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MAY/JUNE 1991
VOL. 74, NO. 3

JANCA 74(3) 435–574 (1991)
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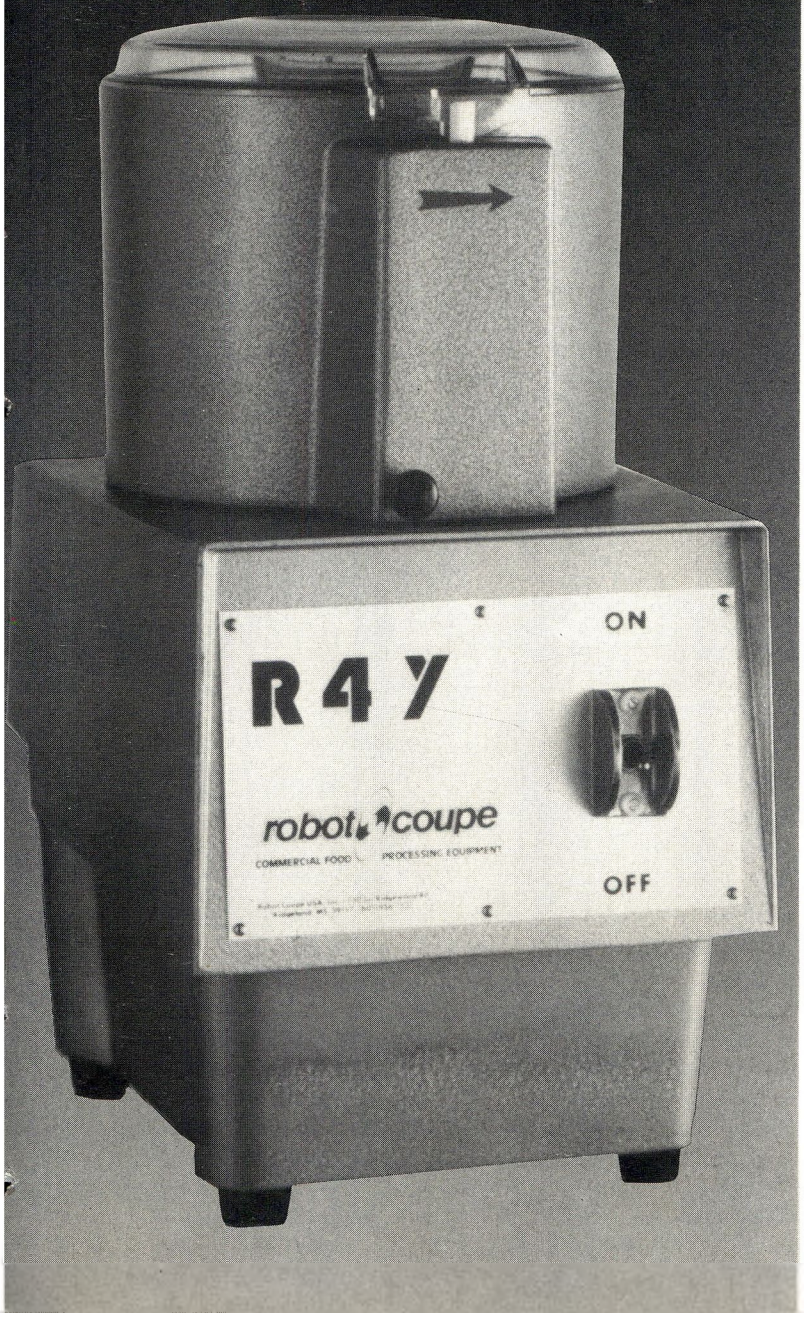

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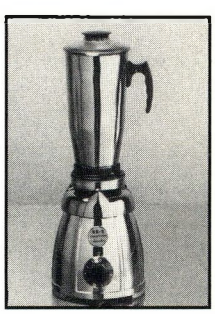

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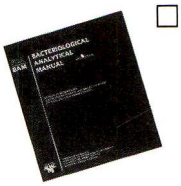

☐ **FDA Bacteriological Analytical Manual, 6th Ed.**
1984, 448 pp. Looseleaf.
ISBN 0-935584-29-3.
Members: $76 in U.S., $82 outside U.S.
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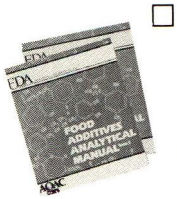
☐ **Principles of Food Analysis for Filth, Decomposition, and Foreign Matter-FDA Technical Bulletin No. 1**
1981. 286 pp. 2nd printing, 1985. Illustrated. Softbound. ISBN 0-935584-33-1.
Members: $54 in U.S., $60 outside U.S.
Nonmembers: $59 in U.S., $65 outside U.S.

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



☐ **FDA Food Additives Analytical Manual (FAAM) Volumes I and II**
Volume I - 1983. 2nd printing, 1988. xv + 364 pp. Illustrated. Softbound.
ISBN 0-935584-22-6. Volume II - 1987. xiv + 346 pp. Illustrated. ISBN 0-935584-34-X.
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☐ **Training Manual for Analytical Entomology in the Food Industry-FDA Technical Bulletin No. 2.**
1978. 184 pp. Looseleaf. ISBN 0-935584-11-0.
Members: $36 in U.S., $42 outside U.S.
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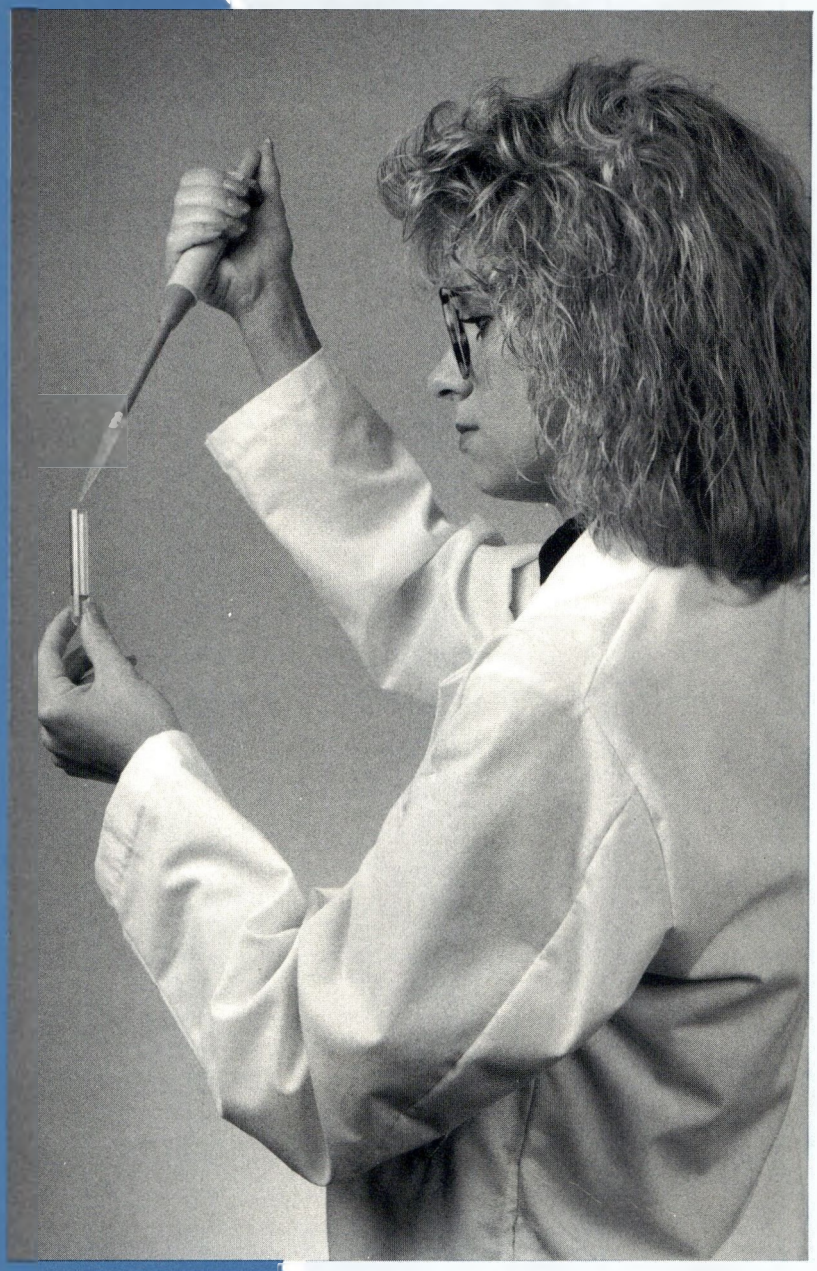

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J A O A C

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MAY/JUNE 1991 VOL. 74, NO. 3 JANCA 74(3)435–574 (1991)

 The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by the Association of Official Analytical Chemists, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 74 (1991) are: Members, $110.00 in U.S., $145.00 outside U.S.; Nonmembers, $160.00 in U.S., $190.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.

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Microbeam Technology for Organic Microanalysis and Chemical Mapping

Designed for use in a wide range of analytical as well as production monitoring and QA/QC applications, the IRus IR-based microprobe provides the ability to correlate molecular structure with physical morphology. Full integration of the microscope and spectrometer functions permit automated sequential data collection, interpretation, and display functions. The system's software and set up viewing makes sampling easy, accurate, and efficient. Spectra-Tech, Inc.

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Compact Microplate Reader

The compact Model 450 microplate reader incorporates solid optical performance, convenient onboard software, and a broad range of standard features in a new space-saving design. The state-of-the-art 12 channel fiber optic and lens system provides precise results, with automatic reading in both single and dual wavelength modes. The easy-to-use onboard software provides fast data analysis. The software will produce and print 4 different analysis reports after reading. A data buffer retains the last set of data for reanalyzing and printing. Bio-Rad Laboratories.

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Chromatography Software

Justice Innovation has released Version 4.0 of Chrom Perfect, its PC-based chromatography software package that acquires data from Hewlett-Packard 3396 or 3393 integrators and/ or PE-Nelson interfaces. Among the numerous improvements are: high speed plotting of chromatograms on the new HP Laserjet III, in addition to the older laser printers and 24-pin or 9-pin Epson printers; on-screen HELP messages for each prompt that can be customized by the chemist; calibrations with equally weighted repetitions of the standard; 2 response factors providing results in 2 separate units. Designed to run on all IBM-compatible PCs and displays, Chrom Perfect offers extensive features for multitasking during data acquisition, integration, plotting, report generation, on-screen peak expansion, calibration, methods development, and data archiving. Justice Innovations Inc.

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Bottle and Vial Crimping Workstation

The Crimpmate benchtop crimping station enables operators to effortlessly crimp even the largest autosampler load quickly and easily for consistent high quality seals. Simply place vial or bottle in the adjustable jaw, then crimp in an easy, single swift action by pulling the Crimpmate lever. Four jaw sizes are available for 8, 11, 13, and 20 mm caps, allowing almost all seal thicknesses and collar sizes to be crimped. Chromacol.

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Improved Reagent Dispenser

The Akrofil II reagent dispenser removes the danger of spilling and burns when filling test tubes and beakers with acid while protecting sinks, surfaces, and plumbing components. It accurately dispenses any amount of fluid from 0 to 20 cc with a single lever stroke. The self-contained portable unit has a stainless steel case. Interior components are crafted of stainless steel, glass, and Teflon. All moving parts are impervious to sulphuric acid and a host of other industrial grade chemicals. SynSat Industrial Sales.

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Low Pressure Chromatography Recorder

Bio-Rad's Model 1325 Econo Recorder's compact size and rugged design make it perfect for crowded laboratories and cold rooms. Its wide range of features makes it ideal for use with virtually any detection device. Features include voltage inputs from 1 mV to 20 V to accommodate any UV/Vis, pH, RI, or conductivity monitor; 12 different chart speeds to allow recording by seconds, minutes, hours, or days; recording parameters plotted automatically on the chart paper; and integral batteries to allow uninterrupted operation without an external power source. Bio-Rad Laboratories.

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High-Purity Standards for Food Analysis

A line of high-purity standards detects residues and impurities found in foods derived from animals, including antibiotics, hormones, organic phosphoric esters, and sulphonamides. The great majority of these standards assay at more than 99%. All are subjected to extensive identification and purity testing and will satisfy the most exacting demands of instrumental analysis. Crescent Chemical Co., Inc.

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C.A.P. LC Columns

Continuously adjustable piston (C.A.P.) columns eliminate void formation in the packed bed without opening or repacking the columns. With operating pressures up to 6000 psig and diameters including 4.6, 20, and 40 mm, these columns have bed pressure adjustment at both ends, are easily repacked, and feature 316 SS PEEK or KEL-F as the only wetted surfaces. Varex Corp.

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Hand-Held Laboratory Computer

Routine calculations, such as the amount of solute or solvent needed for solutions or dilutions, and computations of weights, volumes, concentrations, and activities are made quickly and ac-

curately with Lab Partner Plus. This sophisticated, pre-programmed computer makes difficult calculations, even mixtures involving radioactive materials, as easy to calculate as the push of a button. In fact, all computations are initiated by simply pressing a button. Easily understandable "plain English" messages are shown on the 16-character, liquid crystal display, prompting the user to enter data just as he would say or write the information. Calculated Solutions, Inc.
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Monitor for Trace Organic Vapor in Ambient Air

The MSI-301A portable organic vapor monitor is an easy-to-use, cost-effective instrument for continuously or manually analyzing trace quantities of benzene, toluene, xylene (BTX), or other organics in ambient air. Its low maintenance solid-state detector and onboard microcomputer provide exceptional measurement capability for on-site analysis of a single sample or unattended, long-term monitoring of hazardous organic vapors. Extensive data reporting features are available with the MSI-301A, as well as full remote control capability. Micro-sensor Systems, Inc.
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Chemistry Graphics Software for Mac

The language of chemistry is foreign to most word processors. To bridge the communication gap, chemists can now use ChemIntosh II, a software drawing program designed to create publication-quality graphics for chemical reports and papers. The program is compatible with other MacIntosh drawing applications and word processors. ChemIntosh II updates the original ChemIntosh with more than 45 new features. Two new features (the album and template tools) let the user place commonly used structures directly on the drawing palette for easy access and multiple use. With these 2 features, ChemIntosh offers the user an unlimited number of drawing tools and can be customized for other scientific applications. SoftShell International, Ltd.
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Low Volume FTIR Gas Cell

The LV7 FTIR gas cell is designed for use with FTIR analyzers to measure gas concentrations in small volume samples. The LV7 gas cell is exceptionally compact, easy-to-use, and has a large pathlength-to-volume ratio. Cell volume is 222 cc, with a maximum optical pathlength of 7.25 m. Pathlength is adjusted with a direct reading dial. A solid aluminum housing permits application of vacuum, pressure, and heat. The Foxboro Co.
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Modular Process and Furnace Controller

A modular process and furnace temperature controller, Model MTCP, is designed for the complete temperature and power control of electric reactor furnaces. The system is a combination of all the control and power equipment needed for safe, accurate process and furnace control. The process temperature and setpoint are digitally displayed on the instrument face of the unit. Adjustment to setpoint and tuning parameters are made by keys on the controller's face. The control is 3 mode (PID); self-tuning is standard. Several safety features are included in the instrument, which will disconnect (shut down) furnace power, if necessary. Autoclave Engineers, Inc.
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Glyphosate Analysis LC Columns

The Aminex glyphosate analysis columns are specifically tailored for the quantitative analysis of environmental residues of the herbicide glyphosate. The columns are available in 3 sizes, each useful in a specific type of analysis. The 300 × 4.6 mm column is used to analyze samples with high background interference. To increase sample throughput, the 300 × 4.6 mm column can be used with the 100 × 4.6 mm column in a column switching technique. The 250 × 4.6 mm column can be used to analyze samples with low background interference, such as groundwater or drinking water. Bio-Rad Laboratories.
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For Your Information

Meetings

June 3–5, 1991: Midwest AOAC Regional Section Meeting, Ramkota Inn, Sioux Falls, SD. Contact: Nancy Thiex, Olson Biochemistry Laboratories, South Dakota State University, PO Box 2170, Brookings, SD 57007, telephone 605/688-6171.

June 19, 1991: Mid-Canada AOAC Day, Winnipeg, Manitoba, Canada. Contact: Wanda Young, Technical Services Laboratory, 745 Logan Ave, Winnipeg, Manitoba 23E 1M8, Canada, telephone 204/945-2280.

June 27–28, 1991: Pacific Northwest AOAC Regional Section Meeting, Evergreen State College, Olympia, WA. Contact: Charles A. Laubach, Puget Sound Naval Shipyard, Code 134.3, Bremerton, WA 98314, telephone 206/476-8092.

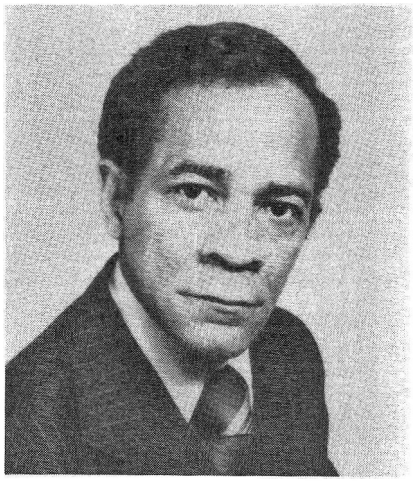
August 12–15, 1991: 105th AOAC Annual International Meeting and Exposition. The Pointe at South Mountain, Phoenix, AZ. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

November 6–8, 1991: Central AOAC Regional Section Meeting, Ramada Hotel, Sharonville, North Cincinnati, OH. Contact: Thomas C. Cronau, Indiana State Board of Health, Bureau of Laboratories, 1330 W Michigan St, PO Box 1964, Indianapolis, IN 46206-1964, telephone 317/633-0224.

November 12–13, 1991: AOAC/Europe Regional Section Meeting, State University of Limburg, Maastricht, The Netherlands. Contact: Ellen Jan De Vries, Duphar B.V., PO Box 900, 1380 DA Weesp, The Netherlands, telephone 31-2940-79296.

Reginald Bennett to Receive Wiley Award

Reginald W. Bennett, a research microbiologist and deputy chief at the U.S. Food and Drug Administration (FDA) Food Microbiology Methods Development Branch, Washington, DC, has been named to receive AOAC's 1991 Harvey W. Wiley Award, the most prestigious recognition of achievement extended by AOAC. Bennett will receive the award



at the opening session of the 105th Annual International Meeting in Phoenix, AZ, on August 12, 1991.

In a career spanning 35 years, the last 25 with FDA, Bennett repeatedly demonstrated skill at producing highly reliable methods that helped further FDA's mission of consumer protection. His efforts have been specifically directed toward detecting microbial contamination of foods and uncovering the epidemiological causes. Bennett received numerous letters of commendation and achievement, among them the U.S. Public Health Service EEO Achievement Award. He also is a 1990 recipient of an FDA Group Recognition Award (Chinese Canned Mushroom Group) and served on the FDA Commissioner's Employee Advisory Council.

Among his many accomplishments, 4 are particularly noteworthy for their contributions to the advancement of microbiology: (1) Bennett refined a microslide gel-diffusion method for detecting and semiquantifying contamination with staphylococcal enterotoxin; (2) he refined extraction methods for microbial toxins and developed analytical methodology for purifying *Bacillus* toxins; (3) he schematized *Listeria* serotyping; and (4) he developed methodology for serological renaturing (with urea) of heat-treated *S. aureus* enterotoxin.

