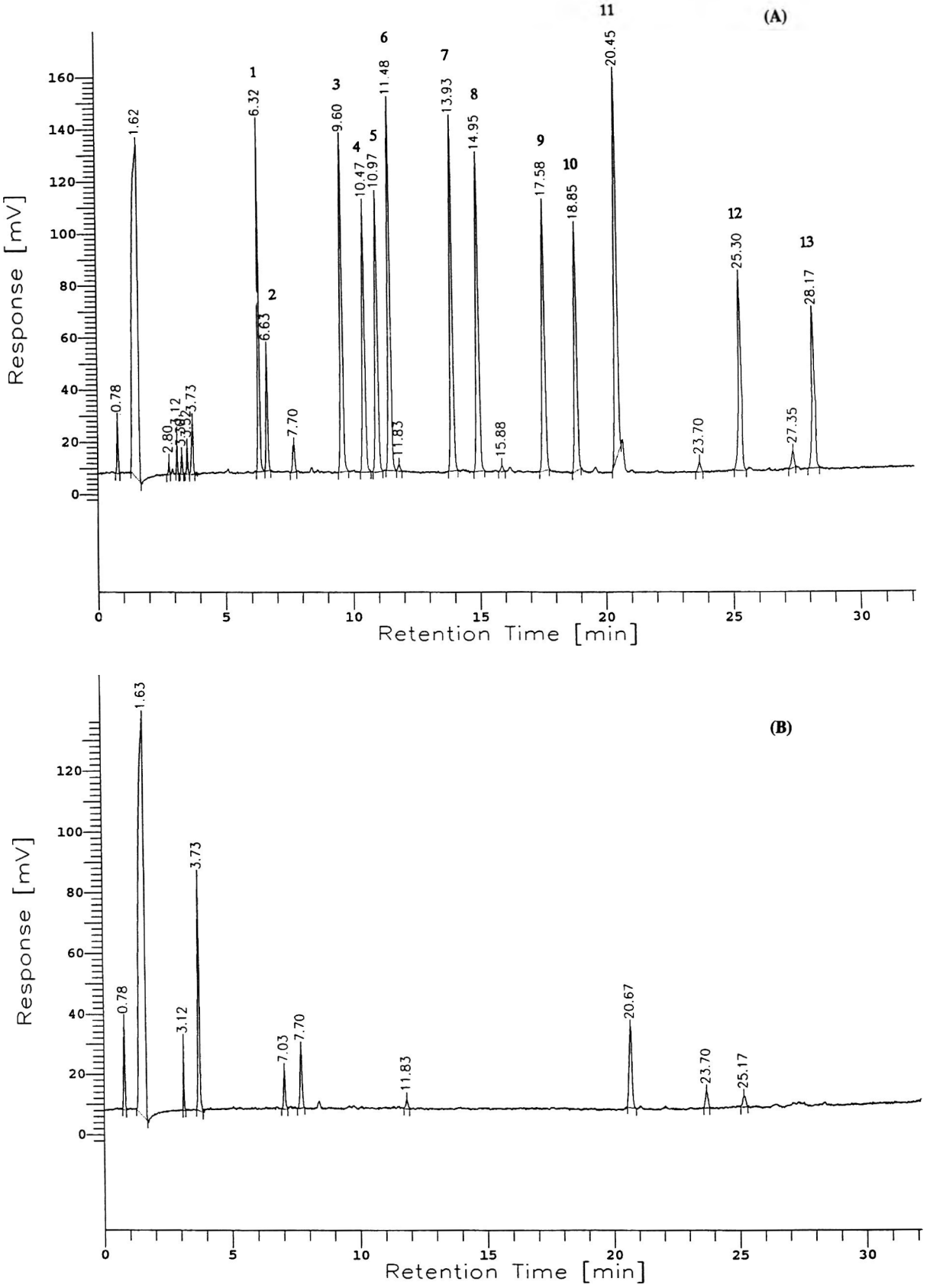


Figure 3. (A) GC/FPD chromatogram of bovine liver extract fortified at 0.125 ppm with OP standard mix A. A 2 μL volume of a 2.0 g/mL sample extract was injected under standard GC conditions. Peaks: (1) mevinphos E, (2) mevinphos Z, (3) phorate, (4) terbufos, (5) diazinon, (6) fonofos, (7) methyl parathion, (8) parathion, (9) DEF, (10) methidathion, (11) ethion, (12) phosalone, and (13) coumaphos. (B) GC/FPD chromatogram of control bovine liver extract. A 2 μL volume of a 2.0 g/mL sample extract was injected under standard GC conditions.

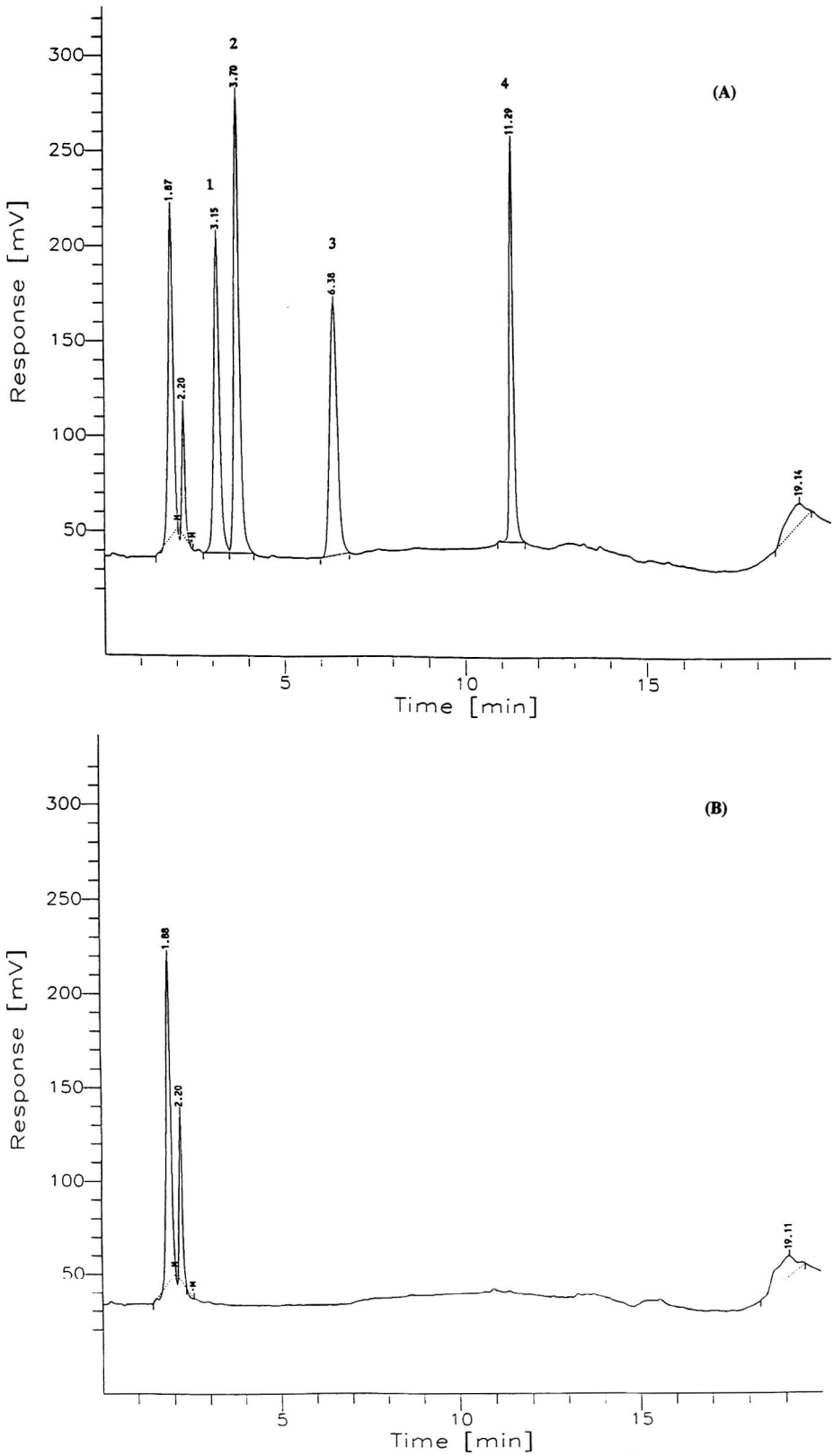


Figure 4. (A) LC chromatogram of bovine liver extract fortified at 0.5 ppm with MC standard mix B. A 10 μL volume of a 0.5 g/mL sample extract was injected under standard LC conditions. Peaks: (1) aldicarb sulfone, (2) methomyl, (3) 3-hydroxycarbofuran, and (4) propoxur. (B) LC chromatogram of control bovine liver extract. A 10 μL volume of a 0.5 g/mL sample extract was injected under standard LC conditions.

Conclusion

The OP MRM developed previously for use in this laboratory was greatly improved. The number of compounds was increased, and 3 classes of insecticides were analyzed from the same sample extract. The sample size was doubled from 5 to 10 g, but the extraction solvent volume was kept constant. All halogenated solvents were eliminated. The GPC parameters were refined by changing from 40% ethyl acetate in hexane to 30% ethyl acetate in hexane. The lower viscosity of the new GPC solvent gives greater resolution and allows use of shorter columns and smaller collection volumes.

This multiresidue insecticide screen extracted, cleaned up, and analyzed 3 classes of insecticides and over 71 compounds in bovine liver tissue, alfalfa, and tomatoes. The method performed well for a high-fat-content matrix (liver), an extremely pigmented matrix (alfalfa hay), and a matrix with high water content (tomato). Good fortification recoveries and coefficients of variation for all 71 insecticides were obtained with all matrixes. The procedure is especially suited to veterinary diagnostic laboratory situations, where sample size is limited, many difficult samples must be analyzed rapidly, and extremely low detection limits (<0.05 ppm) in source material are not required. The method has been used over the past 2 years in routine sample analysis with good spike recoveries from various matrixes, including various animal feeds and tissue types.

Acknowledgments

We thank Miné Palazoglu, Traci Francis, and Bridget Moran-Bradford for their assistance in validating the *N*-methyl carbamate portion of the fortification study.

References

(1) Holstege, D.M., Scharberg, D.L., Richardson, E.R., & Moller, G. (1991) *J. Assoc. Off. Anal. Chem.* **72,** 394–399

(2) Sasaki, K., Takashi, S., & Saito, Y. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 460–464

(3) Lawrence, J.F. (1987) *Int. J. Environ. Anal. Chem.* **29,** 289–303

(4) Blaha, J.J., & Jackson, P.J. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 1095–1099

(5) Ault, J.A., Schofield, C.M., Johnson, L.D., & Waltz, R.H. (1979) *J. Agric. Food Chem.* **27,** 825–828

(6) Sasaki, K., Takashi, S., & Saito, Y. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 460–464

(7) Ault, J.A., & Spurgeon, T.E. (1984) *J. Agric. Food Chem.* **67,** 284–289

(8) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **970.52, 983.21, 984.21**

(9) Ali, M.S. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 586–592

(10) Krause, R.T. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 726–733

(11) Ambrus, A., & Thier, H.-P. (1986) *Pure Appl. Chem.* **58,** 1035–1062

(12) Luke, M.A., Froberg, J.E., & Masumoto, H.T. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 1020–1026

(13) Luke, M.A., Froberg, J.E., Doose, G.M., & Masumoto, H.T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1187–1195

(14) Specht, W., & Tillkes, M. (1985) *Fresenius' Z. Anal. Chem.* **322,** 443–455

(15) Lee, S.M., Papathakis, M.L., Feng, H.C., Hunter, G.F., & Carr, J.E. (1991) *Fresenius' J. Anal. Chem.* **339,** 376–383

(16) Hopper, M.L. (1982) *J. Agric. Food Chem.* **30,** 1038–1041

(17) *The Analysis of Chlorinated Pesticides Using DB-608, DB-5 and DB-1701* (1991) J & W Scientific GC Environmental Application Series GE-6

RESIDUES AND TRACE ELEMENTS

Integration of Liquid Chromatography with Immunoassay: An Approach Combining the Strengths of Both Methods

PETRA M. KRÄMER, [1] QING X. LI, [2] and BRUCE D. HAMMOCK [3]
University of California, Departments of Entomology and Environmental Toxicology, Davis, CA 95616

The integration of liquid chromatography (LC) with immunochemical detection combines the superior separation power of LC and the sensitivity and specificity of immunoassays. This approach is shown with 3 LC systems (Perkin-Elmer, C_{18} RP, 4.6 mm; Varian, C_{18} RP, 1 mm microbore; Michrom, C_{18} RP, 1 mm microbore) integrated with an enzyme-linked immunosorbent assay (ELISA) selective for five 4-nitrophenols. The nitrophenols were separated with the 3 LC systems with isocratic runs of 15 to 20 min. Microbore LC separation showed a 10–20 times reduction in solvent amount compared to conventional separation. LC–immunoassay was about 8- to 10-fold more sensitive compared with LC with UV detection. Integrated LC–immuno-assay proved to be a very selective method when 2-methylphenol was injected with an equimolar mixture of 2-amino-4-nitrophenol and 3-methyl-4-nitrophenol; 2-methylphenol does not cross-react with the serum used. Only 2 peaks could be seen in the detection, even when 2-methylphenol was present in very high amounts (3000 pmol). Further, the ELISA–LC detection proved to be selective and sensitive for complex matrixes. 2-Amino-4-nitrophenol was clearly identified in spiked extracts of soil and plant, even when a very small amount (2.4 ng) was injected. Although LC–immunoassay is more labor intensive than LC with UV detection, it offers great advantages in multiresidue analysis and is generally applicable for peak confirmation.

The use of ELISA (enzyme-linked immunosorbent assay) to detect pesticides and other environmental chemicals has been demonstrated over the past 15 years (1, 2). In many cases, ELISA has proven to be a sensitive and selective analytical method. Although some immunoassays can be very selective, a drawback can be that antibodies cross-react with structurally related compounds; therefore, the signal obtained in an ELISA may not strictly relate to only one compound. This is particularly the case in environmental analysis, where a number of structurally related compounds could be present in the matrix. Multianalyte analysis, therefore, could be done by combining different antibodies, as shown by Muldoon et al. (3) for the analysis of *s*-triazines, or by using one antiserum of different bleeds combined with LC (liquid chromatography), as shown for the screening of β-agonists (4). A step further in this direction is the integration of ELISA with chromatographic procedures, which is demonstrated in this paper. LC is an analytical method that can be related, in many cases, to a single compound. Some uncertainties are associated with this method, too, for example, when standards and/or specific detectors are not available or when complex matrixes are determined. As a result, the idea was to combine the strength of both methods: to use the ELISA as a sensitive and selective detector after the LC separation. As pointed out by de Frutos and Regnier (5), these 2 techniques with their complementary features are very well suited to combine. This paper presents one example for such a union of technologies: taking a polyclonal antiserum that cross-reacts with different 4-nitrophenols (6). These compounds were selected to study the integration of LC and ELISA because (*1*) they are common metabolites or degradation products of pesticides and explosives; (*2*) they have strong UV absorbance, facilitating rigorous comparison of UV and ELISA detection; and (*3*) they are detected by a well-characterized antiserum that showed cross-reactivity in nearly equal percentages with the 4-nitrophenols separated, but does not cross-react with various other compounds that are likely to occur in samples. By comparing 3 LC systems (Systems A, B, and C; Perkin-Elmer LC, Varian LC, and Michrom LC, respectively), which were combined with off-line ELISA detection, this paper will demonstrate some directions in which this field is most likely to evolve.

Received June 18, 1993. Accepted by JS September 13, 1993.
This paper was presented in part at the 105th AOAC INTERNATIONAL Annual Meeting in Phoenix, AZ, August 10–15, 1991.

[1] Current address: GBF, Department of Enzymology, Mascheroder Weg 1, 38124 Braunschweig, Germany.
[2] Current address: University of California, Department of Environmental Science, Policy and Management, Environmental Chemistry and Toxicology Laboratory, Berkeley, CA 94720.
[3] Author to whom correspondence should be addressed.

Experimental

Apparatus

(a) *Liquid chromatographs.—System A.*—Perkin-Elmer Series 410 BIO LC (San Jose, CA) was equipped with an LC-

235 diode array detector and a GP-100 graphics printer. An Everex computer equipped with Perkin-Elmer Omega-4 software controlled the LC run and stored all chromatograms. A Rheodyne sample injector (Cotati, CA) with a 10 µL injector loop was used. *System B.*—Varian 9010 LC (Sugar Land, TX) was equipped with a Varian 9050 UV–VIS detector. Chromatograms were recorded with an HP 3394A integrator (Hewlett-Packard, Avondale, PA). A Rheodyne Model 7125 syringe-loading sample injector with an injection loop of 10 µL was used. *System C.*—Michrom Microbore LC (Michrom BioResources, Pleasanton, CA) was equipped with a UV–VIS detector. The injection loop was 10 µL. Chromatograms were printed on an Epson LQ-570.

(**b**) *LC columns.*—*System A.*—C_{18} RP column (10 µm, 25 cm × 4.6 mm; Vydac, Hesperia, CA). *System B.*—Aquapore OD-300 microbore C_{18} RP column (7 µm, 25 cm × 1 mm; Applied Biosystems, Inc., Foster City, CA). *System C.*—Reliasil C_{18} RP column (3 µm, 15 cm × 1 mm, Michrom BioResources).

(**c**) *Syringes.*—25 and 50 µL syringes (Hamilton Co., Reno, NV).

(**d**) *Fraction collector.*—Microfraction Model 203 collector (Gilson Medical Electronics, Inc., Middleton, WI).

(**e**) *Microtiter plates.*—96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) for fraction collection and microtiter plates (Nunc A/S, Roskilde, Denmark) for assay performance.

(**f**) *ELISA reader.*—V_{max} Microplate reader with software package SOFTmax (Molecular Devices Corp., Menlo Park, CA) was used to measure in dual-wavelength mode, either at 405 – 650 nm or at 450 – 650 nm.

Reagents

(**a**) *Solvents.*—LC grade acetonitrile and methanol (Fisher Scientific, Pittsburgh, PA) were filtered and degassed prior to use. Water was filtered through a Sybron/Barnstedt Nanopure II water system set at 17.8 MΩ-cm. It was used for mobile phase in LC and for aqueous solutions in immunochemistry.

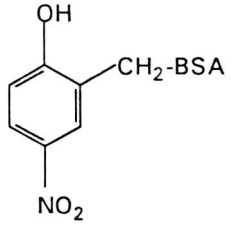
(**b**) *Filters.*—FP-Vericel membrane filter, 0.2 µm pore size (Gelman Sciences, Ann Arbor, MI). Cameo 3N or 25F syringe filters, nylon or Teflon, respectively, 0.45 µm (Micron Separations, Inc., Westboro, MA).

(**c**) *Standards.*—2-Amino-4-nitrophenol [1; molecular weight (MW), 154], 4-nitrophenol (2; MW, 139), 2,4-dinitrophenol (3; MW, 184), 3-methyl-4-nitrophenol (4; MW, 153), 2-chloro-4-nitrophenol (5; MW, 173) (the numbers 1–5 refer to sequence of peaks in LC separation, except for System B(2)), and 2-methylphenol (6; MW, 108) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Stock solutions were prepared in methanol and stored at 4°C. Diluted standards in equimolar concentrations of multianalytes (5 or 3 compounds) in a mixture were prepared and filtered prior to use in the LC system.

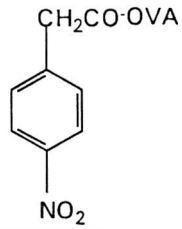
(**d**) *Coating antigen.*—C-OVA (4-nitrophenylacetic acid conjugated to ovalbumin [OVA] [6]; Figure 1).

(**e**) *Immunochemicals.*—Antibody (ab) 1812 (raised against a conjugate of 2-bromomethyl-4-nitrophenol coupled to bovine serum albumin [BSA] [6]; Figure 1), goat anti-rabbit IgG with alkaline phosphatase (AP; ICN Biomedicals, Inc., Diagnostic Div., Costa Mesa, CA, or Sigma Chemical Co., St. Louis, MO), goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP; Sigma Chemical Co.).

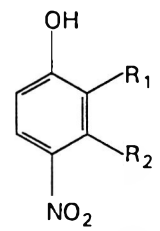
(**f**) *Buffers.—Coating buffer.*—0.5M carbonate buffer, pH 9.6. *Assay buffer.*—0.2M, 0.3M, or 0.4M phosphate-buffered saline solution (pH 7.5) with 0.05% Tween 20 and 0.02% sodium azide (PBSTA); 0.2M phosphate buffered saline solution (pH 7.5) with 0.05% Tween 20 (PBST). *Washing buffer.*—*see* PBST. *Substrate buffer.*—(*1*) 10% diethanolamine buffer (pH 9.8) for AP; (*2*) 0.1M sodium acetate (pH 5.5) for HRP.



Immunogen



Coating Antigen



Cross reactants

No.	R_1	R_2	Chemical name
1	NH_2	H	2-amino-4-nitrophenol
2	H	H	4-nitrophenol
3	NO_2	H	2,4-dinitrophenol
4	H	CH_3	3-methyl-4-nitrophenol
5	Cl	H	2-chloro-4-nitrophenol

Figure 1. Relevant structures for the assay for nitrophenols. BSA, bovine serum albumin; OVA, ovalbumin.

(g) *Substrates for enzyme reaction.*—*p*-Nitrophenyl phosphate (1 mg/mL) and tetramethyl benzidine (6 mg/mL in dimethyl sulfoxide; Sigma Chemical Co.) with 1% H_2O_2 (Fisher Scientific) were used as substrates for AP and HRP, respectively.

Extraction of Soil and Plant Material

(a) *Soil.*—Yolo silt loam (25 g; typic xerorthenl, 1.3% organic carbon) was boiled in 150 mL methanol for 30–40 min. After cooling, the mixture was filtered through Whatman filter No. 1 (Clifton, NJ). The solvent was evaporated with a Buchler rotary evaporator (Kansas City, MO), and the residue redissolved in 1 mL methanol. Before injection, this concentrate was filtered through Cameo 3N, nylon, 0.45-μm filters.

(b) *Plant.*—Maize leaves (90 g; *Zea mays*, Pioneer hybrid, 3379) were homogenized in 600 mL methanol–chloroform (4 + 1) for 10 min (Polytron, Brinkmann Instruments, Westbury, NY) and boiled for 30–40 min. Roughly 100 mL of the extract was filtered and evaporated as described for soil extracts, and the residue after evaporation of solvent redissolved in 3 mL methanol.

Liquid Chromatography

(a) *System A.*—The separation was performed at room temperature (RT, 22°–24°C). The mobile phase was a mixture of acetonitrile (16%, v/v), methanol (16%, v/v), and H_2O (68%, v/v), containing 0.5% (v/v) acetic acid. It was run at a flow rate of 1 mL/min. A mixture of equimolar amounts of standards (ranging from 78 to 10 000 pmol) in methanol was injected onto the column and detected by a UV detector at 280 nm.

(b) *System B.*—Equimolar amounts (ranging from 15.6 to 2000 pmol) of 5 nitrophenols (*see* standards) in methanol were injected. The mobile phase used was either H_2O–acetonitrile–methanol (78 + 11 + 11, v/v) with 0.1% trifluoroacetic acid (TFA; v/v) (B1) or H_2O–methanol–acetonitrile (81 + 9.5 + 9.5, v/v) (B2) and was held isocratic. The flow rate was kept constant either at 0.12 or 0.14 mL/min. The pressure during separation was 8.1–10.1 MPa. Analyses were performed at RT. The output of the UV detector (set at 280 nm) was monitored with an integrator that recorded peak areas (counts) and retention times.

(c) *System C.*—The separation of the five 4-nitrophenols (*see* System A) or a mixture of two 4-nitrophenols (2-amino-4-nitrophenol and 2-chloro-4-nitrophenol) and 2-methylphenol was performed at 40°C (temperature controlled). The mobile phase was H_2O–acetonitrile–methanol (60 + 20 + 20, v/v) with 0.04% (v/v) TFA and was kept pressurized. An isocratic run was used with a flow rate of 50 μL/min. The UV detector was set at 280 nm. The pressure during separation was between 13 and 15 MPa.

Eluate Collection

(a) *Systems A and B.*—The microfraction collector, connected to the LC detector, was used to collect either fractions in microtiter plates or whole peaks in tubes. For System A, the collection volume (1170 μL) of each peak was determined by flow rate and time. For System B, the volume for a single peak varied (170–400 μL), and each corresponding volume was taken into account for the determination of detected amounts.

For off-line monitoring of the separation of the nitrophenols, the eluate was collected with the fraction collector either in time mode (*System A.*—0.14 min/well or 140 μL; *System B.*—0.24 min/well or 35 μL) or in drop mode (2 or 3 drops/well, System B only) directly into the wells of a microtiter plate. For System A, collection was started 4 min after the start of the LC run.

(b) *System C.*—The fraction collector was connected to the LC detector and controlled by the LC. Whole peaks were collected

Table 1. Summary of LC Systems A, B, and C[a]

Item	System A	System B	System C
Liquid chromatograph	Perkin-Elmer LC Series 410 Bio LC	Varian 9010	Michrom Microbore LC
Detector	LC-235 diode array 280 nm	UV–Vis 280 nm	UV–Vis 280 nm
Injector	10 μL loop (Rheodyne)	10 μL loop (Rheodyne, Model 7125)	10 μL loop
LC column	C_{18} RP, 10 μM, 25 cm × 4.6 mm (Vydac)	C_{18} RP Aquapore OD-300 microbore, 7 μM, 25 cm × 1 mm (Applied Biosystems)	Reliasil C_{18} RP microbore, 3 μM, 15 cm × 1 mm (Michrom BioResources)
Temperature	Room temperature (22–24°C)	Room temperature (22–24°C)	40°C
Mobile phase	ACN–MeOH–H_2O (16 + 16 + 68, v/v) Acetic acid, 0.5%	B1: ACN–MeOH–H_2O (11 + 11 + 78); TFA, 0.1% B2: ACN–MeOH–H_2O (9.5 + 9.5 + 81)	ACN–MeOH–H_2O (20 + 20 + 60) TFA, 0.04%
Flow rate, μL/min	1000 Isocratic	120 or 140 Isocratic (Pressure, 8.1 to 10.1 MPa)	50 Isocratic (Pressure, 13 to 15 MPa)
Injection volume, μL	10	2	2
Eluate collection			
Whole peak collection, μL/peak	1170 in tubes	170 to 400 in microtiter plates	50 to 90 in microtiter plates
Collection for monitoring, μL/well	140	35 (2 or 3 drops per well)	10

[a] ACN, acetonitrile; MeOH, methanol; TFA, trifluoroacetic acid.

in wells of a microtiter plate (50–90 µL). To monitor the analytes after the LC separation, the eluate was collected in time mode (0.2 min/well or 10 µL). The collection was started either from start or 2.5 min after the start of LC separation.

All details for the performance of the LC systems are summarized in Table 1.

ELISA

The assay was performed as described by Li et al. (6), with modifications based on this special purpose as a detector for LC. Details of the ELISA-LC are described in Table 2. Both quantification and post-column monitoring by ELISA-LC were performed off-line. For quantification of whole peaks, the corresponding standards were run at the same time on the same plate in quadruplicate. They were set up in the mobile phase that was used for LC separation.

At least 4 zero concentrations (A_{Max}) and 4 background concentrations ($A_{Background}$) were added to each plate for monitoring of the LC separation with ELISA–LC. This made it possible to transform the absorbance values into percent inhibition values (= 100 – % control) and allowed conversion of valleys to peaks for better comparison with the LC separation:

$$\% \text{ inhibition} = 100 - \% \text{ control}$$

$$= 100 - \left[\frac{(A - A_{Background})}{(A_{Max} - A_{Background})}\right] \times 100$$

where A = absorbance of samples (eluate fractions), $A_{Background}$ = absorbance of background (mobile phase for LC separation; *no* ab 1812; *no* inhibitor), and A_{Max} = maximum

Table 2. Summary of ELISA-LC after LC separation

Item	System A		System B		System C
	Quantitative ELISA-LC	Off-line monitoring	Quantitative ELISA-LC	Off-line monitoring	Off-line monitoring
Coating antigen: C-OVA					
Volume, µL/well; concentration,, µg/mL	100; 2.5	100; 2.5	100; 2	100; 2.5	100; 2
Coating buffer: 0.5M carbonate, pH 9.6 incubation	Overnight, 4°C	Overnight, 4°C	Overnight, 4°C	Overnight, 4°C	Overnight, 4°C
Eluate volume for ELISA, µL	55[a]	55[a]	50	20	10
Standard in mobile phase: volume, µL	55[a]		50		
Specific antibody: 1812					
Volume, µL/well; dilution	103; 1:400	103; 1:400	200; 1:1600	80; 1:800	40; 1:1600
Buffer, pH 7.5	0.4M PBSTA	0.4M PBSTA	0.4M PBSTA	0.2M PBSTA	0.2M PBSTA
Incubation, together with eluate and standard	Overnight, RT	Overnight, RT	Overnight, RT	Overnight, RT	Overnight, RT
Specific antibody and eluate or standard on coated plate: volume,, µL/well	50[b]	50	50[b]	50	45
Incubation	2 h, RT	2 h, RT	2 h, RT	2 h, RT	2 h, RT
Second antibody: goat anti-rabbit IgG	Conjugated with AP	Conjugated with AP	Conjugated with AP	Conjugated with AP	Conjugated with HRP
Dilution; volume, µL/well	1:2500; 50	1:2500; 50	1:5000; 50	1:5000; 50	1:8000; 50
Buffer, pH 7.5	0.2M PBSTA	0.2M PBSTA	0.2M PBSTA	0.2M PBSTA	0.2M PBSTA
Incubation	1 h, RT	1 h, RT	2 h, RT	2 h, RT	1.5 h, RT
Substrate for enzyme reaction	*p*-Nitrophenyl phosphate	*p*-Nitrophenyl phosphate	*p*-Nitrophenyl phosphate	*p*-Nitrophenyl phosphate	TMB/1% H_2O_2
Volume, µL/well	100	100	100	100	100
Buffer	10% Diethanol-amine[c]	10% Diethanol-amine[c]	10% Diethanol-amine[c]	10% Diethanol-amine[c]	0.1M sodium acetate[d]
Incubation	1 h	1 h	1 h	20 min	9–10 min
Wavelength, nm	405 – 650	405 – 650	405 – 650	405 – 650	450 – 650

Note: Between each step during the assay, the plates were washed with washing buffer (0.2M PBST, pH 7.5). RT, room temperature (22–24°C); AP, alkaline phosphatase; HRP, horseradish peroxidase; IgG, immunoglobulin G.

[a] 55 µL H_2O and 6.6 µL 0.83N NaOH were added to adjust the pH.

[b] Quadruplicate determination.

[c] pH 9.8.

[d] pH 5.5.

absorbance (mobile phase for LC separation; ab 1812; *no* inhibitor).

Results

ELISA for 4-Nitrophenols

The general assay for nitrophenols developed by Li et al. (6) requires the presence of both a 4-nitro and a 1-phenolic group for detection (Figure 1). It will detect a wide variety of 4-nitrophenols mono- or disubstituted in the 2,3 positions, but it will not cross-react with symmetrical di- or polysubstituted 4-nitrophenols, if the 5 and/or 6 positions are substituted. To evaluate the LC systems, five 4-nitrophenols were selected. These nitrophenols are recognized by this assay in a similar concentration range (50–150nM; Figure 2). The calculated data (I_{50} = concentration of analyte that gives 50% inhibition) from SOFTmax were used to determine the cross-reactivities: 100% for 4-nitrophenol, 67% for 2-amino-4-nitrophenol, 100% for 2,4-dinitrophenol, 95% for 3-methyl-4-nitrophenol, and 230% for 2-chloro-4-nitrophenol [%CR, = (concentration of 4-nitrophenol at i_{50}/concentration of other 4-nitrophenols at i_{50}) × 100]. These values will change slightly, depending on the assay setup. In this study, the assay was run in the mobile phase that was used for LC separation. Cross-reactivity values can be considered analogous to response factors used in quantitative gas–liquid chromatographic or LC analysis. As with these analytical methods, the quality of the resulting data is improved if the response factors and standard curves are determined at the same time as the actual analysis. In this case, it means that the standard curve for the compound of interest should be put on the same plate with the collected eluate for this compound.

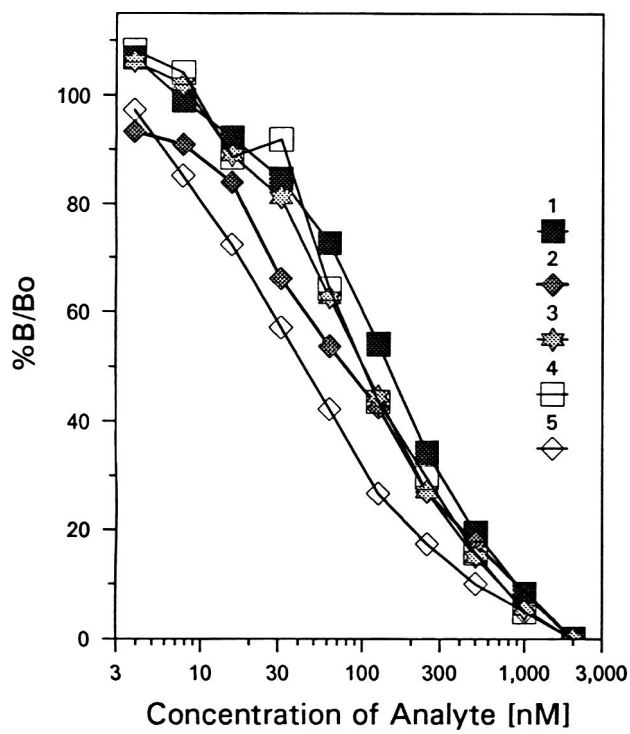


Figure 2. Standard curves for five 4-nitrophenols expressed as follows:

$$\%B/B_0 = \frac{A - A_{excess}}{A_{max} - A_{excess}} \times 100$$

where B = absorbance with an excess concentration of analtye and B_0 = absorbance without analyte. Standards were prepared in the mobile phase used for LC separation, in this case, 11% methanol, 11% acetonitrile, 78% H_2O with 0.1% trifluoroacetic acid. Standard in mobile phase (50 µL) was diluted with 200 µL ab solution (0.3M PBSTA), and the assay was performed as described.

Integrated LC–Immunoassay Setup

The scheme of the LC–immunoassay is shown in Figure 3. Standard amounts of nitrophenols were injected, separated, and monitored with a UV detector in 3 LC systems (lower part) and then collected via a fraction collector either in tubes or in microtiter plates and monitored off-line with ELISA (upper part). LC System A (Perkin Elmer LC, C_{18} RP, 4.6 mm column), B (Varian LC, C_{18} RP, 1 mm microbore column), and C (Michrom Microbore LC, C_{18} RP, 1 mm microbore column) were used and combined with an off-line ELISA detection. The separations with all systems were monitored either with a computer system (Systems A and C) or with an integrator (System B) that was connected to the detector. As an option, a Speed Vac or similar equipment might be useful for evaporation of the solvent after eluate collection, by using either tubes or 96-well-plate racks. In this study, preliminary experiments to evaporate the solvent did not show good reproducibility.

LC Separation of 4-Nitrophenols

With the 3 LC instruments, five 4-nitrophenols were separated with isocratic runs to about 15–20 min. The injected equimolar amounts of the five 4-nitrophenols ranged from 10 to 1800 ng for System A and from 2 to 360 ng for Systems B and C. The retention times of 2-amino-4-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 3-methyl-4-nitrophenol, and 2-chloro-4-nitrophenol with system A (average of 10 LC runs) were 4.26, 7.40, 9.55, 11.21, and 16.38 min, respectively, with a coefficient of variation (CV) ≤3.3%. The volume of the eluate for each peak was 1170 µL, which was determined by flow rate and collection time (Table 3, System A). Because of the relatively large volume, the eluates of the whole peaks had to be collected in tubes and only 55 µL were taken off-line for analysis by ELISA.

For System B, the mobile phase was used with and without acid. The retention times of the separation with 0.1% TFA were 2.33, 7.14, 9.34, 12.96, and 20.19 min, respectively, for the 5 compounds listed earlier (average of 10 runs; CV ≤5.1%; Table 3, System B[1]). After the separation was run without acid, the retention times changed: 2.96 min for 2,4-dinitrophenol, 4.42 min for 2-amino-4-nitrophenol, 6.14 min for 4-nitrophenol, 10.19 min for 3-methyl-4-nitrophenol, and 16.09 min for 2-chloro-4-nitrophenol (average of 10 runs; CV ≤3.5%; Table 3, System B[2]).

Separation with System C showed the least volume for each peak (50–90 µL/peak; Table 3). The retention times (average of 8–15 runs) were 3.92 min for 2-amino-4-nitrophenol,

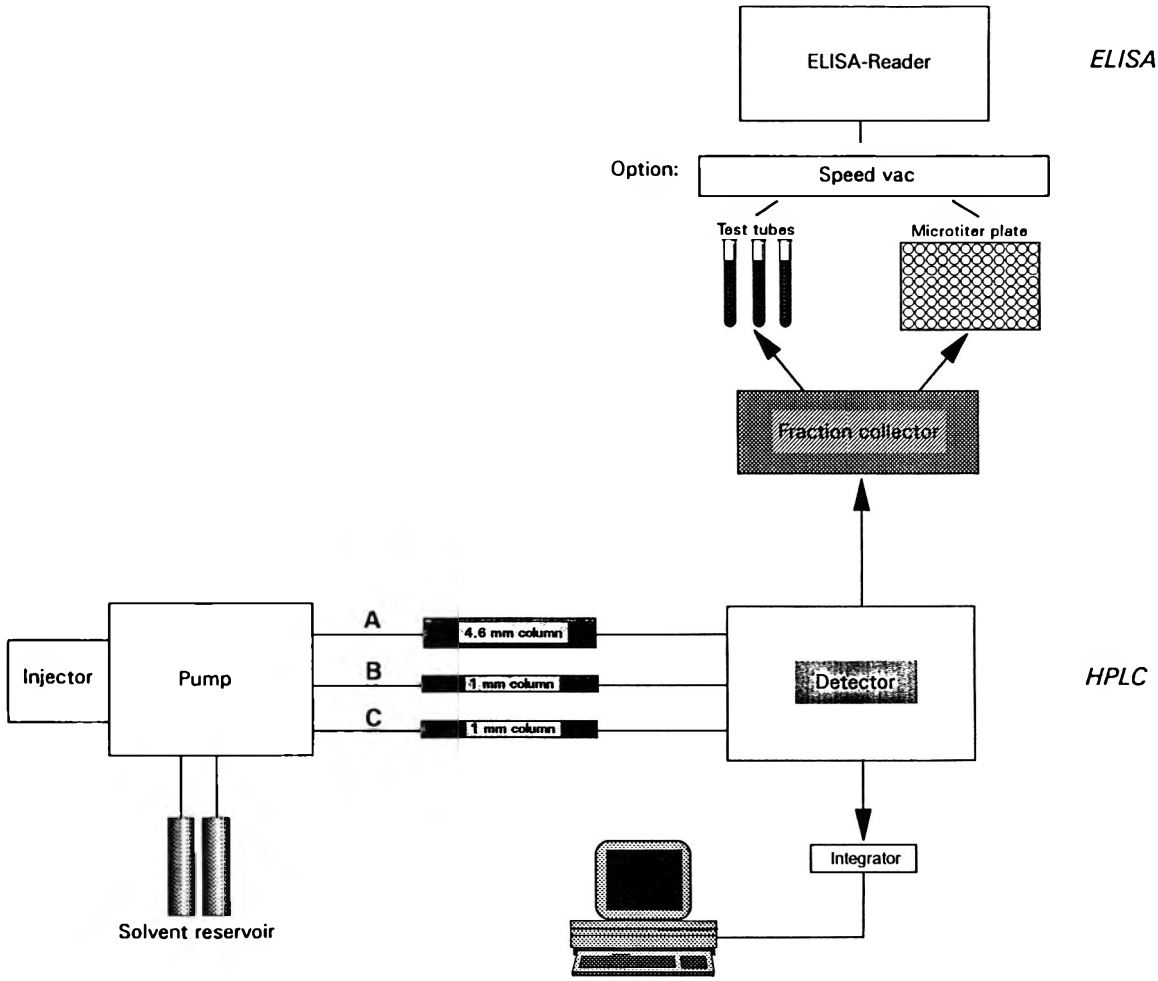


Figure 3. Scheme of integrated LC-immunoassay. Three LC systems were used to separate five 4-nitrophenols: Systems A: Perkin-Elmer LC, C_{18} RP, 4.6 mm column; System B: Varian LC, C_{18} RP, 1 mm microbore column; and System C: Michrom LC, C_{18} RP, 1 mm microbore column. The fraction collector was connected to the outlet of the detector (UV–Vis) and was able to collect either in plates or in tubes. As an option for future projects, a Speed Vac can be used to evaporate the solvent of the eluate after collection and prior to ELISA detection.