Three of Bennett's accomplishments deserve special attention: He co-developed a highly specific miniaturized serological radial system for the detection and serotype identification of *S. aureus* enterotoxin produced in laboratory media and in foods. In demonstrating the presence of toxin-antitoxin reactivity by direct precipitation, this system eliminated the need for the use of tube type in vitro linear systems which lack simultaneous controls. The method, which was adopted by AOAC, is considered internationally to be the most applicable, economical, specific, and sensitive precipitation tool for the detection of staphylococcal entero- toxin.

He also co-developed a simplified medium and methods for the production of staphylococcal enterotoxin in the laboratory; both the medium formulations as well as the methods are applicable to the production for all enterotoxin serotypes (A–E) and have been used by laboratories throughout the world. One of the developed methods has been adopted by AOAC and is recommended by the American Public Health Association, the International Commission for the Microbiological Specifications for Foods, and by FDA whose specifics are published in the *Bacteriological Analytical Manual for Foods* published by AOAC.

After co-developing a method that uses gel filtration and cation exchange chromatography to separate enterotoxin from food polyelectrolytes, Bennett established pH and ionic strength buffer conditions so that food proteins simultaneously eluting with the enterotoxin would not interfere with the interpretation of the serologic tests. This method is the most sensitive tool that has been developed (detection range 30–60 ng/g of food) employing column chromatography and has been adopted by AOAC for separation and subsequent detection of staphylococcal enterotoxin in foods.

In all, Bennett's work on increasing the sensitivity of serology, facilitating the extraction of antigens, and their renaturation after food processing have been major factors in changing the status of staphylococcal enterotoxin from "incriminated" to "causing" foodborne illness. His work has had wide application in other

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Contact: Charles A. Laubach, 206/476-8092

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Contact: Ellen Jan De Vries, 31/2940-79296

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areas of analytical microbiology as well.

Currently, Bennett heads the research group on serological methods in the FDA's Food Microbiology Methods Development Branch. A member of AOAC since 1971, Bennett serves as an Associate Referee in the area of *S. aureus* and *B. cereus*. He is also a Fellow of the American Academy of Microbiology, and is a member of the American Society for Microbiology, Institute of Food Technologists, Association of Food and Drug Officials of Southern States, and the International Association of Milk, Food, and Environmental Sanitarians.

Bennett's career in microbiology has been further distinguished by the publication of over 85 articles and by numerous presentations at professional meetings. In addition, he has conducted training course on the detection of *S. aureus* and *B. cereus* for microbiologists and chemists from FDA district laboratories, state and municipal laboratories, other government agencies, industry, and foreign health agencies. Prior to FDA, he worked as a clinical microbiologist in hospital settings (1956–1959) and was a university faculty member at Benedict University in Columbia, SC, in microbiology (1959–1960). He holds both undergraduate and graduate degrees form the University of Pittsburgh, Pittsburgh, PA.

Outside this professional career, Bennett is a recipient of the Silver Beaver award, the highest service award given by the Boy Scouts of America to adult male scouters, and received an award from the National Association for the Advancement of Colored People for this community service.

Method Validation: What You Pay For Is What You Get

The collaborative study method validation and adoption process for the AOAC Official Methods of Analysis (AOAC-OMA) has evolved over a period of more than 100 years. The process is quite involved and requires considerable time and resources from the scientific regulatory community to have a method adopted to the highest recognized status of "Official Final Action."

The validity of the AOAC process has been recently reinforced by the International Union of Pure and Applied Chemistry's (IUPAC) adoption of the harmonized procedures for collaborative studies that are very similar to those developed by AOAC.[1] In addition, for many years, the U.S. Food and Drug Administration (FDA) has recognized the value of the process by requiring the use of AOAC-OMA methods in some of its regulations.

The first such FDA regulation appeared on August 11, 1972. The preamble states that: "...it is essential that regulatory agencies use standardized, reliable methods of analysis with demonstrated accuracy and reproducibility. Since even before the passage of the original Food and Drug Act of 1906, regulatory agencies developed a mechanism of choosing, validating, and publishing approved and standardized methods of analysis through AOAC. The mechanism, designated as a collaborative study, requires the demonstration of reliability of a method by a number of different laboratories analyzing a number of unknown (to the laboratory) samples...The Commissioner, therefore, wishes to indicate formally by a statement of policy and interpretation what has been the informal practice in the past—that unless otherwise indicated by the statute or regulation, the Food and Drug Administration will utilize the methods of analysis of AOAC in its enforcement programs...."[2]

The general regulation, which is currently presented in 21 CFR 2.19, states: "Where the method of analysis is not prescribed in a regulation, it is the policy of FDA in its enforcement programs to utilize the methods of analysis of AOAC as published in its latest edition...of its publication "Official Methods of Analysis of AOAC"...when available and applicable...." It further states: "...Other methods may be used for quality control...but it is expected that they will be calibrated in terms of the method which FDA uses in its enforcement program...." This regulation acknowledges that AOAC through its *Official Methods of Analysis* participates in the establishment of scientifically well-founded methods of analysis for FDA regulated commodities and products where methods are not specifically legislated or established through regulation. Further, it is noted in the FDA's pharmaceutical Good Manufacturing Practices regulations [21 CFR

COMING IN THE NEXT ISSUE

DRUG FORMULATIONS

- Liquid Chromatographic Determination of Tolnaftate in Commercial Products—R.D. Thompson and M. Carlson

FOOD ADULTERATION

- A Rapid $^{13}C/^{12}C$ Test for Determination of Corn Syrups in Honey—S.T. Brookes, A. Barrier, and J.E. Davies

and

PROCEEDINGS OF THE SYMPOSIUM ON MICROBIOLOGY UPDATE: OLD FRIENDS AND NEW ENEMIES

HELD AT THE 104TH AOAC INTERNATIONAL MEETING SEPTEMBER 10–13, 1990, NEW ORLEANS, LA

211.165(e) and 21 CFR 211.194 (a)(2)] that if a firm is using AOAC-OMA or USP/NF methods of analyses, only minimal additional validation data is required.

The general regulation has remained essentially unchanged for almost 20 years because the FDA scientists and statisticians, and regulated industries are satisfied that the AOAC development, validation, collaborative study, and adoption procedures continue to produce methods that are suitable for law enforcement activities. In addition, many U.S. state, Canadian federal and provincial, and other national governments also have adopted AOAC-OMA methods for the regulation of commodities and products within their jurisdictions. The AOAC-OMA methods frequently are adopted for use by the Codex Alimentarius, the International Organization for Stan- dardization, the International Dairy Federation, as well as other organizations. This continuing worldwide recognition and participation is due to AOAC's efforts to preserve and improve the scientific and statistical bases of the AOAC-OMA method validation and adoption process.

Because of this acceptance, the existence of an AOAC method results in considerable savings for regulated industries and commerce, and the agencies that monitor them. If AOAC-OMA methods were not available, each firm and, in some instances, different sites within a firm would have to establish and maintain extensive method validation data for each of the methods used in the control of their commodities and products. In addition, the costs to the regulatory agencies to audit and, in some instances, test each of these individually validated methods to ensure suitability would be substantial.

The AOAC-OMA method development and adoption process can be summarized as follows:

1. Method concept, development, and validation, which is conducted by scientist(s) in their own laboratories prior to submission to AOAC;
2. Submission of the method and proposed collaborative study (protocol design) to AOAC, which provides safety, statistical, and expert scientific review;
3. Conduct of the collaborative study;
4. Preparation by the method developer of the collaborative study report and recommendation for method approval; the report receives safety, statistical, and expert scientific review;
5. Approval of the method for first action status;
6. Publication of the method and collaborative study report in the AOAC *Journal*, and inclusion of the method in the *Official Methods of Analysis*; and
7. Elevation to final action status by a vote of the membership at least 2 years after publication.

This apparently lengthy process between method development and method adoption is often questioned.

The time requirements can best be presented as follows: Step 1 involves the amount of time required to establish the integrity and statistical validity of a method for the control of a commodity or product. This activity is conducted outside the AOAC process and is independent of AOAC requirements.

To estimate the time required for the AOAC methods review and approval process, several Methods Committee chairpersons were asked to give examples of reasonable current processing times. The Committee on Environmental Quality, working with the U.S. Environmental Protection Agency, has organized, conducted, and passed collaborative studies of multiresidue methods for pesticides in water in approximately 8 months (steps 2 through 5). The committee members typically complete their review of these studies in approximately 1 month. The recently completed collaborative studies included: The determination of 45 pesticides by 10 laboratories, the determination of 29 chlorinated pesticides by 11 laboratories, and the determination of 19 pesticide residues by 10 laboratories.

The Committee on Microbiology and Extraneous Materials estimates that the pre-collaborative study and design requires between 12 and 18 months (step 1); the AOAC portion of the approval time (steps 2 through 5) ranges from 4 to 9 months.

The Committee on Pesticide Formulations and Disinfectants reported that, after a well-validated pesticide formulation method had been developed and tested in-house, the AOAC portion of the approval time could be completed in approximately 6 months. Methods on disinfectants may require much more time because culture preparations sometimes take many weeks to prepare.

The Committee on Residues also observed that the AOAC portion of the approval time could be completed in 6 months.

The Committee on Drugs and Related Topics estimated that the overall process, including the method development and validation, and the AOAC portion, required about 21 months (steps 1 through 5), although a number of less complex methods had been completed in 10 months.

As one reviews these estimates, the approximate time frames for the methods development process are as follows:

- Developing a well-validated method in-house, which is outside the AOAC process, may require a year or more (step 1);
- After submission to AOAC, the protocol development and approval requires from 2 to 4 months (steps 2 and 3); and
- The conduct of the collaborative study followed by the review and committee approval requires between 3 and 8 months (steps 3 and 4).

To shorten the length of time necessary to develop and collaboratively study a method to the point of adoption as First Action, more time must be made available to the parties conducting the collaborative study. As indicated above, the protocol development to adoption as First Action generally takes between 5 and 10 months, and I am confident that time could be further shortened if the individuals involved were able to apply more effort.

All our volunteers are employed and, although many of them spend much personal time on AOAC activities, few of them have laboratory resources at home where they can perform these studies. The burden for this process then falls not only to our volunteers, but also to their employers to provide time for participation in this pursuit of good analytical science. Our industries, governments, and universities must support this enterprise not

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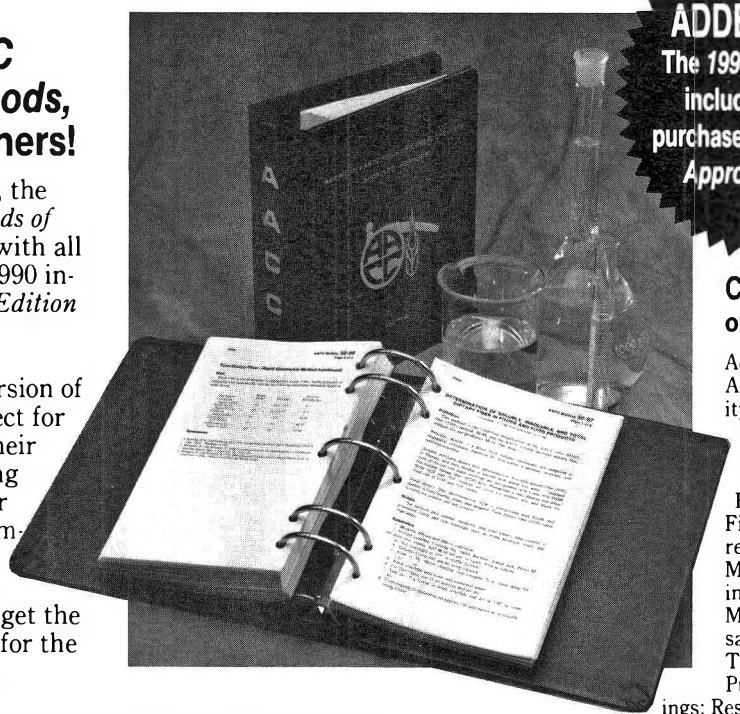

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only with their much needed financial support, but also with personnel and laboratory support. As in all things, what you pay for is what you get. If we do not provide the support for our methods adoption process, we will have to supply the time and resources to individually demonstrate the validity of methods on a laboratory by laboratory basis. This inefficiency in our industry and government will result in costs to society that will greatly exceed the maintenance of our well-founded and accepted method development, validation, and adoption process.

If there is no AOAC-OMA or USP/NF method, then the regulated U.S. pharmaceutical firm must supply all of the validation data for each method. This effort would be similar to step 1. Also, in lieu of the scientific and technical review by the AOAC Method Committee experts, FDA investigators and analysts will have to determine the adequacy of the firm's validation data. Without the well-founded consensus based AOAC-OMA methods, regulated firms will have to establish piecemeal validation processes and maintain that data at each laboratory site for scrutiny. This piecemeal approach will be very expensive to industry and regulatory agencies.

It is in everyone's best interest to protect the integrity of the AOAC-OMA methods and to help generate additional personnel to speed the method adoption process. If the scientific regulatory community fails to adequately support the AOAC-OMA process, the cost of method validation will be paid on a firm by firm and agency by agency basis instead of once through the AOAC-OMA validation process.

It is not sufficient to merely discuss this matter and bewail the time lines and costs. We as scientists must focus on and defend the practice of organized and recognized interlaboratory validation of analytical methods. If we, the scientists, fail to convince our institutional leaders that this element of good science is necessary and is not free, we fail ourselves and our society, and we, the consumers, ultimately will pay the price again and again for the resulting inefficiencies in the regulatory agencies, the regulated industries, and commerce.

[1] Pocklington, W.D. (1990) *Pure and Appl. Chem.* **62**, 149–162
[2] *Fed. Regist.* (Aug. 11, 1972) Vol. 37, No. 156, p. 16174

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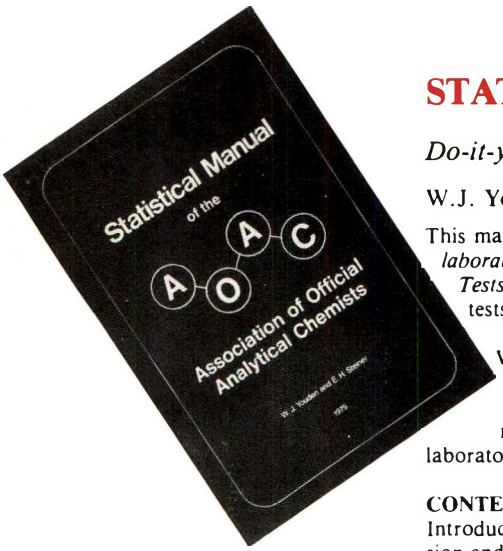

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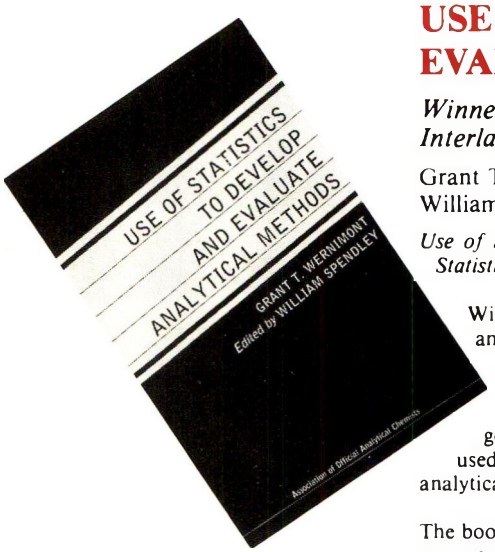

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Books in Brief

Statistical Techniques for Data Analysis. By John Keenan Taylor. Published by Lewis Publishers, Inc. 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1990. 300 pp. Price: U.S. $65.00/Outside U.S. $77.00. ISBN 0-87371-250-1.

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EPA's Sampling & Analysis Methods Database. Compiled by William Mueller and David Smith. Edited by Lawrence H. Keith. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1991. 465 pp. Price: U.S. $95.00/Outside U.S.$113.00. ISBN 0-87371-433-4.

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Computer-Assisted Method Development for High-Performance Liquid Chromatography. Edited by J.L. Glajch and L.R. Snyder. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1990. 676 pp. Price: U.S. $79.75/Dfl. 175.00. ISBN 0-444-88748-2.

This book deals with the use of the computer as an aid in selecting adequate or optimum conditions for a given analytical separation. Originally published as Volume 485 of the *Journal of Chromatography*, it has now been reprinted in book form because the information is so useful that many chromatographers want a copy readily available in the laboratory. An extensive introduction is added to the book edition. This surveys the field and refers to the pages where particular items are discussed in the book. The addition of a Glossary of Terms, an Author Index, and a Subject Index make this book an invaluable source of easily consulted information for the practicing chromatographer.

Practical Handbook of Spectroscopy. By James W. Robinson. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1991. 880 pp. Price U.S. $47.95/Outside U.S. $57.50. ISBN 0-8493-3708-9.

This valuable handbook is based on topics presented in the *CRC Handbook of Spectroscopy*, Volumes I and II, published in 1974, and Volume III, published in 1981. The information has been condensed (by the original contributor, when possible) so that only the most important information from the original 3 volumes has been retained and updated. The topics covered include ESCA flame photometry; atomic absorption and emission spectroscopy, including plasma emission; infrared spectroscopy; Raman spectroscopy; ultraviolet absorption spectroscopy; electron spin resonance, mass spectroscopy; nuclear magnetic resonance, X-ray spectroscopy, Mossbauer spectroscopy, mass photoelectric absorption coefficients, appearance potential spectroscopy, thermal neutron cross sections and resonance integrals for activation analysis, tables for experimental values of X-ray fluorescence and Coster-Kronig yields of the K-,L-, and M-shells.

Interactions of Food Proteins. Edited by Nicholas Parris and Robert Barford. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1991. 304 pp. Price U.S. $59.95: ISBN 0-8412-1935-4.

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Trends in Drug Research. Edited by V. Claassen. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1990. 430 pp. Price: U.S. $160.00/Dfl. 280.00. ISBN 0-444-88614-1.

This volume comprises the invited lectures of the Seventh Noordwijkerhout Camerino Symposium. The authors of the 28 papers give a detailed account of recent advances in the fields of receptor-drug interactions, structure-activity relationships, molecular toxicology, drug targeting, peptidergic drugs, inhibitors of cholesterol biosynthesis, antimalarial and antileprosy drugs. Containing up-to-date surveys of recent trends in synthetical and biological investigations in drug design, this volume should provide invaluable to medicinal chemists and to pharmacologists and toxicologists working in the field of drug design and development.

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DISCUSSIONS IN ANALYTICAL CHEMISTRY

Comparison of Thin Layer Chromatography and Liquid Chromatography

JOSEPH SHERMA
Lafayette College, Department of Chemistry, Easton, PA 18042

Although thin layer chromatography (TLC) is applied in almost every area of analysis ranging from biochemistry, clinical chemistry, and pharmaceutical chemistry to environmental, agricultural, and food sciences, it is not as widely used or highly regarded as gas chromatography (GC) or liquid chromatography (LC)[1]. This is true especially in U.S. laboratories and in regard to modern instrumental techniques of high performance TLC (HPTLC). The relative lack of use in the United States can be ascribed to many factors, including shortage of education in and equipment for TLC in universities and colleges, the lack of available funding for TLC research, and the small number of companies manufacturing TLC equipment and supplies, with corresponding deficiencies of marketing, customer support, and user workshops or symposia.

In its basic mode, TLC is very simple and inexpensive, and this may cause some workers to dismiss it because of the rather widespread fascination with complex, expensive instrumentation. On the other hand, the sophisticated, costly, automated instrumentation introduced by some companies for certain HPTLC steps (e.g., sample application, overpressured development, and spectrometric and radioactivity scanning) has been ignored by others to preserve a technique that traditionally has been regarded as uncomplicated and economical. This is obviously true in poorer countries, where manual methods predominate. However, even in countries where cost is not a factor, use of instrumental HPTLC has increased very slowly. The widespread perception is still that TLC is not truly quantitative and that it cannot begin to compete with LC in terms of resolution and sensitivity, despite many published articles stating that this is not the case in modern HPTLC using optimized techniques and equipment. One of my foreign colleagues has said that many in his country believe TLC is practiced only by analysts who cannot properly perform LC analyses.

Modern HPTLC differs from conventional TLC in several important respects. HP plates are smaller (10 × 10 cm) and have a thinner layer (200 μm) with smaller, more uniform particles (5–15 μm). The sample volume is 0.1–0.2 μL (except for HPTLC on preadsorbent plates), leading to initial spot diameters of 1.0–1.5 mm and developed zones of 2–6 mm. Development distance is 3–6 cm, which typically requires from 3 to 20 min. Detection limits are in the low nanogram range for colored or UV absorbing compounds and the low picogram range for fluorescent compounds, which is roughly 10 times better than for TLC. As many as 19 samples can be applied to a TLC plate and 36 for HPTLC (72, if convergent horizontal development from both ends of the plate is used). In general, HPTLC gives lower analysis times, more compact zones, and better resolution and sensitivity compared to TLC.

TLC should be recognized as a liquid chromatographic technique that competes with and complements LC. Both should be standard techniques available in every analytical laboratory for application to appropriate samples and analytes. In many cases, samples can be analyzed by normal phase TLC and reverse phase LC to confirm qualitative and quantitative analytical results.

Although the physical phenomena causing differential migration of sample components in TLC and LC are the same, there are basic differences in the operational methodologies that lead to various advantages and disadvantages for each technique. TLC is an open-bed system in which multiple samples are applied to the dry stationary phase (layer) concurrently, and the samples are separated by development with the mobile phase. Solutes are detected statically after removal of the mobile phase, and separated zones can be measured by densitometric scanning. Detection and scanning can be carried out without time constraints.

In contrast to TLC, LC is a closed system in which single aliquots of standards and samples are sequentially introduced into the mobile phase, which is moved by external pressure through an equilibrated column. Solutes are eluted from the column by the mobile phase and detected in a dynamic, time-dependent fashion in the presence of this solvent. These diverse characteristics make TLC (often termed development chromatography as opposed to elution chromatography for LC) much more flexible in its operation.

The following are some comparisons of TLC and LC. Although the term TLC is used below, modern HPTLC is the method to be compared to column LC. However, in many instances, conventional TLC and HPTLC share the advantage or disadvantage described.

LC is more efficient (in terms of available total theoretical plates) than 1-dimensional TLC.—It is often possible to more fully separate complex mixtures in a single run by LC. However, most practical LC separations require fewer than 20 000 theoretical plates, and many can be performed by TLC as well as by LC. Resolution is squared in 2-dimensional TLC, and studies indicate that this method can separate complex mixtures at least as well as LC.

The entire LC process has been automated for unattended operation.—In TLC, automated instruments are available for most individual steps (e.g., sample application, development, detection, scanning); however, the plate must be manually handled between steps. Until a robotic, fully automated TLC system is perfected, LC will continue to be less labor intensive.

A greater variety of stationary phases and operational mechanisms has been used for TLC.—These include both commercially available layers, such as silica gel, bonded phases, and cellulose, and layers impregnated with reagents to improve selectivity. Sorbents used to prepare layers usually contain a binder to improve adherence to the support material (most often glass), while LC sorbents do not. The binder does not usually affect the separation obtained. The availability of similar commercial phases for LC and TLC allows the latter to be used to quickly pilot stationary and mobile phase conditions with a minimum of apparatus and materials for transfer to column separations.

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[1]Modern column high performance liquid chromatography.

TLC offers much higher sample throughput (lower analysis time) and lower cost per analysis than LC.—This is because of the ability to apply multiple samples and standards to the same plate and perform their separations in parallel. Even with total automation, LC cannot compete with TLC in terms of number of samples processed in a given time period.

The ability to process samples and standards simultaneously yet independently on a single plate under the same conditions leads to statistical improvements in data handling and better analytical precision and accuracy.—Because samples and reference standards are directly compared, there is less need for internal standards in TLC than in GC or LC.

Cruder samples can be analyzed by TLC because each plate is used only once.—The presence of strongly sorbed impurities, and even solid particles in samples, is of no concern. These materials can build up on an LC column and destroy its performance. In TLC, every sample is separated on fresh stationary phase, without carryover or cross-contamination. Therefore, TLC can require fewer cleanup steps during sample preparation, saving both time and expense.

The choice of the sample solvent for TLC is not as critical because it is evaporated before development of the layer.—In LC, the sample solvent chosen must be compatible in terms of composition and strength with the column and mobile phase.

Because TLC is an open bed development technique, the mobile phase velocity is determined by capillary forces and is not controlled.—Exceptions include the U-Chamber and overpressured TLC techniques. The mobile phase velocity in the closed LC system is carefully controlled and accurately adjustable.

Multicomponent mobile phases may separate (demix) during migration through the layer, producing an undefined solvent gradient.—In LC, well-defined gradients are applied to an equilibrated column to improve separations. System equilibration time in TLC is small, and small to large (for gradient elution) in LC. Mobile phase vapors can preload the layer during equilibration and affect the resultant separation.

Many more solvents can be used for preparation of TLC mobile phases because the phase is completely evaporated before detection and the plate is used only once.—Therefore, UV absorbing properties, purity, or acid/base properties of the mobile phase are not as critical as with LC. However, mobile phases that do not wet the stationary phase can be used in LC because the external pressure will serve to transport the solvent, whereas the capillary forces in TLC will not.

The availability of conventional linear, continuous, multiple, circular, anticircular, and 2-dimensional development methods and the great variety of available mobile phases allow the TLC system to be optimized for separation of only the compound(s) of interest from mixtures containing solutes of varying polarities.—The remainder of the sample can be left at the origin or moved near the solvent front, away from the center plate region of maximum resolution. This can lead to a considerable saving in time compared to LC, in which the most strongly sorbed materials have to be eluted for each sample.

Using TLC, it is possible to apply sequential, compatible detection techniques to a single plate, including inspection under short- and long-wavelength UV light and application of a series of chemical reagents, for greater detection sensitivity and selectivity.—Hundreds of selective or universal chromogenic and fluorogenic post-chromatography spray, dip, and gaseous chemical detection reagents have been described, as well as enzymatic and biological detection methods.

The entire sample is contained on a TLC plate and every component can be detected and observed, including irreversibly sorbed fractions at the origin. In LC, there can be loss of peaks or unexpected appearance of peaks from previous samples. Zone identification is facilitated in TLC by the visual nature of detection using colors and shapes and many different reagents and temperatures, and inspection in daylight and under short- and long-wavelength lamps.

TLC quantification can be carried out in different ways because of the absence of time constraints in development chromatography compared to column chromatography.—Multiple scanning of the same chromatogram at different optimal wavelengths can be used to enhance sensitivity and selectivity. Multiple development can be applied to separate certain solutes in sequence, with scanning after each step. Spots can be measured both before and after in situ reaction with a derivatizing reagent. Complete visible, UV, or fluorescence spectra can be obtained in situ for each separated zone. Although the literature contains many more examples of successful quantitative analysis by LC, there is no doubt that TLC is a truly quantitative method with precision as good as for LC, if proper techniques and equipment are used.

Detection limits are approximately the same for TLC and LC.—These range from picogram to microgram levels.

Highly retained substances (low R_f) in TLC form the most compact zones and are detected with the highest sensitivity.—In LC, highly retained substances (high k') form the widest peaks and are most poorly resolved and detected.

Solvent use is much lower for TLC than for LC.—Use is lower both absolutely and, especially, on a per-sample basis, leading to reduced operating and disposal costs and safety concerns.

Conventional TLC is inexpensive compared to LC.—However, the cost of computer controlled instruments can increase the cost of performing modern instrumental quantitative TLC to an amount at least equivalent to LC instrumentation.

There is great promise for increased future use of TLC for screening, qualitative identification, semiquantitative estimation, and accurate and precise quantitative analysis. Likely application areas include environmental analyses, such as determination of pesticides and metabolites to satisfy the EEC drinking water directive, and analysis of pharmaceutical formulations and raw materials. The methodology of basic TLC is so simple and fool-proof that it can be used successfully for routine separations and approximate quantification by laboratory workers with only a minimum of experimental skills, and it will certainly remain widely used in this way. It is hoped that the advances that have been made beyond this basic level will be appreciated by many more analysts, who will then apply TLC more widely to analytical problems.

The following list of suggested reading can provide additional information about the theory, practice, and applications of modern TLC.

Suggested Reading

(1) *Journal of Planar Chromatography* (1988–present)
(2) *Analytical Chemistry* (1976–present). Reviews of planar chro-

matography, written biennially since 1976 by J. Sherma, appear in even-numbered years in the fundamental reviews issues.

(3) Poole, C.F., & Poole, S.K. (1989) "Modern Thin Layer Chromatography," *Anal. Chem.*, **61,** 1257A–1259A

(4) Fried, B., & Sherma, J. (1986) *Thin Layer Chromatography—Techniques and Applications*, 2nd ed., Marcel Dekker, Inc., New York, 394 pp.

(5) *Modern Thin Layer Chromatography* (1990) N. Grinberg (Ed.), Marcel Dekker, Inc., New York, 490 pp.

(6) *Quantitative Thin Layer Chromatography and Its Industrial Applications* (1987) L.R. Treiber (Ed.), Marcel Dekker, Inc., New York, 353 pp.

(7) *Handbook of Thin Layer Chromatography* (1991) J. Sherma & B. Fried (eds.), Marcel Dekker, Inc., New York, 1047 pp.

CHEMICAL CONTAMINANTS MONITORING

State Findings on Pesticide Residues in Foods—1988 and 1989

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Findings of pesticide and related chemical residues are presented for 27 065 samples of foods collected and analyzed in 10 state food laboratories over 1988 and 1989 (fiscal years (FY) 88 and 89). These laboratories conduct food regulatory programs compatible with national programs of the U.S. Food and Drug Administration. Of the findings, 6325 samples contained detectable levels of 1 or more pesticide analytes and 418 (or 1.5%) of the total number of samples were deemed to be of regulatory significance.

FOODCONTAM is a national database of information generated by state agencies involved in regulation of agriculture, public health, and the environment. The database focuses on pesticide residues and industrial chemical residues in human foods as well as the health hazards related to these substances (1–6). Coded data fields are organized into datalines describing various attributes on a given physical sample so that these data are compatible with data generated in U.S. Food and Drug Administration (FDA) laboratories in Washington, DC, and their district laboratories throughout the United States.

Every state has regulatory agencies that are comparable to FDA, the U.S. Department of Agriculture (USDA), the U.S. Environmental Protection Agency (EPA), and other similar federal agencies. These state food quality regulatory agencies conduct field sampling, analysis, and problem correction programs both independently and in cooperation with their corresponding federal counterparts. Because state programs generate food quality data using quality assurance/quality control (QA/QC) protocols that are comparable or identical to those used by FDA, USDA, and EPA, it is now possible for states to share findings on food quality and safety with FDA.

The FOODCONTAM database, combined with FDA data, provides a more comprehensive information base that food scientists, regulatory agencies, and scientific bodies can use in regulatory risk assessment and tolerance-setting processes. Scientific and regulatory concerns can, thus, be addressed by responsible agencies in a more effective manner with a much larger information base.

U.S. consumers have become more aware recently of potential health risks and are concerned with proper management and avoidance of excessive levels of toxic chemical residues in foods. Such public and governmental concerns have produced critical public policy and raised certain legal issues. Despite such intensified public concern about safety issues in foods, data (6–10) from the Division of Contaminants Chemistry of FDA and state food regulatory sources show that, overall, U.S. foods are safe.

Annual state data collected and published in FOODCONTAM match the size of FDA's current annual database on foods grown in the United States. Such added data reaffirm past and current conclusions by FDA and the states about the generally high quality and safety of the national supply of vegetables, grains, fruits, seafoods, dairy products, and other food types. FOODCONTAM broadens the current knowledge base, and helps link state and federal regulatory programs of FDA, USDA, EPA, and other agencies. Such information may provide food producers and regulatory agencies with insight into actual and potential contamination routes and aid in the formulation of more uniform state regulatory enforcement criteria to minimize food contamination risks and ensure a safer food supply.

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Results

State departments of agriculture, health, and public safety have been recruited as data contributors by managers at FDA's Office of Federal-State Relations (FDA/FSR), Rockville, MD, and by the Mississippi State Chemist over the past 4 years. The program has been widely publicized. States were slow to respond at first, perhaps because of fear of the public's reaction to specific findings of toxic residues in the food supply of their state, but also because of the effort required to code state data files so that data from all states would be compatible, both among states and with the established file formats of the massive current FDA database.

Some states contract with FDA/FSR to send data from their food quality programs on a reasonable cost/dataline or cost/sample basis; others contribute data without charge. Discussions with state food quality scientists and agency directors about data protocols have improved the compatibility of data files.

Cooperating states [California (CA), Florida (FL), Indiana (IN), Massachusetts (MA), Michigan (MI), North Carolina (NC), New York (NY), Oregon (OR), Virginia (VA), and Wisconsin (WI)] sent more than 32 000 datalines/year (ca 13 500–14 000 samples/year) for federal years FY88 and FY89 (see Figure 1). A dataline is a related set of data on a sample (with lab results) showing sample identification, source, food type, name, analytical methods, and quantitative findings. A sample is a physical amount of a food commodity taken in the field or commercial channels from grower sheds to wholesale or retail levels. A positive (+) finding is a detectable and quantifiable value, with units of measure,

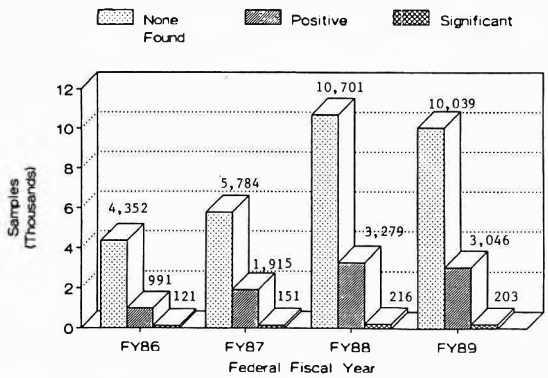

Figure 1. FOODCONTAM growth from FY86 to the present.

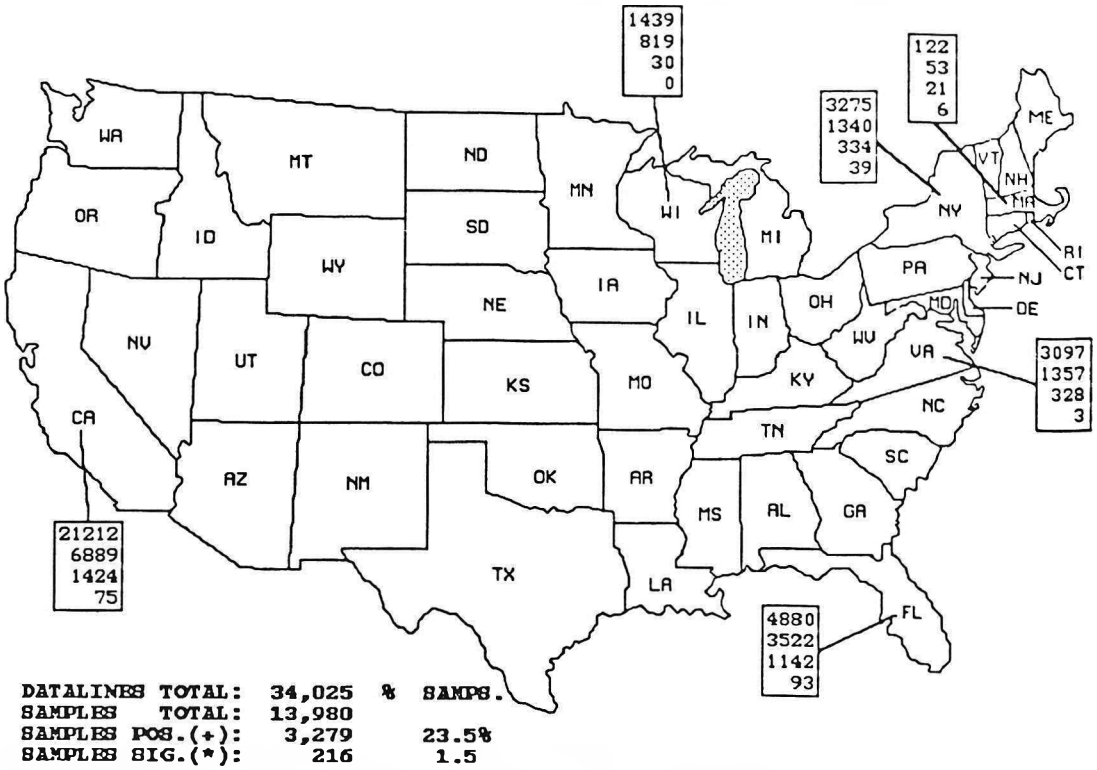

Figure 2. National distribution of FY88 data by states.

given in a single dataline. A significant (*) value is a regulatory statement (e.g., "residue exceeds federal or state tolerance" or "no tolerance established for that chemical/crop combination").