6.52 min for 4-nitrophenol, 8.32 min for 2,4-dinitrophenol, 9.66 min for 3-methyl-4-nitrophenol, and 14.9 min for 2-chloro-4-nitrophenol (CV ≤5.3%). The retention time of *o*-cresol (2-methylphenol), which was added as a compound that does not cross-react in the ELISA detection, was 7.87 (average of 8 runs; CV = 3.2%).

Peak Monitoring with UV–LC and ELISA–LC

The separations with the 3 systems were monitored by UV–LC and by ELISA–LC. As an example, Figure 4 shows the monitoring for System A (top, UV–LC; bottom, ELISA–LC). Here an equimolar amount of 625 pmol was injected. Eluate collection for ELISA–LC was started 4 min after the start of chromatography and collection was interrupted for 3.4 min between peaks 4 and 5. Eighty-eight fractions of 140 µL were collected (0.14 min/well). The retention times with UV–LC corresponded very well with those in ELISA–LC, with a delay of about 30 s for the ELISA–LC due to the connection between detector and collector. With the amount injected, UV–LC and ELISA–LC show comparable performance. When only 15.6 pmol was injected (Figure 5; System C), the ELISA–LC still monitors the LC separation, but the UV–LC is at its limit of detection. Because all compounds were injected in equimolar amounts, the peak heights seen in UV–LC depend on the molar absorption coefficients of these compounds at 280 nm; in ELISA–LC, however, the peak heights depend on the binding of the antibody to each compound. An aspect observed quite often in the ELISA–LC is negative inhibition (for example, *see* Figures 4 and 5), although the inhibition values were determined against the mobile phase of the LC separation. An explanation might be an edge effect on the plate because the control values (background and zero concentration) were always located in the right row of the plate.

Quantitation with UV–LC and ELISA–LC

For quantitative analyses, each peak was collected in a separate tube and the nitrophenol concentration was determined. Table 4 shows a comparison of the injected amounts and the detected amounts with System A. In most cases, the ELISA data show good agreement with the amounts injected. Discrep-

Table 3. Retention times and volumes with different LC systems

	Peak 1 2-Amino-4-nitrophenol	Peak 2 4-Nitrophenol	Peak 3 2,4-Dinitrophenol	Peak 4 3-Methyl-4-nitrophenol	Peak 5 2-Chloro-4-nitrophenol
System A: Perkin-Elmer LC; column: C_{18} RP, 4.6 mm; mobile phase: 16% methanol, 16% acetonitrile, 68% H_2O, 0.5% acetic acid					
Retention time[a], min	4.26 ± 0.14	7.40 ± 0.15	9.55 ± 0.16	11.21 ± 0.19	16.38 ± 0.24
Peak volume[b], µL	1170	1170	1170	1170	1170
System B(1): Varian LC; column: C_{18} RP, 1 mm microbore; mobile phase: 11% methanol, 11% acetonitrile, 78% H_2O, 0.1% trifluoroacetic acid					
Retention time[a], min	2.33 ± 0.12	7.14 ± 0.16	9.34 ± 0.21	12.96 ± 0.30	20.19 ± 0.43
Peak volume[c], µL	338 ± 31	255 ± 24	399 ± 47	286 ± 52	343 ± 121
System C: Michrom LC; column: C_{18} RP, 1 mm microbore; mobile phase: 20% methanol, 20% acetonitrile, 60% H_2O, 0.04% trifluoroacetic acid					
Retention time[d], min	3.92 ± 0.09	6.52 ± 0.17	8.32 ± 0.44	9.66 ± 0.34	14.9 ± 0.38
Peak volume[b,d], µL	54 ± 15	62 ± 14	73 ± 10	73 ± 7	90 ± 11
	Peak 1 2,4-Dinitrophenol	Peak 2 2-Amino-4-nitrophenol	Peak 3 4-Nitrophenol	Peak 4 3-Methyl-4-nitrophenol	Peak 5 2-Chloro-4-nitrophenol
System B(2): Varian LC; column: C_{18} RP, 1 mm microbore; mobile phase: 9.5% methanol, 9.5% acetonitrile, 81% H_2O					
Retention time[a], min	2.96 ± 0.09	4.42 ± 0.10	6.14 ± 0.18	10.19 ± 0.35	16.09 ± 0.56
Peak volume[c], µL	173 ± 63	183 ± 17	266 ± 48	302 ± 75	404 ± 77

Note: Flow rates were 1 mL/min for System A, 0.12 mL/min for Systems B(1) and B(2), and 0.05 mL/min for System C. The separation with the Varian LC was done with (B1) and without (B2) addition of trifluoroacetic acid in the solvent. By eliminating the acid, the first 3 peaks changed their sequence during elution.

[a] Values are means ± standard deviations (SDs) of 10 LC runs.

[b] Values are determined by collection time and flow rate.

[c] Values are means ± SDs of 8 LC runs with injected amounts from 15.6 to 1000 pmol.

[d] Values are means ± SDs of 8–15 LC runs with injected amounts from 125 to 1000 pmol.

ancies in some cases might be related to the peak collection mode, which did not allow variation in peak volume. The dilutions during the separation and within the performance of the assay are still a drawback, especially with this system, which uses a relatively large solvent flow and, therefore, dilutes the analytes during the separation by about 100 times.

In Figure 6, a comparison between UV–LC and ELISA–LC quantitations is shown for the compounds 2,4-dinitrophenol, 3-methyl-4-nitrophenol, and 2-chloro-4-nitrophenol separated by System B (1). For each compound, a standard curve was generated in LC (data not shown) and the concentration measured was plotted against the amount injected. The same separation was also measured by ELISA–LC, which gave better results, especially at lower concentrations (compare with R^2 values). Only one-fifth of the amount injected in System A was injected onto this column.

ELISA–LC as a Selective Detector

An important feature of ELISA–LC is its selectivity. Figure 7 shows an example demonstrating how ELISA–LC selectively detects the compounds that cross-react with the antiserum. Here 2-methylphenol was added in 2 different amounts (A, 250 pmol; B, 3000 pmol) to an equimolar mixture of 2-amino-4-nitrophenol and 2-chloro-4-nitrophenol (A, 250 pmol; B, 1000 pmol). Even with the very high amount of 2-methylphenol in the sample, the ELISA–LC did not give a response. ELISA–LC, therefore, is potentially a very useful tool either to monitor selectively only a small set of compounds out of a mixture or to verify a compound in a mixture. The latter case is particularly helpful when a compound of interest has only low UV absorbance (7). This selectivity also will be important when the compound of interest must be analyzed in a UV-dense matrix. The value of high selectivity was shown by Harris et al. (8) for analysis of nitrophenols in urine and is demonstrated here with the example of soil and plant extracts (Figure 8). 2-Amino-4-nitrophenol was added in a low amount (2.4 ng) to both extracts. It was not possible to detect this compound in soil or in plant extract with UV–LC (Figure 8, top). Rigorous extraction conditions were used with both matrixes to give a worst case situation. Only when the fractions were collected and analyzed by ELISA–LC could the compound be clearly identified by cochromatography of the standard in methanol (Figure 8, bottom). Unlike with UV–LC, the extracts did not affect the ELISA–LC performance (traces C-1 and C-2, respectively).

Microbore LC–Immunoassay

The use of a microbore column for separation offers the great advantage of saving solvent and of creating less solvent waste. The small solvent flow is advantageous for integrated LC–immunoassays. System B (Varian LC) is not a dedicated microbore LC; however, a microbore column allowed a 3- to

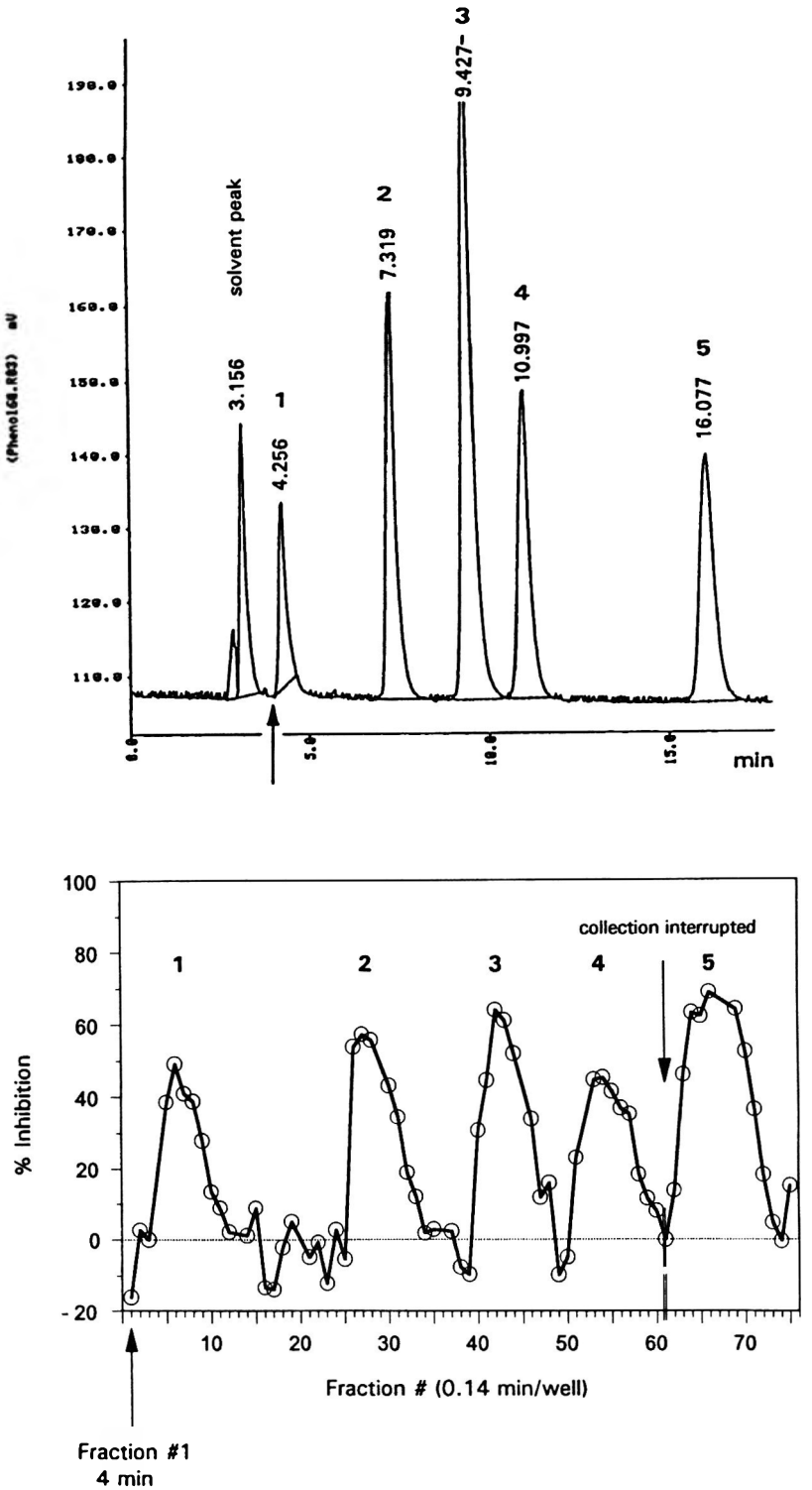


Figure 4. Separation and detection of five 4-nitrophenols by LC-immunoassay using the Perkin-Elmer LC (System A). (Top) LC chromatogram of five 4-nitrophenols. Equimolar amounts (625 pmol) of all five 4-nitrophenols were injected onto a 4.6 mm column, C_{18} RP. Mobile phase contained 16% (v/v) acetonitrile, 16% (v/v) methanol, and 68% (v/v) H_2O with 0.5% acetic acid and was held isocratic. Peaks: (1) 2-amino-4-nitrophenol, (2) 4-nitrophenol, (3) 2,4-dinitrophenol, (4) 3-methyl-4-nitrophenol, and (5) 2-chloro-4-nitrophenol. The first peaks were solvent peaks. The arrow indicates start of fraction collection for ELISA–LC. (Bottom) Peak monitoring with ELISA–LC. Eluate collection was started 4 min after the start of UV-LC, and collection was interrupted for 3.4 min between peaks 4 and 5. Eighty-eight fractions of 140 μL each were collected (0.14 min/well). The eluate–ab mixture was split between 2 plates, and the average of both plates was taken for this ELISA–LC detection. Detection by UV–LC depends on the molar absorbance coefficient of the compound, whereas detection by ELISA–LC depends on the binding and cross-reactivity of the antiserum used.

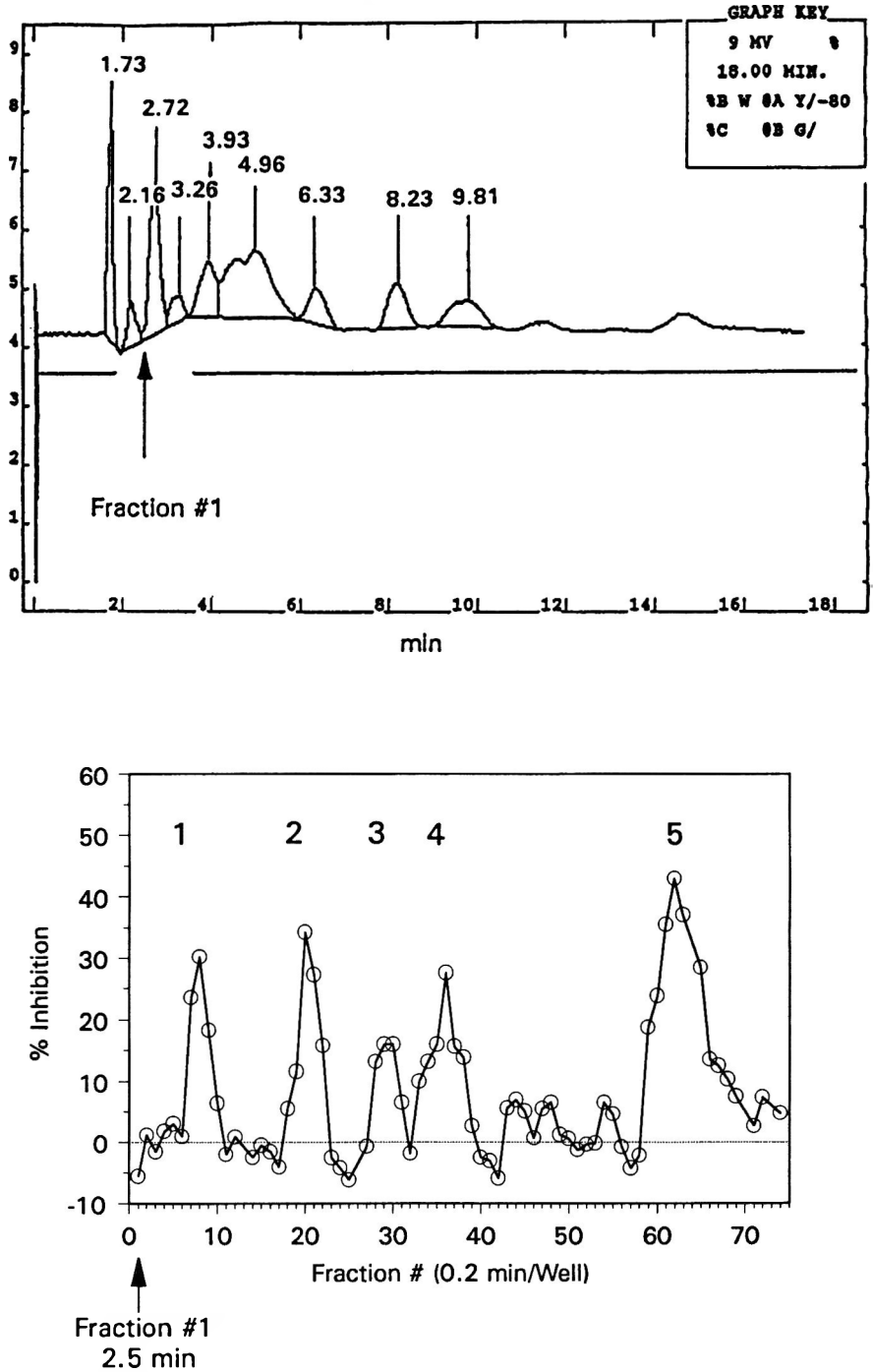


Figure 5. ELISA as a sensitive detector for LC. With the amount injected (equimolar amounts of 15.6 pmol for compounds 1–5), detection with UV–LC (top) is near its limit, whereas detection with ELISA–LC (bottom) is still possible (System C). In UV–LC, a number of peaks were integrated that exceeded the number of compounds injected. When the eluate was detected with ELISA–LC, only 5 peaks were clearly detected. Retention times were 3.9 (1), 6.3 (2), 8.2 (3), 9.5 (4), and 14.7 (5) min, which clearly identified the compounds. The arrow (fraction No. 1) identifies the start of fraction collection.

6-fold reduction in solvent use. Using a microbore LC reduces the amount of solvent sufficiently for one peak to be collected in about 50–90 μL eluate (Table 2, System C). Thus, the ELISA could be run directly without transfer to another plate. With the equipment described in this paper, transferring a definite volume, especially for quantitative data, gave more accurate results because the elution volume for different compounds may differ slightly (*see* Table 2, C).

Discussion

Immunochemical technologies are often considered as competitors of chromatographic-based systems as discussed

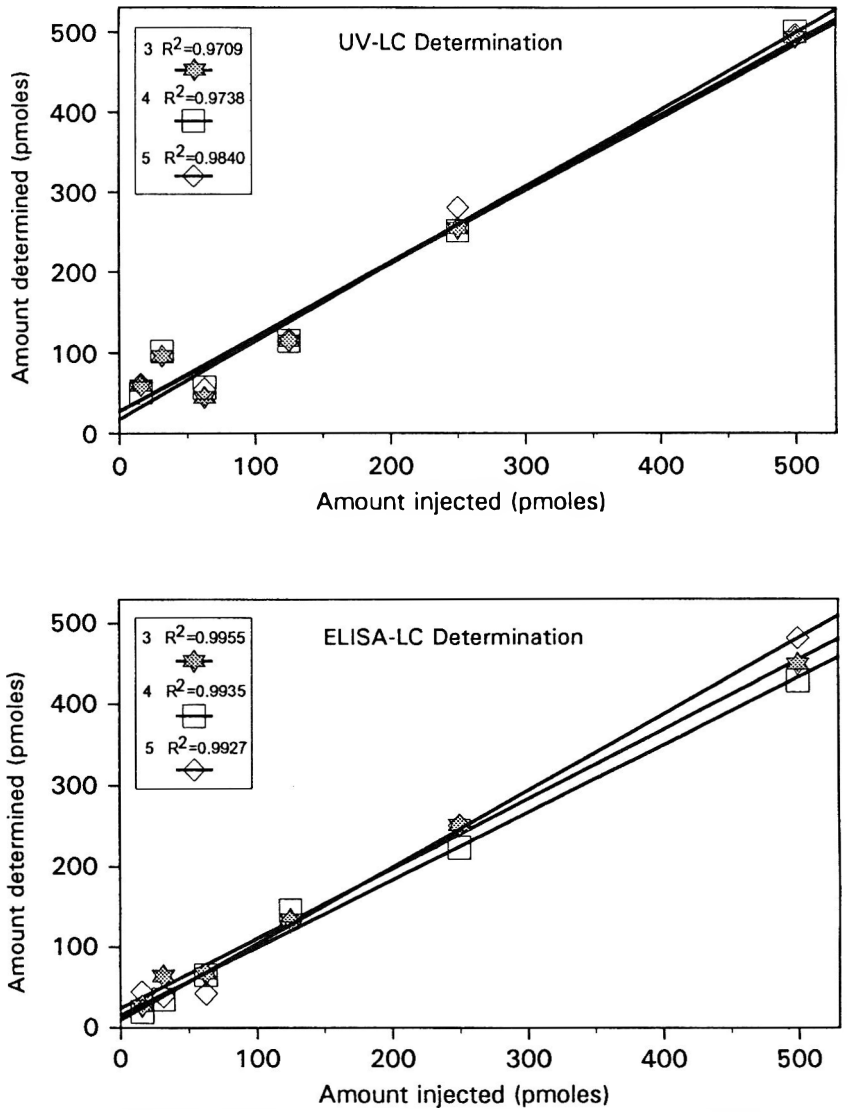


Figure 6. Comparison of quantitative detection by UV–LC and ELISA–LC. System B(1) was used to compare UV–LC detection and ELISA–LC detection. Equimolar amounts (15.6 to 500 pmol) were injected onto the LC column. For UV–LC, areas (counts) were transferred into amounts (picomoles). These values were plotted against amounts injected and R^2 values were determined. For ELISA–LC, the amounts were determined and compared also with the amounts injected onto LC, and R^2 values were determined. Compounds: 3, 2,4-dinitrophenol; 4, 3-methyl-4-nitrophenol; 5, 2-chloro-4-nitrophenol.

here for spectral systems. We feel that a future trend will include integration of analytical technologies, with immunochemical cleanup being a common procedure prior to mass spectral analysis and immunoassays increasingly used as highly sensitive and selective detector systems for chromatographic analysis. Immunoassays long have been used as detectors for chromatographic systems (9, 10). Because of the complexities of integrating the 2 technologies, however, immunoassay detectors have not been widely used. The increasing simplicity of non-radiochemical immunoassays and advances in LC, particularly microbore LC, make the integration of LC and immunoassay increasingly attractive. By designing the analysis to take advantage of the strengths of both technologies, analyses may be optimized. For example, the separation shown in Figure 3 could be optimized by shortening the retention time between peaks 4 and 5. This change would require a change from an isocratic run to a gradient system, which would complicate the immunochemical detection unless the organic cosolvent is removed or volumes are small. Even though most immunoassays tolerate at least 10% of solvent, standardization is difficult with assays sensitive to solvents. Another factor to consider is the eluate pH, which can be adjusted by addition of base, as was done with System A. Another case investigated was avoidance of acid in the mobile phase [*see* Table 2, Systems B (1) and B (2)].

A combination of technologies offers various advantages depending on the configuration of the system. Highly specific immunoassays can be considered as confirmation systems for analyses based on a compound's retention time on LC. With single compounds, the active fraction is easily collected for analysis by one or even a battery of immunoassays. If quantification by immunoassay matches that by a chromatographic

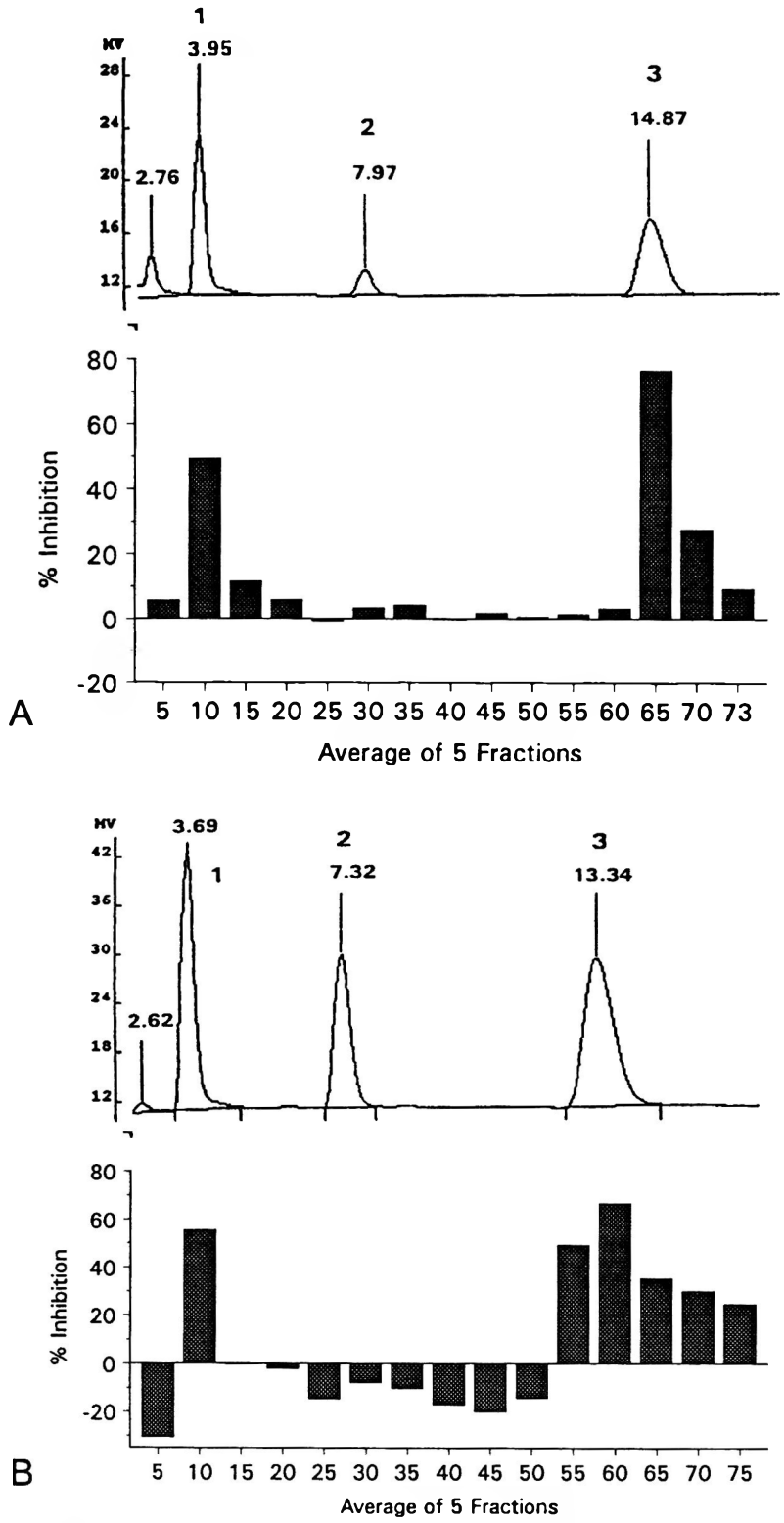


Figure 7. ELISA-LC as a confirmation method. A mixture of 2-amino-4-nitrophenol (A, 250 pmol; B, 1000 pmol), 2-methylphenol (A, 250 pmol; B, 3000 pmol), and 2-chloro-4-nitrophenol (A, 250 pmol; B, 1000 pmol) was injected onto System C and detected by UV-LC (upper parts of A and B, respectively). 2-Methylphenol does not cross-react with the antiserum used; therefore, no immunoreactive material is seen in ELISA-LC (lower parts in A and B, respectively), even when an amount as high as 3000 pmol is present in the sample (B). 2-Chloro-4-nitrophenol on this run was injected at levels that are beyond the linear detection range for the ELISA. Because the ELISA data are expressed as percent inhibition rather than analyte concentration, the ELISA peak appears to tail. Eluate collection with fraction collector for ELISA-LC was started 2.5 min later than that for UV-LC. Peaks at 2.76 (A), and at 2.62 (B) min are solvent peaks; peak 1, 2-amino-4-nitrophenol; peak 2, 2-methylphenol; and peak 3, 2-chloro-4-nitrophenol.

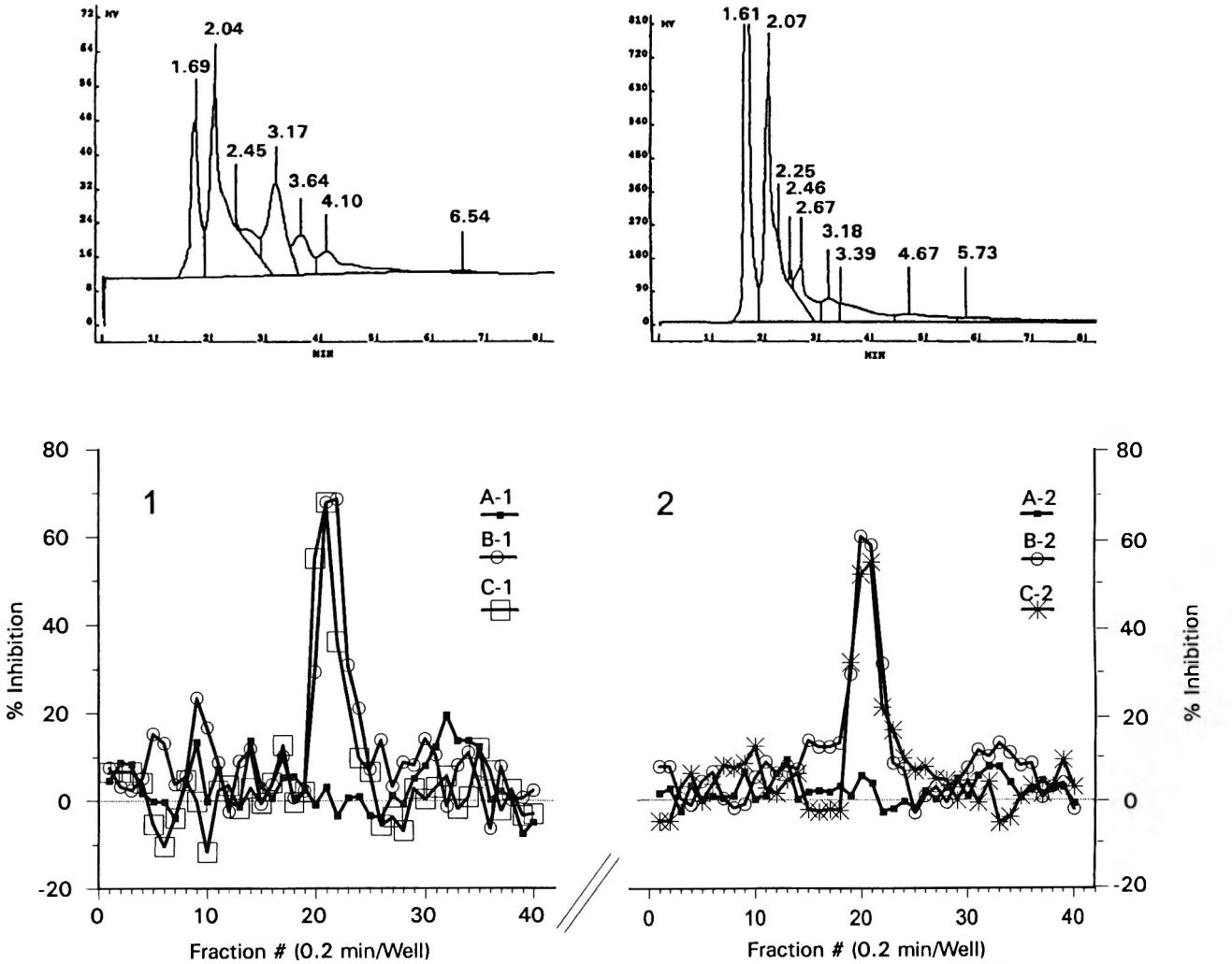


Figure 8. Immunoassay as a highly selective detector for LC separation. The UV-LC tracings are from a 1-mm microbore LC column (System C). (Top) Tracings give the UV profile of a sample of 2-amino-4-nitrophenol (2.4 ng) in soil extract (left) or in plant extract (right). The UV tracing of this compound in methanol shows a retention time of 4.1 min. (Bottom) Tracings show the ELISA response. A-1 shows the detection of the soil extract alone with ELISA-LC. The ELISA response for both chromatograms were identical whether the 2-amino-4-nitrophenol was injected in methanol or in soil extract (B-1 or C-1, respectively). (1 Left) A-1, soil extract; B-1, soil extract spiked with 2-amino-4-nitrophenol; C-1, 2-amino-4-nitrophenol in methanol. (2 Right) A-2, plant extract; B-2, plant extract spiked with 2-amino-4-nitrophenol; C-2, 2-amino-4-nitrophenol in methanol.

detector and if the unknown shows an immunoassay response curve parallel to the standard, there is strong evidence for the assigned structure.

For most environmental needs, immunoassays offer adequate sensitivity when performed directly on crude samples after a single partition or solid-phase purification. However, when high sensitivity is needed, immunoassays can be used as post-column detectors for LC systems. For example, large sample volumes first can be concentrated on a reversed-phase system with low levels of organic cosolvent and then eluted with increasingly powerful solvents. The LC acts both to concentrate the sample and to remove materials that may interfere with the immunoassay. If a standard 4.6 mm column is used, the fractions can be reduced over an order of magnitude in volume for greater sensitivity. Such reduction in volume removes solvents that may interfere with the assay and allows more material to be added. If an appropriate trap solvent such as propane 1,2-diol is used, the sample can be reduced even to dryness and the immunoassay can be run in the same plate or tube used for sample collection. Under these conditions, integrated LC–immunoassays can lead to a 10- to 100-fold increase in assay sensitivity over that with the ELISA alone.

Several techniques are available for collecting samples for post-column immunoassay. The approach that yields the greatest accuracy by minimizing potential errors in measuring volumes and requiring the fewest numbers of assays to be run involves collecting the entire volume of the desired peak as a single sample. Most modern fraction collectors can be integrated with LC detectors to initiate collection as a peak begins to appear and to terminate collection as the peak ends. If the sample is not sufficiently concentrated to be detected spectrally, it can be collected on the basis of retention time.

As a prelude to subsequent projects involving on-line immunoassay, we used mainly off-line collection methods where numerous fractions were collected in tubes or 96-well plates prior to immunoassay. This method is much more labor intensive and slightly less accurate and sensitive. However, it allows one to determine if the immunoreactive material coelutes with the UV peak, providing additional evidence in support of the identity of the unknown.

Immunoassays often are thought of as single-analyte methods, and many laboratories have developed assays that are extraordinarily specific. However, with careful hapten design, methods that can detect a class as a whole or parent molecule plus metabolites can be developed. These assays are very powerful for screening, but compound identification must depend on subsequent steps, including independent methods such as GLC or GLC/MS, an immunoassay based on a specific antibody, or a battery of immunoassays of varying specificity with detection based on pattern recognition of an analogous mathematical approach. This study illustrates that a class-specific immunoassay can be used with LC to provide both quantitative and qualitative information. The nitrophenol assay detects a variety of similar materials present as biomarkers of exposure to a variety of organophosphorus pesticides or as biomarkers or environmental markers of the presence of numerous nitroaromatics. As shown in this study, the ELISA–LC data compare very favorably with the UV–LC data, even when several compounds are present in a single run. The ELISA as a post-column detector is more sensitive than UV even without sample concentration, and of course, it can be applied to materials lacking UV chromophores or other groups easily detected by LC detectors. The technology is applicable to standard LC systems. However, the enhanced resolution and less waste generation made possible by microbore systems probably will drive the field in this direction. This trend makes immunoassay even more attractive because it takes advantage of its great sensitivity as a detector and the reduced organic solvent allows samples to be run directly. With these low volumes, the development of on-line immunochemical detectors for LC becomes increasingly attractive and soon should evolve as a technology integrating these 2 systems.

Acknowledgments

Thanks to D.A. Jones of the Facility for Advanced Instrumentation, University of California, for the use of the LC (Varian) and for technical advice. This work was funded by EPA Cooperative Research Contract CR 814709-01, NIEHS Superfund Grant ES04699-03, and the University of California Toxic Substance Research and Teaching Program. P.M. Krämer is supported by a fellowship from *Deutsche Forschungsgemeinschaft* (Germany). Adam S. Harris in this laboratory provided useful discussion.

References

(1) Hammock, B.D., & Mumma, R.O. (1980) *Recent Advances in Pesticide Analytical Methodology*, J. Harvey, Jr, & G. Zweig (Eds) American Chemical Society, Washington, DC, pp. 321–352

(2) Jung, F., Gee, S.J., Harrison, R.O., Goodrow, M.H., Karu, A.E., Braun, A.L., Li, Q.X., & Hammock, B.D. (1989) *Pestic. Sci.* **26**, 303–317

(3) Muldoon, M.T., Fries, G.F., & Nelson, J.O. (1993) *J. Agric. Food Chem.* **41**, 322–328

(4) Dürsch, I., & Meyer, H.H.D. (1992) *Food Agric. Immunol.* **4**, 211–218

(5) de Frutos, M., & Regnier, F.E. (1993) *Anal. Chem.* **65**, 17A–25A

(6) Li, Q.X., Zhao, M.S., Gee, S.J., Kurth, M., Seiber, J.N., & Hammock, B.D. (1991) *J. Agric. Food Chem.* **39**, 1685–1692

(7) Lucas, A.D., Jones, A.D., Goodrow, M.H., Saiz, S.G., Blewett, C., Seiber, J.N., & Hammock, B.D. (1993) *Chem. Res. Toxicol.* **6**, 107–116

(8) Harris, A.S., Kraemer, P.M., & Hammock, B.D. (1992) Poster presented at the 203rd American Chemical Society National Meeting, San Francisco, CA, April 5–10

(9) Langone, J.J., Van Vunakis, H., & Bachur, N.R. (1975) *Biochem. Med.* **12**, 283–289

(10) Riceberg, L.J., & Van Vunakis, H. (1978) *J. Pharm. Exp. Ther.* **206**, 158–166

RESIDUES AND TRACE ELEMENTS

Graphite Furnace Atomic Absorption Method for the Determination of Lead in Sugars and Syrups

NANCY J. MILLER-IHLI
U.S. Department of Agriculture, BHNRC, Nutrient Composition Laboratory, Bldg 161 BARC-East, Beltsville, MD 20705

A method was developed for the determination of lead in sugars and syrups. Samples are wet-ashed with a nitric acid–hydrogen peroxide procedure and analyzed by graphite furnace atomic absorption spectrometry. The method involves the use of magnesium nitrate as a matrix modifier, air ashing, platform atomization, and quantitation by peak area measurements with direct calibration against aqueous standards. The instrumental detection limit (based on 3.29σ) was 10 pg, or 0.5 μg/L for a 20 μL injection, corresponding to a method detection limit of 3.3 ng/g sugar. The characteristic mass was approximately 12 pg. This method was validated by analyzing sucrose and high-fructose corn syrup samples spiked with known quantities of lead. The average recovery was 101 ± 6%.

Overall exposure to lead is a public health concern. There has been renewed concern about the adverse health effects in individuals exposed to lead levels previously thought to be safe (1, 2). In the United States, the Centers for Disease Control recently lowered the concentration of lead in blood considered to be harmful in young children from 25 μg/dL to 10 μg/dL (3). Because food is a potential source of lead, there is an increasing interest in lowering the acceptable lead limits, particularly for foods that are frequently consumed. One objective of the Committee on Food Chemicals Codex (FCC) is to reduce lead and other heavy metal limits to the lowest extent feasible. In setting acceptable limits for FCC substances, the Committee considers the amount consumed, the feasibility of manufacturing a product within these limits, and the availability of analytical methods to ensure compliance. The current limits for lead in sugars are 0.1 ppm for dextrose and fructose and 0.5 ppm for glucose syrup, sucrose, and high-fructose corn syrup (4).