The number of datalines has grown rapidly over the last 4 years. A small decrease (ca 0.5% absolute) has occurred in the percent of "significant samples" found in FY88–89 compared to FY86–87, as sample numbers more than doubled. The number of positive findings (nonzero analyte values) has ranged from 10–33% of all samples. These are increasing

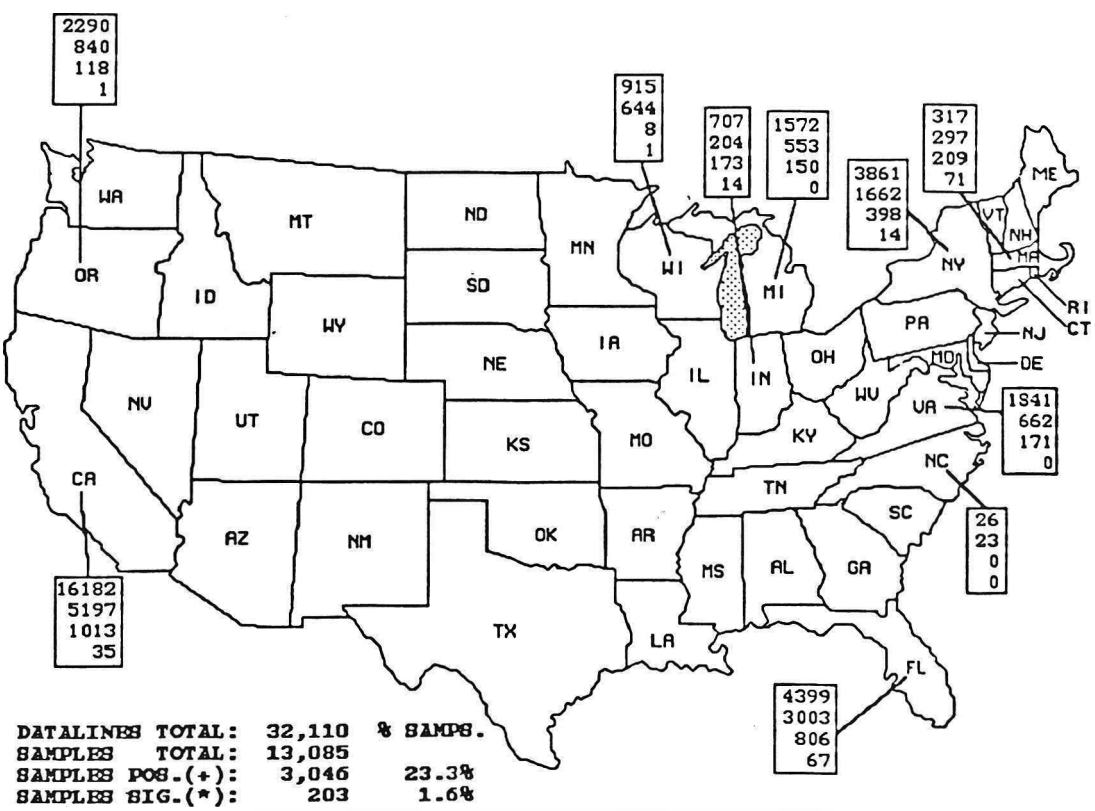

Figure 3. National distribution of FY89 data by states.

Table 1. Pesticides and toxic chemicals in foods (FY88) (by frequency of occurrence in 13 980 samples)

No. positive samples (+)	Samples positive, % (+)	No. significant samples (*)	Analyte found
587	4.198	9	Dicloran
328	2.346	2	Permethrin; see 222, 223
309	2.210	16	DCPA
282	2.017	35	Endosulfan; see 900, 901, 902
207	1.480	6	DDE; see 910, 911
160	1.144	31	Methamidophos
153	1.094	25	Mevinphos; see 578, 579
152	1.087	7	Methomyl
148	1.058	16	Chlorothalonil
135	0.965	2	Malathion
121	0.865	4	Chlorpyrifos
108	0.772	3	Folpet
100	0.715	1	Captan
97	0.693	4	Dimethoate
90	0.643	22	Acephate
90	0.643	2	Carbaryl
89	0.636	10	Diazinon
77	0.550	0	Chlorpropham
71	0.507	4	Endosulfan I
68	0.486	0	Vinclozolin
64	0.457	0	Benomyl
62	0.443	1	Methidathion
48	0.343	4	Permethrin, cis
48	0.343	0	Dieldrin
41	0.293	3	Endosulfan II
41	0.293	7	Parathion oxygen analog
39	0.278	3	Endosulfan sulfate
39	0.278	0	Chloroform
38	0.271	1	2-Chloroethyl stearate
37	0.264	2	Parathion
32	0.228	3	Fenvalerate
29	0.207	0	Dicofol; see 253, 254
26	0.185	0	Lindane
25	0.178	0	DDE, *p,p'*-
24	0.171	0	Phosmet
23	0.164	0	Iprodione
20	0.143	0	Toxaphene
18	0.128	0	Polychlorinated biphenyls
17	0.121	1	Oxamyl
16	0.114	1	Mercury
15	0.107	1	Ethion
14	0.100	1	Propoxur
13	0.092	1	Methyl mercury
12	0.085	0	Daminozide
12	0.085	0	Fluoride
11	0.078	0	Thiabendazole
10	0.071	0	TDE; see 908, 909
10	0.071	0	Chlorobenzilate
9	0.064	0	Azinphos-methyl
8	0.057	5	Sodium
8	0.057	0	Arsenic
8	0.057	0	Phosalone
8	0.057	1	Zineb
7	0.050	0	Aldicarb sulfone nitrile
7	0.050	3	Carbofuran
7	0.050	0	Parathion-methyl
7	0.050	1	Anilazine
7	0.050	0	Chlordane
6	0.042	0	Zinc
6	0.042	0	Aroclor 1260
6	0.042	0	Omethoate
6	0.042	0	Aldicarb
5	0.035	0	TDE, *p,p'*-
5	0.035	0	Aroclor 1254
5	0.035	0	Methoxychlor, *p,p'*-

Table 1. Continued

No. positive samples (+)	Samples positive, % (+)	No. significant samples (*)	Analyte found
4	0.028	0	DDT, *p,p'*-
4	0.028	0	Iodine
4	0.028	1	Manganese
4	0.028	0	Nonachlor, *trans*
4	0.028	0	Trifluralin
4	0.028	0	Methoxychlor
3	0.021	2	Procymidone
3	0.021	0	Naled
3	0.021	2	Fonofos
3	0.021	2	Simazine
2	0.014	0	Naphthalene
2	0.014	0	Pirimiphos-methyl
2	0.014	0	Cyhexatin
2	0.014	0	Hexachlorobenzene
2	0.014	0	Methiocarb
2	0.014	0	Heptachlor epoxide
1	0.007	0	BHC, alpha
1	0.007	0	Propetamphos
1	0.007	0	Metalaxyl
1	0.007	0	Cypermethrin
1	0.007	0	Pyrazon
1	0.007	0	Pronamide
1	0.007	0	Diquat
1	0.007	0	Bromopropylate
1	0.007	0	Iron
1	0.007	0	Copper
1	0.007	0	Selenium
1	0.007	0	Lead
1	0.007	0	Pentachloroaniline
1	0.007	0	Quintozene
1	0.007	0	Dicofol, *p,p'*-
1	0.007	0	Iprodione metabolite isomer
1	0.007	0	Malathion oxygen analog
1	0.007	1	Ethoprop
1	0.007	1	Terbacil
1	0.007	1	Phorate
1	0.007	1	Trichlorfon
1	0.007	0	Thuricide
1	0.007	0	Phenylphenol, o-
1	0.007	0	Pyrethrins
1	0.007	0	Ferbam
1	0.007	0	Ethylene dibromide
1	0.007	1	EPN
1	0.007	0	2,4-D
1	0.007	0	Calcium cyanide
1	0.007	1	Aldrin

with time, probably due to the greater scope of potential contaminants, wider ranging analytical methods, greater method sensitivities, and numbers of samples examined. The trend of decreasing significant samples will continue, we believe, as more regulatory controls are implemented, more EPA pesticide registrations for "older products" are canceled, and more new biotechnology products for controlling weeds, fungi, and insects are substituted for currently used chemicals.

Figures 2 and 3 show the origin of the analytical data by states. Some of these values (ca 5%, average) are for samples grown outside the state of the data contributor, but sampled in that state's programs. A small number of foods are imported from Canada, Mexico, Central America, and Caribbean nations via Florida, California, and perhaps other ports.

Table 1 shows the number of positive (+) (i.e., nonzero findings) and significant (*) (i.e., above tolerance or no tolerance set) values for each analyte in the 13 980 samples of foods analyzed by 6 state contributors (California, Florida, Massachusetts, New York, Virginia, and Wisconsin) for FY88. Table 2 gives the same information on 13 085 samples for FY89 from 10 states (the FY88 group plus Indiana, Michigan, North Carolina, and Oregon).

Table 3 shows pesticides detectable and quantifiable in contributor's programs for both years as well as those actually found. No data were received from other interested potential state participants. About 5–8% of the total data reported in the regulatory survey programs of California, Florida, Massachusetts, New York, Virginia, and Wisconsin were from foods grown in states adjoining or nearby these FY88

Table 2. Pesticides and toxic chemicals in foods (FY89) (by frequency of occurrence in 13 085 samples)

No. positive samples (+)	Samples positive, % (+)	No. significant samples (*)	Analyte found
482	3.683	4	Dicloran
221	1.689	14	Dieldrin
212	1.620	13	DCPA
199	1.520	5	Endosulfan; see 900, 901, 902
182	1.391	70	Lead
145	1.108	5	Captan
143	1.092	20	Mevinphos; see 578, 579
133	1.016	0	Daminozide
131	1.001	0	Zinc
130	0.993	0	DDE; see 910, 911
128	0.978	7	Chlorpyrifos
124	0.947	19	Methamidophos
122	0.932	3	Permethrin, *cis*
114	0.871	0	Endosulfan I
109	0.833	7	Chlorothalonil
96	0.733	3	Acephate
94	0.718	0	Malathion
91	0.695	4	Methomyl
76	0.580	0	Carbaryl
74	0.565	0	Permethrin; see 222, 223
70	0.535	1	Endosulfan II
70	0.535	5	Dimethoate
58	0.443	3	Diazinon
57	0.435	1	Vinclozolin
51	0.389	1	Polychlorinated biphenyls
49	0.374	1	Endosulfan sulfate
48	0.366	0	Methidathion
45	0.343	1	Mercury
44	0.336	0	Phosmet
37	0.282	0	Chlorpropham
33	0.252	0	DDE, *p,p'*-
33	0.252	0	Dicofol; see 253, 254
31	0.236	0	Parathion
27	0.206	0	Benomyl
27	0.206	1	Folpet
25	0.191	1	Methyl mercury
25	0.191	2	Ethion
25	0.191	0	DDT; see 906, 907
24	0.183	10	Chlordane
23	0.175	0	Iprodione
23	0.175	0	Lindane
22	0.168	2	Fenvalerate
22	0.168	0	EBDC (identity unknown)
22	0.168	0	Toxaphene
21	0.160	0	Propetamphos
21	0.160	0	2-Chloroethyl stearate
20	0.152	0	Chlorobenzilate
19	0.145	0	Methyl bromide
18	0.137	3	Parathion oxygen analog
18	0.137	0	Aldicarb sulfoxide
16	0.122	0	DDT, *p,p'*-
15	0.114	0	Aldoxycarb
14	0.107	0	Thiabendazole
14	0.107	0	Heptachlor epoxide
13	0.099	0	Copper
13	0.099	0	Methoxychlor
12	0.091	0	Chromium
12	0.091	0	Arsenic
12	0.091	0	Hexachlorobenzene
12	0.091	0	Nonachlor, *cis*
12	0.091	0	Nonachlor, *trans*
12	0.091	0	Chlordane, *cis*
12	0.091	0	Chlordane, *trans*
12	0.091	0	Anilazine
11	0.084	0	Methoxychlor, *p,p'*-

Table 2. Continued

No. positive samples (+)	Samples positive, % (+)	No. significant samples (*)	Analyte found
11	0.084	0	Endrin
10	0.076	0	BHC, alpha
10	0.076	0	Tetrachloroethylene
9	0.068	0	TDE; see 908, 909
9	0.068	0	Pyrethrins
8	0.061	0	BHC, delta
8	0.061	0	Imazalil
8	0.061	0	Parathion-methyl
7	0.053	0	Unsymmetrical dimethylhydrazine (UDMH)
7	0.053	0	Quintozene
7	0.053	2	Phenylphenol, o-
6	0.045	0	Azinphos-ethyl
6	0.045	2	Pronamide
6	0.045	0	Azinphos-methyl
5	0.038	0	TDE, *p,p'*-
5	0.038	0	Oxamyl
4	0.030	2	Ethoprop
3	0.022	0	Aldicarb sulfone nitrile
3	0.022	1	Pirimiphos-methyl
3	0.022	1	Monocrotophos
3	0.022	0	BHC; see 903, 904, 905, 050
2	0.015	0	Nickel
2	0.015	0	Dichlorvos
2	0.015	1	Iprodione metabolite isomer
2	0.015	1	Methiocarb
2	0.015	0	Propoxur
2	0.015	0	Diphenylamine
2	0.015	0	Tetradifon
2	0.015	0	Maneb
2	0.015	0	Magnesium arsenate
2	0.015	0	Heptachlor
1	0.007	0	BHC, beta
1	0.007	1	Mecarbam
1	0.007	0	Endrin ketone
1	0.007	0	Cypermethrin
1	0.007	0	Monohydrokepone
1	0.007	0	Cadmium
1	0.007	0	Chlordecone
1	0.007	0	Naled
1	0.007	0	Propiconazole (Tilt)
1	0.007	0	TDE, *p,p'*-olefin
1	0.007	1	Profenofos
1	0.007	0	Malathion oxygen analog
1	0.007	0	Carbofuran
1	0.007	0	Aldicarb
1	0.007	0	Fonofos
1	0.007	0	Tecnazene
1	0.007	0	Aldrin

FOODCONTAM data sources. Vegetables and fruits with high surface areas and "fuzzy or waxy" coatings, and some root foods such as beets and turnips, tend to have a higher frequency of positive reports. Some may have been higher than the average for all foods because of more intensive sampling, or because of special programs focused on some state's concerns about specific chemicals. State data correlate well with data published by FDA in similar national food quality assurance activities (7–11).

State data are usually derived from analyses of raw, unwashed, and unprocessed fruits and vegetables, among other foods. Washing, grading, and discarding procedures for blemished produce, routinely done in wholesale fruit and vegetable processing, packing, and market preparation, removes much of the external residues found on some of the samples reported here (12).

Some cooperating states conduct "special studies" on certain matrixes such as fish, bottled water, and milks, or on classes of chemicals for their own regulatory purposes. Such data are reported with all other data, in this and other years (6). These may bias findings positively or negatively for a given chemical and matrix combination for that report period, and can bias findings for a chemical (e.g., daminozide and UMDH) or a matrix (such as drinking water or apples) for that report period. Tables 4 and 5 list state analyses by food commodity groups. These clearly show the raw food

Table 3. Pesticides detectable by methods used or found in FY88 and FY89 state regulatory monitoring

Acephate*	2-Chloroethyl stearate*	Dieldrin*	Iron*	Phenothiozine
Aldicarb*	Chloroneb	Diflubenzuron	Lead*	Phenylphenol, o-*
Aldicarb sulfone nitrile*	Chloroform*	Dimethoate*	Lindane*	Phosphamidon
Aldicarb sulfoxide*	Chlorothalonil*	Dinoseb	Linuron	Profenfos*
Aldoxycarb*	Chlorpropham*	Dioxathion	Magnesium arsenate*	Propetamphos*
Aldrin*	Chlorpyrifos*	Diphenylamine*	Malathion*	Propoxur*
Allethrin	Chlorpyrifos-methyl	Diquat*	Malathion oxygen analog*	Pronamide
Ametryne	Chlorthion	Disulfoton	Mancozeb	Pyrethrins*
Ametraz	Chlorthiophos	Diuron	Maneb*	Pyrimiphos methyl*
Anilazine*	Chlortetracycline	DNOC	Manganese	Quintozene*
Antimony	Chromium*	EBDC (Ident. unknown)*	Mecarbam*	Resmethrin
Aroclor 1242*	Command	Endosulfan, unspec.*	Melamine	Selenium*
Aroclor 1254*	Copper*	Endosulfan I*	Mercury	Silver
Aroclor 1260*	Coumaphos	Endosulfan II*	Metalaxyl	Silvex
Arsenic*	Crotoxyphos	Endosulfan sulfate*	Methamidophos*	Simizine*
Atrazine*	Cyanazine	Endrin*	Methidathion*	Sodium*
Azinphos-ethyl*	Cyhexatin*	Endrin ketone*	Methiocarb*	Sulfallate*
Azinphos-methyl*	Cypermethrin*	EPN*	Methomyl*	Sulfur
Bendiocarb	Cyromazine	EPTC	Methoxychlor, *p,p'*-*	TDE, any isom.*
Benfluralin	Cyanazine	Ethion*	Methyl bromide*	TDE, *p,p'* olefin*
Benomyl*	2,4-D*	Ethylene dibromide*	Mevinphos & isomers*	Technazine
Bensulide	2,4-DB	Ethoprop*	Methyl mercury	Terbacil
BHC, alpha*	Daminozide*	Fensulfothion	Mirex	Tetrachloro(methylthio)-benzene
BHC, any isomer*	DCPA*	Fenthion	Monocrotophos*	Tetradifon*
Bromophos	DDE, *p,p'*-*	Fenvalerate*	Monohydrokepone*	Tetrachloroethylene*
Bromopropylate	DDE, any isomer*	Ferbam*	Monuron	Thiabendazole*
Bufencarb	DDT, *o,p'*-*	Fluchloralin	Naled*	Thiodicarb
Cadmium*	DDT, *p,p'*-*	Flucythrinate	Naphthalene*	Thiram
Calcium cyanide*	DDT, total*	Fluoride*	Nickel*	Thuricide*
Captan*	Demeton	Fluvalinate	Nonachlor, *cis**	Toxaphene*
Captafol	Diallate	Folpet*	Nonachlor, *trans**	Triadimefon
Carbanolate	Diazinon*	Fonofos*	Omethoate*	Triadimenol
Carbaryl*	Dibromochloropropane	Gardona	Oxamyl*	Trichlorfon*
Carbofuran*	Dicapthon	Heptachlor*	Parathion*	Trifluralin*
Carbophenothion	Dichlone	Heptachlor epoxide*	Parathion, O analog*	Unsym. dimethylhydrazine (UMDH)*
Chlordane*	Dichloran*	Hexachlorobenzene*	Parathion, methyl*	Vanadium
Chlordecone*	Dichlorvos*	Imazalil*	Pentachloroaniline*	Vinclozolin*
Chlordimeform	Dicofol, *p,p'*-*	Iodine	Pentachlorophenol	Zinc*
Chlornitrofen	Dicofol, total*	Iprodione*	Permethrin, *cis-**	Zineb*
Chlorobenzilate*	Dicrotophos	Iprodione metab.*	Permethrin, any*	Ziram*

* Denotes substances found.

types with the greatest likelihood of contamination. Figures 4 and 5 show these data graphically, organized by product commodity groups for FY88 and FY89.

Analytical methods used by state contributors are the same as those used by FDA's headquarters and field laboratories, typically AOAC official methods, or modifications of these that have been validated in the user's laboratory in collaboration with other state or industry laboratories. Most sample extraction cleanup/preparations for liquid chromatography or gas chromatography used by California and other state laboratories are slight modifications of Luke's procedure. Appendix A lists the methods most widely used by the 10 state laboratories now providing data on food pesticide residue analyses.

Discussion

Violative levels of pesticide residues in foods (i.e., above federal tolerances) are low for most food categories and account for only 1.5% of all samples in FY88 and 1.6% in FY89. The largest number of violative or significant samples was found in the "Vegetables" food commodity group and in the group termed "Other," which includes spices, alcoholic and nonalcoholic beverages, and water. Cheese and egg products, some tropical fruits, and leafy vegetables such as parsley, chinese cabbage, broccoli, eggplant, peppers, and turnips tend to have higher contamination frequencies (above federal tolerances) than other foods. Overall, the food supply seems to be free of significant levels of pesticide and industrial chemical residues, although many items contain trace levels at the parts per billion and lower levels. These findings are in marked contrast to current levels of public alarm, perceptions of increasing health risks from "toxins" in foods, and continuing concerns expressed about "pervasive contamination" of the national food supply.