Graphite furnace atomic absorption spectrometry (GFAAS) is a highly sensitive technique that can be used to determine lead at sub-part-per-billion concentrations. This analytical technique is used routinely in the Nutrient Composition Laboratory for the determination of very low concentrations of elements in foods and biological fluids (5, 6). This paper describes an optimized method for the routine determination of low levels of lead in sugars and syrups. Lead can be quantitated easily by this method at about the 20 ng/g level, which is significantly lower than the FCC limits. The most recent FCC method (4) is also a GFAAS method but uses antiquated technology and does not provide reliable performance because it employs in situ tungsten coating of the graphite tubes. A collaborative study of that method showed poor performance with low average recoveries (86%) and poor average within-laboratory (26%) and between-laboratories (42%) precisions (Elkins, E., personal communication regarding 1992 NFPA/ILSI interlaboratory comparison).

The method described herein was developed as an alternative to the FCC method and was designed for use with a wide range of modern GFAAS instruments. It uses platform atomization, oxygen ashing, peak area measurements, and calibration against aqueous standards. Zeeman background correction was used to ensure adequate compensation for background from complex sample matrixes, but deuterium background correction should also provide adequate background correction. No time-consuming matrix matching of standards or method of standard additions was required. The method was developed by starting with a basic wet ash digestion procedure. Next, furnace atomization conditions were optimized, and calibration range studies were performed. Finally, the method was validated. Analytical figures of merit for the method are presented and validation data for method accuracy are reviewed.

Experimental

Apparatus

A Perkin-Elmer Model 5100PC with Zeeman background correction was used for this study (Perkin-Elmer Corp., Norwalk, CT). The spectrometer was equipped with an AS-60 autosampler and an HGA-600 furnace. An electrodeless discharge lamp was operated at 10 W, and all data were taken at the 283.3 nm line by using a 0.7 nm slit. All analyses were done with solid, pyrolytic graphite-forked platforms inserted in pyrolytically coated graphite tubes (Perkin-Elmer part B050-5057). A 2% HNO_3 rinse was used for the autosampler to avoid carryover from one injection to the next. Breathing-quality air

Received June 14, 1993. Accepted by JS September 24, 1993.

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

was hooked up as the alternative gas for the furnace controller to facilitate oxygen ashing of samples. Argon was used as the normal purge gas.

Reagents

Care was taken to avoid sample contamination by the reagents. Nitric acid used to prepare samples and standards was sub-boiling distilled (Seastar, Seattle, WA) and was certified as containing no more than 0.04 μg lead/L. Ultrapure magnesium nitrate [$Mg(NO_3)_2 \cdot 6H_2O$; Johnson Matthey, Materials Technology, UK] was used to prepare a 20% solution that was diluted 1:10 prior to use as a matrix modifier. A cleaning solution for glassware and plasticware consisted of 5% sub-boiling distilled hydrochloric acid (Seastar, Seattle, WA) and 5% sub-boiling distilled nitric acid. Hydrogen peroxide (50%) (Fisher, Fair Lawn, NJ) was used for the wet ash digestion.

Contamination Control

Samples were prepared in a class 100 clean room, and the spectrometer was located under laminar flow. To avoid contamination, Teflon autosampler cups (Perkin-Elmer part B008-7600), class A volumetric glassware, and polyethylene test tubes used for sample digestion were cleaned with a mixture of 5% sub-boiling distilled nitric acid and 5% sub-boiling distilled hydrochloric acid and rinsed thoroughly with 18 MΩ water prior to use. Disposable micropipet tips (Brinkmann Instruments Inc., Westbury, NY) for Eppendorf pipets were tested to ensure that they were free of lead contamination.

Wet Ash Digestion

Sample dissolution was performed in a clean hood. For crystalline sucrose samples, a more representative subsample may be obtained from a sucrose solution prepared by weighing equivalent amounts of sucrose and 18 MΩ water and mixing them. A minimum of 5 g each was used, and the sucrose was completely dissolved. When working with high-fructose corn syrups, ultrasonic mixing and/or vortex mixing was used prior to weighing samples for analysis to ensure that a representative subsample was used. Approximately 1.5 g sugar was weighed into a polyethylene test tube (Sarstedt, Princeton, NJ), and the weight was recorded to the nearest milligram. For sucrose solutions, 3 g of the solution was used to provide the specified 1.5 g sucrose for analysis. A solution reagent blank was prepared with 1.5 g 18 MΩ water. Next, 0.75 mL sub-boiling distilled nitric acid was added to all samples. Plastic test tubes were heated in a water bath. Samples were heated at 90°–95°C with a ramp to the final temperature to avoid spattering. A 200 mL beaker containing approximately 125 mL water was used as the water bath for 6–8 polypropylene test tubes, and the temperature was monitored by using a dummy sample. Samples were heated until all brown vapors had dissipated and samples no longer had a rust-colored tint. This step typically took 20–30 min. Samples were cooled, 0.5 mL 50% hydrogen peroxide was added dropwise to each sample, and samples were heated at 90°–95°C for approximately 5 min and then allowed to cool. A second 0.5 mL portion of 50% hydrogen peroxide was added dropwise to each sample, and the samples were heated at 90°–95°C for an additional 10 min or until clear. Once cooled, the samples were diluted to a final volume of 10 mL and were ready for direct analysis.

Standards

A 10 000 mg/L single-element lead stock standard solution (NIST SRM 3128, Gaithersburg, MD) was used to prepare an intermediate 10 mg/L standard weekly. All standards were prepared in 5% sub-boiling distilled nitric acid. A 1.0 mg/L standard was prepared daily, and working calibration standards of 10.0, 25.0, 50.0, and 100.0 μg/L were prepared from this. All standards were stored in acid-cleaned polyethylene bottles. In some instances, the autosampler was used to dilute standards. In those instances, the amount of standard pipetted was not less than 3 μL.

Results and Discussion

GFAAS Program Optimization

Oxygen ashing (7) can be used during the char step to avoid buildup of residue and to stabilize the Pb to higher temperatures. Thus, it acts as a matrix modifier (8). An additional benefit of oxygen ashing is that incomplete sample digestion is not a problem because samples are ashed in the furnace. Oxygen ashing even facilitates direct analyses by making it possible to avoid sample digestion altogether (9).

Charring and atomization temperature studies were performed to identify optimum GFAAS conditions. The optimum charring temperature is the maximum temperature that may be used without loss of analyte prior to atomization, and the optimum atomization temperature is the lowest temperature that can be used and still provide an absorbance in the plateau region of the curve. Atomization temperature studies were conducted at 1700° to 1900°C. The optimum atomization temperature was 1800°C. A charring study also was conducted with temperatures ranging from 650° to 850°C. Peak area measurements and background measurements were virtually identical for a 25 μg/L standard analyzed at various char temperatures, but analysis of a sugar digest showed a significant decrease (approximately by a factor of 3) in the background at 750°C compared with 650°C with no loss of analyte. As a result, a 750°C char was used. Although higher char temperatures can provide lower background signals, oxygen ashing required the use of lower temperatures to avoid unnecessary oxidation of the graphite tube. Also, a 850°C char temperature significantly shortened tube lifetime.

Table 1 shows the optimized GFAAS program used for this work. The 60 s cooldown prior to atomization ensures that the air used for oxygen ashing is cleared from the furnace. This step prevents unnecessary oxidation of the graphite tube at high temperatures. An 8 s atomization time was selected to make the method applicable to a wide range of instruments and various matrix modifiers. The optimum integration time for the system described would have been 5 s because the analyte signal had returned to baseline by that time. Sample volumes were 20 μL

Table 1. GFAAS conditions

Step	Temp., °C	Ramp, s	Hold, s	Gas flow, mL/min	Read
Dry	200	20	30	Ar, 300	
Char	750	40	40	Air, 300	
Cool down	20	1	60	Ar, 300	
Atomize	1800	0	8	Ar, stop flow	8 s
Clean	2600	1	7	Ar, 300	
Cool down	20	1	5	Ar, 300	

for all analyses, and 5 µL 2% modifier solution was used to provide 0.06 mg magnesium nitrate.

Method Optimization

Next, the calibration working range was optimized. The concentration range considered was 10.0–100.0 µg/L because data analysis will not be constrained to a linear least-squares fit. Standards (20 µL volumes) were analyzed and typical reagent-blank-corrected, integrated absorbance signals for various standard concentrations were 10.0 µg/L, 0.067 abs-sec; 25.0 µg/L, 0.166 abs-sec; 50.0 µg/L, 0.311 abs-sec; and 100.0 µg/L, 0.588 abs-sec. A calibration curve appears in Figure 1. The nonlinear rational calibration function provided in the Perkin-Elmer software (10) gave a correlation coefficient of 0.99993. The relative concentration precision (based on the precision for 5 measurements) was better than 2% over the whole concentration range. Throughout the study, characteristic mass (m_o) values and detection limits were monitored. The m_o, defined as the amount of analyte required to produce an integrated absorbance measurement of 0.0044, was typically 12–13 pg, based on a 20 µL injection of the 25 µg/L standard. This value of m_o is in good agreement with the manufacturer's specification for lead (12.0 pg). A well-characterized

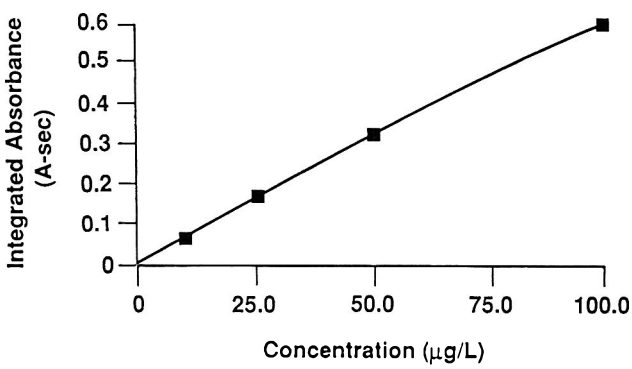


Figure 1. Calibration curve for lead (20 µL sample volumes). Nonlinear rational fit: correlation coefficient, 0.99993; slope, 0.0068.

Table 2. Analytical results for NIST water SRMs

Material	Concentration determination, ng/g	Certified concentration, ng/g
SRM 1643 (direct)	20.6 ± 0.4	20 ± 1
(×2 diln)	19.7 ± 0.6	
SRM 1643c (direct)	34.9 ± 0.9	34.7 ± 0.9
(×2 diln)	33.6 ± 0.6	

blank was used to compute the standard deviation (σ). The calculation of the detection limit was based on 3.29σ. The average detection limit was 10 pg or 0.5 µg/L for a 20 µL injection.

Method Validation

Prior to analysis of digested sugar samples, NIST SRM 1643 and SRM 1643c acidified water samples were analyzed to ensure analytical accuracy. The results appear in Table 2. Both acidified water samples were analyzed before and after dilution (1:2). This ensured accuracy over the 10–35 µg/L range. Next, spiked sucrose and fructose samples were wet-ashed and analyzed. Typical absorbance profiles for a lead standard and a sugar digest appear in Figure 2. Although the peak shapes are not identical, accurate results can be obtained by using peak area measurements for quantitation. The analytical results appear in Table 3. The unspiked sucrose sample E contained a very low level of lead. As a result, the method was modified to use larger quantities of the sucrose suspension for analysis, as well as smaller final volumes. This modification resulted in solutions with lead concentrations in excess of the detection limit. Both sucrose and fructose results were blank-corrected for the amount of lead in the unspiked material. The

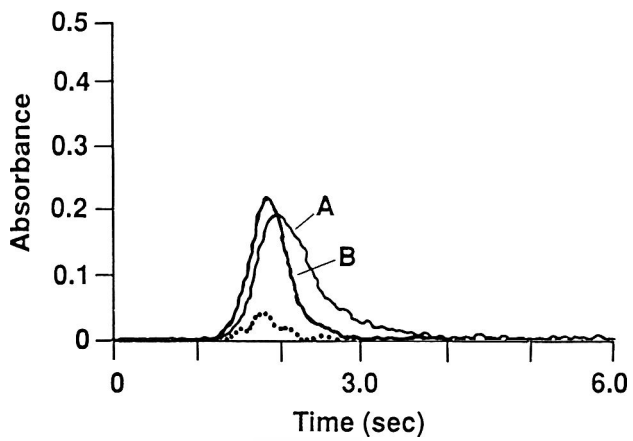


Figure 2. Typical atomization profiles for a lead standard and a sugar digest (first 6 s, 20 µL sample volumes). Solid lines represent the background corrected atomic absorption signals for (A) 25 µg/L Pb standards and (B) sugar A digest. Broken line represents background absorbance for the sugar sample. The calculated concentration for sugar A was 106 ng/g, compared with the known spiked concentration of 105 ng/g.

Table 3. Analytical results for spiked sugar samples

Material	Concentration determined	ng/g[a]	Added spike, ng/g
Unspiked Sucrose E		0	
5 g samples	0.57		
7 g samples	0.98		
Average	0.8		
Sucrose A	99.0 ± 2.2 [98.2]	105	94
Sucrose B	482 ± 16 [481.2]	508	95
Sucrose C	53.7 ± 0.1 [52.9]	53.7	99
Sucrose D	202 ± 3 [201.2]	214	94
Sucrose F	35.0 ± 0.1 [34.2]	32.2	106
Unspiked fructose I	18.8 ± 1.0		
Fructose G	483 ± 13 [464.2]	454	102
Fructose H	61.9 ± 4.8 [43.1]	44.8	96
Fructose J	51.8 ± 1.3 [33.0]	31.8	104
Fructose K	121 ± 4 [102.2]	93.3	109
Fructose M	182 ± 15 [163.2]	151	108

[a] Values are means ± standard deviations (n = 5). Values in brackets are blank-corrected.

blank-corrected data appear in brackets. Recoveries ranged from 94 to 109%, (average recovery, 101%). The overall average accuracy was 101 ± 6%. These data suggest that lead may be determined accurately in the 15–500 ng/g concentration range. To verify the accuracy over the whole calibration range, some of the digests were diluted 1:10 and results were compared with those from analysis of the straight digests. Results were not statistically significantly different.

Precisions, expressed as percent relative standard deviation (% RSD), were 0.2–3.3% RSD for the spiked sucrose samples and 2.5–8.2% RSD for the spiked fructose samples. The average precision was approximately 4% RSD for n = 3 digests with triplicate instrumental readings. The spiked samples were prepared for use in a collaborative study of this method, and on the basis of these data, it was concluded that the spiked samples could be used to assess the performance of this method in collaborating laboratories (11).

Matrix Modifiers

In addition to magnesium nitrate with oxygen ashing, 2 other commonly used modifiers were evaluated by using the specified furnace program. One was a combination phosphate and magnesium nitrate modifier [0.2 mg PO_4^{2-} and 0.01 mg $Mg(NO_3)_2$], and the second was palladium (0.008 mg Pd). The use of magnesium nitrate without oxygen ashing also was studied. Although furnace conditions could be optimized for each modifier to obtain optimum performance, the same program was used because with oxygen ashing, only the 750°C char could be used to avoid tube degradation, and the differences in analytical performance were slight when the atomization temperature was varied from 1700° to 1900°C. Table 4 contains data obtained with the various modifiers. All the modifiers provided reasonable results. Characteristic mass values (m_o) were all in good agreement with the manufacturer's recommended value of 12 pg. The data for the sugar A digest showed good recoveries compared with the known spiked concentration, and the data for NIST SRM 1643 showed good agreement compared with the reference concentration (20 ± 1 ng/g), regardless of the choice of modifier as long as peak area measurements (abs-sec) were used for quantitation.

The profiles of absorbance vs time and the detailed data provided some interesting insights. Background absorbances were highest for the combination phosphate and magnesium nitrate modifier and significantly lower for the palladium and the magnesium nitrate modifiers used with oxygen ashing. The peak shapes for the sugar digest indicate that the palladium and magnesium nitrate modifiers used with oxygen ashing provided somewhat broader peaks, with peak maxima occurring 0.2–0.3 s later than peak maxima when magnesium nitrate with no oxygen ashing was used or when the phosphate and magnesium nitrate combination modifier was used with oxygen ashing. Although peak shapes were slightly different with the various modifiers, all modifiers clearly provided accurate results as long as peak area measurements were used for quantitation.

Table 5 contains peak height data for the same study of the various modifiers. As expected, peak height data generally provide poorer analytical results. Current GFAAS practice, as reported by Slavin (12) when he described the stabilized-temperature platform furnace (STPF) concept, recommends the use of peak area measurements for quantitation. Peak height measurements are more prone to interference effects seen in the nonisothermal conditions of the standard Massmann-style furnace. The peak height data suggest that sensitivity varies greatly with the choice of modifier, and yet, characteristic mass values based on peak area measurements indicate that all modifiers perform equally well. If a modifier is selected on the basis

Table 4. Comparison of matrix modifiers: results based on peak area measurements

Matrix modifier	25 µg/L, A-sec	m_o, pg	Sugar A, A-sec	Sugar A, ng/g[a]	SRM 1643 A-sec	SRM 1643, ng/g[b]
0.06 mg $Mg(NO_3)_2$	0.184	11.9	0.121	109	0.143	19.4
0.06 mg $Mg(NO_3)_2$, no O_2 ashing	0.190	11.6	0.119	104	0.143	18.8
0.2 mg PO_4^{2-} + 0.01 mg $Mg(NO_3)_2$	0.194	11.3	0.113	96.8	0.143	18.4
0.008 mg Pd	0.177	12.4	0.102	95.8	0.127	17.9

[a] Reference value, 105 ng/g.
[b] Reference value, 20 ± 1 ng/g.

Table 5. Comparison of matrix modifiers: results based on peak height measurements

Matrix modifier	25 µg/L, Abs	Sugar A, Abs	Sugar A,[a] ng/g	SRM 1643, Abs	SRM 1643,[b] ng/g
0.06 mg $Mg(NO_3)_2$	0.175	0.169	161	0.148	21.1
0.06 mg $Mg(NO_3)_2$, no O_2 ashing	0.199	0.173	145	0.196	24.6
0.2 mg PO_4 + 0.01 mg $Mg(NO_3)_2$	0.304	0.203	111	0.232	19.1
0.008 mg Pd	0.089	0.050	93.3	0.048	13.5

[a] Reference value, 105 ng/g.
[b] Reference value, 20 ± 1 ng/g.

of peak height data, only the combination phosphate and magnesium nitrate modifier might be considered suitable, and yet, this modifier produces a significantly higher background signal compared with those of other modifiers studied. The data in Tables 4 and 5 illustrate why peak area measurements provide more versatility and should be used for quantitation and why peak height measurements often provide significantly biased analytical results.

Conclusions

A method was developed to facilitate the quantitation of lead levels in sugars. The graphite furnace method developed uses oxygen ashing, platform atomization, and quantitation using peak area measurements with aqueous standards. Incomplete sample digestion is not a problem because oxygen ashing is used during the char step. A char temperature of 750°C provided significantly lower background than 650°C. The nonlinear calibration function provided by the instrument allows accurate calibration up to 100 ng/mL, avoiding the limitation of working in the linear range. Accuracy data ranged from 94 to 109%, with an average accuracy of 101%. Although magnesium nitrate was used as the matrix modifier, results indicated that palladium could be used as an alternative and that a combination phosphate and magnesium nitrate modifier also provides good results. This method was developed for widespread use and was recently tested collaboratively by 8 laboratories (11).

Acknowledgment

I thank Jon DeVries for preparing the spiked fructose and sucrose materials and gratefully acknowledge the technical support provided by F. Ella Greene.

References

(1) Baghurst, P.A., McMichael, A.J., Wig, N.R., Vimpani, G.V., Robertson, E.F., Roberts, R.J., & Tong, S.L. (1992) *N. Engl. J. Med.* **327**, 1279
(2) Parsons, P.J., & Slavin, W. (1993) *Spectrochim. Acta* **48B**, 925
(3) Centers for Disease Control (1991) *Preventing Lead Poisoning in Young Children*, U.S. Department of Health and Human Services, Atlanta, GA
(4) *Food Chemicals Codex*, 3rd Supplement to the 3rd Edition, November 1992, pp. 109–169
(5) Miller-Ihli, N.J. (1988) *J. Res. Nat. Bureau Stand.* **93**, 350–354
(6) Miller-Ihli, N.J. (1992) *J. AOAC Int.* **75**, 354–359
(7) Eaton, D.K., & Holcombe, J.A. (1983) *Anal. Chem.* **55**, 946
(8) Hahn, L., Muller-Vogt, G., & Wendl, W. (1993) *J. Anal. At. Spectrom.* **9**, 223–227
(9) Miller-Ihli, N.J. (1993) *At. Spectrosc.* **14**, 85–89
(10) Barnett, W.B. (1984) *Spectrochim. Acta Part B* **39**, 829–836
(11) Miller-Ihli, N.J. *J. AOAC Int.*, in preparation
(12) Slavin, W., Manning, D.C., & Carnrick, G.R. (1981) *At. Spectrosc.* **2**, 137

RESIDUES AND TRACE ELEMENTS

Capillary Gas Chromatographic Determination of Thiabendazole in Citrus and Apple Juices

MITSUO OISHI and KAZUO ONISHI
Tama Branch Laboratory, The Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan
ITSU KANO
The Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan
HIROYUKI NAKAZAWA
National Institute of Public Health, Tokyo, Japan
SHINZO TANABE
Meiji College of Pharmacy, Tokyo, Japan

A rapid and simple procedure for the determination of thiabendazole (TBZ) residue in citrus and apple juices is described. A juice sample is made basic with 2M NaOH and applied to a disposable Extrelut prepacked column. TBZ is eluted with hexane–ethyl acetate (3 + 1) from the column. The eluate is evaporated to dryness under reduced pressure and then dissolved in an internal standard solution. TBZ is monitored without derivatization by capillary gas chromatography with nitrogen–phosphorus detection. The recoveries of TBZ added to fruit juices at 0.05–1.0 μg/g were 90–96%. The limit of detection of the method for TBZ was 0.01 μg/g. The proposed method is rapid, simple, and sensitive and is applicable to the determination of TBZ in commercial fruit juices.

Thiabendazole (TBZ) is widely used as a fungicide on various food crops. In Japan, it is approved for use on citrus fruits and bananas, but it is prohibited for use in fruit juices. Hence, a low level of TBZ in fruit juices must be determined. Analytical methods for the determination of TBZ in fruits include spectrophotometry (1–3), fluorometry (4–6), thin-layer chromatography (7), liquid chromatography (8–13) and gas chromatography (GC) (14–20). However, these methods involve tedious steps such as liquid–liquid extraction and column cleanup to eliminate interfering substances. In addition, gas chromatographic analysis is usually carried out after formation of methyl (14), pentafluorobenzoyl (15), or pentafluorobenzyl derivative (16). To quantify a low level of TBZ, a highly sensitive method is required and losses during derivatization reactions must be avoided. A procedure for determining TBZ residues in citrus and apple juices was developed. The procedure uses a disposable Extrelut prepacked solid-phase extraction column and capillary gas chromatography equipped with a nitrogen–phosphorus detector.

Received May 26, 1993. Accepted by JS September 14, 1993.

Experimental

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5890 equipped with a nitrogen–phosphorus detector (NPD). Operating conditions: injector, 300°C; splitless injection; initial column temperature, 100°C, hold for 1 min, then increase from 100° to 200°C at 20°C/min and from 200° to 250°C at 10°C/min, hold at final temperature for 8 min. hold at post-run temperature of 270°C for 2 min; detector, 300°C. Gas flow rate: helium carrier gas, 0.75 mL/min; nitrogen makeup gas, 30 mL/min; hydrogen, 3 mL/min; and air, 130 mL/min. Injec-

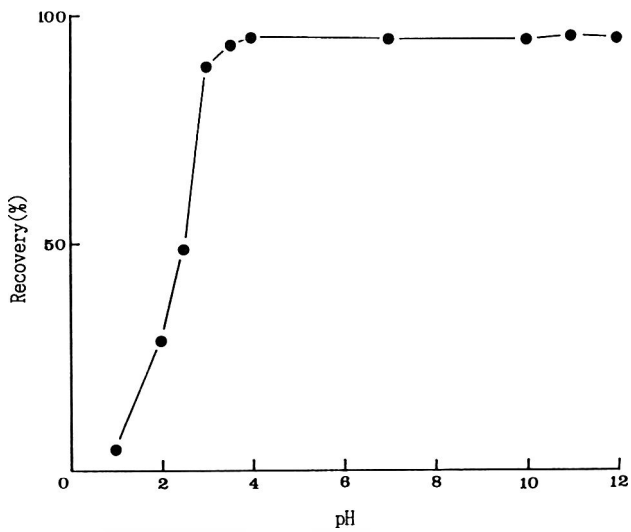


Figure 1. Effect of sample pH on recovery from Extrelut column. Eluting solvent, hexane–ethyl acetate (3 + 1); sample, orange juice fortified with TBZ at 0.5 μg/g.

Table 1. Effect of eluting solvent on recovery of TBZ from Extrelut column

Solvent ratio (hexane–ethyl acetate)	Recovery[a]
0 + 1	97.4
1 + 1	95.5
2 + 1	95.0
3 + 1	93.1
4 + 1	87.3
5 + 1	83.4

[a] Orange juice (20 mL) fortified with TBZ at 0.5 μg/g was applied to the column and eluted with each solvent (100 mL). Results are averages of duplicate determinations.

tion volume, 2 μL. Capillary column: 30 m × 0.25 mm id with 0.25 μm DB-5 (J&W Scientific).

(**b**) *Centrifuge.*—Model M-160-IV (Sakuma Seisakusyo Co., Ltd, Tokyo, Japan).

(**c**) *Rotary evaporator.*—Rotary vacuum evaporator N (Tokyo Rikakikai Co., Ltd, Tokyo, Japan).

Reagents

(**a**) *Ethyl acetate and hexane.*—Pesticide grade (Wako Pure Chemical Industries Ltd, Osaka, Japan).

(**b**) *2M NaOH.*—Dissolve 20 g NaOH (Wako) in 250 mL water.

(**c**) *Phenolphthalein, 0.1%.*—Dissolve 0.1 g phenolphthalein (Wako) in 100 mL ethanol (Wako).

(**d**) *Standard stock solution.*—Accurately weigh 10 mg TBZ (Wako) into a 100 mL volumetric flask and dilute to volume with ethyl acetate to give a solution concentration of 100 μg/mL.

(**e**) *Intermediate standard solution.*—Add 1 mL standard stock solution to a 10 mL volumetric flask and dilute to volume with ethyl acetate to give a final concentration of 10 μg/mL.

(**f**) *Internal standard solution.*—Weigh 10 mg phenazine (Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan) into a 100 mL volumetric flask and dilute to volume with ethyl acetate (stock solution, 100 μg/mL). Dilute 1 mL of the solution to 200 mL with ethyl acetate to obtain a final concentration of 0.5 μg/mL.

(**g**) *Working standard solutions.*—Working standard solutions (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 μg/mL) are prepared by dilution of the intermediate standard solution with the internal standard solution.

(**h**) *Extraction column.*—Extrelut 20, No. 11737 (E. Merck, Darmstadt, Germany), packed with disposable diatomaceous earth.

Preparation of Sample

Weigh 20 g sample into a 25 mL graduated test tube equipped with a glass stopper. Add 1 drop 0.2% phenolphthalein solution and 2M sodium hydroxide solution until the color is developed (pH 8–9). Dilute to 25 mL with water and mix well. Transfer the solution to a 50 mL centrifuge tube equipped with a glass stopper. Centrifuge at 3000 rpm for 10 min. Apply 20 mL supernatant to the Extrelut column. Allow column to stand for 20 min, and then add 100 mL hexane–ethyl acetate (3 + 1, v/v) to the column and collect eluate in a 200 mL round-bottom flask. Evaporate the eluate to dryness on a rotary evaporator under reduced pressure at 45°C. Dissolve the residue in 2 mL internal standard solution.

Results and Discussion

Various methods have been reported for the quantitation of TBZ in fruits. For the efficient extraction of TBZ from acidic samples, such as citrus fruits, with organic solvents, samples have been pretreated with buffer solutions (14, 15, 17) or alkali solutions (20). In this study, we used prepacked Extrelut columns for rapid and simple preparation of samples. Because sample pH seems to be important, TBZ recovery from Extrelut columns was investigated at various pH values. Citrus and apple juices are usually acidic, that is, pH 3–4. At pH 3, TBZ recovery was 89%, and at pH 4, it increased to 95%. High recoveries were attained at pH 4 and over (Figure 1). However, TBZ

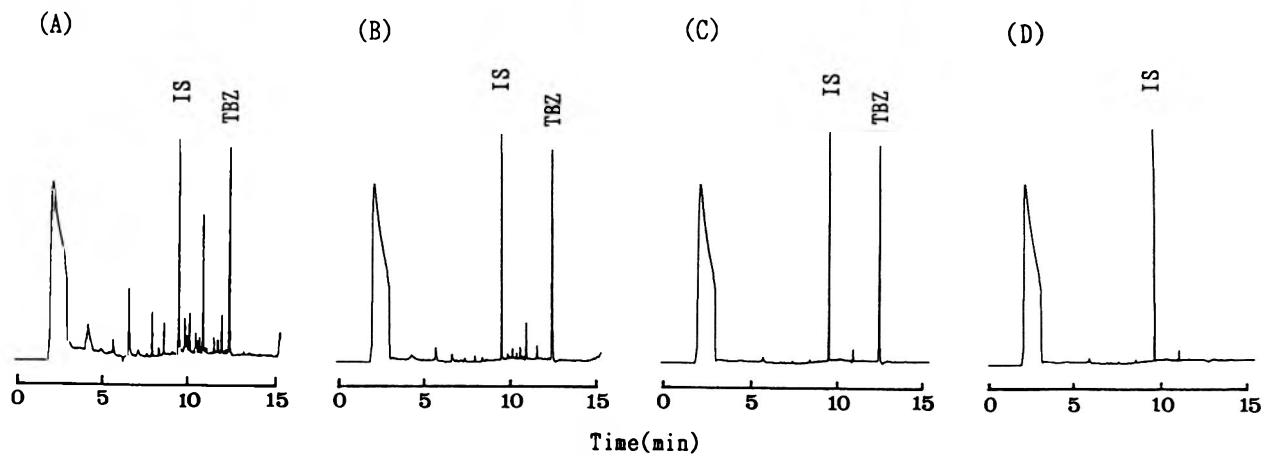


Figure 2. Gas chromatograms of extracts of orange juice fortified with TBZ at 0.5 μg/g and extracted with (A) ethyl acetate, (B) hexane–ethyl acetate (1 + 1), (C) hexane–ethyl acetate (3 + 1), and (D) control sample extracted with hexane–ethyl acetate (3 + 1). IS, internal standard phenazine.

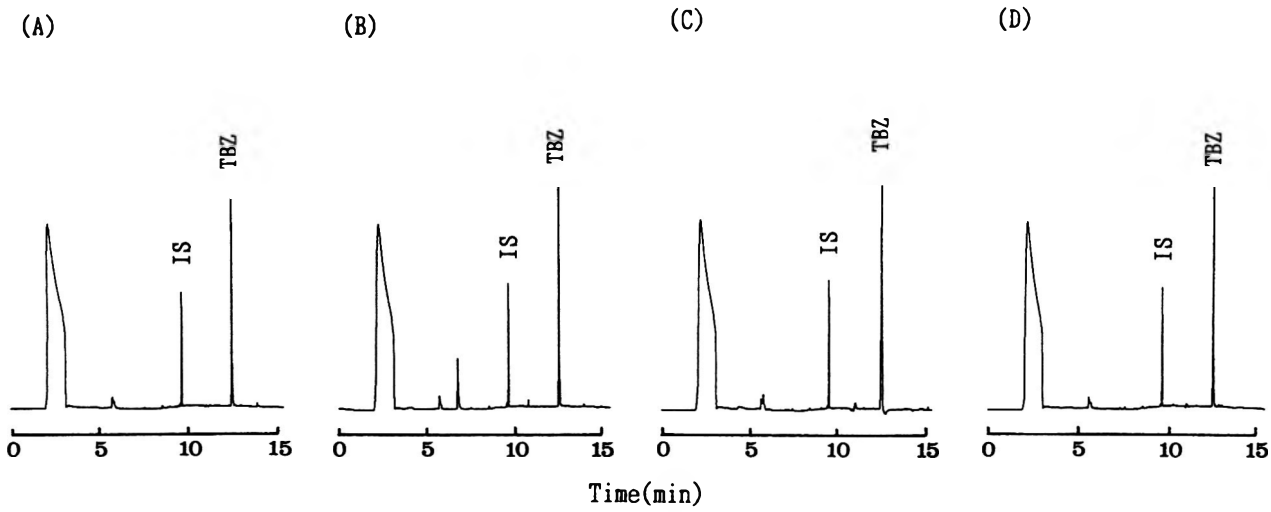


Figure 3. Gas chromatograms of extracts of fruit juices fortified with TBZ at 0.5 μg/g: (A) orange juice; (B) grapefruit juice; (C) lemon juice; (D) apple juice. IS, internal standard phenazine.

recovery from lemon juice was low (82%) without addition of alkali because the pH of the sample was about 2.7. Therefore, all juice samples were adjusted to pH 8–9 with sodium hydroxide solution.

Ethyl acetate has been frequently used as the extraction solvent for TBZ (1, 4, 5, 8, 10–19). However, ethyl acetate was not suitable for elution from the Extrelut column because it also eluted interfering materials from the sample. Therefore, a nonpolar solvent, hexane, was mixed with ethyl acetate at various ratios. Although TBZ recovery decreased with increasing ratios of hexane to ethyl acetate (Table 1), interfering peaks disappeared (Figure 2). Hexane–ethyl acetate (3 + 1) gave the best results in specific elution of TBZ. TBZ was almost completely eluted from the column with 70 mL of this solvent.

Several nitrogen-containing compounds for NPD monitoring were examined as the internal standard (IS) under the GC conditions described earlier. On the basis of peak height and adequate retention time, phenazine proved to be acceptable. Use of an IS is indispensable for accurate determination because the reproducibility of injection with IS (coefficient of variation [CV] = 1.2%; $n = 4$) was better than that without IS (CV = 5.2%; $n = 4$). The chromatograms of TBZ and IS in extracts of orange juice monitored by NPD, flame ionization detector (FID), and flame photometric detector S mode (FPD-S) were compared. TBZ and IS are hardly discriminated by FID because of interfering peaks. The response of TBZ monitored by FPD-S was low. Compared with FID and FPD-S, NPD proved to be suitable for the determination of TBZ because of the specific response to nitrogen compounds.

Table 2. Recovery of TBZ from fortified fruit juices

Sample	Added, μg/g	Found, μg/g	Rec.,[a] %	CV, %
Grapefruit juice	0.05	0.045	90	2.5
	1.0	0.93	93	3.3
Orange juice	0.05	0.045	90	1.9
	1.0	0.92	92	1.9
Lemon juice	0.05	0.045	90	3.2
	1.0	0.95	95	2.0
Apple juice	0.05	0.045	90	3.4
	1.0	0.96	96	0.8

[a] Values are means of 5 trials.

Figure 3 shows typical gas chromatograms of extracts of orange, grapefruit, lemon, and apple juices fortified with TBZ at 0.5 μg/g. TBZ was detected without interference from other peaks.

TBZ recoveries from grapefruit, orange, lemon, and apple juices fortified at 0.05 and 1 μg/g are shown in Table 2. TBZ recoveries from juices fortified at 0.05 μg/g were 90% regardless of the kind of fruit juice. Recoveries from juices fortified at 1 μg/g were more than 92%. CVs were less than 3.4%. These results show satisfactory reproducibility. A linear calibration curve for TBZ at concentrations ranging from 0.05 to 2.0 μg/mL was obtained by the internal standard method. The limit of detection of the method was 0.01 μg/g for TBZ (signal-to-noise ratio = 5). The combination of Extrelut column cleanup and capillary gas chromatography with NPD gives a rapid, simple, and sensitive method for analyzing TBZ in fruit juices. The proposed method can be applied to other processed fruits.

References

(1) Pifarre, A.M., & Vayreda, M.X. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 596–598

(2) Romminger, K., & Hoppe, H. (1976) *Nahrung* **20**, 407–417

(3) Hey, H. (1972) *Z. Lebensm. Unters. Forsch.* **149**, 79–86

(4) Norman, S.M., Fouse, D.C., & Craft, C.C. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 1239–1244
(5) Mihara, M., Konodo, T., & Tanabe, H. (1973) *J. Food Hyg. Soc. Jpn.* **14**, 179–185
(6) Aharonson, N., & Ben-Aziz, A. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 1330–1334
(7) Ottender, H. (1975) *J. Chromatogr.* **109**, 181–187
(8) Tafuri, F., Marucchini, C., Patumi, M., & Businelli, M. (1980) *J. Agric. Food Chem.* **28**, 1150–1153
(9) Isshiki, K., Tsumura, S., & Watanabe, T. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 747–749
(10) Maeda, M., & Tsuji, A. (1976) *J. Chromatogr.* **120**, 449–455
(11) Kitada, Y., Tamase, K., Inoue, M., Sasaki, M., & Tanigawa, K. (1982) *J. Food Hyg. Soc. Jpn.* **23**, 21–24
(12) Kitada, Y., Sasaki, M., & Tanigawa, K. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1302–1304
(13) Nakazato, M., Saito, K., Kikuchi, Y., Ibe, A., Fujinuma, K., & Nishima, T. (1988) *Eisei Kagaku* **34**, 401–407
(14) Tanaka, A., & Fujimoto, Y. (1976) *J. Chromatogr.* **117**, 149–160
(15) Nose, N., Kobayashi, S., Tanaka, A., Hirose, A., & Watanabe, A. (1977) *J. Chromatogr.* **130**, 410–413
(16) Tjan, G.H., & Jansen, J.T.A. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 769–773
(17) Isshiki, K., Tsumura, S., & Watanabe, T. (1980) *Nippon Nogei Kagaku Kaishi* **54**, 1045–1050
(18) Isshiki, K., Tsumura, S., & Watanabe, T. (1982) *Agric. Biol. Chem.* **46**, 993–999
(19) Bardalaye, C.P., & Wheeler, B.W. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 114–116
(20) Lafuente, M.T., Tadeo, J.L., & Tuset, J.J. (1987) *J. Chromatogr. Sci.* **25**, 84–87

RESIDUES AND TRACE ELEMENTS

Analysis of Methyl Isothiocyanate in Wine by Gas Chromatography with Dual Detection

ISAO SAITO, SADAJI YAMADA, HARUMI OSHIMA, YOSHITOMO IKAI, HISAO OKA, and JUNKO HAYAKAWA
Aichi Prefectural Institute of Public Health, 7-6, Nagare, Tsuji-machi, Kita-ku, Nagoya 462 Japan

Methyl isothiocyanate (MITC) was extracted from a 5 mL wine sample with 2 mL dichloromethane, and the extract was washed with 5% sodium chloride solution. MITC was determined by gas chromatography with a dual nitrogen–phosphorus detector (NPD) and a flame photometric detector (FPD, S mode) (DB-210 column, 30 m × 0.53 mm id, column temperature, 70°C). The limit of detection and quantitation of MITC with NPD–GC were 0.003 and 0.01 ppm, respectively. Average recoveries of MITC added to wine at 1 and 0.1 ppm were over 95%. This method is simple and rapid for routine analysis of MITC in wine.

Methyl isothiocyanate (MITC) is used as a soil fumigant for nematodes, fungi, and other diseases in vegetables, fruits, etc. MITC residues in crops were regulated to be less than 0.05 ppm for fruits, 1.0 ppm for vegetables and tea, and 0.5 ppm for potatoes by the Environment Agency in Japan.

Recently, MITC was detected in imported Italian wine as an illegal antifermentative substance (1, 2).

MITC in wine is usually extracted with an organic solvent. The extract is concentrated under reduced pressure or cleaned up by distillation and determined by gas chromatography (GC) or GC/MS (mass spectrometry) (3–6). However, concentration of extract under reduced pressure results in poor recovery of MITC because MITC is volatile (7). Use of different GC detectors like the flame photometric detector (FPD) and the nitrogen–phosphorus detector (NPD) is sufficient for peak identification. The method is based on extraction of MITC with dichloromethane without extract concentration and determination of MITC by GC with dual detection.

Received March 12, 1993. Accepted by AP September 3, 1993.

METHOD

Reagents

All reagents used were analytical grade (Wako Pure Chemical Industries, Osaka, Japan). Deionized, distilled water was used throughout.

(a) *Methyl isothiocyanate solution.*—Dissolve in methanol to obtain a 1 mg/mL solution and dilute with dichloromethane for working solutions.

(b) *Sodium chloride solution, 5%.*—Dissolve 50 g sodium chloride in distilled water and bring up to 1 L with water. Wash the solution in n-hexane before use.

(c) *Anhydrous sodium sulfate.*—Extra pure grade.

(d) *Extracting solvent.*—Dichloromethane containing isobutyl isothiocyanate at 0.5 μg/mL as internal standard.

(e) *Test tubes.*—Graduated glass test tube (10 mL) with stopper and round bottom.

Apparatus

(a) *Gas chromatography.*—Hewlett-Packard 5890 series II (Yokogawa Analytical Systems, Tokyo, Japan) equipped with NPD and FPD (S mode). The column was DB-210 (30 m × 0.53 mm; film thickness, 1 μm; 50% trifluoropropyl methyl polysiloxane; J&W Scientific, Inc., Folsom, CA). The column outlet was connected to a 2-way separator for dual detection. The separation ratio was 50.3:49.7. The column temperature was programmed as follows: 70°C for 6 min, increase from 70°C to 170°C at 20°C/min, and 170°C for 5 min. The injection port column temperature was 160°C; the detection port temperature was 200°C. The flow rate of carrier gas (helium) was 20 mL/min (NPD). Other flow rates were as follows: hydrogen, 3 mL/min; air, 100 mL/min (FPD); hydrogen, 75 mL/min; and air, 100 mL/min.

(b) *GC/MS.*—Hewlett-Packard GC 5890 series II and JEOL JMS-AX 505W (JEOL, Tokyo, Japan) operated in the electron impact mode at an ionization voltage of 70 eV and an ionization current of 300 μA. Operating conditions were as follows: columns, 15 m × 0.53 mm id DB-WAX and 30 m × 0.53 mm id DB-17 (film thickness, 1 μm; J&W Scientific, Inc.); injection port, 200°C; column, 100°C; and carrier gas (helium) 10 mL/min. Selected ion chromatograms of MITC were monitored at *m/z* 73 (M^+), 72, and 45.

(c) *Shaker.*—Yayoi SD-1 (Tokyo, Japan).

(d) *Centrifuge.*—Kubota RS-6000 (Tokyo, Japan).

Extraction

Weigh 5 g wine (white, red, and rosé) into a glass test tube (10 mL). Add 2 mL dichloromethane containing internal standard and shake for 5 min. Centrifuge at 3000 rpm for 5 min. Discard upper aqueous layer by suction and add 2 mL 5% sodium chloride solution to the organic layer and shake for 1 min. Discard aqueous layer by suction and dry the organic layer with anhydrous sodium sulfate.

Determination

Inject 2 μL of the extracted solution into a gas chromatograph with splitless mode. An internal standard is used to decrease the variability during GC analysis. Calculate the concentration of MITC in wine samples by using the MITC/internal standard peak ratio and referring to the appropriate MITC standard curve.

Results and Discussion

Although a large amount of sample is generally used to analyze MITC in wine (1, 4, 5), a small amount of wine is sufficient with the present method. Therefore, we chose 5 g as the sample weight. MITC is easily extracted with many organic solvents, such as diethyl ether, ethyl acetate, and dichloromethane. We chose dichloromethane (2 mL) as extracting solvent. MITC extracts easily into the lower dichloromethane layer after shaking with wine, and the upper wine layer is discarded; 5% sodium chloride solution is used as a washing solution. Furthermore, dichloromethane has less water than other solvents. The efficiency of MITC extraction was over 95% for an ethanol content of up to 30%. Therefore, the ethanol content of wine does not effect MITC extraction. As described previously, MITC may be lost during concentration of the extract, so that the resulting recoveries are low (7). Therefore, the extract was injected directly into the gas chromatograph.

A polar, liquid-phase column-like poly(ethyleneglycol) is usually used for determining MITC. We used DB-210 (50% trifluoropropylmethyl polysiloxane), which is more stable than the polar, liquid-phase column and shows good peak separation. MITC has one nitrogen and one sulfur atom in the structure. We used NPD and FPD (S mode) as GC detectors. The limits of detection for MITC in wine by NPD and FPD were 0.003 and 0.03 ppm, respectively ($As - Ab > 3\,Sb$; As = average of sample signal, Ab = average of blank signal, Sb = standard deviation of blank signal). The limits of quantitation for MITC in wine by NPD and FPD were 0.01 and 0.1 ppm, respectively ($As - Ab > 10\,Sb$). A disadvantage of the FPD (S mode) is that the response is not linear with respect to sulfur concentration; thus, the quantitation was done by using the square root of the peak height or peak area. Therefore, NPD was used mainly for quantitative calculation and FPD was used for confirmation of MITC. The 2-way separator on the detector did not affect quantitation because the variation coefficients of the peak ratio between 2 detectors were below 5% when the same sample was injected 10 times. The variation coefficient of the MITC peak and MITC/internal standard peak ratio on 10 injections were 6.7 and 1.6%, respectively. Therefore, we chose the peak ratio for MITC quantitation. Figure 1 shows calibration curve of the MITC/internal standard peak ratio. This curve gives the concentration of MITC by dual detection. The calibration curve showed good linearity (NPD: $r = 0.999$, FPD: $r = 0.998$).

The average recoveries (3 trials) of MITC added to wine at 1.0 and 0.1 ppm were 97.6 ± 0.65 and 98.0 ± 1.74%, respectively. Recoveries and standard deviations were satisfactory. MITC concentrations in contaminated wine analyzed by this method were 0.077 ± 0.004 ppm by NPD and 0.080 ± 0.001 ppm by FPD (6 trials). The results based on dual detection agreed well.

Figure 2 shows typical chromatograms of MITC detected in wine. Interfering peaks were not observed on the chromatogram of a blank sample. With FPD, interference by sulfites was not observed. If chromatographic conditions include dual columns and dual detectors, separation and confirmation are better and more precise. The MITC peak detected in wine was con-

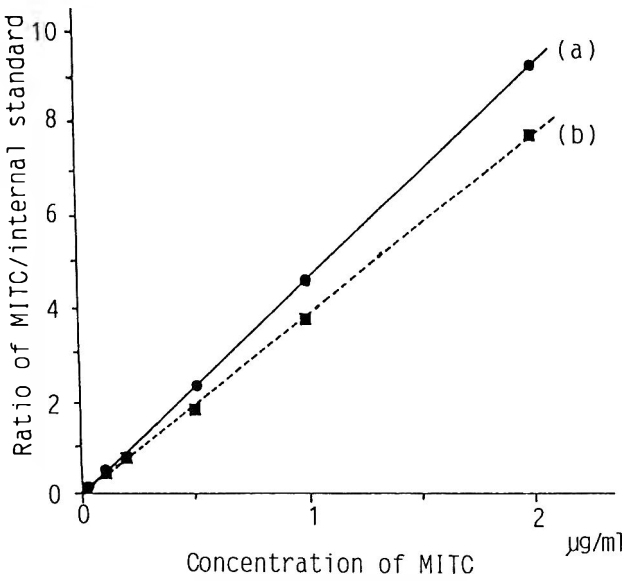


Figure 1. Calibration curve of MITC: (a) NPD, (b) FPD (S mode).

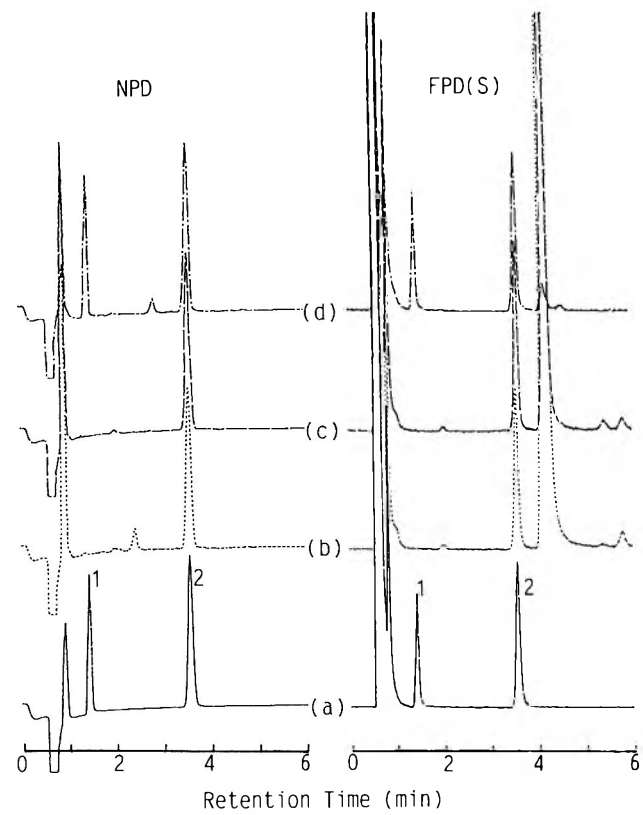


Figure 2. Gas chromatograms of MITC in wines: left column, NPD; right column, FPD (S mode); peak 1 is MITC, peak 2 is internal standard; (a) standard solution 0.2 ppm of MITC, (b) red wine extract, (c) white wine extract, (d) white wine extract detected MITC.

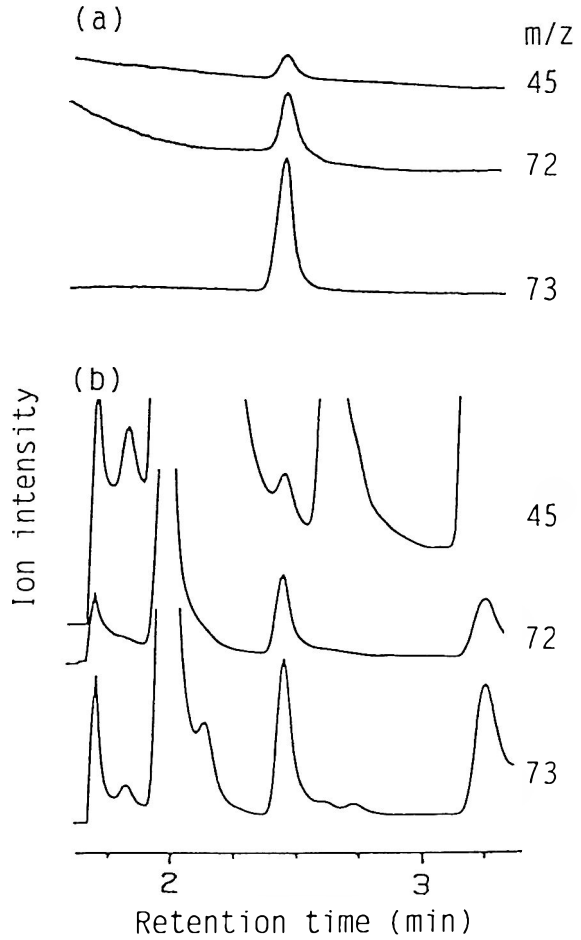


Figure 3. Selected ion monitoring of MITC detected in white wine: monitored ions are M^+ 73, 72, and 45 *m/z* of MITC; (a) standard solution of MITC, (b) MITC detected in white wine.

firmed by selected ion mass chromatograms monitored at *m/z* 73 (M^+), 72, and 45. (Figure 3).

This method is good for separation and is accurate for the determination of MITC in wine.

Acknowledgments

We thank the staff of Tokyo Metropolitan Research Laboratory of Public Health, Nagoya City Public Health Research Institute, and National Institute of Hygienic Science for providing a wine sample and MITC standard.

References

(1) Uchiyama, S., Takeda, H., Kobayashi, A., Ito, S., Sakurai, H. et al. (1992) *J. Food Hyg. Jpn.* **33**, 603–608

(2) Terada, H., Nagai, Y., & Miyabe, M. (1992) *J. Food Hyg. Jpn.* **33**, 586–592

(3) Carbone, D. (1987) *Rev. Vitic. Enol.* **40**, 478–485; Chem. Abstr. **108**, 185145r

(4) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, pp. 1001–1002

(5) *Analytical Methods of Pesticide Residues: Addendum* (1987) S. Goto & S. Kato (Eds) Soft Science Co., Tokyo, pp. 108–109

(6) Hasse-Aschoff, K., & Hasse-Aschoff, I. (1992) *Dtsch. Lebensm. Rundsch.* **88**, 144–146

(7) Sirons, G.J. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 41–44

RESIDUES AND TRACE ELEMENTS

Analysis of Calcium and Lead in Calcium Supplements by Inductively Coupled Plasma–Atomic Emission Spectrometry and Graphite Furnace Atomic Absorption Spectrophotometry

PAUL H. SIITONEN and HAROLD C. THOMPSON, JR
U.S. Food and Drug Administration, National Center for Toxicological Research, Office of Research, Division of Chemistry, Jefferson, AR 72079-9502

A method was developed to analyze various calcium supplements for Ca and Pb content. The analysis involves a dry ash of the supplements followed by wet digestion. The Pb is determined by graphite furnace atomic absorption spectrophotometry (GFAAS). Analysis of Ca is by inductively coupled plasma–atomic emission spectrometry (ICP-AES). Ca supplements fortified with Pb at levels ranging from 0.25 to 10.0 μg/g yielded recoveries ranging from 82.7 ± 4.2 to 105.0 ± 1.7%. To test accuracy, the method was applied to National Institute of Standards and Technology standard reference materials (NIST SRMs) 1572 citrus leaves and 1486 bone meal. GFAAS analysis of SRM 1572 averaged 13.1 ± 0.6 μg Pb per g (certificate value, 13.3 ± 2.4 μg Pb per g), and analysis of SRM 1486 averaged 1.34 ± 0.11 μg Pb per g (certificate value, 1.335 ± 0.014 μg Pb per g). ICP-AES analysis of SRM 1572 averaged 3.12 ± 0.01% Ca (certificate value, 3.15 ± 0.10% Ca by weight), and analysis of SRM 1486 averaged 27.63 ± 0.27% Ca (certificate value, 26.58 ± 0.24% Ca). The method's limit of quantitation (LOQ), on supplement Ca basis and a 1 g sample, averaged 0.75 μg Pb per 1 g Ca for supplements containing 9 to 35% Ca by weight. At a Pb level of 0.663 μg/g Ca, the reproducibility relative standard deviation (RSD_R) averaged 7.3% and the repeatability relative standard deviation (RSD_R) averaged 8.0%. It is recommended that the method be studied collaboratively.

Received March 9, 1993. Accepted by JS September 28, 1993.

Lead has been known to be a toxic element for centuries. The low Pb exposure levels necessary to produce toxic effects, however, have been realized only recently. The outward clinical manifestations of lead overexposure are evident only at levels that produce behavioral and neurological effects that are thought to be permanent. The effects of low-level exposure to lead can be assessed only by neuropsychological behavior tests, which indicate decrements in IQ scores in group studies, hyperactivity, poor attention span, and other significant manifestations (1–4). These effects occur at levels of 15 to 50 μg Pb/dL blood. It is not clear at what levels in blood Pb has no effect.

Lead is sometimes found at elevated levels in Ca supplements that are often prescribed to expectant women to meet the increased Ca needs of pregnancy. This Pb in Ca supplements is of concern because the developing fetus is especially sensitive to Pb, which readily crosses the placenta (5). Studies indicate that infants and children absorb and retain a greater percentage of Pb than do adults (6–8). The consensus is that exposure to Pb should be minimized.

The analysis of Ca supplements for Pb until now has involved digestion with perchloric acid and detection of Pb by differential pulsed anodic stripping voltammetry (DPASV) (9). The use of perchloric acid in digestions should be minimized, because it presents an explosive hazard. DPASV involves the use of a hanging mercury drop electrode, which presents chemical waste disposal and toxicity problems. Recently, wet

digestion was used in combination with a dry ash for analysis of minerals in biological materials (10).

The United States Pharmacopeia (USP) recently lowered the level of Pb allowed in $CaCO_3$ from 10 to 3 μg/g. The USP action became official on January 1, 1990 (11). The U.S. Food and Drug Administration (USFDA) recently began a program to minimize exposure to Pb from all sources and is considering a proposed regulation that would extend the USP limit for Pb in $CaCO_3$ to other types of Ca supplements. The USFDA provisional tolerable daily intake (TDI) for infants and youth of age 6 and under is 6 μg Pb per day (12). Commonly available Ca supplements vary in composition from about 9 to 36% Ca by weight. The various manufacturers' recommended daily intakes also vary for different supplement types and sometimes within a supplement type. To uniformly evaluate the contribution of Ca supplements to daily exposure to Pb, it is necessary to express the Pb content of supplements on the basis of Ca content. The USP specification of 3 μg Pb per 1 g $CaCO_3$ is equivalent to 7.5 μg Pb per 1 g Ca.

The preceding information and concerns led to the development of the method described in this paper.

Materials and Methods

Principle

Samples of calcium supplements are mineralized by dry ashing and wet digestion. The sample digest matrix is modified by addition of $(NH_4)_2HPO_4$ to 0.5% w/v, and the resulting solution is analyzed for Pb by graphite furnace atomic absorption spectrophotometry (GFAAS). Ca analysis is performed by inductively coupled plasma–atomic emission spectrometry (ICP-AES).

Apparatus

Initially all laboratory glass and plasticware were washed with a neutral detergent, rinsed with deionized water, soaked in 1N HNO_3 overnight, rinsed with deionized water, and air dried. Thereafter, laboratory ware were thoroughly cleaned with 1N HNO_3, rinsed with deionized water, and air dried to avoid Pb contamination.

(**a**) *GFAAS.*—Capable of an instrument detection limit (IDL) of 2 ng Pb per mL with background correction enabled; Thermo Jarrell Ash Corp. (Franklin, MA) model video 22 with Smith-Hieftje background correction and model 755 furnace atomizer or equivalent.

(**b**) *Inductively coupled plasma–atomic emission spectrometer (ICP-AES).*—Capable of analysis of calcium, Thermo Jarrell Ash Corp., model ICAP 61 or equivalent.

(**c**) *Programmable muffle furnace.*—Lindeburg type 51668 furnace with a model 59564 control console or equivalent.

Materials

(**a**) *Calcium Standard Stock Solution.*—10.00 mg/mL, National Institute of Standards and Technology (Gaithersburg, MD), SRM 3109 or equivalent.

(**b**) *Lead standard stock solution.*—10.00 mg/mL, NIST SRM 3128 or equivalent.

(**c**) *Ammonium phosphate dibasic.*—Mallinckrodt, analytical reagent; meets ACS specifications (Mallinckrodt Chemical Works, St. Louis, MO) or equivalent.

(**d**) *Nitric acid (70.0–71.0%).*—Baker Instra-Analyzed reagent for trace metals analysis (J.T. Baker, Inc., Phillipsburg, NJ) or equivalent.

(**e**) *Hydrogen peroxide, 30%.*—Baker-Analyzed reagent (stabilized) (J.T. Baker, Inc.) or equivalent.

(**f**) *Reagent grade water ASTM type II.*—Barnstead (Thermolyne Corp., Dubuque, IA) nanopure deionized water system or equivalent.

Quality Control

(**a**) *Reagent blank.*—A blank sample is carried through the method to monitor reagent contamination. Prior to analysis of samples and with each analytical batch, a reagent blank is evaluated for potential Ca or Pb contribution. A Ca level of 0.5 μg/mL or a Pb level of >50% of the low Pb quantitation standard in the method blank is the basis for identification and elimination of the source(s) of contamination.

(**b**) *Calibration blank standard (CBS).*—This blank is used to establish the analytical curve. For GFAAS, the CBS is 0.5% w/v $(NH_4)_2HPO_4$ in 1N HNO_3. The ICP-AES CBS is 1N HNO_3. During Ca analysis, the CBS is read after analysis of each batch of 10 samples. If the result is more than 3 standard deviations from the initial blank value, the CBS is read 2 more times and the results are averaged. If the averaged CBS value is more than 3 times the standard deviation of the initial CBS value, the instrument is restandardized, and the previous batch of 10 samples are analyzed again.

(**c**) *Instrument check standard (ICS).*—This midrange standard is analyzed after each batch of 10 samples. For ICP-AES analysis, if the ICS result is $> \pm 10\%$ of the known value, restandardization and reanalysis of the previous batch of 10 samples is required. For GFAAS analysis, the ICS is required to be $\leq \pm 20\%$ of the expected value.

(**d**) *Duplicate sample.*—Two similar samples from a single source are brought through the entire sample preparation and analytical process. The results of duplicate sample analyses must agree to $\pm 20\%$. The relative percent difference (RPD) of duplicate results should be $\leq \pm 20\%$, where $RPD = 100 \times (S_1 - S_2)/[\frac{1}{2}(S_1 + S_2)]$. If the RPD is greater than $\pm 20\%$, the sample is reanalyzed. However, if the RPD for the reanalyzed sample remains $> \pm 20\%$, sample homogeneity is suspect and the mean sample value ($n = 4$) is reported.

(**e**) *NIST/SRM.*—The analysis of NIST SRM for Ca or Pb must be accurate to $\leq \pm 10\%$ of the certificate value or within the uncertainties stated in the NIST SRM certificate of analysis, whichever is greater. For example, analysis of NIST SRM 1572 for Pb should yield 13.3 ± 2.4 μg Pb per g or $\pm 18\%$ (the certificate value). Analysis of NIST SRM 1486 for Pb should yield 1.34 ± 0.13 μg Pb per g (certificate value, 1.335 ± 0.014 μg Pb per g).

(**f**) *Instrument detection limit (IDL).*—This limit is the concentration or mass of analyte that yields an absorbance equal to 3 times the standard deviation of a series of measurements of a solution (minimum of 3 measurements). It is the concentration

or mass of analyte that is distinctly detectable above the background.

(g) *Limit of quantitation (LOQ).*—The LOQ for analyte with respect to supplement is based on 5 times the standard deviation of a series of measurements of a solution.

(h) *Sensitivity.*—For GFAAS, the sensitivity is the mass of analyte that results in an absorbance of 0.0044 absorbance unit when analyzed. For this method, sensitivity is calculated from a midrange standard absorbance (20 ng Pb per mL). The sensitivity should be within 50% of the manufacturer's specification and should remain consistent from day to day.

Procedure

(a) Weigh duplicate 1-g samples of Ca supplement to 4 significant figures into suitable precleaned borosilicate glass vessels.

(b) Analyze a reagent blank and NIST SRM 1486 bone meal (to monitor reagent contamination, method LOQ, and method performance) with each batch of samples.

(c) Place samples in programmable muffle furnace and allow furnace to reach an initial temperature of 200°C. Initialize the following temperature program: heat from 200° to 450°C linearly over 4 h (to avoid ignition of sample), hold at 450°C for 8–16 h, and then allow to cool to room temperature.

(d) Wet each sample with 5 mL H_2O and cautiously add 2 mL HNO_3 (because of foaming). Heat at 125°C until near dryness.

(e) Add 30% H_2O_2 dropwise until all carbonaceous material is mineralized (up to 4 mL).

(f) Transfer mineralized sample to 50 mL volumetric flask with several rinses of ca 5 mL 1N HNO_3, add 1.0 mL 25% w/v ammonium phosphate as a matrix modifier for GFAAS, and adjust to volume with 1N HNO_3.

(g) Dilute the sample concentrate 1:200 with 1N HNO_3 for a 1:10 000 final dilution for ICP-AES Ca analysis. The reagent blank is not diluted prior to Ca analysis.

(h) Perform additional dilutions required for GFAAS with 1N HNO_3 containing 0.5% w/v ammonium phosphate.

GFAAS Determination

The GFAAS instrument was configured and optimized for Pb analysis per manufacturer's specifications. Typical general conditions are listed in Table 1. Quantitation standards containing 5.0, 10.0, 20.0, 40.0 and 80.0 ng Pb per mL and 0.5% w/v ammonium phosphate in 1N HNO_3 were analyzed just prior to and immediately after sample analysis. The IDL was determined. An ICS was analyzed after each batch of 10 samples. At least 3 readings of each sample were taken, and the absorbance area was averaged for each. A linear regression of the data for quantitation standards was performed, with Pb concentration as the x axis and absorbance as the y axis. Absorbance data for unknown samples were then used to calculate the Pb concentration in sample digests. Reagent blank contribution, dilution factors and the amount of Ca in sample (%) were applied to determine Pb content based on Ca content of supplement (μg Pb per 1 g Ca).

Table 1. Typical instrumental conditions for GFAAS

Item	Setting
Furnace geometry	Delayed atomization cuvette
Purge gas	Argon
Smith-Hieftje background correction settings	
Hollow cathode lamp	Pb
Wavelength	283.3 nm
Bandpass	1 nm
Signal current	1.5 mA
Background current	3.0 mA
Temperature program	
Sampling and drying	120°–140°C; 5 s
Pyrolysis	250°C; 10 s
	680°C; 20 s
Ramp atomization	2300°C; 10 s

ICP-AES Determination

The ICP-AES instrument was initialized and allowed to achieve thermal equilibrium over 30 min. Instrumental parameters for analyses were coolant flow, 22 L/min; auxiliary flow, 0.4 L/min; nebulizer flow, 0.5 L/min; incident power, 1.2 kW; reflected power, <5 W; and observation height, 13 mm above work coil. The sample uptake rate was 1.5 mL/min. For sample solutions containing ≤10 μg Ca/mL, the 393.37 nm line was used for quantitation; for solutions containing >10 μg/mL, the 315.89-nm line was used.

Spectral scans (wavelength vs intensity) were obtained by scanning 63 steps from −31 to +31 positions across the analytical lines monitored. The instrument was calibrated and standardized per manufacturer's specifications. Four 5 s integrations were obtained for each sample or standard for signal averaging. Before sample analysis and after each batch of 10 samples, the CBS and ICS were read to evaluate instrument stability. If the CBS differed by more than 3 standard deviations from the initial value or if the ICS differed from the known value by more than ± 10%, corrective action was warranted (*see* Quality Control).

Experimental

Calcium supplements were obtained from area health food stores and chemical supply stores. For samples in tablet form, 10 tablets were ground to a fine powder in a prewashed, HNO_3 (1N)-soaked, water-rinsed and air-dried alumina mortar and pestle. The powdered sample was air dried for 2 h at 95°C. One supplement of the gel capsule type was analyzed as received.

Several digestion methods (wet digestion, dry ashing, and microwave digestion) were attempted before the present method was chosen.

Recovery experiments were performed by fortification of a $CaCO_3$ sample (SP1A) with 0.25, 0.50, 1.0, 5.0 and 10.0 μg Pb per 1 g supplement. Fortification was performed by adding the respective Pb levels, each contained in 1 mL 1N HNO_3, to quadruplicate 1 g subsamples of supplement. Additionally,

during analysis of various types of Ca supplements, supplements were spiked at 5 μg Pb per g to monitor recovery from a full range of supplement types.

A comparative study, undertaken by the FDA Center for Food Safety and Applied Nutrition (CFSAN) Neutron Activation Analysis Laboratory and the FDA National Center for Toxicological Research (NCTR), provided an independent indication of the validity of the Ca levels determined in the sample digests by ICP-AES at the NCTR.

Subsamples of Ca supplements were analyzed in parallel. At the NCTR, Ca analysis was performed by the current method. At the CFSAN, Ca analysis of intact Ca supplements was performed by instrument neutron activation analysis (INAA). For ICP-AES analysis, quadruplicate readings were taken of duplicate supplement samples. For INAA, 5 analytical portions of each supplement type were analyzed.

Method accuracy and precision for Pb were assessed by concurrent analysis of NIST SRM 1572 with each batch of samples analyzed. Although not a Ca supplement, this material contains 3.15% Ca by weight and was the most readily available material for repeated monitoring of method performance. Samples of animal bone reference material (H-5) from the International Atomic Energy Agency, which were also analyzed during the recovery experiment, provided additional information on the accuracy of Pb analysis. Because the H-5 reference material had limited availability, it was not used routinely for performance evaluations.

NIST SRM 1486 bone meal became available during the latter stages of method development. Analysis of SRM 1486 and spikes (5 μg Pb per g) were performed in quadruplicate to assess further the accuracy and precision of the method.

Results and Discussion

Recovery Experiments

The results of recovery experiments with Pb-fortified supplement appear in Table 2. The recovery increased and the coefficient of variation (CV) decreased as the fortification level increased. The recoveries ranged from 85.7 to 105%, whereas the CV values decreased from 5.1 to 0.8 as spike levels increased. The recoveries of Pb from supplement spiked at 5 μg Pb per g ranged from 85 to 115% for supplements containing 0.103 to 6.39 μg Pb per g. Analysis of H-5 reference material yielded 3.20 ± 0.10 μg Pb per g, compared with the provisionally certified value of 3.1 mg Pb per kg with a 95% confidence interval of 2.6–3.7 mg Pb per kg.

Analyses of NIST SRM 1486 gave an average value of 1.34 ± 0.11 μg Pb per g compared with the certified value of 1.335 ± 0.014 μg Pb per g.

Supplement Analyses

The results of analyses of various Ca supplements appear in Table 3. The analyses yielded Pb contents of 0.326 to 19.3 μg Pb per 1 g Ca, which translate to 0.103–6.39 μg Pb per 1 g supplement. The method LOQ ranged from 0.34 to 1.5 μg Pb per 1 g Ca, which translates to 0.14 μg Pb per 1 g supplement.

Table 2. Recovery of Pb from fortified Ca supplement[a]

Fortification, μg of Pb per g	Pb determined, μg of Pb per g	Recovery, %	CV
—	0.295 ± 0.026	—	—
0.250	0.502 ± 0.011	82.7 ± 4.2	5.1
0.500	0.750 ± 0.027	90.3 ± 5.9	6.5
1.00	1.18 ± 0.02	89.4 ± 1.9	2.1
5.00	5.52 ± 0.08	105 ± 1.7	1.6
10.0	10.4 ± 0.2	103 ± 0.8	0.8

[a] Results are expressed as the mean ± 1 standard deviation for n = 4 replicates.

The high LOQ on a Ca basis was obtained only for Ca lactate, which contains 9.25% Ca by weight. The average LOQ for 10 types of supplements analyzed was 0.75 μg Pb per 1 g Ca, which is below the USP limit by a factor of 10.

For methods that may be used for regulatory purposes, LOQs that are a factor of 10 below the regulatory limit are desirable. Because the USP specification for $CaCO_3$ based on Ca content is equivalent to 7.5 μg Pb per 1 g Ca, these LOQs are adequate. The IDL and LOQ for Ca by ICP-AES were far below the levels seen in the diluted supplement samples. The diluted digests contained 3.2 to 40 μg Ca per mL, compared

Table 3. Pb content of various Ca supplements

Ca supplement	Type[a]	Pb Content μg/g of Ca[b]	Pb Content μg/g of supplement	CV
PM1B	1	2.50 ± 0.18	1.00 ± 0.07	2.5
SG1A	1	0.415 ± 0.090	0.166 ± 0.036	22
SP1A	1	0.663 ± 0.053	0.265 ± 0.021	8.0
SP2A	1	3.15 ± 0.20	1.26 ± 0.08	6.3
SG1C	2	9.15 ± 0.23	1.19 ± 0.03	2.5
SP2C	2	<LOQ[c]	—	—
HT1B	3	19.3 ± 3.9	6.39 ± 1.29	20
PB1B	3	3.67 ± 0.12	1.21 ± 0.04	3.3
PB2B	3	15.7 ± 0.45	5.21 ± 0.15	2.9
HT1D	4	2.68 ± 0.42	0.545 ± 0.085	16
PB1A	4	7.02 ± 0.33	1.51 ± 0.07	4.6
PB2A	4	3.59 ± 0.22	0.753 ± 0.046	6.1
HT1A	5	2.62 ± 0.11	0.633 ± 0.027	4.3
HT1C	5	2.11 ± 0.27	0.280 ± 0.036	13
HT1E	5	1.73 ± 0.09	0.280 ± 0.015	5.4
PB2C	5	2.06 ± 0.07	0.817 ± 0.026	3.2
PM1A	5	0.326 ± 0.044	0.103 ± 0.014	14
SG1B	5	<LOQ[c]	—	—
SP2B	5	<LOQ[c]	—	—

[a] 1, $CaCO_3$ or oyster shell; 2, Ca lactate; 3, bone meal; 4, dolomite; and 5, other.

[b] Pb level divided by the fractional percent Ca in the supplement determined by instrumental analysis or from manufacturer's labeling information.

[c] The limit of quantitation (LOQ) ranged from 0.34 to 1.5 μg of Pb per g of Ca supplement.

Table 4. Ca content of various Ca supplements determined by ICP-AES and INAA

Sample/type	Ca content per manufacturer label, %	Ca content, %		Differences,[a] %
		ICP-AES	INAA	
PS1A/tribasic Ca phosphate	33.5	32.32 ± 0.43	34.02 ± 0.38	5.0
PS2D/oyster shell	35.1	33.88 ± 0.24	36.37 ± 0.50	6.9
PS2E/$CaCO_3$	32.8	32.77 ± 0.75	35.33 ± 0.96	7.2
PS3A1/Ca lactate	13.3	15.22 ± 0.12	17.47 ± 0.53	13.
PS4H/bone meal	29.6	28.63 ± 0.25	30.75 ± 0.32	6.9
HT1A/chelated Ca Mg	23.1	23.95 ± 0.13	24.52 ± 0.38	2.3
HT1C/calcium soft gel	13.6	13.25 ± 0.12	13.38 ± 0.14	0.97
HT1D/dolomite	18.0	19.82 ± 0.18	20.81 ± 0.50	4.8
HT1E/Ca malted milk balls	17.9	15.82 ± 0.16	16.56 ± 0.37	4.5
SP2B/Ca gluconate	9.12–9.50	8.838 ± 0.061	9.670 ± 0.27	8.6
NIST SRM 1572	3.15 ± 0.10	3.14 ± 0.02	ND[b]	0.32
NIST SRM 1549	1.30 ± 0.05	ND	1.266[c]	2.7

[a] Difference, % = [(INAA – ICP–AES)/INAA]100. NIST SRM values are the difference from certified values.
[b] ND, value not determined.
[c] The historical average by INAA is 1.258 ± 0.030%.

with IDLs of 0.004 and 0.04 μg of Ca per mL for the 393.37- and 315.89-nm Ca lines, respectively. Spectral scans of the diluted supplements indicated that background correction was not necessary.

Three supplements contained greater than the USP $CaCO_3$ equivalent of Pb: 2 bone meal supplements and 1 Ca lactate supplement. These supplements would contribute 6.1 to 38.3 μg Pb per day if taken as recommended. On the basis of the recommended daily allowance (RDA) of 1000 mg Ca per day, 4 supplements would contribute 7.0 to 19.3 μg Pb per day. These levels all exceed the FDA provisional TDI for youth and would not be recommended to expectant women. There was nonhomogeneity evident in one bone meal sample analyzed repeatedly (HT1B).

Comparative Study

The results of the comparative study of Ca analyses by ICP-AES and INAA appear in Table 4. The results indicate a less than 10% difference between the two methods, except for the Ca lactate sample. If the Ca lactate result is eliminated, the average difference in Ca content determined was 5.2 ± 2.5% for 9 supplement types.

The 13% difference observed for the Ca lactate sample may have been due to different levels of hydration. The manufacturer indicated that this sample contained 13.3% calcium. Ca lactate monohydrate contains 17% Ca, whereas Ca lactate pentahydrate contains 13% Ca. Although the analyses were performed on dried materials, which were desiccated after drying, various degrees of hydration seems the best explanation.

In each case, ICP-AES analysis gave somewhat lower results compared with INAA analysis for identical supplements. This difference may reflect differences in sample handling or lower recovery by ICP-AES, because INAA was performed on intact supplement samples. However, ICP-AES analyses of NIST SRM materials did not reflect this bias.

During INAA, NIST SRM 1549 nonfat milk powder was analyzed concurrently with Ca supplements. A mean value of 1.27% Ca was obtained, compared with the certificate value of 1.30 ± 0.05% Ca. Analysis of NIST SRM 1572 citrus leaves by ICP-AES yielded 3.14 ± 0.02% Ca, compared with the certificate value of 3.15 ± 0.10% Ca. Likewise, ICP-AES analysis of NIST SRM 1486 yielded 27.63 ± 0.27% Ca, compared with the certificate value of 26.58 ± 0.24% Ca. The Ca contents of supplements given in manufacturers' labeling information were accurate in most cases, on the basis of INAA and ICP-AES results.

These data support the conclusion that analysis of digests by ICP-AES evaluates the Ca content of various Ca supplements with acceptable accuracy.

Method Performance

In-house testing of method ruggedness and performance was performed to evaluate accuracy and precision. At a level of 0.663 μg Pb per 1 g Ca, the reproducibility relative standard deviation (RSD_r) averaged 7.3% and the repeatability relative standard deviation (RSD_R) was 8.0%. The Pb analyses of NIST SRM 1572 yielded an average RSD_r of 5.7% and an RSD_R of 4.7%. These data support the recommendation that the method be studied collaboratively.

Acknowledgments

We thank Stephen G. Capar of CFSAN for his suggestions and support. We also thank William C. Cunningham of the FDA Neutron Activation Analysis Laboratory at NIST for his analyses.

References

(1) Rutter, M. (1983) in *Lead Versus Health*, M. Rutter & R.R. Jones (Eds), John Wiley & Sons, Ltd, London, UK, p. 333

(2) Needleman, H.L., Gunnoe, C., Leriton, A., Reed, R., Peresis, H., Maher, C., & Barrett, P. (1979) *N. Engl. J. Med.* **300**, 689

(3) Burchfield, J., Duffy, F., Bartels, P.H., & Needleman, H.L. (1980) in *Low Level Lead Exposure*, H.L. Needleman (Ed.), Raven, New York, p. 75

(4) Benignus, V.A., Otto, D.A., Muller, K.E., & Seipk, J.J. (1981) *Neurophysiology* **52**, 240

(5) Barltrop, D. (1969) in *Mineral Metabolism in Pediatrics*, D. Barltrop & W.L. Burland (Eds), p. 139

(6) Rabinowitz, M.D., Wetherill, G.W., & Kopple, J.D. (1974) *Environ. Health Perspect.* **7**, 145

(7) Alexander, F.W., Clayton, B.E., & Delves, H.T. (1974) *Quart. J. Med.* **43**, 89

(8) Ziegler, E.E., Edwards, B.B., Jensen, R.L., Mahaffey, K.R., & Fomon, S.J. (1978) *Pediatr. Res.* **12**, 29

(9) Capar, S.G., & Gould, J.H. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1054–1061

(10) Hill, A.D., Patterson, K.Y., Veillon, C., & Morris, E.R. (1986) *Anal. Chem.* **58**, 2340–2342

(11) USP-NF XXII (1990) United States Pharmacopeial Convention, Inc., Rockville, MD, p. 2096

(12) U.S. Food and Drug Administration *Memorandum: Provisional Tolerable Exposure Levels for Lead*, U.S. Public Health Service, Contaminants Team (HFF-156), Nov. 16, 1990

RESIDUES AND TRACE ELEMENTS

Analysis of Chlorophenols in Air at the Nanogram-per-Cubic-Meter-Level

KAMAL SWAMI and RAJINDER S. NARANG
New York State Department of Health, Wadsworth Center for Laboratories and Research, Empire State Plaza, PO Box 509, Albany, NY 12201-0509

A gas chromatographic (GC) method for the analysis of chlorophenols in air at the nanogram-per-cubic-meter level was developed. Chlorophenols were trapped on Porapak-N, eluted with methanol, and then derivatized before GC analysis.