Many pesticides currently of concern to the public have had EPA registrations canceled within the last several years. Many more are under careful reevaluation for the current uses allowed on product labels. We believe that this is an international trend and that it will continue to result in more foods being essentially free (parts per billion or trillion level) of agrochemical and industrial chemical residues, barring accidents in food distribution channels. Continued scientific studies in these vital areas of public health and safety are certainly justified. They should help provide adequate data

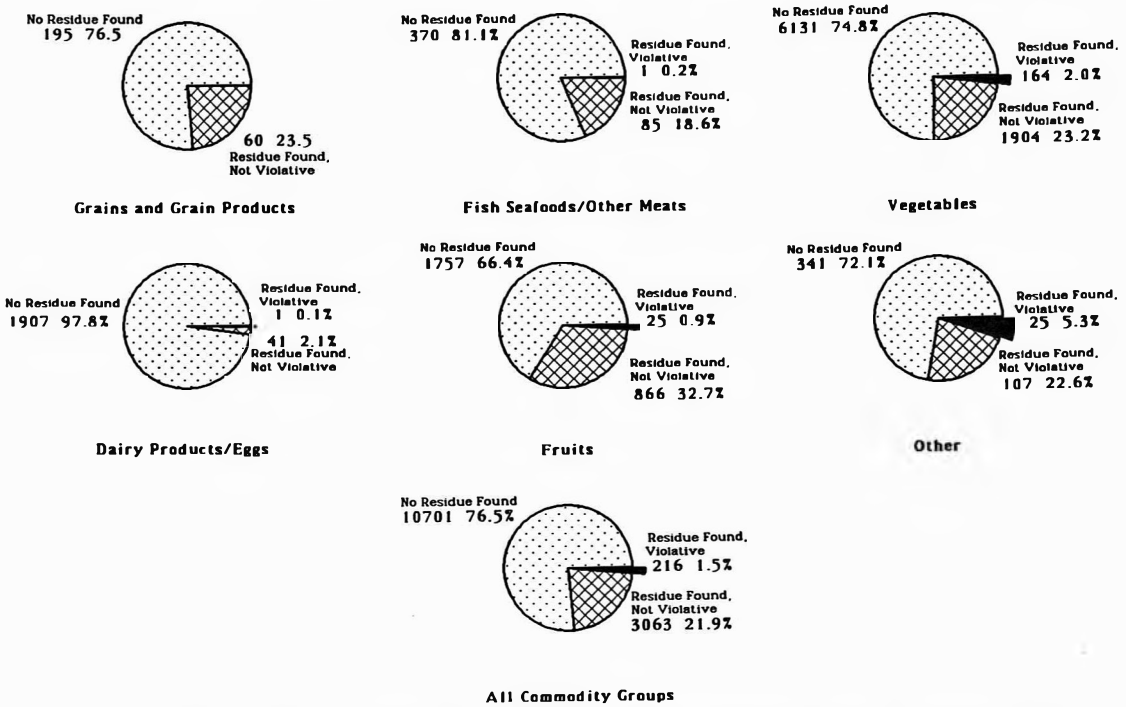

Figure 4. Summary of results of FY88 sample analyses of pesticide residues by commodity groups.

for governments to respond rapidly and realistically to those problems of true toxicological significance, and help allay public concern about food safety.

Acknowledgments

All activities of the FOODCONTAM program are supported under contracts from the U.S. Department of Health and Human Services, Food and Drug Administration Nos. 223-84-4230, 223-87-4230, 223-90-4230, and continuations. We thank all state agencies that provide data and appreciate the encouragement of state regulatory officials, especially the board of directors of the Association of Food and Drug Officials, for publicity for this program.

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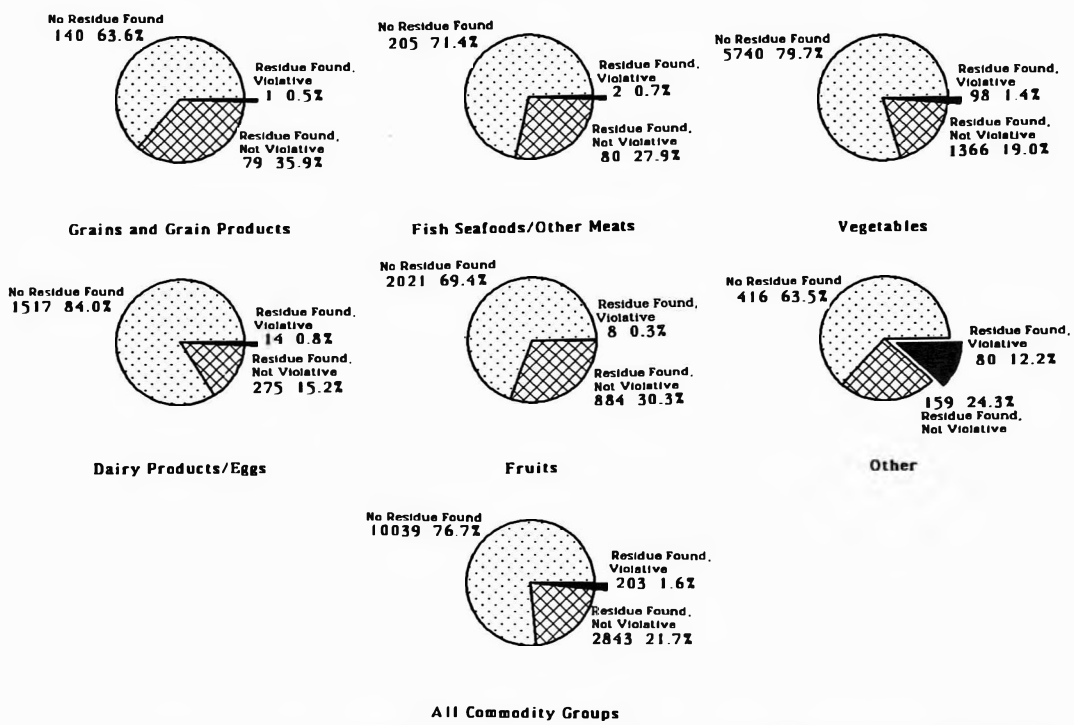

Figure 5. Summary of results of FY89 sample analyses of pesticide residues by commodity groups.

Table 4. Analysis of all findings in United States by commodity group (FY88)

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
A. Grains and Grain Products				
Rice	3	2	0	0
Wheat	3	2	0	0
Other whole grains	39	27	0	0
Bakery & cereal products/snack foods	89	81	0	0
Pasta products	5	5	0	0
Rice products	8	8	0	0
Wheat products	4	3	0	0
Other whole grain products	104	67	0	0
Total	255	195	0	0
B. Dairy Products/Eggs				
Milk and cream	1760	1725	1	<1
Cheese/cheese products	2	2	0	0
Eggs/egg products	140	134	0	0
Butter	15	15	0	0
Ice cream	32	31	0	0
Total	1949	1907	1	<1
C. Fish Seafoods/Other Meats				
Fish & shellfish	368	299	1	<1
Other meats	39	25	0	0
Poultry	48	45	0	0
Vegetable protein products, NEC	1	1	0	0
Total	456	370	1	<1
D. Fruits				
Blackberries	3	3	0	0
Blueberries	13	8	0	0
Boysenberries	1	0	0	0
Grapes	319	246	1	<1
Raspberries	18	9	0	0
Strawberries	216	79	10	5
Other berries	9	6	0	0
Grapefruit	1	1	0	0
Lemons	124	85	2	2
Limes	24	23	0	0
Oranges	251	147	0	0
Tangerines	41	39	0	0
Other citrus fruits	39	34	1	3
Apples	315	230	1	<1
Pears	126	102	3	2
Core fruits, NEC	11	10	1	10
Apricots	31	28	1	3
Avocados	112	107	0	0
Cherries	44	15	1	2
Nectarines	146	19	1	<1
Olives	1	1	0	0
Peaches	165	40	1	<1
Plums and prunes	157	75	0	0
Other pit fruits	18	15	0	0
Mangoes	14	11	0	0
Papaya	10	10	0	0
Pineapples	10	9	0	0
Plantain	3	3	0	0
Other tropical fruits	48	47	0	0
Cantaloupe	100	92	1	1
Honeydew	26	24	0	0
Watermelon	37	37	0	0

Table 4. Continued

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
Bitter melons	3	3	0	0
Other vine fruits	26	25	0	0
Other fruits	27	23	1	4
Fruit jams & jellies	31	31	0	0
Fruit juices	25	23	0	0
Fruits, dried or paste	103	97	0	0
Total	2648	1757	25	<1
E. Vegetables				
Blackeyed peas	9	8	0	0
Corn	151	149	0	0
Garden/green/sweet peas	91	75	1	2
String beans	190	154	1	<1
Other beans, peas, corn	88	78	1	1
Cucumbers	217	173	1	<1
Eggplant	76	67	3	4
Okra	54	50	0	0
Peppers	272	220	3	1
Pumpkins	5	5	0	0
Squash	400	370	3	1
Tomatoes	340	302	3	1
Other fruits used as vegetables	1	0	0	0
Asparagus	62	60	0	0
Bamboo sprouts	2	2	0	0
Broccoli	237	198	2	1
Broccoli raab	18	12	0	0
Brussels sprouts	84	76	0	0
Cabbage	412	359	2	1
Cauliflower	126	120	0	0
Celery	327	132	0	0
Chinese cabbage	206	154	11	5
Collards	207	119	15	7
Endive/chicory	380	241	14	4
Kale	201	104	6	3
Lettuce	1502	1006	33	3
Mustard greens	107	63	8	8
Parsley	94	65	5	5
Spinach	415	292	5	1
Turnip greens	95	66	1	1
Other leaf/stem vegetables	466	387	13	3
Mixed vegetables	10	8	0	0
Mushroom/truffle products	95	77	1	2
Carrots	91	66	2	3
Leeks	43	40	0	0
Onions	197	186	0	0
Potatoes	146	53	20	14
Radishes	137	107	0	0
Red beets	84	66	6	7
Sweet potatoes	148	63	0	0
Turnips	37	19	5	14
Water chestnuts	4	4	0	0
Other root/tuber vegetables	327	294	0	0
Vegetables, dried or paste	2	2	0	0
Vegetables with sauce	7	6	0	0
Vegetable juice	35	32	1	3
Vegetables, NEC	1	1	0	0
Total	8199	6131	164	2

Table 4. Continued

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
F. Other				
Whole coriander	24	20	3	13
Other whole spices	195	163	14	8
Other spices & flavorings	12	10	1	8
Condiments	1	1	0	0
Cashews	2	2	0	0
Other nuts & related products	127	80	0	0
Edible seeds & related products	1	1	0	0
Other vegetable oil products	1	1	0	0
Alcoholic beverages	1	0	0	0
Coffee & tea	4	4	0	0
Waters & nonalcoholic beverages	58	18	5	9
Chocolate & cocoa products	5	5	0	0
Baby food products	14	12	1	7
Other food products	28	24	1	4
Total	473	341	25	5
A–F Grand Total	13980	10701	216	1.5

Table 5. Analysis of all findings in United States by commodity group (FY89)

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
A. Grains and Grain Products				
Rice	1	1	0	0
Wheat	4	4	0	0
Other whole grains	15	11	0	0
Bakery & cereal products/snack foods	155	102	0	0
Pasta products	11	9	0	0
Rice products	7	4	0	0
Wheat products	12	4	0	0
Other whole grain products	15	5	1	7
Total	220	140	1	<1
B. Dairy Products/Eggs				
Milk and cream	1584	1338	14	<1
Cheese/cheese products	40	15	0	0
Eggs/egg products	117	116	0	0
Butter	37	34	0	0
Ice cream	28	14	0	0
Total	1806	1517	14	<1
C. Fish Seafoods/Other Meats				
Fish & shellfish	223	145	2	<1
Other meats	32	29	0	0
Poultry	32	31	0	0
Total	287	205	2	<1
D. Fruits				
Blackberries	3	2	0	0
Blueberries	19	16	0	0
Boysenberries	3	3	0	0
Grapes	297	220	0	0

Table 5. Continued

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
Raspberries	6	6	0	0
Strawberries	163	62	2	1
Other berries	18	10	0	0
Grapefruit	40	20	3	8
Lemons	79	51	0	0
Limes	7	6	0	0
Oranges	214	121	0	0
Tangerines	32	30	0	0
Other citrus fruits	32	22	0	0
Apples	568	434	0	0
Pears	149	108	0	0
Mixed fruits	1	0	0	0
Core fruits, NEC	5	4	0	0
Apricots	41	36	0	0
Avocados	97	97	0	0
Cherries	82	57	0	0
Nectarines	112	18	2	2
Peaches	155	55	0	0
Plums and prunes	135	76	0	0
Other pit fruits	21	12	0	0
Mangoes	13	13	0	0
Papaya	6	6	0	0
Pineapples	6	6	0	0
Plantain	3	3	0	0
Other tropical fruits	68	64	1	2
Cantaloupe	112	108	0	0
Honeydew	21	20	0	0
Watermelon	51	51	0	0
Bitter melons	6	6	0	0
Other vine fruits	23	21	0	0
Other fruits	36	34	0	0
Fruit jams & jellies	36	26	0	0
Fruit juices	147	110	0	0
Fruit toppings	3	3	0	0
Fruits, dried or paste	103	84	0	0
Total	2913	2021	8	<1
E. Vegetables				
Blackeyed peas	4	4	0	0
Corn	113	113	0	0
Garden/green/sweet peas	84	66	1	1
String beans	184	148	1	<1
Other beans, peas, corn	37	33	0	0
Cucumbers	256	208	0	0
Eggplant	70	60	4	6
Okra	26	26	0	0
Peppers	328	250	2	<1
Pumpkins	8	8	0	0
Squash	373	310	15	4
Tomatoes	388	334	2	<1
Other fruits used as vegetables	8	8	0	0
Asparagus	102	101	0	0
Bamboo sprouts	2	2	0	0
Broccoli	284	250	3	1
Broccoli raab	4	2	0	0
Brussels sprouts	49	45	0	0

Table 5. Continued

Commodity group	Total no. of samples	No. samples with no residues found	No. samples significant: Over tol. or no tol.	Percent samples significant: Over tol. or no tol.
Cabbage	337	304	1	<1
Cauliflower	177	169	1	<1
Celery	319	130	0	0
Chinese cabbage	154	108	17	11
Collards	149	109	1	<1
Endive/chicory	217	148	6	3
Kale	116	75	5	4
Lettuce	1144	863	14	1
Mustard greens	85	65	2	2
Parsley	54	34	0	0
Spinach	335	239	8	2
Turnip greens	21	16	0	0
Other leaf/stem vegetables	251	215	6	2
Mixed vegetables	9	9	0	0
Mushroom/truffle products	55	50	0	0
Carrots	238	203	0	0
Leeks	30	27	0	0
Onions	186	173	0	0
Potatoes	357	300	2	<1
Radishes	102	96	1	<1
Red beets	70	62	1	1
Sweet potatoes	135	80	1	<1
Turnips	55	36	2	4
Other root/tuber vegetables	253	229	0	0
Vegetables, dried or paste	9	8	0	0
Vegetables with sauce	10	10	0	0
Vegetable juice	16	14	2	13
Total	7204	5740	98	1
F. Other				
Dressings	1	1	0	0
Whole coriander	2	1	0	0
Other whole spices	152	124	7	5
Other spices & flavorings	19	16	1	5
Cashews	4	2	0	0
Coconuts	2	2	0	0
Other nuts & related products	25	22	0	0
Edible seeds & related products	5	5	0	0
Refined vegetable oil	13	13	0	0
Other vegetable oil products	5	4	0	0
Coffee & tea	8	8	0	0
Waters & nonalcoholic beverages	268	86	70	19
Chocolate & cocoa products	3	3	0	0
Baby food	88	72	2	3
Other food products	60	57	0	0
Total	655	416	80	12
A–F Grand Total	13085	10039	203	1.6

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Table A-1. Methods used by 10 state laboratories in state food quality assurance, 1989–90

FDA method codes	Extraction	Detector	No. of samples	State(s) using
999 55	"Other" multiresidue methods	ICP-FE	712	CA
999 58	"Other" multiresidue methods	GLC	718	CA
999 54	"Other" multiresidue methods	FE	733	CA
999 57	"Other" multiresidue methods	Fluorescence spectroscopy	734	CA
000 60	No extraction necessary	Spectrophotometric	770	VA
000 99	No extraction necessary	Other	806	MI
999 56	"Other" multiresidue methods	DCP-FE	870	CA
001 99	*PAM I*, 211.1 w/6% EtO/PE eluant only	Other	950	WI
999 12	"Other" multiresidue methods	LC post-column deriv. & fluorometric detection (Krause method, carbamates)	1094	CA
999 13	"Other" multiresidue methods	GLC, P selective detector, *LIB 2166* (aryl phosphates)	1112	CA
999 18	"Other" multiresidue methods	GLC, S selective detector, polar column, e.g., DEGS	1184	CA
999 17	"Other" multiresidue methods	GLC, halogen selective detector, *LIB 1710/1710A*, early eluters	1198	CA
999 14	"Other" multiresidue methods	GLC, P selective detector, polar column, e.g., DEGS	1214	CA
999 11	"Other" multiresidue methods	LC, UV detector	1236	CA
999 59	"Other" multiresidue methods	LC	1584	CA
999 15	"Other" multiresidue methods	GLC, N selective detector, polar column, e.g., DEGS	1634	CA
999 16	"Other" multiresidue methods	GLC-AFID (N/P or RbCl TD)	1661	CA
999 01	"Other" multiresidue methods	GLC-ECD	1735	CA
999 20	"Other" multiresidue methods	OV-101 column at 240°C, ECD (*LIB 3092*), or equiv., for syn. pyrethroids	1934	CA, IN
999 10	"Other" multiresidue methods	Paper chromatography	2067	CA, IN, NC
999 02	"Other" multiresidue methods	GLC, phosphorus selective detector (FPD)	2244	CA, IN
999 03	"Other" multiresidue methods	GLC, sulfur selective detector (FPD)	2244	CA, IN, NC
999 08	"Other" multiresidue methods	GLC-ECD, *LIB 1710/A*, early eluters	2988	CA, IN
999 04	"Other" multiresidue methods	GLC, nitrogen selective detector (HALL 700A)	2996	CA, IN, NC
999 07	"Other" multiresidue methods	GLC-MS	3007	CA, IN, NC, WI

Table A-1. Continued

FDA method codes	Extraction	Detector	No. of samples	State(s) using
999 05	"Other" multiresidue methods	GLC. halogen selective detector (HALL 700A or Microcoulometric)	3331	CA, IN
999 06	"Other" multiresidue methods	GLC-FID	3485	CA, IN
999 09	"Other" multiresidue methods	TLC	3631	CA, IN, NC, WI
001 50	*PAM I*, 211.1 with 6% EtO/PE eluant only	Flame AAS	5965	FL, NY, OR, VA
999 99	"Other" multiresidue methods	Other	24803	CA, FL, IN, NC, OR, VA, WI

Note: Abbreviations for states are as shown in text. AAS = atomic absorption spectrophotometry; AFID = alkali flame ionization detector; DCP = direct current plasma; DEGS = diethylene glycol succinate; ECD = electron capture detector; FE = flame emission; FID = flame ionization detector; FPD = flame photometric detector; GLC = gas liquid chromatography; ICP = inductively coupled plasma; LC = liquid chromatography; *LIB = Laboratory Information Bulletin;* MS = mass spectrometry; *PAM = Pesticide Analytical Manual*; RbCl TD = Rubidium chloride thermionic detector; TLC = thin layer chromatography; and UV = ultraviolet.