Chlorophenols are or have been extensively used as wood preservatives, herbicides, and antiseptics (1) and in dyes (2, 3) and petrochemicals (4, 5). They are also byproducts of the chlorine-bleaching process used in the pulp and paper industries (6, 7). Chlorophenols, a group of semivolatile organic compounds, are potential sources of environmental contamination and were identified in ambient air samples at low nanogram-per-cubic-meter concentrations (8–10).

A method was reported (8) for the analysis of chlorophenols in ambient air by trapping chlorophenols on cartridges filled with Tenax GC, thermal desorption to elute the respective compounds onto a gas chromatographic (GC) column, and either electron capture or mass spectrometric detection. However, this method is slow, and the thermal desorbtion of all the chlorophenols is difficult. Polyurethane foam alone (8) or Tenax GC sandwiched between 2 polyurethane foam plugs (11) was used to collect the chlorophenols, followed by Soxhlet extraction of polyurethane foam and Tenax GC. GC analyses of chlorophenols were carried out directly (8) or after derivatization (11). The advantage of this method is that large sample volumes can be collected, and the potential for achieving low detection limits exists. However, the whole procedure involves too much solvent and glassware. Grob and Newkom (12) carried out the analysis of pentachlorophenol by trapping and derivatizing it on a precolumn, which is then attached to a GC column to achieve the required separation. This method may or may not work for more volatile chlorophenols. It also has the disadvantage that separation from other organic compounds trapped along with chlorophenols may become a difficult process. We describe a method that involves trapping of the various chlorophenols on Porapak-N, desorption with methanol, derivatization of chlorophenols, and subsequent analysis by GC–electron capture detection (ECD).

Received November 2, 1992. Accepted by JS October 22, 1993.

Experimental

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard gas chromatograph (Model 5830A) equipped with an electron capture de-

tector (Model HP 18803-60520), a capillary injection port, and an autosampler (Model HP 7671A); DB-5 column (0.32 mm × 30 m; film thickness, 0.25 μm) and DB-225 column (0.32 mm × 30 m; film thickness, 0.25 μm), J&W Scientific (Rancho Cordova, CA). DB-5 column was used for the analysis of chlorophenols, and DB-225 column was used for the analysis of chlorophenol acetates. Samples were injected in a splitless mode (2.2 μL) by using helium as carrier gas and a column head pressure of 20 psi. ECD make-up gas was argon–methane (95 + 5), with a flow rate of 45 mL/min. The general GC conditions were as follows: detector, 300°C; injector, 200°C; oven programmed from 60° to 220°C at 2°C/min, with hold at initial temperature of 5 min and at a final temperature of 60 min.

(b) *Sampling cartridges.*—Glass tubing (16 cm long and 2 cm wide) was drawn at one end to give a 6 mm opening (3 cm long), as shown in Figure 1. Cleaned and conditioned Porapak-N (4.5 g) or XAD-2 (5 g) was placed in the tube and held in place with glass wool plugs at each end. The Porapak-N cartridges were washed with methanol (50 mL) and reconditioned at 170°C for 1 h under a nitrogen stream (20 mL/min). After conditioning, the cartridge was allowed to cool to room temperature, and the ends were sealed with polyethylene plugs on the 6-mm end and aluminum foil at the 2 cm end. The XAD-2 cartridge was washed with methanol (50 mL) and dried at room temperature under a nitrogen stream (20 mL/min).

(c) *Glass wool.*—Cleaned by Soxhlet extraction overnight with hexane–acetone (1 + 1).

Reagents

(a) *Methanol and benzene.*—Reagent grade (J.T. Baker, Inc.).

(b) *Hexane and ethyl ether.*—Nanograde (Mallinckrodt, Inc.).

(c) *Potassium hydrogen carbonate, anhydrous sodium sulfate, and acetic anhydride.*—(Aldrich Chemical Co.). Distill acetic anhydride 3 times, and use fraction boiling at 138°-140°C.

(d) *XAD-2 resin.*—(Supelco, Inc.). Wash with water followed by successive overnight Soxhlet extraction with methanol, methylene chloride, and benzene.

(e) *Porapak-N.*—(Alltech Assoc.). Extract by Soxhlet overnight with methanol, pack in a conditioning column, dry, and condition at 170°C overnight under nitrogen stream (20 mL/min).

(f) *Chlorophenols.*—2,6-Dichlorophenol, 2,3,6-trichlorophenol, 2,3,5,6-tetra-chlorophenol, and 2,3,4,5,6-pentachlorophenol, analytical grade (Aldrich, Inc.).

(g) *Florisil.*—Activate 30-60 mesh (Aldrich, Inc.) at 450°C overnight and then deactivate (2%) with interference-free water.

Chlorophenol Standards

Stock solutions were prepared at 5000 μg/mL in methanol. These were diluted to prepare other standards as required.

Extraction

Method A.—The cartridge was placed vertically on a ring stand and eluted with methanol (20 mL) by gravity.

Method B.—The resin from the cartridge was emptied into a Soxhlet apparatus and extracted overnight with methanol.

Derivatization

Acetates were prepared by the procedure of Lee et al. (13). A mixture containing from 50 ng to 10 μg of each chlorophenol in methanol (20 mL) was diluted with water (20 mL) in a separatory funnel, and potassium hydrogen carbonate (750 mg) was then added. The mixture was shaken until all the solid was dissolved, and acetic anhydride (250 μL) was added. The mixture was allowed to stand for 5 min and then extracted with 3 × 2 mL hexane. The organic extracts were combined, dried by passing through anhydrous sodium sulphate, and concentrated to 1 mL under a stream of nitrogen.

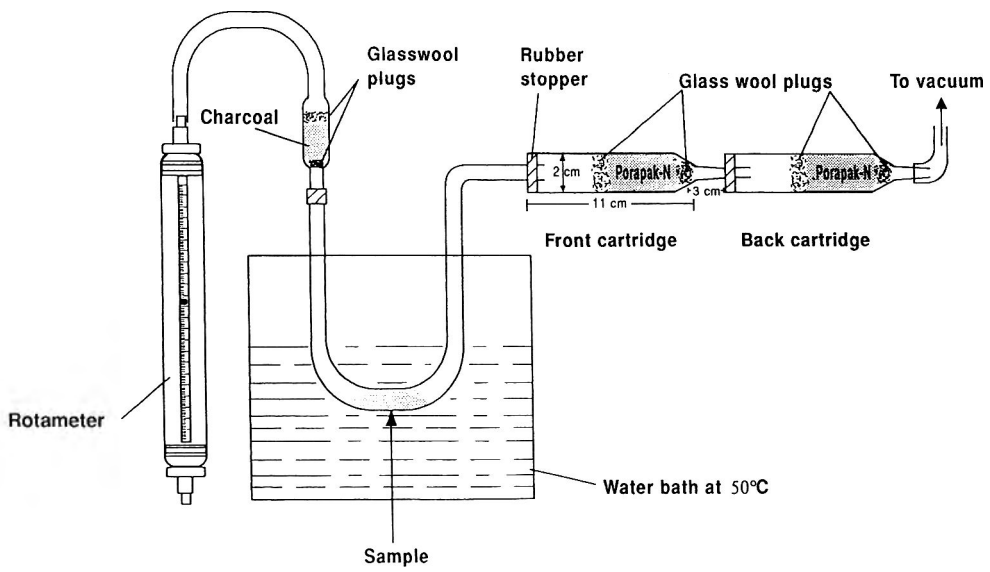


Figure 1. Apparatus used for safe sampling of phenols in air.

Table 1. GC—ECD analysis of a mixture of 4 chlorophenols at different levels[a]

Sample, µg/mL	µg/mL			
	Cl_2-Phenol, µg/mL	Cl_3-Phenol, µg/mL	Cl_4-Phenol, µg/mL	Cl_5-Phenol, µg/mL
10	10.000 ± 7	10.000 ± 6	10.000 ± 11	10.000 ± 17
5	5.066 ± 6	4.922 ± 6	4.260 ± 7	2.131 ± 15
1	1.165 ± 10	0.822 ± 10	0.215 ± 35	—
0.5	0.679 ± 9	0.291 ± 17	0.007 ± 61	—
0.1	0.134 ± 5	0.008 ± 49	—	—
0.05	0.059 ± 3	0.002 ± 28	—	—

[a] $n = 3$. Values are mean (µg/mL) ± RSD (%).

Cleanup

A glass column (30 × 1 cm) was packed with deactivated Florisil (7.5 g). The acetates in hexane (1 mL) were transferred onto the top of the column with disposable glass pipets. The column was first eluted with hexane (50 mL) and then 15% ether in hexane (50 mL). The first fraction was rejected, and the second fraction was concentrated by using K-D apparatus over steam bath to ca 5 mL and then under a stream of nitrogen to 1 mL. This fraction was then analyzed by GC.

Collection Efficiencies

The apparatus was assembled as shown in Figure 1 and consists of a 6 mm od U-tube. One end of the tube was attached to a set of 2 sampling cartridges, front and back, while the end of the back cartridge was attached to the house vacuum system. On the other end, a charcoal-filled cartridge was attached to purify the room air; the cartridge was attached to a rotameter. A known amount of the chlorophenols in a small amount of methanol was placed in the U-tube. The solvent was allowed to evaporate, leaving the chlorophenols coated on the walls of the U-tube. The tube was immersed in the hot water bath, and the vacuum cock was opened. The reading on the rotameter was recorded at the beginning of the experiment, after 12 h, and at the end of the experiment (average flow rate, 11.0–15.0 L/min). At the end of the required sampling time, the vacuum cock was closed. The sampling cartridges were removed and chlorophenols extracted by following Method A. After derivatization and cleanup, the extract was analyzed for the chlorophenols. The U-tube was washed with methanol (10 mL), and after derivatization and cleanup the wash was analyzed for any residual chlorophenols.

Results and Discussion

With the advances in the commercially available capillary columns and hardware (deactivated liners, etc.), we believed one might be able to carry out the analysis of the parent chlorophenols at very low levels. We tried a number of commercially prepared columns (DB-1, DB-5, DB-225, etc.) along with commercially available hardware. Table 1 shows the results from repetitive injections of 4 chlorophenols at different levels on a DB-5 capillary column. The response of various phenols falls off very rapidly; pentachlorophenol could not be detected at 1 µg/mL levels. Apparently, the difficulty arrises from getting rid of all the active sites in the injection port liners, the columns, or both, to achieve reproducible results at low levels over any length of time. This problem was confirmed early on by Grob & Newkom (12) and also more recently by other workers; Patton et al. (11) preferred to carry out the GC analysis of chlorophenolics only after derivatiation. We observed that it was easy to chromatograph chlorophenol acetates even at 0.005 µg/mL levels with almost any capillary column. We decided to make acetate derivatives of these phenols, as these are stable and are easy to make and no toxic reagents such as diazomethane are required. Also, we were able to carry out

Table 2. Extraction efficiencies of chlorophenols from adsorbents

Adsorbent	Amount, ng	Method	Recovery, %[a]			
			Cl_2-Phenol	Cl_3-Phenol	Cl_4-Phenol	Cl_5-Phenol
XAD-2	50	A	50.0 ± 7.9	28.0 ± 54	21.0 ± 24	9.0 ± 36
XAD-2	50	B	50.0 ± 1	59.5 ± 6	65.0 ± 0.1	70.0 ± 2
Porapak-N	50	A	101 ± 4	102 ± 2	108 ± 5	106 ± 6
Porapak-N	20	A	104 ± 6	100 ± 6	99 ± 7	115 ± 9
Porapak-N	1000	A	103.0 ± 11	71.0 ± 13	93.0 ± 19	109.0 ± 26
Florisil[b]	20	—[c]	91.0 ± 5	91.0 ± 6	80.0 ± 4	100.0 ± 6

[a] $n = 3$. Values are mean ± RSD (%).
[b] A mixture of 4 chlorophenol acetates was spiked.
[c] Recoveries of chlorophenol acetates from the cleanup procedure are reported here.

Table 3. Collection efficiencies of chlorophenol mix spiked in a glass tube at 50°C and collected in Porapak-N cartridges

Sample	Amount of air sampled, L	Recovery, %[a]			
		Cl_2-Phenol	Cl_3-Phenol	Cl_4-Phenol	Cl_5-Phenol
4-Phenol mix, 1 μg each	100 ± 0	104 ± 24	100 ± 7	85 ± 8	84 ± 4
4-Phenol mix, 1 μg each	1000 ± 0	80 ± 9	87 ± 3	97 ± 8	98 ± 10
4-Phenol mix, 1 μg each	2000 ± 0	100 ± 5	92 ± 7	94 ± 9	88 ± 8
4-Phenol mix, 1 μg each	19300 ± 12	79 ± 10	84 ± 12	80 ± 11	87 ± 4
4-Phenol mix[b], 20 ng each	31000 ± 11	104 ± 8	89 ± 9	94 ± 8	104 ± 8
4-Phenol mix, 10 ng each	19400 ± 15	110 ± 74	120 ± 36	70 ± 39	43 ± 13

[a] $n = 3$. Values are mean ± RSD (%).
[b] $n = 7$.

the derivatization of the chlorophenols at low nanogram levels. The acetates show very good linearity on an electron-capture detector (0.005–1 μg/mL).

To check which adsorbent releases various chlorophenols completely, cartridges filled with Porapak-N or XAD-2 were spiked directly with known amounts of a mixture of chlorophenols consisting of pentachlorophenol and one isomer from each group of chlorophenols (di- through tetra-), and eluted with methanol. The results are shown in Table 2. Everything elutes from Porapak-N in the first 20 mL, whereas the recoveries from XAD-2 were poor even after overnight Soxhlet extraction. Table 2 also shows the recoveries of various chlorophenol acetates from a Florisil column.

On the basis of the above findings, we studied the collection efficiencies of chlorophenols from Porapak-N only. The U-tube (Figure 1) was spiked with a mixture of chlorophenols containing from 20 ng to 1 μg of each phenol. Air, 0.1–19 m^3, was pulled through the cartridges at 11–15 L/min. Results are shown in Table 3. In every case, all of the chlorophenols were trapped on the first cartridge; therefore, no breakthrough of any chlorophenol occurs up to at least 19 m^3 of sampling volume.

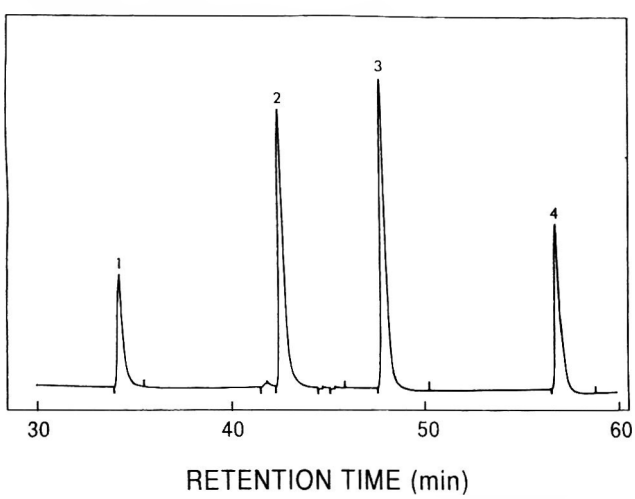


Figure 2. GC ECD chromatogram of chlorophenol acetates: (1) 2,6-dichlorophenol acetate, (2) 2,3,6-trichlorophenol acetate, (3) 2,3,5,6-tetrachlorophenol acetate, and (4) 2,3,4,5,6-pentachlorophenol acetate.

The analysis of the spiked U-tube did not show the presence of any residual chlorophenols (<2.5 ng). Also, the quantitative recoveries of various phenols show that, once trapped, these were not further oxidized. To check the method detection limits, the U-tube was spiked with 10 and 20 ng of each chlorophenol and the sampling carried out in the normal fashion. The results, shown in Table 3, show that even at approximately 1 ng/m^3 the method works very well. A typical ECD gas chromatogram of a mixture of 4 chlorophenol acetates is shown in Figure 2. Currently, we are in the process of using this method for the analysis of other chlorophenol congeners.

Conclusion

The chlorophenols can be efficiently trapped as well as desorbed from Porapak-N cartridges with a minimal amount of solvent. On the basis of a 20 m^3 sample, detection limits of 1 ng/m^3 can be achieved.

References

(1) Xie, T.M., Abrahamsson, K., Fogelgvist, E., & Josefsson, B. (1986) *Environ. Sci. Technol.* **20**, 457–463

(2) Hunter, J.V. (1971) in *Organic Compounds in Aquatic Environments*, Marcel Dekker, New York, NY, p. 51

(3) Games, L.M., & Hites, R.A. (1977) *Anal. Chem.* **49**, 1433

(4) Baird, R.B., Carmona, L.G., & Jenkins, R.L. (1977) *Bull. Environ. Contam. Toxicol.* **17**, 764

(5) Alford, A. (1974) *Environmental Application of Advanced Instrument Analyses: Assistance Projects*, FY '73, EPA-660/2-74-078, Southeast Environmental Research Laboratory, Athens, GA

(6) Keith, L.H. (1976) in *Identification and Analysis of Organic Pollutants in Water*, Ann Arbor Science, Ann Arbor, MI, p. 671

(7) Kringstad, K.P., & Lindstrom, K. (1984) *Environ. Sci. Technol.* **18**, 236A–248A

(8) Leuenberger, C., Ligocki, M.P., & Pankow, J.F. (1985) *Environ. Sci. Tech.* **19**, 1053–1058

(9) Dann, T. (1990) *Detroit Incinerator Monitoring Program Data Report #4*, PMD-90-8, River Road Environmental Technology Center, Environment Canada Conservation and Protection, Ottawa, ON, Canada

(10) Bruckmann, P., Kersten, W., Funcke, W., Balfanz, E., Konig, J., Theisen, J., Ball, M., & Papke, O. (1987) *Chemosphere* **17**, 2363–2380

(11) Patton, G.W., McConnell, L.L., Zaranski, M.T., & Biddleman, T.F. (1992) *Anal. Chem.* **64**, 2858–2861

(12) Grob, K., & Newkom, H.P. (1984) *J. Chromatogr.* **295**, 49–54

(13) Lee, H.B., Weng, L.D., & Chau, A.S.Y. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 789–794

RESIDUES AND TRACE ELEMENTS

Determination of Cholinesterase Activity in Brain and Blood Samples Using a Plate Reader

ELIZABETH R. TOR, DIRK M. HOLSTEGE, and FRANCIS D. GALEY
University of California, Toxicology Laboratory, California Veterinary Diagnostic Laboratory System, Davis, CA 95616

A rapid method is described for the quantitative determination of cholinesterase activity in large batches of blood and brain samples. The technique is an adaptation of the Ellman procedure for a 96-well microtiter plate reader. Ten samples can be analyzed simultaneously in 5 min, with all calculations, including statistical analysis, done automatically. The method detection limit is 0.1 μM/mL/min for blood and 0.1 μM/g/min for brain samples. The procedure has been applied to the routine analyses of samples presented to the veterinary diagnostic laboratory. Method performance, quality control, and normal ranges of cholinesterase activity in livestock and other animals are described.

Exposure to organophosphorus and carbamate insecticides can be assessed by analyzing intact compounds in feed and tissue samples (1–3), alkyl phosphate metabolites in urine or tissues (4, 5), or the depression of cholinesterase (ChE) activity in blood and brain.

Determination of ChE activity as a rapid screen for exposure to these insecticides has been widely used (6–8). Two related ChE enzyme classes, capable of hydrolyzing acetylcholine, exist in the blood and brain of animals in various proportions. These are pseudocholinesterase (PChE; EC3.1.1.8), found in plasma, liver, and muscles of vertebrates, and acetylcholinesterase (AChE; EC3.1.1.7), found in nerve endings, erythrocytes, and the gray matter of brain (9). Following inhibition, PChE activity in plasma recovers more rapidly (8). Specific substrates or inhibitors can be used to distinguish these 2 classes of ChEs (10, 11). Most analytical procedures measure the total ChE activity in whole blood or brain samples. The use of retinal ChE measurements in veterinary diagnostic medicine has also been evaluated (12).

Various analytical methods are used by veterinary and clinical diagnostic laboratories for determination of ChE activity. These include measurement of changes in pH over a 90 min incubation time (13), assays of radiolabeled acetylcholine (14), and spectrophotometric methods based on the Ellman reaction (15). Results obtained from these incompatible methods cannot be compared reliably from one study to another (16). Interpretation of results is further complicated by the large variability of ChE activities among species (17). The compilation of baseline, normal ChE levels for different species of animals is necessary prior to use of the test as a diagnostic tool. Improved uniformity of the analytical method would aid veterinary practitioners in diagnosing cases of organophosphorus and carbamate insecticide poisonings.

In recent years, the Ellman procedure has been used widely to measure total ChE activity in blood, plasma, and homogenates of tissues from domestic and wild species (18, 19). This wide use is due to rapid analysis time, convenience, good sensitivity, reproducibility, and suitability for automation. Short analysis time permits detection of ChE depression by reversible ChE inhibitors such as carbamate insecticides, which dissociate from ChE during the long incubation time required by the pH method. The principle of the Ellman method is measurement of the rate of production of thiocholine as the substrate acetylthiocholine is hydrolyzed by the ChE present in the sample. Thiocholine reacts with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to produce a yellow ion of 5-thio-2-nitrobenzoic acid. The rate of color production is measured at 412 nm by a spectrophotometer. The original Ellman procedure has been

Received June 18, 1993. Accepted by JS September 29, 1993.

modified for field applications and to accommodate larger batches of samples (20–22).

In clinical and environmental situations, a quick screening method with good quality control is needed to assess exposure to ChE-inhibiting insecticides. The recent introduction of the plate reader for colorimetric measurements offers a significant advance in data analysis and management. The method described here, an adaptation of the Ellman assay for the plate reader, was used for rapid and reliable determination of total ChE levels in large batches of blood and brain samples from many species of animals presented for routine testing to the diagnostic laboratory.

METHOD

Apparatus

(**a**) *Kinetic microplate reader.*—Model UVmax, (Molecular Devices Corp., Menlo Park, CA) set at 405 nm, with SOFTmax version 2.01 computer software program.

(**b**) *Microtiter plates.*—Falcon 3915, 96-well polystyrene (Becton and Dickinson Co., Lincoln Park, NJ).

(**c**) *Multi-channel pipet.*—Eppendorf Plus/8 repeater, variable volume, 25–125 µL, with 8-channel Eppendorf Plus/8 adapter (Brinkmann Instruments, Inc., Westbury, NY).

(**d**) *Eppendorf combitips and cartridges.*—1.25 mL capacity, disposable (Brinkmann Instruments, Inc.).

(**e**) *Eight-well reservoirs.*—Polypropylene (Titertek, Flow Laboratories, Finland).

(**f**) *Drummond pipetter.*—10 µL, with disposable glass tips (Drummond Scientific Co., Broomall, PA).

(**g**) *Tissue grinder.*—Potter-Elvehjem, Wheaton, 55 mL (Fisher Scientific).

(**h**) *Reagent dispenser.*—Oxford type S-A (Fisher Scientific).

(**i**) *Centrifuge tubes.*—Polypropylene, 50 mL (Corning, Inc., Corning, NY).

(**j**) *Microcentrifuge tubes for storage of standards and controls.*—1.5 mL polypropylene with caps (American Scientific Products, McGaw Park, IL).

(**k**) *Disposable tubes.*—Polystyrene with caps, 5 and 10 mL (Falcon, Becton Dickinson Co., Lincoln Park, NJ).

(**l**) *Electric thermometer with water-proof probe.*—TEGAM 132C TRMS multimeter.

Reagents

(**a**) *Substrate solution.*—Weigh 0.067 g acetylthiocholine iodide (ATCI; Sigma Chemical Co., St. Louis, MO) into a 10 mL volumetric flask and make up to volume with deionized water. Mix well, divide into five 2 mL portions, and keep frozen in polystyrene screw-capped vials. This solution is stable for 3 weeks.

(**b**) *Ellman reagent solution.*—Weigh 0.0205 g 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) into a 25 mL volumetric flask and make up to volume with 0.1M, pH 7.00 phosphate buffer. Store in the same manner as the substrate solution.

(**c**) *Phosphate buffers (0.1M).*—(*1*) *Sodium phosphate, dibasic.*—Weigh 26.8 g $Na_2HPO_4 \cdot 7H_2O$ (Fisher Scientific) into a 1.0 L volumetric flask, and make to volume with deionized water. (*2*) *Potassium phosphate, monobasic.*—Weigh 13.6 g anhydrous KH_2PO_4 (Fisher Scientific) into a 1.0 L volumetric flask, and make to volume with deionized water. To buffer (*1*) add enough buffer (*2*) to obtain solutions of pH 7.00 or 8.00.

(**d**) *Bovine serum albumin (BSA), 3 mg/mL.*—Weigh 0.075 g BSA (Sigma) into a 25 mL volumetric flask, dissolve in 0.1M, pH 8.00 phosphate buffer, and make up to volume. Keep refrigerated (4°C).

Preparation and Storage of Controls and Standards

(**a**) *Control serum.*—SeraChem level 1, clinical chemistry control serum (human), assayed, (Fisher Scientific). Prepare according to manufacturer's instructions. Store 250 µL portions at –4°C in capped vials. Use a fresh portion of the control for each day of analysis. ChE activity in serum stored as described is stable for 4 weeks.

(**b**) *Standard AChE, type III, 10 units/mL.*—Sigma. Pipette 52.63 µL (50 units) of standard into a 5 mL volumetric flask and make up to volume with 3 mg/mL BSA. Use this stock solution to prepare standards containing 1–5 units/mL by diluting with 3 mg/mL BSA.

(**c**) *Standard AChE, type XII-S, 2 units/mL.*—Sigma. Weigh 20 mg 0.5 unit/mg standard into a 5 mL volumetric flask and make up to volume with 3 mg/mL BSA. Make dilutions as above. Store standards as previously described for controls.

Sample storage

(**a**) *Brain.*—Fresh brain samples should be stored on ice or refrigerated if the analysis is to be done within 24 h. Frozen samples should be placed in the freezer immediately after receipt and thawed just before analysis.

(**b**) *Blood.*—Only unclotted blood samples can be used for analysis. Fresh, whole blood should be kept in ethylenediaminetetraacetic acid or heparinized Vacutainer tubes at 4°C. Blood samples should not be frozen.

Sample Preparation

(**a**) *Brain.*—Homogenize the whole brain sample (use a glass sample blender for large brains or a spatula for small brains). Weigh 5 g of homogeneous sample into a Wheaton homogenizer. Add approximately 10 mL 0.1M, pH 8.00 phosphate buffer. Homogenize for 5 min. Transfer quantitatively into a 50 mL, screw-cap centrifuge tube, and adjust the volume to 25 mL with the same buffer. Place the homogenate on ice and use for further dilutions. Make subsequent dilutions of homogenate. The most convenient dilutions are 1:1000 to 1:2500 for mammals and 1:5000 for birds. Dispense 10 mL pH 8.00, 25°C phosphate buffer into a 10 mL Falcon, polystyrene screw-cap tube by using an Oxford type S-A dispenser. Using a disposable Eppendorf combitip, pipette an aliquot of the brain homogenate into the tube containing 10 mL buffer to obtain the desired dilution. Mix well by inverting the tube.

(b) *Blood.*—Dilute 10 µL of well-mixed whole blood to 10 mL with pH 8.00, 25°C phosphate buffer. Mix well before analysis.

Determination

Analysis for ChE activity is done at 25°C. Allow all reagents to reach this temperature before performing the assay.

Assign wells on the plate to controls, samples, and duplicates. Allow at least 3 wells for each sample and 2 wells for its substrate blank. Include control and duplicate samples on each plate. Using the multichannel pipet, transfer 250 µL of well-mixed, diluted brain, blood, or control serum from the 8-well reservoir into the wells on the plate. Add 25 µL DTNB reagent to each well. Ensure that the temperature in the wells is 25°C. Add 25 µL ATCI to the "sample" wells only. Add 25 µL phosphate buffer to "substrate blank" wells. Wait for 1 min for the reaction to equilibrate. With the plate reader in the automix mode, measure the changes in absorbance at 405 nm every 8 s for 5 min. Repeat the analysis if the reaction is not linear or if the ChE activity of control serum is not within the acceptable range (10% coefficient of variation [CV] from the established value).

Calculations

ChE activity is expressed as micromoles of acetylthiocholine iodide hydrolyzed per milliliter of blood or gram of brain sample per minute (µM/mL/min or µM/g/min). The average absorbance increase per minute (*dA*/min), standard deviation, and coefficient of variation on the replicates of the sample (CV, %) is calculated by the Soft Max program and presented in the report form. From Beer's law, $C = (A/l) \times E$, where C = concentration, l = 1.05 cm (path length for Falcon 3915 plate), and E = 13 600 $M^{-1}cm^{-1}$ (extinction coefficient). Calculate ChE activity as follows: µM/mL/min = *dA*/min × 0.07003 µM/mL × dilution factor. Subtract the "substrate blank" rate to obtain the activity of ChE in the sample. *If using a microplate different from that specified (Falcon 3915), the path length must be determined for that plate before the analysis.*

Recovery Experiments

Blood and brain samples from diagnostic cases were fortified with AChE type XII-S (blood) and AchE type III (brain). Fortification levels and species of animals involved are shown in Table 1. Samples were analyzed by the method described, along with corresponding standards and unfortified samples. Recovery was calculated by subtracting the ChE activity of the unfortified sample from that of the fortified sample and dividing the difference by the activity of the corresponding standard.

Results and Discussion

Thirty-four samples of bovine blood and 11 samples of equine brain were assayed by our technique and the original Ellman method (15). The results showed an excellent correlation between the 2 methods, with a correlation coefficient of 0.9772 for blood and 0.9959 for brain samples. The values from the plate reader method differed from those from the Ellman method only by an average of 8.5% for blood and 3.5% for brain samples.

The method was tested by running the recovery experiments on diagnostic blood and brain samples from different species of animals. Table 1 summarizes the recoveries of AChE type XII-S from fortified blood and of AchE type III from fortified brain samples. Variations in recoveries are generally small despite different concentration ranges or species of animals tested.

The observed rate of substrate hydrolysis was a linear function of enzyme concentration with r^2=0.9998 for AChE type III and 0.9995 for bovine erythrocyte AChE type XII-S in the range of 0.1 to 10 units of activity.

Concentrations of substrate and DTNB reagent in sample wells needed to assure maximum rate of reaction (V_{max}) during the 5 min assay were determined to be 1.93×10^{-3}, and 1.75×10^{-4}M, respectively.

During a typical 5 min kinetic assay, the plate reader performs 39 absorbance measurements in each of the 96 wells. Plots of optical density (OD) vs time were linear, with r^2 of 0.998 to 1.000 for blood and 0.999 to 1.000 for brain samples.

The rate of background, nonenzymatic hydrolysis of acetylthiocholine at 25°C was 0.003 µM/mL/min (Table 2). Blood samples have a considerable background (up to 40% of total ChE level in dogs); therefore, a blank consisting of diluted blood, DTNB, and buffer is required to correct for the release of thiol material from the cells and the absorbance of other materials in the suspension. The rate of the enzyme activity is, thus, the net rate after the background rate is subtracted from the observed rate. The background of brain samples is usually

Table 1. Averge recoveries of bovine erythrocyte AChE type XII-S from fortified blood and AChE type III from fortified brain samples[a]

Fortification level, units/g	Species	Recovery from blood, %	CV, %	Fortification level, units/g	Species	Recovery from brain, %	CV, %
0.1	B, E	86	8	0.1	B, E	92	6
1.0	A, F, B	83	6	1.0	E	96	5
2.0	B, E	101	3	2.0	B, C, F, P	100	2
5.0	K9, C, B	96	5	5.0	B, E, F	99	1

[a] n = 4. A, avian; B, bovine; C, caprine; E, equine; F, feline; K9, canine; P, porcine.

Table 2. Background activity of cholinesterase in bovine brain and blood samples

Sample	Well contents, + or −[a]			ChE activity	% sample activity
	Sample	DTNB	ATCI		
Reagent blank	−	+	+	0.003	
Brain	+	+	+	2.49	
	+	+	−	0.009	0.4
	+	−	+	0.005	0.2
	+	−	−	−0.012	−0.5
Blood	+	+	+	2.1	
	+	+	−	0.26	15
	+	−	+	0.14	9
	+	−	−	0.15	9

[a] +, present in wells; −, absent in wells. DTNB, 5,5′-dithiobis(2-nitrobenzoic acid). ATCI, acetylcholine iodide.
[b] In μM/mL/min for blood and μM/g/min for brain samples.

Table 3. Repeatability of rate measurements by plate reader method[a]

Statistical parameter	ChE activity of standards		ChE activity of bovine samples	
	AChE type III	AChE type XII-S	Blood	Brain
Average ChE activity	3.31	1.79	2.14	3.56
Repeatability				
SD	0.06	0.04	0.09	0.03
CV, %	1.81	2.23	4.21	0.84

[a] Statistical data on multiple determinations (n = 12) of a sample within 1 day are presented. Values for ChE activity are in μM/mL/min for standards and blood and in μM/g/min for brain samples.

below 1% of the sample activity and is considered to be negligible.

The sensitivity of the assay is comparable with that of the conventional spectrophotometric method. The method detection limit is 0.1 μM/mL/min for blood and 0.1 μM/g/min for brain samples, as determined by instrument noise and spike recoveries.

ChE activity depended on the temperature of reaction in a linear fashion, with r^2 of 0.9989. For every degree (°C) of increased assay temperature in the 19°–28°C range, the ChE activity of control serum increased by approximately 4%. There was a 1°C temperature difference between the outer rows of wells on the plate and the inner wells; therefore, outer rows were not used for sample analysis. Pipetting 300 μL phosphate buffer into unused, outer rows of wells on the plate considerably reduced temperature fluctuations and, therefore, improved the reproducibility of the results.

The overall precision of the assay was better than that of the traditional spectrophotometric method. Results of multiple determinations of AChE type XII-S and AchE type III standards and bovine blood and brain samples are summarized in Table 3. Duplicate analyses obtained on 2 different plates were very similar to those obtained on the same plate. Twenty-five chicken brain samples analyzed in duplicate on different plates showed a CV of less than 2%. The method demonstrated good reproducibility during routine analyses of diagnostic samples. The average relative standard deviations of 130 blood and 205 brain samples analyzed in duplicate during a 2 year period were 3.3 and 1.8%, respectively.

The SeraChem 1 used as control for this assay had a stable ChE activity for up to 5 weeks if stored as described. Mean ChE activity of lot number 991524 was 1.76 μM/mL/min with a CV of 3.5% for 287 consecutive measurements during a 1 year period.

Storage of blood samples with normal and depressed ChE activities for up to 120 h at 4°C did not significantly change enzyme activity (Table 4), in agreement with the findings of other authors (18, 19, 23).

A wide range of blood ChE activities was observed in clinically normal animal populations among and within species. The ChE activities in brain and blood samples from animals where ChE inhibiting agents were not suspected are presented in Table 5. Normal blood ChE activities in common species of livestock were comparable with findings of other authors, as shown in Table 6.

The method described here is, in principle, similar to that of Ellman et al. (15). However, the plate reader method has several significant advantages over traditional spectrophotometric assays. (*1*) Speed of analysis is much greater. With a classical spectrophotometer, an analyst can run only one measurement at a time. With the plate reader, 10 samples can be analyzed simultaneously on one plate, and productivity is increased greatly. The limiting factor is the sample preparation time, not the measurements themselves. The use of buffer distributors and multichannel pipettes also reduces sample preparation time. (*2*) Improved quality control is achieved by making 5 separate measurements for each sample analyzed. This practice diminishes the possibility of random error. All quality control samples are run concurrently with the diagnostic samples to eliminate many analytical variables. (*3*) Results obtained with this method are directly comparable with those obtained with the original Ellman method and modifications by Harlin (12) and Halbrook et al. (18). This comparability enables laborato-

Table 4. Storage stability at 4°C of blood fortified with selected *N*-methyl carbamate insecticides at 0.1 μg/g

Time, h	Average ChE activity, μM/mL/min			
	Control (n = 10)	Methomyl (n = 3)	Aldicarb (n = 3)	Carbofuran (n = 3)
0	2.08 (2.1)[a]	1.37 (2.2)	1.71 (4.2)	0.60 (3.3)
48	2.18 (3.7)	1.26 (1.7)	1.70 (3.5)	0.67 (0.9)
120	2.00 (4.3)	1.16 (6.2)	1.62 (2.1)	0.67 (4.8)

[a] CV values (%) are given in parentheses.

Table 5. Cholinesterase (ChE) activities in brain and blood samples from animals where ChE inhibiting agents were not suspected

Species	Sample	Average activity[a]	Standard deviation	*n*
Avian				
Chicken	Brain	15.99	3.18	26
Turkey	Brain	19.08	3.60	26
Bovine				
	Blood	2.26	0.47	120
	Brain	3.78	1.25	82
Canine				
	Blood	1.63	0.34	28
	Brain	1.96	0.97	3
Caprine				
	Blood	0.71	0.20	4
	Brain	5.23	0.90	5
Deer				
	Brain	5.45	0.15	2
Equine				
	Blood	2.21	0.40	35
	Brain	3.75	1.03	61
Feline				
	Blood	1.28	0.13	4
	Brain	7.11	0.65	2
Fish				
	Brain	12.51	1.31	2
Llama				
	Blood	0.82	0.12	6
	Brain	4.10	1.48	5
Ovine				
	Blood	1.01	0.17	70
	Brain	5.42	2.51	27
Porcine				
	Blood	1.69	0.15	3
	Brain	5.50	1.24	14
Rabbit				
	Brain	13.65	2.09	4
Sea lion				
	Blood	0.91	0.29	8
	Brain	2.50	1.44	10
Sea turtle				
	Brain	4.92	1.53	11
Seal				
	Blood	0.75	0.12	2
	Brain	1.51	0.35	2

[a] In μM/mL/min for blood and μM/g/min for brain.

ries to combine or exchange databases of normal ChE activities in various animal species. (*4*) The method is more convenient. All the calculations including statistical analysis are done automatically by the SoftMax program, which greatly speeds up report preparation. (*5*) All results are automatically archived and easy to reprocess. (*6*) The method can be extended for other applications such as a quick carbamate and organophosphorus insecticide screen on crop or tissue samples based on inhibition

Table 6. Comparison of normal blood cholinesterase activity (μM/mL/min) in livestock as reported by various authors

Species	*n*	Average activity	Standard deviation	Reference
Bovine	120	2.26	0.47	This method
	29	2.65	0.30	Halbrook et al. 1992 (18)
	73	2.45	0.63	Harlin 1991 (12)
Equine	35	2.21	0.40	This method
	27	2.01	0.16	Halbrook et al. 1992 (18)
	44	1.88	0.31	Harlin 1991 (12)
Ovine	70	1.01	0.17	This method
	35	1.20	0.11	Halbrook et al. 1992 (18)
	4	1.38	0.35	Harlin 1991 (12)

of ChE activity as previously tested by the ChE-thin-layer chromatographic method (24). Some preliminary data on this application is presented in Table 7. This method is a valuable diagnostic tool for veterinary toxicology investigations, where large sample throughput and rapid analysis time are common.

Conclusions

The analytical method described here provides a rapid, reproducible, convenient, and efficient procedure for determining ChE levels in brain and blood samples. The method performed well in routine sample analysis, with good spike recoveries from blood and brain samples from a variety of animal species. This automatic technique enables simultaneous processing of many samples and is suitable for use by clinical and veterinary diagnostic laboratories.

Table 7. Preliminary results on the application of the method for quick carbamate screen in tissues: inhibition of cholinesterase (ChE) activity in chicken brain by liver extracts fortified with carbofuran at 1.25 and 0.1 μg/g

Sample	*n*	Average ChE activity, μM/g/min	Inhibition, %
Brain control	6	13.28	
Reagent blank	1	13.36	
Liver control (1 g)	3	15.32	
Liver spiked at 1.25 μg/g	6	0.53	93
Liver spiked at 0.1 μg/g	6	6.35	59

References

(1) Osweiler, G.D., Carson, T.L., Buck, W.B., & Van Gelder, G.A. (1985) *Clinical and Diagnostic Veterinary Toxicology,* 3rd Ed., Kendall Hunt Publishing Co., Dubuque, IA

(2) Holstege, D.M., Scharberg, D.L., Richardson, E.R., & Moller, G. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 394–399

(3) Holstege, D.M., Scharberg, D.L., Richardson, E.R., Hart, L.C., & Galey, F.D. (1994) *J. AOAC Int.* **77**, 1263

(4) Mount, M.E. (1984) *American Association for Veterinary Laboratory Diagnosticians, 27th Annual Proceedings*, pp. 383–402

(5) Richardson, E.R., & Seiber, J.N. (1993) *J. Agric. Food Chem.* **41**, 416–422

(6) Harlin, K.S., & Ross, P.F. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 616–619

(7) Martin, A.D., Norman, G., Stanley, P.I., & Westlake, G.E. (1981) *Bull. Environ. Contam. Toxicol.* **26**, 775–780

(8) Meerdink, G.L. (1989) *Vet. Clin. North Am. Food Anim. Pract.* **5**, 375–389

(9) Whittaker, M. (1986) in *Monographs in Human Genetics,* Vol. 11, L. Beckman (Ed.) Karger Publishing Co., New York, NY

(10) Wilson, B.W., Hooper, M.J., Littrell, E.E., Detrich, P.J., Hansen, M.E., Weisskopf, C.P., & Seiber, J.N. (1991) *Bull. Environ. Contam. Toxicol.* **47**, 717–724

(11) Thomsen, T., Kewitz, H., & Pleul, O. (1989) *Enzyme* **42**, 219–224

(12) Harlin, K.S. (1991) M.S. Dissertation, University of Illinois at Urbana-Champaign, IL

(13) Michel, H.O. (1949) *J. Lab. Clin. Med.* **34**, 1564–1568

(14) Thomsen, T., Kewitz, H., & Pleul, O. (1988) *J. Clin. Chem. Biochem.* **26**, 469–475

(15) Ellman, G.L., Courtney, K.D., Andres, V., & Featherstone, R.M. (1961) *Biochem. Pharmacol.* **7**, 88–95

(16) Munro, N.B., Shugart, L.R., Watson, A.P., & Halbrook, R.S. (1991) *J. Am. Vet. Med. Assoc.* **199**, 103–115

(17) Hill, E.F., & Fleming, W.J. (1982) *Environ. Toxicol. Chem.* **1**, 27–38

(18) Halbrook, R.S., Shugart, L.R., Watson, A.P., Munro, N.B., & Linnabary, R.D. (1992) *J. Am. Vet. Med. Assoc.* **201**, 714–725

(19) Hill, E.F. (1989) *J. Wildlife Diseases* **25**, 580–585

(20) Garcia, I.L., Abellan, F.R., & Cordoba, M.H. (1992) *Anal. Biochem.* **200**, 176–179

(21) Galgani, F., & Bocquene, G. (1991) *Water Res.* **25**, 147–150

(22) Khan, A.A., Coppock, R.W., Schuler, M.M., & Lillie, L.E. (1988) *Am. J. Vet. Res.* **49**, 1184–1186

(23) Plumlee, K.H., Richardson, E.R., Gardner, I.A., & Galey, F.D. (1993) *J. Vet. Diagn. Invest.* **6**, 247–249

(24) Zoun, P.E., & Spierenburg, T.J. (1988) *J. Chromatogr.* **462**, 448–453

STATISTICAL ANALYSIS

Validation of Computerized Liquid Chromatographic Systems

WILLIAM B. FURMAN and THOMAS P. LAYLOFF
U.S. Food and Drug Administration, Division of Drug Analysis, 1114 Market St, Room 1002, St. Louis, MO 63101
RONALD F. TETZLAFF
U.S. Food and Drug Administration, 60 Eighth St, NE, Atlanta, GA 30309

Almost all vendors of liquid chromatographs offer dedicated computer-based controller/data-processing systems. These systems are integrated into the operation of the equipment and frequently use proprietary software systems for which complete documentation and program listings are not available to the user. In some instances, data in magnetic media are considered to be "raw data" for regulatory retention requirements. In addition, printed copies of final data used to make decisions and the procedures used to generate these data from raw data must be prepared and retained. In all cases, the systems must be validated to demonstrate that they are suitable for their intended uses. Approaches to validation of these systems are described and discussed.

Validation of computerized liquid chromatographic (LC) systems is of interest from scientific and regulatory viewpoints. A scientist wants assurance of reliable data and computed results. A regulator wants tangible evidence of validation, such as written standard operating procedures (SOPs), raw data, guidelines, and policy documents that may be reviewed during inspections of manufacturing facilities. We present and discuss 2 approaches to such validation—modular and holistic—and describe procedures to satisfy the needs of scientists and regulators. Our remarks are addressed to quantitative analysis of products intended for the marketplace, or components of such products, and exclude qualitative or preliminary investigational work.

Modular Validation

By modular validation, we mean individual calibration or validation of each distinct, important part of a computerized laboratory system. This general approach was discussed by Tetzlaff (1) in a review of U.S. Food and Drug Administration (FDA) requirements and expectations during inspections of such systems. Our concept of modular validation of computerized LC systems is outlined in Table 1, which is meant to be illustrative, not comprehensive. Although calibration of each module may be useful for troubleshooting purposes, such tests alone cannot guarantee the accuracy and precision of analytical results.

Detector Linearity

An attempt could be made to verify that the detector's output voltage is linear with concentration of a standard material. For a UV-VIS LC detector, this test would have to be performed at several wavelengths to determine boundaries of reliability; for example, at which upper and lower wavelengths does stray light start to reduce the concentration range in which the output is linear? Even if these measurements could be made, there would be little assurance they would guarantee linearity when the detector is used for analytical measurements on compounds different from those selected for the detector calibration. Similar arguments apply to other types of LC detectors (fluorometric, electrochemical, etc.).

Detector Wavelength Accuracy

We have often encountered variable-wavelength UV-VIS LC detectors whose indicated wavelength differs from the actual wavelength by considerable amounts, as much as 20 to 30 nm. Loss of wavelength calibration can occur from mechanical shocks during shipment, and we have observed such errors in detectors newly received from manufacturers. Wavelength inaccuracy is often not apparent to the person operating the detector and can lead to substantial errors in interpretation of data. The operator's manual usually gives a quick way to adjust the detector to meet the manufacturer's specification for wavelength accuracy; we urge all laboratories to perform this calibration.

Pump Flow Rate

An attempt could be made to measure flow rates at several pump settings with various back pressures. Such tests might be repeated over several days to evaluate the reproducibility of the pump's flow rate. Yet, such measurements are fleeting and of limited value for 2 reasons: Critical pump parts—valves, especially—deteriorate with time, and pump performance is often influenced by deposits of buffer salts on critical parts. Neither of these effects can be reliably duplicated in standardized tests.

Received July 28, 1993. Accepted by GL August 31, 1993.
Presented in part at the Workshop on Antibiotics and Drugs in Feeds, 106th AOAC Annual International Meeting and Exposition, August 30, 1992, at Cincinnati, OH.

Table 1. Modular validation of computerized LC systems

Detector (UV-VIS)
Is output voltage linear with concentration?
For variable-wavelength detectors, are wavelength settings accurate?
Pump
Are flow rates accurate and reproducible?
Injector
Are injected volumes accurate and reproducible?
Data Processor (Integrator, Computer)
Is analog-to-digital converter accurate?
Is program code correct? Does it give correct calculated results (plots, peak heights or areas, assays, etc.)?
Are the macros coded correctly? Validated? Documented?
Controller (Computer)
Is program code correct?
System Documentation
Is system documentation in place?

Injection Volume

Similarly, measuring within-day and between-day accuracy and precision of the amount of solution injected automatically or manually might be attempted. And again such measurements could not be assumed to hold up over useful periods of time due to gradual deterioration in injector parts or due to differences in viscosity or surfactant levels among the solutions used to test the injector and those actually used during analyses.

Data Processor: Analog-to-Digital Converter

In a rigorous modular validation, an attempt would be made to measure the accuracy of the analog-to-digital converter's output (numbers) vs the input signal (detector voltage). However, by connecting the voltage source, electromagnetic interference might be introduced from the test leads, or the load on the input of the analog-to-digital converter might change enough to affect its output accuracy. It is extremely difficult to design electrical test protocols that accurately simulate the conditions present within the instrument when the detector, not the test device, is connected to the analog-to-digital converter. This approach is beyond the capability reasonably to be expected of many laboratories.

Data Processor and System Controller: Correctness of Program Code

If a laboratory has written its own program code or has purchased a custom-written program code for data processing or system control, the source code should be available for scrutiny and validation. Included here are any "macros" that may be written for an LC system; they must be documented and available for review. Minimum documentation of macros (and other custom-written routines) must include (*1*) a statement of what the macro is supposed to do; (*2*) a written description of how it performs its task, including a clear explanation of each step, the formulas developed for custom-written calculations, and references to the vendor's user manual for vendor routines utilized; and (*3*) name(s) of the person(s) who prepared the macro, documented approval of the macro, date of its installation, date(s) of revision, and validation tests.

Let us now consider the programs supplied by the vendor of a chromatographic workstation—the programs that process data and control the LC system. Most practicing chemists do not have the ability or inclination to perform systematic software review and validation of the vendor's programs, which may consist of thousands of lines of code. Further, most chemists use integrators or workstations that contain proprietary algorithms that the manufacturer will not share. The "logic" or the "applicability" of these internal programs cannot be verified because a source listing of the code is not available. Even if the manufacturer supplied the source code, it is unlikely that even the most competent programmer could discern all the faults in all the branches and loops of an unfamiliar program.

All equipment manufacturers should have validated their software before releasing it. Some vendors will provide useful information on the behavior and performance of their programs. Inquire about tests the vendor has run on the program code and request copies of the results.

Critique of the Modular Approach

Most chemists cannot evaluate the proprietary programs running in their computerized LC systems, so they require other procedures for system validation. Individual calibrations of injectors, pumps, detectors, analog-to-digital converters, and the like are admirable but difficult. Although they may be useful for diagnosing problems with LC systems, they alone cannot guarantee reliable analytical results for the reasons given earlier.

Holistic Validation

In a holistic validation (Table 2), we use a series of tests to measure and evaluate the performance of the entire computerized LC system under the conditions of its intended use: chemical analyses. The minimum holistic validation is a 2-step process: initial calibration and running calibration. Each laboratory should develop and include other tests to cover its own special requirements.

Initial Calibration—Linearity

Before any samples are run, the entire system—injector, pump, column, detector, data-acquisition device, and data-output device—must be calibrated for linearity (or for reproducible nonlinearity) by injecting several standard solutions of different concentrations. Use at least 4 standard solutions to generate this linearity curve; 0 must not be used as a "data point." Initially, the linearity test must be run daily. If the linearity curve is proven reproducible over several days, the test interval may be lengthened: every other day, weekly, or as specified in SOPs. Documented evidence must be obtained to establish that the specified test interval keeps the LC system in control.

Table 2. Holistic validation of computerized LC systems

Initial Calibration—Linearity
Use at least 4 standard solutions.
Concentration range of standards must span anticipated results, plus safety margin.
Run standards daily[a] before starting sample analysis.
Initial Calibration—System Precision
Calculate precision daily[a] from at least 6 replicate injections of a standard solution before starting sample analysis.
Running Calibration
Run a standard solution at specified time intervals or after a specified number of sample solutions.
Data Processor (Integrator, Computer)
Are the macros coded correctly? Validated? Documented?
System Documentation
Is system documentation in place?

[a] Or at intervals specified in SOP.

How shall the span of concentrations be chosen for these standard solutions? For routine work, in which the result is expected to fall within a known range, the standard curve must cover this range plus a bit more for safety. As an example, when analyzing individual tablets by the United States Pharmacopoeia (USP) procedures, results are normally expected to fall between 85 and 115% of the labeled amount. Linearity standards could be set up to cover 50 to 150% of label, for example. Each laboratory must prepare its own written requirements for calibration span of linearity standards and must not report analytical results calculated from data that fall outside this span.

In research work, the result cannot always be anticipated. Examine the linearity of the system over several concentration decades to ensure usable linearity or to set up guidelines for dilutions or concentrations that are within the acceptable range.

Initial Calibration—System Precision

At first, measure the system precision daily by making at least 6 replicate injections of a single standard solution and by calculating the standard deviation of the standard responses. Only after acceptable system precision with replicate injections of a standard solution is obtained, should sample analysis proceed. If the system precision is proven reproducible over several days, the test interval may be lengthened—every other day, weekly, or as specified in the SOPs—as long as documented evidence is available that adequate system precision is maintained over the chosen interval.

Legally recognized methods, such as those in the *United States Pharmacopoeia* (2) or *Official Methods of Analysis* (3), often give requirements for system precision and procedures to measure it. If these guidelines are lacking, the "acceptable precision" of the process to be controlled must be compared to the precision of the LC system. Unless the latter is much better than the former, the LC system will be of little use. As a rough guide, currently at the Division of Drug Analysis, we routinely obtain precision, expressed as a relative standard deviation, of 1% or less from a given LC system and operator.

Running Calibration

After satisfactory linearity and precision data are obtained and sample analysis has begun, how can quality control be sustained? A standard solution should be run at regular intervals—every 2 h or every 5 samples—or at the interval given in the written SOP. Calibration intervals are arguable, but documented evidence must be provided to establish that the interval selected and specified keeps the LC system in control.

The goal is to document that the system is not drifting or has not experienced a catastrophe. The basic equation is as follows: The more often the system is calibrated, the less likely loss of quality measurements will occur. If the system does develop a problem, how soon will it be discovered? How many samples will have to be rerun since the last good calibration? Will those reruns cost more than the additional calibrations would have? Each laboratory has to balance this quality assurance equation in the most cost-effective way.

Definition and Storage of "Raw Data"

The term "raw data" is broadly defined in FDA's Good Laboratory Practices (21 CFR 58.3(k)) (4). We excerpt the following portions of interest:

Raw data means any laboratory ... records ... that are the result of original observations ... and are necessary for the reconstruction and evaluation of the report of [a] study ... *Raw data* may include ... computer printouts, magnetic media, ... and recorded data from automated instruments.

In the context of computerized LC systems, we further propose the following definition of raw data: Of the data captured by the data processor, raw data are all those that can be saved and accessed later.

First, consider the simplest data processor, an ordinary integrator that furnishes at least 3 items in a printed report: a chromatogram, a list of peak heights or areas vs retention time, and a table of "parameters" used to obtain the chromatogram and data (attenuation, noise rejection, slope sensitivity, construction of baseline, etc.). This simple printout constitutes all the raw data that can be saved and accessed later.

Now consider the other end of the scale—a computerized workstation. The workstation can give a very abbreviated output—a chromatogram, a list of peak heights or areas vs retention time, and a table of parameters—just as with the simple integrator. But now not only this "finished presentation" is available but also additional raw data residing on the hard drive—the detector readings and corresponding times from which the computer program calculated and printed the report. The workstation could capture much more data; obtain UV-VIS spectra of each compound eluting from the column; and obtain spectra taken at the beginning, middle, and end of each emerging peak, etc. If the workstation is instructed to do this, much more raw data will be available on the hard drive for each run.

How long must raw data be saved? The minimum period is the time specified by the current FDA regulations applying to each project. When considering computerized workstations and digitized raw data (and with floppy disks or tape cartridges so cheap these days), it does not make sense to throw away any raw data, especially not the raw data that led to the results reported as final and correct.

Consider the following scenario: a couple of small peaks are detected eluting ahead of the main compound. The current method does not mention these peaks, so they are ignored. A year later, the research group announces it has identified those 2 compounds as highly obnoxious materials, and management wants to know how to determine how much of these materials was in all those lots analyzed last year. What is preferable: rerunning all the samples to integrate those little peaks, or having the computer retrieve the old files of raw data and integrate them?

So, our attitude toward saving raw data is very conservative: save all of it. If all that is available is a simple printed chromatogram and table of results from the integrator, save them. If the computerized workstation was instructed to get a chromatogram at a given wavelength but also to obtain and save the complete UV spectrum of each eluant, save all of those raw data as well.

If possible, equip each computerized workstation with a tape backup card and purchase a tape backup drive. The drive is the most expensive component, but one drive can serve many workstations. Adopt a consistent way of naming files that matches file names with sample numbers or research projects and that prevents overwriting of old files by new files. Then, at the interval specified in written procedures, save all raw data onto a tape cartridge. It is like having a time machine; the workstation can be put back the way it was on January 7, 1991, and again examine the raw data saved.

Definition and Storage of Data Used To Make Decisions

An analyst frequently works with the raw data and adjusts parameters to generate a suitable chromatogram and to obtain acceptable measurements of retention times and peak heights or areas. From these intermediate results, the analyst (or the workstation) calculates the final results used for decision making. Examples of such decisions include whether to recalibrate the LC system, whether a lot passes or fails requirements, whether to adjust a manufacturing process, etc. These data and the conditions used to obtain them should also be saved as paper copies for the length of time specified by the current FDA regulations applying to each project.

Although the trend is toward "paperless laboratories," there are several strong arguments for retaining printed copies of all data used to make decisions. First, given a set of raw data, it is unlikely that 2 qualified independent operators of a computerized LC system will make exactly the same choices about how to treat the raw data to obtain finished results. The operators might choose different data-measurement parameters for smoothing data, noise-rejection levels, points to start and stop integration of peak areas, etc. Unless chromatograms, data-measurement parameters, final data (retention times, peak areas or heights, etc.), and methods of calculating final results are saved as paper copies, it may not be possible to fully reconstruct how the final decision was made. It is also wise to document any problems operators might have encountered in generating final data and results from raw data, such as integration difficulties or any other comments. Second, the LC system, including its workstation, will eventually become obsolete or defective. If the computer or its programs are lost, regenerating final data and results from raw data may be impossible. Third, review by supervisors, quality assurance staff, and FDA investigators will be greatly facilitated by available paper records that document all final decisions.

Responsibilities

Although regulatory liability for failure to calibrate equipment is shared by management within a firm, the primary responsibility for instrument calibration resides with the chemist or technician who produces the data. He or she may receive assistance from repair personnel, but the ultimate responsibility stays with the person generating the data. Management's responsibility includes guidance on what is acceptable; an individual chemist or technician must not have free rein to decide what constitutes good data and what does not.

Why is responsibility placed with the person acquiring the data and with the supervisors to follow SOPs and not with repair personnel or the manufacturer of the equipment? Let us first dispense with "manufacturer responsibility." If the company who made our scientific apparatus could be sued because we cannot control the quality of the data it gave us, we would soon be forced to build our own liquid chromatographs and write our own computer programs to run them. The manufacturers would quickly abandon us for greener, less litigious pastures. Besides, no self-respecting laboratory manager would abrogate his or her responsibility in this manner.

Next, repair personnel do not produce the data and provide the final results to management. How can we hold repair personnel responsible for calibration of the LC system? Even if we did, there might be subtle differences between the test conditions used by repair personnel and the conditions existing when the LC system is being used to analyze samples, as detailed earlier under *Modular Validation*.

Documentation

For an institution to make quality measurements, the very highest management levels must support this commitment. If such support is present, an institution will manifest this commitment in several ways, among them written SOPs that clearly state what management expects and how the staff fulfills these expectations.

As a minimum, a laboratory should have a written SOP for each element of validation it elects to implement (*see* Tables 1 and 2 for example elements). Management must approve these procedures in writing; the procedures must be in the hands of

the operating staff; and the institution's quality assurance unit must check to see that the procedures are being followed.

Granted, reviewing and validating proprietary computer programs purchased from commercial vendors is usually not possible. But, it is best to have documentation on the applicability and limitations of such programs. As a minimum, the user manuals would suffice that were supplied by the firm who sold the proprietary programs. These manuals, plus additional documentation on program behavior and performance tests if supplied by the manufacturer, should be referenced in the written SOP. State that these programs are to be operated as intended and within the limitations specified in the manufacturer's manuals and documentation.

As program revisions are received from the equipment manufacturer or from other program vendors, their installations should be documented: date installed, which systems were upgraded, etc. References to new user manuals and new manufacturer's documentation on program behavior and performance tests should also be updated in the laboratory's written operating procedures. If a laboratory has several similar instruments, it is desirable to use the same revision of the program on all of them. If there are "compatibility problems" among revisions, using the same revision throughout an installation may become a necessity.

Regardless of the data processor employed, it is important to describe raw data and to include a definition of raw data in the operating procedures for the specific types of testing conducted.

Summary

Even if all elements of a modular validation were passed, there would be no direct assurance that final analytical results are correct. We urge all laboratories to test variable-wavelength LC detectors for wavelength accuracy. Otherwise, we suggest the holistic approach to calibration and validation of computerized LC systems. Because reviewing and validating proprietary programs is usually unfeasible, other methods must be used to verify them. Chemists should use chemical methods, as outlined, to achieve these goals.

Acknowledgments

We thank Henry Avallone, David Barr, Paul Motise, Frank Kerner, and John Reepmeyer, U.S. Food and Drug Administration, for review and useful comments.

References

(1) Tetzlaff, R.F. (1992) *Pharm. Technol.* **16** (5), 70, 72, 74–78, 80–83

(2) *United States Pharmacopoeia* (1990) 22nd Rev., U.S. Pharmacopeial Convention, Rockville, MD

(3) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA

(4) *Code of Federal Regulations* (1993) Title 21, Part 58, Section 3(k), pp. 247–248

STATISTICAL ANALYSIS

Display Methods for Visual Comparison of the Results of Two Measurement Methods

CHRISTA HARTMANN and DESIRÉ L. MASSART
Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

The use of a plot originally proposed by Bland and Altman (1986, *Lancet* 8, 307–310) for the comparison of 2 clinical measurement methods was investigated and compared with a new visual display based on principal component analysis. The characteristics of both methods are demonstrated for several computer-simulated situations. For visual comparison of 2 methods, it is recommended to use the 2 methods simultaneously, together with a plot of the results of method 2 against method 1.

Received August 9, 1993. Accepted by GL September 8, 1993.

In method validation, a new measurement method is often validated by comparing it with a formerly used, already validated method. This comparison is done by analyzing a set of samples covering the concentration range to be validated.

Table 1. Fat in pork products[a]

	Fat, %			
	AOAC		Instrument	
Product	X1	|d1|	X2	|d2|
1	4.63	0.00	4.83	0.00
2	7.39	0.80	6.74	0.14
3	8.76	0.24	8.46	0.09
4	11.85	1.30	12.60	0.33
5	13.67	0.49	13.52	0.14
6	15.80	1.31	15.72	0.62
7	16.05	0.12	15.83	0.24
8	18.22	0.74	18.37	0.54
9	18.79	0.90	18.90	0.34
10	22.80	0.15	23.10	1.03
11	32.53	0.39	32.45	0.05
12	41.03	0.82	40.89	0.64
13	41.52	0.26	41.42	0.77
14	43.70	0.16	43.36	0.49
15	45.89	0.03	45.96	0.59
16	47.81	1.16	47.70	0.04
17	50.11	0.56	50.02	0.07
18	57.51	0.65	57.20	0.39
19	79.38	0.09	78.48	0.40

[a] Data adapted from reference 1. X1, mean of duplicate analyses with AOAC method; X2, mean of duplicate analyses with instrumental method; d1, differences between 2 replicates of AOAC method (variance, 0.2302); d2, difference between 2 replicates of instrumental method (variance, 0.1047).

In general, a statistical test is applied to compare the results of the samples analyzed with both methods.

As an example consider Table 1, derived from reference 1. The data concern the validation of methods for determining fat in meat products. A newly available instrumental method is compared with an existing AOAC method. The results for 19 pork products, analyzed with both methods in replicate, are given in Table 1. After testing that the variances of both methods are not significantly different (F-test), the authors applied a paired t-test to conclude that there is no significant difference between the 2 measurement methods.

Another approach is to compare the results of both methods by regression and to check if there is a significant difference between the coefficients of the regression line and the line that would have been obtained when both methods give the same results. When both measurement methods differ only by random error, then the regression line has a slope $\beta_1 = 1$ and an intercept $\beta_0 = 0$. β_1 and β_0 are estimated by the calculated coefficients b_1 and b_0, and the test consists of deciding whether they differ significantly from 1 and 0. When the deviations from 1 or 0 are significant, the presence of a proportional ($\beta_1 \neq 1$) or an absolute ($\beta_0 \neq 0$) systematic error is indicated.

However, as in every other application field, when such statistical techniques are applied in method comparison, it is not recommended to use only numerical results. Visual evaluation of results is recommended both to observe sources of variation in the data and to detect possible problems with the statistical hypotheses.

The most common visualization method is a simple plot of the results of method 2 against method 1 (Figure 1a). However, because of the scale effect, it is sometimes difficult to detect problems with this plot. Bland and Altman (2) proposed a simple but efficient visualization procedure to evaluate results of 2 measurements. The differences between 2 results are plotted against the means of these 2 measurements. Hartley (3) slightly simplified their plot. To detect a trend in measurement results, he suggests to plot only the differences between 2 measurement results in increasing order of the means. With the visual display, attention is drawn to the extreme values and small biases, which probably would be unnoticed with only a statistical test.

As an example to illustrate the method of Bland and Altman, we applied it to the data of the fat determinations given in Table 1. The differences between the instrumental method and the AOAC method are plotted against the means of both methods. As Figure 1b shows, the measurement results are more or less randomly scattered around the horizontal line zero, and it may be assumed that there is no systematic difference between both methods. The result for product 19 seems rather far from the

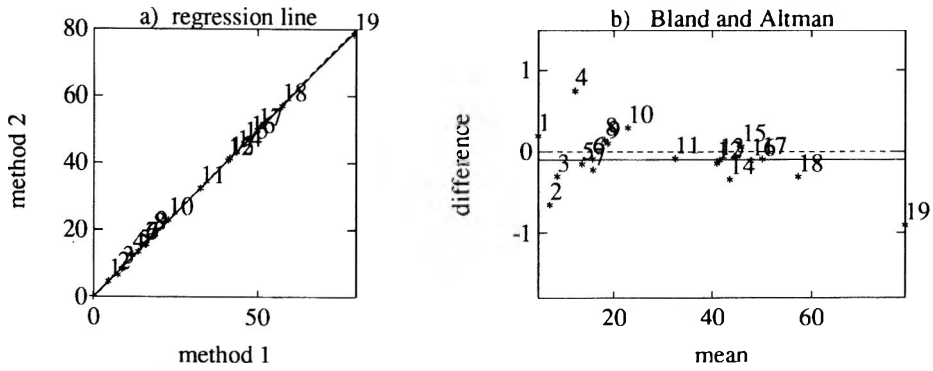


Figure 1. Fat determination in meat products (data in Table 1). (a) Regression line of the results of the 2 measurement methods: ———, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ———, mean of the differences; – – – –, line with difference 0.

line of no difference in comparison with the other points. It is not impossible that, at this extreme concentration, the instrumental method gives a low result. Therefore, the quality of the methods at this concentration level should be investigated.

Recently, Lyon et al. (4) described the use of Bland and Altman's visualization procedure to detect a small bias and an incorrect model selection that was not noticed with regression analysis. We wanted to investigate whether it would be useful to apply another type of visual display based on principal component analysis (PCA).

Experimental

Description of the PCA Method

The concept of the new visual display based on PCA is illustrated in Figure 2. With PCA, the original variables are transformed (by linear transformation) to new variables, summarizing the data in a more informative way. To capture as much information as possible, the first of these new variables (PC1) is chosen to lie in the direction with most variation of the data. This first principal component, therefore, describes the general size of the data and is sometimes called a size component (Figure 2). The second variable (PC2), orthogonal to PC1, then shows mainly a contrast between variables (5). The value of a data point projected on a principal component is called the score for this principal component. Consequently, a plot of the scores on PC1 against the scores on PC2 is a plot that compares size (PC1) and contrast or difference (PC2). A PCA plot, therefore, resembles in principle somewhat the original plot of Bland and Altman because the mean of the methods describes the general size of the data and the difference can be viewed as a contrast. It seemed, therefore, indicated to investigate to what extent PCA can be applied as a visualization procedure.

An additional useful characteristic of the PCA method is that the slope of PC1 is identical with the linear regression line

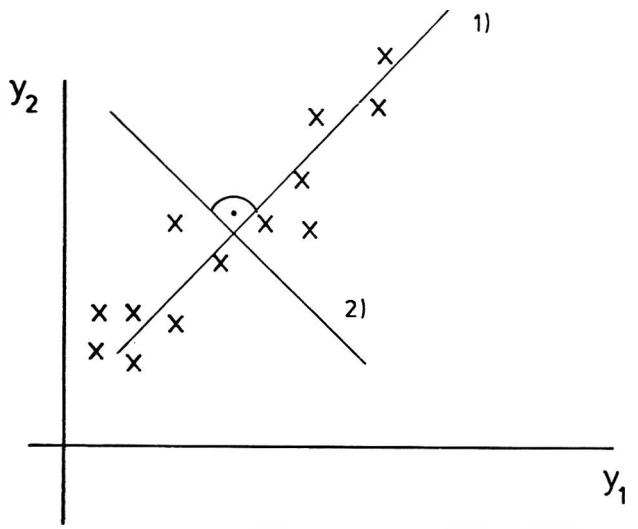


Figure 2. Principal components 1 and 2. PC1 and PC2 through a scatter of points: (1) PC1; (2) PC2.

that takes into account the fact that there is measurement error in both variables (6). Usually, ordinary least squares (OLS) is applied to calculate such a regression line. However, in method comparison studies with a restricted measurement range, as for example in clinical chemistry, OLS significantly underestimates the slope of the linear regression line (6). This underestimation can be explained by the fact that OLS is strictly correct only for calculations with only one variable subject to error. To take into account the measurement error of both variables, the residuals should be calculated orthogonal to the linear regression line and not parallel to the y axis, as in OLS. When both variables have the same measurement error, this orthogonal regression line is identical with the line of the first principal component (PC1) of a column mean-centered $n \times 2$ data matrix, where n is the number of measurements (6). The residuals, orthogonal to PC1, are in the second principal component (PC2).

Consequently, the score plot of PC1 against PC2 is not only a graph relating size and difference but also a visual display of the residuals to the appropriately calculated regression line, when both variables are equally subject to error.

Simulated Data

To demonstrate the different possibilities of these visualization procedures and their advantages for checking measurement results and finding disagreement, the following situations were simulated:

(*1*) There are no blank problems or matrix interferences; that is, there is no bias.

(*2*) The measurement results of the 2 methods still agree well as in case 1. However, the variance of the measurement error of both measurement methods is not constant over the measurement range (heteroscedasticity); that is, repeatability is not a constant function of concentration. A special case of heteroscedasticity, a constant relative standard deviation, is chosen because of its importance in analytical chemistry.

(*3*) The method to be tested has a blank problem. The results of this method differ from the reference method by a constant value.

(*4*) There are matrix interferences. Systematically, higher results are obtained with the method to be validated than with the other. The differences are proportional to the concentration.

(*5*) The chosen model of a linear relationship between the methods is not correct. There is a disagreement between the 2 methods at higher concentrations. Therefore, the new method is valid only in a restricted concentration range.

The computer simulations were made with a computer program written in Visual Basic (7). Nineteen values were randomly generated following a rectangular distribution in the range 1–5. The biases were introduced as follows: In case 3, a constant value of 0.3 was added to the points of method 2. In case 4, a proportional error of 15% was added to the points of method 2. In case 5, a quadratic term (15% of the square of the value) was added to all points of method 2. A randomly generated, normally distributed measurement error was superimposed for the 2 methods. This random measurement error had either a constant variance (the standard deviation was 2% of the

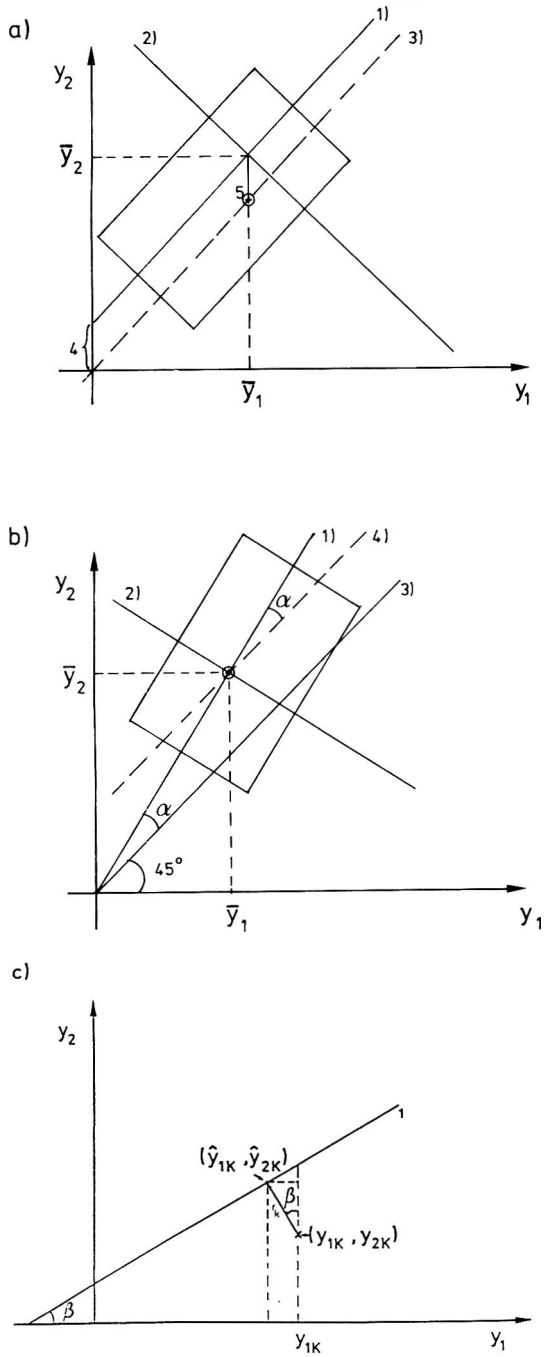


Figure 3. Display of the position of the score plot (⊡) in the (y_1,y_2) variable space. (a) Situation with a constant systematic error: ⊕, point where the origin of PC1, PC2 would lie with zero intercept; (1), PC1; (2), PC2; (3), expected line with slope 1 and intercept 0; (4), intercept in y_1,y_2; (5), scores of intercept for ($\bar{y}_1$, $\bar{y}_2$). (b) Situation with a proportional systematic error: (1), PC1; (2), PC2; (3), expected line (slope 1 and intercept 0); (4), in score plot indicated line with slope 1 through point ⊕. (c) Orthogonal residuals: r_k, orthogonal residual for point k; (1), PC1, y_1: method 1, y_2: method 2, $\hat{y}_{1k}$, $\hat{y}_{2k}$: predicted values for methods 1 and 2, respectively.

mean of the range) (cases 1, 3, 4, and 5) or a constant relative standard deviation (2% of the original value) (case 2).

The calculations for the plots and the plots themselves were made with Matlab (8). The singular value decomposition (svd) function of Matlab was used to calculate PC1 and PC2 of the $n \times 2$ column mean-centered data matrix consisting of the n results of both measurement methods. The slope was in all cases calculated with the assumption of the same random measurement error in both methods. To visualize a systematic error between 2 measurement methods, the following 2 elements were added to the score plot (Figures 3a and 3b). A constant error is visualized with the distance between the origin (0,0) of PC1,PC2 and the point ⊕ (Figure 3a). The point ⊕ represents the point where the origin of PC1,PC2 would lie with $b_0 = 0$ (i.e., if no constant error occurred). Through mean centering during the svd computation, the origin of PC1,PC2 corresponds to the means of both measurement methods, that is, $(\bar{y}_1, \bar{y}_2)$. The point ⊕ can, therefore, be computed as the negative value of the intercept $b_0 (= \bar{y}_2 - \text{slope}_{pc1} \times \bar{y}_1)$ expressed in scores for PC1 and PC2 and illustrated for the point $(\bar{y}_1, \bar{y}_2)$. A proportional error is illustrated through the deviation of the line of PC1 and a dashed line (Figure 3b). The dashed line has a slope of 1. With this dashed line, the deviation angle (α) of PC1 from the 45° line is indicated. The line is drawn through the origin of PC1,PC2 with $b_0 = 0$. To have PC1 with a 45° angle to the x axis (parallel to a slope $b_1 = 1$, $b_0 = 0$), PC1 has to be rotated with α round the point ⊕ in the direction of the dashed line.

The residual plot of the calculated orthogonal residuals against the mean of the predicted values for both measurement methods is also given. The calculation of the residuals is illustrated in Figure 3c. To avoid unclear situations due to scale effects, the expected line with $b_1 = 1$ and $b_0 = 0$ is not given in the same plot.

Results and Discussion

For illustration, the score plot of the data of the fat determination (Table 1) is given in Figure 4c. For convenience, Figures 1a and 1b are repeated as Figures 4a and 4b, respectively. The score plot is quite similar to the plot of Bland and Altman (Figure 4b). The residual plot (Figure 4d) gives the same scatter of the points around the regression line as the score plot. Again, the plot leads to the conclusion that point 19 may be wrong or that nonlinearity might occur as the central residuals more or less fall on the same side of the zero line. This conclusion indicates that further statistical or experimental work is required. In the present instance, an F-test on the coefficient of the quadratic term was carried out. Because this test was not significant, it can be concluded that nonlinearity cannot be shown.

Figures 5a–5d through 9a–9d show that the position of the calculated orthogonal regression line relative to the expected line with slope 1 and intercept 0 is best visually represented with the regression plot. However, it is easier to interpret visually the data structure with one of the more complex display methods (Bland and Altman's method, PC [principal compo-

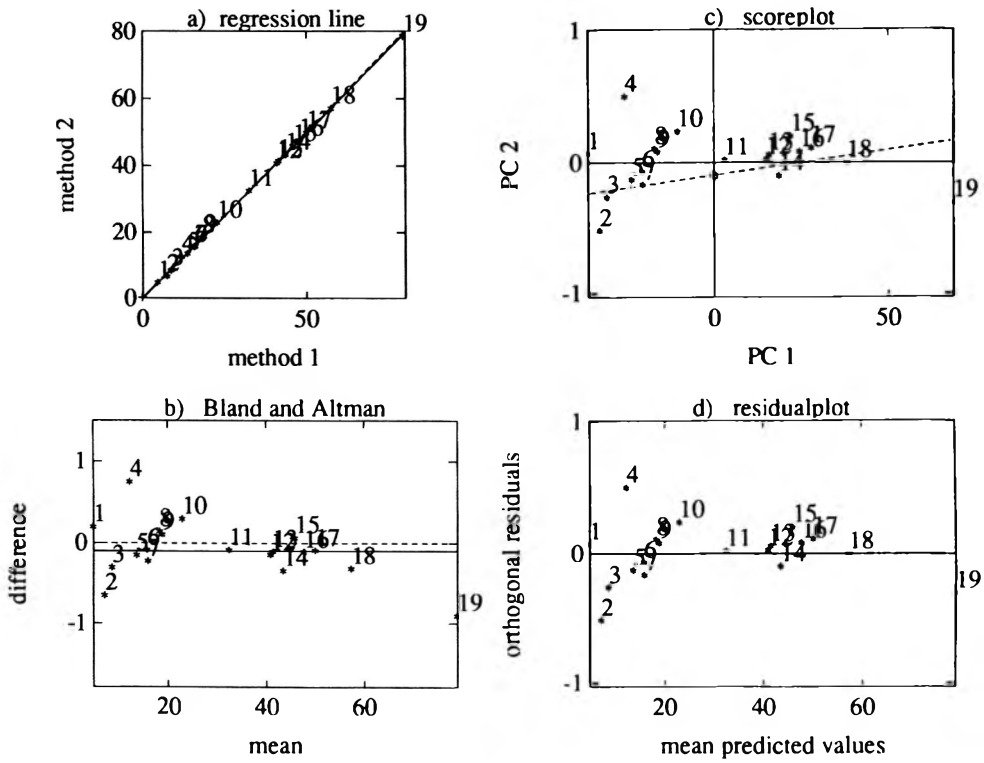


Figure 4. Fat determination in meat products (data in Table 1). (a) Regression line of the results of the 2 measurement methods: ——, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ——, mean of the differences; – – – –, line with difference 0. (c) PC score plot for the results of the 2 measurement methods. (d) Plot of the orthogonal residuals.