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Appendix A. Analytical Method Codes used by State Food Regulatory Agencies

In Table A-1, the first column contains FDA method codes as a 3-digit numeric code for extraction/cleanup of samples, plus a 2-digit code defining the separating instrument/detector combination. The second column contains the extraction/cleanup technique and the third column, the separative/detector unit. The fourth column contains the number of samples run by the state or states, and the state(s) using each method are listed in the last column.

The ubiquitous use of the code "999" to designate the extraction and preliminary cleanup of the sample extract is unfortunate, but reflects the extreme diversity of sample classes tested, as well as modifications of these procedures from standard AOAC or FDA methodologies by state laboratory chemists. Most liquid chromatographic or gas chromatographic separations are preceded by a "Luke extraction" (or similar) technique, with varying ratios and kinds of solvents that have shown to produce best recoveries of analytes for given types of matrixes. A wide variety of "cleanup" columns such as silica gel, alumina, or ion exchange resins is used. Each laboratory is responsible for demonstrating recovery, precision, and accuracy in such modified analyses by participation in many "check-sample" QA/QC programs, etc. Some laboratories are using inductively coupled argon or helium plasma mass spectrometry, or similar specialized detectors on capillary or packed column gas chromatographic separative units.

DRUG FORMULATIONS

Liquid Chromatographic Method for Selegiline Hydrochloride and Related Compounds in Raw Materials and Tablets

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A liquid chromatographic method has been developed for determination of selegiline hydrochloride and related substances in drug raw material and tablet formulations. The method resolves 3 known related compounds from the drug with a limit of quantitation of less than 0.05%. Drug raw material and tablet samples contained ca 0.2 and 0.6% impurities, respectively. These included some related compounds, the identities of which were confirmed by gas chromatography with a Fourier transform infrared detector.

Selegiline hydrochloride is used in the management of parkinsonism. It is available as 5 mg tablets in a number of European countries (1), Canada, and the United States. There are no monographs for selegiline hydrochloride in either the British or United States pharmacopeias.

Figure 1 shows structures and chemical names of selegiline hydrochloride (IV) and a number of its related compounds, based on the route of synthesis (2). Compound II is a probable reaction intermediate; compounds I and III are possible side reaction products.

No methods for determination of selegiline were found in the literature. The present paper describes a method for drug content and impurities in raw materials and tablets.

Experimental

Equipment

The liquid chromatographic (LC) system consisted of a Varian Model 2010 pump, a Waters Model WISP 710B autosampler, a Varian Model 2050 variable wavelength detector set at 254 nm and a Perkin-Elmer Model LCI-100 integrator. A Spherisorb® CSC-S 3 μm cyano bonded column 150 × 4.6 mm (Chromatographic Sciences Company No. 048943) was used at ambient temperature with a mobile phase flow rate of 1 mL/min. Infrared spectra were obtained using a Digilab FTS-40 Fourier transform infrared (FTIR) spectrometer coupled to a Hewlett Packard 5890 gas chromatograph equipped with a 7 m × 0.53 mm column coated with a 2.65 μm film of dimethyl polysiloxane. Other equipment used was a Varian DMS 90 UV/Vis spectrophotometer connected to an HP 85 computer with a plotter and disk drive, a Fisher Accumet Model 425 pH meter and an Eberbach shaker.

Determination of Selegiline HCl and Related Compounds

Chemicals

The following LC grade chemicals were used: acetonitrile (J.T. Baker Co, Phillipsburg, NJ); ammonium phosphate monobasic; 85% phosphoric acid (Fisher Scientific, Fair Lawn, NJ). Deionized water was prepared using a Sybron/Barnstead system. Samples of selegiline hydrochloride and related compounds were obtained from the internal sample library of the Health and Welfare Canada Bureau of Drug Research; the nuclear magnetic resonance (NMR), mass, and infrared spectra of these samples corresponded to the known structures. Selegiline tablets were purchased from a local pharmacy.

Mobile Phase

Transfer acetonitrile (150 mL) to a 1 L volumetric flask, make up to volume with 0.1M ammonium phosphate buffer. Mix, filter, and degas under vacuum. The buffer consisted of 0.1M ammonium phosphate monobasic solution, adjusted to pH 3.1 with 85% phosphoric acid.

Preparation of Solutions

Prepare all solutions in dissolution solution consisting of 15% acetonitrile in water. *Resolution solution.*—0.05 mg/mL each of selegiline hydrochloride and III. *Related compounds standard solution.*—0.01 mg/mL selegiline hydrochloride. *Assay standard solution.*—0.5 mg/mL selegiline hydrochloride. *Related compounds test solution.*—5 mg/mL selegiline hydrochloride. *Tablet-related compounds test solution.*—Powder and mix 20 tablets. Transfer a portion of mix equivalent to 10 mg selegiline hydrochloride to a 6 mL centrifuge tube fitted with Teflon-lined screw cap and add 2 mL dissolution solution. Shake tube for 30 min, centrifuge at 3000 rpm for 10 min, and filter through a 0.45 μm filter (LD/X Syringeless Filter AQOR 0.45 μm Nylon 66). *Tablet assay test solution.*— Follow procedure above but use 30 mL test tube and 20 mL dissolution solution.

System Suitability

A 20 μL aliquot of the resolution solution was injected. The system was deemed satisfactory if the resolution between

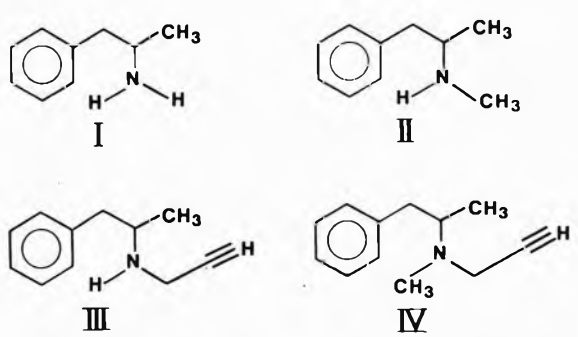

Figure 1. The chemical structures of selegiline and related compounds. (I) 1-phenyl-2-aminopropane (amphetamine). (II) 1-phenyl-2-(*N*-methyl)aminopropane (methamphetamine). (III) 1-phenyl-2-(*N*-2′-propyne)aminopropane (desmethyl selegiline). (IV) 1-phenyl-2-(*N*-methyl, *N*-2′-propyne)aminopropane (selegiline).

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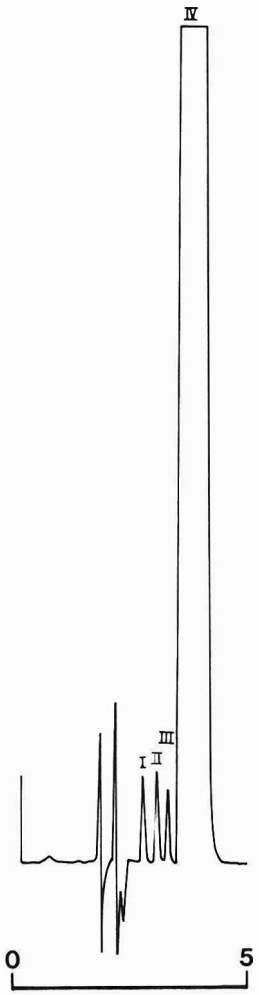

Figure 2. LC chromatogram of selegiline HCl raw material to which has been added 0.2% of related compounds I, II, and III.

the peaks was greater than 3, the efficiency of the column (drug peak) was more than 40 000 plates/m, the tailing factor was less than 2, and 6 replicate injections of the related compounds and assay standard solutions produced coefficients of variation (CVs) of less than 3% and 1%, respectively, all calculated on the selegiline peak using USP procedures (3). Retention times of selegiline and III were about 3.9 and 3.2 min, respectively (Figure 2).

Procedures

Related compounds.—Inject 20 μL of related compounds standard and test solutions into chromatograph and run for 30 min. Calculate percentage of each impurity from $100(A_i/A_s)(C_s/C_u)$ where A_i = peak area response due to each impurity, A_s = peak area response of the selegiline hydrochloride standard, C_s = concn of selegiline hydrochloride in the standard solution and C_u = concn of selegiline hydrochloride in the test solution.

Drug assay.—Inject 20 μL of assay standard and test solutions into chromatograph and run for 6 min. Calculate percentage of selegiline hydrochloride from $100(A_u/A_s)(C_s/C_u)$ where A_u and A_s = the areas of the selegiline hydrochloride assay test and assay standard solutions, respectively, and C_s and C_u = the corresponding concentrations.

Table 1. LC and UV characteristics of selegiline HCl and related compounds

Compound	RRT[a]	Slope[b]	Intercept[c]	Relative LC response	Relative[d] UV absorptivity
I	0.675	162	779	1.37	1.20
II	0.75	157	2058	1.33	1.25
III	0.825	148	435	1.25	1.10
IV	1	118	853	1.0	1.0

[a] Retention time relative to selegiline HCl at 3.9 min.
[b] Area counts/nanogram on column.
[c] Area counts. Amounts on column ranged from 50 to 1000 ng; corr. coeff. were 0.999.
[d] Absorptivity at the UV maximum of 257 nm.

Identification of Drug-Related Impurities

Sample Solution

Shake 5 powdered tablets for 10 min with 10 mL of 0.1M HCl; adjust pH to ca 9.5 with 0.1M NaOH and extract mixture with three 4 mL portions of dichloromethane. Combine extracts, filter, and evaporate to ca 1 mL on a rotary evaporator. Transfer residue to a suitable tube and reduce to 100 μL under a stream of dry nitrogen. The approximate concentration is 250 mg/mL.

Procedure

Program gas chromatograph as follows: 40°C for 2 min, 15°C/min to 200°C and hold for 10 min. Set injection port at 200°C and transfer line and light pipe at 260°C. Adjust helium carrier gas flow to 3 mL/min. Inject 2 μL aliquots of sample as prepared above and record results.

Results and Discussion

A chromatogram showing the resolution of I, II, and III from selegiline hydrochloride is presented in Figure 2; UV and LC characteristics are listed in Table 1. The system was linear for amounts on column, determined by varying the solution concentration, corresponding to 0.05 to 1.0% of each related compound in the drug raw material. The correlation coefficient of the selegiline response from 6520 to 14 320 ng on column, corresponding to 65 to 143% of assay concentration, was 0.996. Slopes (Table 1) produce the response of the system to the related compounds, for comparison to selegiline.

Extraction from Formulations

Forty tablets were powdered and aliquots equivalent to 10 mg of drug were transferred into 30 mL test tubes fitted with Teflon-lined screw caps; then, 20 mL of 15% acetonitrile in water was added. Tubes were shaken for periods of 10, 20, 30, and 45 min and contents assayed. Recoveries were 99.7, 100.2, 101.0, and 100.8% after 10, 20, 30, and 45 min, respectively. The method requires shaking for 30 min to assure complete extraction.

Precision

The precision of the system was determined by making 6 replicate injections of a solution containing 0.545 mg/mL selegiline hydrochloride in 15% acetonitrile in water. The relative standard deviation (RSD) of the peak area responses was 0.18%. Six replicate injections of compounds I, II, III,

Table 2. Related compounds in selegiline HCl raw material and tablet samples (%)[a]

RRT[b]	A	B	C
0.62	0.12		
0.68			0.02
0.73			0.21
0.78		0.14	0.04
1.55		0.05	
2.08			0.30
Total	0.12	0.19	0.57

[a] Samples A and B = raw materials; C = 5 mg tablets.
[b] Relative to selegiline HCl at 3.8 min.

and IV at concentrations of 10.32, 10.24, 10.20, and 10.16 μg/mL produced RSDs of 1.97, 3.47, 1.29, and 2.41%, respectively. Six portions of a tablet composite produced a mean selegiline content of 101.0% (RSD 1.9%). In another case, 6 portions analyzed on the same day produced an RSD of 1.4%; when analyzed on 5 successive days, the RSD was 2.0%.

Ruggedness

Most method development was done on a Spherisorb CSC-S cyano bonded column 150 × 4.6 mm (Chromatographic Sciences Company No. 048943). Column Nos. 048942 and 039053 from the same source were also successfully used. Increasing the pH from 3.1 to 3.5 had little effect on the retention time of selegiline and related compounds. Increasing acetonitrile content from 15 to 20% decreased all retention times, bringing amphetamine closer to the solvent peak. Decreasing acetonitrile content from 15 to 10% increased the retention time of all compounds without affecting their resolution. A 10 cm cyano column from the same company (No. 078714) produced good resolution of all compounds, with shorter retention times. Lowering the flow rate on this column increases separation and retention times. None of these changes affected the tailing of the drug and related compounds. A solution of selegiline hydrochloride in 15% acetonitrile in water was injected at regular intervals over a period of 5 days. No increase in the level of impurities was detected.

Impurity Levels

Two samples of selegiline HCl raw material and one of 5 mg tablets were analyzed for related compounds. Results are shown in Table 2. The method was checked by an independent analyst using different equipment.

Table 3. Assay results for drug raw materials and 5 mg tablets (%)[a]

Sample	1st Weighing	2nd Weighing	3rd Weighing	Mean
A	99.4	100.6	99.7	99.9
B	99.9	98.3	98.9	99.0
C	101.4	99.7	101.2	100.8

[a] Samples A and B = raw materials; C = 5 mg tablets.

Assay

Raw material and tablet samples were assayed in triplicate. Results and assay means are shown in Table 3.

Identification of Related Compounds by GC/FTIR

Related compounds I, II, and III were present in a tablet product. Identification was confirmed by comparison of GC retention times and infrared spectra obtained by GC/FTIR to similar data obtained from authentic samples, with additional confirmation by gas chromatography/mass spectroscopy (GC/MS). For example, a peak at 8.87 min was identified as II by infrared [700 (s), 635 (m), 1131 (m), 1164 (m), 1454 (m), 1490 (m), 2801 (m), 2859 (m), 2934 (s), 2974 (s), 3034 (s), 3073 (m)] and mass spectroscopy [m/z = 148 (1%), 134 (2), 115 (1), 91 (5), 65 (4), 63 (2), 58 (100), 56 (5), 51 (3), 42 (4), and 39 (3)].

Conclusion

The present methods are useful for evaluation of the purity and drug content of selegiline drug raw materials and tablet products.

Acknowledgments

M. Bos, M. Cléroux, and R.W. Sears assisted with the experimental work. NMR, infrared (in KBr only), and mass spectra were obtained by H. Avdovich, H. Beckstead, and J.-C. Ethier, respectively.

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Methods for Assay, Related Substances, and Organic Volatile Impurities in Triazolam Raw Materials and Formulations

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Liquid chromatographic methods have been developed for determination of triazolam and related compounds in drug raw materials and tablets. The methods resolve 9 available related compounds from the drug with quantitation limits of 0.1% or less. Eight raw materials and 16 formulations were examined for related compounds. Total impurities in raw materials ranged up to 0.6% and up to 1% in formulations, not including a compound in some tablets that is believed to be excipient related. Assay values were between 99.9 and 101.3% for raw materials and between 89.4 and 105.5% for tablets. A gas chromatographic method was developed for organic volatile impurities in drug raw materials. The major solvents found were methyl ethyl ketone (100–750 ppm), ethyl acetate (510 ppm), and *n*-propanol (80–300 ppm), with a trace of benzene (<30 ppm) in several samples.

Drug raw materials are increasingly produced in different facilities around the world. To evaluate the quality of such drugs, analysts need methods for purity and assay that respond to the presence of impurities originating during synthesis or degradation, whether drug- or process-related.

Triazolam is a potent sedative and hypnotic available in Canada and the United States at tablet strengths up to 0.5 mg. Many liquid chromatographic (LC) (1–4) and gas chromatographic (GC) (5) methods have been reported for determination of triazolam in body fluids; however, methods for determination of related compounds and organic volatile impurities in drug raw material and formulations do not appear to have been published.

Figure 1 shows structures of triazolam and a number of related compounds. The *United States Pharmacopeia* (USP) (6) contains monographs for triazolam raw material and tablets. The USP provides a GC method for purity with the requirement that total impurities not exceed 1.5%. Using this method, compounds V and VI were found to elute at the solvent front and compound III gave poor peak shape. The LC assay method was evaluated for possible use in related compound determination. Compounds V and VI were not resolved from the solvent front; however, all related compounds available to us were resolved from the drug. Thus, the method appears to be satisfactory for its intended purpose of drug assay. The present paper describes an LC method for determination of triazolam and related compounds and a GC method for organic volatile impurities.

METHODS

Reagents and Apparatus

(a) *Chemicals.*—Acetonitrile and chloroform (J.T. Baker Co., Phillipsburg, NJ), tetrahydrofuran (Aldrich, Milwaukee, WI), ammonium phosphate monobasic, phosphoric acid (85%), and triethylamine (Fisher Scientific, Fairlawn, NJ) were all LC grade. Dichloromethane (J.T. Baker Co.) was "capillary analyzed." Benzyl alcohol (Aldrich) was "gold label;" trichloroethylene and 1,4-dioxane (Fisher) were certified and ACS reagent grades, respectively; benzene (Caledon, Georgetown, ON, Canada) was ACS reagent grade; and ethylene oxide was from Matheson. Triazolam was USP reference standard (USP, Rockville, MD). Water was deionized on a Sybron/Barnstead system. Triazolam-related compounds were obtained as follows. Compounds II, III, and IV (Fabrica Italiana Sintetici, Milan, Italy); V, VI, and VII (Profarmaco, Milan, Italy); IX (Aldrich, Milwaukee WI). Nuclear magnetic resonance (NMR) and infrared spectra were consistent with respective structures of these compounds. Compound VIII was prepared by refluxing triazolam with sodium borohydride in tetrahydrofuran with recovery by evaporation, dissolution in sodium bicarbonate solution, and extraction with methylene chloride. Mass and infrared spectra were consistent with the structure of VIII.

(b) *Liquid chromatograph.*—Varian Model 5060 fitted with a 10-μL loop injector (Spectra-Physics Model SP8780 XR), a detector set at 220 nm (Varian Model UV-100), and a data station (Varian Model Vista 402). 3-μm Spherisorb octadecylsilane bonded phase columns (ODS-2, 100 × 4.6 mm, Chromatography Sciences Co., Serial Nos 088915 and 078921 and 150 × 4.6 mm, Serial No. 118859) were used at ambient temperature with a mobile phase flow rate of 1 mL/min.

(c) *Gas chromatographs.*—For related compounds: Hewlett Packard Model 5890 was equipped with a flame ionization detector (FID), a 7673A autosampler, a Model 3393A integrator with an HP 9114B disk drive and a capillary column (HP Ultra2: 5% phenyl methylsilicone, 0.33 μm, 12.5 m × 0.2 mm). For organic volatile impurities: Hewlett Packard Model 5890 was equipped with an FID detector and a 1 m deactivated retention gap coupled to a fused silica column (DB-5, J&W Scientific: 5% phenyl dimethyl polysiloxane, 1.5 μm, 30 m × 0.53 mm). For identification of organic volatile impurities: a Fourier transform infrared detector (Digilab FTS-40) with a Sadtler library of vapor phase spectra was coupled to a Hewlett Packard 5890 gas chromatograph equipped with a capillary column (HP-1: dimethylpolysiloxane, 2.65 μm, 0.53 mm × 7 m).