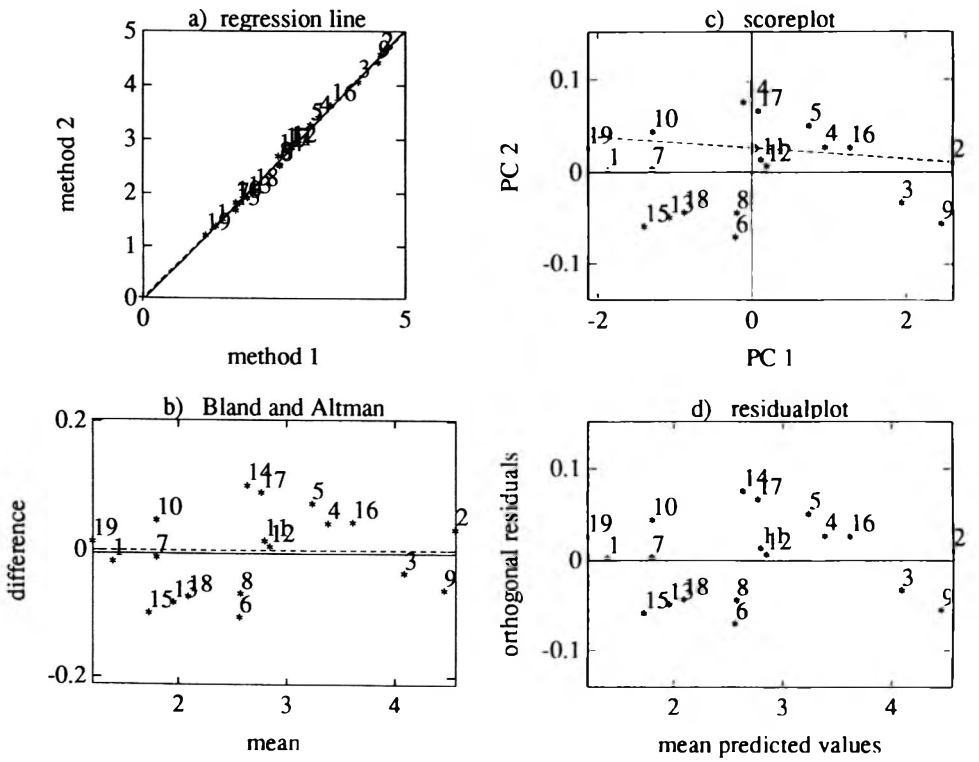


Figure 5. Computer-simulated data with a homoscedastic random measurement error in both methods and no systematic error (case 1; *see* text). (a) Regression line: ——, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ——, mean of the differences; – – – –, line with difference 0. (c) PC score plot. (d) plot of the orthogonal residuals.

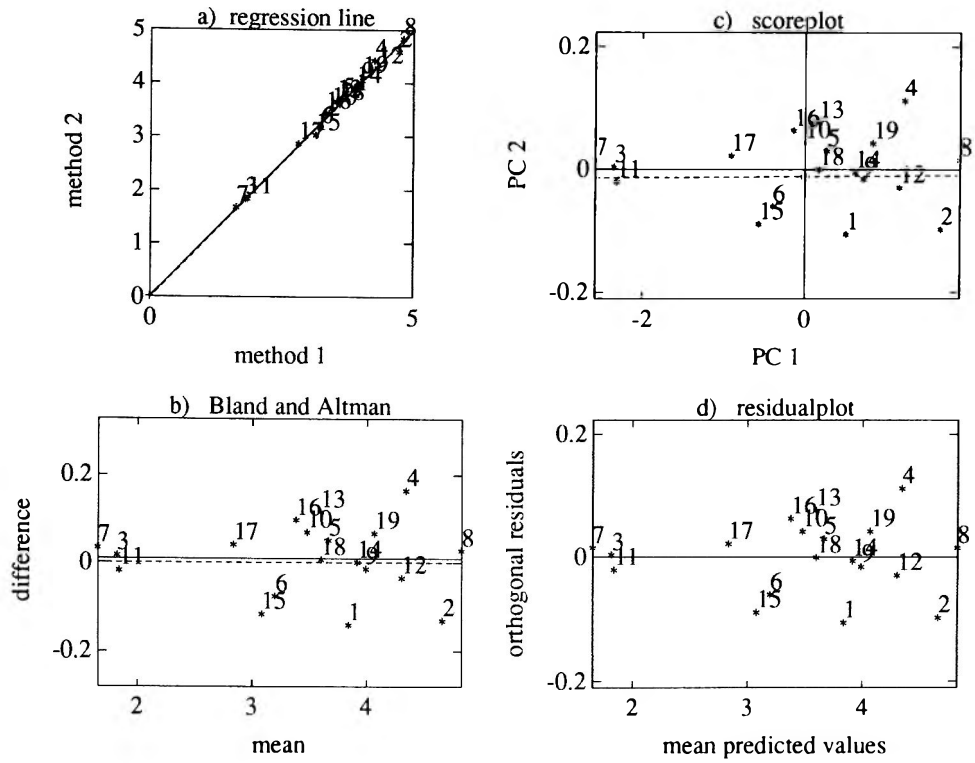


Figure 6. Computer-simulated data with a heteroscedastic random measurement error in both methods and no systematic error (case 2; *see* text). (a) Regression line: ———, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ———, mean of the differences; – – – –, line with difference 0. (c) PC score plot. (d) Plot of the orthogonal residuals.

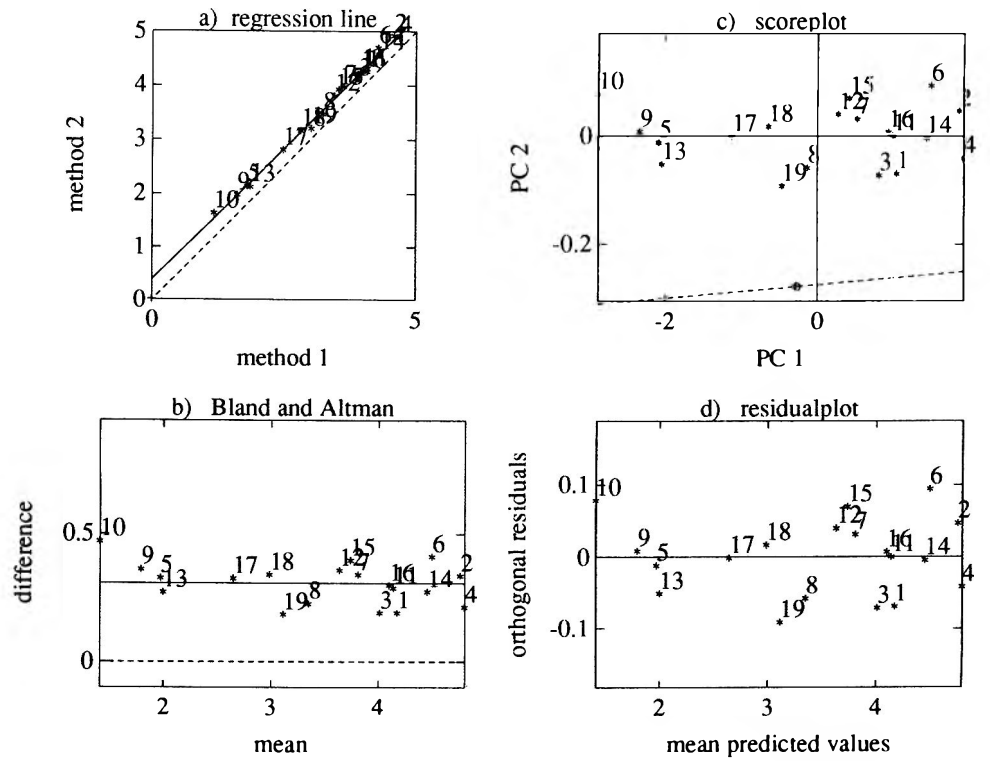


Figure 7. Computer-simulated data with a homoscedastic random measurement error in both methods and a constant bias between methods 1 and 2 (case 3; *see* text). (a) Regression line: ———, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ———, mean of the differences; – – – –, line with difference 0. (c) PC score plot. (d) Plot of the orthogonal residuals.

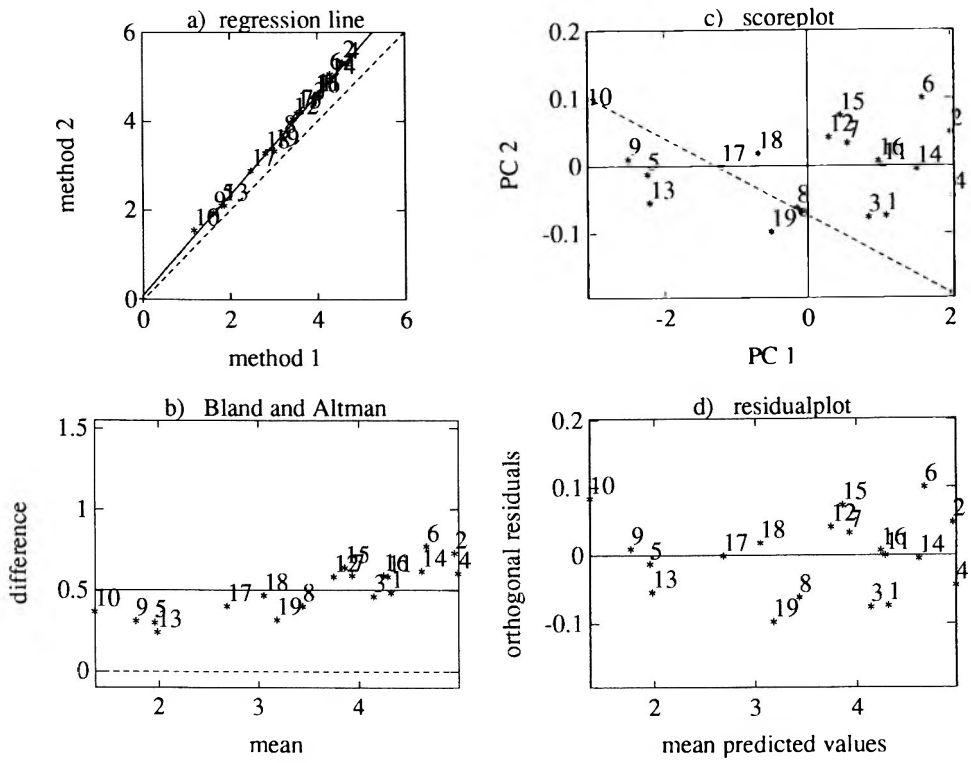


Figure 8. Computer-simulated data with a homoscedastic random measurement error in both methods and a proportional bias between methods 1 and 2 (case 4; *see* text). (a) Regression line: ———, calculated with PCA; – – – –, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ———, mean of the differences; – – – –, line with difference 0. (c) PC score plot. (d) Plot of the orthogonal residuals.

nent] score plot or orthagonal residual plot) than with graphs that simply plot results of one method against another (regression plot).

Although both visualization procedures, Bland and Altman and the one based on PCA, give rather similar graphs, there are some differences due to the data treatment with PCA.

When the results of both measurement methods agree well (Figures 5 and 6), there is no striking difference between the score plot and residual plot and the Bland and Altman plot.

When a systematic bias between both measurement methods occurs, the Bland and Altman plot gives a different picture for an absolute or a proportional systematic error (Figures 7 and 8).

It is in these cases that it is sometimes difficult to decide if the observed increase or decrease of the differences with higher increasing mean values is due to a proportional systematic error (Figure 8) or if this phenomenon is due to a nonlinear relationship (Figure 9).

In the PC score plot, a systematic error cannot be detected over the whole measurement range without additional hints about the position of the expected line with slope 1 and intercept 0. On the other hand, with the additional dashed line in the score plot of PC1/PC2, one has the advantage of the visualized deviation angle between the calculated slope and the expected slope. The estimate of the extent of a constant bias is possible with the indication of the intercept (⊕). A disadvantage of the proposed PC score plot is, however, that visualization of the data scatter and the bias is obtained in units other than those of the original results, which can confuse a user who is inexperienced with PCA. The residual plot (which shows the same data scatter as the score plot) no longer has this disadvantage, and it is easy to judge the importance of the size of a certain residual at a specific concentration level at first sight. Because with the PC procedures residuals to the regression line (calculated by least squares) are obtained, a single outlier can affect this line and, therefore, all residuals. With Bland and Altman's procedure, on the other hand, an outlying point affects only the coordinates of the outlier.

For both procedures, the classification of the plots in one of the cases described becomes much more difficult if the data points are not almost rectangularly spread over the measurement range, especially with a low measurement precision. Moreover, the distinction between homoscedasticity and heteroscedasticity (cases 1 and 2) is possible only if both variables have the same kind of measurement errors; that is, both must have a homoscedastic (or a heteroscedastic) measurement error. In simulated cases with homoscedasticity for method 1 and heteroscedasticity for method 2, the kind of measurement error could not be detected from the plots of Bland and Altman and the PC score plot/residual plot; neither is it detected in cases with more than 19 measurement points. Therefore, it is advisable first to check the measurement error of both methods by applying the visual comparison procedure to 2 replicates of one method instead of applying it to 2 different methods.

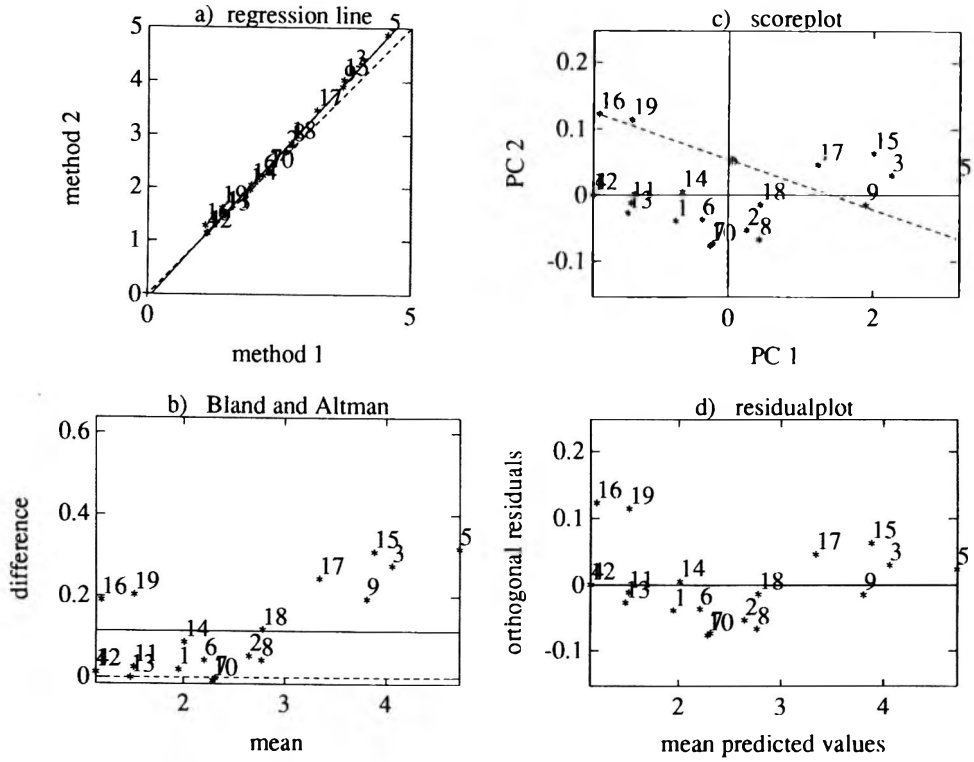


Figure 9. Computer-simulated data with a homoscedastic random measurement error in both methods and a nonlinear relationship between methods 1 and 2 (case 5; *see* text). (a) Regression line: ___, calculated with PCA; —, expected line with slope 1 and intercept 0. (b) Bland and Altman plot: ___, mean of the differences; —, line with difference 0. (c) PC score plot. (d) Plot of the orthogonal residuals.

Conclusion

The residual plot, the more easily interpretable variant of the score plot, enables visualization of the orthogonal residuals toward the linear regression line between the 2 methods being compared. We recommend its use simultaneously with visualization by the classical regression plot and by Bland and Altman's method. Each procedure has its own advantages, which cannot be summarized in one easily understandable graph.

Acknowledgment

We thank Fonds voor geneeskundig wetenschappelijk onderzoek (FGWO) for financial assistance.

References

(1) Wernimont, G.T. (1985) in *Use of Statistics to Develop and Evaluate Analytical Methods*, W. Spendley (Ed.), AOAC, Arlington, VA

(2) Bland, J.M., & Altman, D.G. (1986) *Lancet*, **8**, 307–310

(3) Hartley, T.F. (1990) in *Computerized Quality Control Programs for the Analytical Laboratory*, 2nd Ed., J.F. Tyson & M.M. Masson (Eds), Ellis Horwood, New York, NY

(4) Lyon, T.D.B., Fell, G.S., McKay, K., & Scott, R.D. (1991) *J. Anal. At. Spectrom.* **6**, 559–564

(5) Lewi, P.J. (1992) in *COMETT Course Introduction to Multivariate Techniques for Chemical Applications*, Janssens Pharmaceutica NV, Belgium

(6) Hartmann, C., Smeyers-Verbeke, J., & Massart, D.L. (1993) *Analusis*, **21**, 125–132

(7) Microsoft Visual Basic, Programming System for Windows, Version 1.0, Microsoft Corp.

(8) PC-Matlab for MS-DOS Personal Computers, The Math Works, Inc., Version 3.5k

STATISTICAL ANALYSIS

Detection of Milk Fat Adulteration by Linear Discriminant Analysis of Fatty Acid Data

FRANZ ULBERTH
Agricultural University, Department of Dairy Research and Bacteriology, Gregor Mendel-Str. 33, A-1180 Vienna, Austria

Analysis of the fatty acid (FA) profile of milk fat (MF) by gas-liquid chromatography is widely used to detect adulteration with foreign fats. On the basis of the FA spectra of 352 genuine Austrian MF samples collected over a 4-year period, the effectiveness of concentration ranges of the major FA of MF and of certain FA ratios to identify non-MF/MF mixtures was tested. FA ratios proved useful for the detection of coconut fat in MF and admixture of vegetable oils rich in linoleic acid down to a level of 2%. This approach failed to identify non-MF/MF blends containing beef tallow, lard, olive oil, or palm oil at a level less than 10% commingling. Linear discriminant analysis applied to FA data was successful in distinguishing pure MF from adulterated MF. Computer-simulated data were used to derive the discriminant functions. Saturated and unsaturated FA with 18 C atoms were the most useful discriminating variables selected by a stepwise variable selection procedure. More than 95% of a data set composed of pure MF, and non-MF/MF blends containing 3% of either tallow, lard, olive oil, or palm oil were correctly classified. The validity of the classification rule was also tested by 206 gravimetrically prepared fat mixtures. Mixtures containing >3% foreign fat were detected in all cases.

In most countries, milk fat (MF) is normally higher priced than other edible fats. If the price is high for a certain product and low for another group of different but otherwise related items, a distinct incentive exists for the unscrupulous manufacturer to commingle the cheaper product into the higher priced goods to reduce costs and to improve profits. Among fats and oils, MF, virgin olive oil, and cacao butter are vulnerable to such malpractices. Adulteration of these food commodities has been common since ancient times and, moreover, "...continues as a major challenge to the chemists of modern times" (1). The problem is further intensified by the legalization of butter–margarine blends in most western countries. Therefore, the authority has to verify the correct labeling of these products to protect consumers from fraudulent practices.

Received June 7, 1993. Accepted by JL September 28, 1993.

Different analytical techniques were proposed to state the genuineness of MF (2). Analysis of the sterol fraction is a well-known and widely used approach to detect vegetable fats in MF (3–5). An adulteration is verified if sterols other than cholesterol are present. In addition to sterols, analysis of tocopherols or tocotrienols is another valuable tool for assessing the genuineness of MF (6, 7). However, both methods are only applicable to the detection of foreign fats of vegetable origin.

Infrared spectroscopic and thermoanalytical methods were also proposed as means for detecting adulterated MF (8, 9).

The occurrence of short-chain fatty acids (FA) in MF is a characteristic often used for authentification. Simple methods were devised for the group separation of these constituents, e.g., the Reichart-Meissl and the Polenske value (10). With the advent of gas–liquid chromatography (GLC), the classical fat values were substituted by the analysis of the total FA spectrum. Strategies to detect adulterated MF can be based on either the concentration ranges of individual FA or the concentration ratios of 2 or more FA. Although low limits of detection are obtainable for mixtures of MF with certain fats and oils of vegetable origin, sensitivity toward falsification with animal fats, e.g., beef tallow or lard, is unsatisfactory. In general, the detectability of tallow in MF is in the range of 15–25% admixture (11–13). On the contrary, Toppino et al. (14) reported that blends consisting of 10% tallow in MF were traceable in 83% of the cases tested through a combination of several FA ratios.

Analysis of intact triacylglycerols (TG) was proposed as an alternative to detect adulterated MF. Timms (15) developed an approach combining TG analysis with multivariate evaluation of the results that allowed the determination of non-MF, including animal fats, in MF down to a level of 5%. His basic idea was developed further by Precht and Heine (16, 17) and by Precht (18, 19). With the elaborated "general" TG formula, the detection of 2 to 5% foreign fat in MF is possible. For vegetable fats, slightly better limits of detection (2–4%) than for animal fats (tallow and lard) are dealt with (18). Several collaborative studies organized by the European Community (EC) demonstrated the reliability of the TG formula method (20).

Compared with FA analysis, the techniques necessary for TG separation by GLC are much more stringent with respect to column inertness, carrier gas purity, sealing materials (septa, ferrules), etc., because of the high temperatures used (up to

350°C). Therefore, GLC hitherto was mostly used to analyze the FA composition of MF. This fact resulted in detailed knowledge of the regional and seasonal variations in the FA profile of MF and, moreover, in a higher degree of expertise in MF FA analysis compared to MF TG analysis.

In combination with multivariate evaluation methods, especially linear discriminant analysis (LDA), FA data were successfully applied to problems regarding the distinction of olive oils (21) and fish oils (22) according to their geographical origin, mixtures of MF of different ruminant animals (23), and mixtures of animal depot fats (24). It is hypothesized that this approach should also be of great value for detecting adulterated MF. If the information content of an FA chromatogram is treated adequately, the final outcome, i.e., a statement about the genuineness of the fat, should be as efficient as the result obtained by the TG method. The objective of the present work was to prove the validity of this assumption.

Experimental

Materials

Synthetic TG standards were of highest purity (>99.0%) and were purchased from Sigma (Sigma Chemie, Deisenhofen, Germany). Cyclohexane, *n*-hexane, and diethylether were from E. Merck (Darmstadt, Germany). Diethylether was redistilled before use. Austrian butter and MF samples (in total 352) collected in 1987–1991 represented the set of genuine MF. Butter was molten and the clear fat layer was filtered over anhydrous Na_2SO_4 to remove traces of moisture. Vegetable oils were donated by Unilever-Austria and Ebhardt & Herout, Vienna. Tallow and lard samples were provided by a local rendering plant (Schachinger, Vienna). An MF with certified FA composition (reference material CRM 164) was obtained from the Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium.

Apparatus

A Mega 5160 gas chromatograph equipped with a flame ionization detector (FID) and an on-column autosampler AS 550 (Carlo Erba Strumentazione, Milan, Italy) was used. Chromatogram processing was done with an SP 4270 integrator interfaced to an IBM-compatible PC/AT running the WINner software (Spectra-Physics, San Jose, CA). FA separation was accomplished with a CP-Wax 58 CB fused silica column (25 m × 0.32 mm id, film thickness 0.2 μm, Chrompack, Middelburg, The Netherlands). On-column injection of 1 μL samples was performed at an oven temperature of 35°C. After 3 min, the oven was programmed to a temperature of 110°C at 10°C/min (held there for 1 min), and to 230°C at 6°C/min (held there for 10 min). Hydrogen at 35 kPa was the carrier gas.

Transesterification

Transesterification of fat samples was done according to Christopherson and Glass (25). A 10% solution of fat in *n*-hexane was vigorously shaken with 200 μL 2M KOH in dry methanol for 1 min. To prevent saponification of the formed fatty acid methyl esters (FAMEs), 0.5 g $KHSO_4 \cdot H_2O$ was added after a further reaction time of 5 min. Two microliters of the ester-containing solution were added to 1 mL redistilled diethylether in an autosampler vial and crimp-capped.

Calibration and Validation of the GLC Method

A calibration mixture that consisted of the saturated TG covering the range of the even-numbered FA with 4 to 18 C atoms and resembling the composition of MF was prepared gravimetrically. This mixture was dissolved in cyclohexane, transesterified, diluted, and chromatographed. Correction factors were calculated to account for the uneven FID-response toward short- and long-chain FA. The content of an individual FA was reported as g per 100 g total FA.

The complete method was validated by means of the reference material CRM 164. At the beginning of a workday, the performance of the overall technique was checked by an injection of the transesterified reference material. In addition, reference samples were included in the sampling sequence (every 6th or 7th vial). If the values of the reference sample did not comply with the certified values (mean values ± uncertainty) the instrument setup was checked, recalibrated with the primary standard, if necessary, and the sequence was reanalyzed.

Fat mixtures

Mixtures of MF and non-MF were simulated arithmetically by the relationship:

$$\begin{aligned}\text{Proportion of an individual FA in the mixture} = \\ [\%\ \text{FA in MF}] \times [\%\ \text{MF in the mixture}] \\ + [\%\ \text{FA in non-MF}] \times [\%\ \text{non-MF in the mixture}]\end{aligned}$$

Statistical Analysis

The SAS/STAT Release 6.03 program (SAS Institute, Cary, NC) run on an IBM-compatible PC was used for computations. Texts by Fahrmeier et al. (26), Srivastava and Carter (27), and Lachenbruch (28) aided in interpreting the results.

Results

Fatty Acid Composition and Fatty Acid Ratios

Although a multitude of different FA can be found in MF of ruminant animals, only those FA that contributed to more than 0.2 g/100 g of the FA spectrum were selected for further statistical treatment. Major FA were identified by comparison to authentic standard substances. In cases where standards were not available, equivalent chain length values calculated according to Van den Dool and Kratz (29) and literature data (30, 31) facilitated tentative identification of unknowns. Average concentration values of 21 FA are listed in Table 1. Because of the strict quality control scheme applied, the FA data were highly reliable. This was demonstrated by a comparison of mean FA values of CRM 164 based on 54 replicates analyzed over a period of 12 weeks and the corresponding certified values (mean values ± uncertainty) (Table 2). Only for the very volatile C4:0

Table 1. FA concentration values (g individual FA/100 g of total FA) of 352 Austrian MF samples

Fatty acid	Mean	Std	CV, %	Maximum	Minimum
C4:0	3.71	0.132	3.56	4.02	3.17
C6:0	2.20	0.099	4.52	2.38	1.88
C8:0	1.22	0.077	6.27	1.36	0.99
C10:0	2.63	0.235	8.95	3.07	2.03
C12:0	2.98	0.315	10.58	3.62	2.25
C14:0	10.51	0.761	7.23	11.73	8.66
C14:1	0.88	0.097	10.94	1.13	0.67
C15:0 ISO[a]	0.35	0.023	6.70	0.42	0.29
C15:0 AISO[b]	0.55	0.037	6.74	0.69	0.48
C15:0	1.34	0.065	4.82	1.52	1.18
C16:0	30.00	2.931	9.77	34.74	25.05
C16:1	1.54	0.085	5.56	1.77	1.27
C17:0 ISO	0.59	0.041	6.95	0.72	0.44
C17:0 AISO	0.50	0.039	7.74	0.84	0.43
C17:0	0.81	0.108	13.29	1.04	0.05
C18:0	9.13	0.994	10.88	11.42	7.45
C18:1ω9	19.60	1.724	8.80	23.62	16.79
C18:1ω7[c]	2.75	1.195	43.45	5.85	1.21
C18:2	1.21	0.115	9.54	1.93	0.88
C18:3	0.84	0.186	22.17	1.32	0.31
C18:2 CONJ[d]	0.95	0.411	43.14	2.15	0.38

[a] Iso-branched FA.
[b] Antiso-branched FA.
[c] Mixture of *cis* and *trans* isomers.
[d] 9 *cis*, 11 *trans* octadecadienoic acid.

methyl ester and C18:3 was a relative standard deviation (RSD) >2% observed. (The first figure indicates the number of C atoms in the carbon chain of the fatty acid. The second figure indicates the number of double bonds in the molecule. The distance of a double bond from the methyl end of the FA chain is given by the ω-notation.) As standard deviation values for individual FA were also known from the certification experiment, the obtained values were compared with the reference values by means of Student's *t*-test. In all cases, no significant difference between the reference values and the experimentally obtained values was found ($P > 0.10$, double-sided test).

Various FA ratios are recommended in the literature to authenticate MF (13, 14, 32–34). Some of these ratios and the respective values for Austrian MF are compiled in Table 3.

To test the effectiveness of absolute FA concentration values and FA ratios for identifying adulterated MF, mixtures of pure MF and foreign fats (6 different samples of coconut fat, palm oil, n=5; olive oil, n = 3; lard, n = 6; tallow, n = 10; and a total of 7 vegetable oils including sunflower, soy, corn, safflower, grape kernel, and unlabeled salad oil) were produced by computer simulation. The FA spectra of vegetable oils were very similar. Therefore, they were combined in one group and average composition values were calculated and used in the computations. To prepare samples with a 3% admixture of tallow to MF, e.g., each tallow sample was numerically blended with the 352 MF samples, giving a total of 3520 blends. Out of this, a random sample of 352 tallow/MF mixtures was taken for analysis. Blends with 1 to 5% and 10% foreign fat in MF were

Table 2. Comparison of certified and experimentally found FA concentration values (g individual FA/100 g total FA) of reference material CRM 164

Fatty acid	Certified value[a]	Found value[b]	Maximum	Minimum
C4:0	(3.67)[c]	3.61 ± 0.086	3.83	3.46
C6:0	2.36 ± 0.19	2.26 ± 0.032	2.33	2.17
C8:0	1.36 ± 0.10	1.32 ± 0.018	1.36	1.27
C10:0	2.89 ± 0.12	2.84 ± 0.025	2.90	2.78
C12:0	4.03 ± 0.10	3.98 ± 0.035	4.10	3.92
C14:0	10.79 ± 0.35	10.66 ± 0.091	10.96	10.48
C14:1	(1.07)	1.00 ± 0.016	1.04	0.96
C15 ISO	(0.32)	0.29 ± 0.004	0.30	0.29
C15 AISO	(0.51)	0.51 ± 0.009	0.53	0.50
C15:0	(1.04)	1.05 ± 0.009	1.08	1.03
C16:0	26.91 ± 0.84	26.75 ± 0.192	27.07	26.12
C16:1	(1.48)	1.35 ± 0.015	1.39	1.32
C17 ISO	(0.45)	0.56 ± 0.009	0.58	0.53
C17 AISO	(0.42)	0.46 ± 0.007	0.47	0.44
C17:0	(0.51)	0.61 ± 0.010	0.64	0.58
C18:0	10.51 ± 0.40	10.32 ± 0.087	10.69	10.20
C18:1 (total)	24.82 ± 0.61	24.98 ± 0.177	25.58	24.51
C18:2 (total)	2.68 ± 0.40	2.53 ± 0.037	2.60	2.36
C18:2 CONJ	(0.86)	0.87 ± 0.010	0.90	0.85
C18:3	0.51 ± 0.04	0.52 ± 0.011	0.56	0.51

[a] Mean value ± uncertainty.
[b] Mean value ± standard deviation of 54 injections obtained during a 12-week analysis period.
[c] Values in parentheses are indicative values.

Table 3. FA ratios used for authentification purposes of genuine MF

Fatty acid ratio	Mean	Std	Minimum	Maximum
A C4:0/C6:0	1.688	0.083	1.428	1.923
B C6:0/C8:0	1.806	0.046	1.969	1.698
C C10:0/C8:0	2.148	0.071	2.325	1.985
D C12:0/C10:0	1.132	0.023	1.194	1.090
E C14:0/C12:0	3.542	0.142	3.900	3.181
F C14:0/C18:0	1.173	0.206	1.529	0.771
G C14:0/C18:2	8.838	1.320	13.077	5.708
H C16:0/C12:0	10.086	0.494	11.393	8.641
I C16:0/C14:0	2.849	0.102	3.060	2.573
J C18:0/C8:0	7.551	1.264	10.851	5.650
K C18:0/C12:0	3.134	0.679	4.988	2.101
L C18:1/C18:0	2.153	0.105	2.477	1.836
M C18:2/C8:0	0.994	0.143	1.458	0.705
N C4:0/(C6:0+C8:0)	1.086	0.063	1.265	0.841
O C12:0/(C4:0+C6:0)	0.504	0.048	0.608	0.395
P C18:0/(C6:0+C8:0)	2.687	0.416	3.715	2.054
Q (C18:1+C16:0)/C4:0	13.391	0.718	18.910	11.577
R (C6:0+C8:0+C10:0+ C12:0)/C18:0	1.008	0.181	1.353	0.653
S (C18:0+C18:1)/(C6:0+C8:0)	8.450	1.204	11.605	6.675

produced in this way to test the efficiency of FA data for authentification.

The validity of computer-simulated FA spectra of non-MF/MF mixtures was checked by analyzing gravimetrically prepared tallow/MF mixtures covering the range of 0.94 to 25.97% tallow (8 blends in total). FA values obtained experimentally were compared with the FA values computed from the known FA spectra of the starting materials. In general, differences were <0.1 g individual FA/100 g total FA, with one exception. At an addition level of 16.95% tallow, the C16:0 concentration found was 0.25% (m/m) lower than the computed value.

The admixture of a certain amount of a foreign fat with a high concentration of a particular FA in its spectrum, i.e., C12:0 in coconut fat (ca 45%), C18:2 in vegetable oils (ca 55%), and C18:1 in olive oil (ca 75%) should shift the FA concerned out of the range that is normally encountered with genuine MF. This assumption proved to be true for coconut fat and vegetable oils. Because of the high content of C12:0, the addition of 5% coconut fat to MF moved the minimum value of the blends (4.39% C12:0) well above the maximum value of genuine MF (3.62% C12:0). For vegetable oils, the minimum amount necessary to displace the C18:2 content to abnormal values was 3% (minimum value of the blends 2.44% vs a maximum of 1.93% in genuine MF). Despite the extremely high content of C18:1 in olive oil, additions of 10% olive oil to MF were not detectable with high certainty. This was attributed to the wide intrinsic variation range of C18:1 in unadulterated MF. Palm oil and animal fats added at a 10% level paralleled olive oil in that no discriminating effect was exhibited by at least one FA.

FA ratios (Table 3) were more effective in detecting adulterated MF compared to concentration ranges of certain FA. Admixtures of coconut fat or vegetable oils were traceable down to 2% by ratios D and E (for coconut fat) or M (for vegetable oils). Ratios G, I, M and G, L, M proved useful to detect blends containing 10% palm oil or olive oil, respectively. Whereas at the 10% level all adulterated samples were positively identified with the ratios mentioned, only 47.4, 68.2, and 51.7% of the MF samples mixed with palm oil at the 5% level and 42.3, 73.0, and 42.9% of the samples containing 5% olive oil were correctly classified. Ten percent lard in MF could be detected in all cases by means of ratios G and M, which were also effective in 61.6 and 71.9% at 5% commingling. As expected, the worst situation was observed with MF/tallow mixtures. Ratio I was most useful for detecting adulteration with tallow (63.4% of the samples with 10% tallow, 14.8% with 5% tallow).

Linear Discriminant Analysis

As coconut fat and vegetable oils in mixtures with MF were unequivocally identifiable with excellent sensitivities using FA ratios, these non-MF types were excluded from LDA. A data set was prepared that was composed of genuine MF and computer-simulated mixtures of 3% of tallow, lard, palm oil, and olive oil in MF and was designated MIX3. Mean FA values of MIX3 are listed in Table 4. Although differences between the group means were small, analysis of variance indicated significant differences in most cases ($P < 0.001$). Insignificant ($P > 0.15$) were the group differences of C18:1 ω7, C18:3, and C18:2 CONJ. Variables with high discriminating power were selected with procedure "Stepdisc" of the SAS/STAT software. Minimization of Wilks' Lambda was the optimization criterion chosen in the stepwise variable selection procedure. All variables contributed to the separation of the groups and were, therefore, included in the calculation of the discriminant func-

Table 4. Group means of major FA of genuine and adulterated MF (adulteration level 3%)

Fatty acid	Pure MF	Tallow	Lard	Palm oil	Olive oil
C4:0	3.71	3.60	3.58	3.61	3.60
C6:0	2.20	2.13	2.13	2.14	2.13
C8:0	1.22	1.18	1.18	1.18	1.18
C10:0	2.62	2.54	2.54	2.55	2.53
C12:0	2.98	2.88	2.88	2.89	2.87
C14:0	10.51	10.25	10.22	10.25	10.20
C14:1	0.88	0.86	0.85	0.85	0.85
C15:0 ISO	0.34	0.34	0.33	0.33	0.33
C15:0 AISO	0.55	0.54	0.53	0.53	0.53
C15:0	1.33	1.31	1.29	1.29	1.30
C16:0	29.99	29.79	29.77	30.47	29.37
C16:1	1.53	1.55	1.54	1.49	1.50
C17:0 ISO	0.58	0.58	0.57	0.56	0.57
C17:0 AISO	0.50	0.51	0.49	0.48	0.48
C17:0	0.82	0.84	0.81	0.79	0.80
C18:0	9.13	9.56	9.33	8.98	8.96
C18:19	19.59	19.91	20.22	20.07	21.21
C18:17	2.75	2.84	2.79	2.67	2.72
C18:2	1.20	1.25	1.48	1.44	1.43
C18:3	0.84	0.84	0.84	0.81	0.83
C18:2 CONJ	0.95	0.94	0.93	0.92	0.92

tions that were derived by SAS/STAT procedure "Discrim." C18:2 followed by C18:0, C18:1 ω9, C16:1, and C6:0 were the variables with the highest discriminating power.

To classify a test sample into one of the groups, the FA composition had to be determined by GLC, and the concentration values obtained thereby were inserted in the discriminant functions (coefficients are given in Table 5). The sample was assigned to the group for which the discriminant score gave a maximum.

The classification rule was checked in 2 ways. First, it was checked by cross validation, which meant that the LDA program computed the discriminant functions with *n-1* observations of the training set and then applied them to classify the one case left out. This operation was repeated for each case in the training set. The total error rate was 4.38% (Table 6). Nine samples from the tallow/MF as well as from the lard/MF blends were identified as genuine MF, and 24 pure MF were "false-positively" assigned as adulterated. None of the cases in the remaining vegetable oil/MF groups were incorrectly classified as genuine MF. Canonical discriminant analysis (SAS/STAT procedure "Candisc") was used to visualize the classification of pure MF samples and non-MF/MF mixtures (Figure 1). The first 2 canonical functions that were also used as axes in the 2-dimensional projection accounted for 96% of the total between-groups' variability. As can be seen from Figure 1, vegetable oil/MF blends were very well separated from pure MF and MF in mixtures with animal fats. Thus, we attempted to achieve a better distinction among the animal fat groups by excluding the palm oil and the olive oil group from LDA. Unfortunately, the discrimination was not further improved (total error rate 5.11 vs 4.38% in the original model).