(d) *Mass spectrometer.*—A Finnigan MAT Model 4610B used in the electron impact mode, was fitted with a J&W Scientific, 15 m DB-5 column, film thickness, 0.25 μm. The ionizing energy was 70 eV and the scan rate was 34–550 daltons/s.

(e) *Other equipment.*—Centrifuge (International Equipment Co., Model K); UV/Vis spectrophotometer (Varian DMS 90) connected to an HP 85 computer with plotter and disk drive; horizontal action shaker (Burrel Model 75).

LC Methods

Mobile Phase

Buffer.—Prepare a solution of 0.05M ammonium phos phate monobasic and adjust pH to 3 with 0.05M phosph

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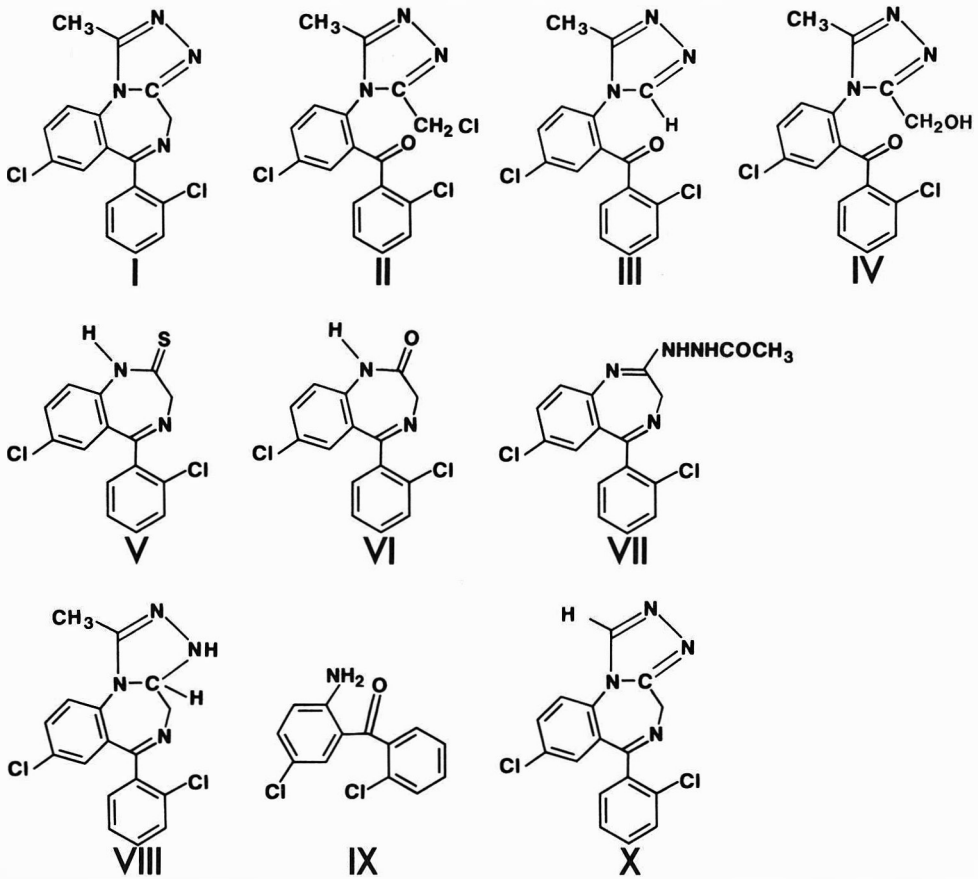

Figure 1. Structures of triazolam and related compounds. (I) 8-chloro-6-(2′-chlorophenyl)-1-methyl-4H-triazolo-[4,3-a][1,4]-benzodiazepine (triazolam). (II) 2′,5-dichloro-2-[3-(chloromethyl)-5-methyl-4H-1,2,4-triazol-4-yl]-benzophenone. (III) 2′,5-dichloro-2-(3-methyl-4H-1,2,4-triazol-4-yl)-benzophenone (methyl-triazolyl-dichlorobenzophenone). (IV) 2′,5-dichloro-2-[3-(hydroxymethyl)-5-methyl)-4H-1,2,4-triazol-4-yl]-benzophenone. (V) 5-(2′-chlorophenyl)-7-chloro-1,3-dihydro-1,4-benzodiazepin-2H-2-thione. (VI) 5-(2′-chlorophenyl)-7-chloro-1,3-dihydro-1,4-benzodiazepin-2H-2-one. (VII) 5-(2′-chlorophenyl)-7-chloro-1,3-dihydro-1,4-benzodiazepin-2H-2-acetyl hydrazine. (VIII) 8-chloro-6-(2′-chlorophenyl)-1-methyl-4H-s-2,3-dihydrotriazolo-[4,3-a][1,4]-benzodiazepine (dihydrotriazolam). (IX) 2-amino-2′,5-dichlorobenzophenone. (X) 8-chloro-6-(2′chlorophenyl)-4H-triazolo [4,3-a][1,4] benzodiazepine (desmethyl triazolam).

acid. Transfer 400 mL acetonitrile to a 1 L volumetric flask, add 1 mL triethylamine, and dilute to volume with buffer. Filter through a 0.45 μm nylon 66 filter.

Preparation of Solutions

Use acetonitrile for all solutions. *Resolution solution.*—0.01 mg/mL triazolam and 0.01 mg/mL VI. *Related compounds standard solution.*—0.001 mg/mL triazolam. *Assay standard solution.*—0.05 mg/mL triazolam. *Related compounds test solution.*—0.5 mg/mL triazolam. *Assay test solution.*—0.05 mg/mL triazolam. *Tablet-related-compounds test solution.*—Powder not less than 20 tablets and mix. Transfer equivalent of 1 mg triazolam, accurately weighed, to a 5 mL centrifuge tube, add 2 mL acetonitrile and shake for 30 min to obtain a final concentration of 0.5 mg/mL. Filter solution through a 0.45 μm disposable filter. *Tablet assay test solution.*—Dilute tablet-related-compounds test solution with acetonitrile to obtain a final concentration of 0.05 mg/mL.

System Suitability

Inject a 10 μL aliquot of resolution solution. Resolution is more than 2, efficiency of the column (based on the triazolam peak) is greater than 40 000 plates/meter, and the tailing factor is 1.5 or less—all calculated using USP procedures (6). Six injections of related-compounds standard solution produce a coefficient of variation (CV) of less than 5%. Retention time of triazolam is between 5 and 6 min; relative retention time (RRT) of VI is ca 1.3. If necessary, adjust concentration of acetonitrile in the mobile phase to meet these requirements. Five injections of the assay standard solution produce a CV of less than 1%.

Procedures

Related compounds.—Inject separately 10 μL of related compounds standard and test solutions into chromatograph and run for 30 min. Calculate percentage of each impurity peak from $100(A_i/A_s)(C_s/C_u)$, where A_i = peak area response of each impurity, A_s = peak area response of triazolam in the standard solution, C_s = concentration of triazolam in the standard solution, and C_u = concentration of triazolam in the test solution.

Drug assay.—Inject separately 10 μL of the assay standard and test solutions into chromatograph and run for 10 min. Calculate percentage of triazolam from $100(A_u/A_s)(C_s/C_u)$, where A_u and A_s = areas of the triazolam peaks and C_s and C_u = concentrations of triazolam in the standard and test solutions, respectively.

GC Methods

Organic Volatile Impurities

Test solution.—Accurately weigh 17 mg triazolam into a 1.7 mL vial, crimp close, and add 1.7 mL benzyl alcohol.

Procedure

Set helium carrier gas flow rate to 6 mL/min, nitrogen makeup gas to 28 mL/min, air flow to 390 mL/min, and hydrogen flow to 34 mL/min. Inject 1 μL each of blank (benzyl alcohol) and test solution in splitless mode with purge at about 70 mL/min, activated at 0.8 min. Temperature program column oven as follows: 35°C for 4 min; 5°C/min to 50°; 20°C/min to 150°C and hold for 15 min. If peaks due to organic volatile compounds are present, make tentative identification by comparison to a table of retention times of common organic volatile impurities. If necessary, confirm identification by the procedure for identification of volatile impurities given below. If the impurity is present at a level that exceeds 100 μg/tablet, quantitate by peak area comparison to an external standard of the compound.

Identification of Volatile Impurities

Transfer 0.5 g triazolam and 50 μL water to a 4 mL vial and heat to 100°C for 15 min. Inject ca 3 mL headspace gas from vial into gas chromatograph coupled to Fourier transform infrared (FTIR) detector. Identify unknown impurity by comparison of its spectra to those in the FTIR library.

Related Compounds

Test solution.—Prepare a solution of drug raw material 2 mg/mL in a 9:1 (v/v) solution of chloroform–methanol.

Procedure.—Set injector temperature to 280°C and detector temperature to 290°C. Program oven temperature as follows: 250°C for 10 min, then 5°C/min to 280°C and hold for 20 min. Use helium carrier gas at a linear flow velocity of 20 cm/s. Inject 4 μL aliquots of test solution with a split injection ratio of ca 50:1.

Results and Discussion

Liquid Chromatography

A chromatogram showing resolution of triazolam from 8 related compounds is presented in Figure 2. The precision of the system was determined by making 5 replicate injections of solutions containing 0.0367, 0.0403, and 0.0528 mg/mL triazolam. CVs were 0.5, 0.51, and 0.4%, respectively.

Sensitivity and linearity.—UV maxima in the range from 200 to 350 nm, absorbance at 220 nm, and LC characteristics are presented in Table 1 for triazolam and the available related compounds. The chromatographic response for all compounds was linear from 1 to 100 ng on column, R^2 0.9983 or greater. In addition, triazolam response was linear from 250 to 800 ng on column, R^2 greater than 0.999. The latter range corresponds to 50 to 160% of assay concentration.

Related compounds.—Nine samples of drug raw material from 4 sources and 16 tablet samples from 5 sources were

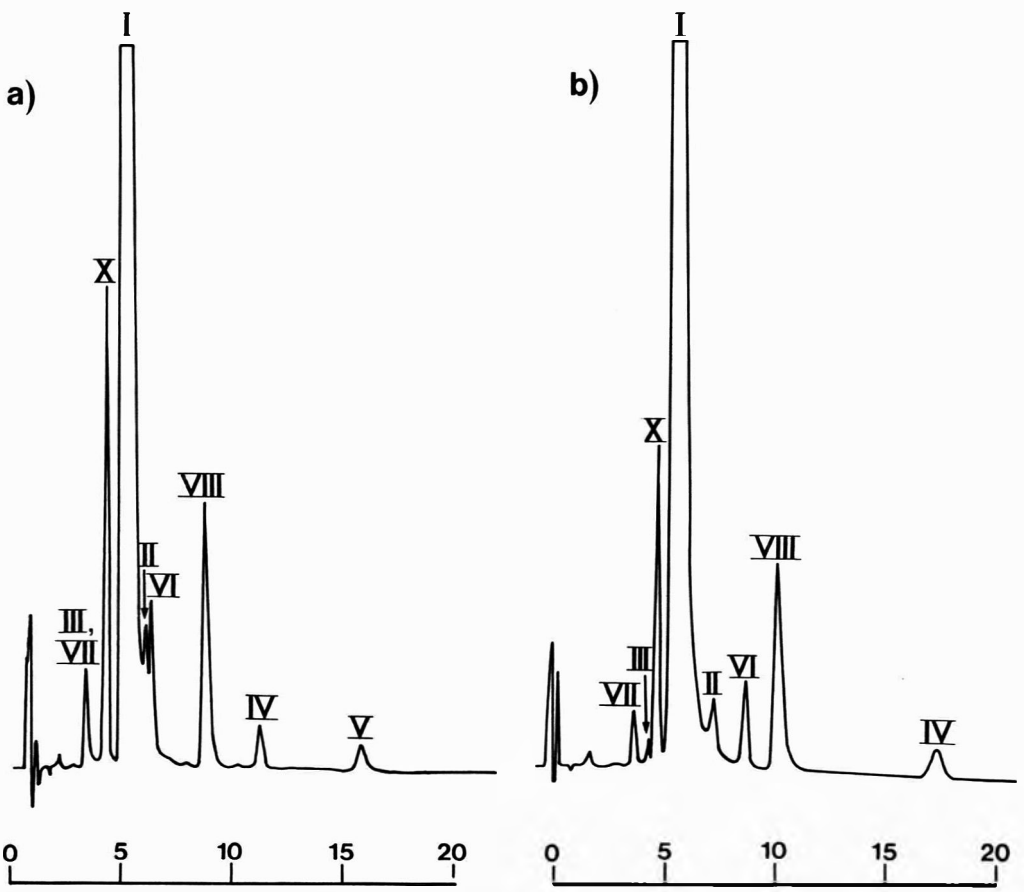

Figure 2. Chromatograms of triazolam in the presence of available related compounds, concentrations in percent relative to 4.97 μg triazolam on column: II (0.22), III (0.20), IV (0.22), V (0.20), VI (0.24), VII (0.16), VIII (0.16). Chromatogram (a) was obtained with column No. 078921 after 3 months use; (b) was obtained with a new column, No. 088915 (V is at 27 min). The scale is in minutes.

Table 1. UV and LC characteristics of triazolam and related compounds

Compound	Maximum, nm	Absorptivity, 220 nm	LC characteristics		
			RRT[a]	Response[b]	Limit, %[c]
I	220	0.122	1.00	1.00	0.02
II	215, 252	0.093	1.22	0.69	0.04
III	215, 252	0.085	0.66	0.61	0.01
IV	215, 253	0.086	2.4	0.62	0.04
V	215, 305	0.075	3.6	0.59	0.09
VI	221	0.137	1.3	0.96	0.04
VII	215	0.067	0.61	0.5	0.09
VIII	203, 235	0.058	1.6	0.36	0.09
IX			4.1	0.55	0.11

[a] Retention time relative to triazolam at 5.1 min.
[b] Slope of LC weight response curve, relative to triazolam.
[c] Quantitation limit is that level of impurity that can be analyzed with a CV of less than 10%.

available for analysis. To code samples, each manufacturer was assigned a code letter and samples from the same manufacturer were distinguished by a number. (Tablet sample codes begin with the letter T). Related compound levels found in raw materials and tablets are shown in Tables 2 and 3, respectively. Impurities found in tablet samples corresponded approximately with those in raw materials. The UV spectrum of an impurity found in some tablets at an RRT of 4.6 (Table 3) is dissimilar to that of the drug. This impurity is thought to be excipient related. None of the related compounds available for method development corresponded to the major impurities found in the samples analyzed.

Drug content.—Drug raw material and tablet samples were assayed in triplicate; sample D1 was used as a standard. For raw materials, results were as follows [sample, mean, % (CV, %)]: A1, 101.1 (0.6); A2, 99.9 (0.7); A3, 100.8 (0.3); B1, 100.5 (0.5); B2, 100.6 (1.2); C, 100.1 (0.8); D2, 101.3 (0.4). For tablets, results were: TF1, 98.9 (0.1); TF2, 96.3 (1.3); TF3, 105.5 (2.2); TG1, 96.4 (0.2); TG2, 91.5 (0.4); TG3, 95.8; TG4, 96.0 (1.4); TG5, 98.0 (1.4); TG6, 99.1 (0.2); TH1, 89.4 (2.0); TH2, 92.7 (0.8); TD1, 94.9 (0.9); TD2, 94.0 (1.1); TD3, 99.5 (0.7); TJ1, 95.1 (1.4); TJ2, 97.5 (1.0). CVs were <1.2% for raw materials and 2.2% for tablets.

Solution stability.—A solution of triazolam in acetonitrile was stable for at least 24 h under laboratory conditions (non-

Table 2. Impurities in triazolam raw material samples by LC (%)

Sample	RRT[a]			Total impurities
	0.83	1.51	1.78	
A1	0.04			0.04
A2	0.03			0.03
A3	0.03			0.03
B1	0.02	0.09	0.29	0.40
B2	0.02	0.09	0.29	0.40
C	0.57			0.57
D1				ND[b]
D2				ND
E		0.01		0.01

[a] Retention time relative to triazolam at 5.1 minutes.
[b] Not detected.

Table 3. Impurities in triazolam tablet samples by LC (%)

Sample	RRT[a]									Total
	0.32	0.54	0.64	0.85	1.5	1.7	2.1	3.6	4.6[b]	
TF1				0.04	0.15	0.25	0.03			0.47
TF2				0.04	0.15	0.22	0.02			0.43
TF3				0.04	0.17	0.18				0.39
TG1				0.71		0.11	0.15			0.97
TG2				0.68			0.07			0.75
TG3				0.68			0.07			0.75
TG4				0.70	0.03		0.09			0.82
TG5				0.71	0.02		0.05			0.78
TG6				0.72			0.04			0.76
TH1	0.05	0.02	0.01	0.03		0.02	0.09	0.03		0.25
TH2			0.01	0.03		0.06	0.02			0.12
TD1									0.98	0.98
TD2									0.67	0.67
TD3									0.33	0.33
TJ1									1.71	1.71
TJ2									0.77	0.77

[a] Retention time relative to triazolam at 5.1 minutes.
[b] May be excipient related.

UV fluorescent light) and only 0.6% of compound VII was transformed to triazolam after 12 h. The choice of extraction solvent was crucial to the success of the method. Compound VII is readily transformed to triazolam in methanol and to IV in dilute acid (0.1N HCl).

Tablet extraction.—Portions of TD3, suitably powdered, were shaken in acetonitrile for 20, 30, 45, and 60 min and assayed. Assays were 100.8, 102.8, 101.6, and 102.4%, respectively. Similarly, portions of TH2 shaken for 10, 20, and 30 min produced assays of 92.7, 97.7, and 97.1% of label claim. The method calls for a 30 min extraction time.

Precision of the method.—Raw material A2 was assayed 6 times on the same day to produce a mean of 99.9% with an intraday CV of 0.7%. The same raw material was assayed in triplicate daily for 5 consecutive days to produce a mean of 100.1% with an interday CV of 0.7%. Formulation TD3 was assayed 6 times on the same day to produce a mean of 99.5% with an intraday CV of 0.7%. The same formulation was assayed in triplicate for 5 consecutive days to produce a mean of 99.2% with an interday CV of 1.5%.

Ruggedness.—Two columns were used during method development (150 × 4.6 mm, Serial No. 118859, and 100 × 4.6 mm, Serial No. 078921). Both resolved all related compounds from the drug. The 100 mm column, which gave shorter retention times, was adopted for analytical work. An increase in pH from 3 to 4 produced peak broadening for III. Increasing triethylamine from 0.1 to 0.4% lead to peak broadening and peak splitting, with loss of resolution of some impurities from each other and triazolam. Without triethylamine, several peaks were broad and difficult to quantitate. An increase of 5% in acetonitrile brings compounds II and VI to the shoulder of the drug peak. After a few weeks of use, retention times were slightly shorter and a small adjustment in acetonitrile content of the mobile phase was needed to meet system suitability requirements. A third column (100 × 4.6 mm, Serial No. 088914) was evaluated; it produced slightly longer retention times. All compounds eluted in less than 30 min on all 3 columns. Compounds III and VII may co-elute; however, this is not critical because they originate in different synthetic processes and are unlikely to be present simultaneously.