The cross-validated error rate improved when a data set (MIX4) containing 4% tallow in MF but otherwise identical to MIX3 was used for LDA. Only 4 tallow blends and 16 genuine MF samples were misclassified (5 as adulterated with tallow, 8 with lard, and 2 with palm oil).

In the second check for the classification rule, the discrimination model was tested with 206 gravimetrically prepared and chromatographically analyzed MF mixtures. Pure MF samples were randomly chosen from the original set and mixed with non-MF to give mixtures that contained between 1 and 30% foreign fat. In addition, 12 pure but otherwise unknown MF samples were also included in the trial. The test data set was classified by the discriminant model obtained with the MIX3 data set. Two in 81 tallow/MF blends were incorrectly classified as genuine MF (Table 7). However, these 2 samples contained only 1.41 and 1.15% tallow, respectively. Other blends misclassified as pure MF contained <3% foreign fat throughout. On the other hand, mixtures containing a very low amount of foreign fat, e.g., 2.02% lard in MF, were sometimes correctly classified. One of the pure test MF samples was false-positively identified as adulterated with tallow.

Discussion

To find out the origin of a certain object or to discern what class it belongs to is a common problem in food or agricultural chemistry. Provided a fundamental difference exists in composition between the 2 assignments, the classification can be

Table 5. Coefficients of linear discriminant functions to distinguish genuine MF and MF in mixtures with other fats

Fatty acid	Pure MF	Tallow	Lard	Palm oil	Olive oil
Constant	−17650.0	−17559.0	−17681.0	−17875.0	−17984.0
C4:0	122.0	126.5	121.2	122.8	109.0
C6:0	976.9	909.6	944.0	959.5	1064.0
C8:0	383.2	341.8	342.5	277.6	235.0
C10:0	−2961.0	−2869.0	−2953.0	−2988.0	−3084.0
C12:0	3767.0	3709.0	3776.0	3886.0	3959.0
C14:0	284.6	269.2	279.0	241.8	263.9
C14:1	−1666.0	−1580.0	−1618.0	−1620.0	−1690.0
C15:0 ISO	62.4	86.7	43.8	87.7	1.8
C15:0 AISO	1376.0	1361.0	1425.0	1499.0	1532.0
C15:0	895.4	847.4	868.0	874.6	912.8
C16:0	425.1	427.7	428.2	441.8	436.4
C16:1	−1320.0	−1284.0	−1341.0	−1535.0	−1563.0
C17:0 ISO	−1064.0	−1043.0	−1033.0	−1058.0	−1063.0
C17:0 AISO	−1073.0	−990.0	−1131.0	−1103.0	−1244.0
C17:0	1350.0	1348.0	1339.0	1376.0	1400.0
C18:0	188.7	206.6	191.1	170.6	153.5
C18:19	523.2	514.4	524.2	541.4	551.7
C18:17	137.6	136.7	137.7	139.2	140.6
C18:2	260.9	262.5	299.0	290.7	291.6
C18:3	765.3	760.8	777.4	766.1	782.5
C18:2 CONJ	1359.0	1351.0	1353.0	1397.0	1389.0

based on the analysis of one or more critical component(s). The distinction between animal and vegetable fats according to the presence or absence of cholesterol and phytosterols in the unsaponifiable is an example for this deductive approach. Unfortunately, in most practical situations, fundamental differences are lacking. Therefore, a classification scheme relying on quantitative differences of properties common to both (all) groups has to be derived empirically. A prerequisite for this inductive approach is that the absolute amounts of the discriminating variables—FA in the present case—are determined with high reliability. Otherwise, concentration ranges of certain criteria used to assess the genuineness of MF are needlessly broadened. To illustrate this aspect, C4:0 concentration values in MF of U.S. origin as low as 1.61% and as high as 5.5–6.0% may be found in the newer literature (1, 35). Validation of the complete technique applied by means of a certified reference material as described in this study seems to be a necessity to ensure between-laboratory comparability of analytical results.

In concordance with other authors, FA data (absolute concentration or FA ratio ranges) could only be used to advantage for the detection of MF adulterated with coconut fat or vegetable oils. Ratio M permitted the detection of 2% vegetable oil in MF, whereas Fox et al. (36) reported 8.2% partially hydrogenated vegetable oil as the limit of detection. They used the ratio of C4:0/C18:1 as a purity criterion.

Table 6. Cross-validated classification results of the data set MIX3 (group assignment based on the linear functions given in Table 5)

	Samples classified in				
Samples	Pure MF	Tallow	Lard	Palm oil	Olive oil
Pure MF	328	15	7	2	0
(%)	93.18	4.26	0.99	0.57	0.00
Tallow	9	336	7	0	0
(%)	2.56	95.45	1.99	0.00	0.00
Lard	9	3	334	6	0
(%)	2.56	0.85	94.89	1.70	0.00
Palm oil	0	0	1	342	9
(%)	0.00	0.00	0.28	97.16	2.56
Olive oil	0	0	0	9	343
(%)	0.00	0.00	0.00	2.56	97.44

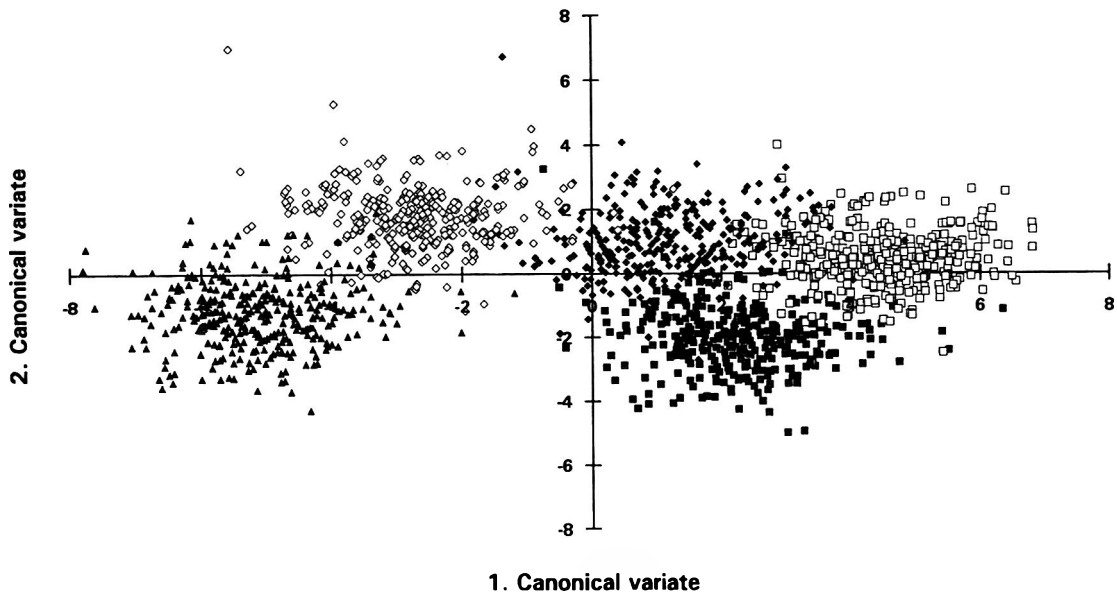


Figure 1. Canonical plot of pure MF (■) samples and MF blends containing 3% tallow (□), lard (◆), palm oil (◇), or olive oil (▲).

Low amounts (<10%) of animal fats, palm oil, or olive oil in MF remained undetected by the FA concentration as well as by the FA ratio approach. In contrast to the findings of Precht (12), additions of ≥10% olive oil or palm oil were traceable in all cases. According to him, the most sensitive ratios for the detection of palm oil, olive oil, lard, and tallow admixture to MF, i.e., C16:0/C14:0, C18:1/C18:0, C18:2/C4:0, and C18:0/C4:0, resulted in detection limits ranging from 16.4% (lard) to 21.3% (palm oil).

Also proposed as means to improve the sensitivity of certain tests was taking into account the variation of MF FA profiles caused by feeding and/or lactation and, moreover, by the dependency of FA purity criteria on the production period (37). To stabilize the natural variability of the MF composition, a "weighted average" of 3 TGs that are typical for MF was introduced by Timms (15). The weights were chosen by multiple regression analysis so as to give a mean value close to 100. Therefore, the dependent variable is a constant rather than a continuous variable; one of the assumptions underlying parametric regression models (normal distribution) is thereby violated. LDA is a technique closely related to regression analysis except that a weighted combination of variables is used to predict the discrete class to which an item belongs. FA data as variables in LDA allowed segregation of olive oils and fish oils according to their geographical origin (21, 22). In the same way, LDA in combination with MF FA analysis was effective to differentiate pure milk from different ruminant species (cow, sheep, and goat) and mixtures thereof (23). The variables C10:0, C12:0, C4:0, C8:0, and C14:1 were most important to discriminate between cow and goat milk mixtures, whereas C4:0, C14:1, C16:0, C14:0, and C10:0 proved to be useful to separate sheep and goat milk mixtures (FA listed according to

Table 7. Classification results of gravimetrically prepared non-MF/MF mixtures used for validation of the discrimination model (group assignment based on the linear functions given in Table 5)

	No. samples classified				
Samples	pure MF	Tallow	Lard	Palm oil	Olive oil
Pure MF	11	1	0	0	0
(%)	91.67	8.33	0.00	0.00	0.00
Tallow	2	79	0	0	0
(%)	2.47	97.53	0.00	0.00	0.00
Lard	4	5	56	0	0
(%)	6.15	7.69	86.15	0.00	0.00
Palm oil	2	0	2	25	1
(%)	6.67	0.00	6.67	83.33	3.33
Olive oil	1	0	1	3	25
(%)	3.33	0.00	3.33	10.00	83.33

their separation power). In general, in ≥90% of the cases, a correct classification was possible of pure milk samples and mixtures composed of pure milks to which 1/10 by volume of milk from a different species was added. The same working group proposed a technique based on principal components analysis to classify depot fat from different animals and their mixtures (24). The approach is primarily aimed at presenting hull boundaries of different classes graphically. The results obtained showed that for the particular application tested the method was valid, though mixtures of 1 part beef and 2 parts horse fat were occasionally misclassified.

Saturated and unsaturated FAs with 18 C atoms, especially C18:2, exhibited the highest discrimination effect for segregating groups of pure MF and non-MF/MF mixtures, respectively (Table 5). C_{18} acids as variables in LDA were also most effective for classifying Spanish olive oil samples regarding their variety assignment (38). To obtain correct classifications of depot fat samples of beef cattle (*Bos taurus*) and Malagasy zebu (*Bos indicus*), minor FA (concentration <0.1%) was considered (39). In the present study, those FAs did not contribute to any greater extent to the group separation of MF and MF mixtures.

With the discriminant coefficients given in Table 5, 95.6% of the 1760 cases contained in the training sample were correctly classified. Although the proportion of the pure MF samples identified "false positively" as adulterated was comparatively high, the validation of the classification scheme with a separate set of test samples resulted in a very high degree of correct classification and excellent sensitivities toward foreign fats admixed to MF. Blends that contained ≥3% of either tallow, lard, olive oil, or palm oil could be distinguished from pure MF by LDA. Compared with the TG formula method (12, 18), the limit of detection of the proposed approach is equivalent. Moreover, LDA offers the additional benefit that, besides providing information about the genuineness of MF, the fat type added to MF can be inferred from the classification outcome.

With the MIX4 training set, the rate of misclassified pure MF samples dropped to 4.26%. Higher levels of non-MF in the mixtures led to a further reduction of false-positively grouped pure MF (data not shown). Unfortunately, this measure also increased the limit of detection for foreign fats in MF when checked by the gravimetrically prepared test data. By using the MIX3 data set for setting up the discriminant functions, a compromise was reached that balanced demands on test sensitivity on one hand with misclassification rates on the other.

A further advantage of the outlined procedure is that routines to perform LDA are implemented in most statistical computation packages (e.g., BMDP, SAS, SPSS) that are, in turn, in widespread use. This fact allows other workers to check whether the discriminant coefficients given in Table 5 apply to their own data without amendments or adaptions caused by regional individualities. Likewise, use of these programs enables a direct classification of unknown samples. The calibration information obtained by the training set is saved and used to classify new observations. In addition to this, the probability that a sample belongs to a certain group is also provided by the computerized evaluation procedure. These group membership probabilities aid in interpreting the classification results. If substantial uncertainty about the group membership exists, further testing of the suspect sample is warranted.

References

(1) Iverson, J.L., & Sheppard, A.J. (1989) *J. Dairy Sci.* **72**, 1707–1712

(2) Collomb, M., & Spahni, M. (1991) *Trav. Chim. Aliment. Hyg.* **82**, 615–662

(3) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 28.096–28.099

(4) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 28.100–28.103

(5) Homberg, E., & Bielefeld, B. (1979) *Z. Lebensm. Unters. Forsch.* **169**, 464–467

(6) Coors, U., & Montag, A. (1985) *Milchwissenschaft* **40**, 470–473

(7) Keeney, M., Bachmann, K.C., Tikriti, H.H., & King, R.L. (1971) *J. Dairy Sci.* **54**, 1702–1703

(8) De Ruig, W.G. (1968) *Neth. Milk Dairy J.* **22**, 104–113

(9) Lambelet, P., & Ganguli, N.C. (1983) *J. Am. Oil Chem. Soc.* **60**, 1005–1008

(10) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 28.039–28.040

(11) Parodi, P.W. (1971) *Austr. J. Dairy Technol.* **26**, 155–158

(12) Precht, D. (1991) *Fat Sci. Technol.* **93**, 538–544

(13) Muuse, B.G., Werdmuller, G.A., Geerts, J.P., & de Knegt, R.J. (1986) *Neth. Milk. Dairy J.* **40**, 189–201

(14) Toppino, P.M., Contarini, G., Traversi, A.L., Amelotti, G., & Gargano, A. (1982) *Riv. Ital. Sost. Grasse* **54**, 592–610

(15) Timms, R.E. (1980) *J. Dairy Res.* **47**, 295–303

(16) Precht, D., & Heine, K. (1986) *Milchwissenschaft* **41**, 329–334

(17) Precht, D., & Heine, K. (1986) *Milchwissenschaft* **41**, 406–410

(18) Precht, D. (1992) *Z. Lebensm. Unter. Forsch.* **194**, 1–8

(19) Precht, D. (1992) *Z. Lebensm. Unters. Forsch.* **194**, 107–114

(20) Precht, D. (1992) *Dtsch. Molkerei Ztg.* **113**, 796–803

(21) Eddib, O., & Nickless, G. (1987) *Analyst* **112**, 391–395

(22) De Jong, S. (1991) *Mikrochim. Acta II*, 93–101

(23) Smeyers-Verbeke, J., Massart, D.L., & Coomans, D. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1382–1385

(24) Thielemans, A., de Brabander, H., & Massart, D.L. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 41–47

(25) Christopherson, S.W., & Glass, R.L. (1969) *J. Dairy Sci.* **52**, 1289–1290

(26) Fahrmeir, L., Häußler, W., & Tutz, G. (1984) in *Multivariate Statistische Verfahren*, Fahrmeir, L., Hamerle, A. (Eds), Walter de Gruyter, New York, NY, pp. 301–370

(27) Srivastava, M.S., & Carter, E.M. (1983) *An Introduction to Applied Multivariate Statistics*, North-Holland, NY

(28) Lachenbruch, P.A. (1979) *Biometrics* **35**, 69–85

(29) Van den Dool, H., & Kratz, P.D. (1963) *J. Chromatogr.* **11**, 463–471

(30) Ackman, R.G. (1986) in *Analysis of Oils and Fats*, Hamiltion, R.J., & Rossell. J.B. (Eds), Elsevier Applied Science Publishers, New York, NY, pp. 137–206

(31) Christie, W.W. (1988) *J. Chromatogr.* **441**, 305–314

(32) Martinez-Castro, I., Juarez, M., & Martin-Alvarez, P.J. (1979) *Milchwissenschaft* **34**, 207–210

(33) Huyghebaert, A., & Hendrickx, H. (1971) *Milchwissenschaft* **26**, 613–617

(34) Timmen, H. (1982) *Proc. XXI. Int. Dairy Congr.* 368–369

(35) Bitman, J., & Wood, D.L. (1990) *J. Dairy Sci.* **73**, 1208–1216

(36) Fox, J.R., Duthie, A.H., & Wulff, S. (1988) *J. Dairy Sci.* **71**, 574–581

(37) Fox, J.R., Duthie, A.H., & Wulff, S. (1989) *J. Dairy Sci.* **72**, 1981–1985

(38) López Sabater, M.C., Boatella Riera, J., & De la Torre Boronat, M.C. (1986) *Rev. Française Corps Gras* **33**, 65–67

(39) Gaydou, E.M., Iatrides, M.-Ch., Ramananarivo, R., Artaud, J., & Pfeiffer, G. (1984) *J. Agric. Food Chem.* **32**, 651–655

TECHNICAL COMMUNICATIONS

Identification of Synthetic Colors in Beverage Alcohol Products by Solid-Phase Extraction and Thin-Layer Chromatography

SUMER M. DUGAR, JEFFREY N. LEIBOWITZ, and RANDOLPH H. DYER
Department of the Treasury, Bureau of Alcohol, Tobacco, and Firearms, National Laboratory Center, 1401 Research Blvd, Rockville, MD 20850

A solid-phase extraction (SPE) sample cleanup and enrichment procedure coupled with thin-layer chromatography (TLC) is described for the enrichment of synthetic colors in all types of beverage alcohol products. SPE followed by TLC provides the identification of synthetic colors at sub-part-per-million levels. Two complementary solvent systems afford detection of all the permitted (and some prohibited) synthetic colors in beverage alcohol products. This method is simple, offers consistency, and has no artifacts in TLC.

Several methods were reported for the detection and identification of synthetic colors in foods and beverages (1–9). However, none of the reported methods is useful for all types of beverage alcohol products. Steele (8) described a thin-layer chromatographic (TLC) procedure for synthetic colors in alcoholic beverages that required an elaborate sample preparation and a large quantity of extraction solvent. Despite the labor-intensive extraction procedure, satisfactory results were often not obtained due to froth formation during extraction, streaking on the plate, and overlapping of color bands. Young (9) described the use of a C_{18} cartridge for solid-phase extraction (SPE) of synthetic colors in foods and drugs. This SPE cleanup and extraction procedure is not suitable for the detection and identification of synthetic colors in alcoholic beverages due to the presence of ethanol in the product. Even after 10- to 15-fold dilutions of the alcoholic beverage with water, the C_{18} cartridge failed to retain the synthetic colors. Neither of these methods specifies TLC conditions, such as the spotting specifications, the concentration of the standard, the amount of sample application required for appropriate visualization, or the detection limits.

We report the use of an amino SPE cartridge for sample cleanup and enrichment, followed by TLC for the detection of 11 synthetic colors encountered in beverage alcohol products (Tables 1 and 2). This quick and simple sample cleanup and enrichment procedure before TLC affords detection and identification of synthetic colors at sub-part-per-million levels (<1 ppm).

Received June 14, 1993. Accepted by JL November 23, 1993.

Experimental

Apparatus

(a) *Centrifuge.*—Sorvall Superspeed RC2-B, or equivalent.

(b) *Heat lamp.*—Fisher Infra-Rediator Model 11-504-5V4, or equivalent.

(c) *Micropipets.*—5 and 10 μL.

(d) *Saturation pad.*—Analtech SP-240, or equivalent.

(e) *Screw-cap centrifuge tubes.*—100 mL.

(f) *SPE cartridge.*—Fisher Scientific PrepSep-NH_2 (Amino), or equivalent.

(g) *SPE vacuum manifold.*—Supelco Visiprep, or equivalent.

(h) *TLC developing chamber.*—Analtech Cat. No. 75-11, or equivalent.

(i) *TLC plates.*—Whatman High Performance LHP-K, 10 × 10 cm, 200 μm layer of silica gel or, equivalent.

(j) *Volumetric flask.*—25 mL.

(k) *Weighing balance.*—Sartorius Model A 200 S, or equivalent.

Reagents

(a) *Acetonitrile.*—LC grade.

(b) *Ammonium hydroxide.*—Reagent grade.

(c) *1-Butanol.*—Certified ACS.

(d) *2-Butanol.*—Certified ACS.

(e) *Deionized (DI) water.*—Type I reagent water.

(f) *Ethanol.*—USP dehydrated, 200 proof.

(g) *Methylene chloride.*—LC grade.

(h) *Methyl ethyl ketone.*—Certified ACS.

(i) *Sodium chloride solution.*—Prepare by dissolving 0.5 g sodium chloride (ACS grade) in 100 mL DI H_2O.

(j) *Standard colors.—System A.*—FD&C Blue No. 1, FD&C Blue No. 2, FD&C Green No. 3, FD&C Red No. 1, FD&C Red No. 2, FD&C Red No. 3, FD&C Red No. 4, FD&C Red No. 40, FD&C Yellow No. 5, and FD&C Yellow No. 6; U.S. Food and Drug Administration certified. *System B.*—Patent Blue V, Sigma Chemical research grade.

(k) *Stock standard solutions.*—Weigh 5 mg of each color into separate 25 mL volumetric flasks, dissolve, and dilute to volume with ethanol–DI water (1 + 1, v/v). Label each solution with appropriate name, concentration, and expiration date

Table 1. Retention factors (R_f) of synthetic colors using solvent system A

Synthetic color	Dye C.I. No.	Dye EEC No.	R_f
Red No. 3 (Erythrosine)	45430	E 127	0.83
Red No. 4 (Ponceau SX)	14700	—	0.70
Red No. 40 (Allura Red)	16035	E 129	0.67
Yellow No. 6 (Sunset Yellow FCF)	15985	E 110	0.65
Blue No. 2 (Indigo Carmine)	73015	E 132	0.64
Red No. 1	15850	—	0.60
Patent Blue V	42051	E 131	0.55
Green No. 3 (Fast Green FCF)	42053	—	0.50
Blue No. 1 (Brilliant Blue FCF)	42090	E 133	0.48
Red No. 2 (Amaranth)	16185	E 123	0.25
Yellow No. 5 (Tartrazine)	19140	E 102	0.18

(6 months from the date prepared except Blue No. 2, which must be prepared fresh, as needed). Keep refrigerated.

(l) *Sulfuric acid.*—1N, certified Fisher Scientific.

(m) *Tetrahydrofuran.*—Certified ACS.

(n) *Working standard solutions.*—Dilute stock standard solutions appropriately with ethanol–DIH_2O (1 + 1) to prepare working standards at the concentrations shown below. Label each working standard appropriately with expiration of 1 month from the date prepared, except Blue No. 2, which must be prepared fresh, as needed. Blue No. 1, Green No. 3, Patent Blue V, Red No. 2, and Red No. 4 were diluted to 25 ppm; Blue No. 2, Red No. 1, Red No. 3, Red No. 40, Yellow No. 5, and Yellow No. 6 were diluted to 50 ppm.

Sample Preparation

Listed below are the SPE sample cleanup and enrichment procedures for various types of beverage alcohol products. With the exception of the dairy and nondairy cream liqueurs (which are emulsions), no sample preparation is required prior to SPE.

Table 2. Retention factor (R_f) of synthetic colors using solvent system B

Synthetic color	R_f
Red No. 3	0.54
Red No. 40	0.40
Yellow No. 6	0.35
Patent Blue V	0.35
Red No. 1	0.33
Red No. 4	0.30
Blue No. 1	0.28
Red No. 2	0.10
Green No. 3	0.10
Yellow No. 5	0.03
Blue No. 2[a]	—

[a] Blue No. 2 fades away on the plate during TLC plate development.

Distilled spirits, coolers, malt beverages, and wines.—Attach SPE-NH_2 cartridge to vacuum manifold and apply a vacuum of –2 in. Hg. This vacuum should be applied throughout the extraction. Deliver 50 mL sample onto SPE-NH_2 cartridge. Wash with 10 mL ethanol, then with 10 mL DI water, and finally with 0.4 mL ethanol–1N sulfuric acid (1 + 1). Discard waste. Elute synthetic colors with 1 mL ethanol–1N sulfuric acid (1 + 1).

Dairy cream liqueurs.—Transfer 25 mL sample to a 100 mL screw-cap centrifuge tube. Add 25 mL ethanol. Cap and vigorously shake for 2 min. Centrifuge at 2500 rpm for 10 min. Attach SPE-NH_2 cartridge to the vacuum manifold and apply a vacuum of –5 in. Hg. This vacuum should be applied throughout the sample delivery and washing steps. Carefully deliver supernatant layer onto SPE-NH_2 cartridge. Repeat the same operation with additional 25 mL sample (to make a total of 50 mL sample delivered onto the SPE-NH_2 cartridge). Wash with 10 mL ethanol, then with 10 mL DI water, and finally with 0.4 mL ethanol–1N sulfuric acid (1 + 1). Discard waste. Elute synthetic colors with 1 mL ethanol–1N sulfuric acid (1 + 1) at –2 in. Hg.

Nondairy cream liqueur.—Transfer 25 mL sample to a 100 mL screw-cap centrifuge tube. Add 25 mL ethanol. Cap and shake for 1 min. Add 25 mL methylene chloride, cap, and vigorously shake for 2 min. Centrifuge at 2500 rpm for 10 min. Attach SPE-NH_2 cartridge to the vacuum manifold and apply a vacuum of –5 in. Hg. This vacuum should be applied throughout the sample delivery and washing steps. Carefully deliver the aqueous layer onto SPE-NH_2 cartridge. Repeat the same operation with additional 25 mL sample (to make a total of 50 mL sample delivered onto the SPE-NH_2 cartridge). Wash with 10 mL ethanol, then with 10 mL DI water, and finally with 0.4 mL ethanol–1N sulfuric acid (1 + 1). Discard waste. Elute synthetic colors with 1 mL ethanol–1N sulfuric acid (1 + 1) at –2 in. Hg.

TLC Determination

Solvent system A.—1-Butanol–2-butanol–acetonitrile–tetrahydrofuran–methyl ethyl ketone–0.5% aq. sodium chloride–ammonium hydroxide (10 + 10 + 25 + 15 + 20 + 18 + 2) (8).

Solvent system B.—1-Butanol–methyl ethyl ketone–ammonium hydroxide–water (5 + 3 + 1 + 1) (9).

Spotting and developing TLC plates.—Place a saturation pad in TLC developing chamber, and add 25 mL of the appropriate solvent system. Let equilibrate for 0.5 h before use. Spot 5 µL working standard and 10 µL sample on TLC plate. Dry TLC plate under heat lamp for 5 min. Develop plate to 7 cm in developing chamber. Dry plate and observe the color bands.

Note: Common safety measures (i.e., use of a fume hood) should be taken to minimize exposure to vapors from solvent systems.

Results and Discussion

The synthetic dyes are retained because of ionic interactions between the dyes and the SPE cartridge. Natural colors and caramel are not retained. The ethanol–sulfuric acid solution eluted the retained dyes from the cartridge. Tables 1 and 2 list some of the common dyes encountered in beverage alcohol products. These colors are identified by this method using the SPE-NH_2 cartridge and TLC solvent system. The retardation factor values for the described TLC solvent systems are given in the tables. In addition, the dyes are listed with the common name, the C.I. number, and the EEC number for complete reference purposes. Table 3 represents some of the samples analyzed. These samples were also analyzed for comparative purposes by the established Alcohol, Tobacco, and Firearms procedure (8). Both methods gave similar results, although the SPE-NH_2 method was more sensitive and detected dyes that where missed by the established method.

As described above, FD&C and other synthetic colors are retained on the SPE-NH_2 cartridge by ion exchange and are eluted only with a strongly acidic elution solvent. This affords an easy cleanup for most types of samples containing high sugar, tannins, and natural coloring materials, except dairy and nondairy cream liqueurs. Cream liqueurs are emulsions and the emulsion needs to be broken, as described in *Sample Preparation* before sample application on to the SPE cartridge. Delivery of 50 mL sample on the SPE cartridge and elution of synthetic colors in 1 mL ethanol–1N sulfuric acid (1 + 1) affords a 50-fold sample enrichment and results in 0.5 ppm or lower detection limits for these colors. The 2 TLC-developing solvent systems complement each other. Synthetic colors that are not well-resolved in solvent system A are well-resolved in solvent system B, and vice versa.

Blue No. 2 is susceptible to fading. Fading can occur in light if the plate is dried for longer than the recommended time. If Blue No. 2 is suspected in the product, the plate should be allowed to dry in the dark for approximately 30 min before developing, without any use of the heat lamp. Also, in solvent system B, synthetic color Blue No. 2 fades away during the TLC plate development. Thus, solvent system B is not recommended for the detection of synthetic color Blue No. 2.

Conclusions

The optimized SPE cleanup and sample enrichment, prior to TLC, described in this work is applicable for all types of beverage alcohol products. The proposed method is simple and offers consistency in chromatography with no artifacts, such as streaking or overlapping of the color bands. A 10 µL spotting of the prepared sample affords sensitivity to a 0.5 ppm detection limit for these synthetic colors (detection limits were established by visual observation using known prepared standard solutions of the dyes). Table 3 represents the synthetic colors that were detected in a number of different types of beverage alcohol products analyzed by this method. This method is not a quantitative procedure but can be used for semiquantitative purposes.

Table 3. Identification of synthetic colors in different types of beverage alcohol products

Product	Visible color	Synthetic color detected
Lime specialty	Green	Yellow No. 5 Blue No. 2
Pepperment liqueur	Green	Yellow No. 6 Blue No. 1
Orange liqueur	Orange	Yellow No. 5
Orange sparkling cocktail	Yellow	None
Cranberry sparkling cocktail	Red	Red No. 40
Pineapple sparkling cocktail	Yellow	None
Daiquiri cocktail	Yellow	Yellow No. 6
Cream liqueur	Cream	None
Coco cream liqueur	White	None

References

(1) Betz, R. (1981) *Wein-Wiss.* **36**, 268–292

(2) Boley, N.P., Bunton, N.G., Crosby, N.T., Johnson, A.E., Roper, P., & Somers, L. (1980) *Analyst* **105**, 589–599

(3) Corradi, C. (1982) *Bol. Chim. Lab. Prov.* **8**, 3–11

(4) Egginger, R. (1988) *Fleischwirtschaft* **68**, 41–43

(5) Graham, R.J.T., & Nya, A.E. (1969) *Fifth International Symposium on Chromatography and Electrophoresis*, P. de Moerlosse (Ed.), Humphery Science Publishers, Inc., Ann Arbor, MI

(6) Martin, G.E., Tenenbaum, M., Alfonso, F., & Dyer, R.H. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 908–910

(7) Noda, A., & Nishiki, S. (1977) *J. Food Hyg. Soc. Jpn. (Shokuhin Eiseigaku Zasshi)* **18**, 321–327

(8) Steele, J. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 540–541

(9) Young, M.L. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 458–461

TECHNICAL COMMUNICATIONS

Hydrolysis of Peptide Binding by Phosphotungstic Acid

ANNE FIALAIRE and ERIC POSTAIRE
Department of Clinical Pharmacy, School of Pharmacy, University of Paris, Rue J.B. Clement, F92290 Chatenay-Malabry, France

A new method for the precipitation of protein and oligopeptides is described. In this method, the selectivity of precipitation is a function of the percentage of alcohol. With an 80:20 (v/v) mixture of ethanol and water at room temperature, the selectivity of oligopeptide precipitation was as good as that using phosphotungstic acid.

A method commonly used for oligopeptide precipitation in bromatology is fractional precipitation by phosphotungstic acid (PTA) at 5%. This method precipitates proteins and oligopeptides in the presence of sulfuric acid (1–3). It is a selective method because only small molecules (MW < 600) such as di- or tripeptides and amino acids remain in solution.

To quantitate the small peptides included in a commercial protein hydrolysate, we used a PTA precipitation-based protocol (Figure 1). This protocol consisted of determining total nitrogen of the initial mixture. We then precipitated proteins and oligopeptides using PTA and measured nitrogen of the soluble fraction. The difference between the 2 nitrogen determinations indicated the amount of proteins and oligopeptides. Finally, we separated di- and tripeptides from amino acids on the soluble fraction. Although this protocol gave us satisfactory results at a quantitative level, we found out that the liquid chromatographic (LC) separation profile for the di- and tripeptides was modified before and after PTA precipitation. This observation was explained by the hydrolysis of a few peptide bonds. Indeed, we identified the constituent amino acids of GlycylLeucine (Gly-Leu) after PTA precipitation (Figure 2) by the same LC method (4).

Table 1. Nitrogen content determined by Kjeldahl method for bags of proteolysate

Precipitation	Nitrogen, g
Soluble nitrogen after PTA precipitation ($n = 6$) ± σ	2.43 ± 0.05
Soluble nitrogen after ethanol precipitation ($n = 6$) ± σ	2.50 ± 0.17
Total nitrogen calculated ($n = 6$) ± σ	3.37 ± 0.06
Total nitrogen manufactured	3

This finding led us to conclude that the PTA precipitation was not suitable to quantitatively determine peptides or amino acids. Indeed, for the amino acids, we obtained a positive error, and for the peptides, we obtained a negative error.

Therefore, we proposed a new precipitation method using ethanol, similar to the one used by Conn for protein precipitation (5). In this method, the selectivity of precipitation is a func-

Received June 15, 1992. Accepted by JW February 18, 1993.

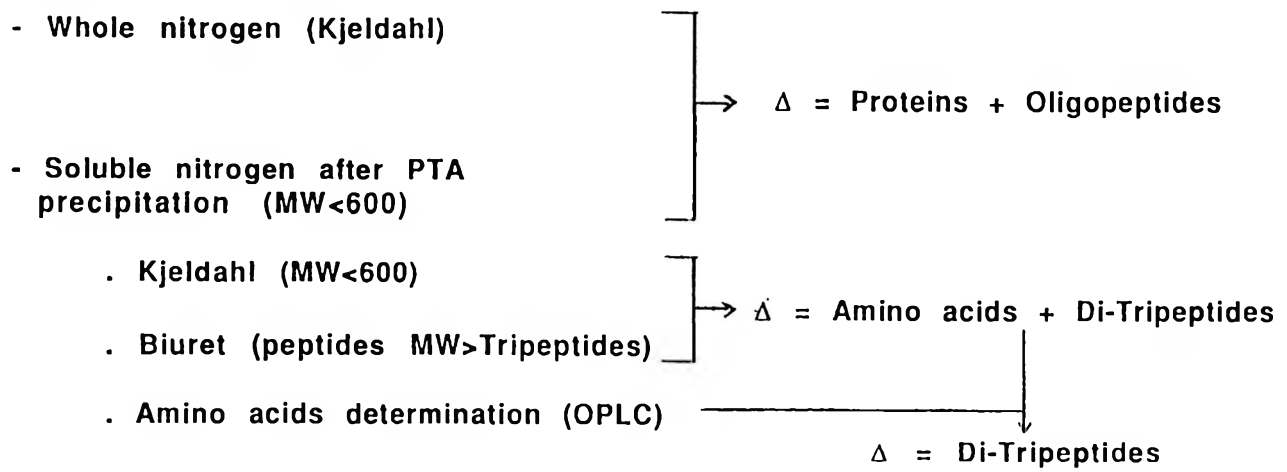


Figure 1. Protocol for the determination of small peptides in a proteolysate.

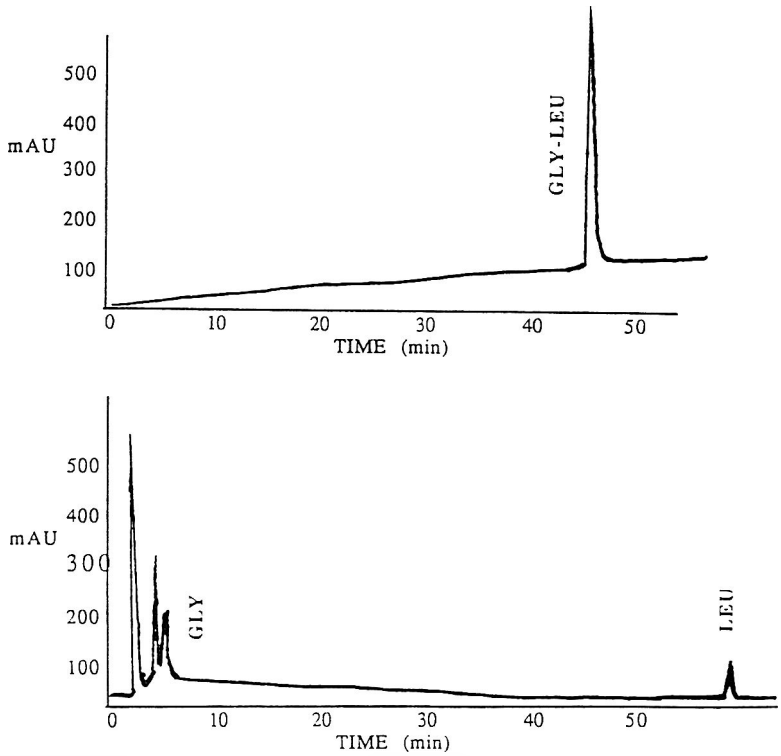


Figure 2. Gly-Leu chromatogram before and after PTA precipitation.

tion of the percentage of alcohol. We have performed 3 successive extractions using an 80:20 (v/v) mixture of ethanol and water at room temperature. The volume of the ethanol–water mixture corresponded to a total weight that was 40 times the dry weight of the sample to be extracted. Each extraction was followed by a centrifugation at 4000 × *g* for 15 min to separate the soluble from the insoluble fraction. The selectivity of oligopeptide precipitation was as good as that using PTA (Ta-

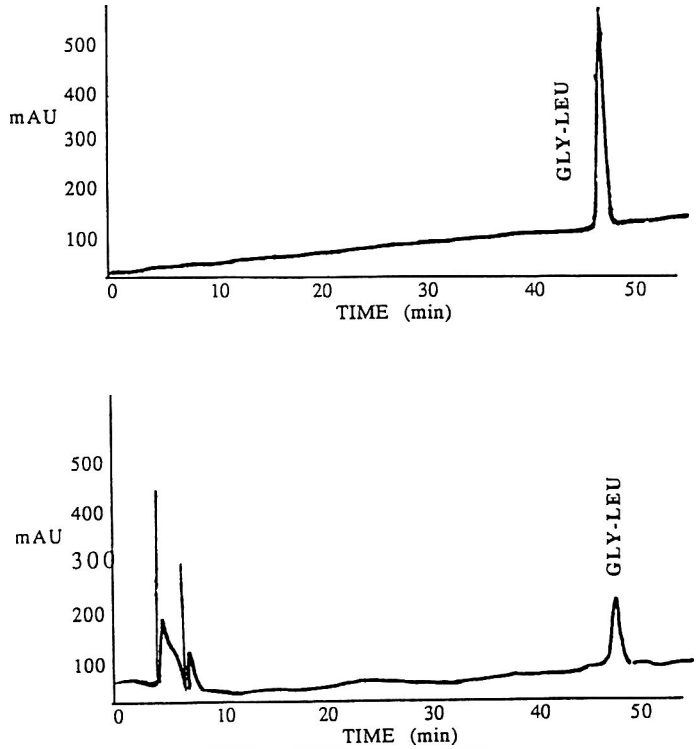


Figure 3. Gly-Leu chromatogram before and after ethanol precipitation.

ble 1). Moreover, by using LC, we showed that the ethanol precipitation method did not damage the Gly-Leu peptide bond (Figure 3).

References

(1) Reiter, B., Sorokin, Y., Pickering, A., & Hall, A.J. (1969) *J. Dairy Res.* 36, 65–74

(2) O'Keeffe, R.B., Fox, P.F., & Daly, C. (1976), *J. Dairy Res.* **43**, 97–107

(3) Rank, T.C., Grappin, R., & Olson, N.F. (1985) *J. Dairy Res.* **68**, 801–805

(4) Martinez, D., Postaire, E., Prognon, P., Ricour, C., Corriol, O., & Pradeau, D. (1988) *Nutr. Clin. Metabol.* **2**, 29–32

(5) Scopes, R.K. (1987) in *Protein Purification: Principles and Practice*, 2nd Ed., C.R. Cantor (Ed.), Springer-Verlag, Berlin, Germany