Table 4. Organic volatile impurities in triazolam raw materials

Sample	Retention time, min	Area (counts)	Amount, ppm	Identity[a]
A1	2.99	3126	100	MEK
A2	2.98	21834	750	MEK
	4.59	1814	30	Benzene
A3	2.99	14931	460	MEK
	4.60	378	6	Benzene
B1	2.42	7832	300	*n*-PrOH
	2.71	911		Unknown
B2	2.42	2088	80	*n*-PrOH
	4.60	689	10	Benzene
C	3.36	10156	510	EtOAc
	4.68	444	10	Benzene
D1	1.58	1927	100	EtOH

[a] EtOAc = ethyl acetate; *n*-PrOH = *n*-propanol; MEK = methyl ethyl ketone.

Table 5. GC retention times and relative sensitivities of triazolam and related compounds

Code	Retention time, min	RRT[a]	Relative sensitivity
I	20.81	1.00	1.00
II	17.02	0.82	0.40
III	13.66	0.66	0.68
IV	17.81	0.86	0.14
V	15.09	0.73	0.32
VI	10.71	0.51	0.56
VII	20.48	0.98	0.48

[a] Retention time relative to triazolam at 20.8 min. Impurity levels calculated by area comparison to an external triazolam standard.

Gas Chromatography

Organic volatile impurities.—Levels found in representative drug raw materials are shown in Table 4. Of the compounds identified, only benzene is on the USP list of organic volatile impurities, with a proposed limit of 100 ppm (7). Where found, levels of benzene were 30 ppm or less.

Related compounds.—Retention times and relative sensitivities of triazolam related compounds are shown in Table 5 and related compound levels found in selected raw material samples are shown in Table 6. Changing column temperature or solvent for dissolving raw materials did not alter relative peak areas. A peak at RRT 0.80 was observed in all raw material samples, but was not observed by the GC/MS procedure, nor was a common peak observed by LC. This peak is believed to be an artifact of the GC method. The compound at RRT 0.91 in product C exhibited a mass spectrum consistent with desmethyl triazolam X. Typical ions were (*m/z*): 328, 326 (3:1), 293 ($M^+ - Cl$), 265, 239, 207, and 137.

Table 6. Related compounds in triazolam raw materials by GC (%)

RRT[a]	A1	A3	B1	B2	C	D1	E
0.36	0.19		0.05				
0.80	0.41	0.35	0.31	0.25	0.43	0.27	0.30
0.91					0.56		
1.02			0.34	0.31			
1.38				0.10			
Total	0.60	0.35	0.70	0.66	0.99	0.27	0.30

[a] Retention time relative to triazolam at 20.8 min. Impurity levels calculated by area comparison to an external triazolam standard.

Conclusions

LC methods for determination of related compounds and drug content and GC methods for determination of related compounds and organic volatile impurities have been described. The LC method for drug and related compounds is preferred to avoid possible problems with an artifact in the GC method and thermal decomposition of VII to triazolam.

Acknowledgments

Mass, nuclear magnetic, and KBr infrared spectra were obtained by J.-C. Ethier, H. Avdovich, and H. Beckstead, respectively.

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Liquid Chromatographic Determination of Clidinium Bromide and Clidinium Bromide-Chlordiazepoxide Hydrochloride Combinations in Capsules

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A specific liquid chromatographic method was developed for determination of clidinium bromide and clidinium bromide-chlordiazepoxide hydrochloride combinations in capsules. The procedure uses a Partisil 10 ODS-3 column and a mobile phase consisting of acetonitrile–0.3M ammonium phosphate (32 + 68) (pH = 4.3). The detection wavelength is 235 nm. Accuracy, reproducibility, and linearity were within accepted criteria. Four commercial samples of the single ingredient were tested; results compared favorably with the compendial method. Two commercial samples of the combination product were tested by the proposed method and results reported. The system was found to be free from any interferences from the 4 known related compounds of the 2 major components and is useful as a screening procedure for 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide, the principal degradation product of chlordiazepoxide hydrochloride.

Clidinium bromide [3-hydroxy-1-methyl-quinuclidinium bromide benzilate] (CB) is a quaternary ammonium antimuscarinic drug (1) commercially available in the United States in capsule form. (See Figure 1.) It is often combined with the anti-anxiety drug, chlordiazepoxide hydrochloride (CP), and used for the treatment of disorders of the bowel and for peptic ulcers.

A number of techniques have been used to determine clidinium bromide in pharmaceutical dosage forms. Abdelkader et al. (2), in a study of quaternary compounds, analyzed clidinium bromide in the presence of chlordiazepoxide hydrochloride by forming a trihalide complex and measuring it spectrophotometrically. Sidhu et al. (3) included clidinium bromide in a study of a general liquid chromatographic (LC) system for determining many pharmaceuticals. More specifically, Honigberg et al. (4), in a study of the LC characteristics of a group of anti-anxiety, antispasmodic mixtures, used 2 types of columns and buffered mobile phases of varying pH to separate clidinium bromide and chlordiazepoxide hydrochloride. Several of these systems, however, suffered from significant tailing and multiple peaks. Recently, Jalal et al. (5) developed a specific LC method for determining clidinium bromide and chlordiazepoxide hydrochloride in tablet formulations; however, the method uses separate sample preparations for each component.

The *U.S. Pharmacopeia* (USP, 22nd Rev.) introduced a new LC method for determination of CB and CP in capsules that has been revised in the third supplement (6). That method uses a reverse-phase cartridge column (8 × 100 mm) and a specialized module not readily available in most laboratories. Limit tests are included for the related compounds of CP, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide (CBO) (3%) and 2-amino-5-chlorobenzophenone (ACB) (0.1%), and the related compounds of CB, 3-hydroxyl-methyl quinuclidinium bromide (1%) and the toxic impurity 3-quinuclidinyl benzilate (0.03%), also known as BZ, a neurotoxin and chemical warfare agent (7). The compendial assay method for clidinium bromide capsules is a nonspecific procedure involving reaction with thymol blue, extraction with chloroform, and spectrophotometric determination (6).

The present paper describes a specific procedure for analysis of clidinium bromide and combinations of clidinium bromide with chlordiazepoxide hydrochloride in capsules. The method was tested for accuracy, reproducibility, and linearity and was well within accepted criteria. The method was applied to 4 commercial samples of the single ingredient formulation and 2 commercial samples of the combination product and found to be free from any interferences. The system is also free from any interference by the 4 known related compounds that may be present as impurities.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—DuPont Model 8800 pump with DuPont variable wavelength UV detector (DuPont Co., Wilmington, DE 19898). Rheodyne model 7125 injection valve, fitted with 10 μL injection loop (Rheodyne, Inc., Berkeley, CA 94710). Operating conditions: flow rate, 1 mL/min; detector wavelength, 235 nm; detector sensitivity, 0.01 absorbance units full scale (AUFS).

(b) *Recorder.*—Spectra Physics 4290 integrator (Spectra Physics, Inc., San Jose, CA 95134). Operating conditions: chart speed, 1 cm/min; attenuation for clidinium bromide, 8, and for chlordiazepoxide hydrochloride, 256.

(c) *Chromatographic column.*—Partisil 10 ODS-3, 4.6 mm id × 25 cm, 10 μm particle size (Whatman, Inc., Clifton, NJ 07014).

Reagents

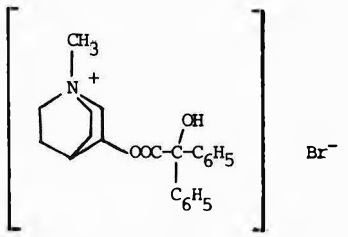
(a) *Solvents.*—LC grade acetonitrile (Mallinckrodt, Inc., Paris, KY 40361) and distilled water.

(b) *Mobile phase.*—Prepare a solution of acetonitrile–0.3M ammonium phosphate monobasic (32 + 68) by adding the appropriate amount of acetonitrile to 0.3M ammonium phosphate. Adjust pH to 4.3 ± 0.1 with 10% phosphoric acid. Degas in vacuum with the aid of an ultrasonic bath.

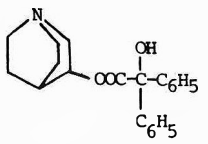
(c) *Standards.*—Clidinium bromide, chlordiazepoxide hydrochloride, 3-quinuclidinyl benzilate, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide (CBO), 2-amino-5-chlorobenzophenone (ACB) and 3-hydroxy-1-methylquinuclidinium bromide were USP reference standards (The U. S. Pharmacopeial Convention, Inc., Rockville, MD 20852).

(d) *Clidinium bromide standard solution.*—Accurately weigh 10 mg clidinium bromide standard and transfer to 50 mL volumetric flask. Dissolve and dilute to volume with water.

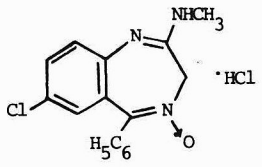
(e) *Clidinium bromide-chlordiazepoxide hydrochloride mixed standard solution.*—Accurately weigh 10 mg clidinium bromide standard and 20 mg chlordiazepoxide hydrochloride standard and transfer to 50 mL volumetric flask. Dissolve and dilute to volume with water.



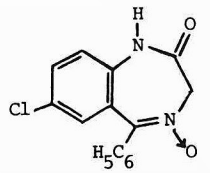
Clidinium Bromide (CB)



3-Quinuclidinyl Benzilate (QB)



Chlordiazepoxide Hydrochloride (CP)



7-Chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide (CBO)

Figure 1. Structures for clidinium bromide (CB), 3-quinuclidinyl benzilate (QB), chlordiazepoxide hydrochloride (CP), and 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide (CBO).

Sample Preparation

Clidinium bromide capsules and clidinium bromide-chlordiazepoxide hydrochloride capsules.—Accurately weigh contents of not less than 20 capsules and determine the average weight/capsule. Mix contents of capsules and accurately weigh a portion of the powder equivalent to 5 mg clidinium bromide and transfer to 25 mL volumetric flask. Add 15 mL water, sonicate 10 min with gentle swirling, and dilute to volume with water. Centrifuge a portion of this solution and use for analysis.

System Suitability

Inject 10 μL of the appropriate standard solution into chromatograph and measure peak areas. In a typical chromatogram, retention times will be ca 7 min for clidinium bromide and 10 min for chlordiazepoxide hydrochloride. The relative standard deviation for 5 consecutive injections of the standard preparation is not more than 2%. The resolution factor between CB and CP under these conditions is not less than 2.

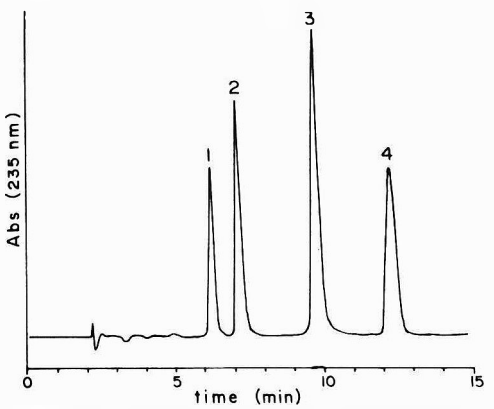


Figure 2. LC separation of synthetic mixture of (1) 3-quinuclidinyl benzilate, (2) clidinium bromide, (3) chlordiazepoxide hydrochloride and (4) CBO. Chromatographic conditions: column, Partisil 10 ODS-3, 10 μm; mobile phase, acetonitrile–0.3M ammonium phosphate monobasic (32 + 68), pH = 4.3; flow rate, 1 mL/min; detection wavelength, 235 nm; chart speed, 1 cm/min; temperature, ambient.

Determination

Separately inject duplicate, 10 μL aliquots of the appropriate standard and sample preparation into chromatograph and measure individual peak areas. For the combination product, change the attenuation of the integrator when going from the peak of clidinium bromide to the peak of chlordiazepoxide hydrochloride. Calculate amount of clidinium bromide in the portion of capsules taken by the following formula:

$$\text{CB, mg/capsule} = (PA/PA') \times C \times D \times (A/W)$$

where PA and PA' = peak areas of clidinium bromide in the sample and standard preparations, respectively; C = concn of clidinium bromide in the standard preparation (mg/mL); D = sample dilution factor; A = average capsule weight (mg); W = weight of sample taken for analysis (mg). For combination products, calculate amount of chlordiazepoxide hydrochloride present in the portion of capsules taken by the same formula, substituting appropriate values for chlordiazepoxide hydrochloride.

Results and Discussion

Figure 2 is a chromatogram showing separation of the 2 principal components and 2 related compounds. The elution sequence is: 3-quinuclidinyl benzilate, 6.2 min; clidinium bromide, 7.1 min; chlordiazepoxide hydrochloride, 9.7 min; and CBO, 12.3 min. Under these conditions, ACB is separated but will elute at >2 h and 3-hydroxy-1-methylquinuclidinium bromide, because of a lack of UV absorption, will not be detected. Thus, neither will interfere with assay results of the 2 major components.

The chromatogram of clidinium bromide and chlordiazepoxide hydrochloride in capsule formulation in Figure 3 illustrates the usefulness of the procedure as a screening method for CBO, which might be present in the combination

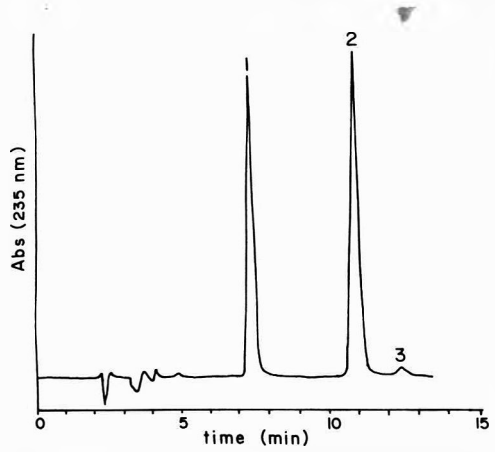


Figure 3. LC separation of (1) clidinium bromide, (2) chlordiazepoxide hydrochloride and (3) CBO in capsule formulations. Chromatographic conditions: column, Partisil 10 ODS-3, 10 μm; mobile phase, acetonitrile–0.3M ammonium phosphate monobasic (32 + 68), pH = 4.3; flow rate, 1 mL/min; detection wavelength, 235 nm; chart speed, 1 cm/min; temperature, ambient.

product. The level of CBO in this sample is approximately 2%.

A number of mobile phase conditions such as acetonitrile content, pH, and salt concentration were investigated to determine their effect on the separation of the 2 principal components and their related compounds. Of these, the most critical factor was found to be pH. As Figure 4 shows, k' for chlordiazepoxide hydrochloride was sharply reduced as pH of the mobile phase was lowered. A pH of 4.3 was chosen because it produced the best combination of separation and speed for analysis of the combination product. The mobile phase pH may have to be adjusted slightly higher or lower than 4.3, depending on the age and history of the column in use. Figure 2 is a good reference point for positioning chlordiazepoxide hydrochloride in the chromatogram for optimum results.

The salt concentration of 0.3M was used to achieve a resolution factor of at least 2 between CB and QB, so this system could be used to determine this toxic impurity at the USP limit of 0.03%. A salt concentration of 0.1M will maintain baseline separation of QB and CB, while slightly increasing k' values for all components in the system.

Table 1. Recovery of clidinium bromide and chlordiazepoxide hydrochloride from commercial products by standard addition

Sample	Drug added[a]	Amt added, mg	Recovered, mg	Recovery, %
		CB capsules		
1	CB	2.518	2.492	99.0
2	CB	2.536	2.524	99.5
3	CB	2.532	2.491	98.4
4	CB	2.518	2.526	100.3
		CB-CP capsules		
1	CB	5.008	4.925	98.3
	CP	9.969	10.183	102.1
2	CB	5.011	5.056	100.9
	CP	9.999	10.128	101.3

[a] CB = clidinium bromide; CP = chlordiazepoxide hydrochloride.

The accuracy of the method was tested by performing recovery studies on commercially available products spiked with clidinium bromide and chlordiazepoxide hydrochloride. Results in Table 1 were obtained by combining an equivalent amount of the appropriate standard with a portion of sample composite that had been previously assayed. Mean recovery values were: clidinium bromide (single), 99.3%; clidinium bromide (combination), 99.6%; and chlordiazepoxide hydrochloride (combination), 101.7%.

To test linearity of the method, clidinium bromide and clidinium bromide-chlordiazepoxide hydrochloride standard preparations representing 50% to 150% of the theoretical sample preparation concentrations were injected. A plot of peak area vs concentration for each component produced a straight line with a correlation coefficient >0.999 in each case.

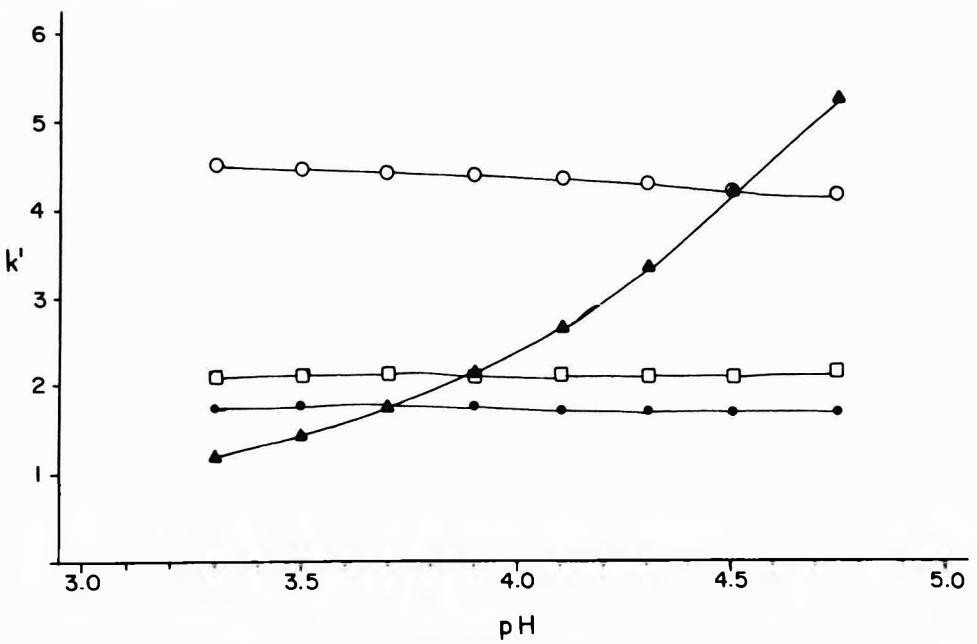


Figure 4. Plot of k' vs pH of mobile phase. ● = 3-quinuclidinyl benzilate, □ = clidinium bromide, ▲ = chlordiazepoxide hydrochloride, ○ = CBO.

Table 2. Assay (% of declared) of clidinium bromide capsules determined by USP and LC methods

Sample	Amt declared, mg	*USP*, 22nd Rev.	LC
1	2.5	101.2	101.8
2	2.5	104.4	102.8
3	5.0	99.0	100.3
4	5.0	101.2	100.2

Reproducibility of the detector response was confirmed by making 10 consecutive injections of each standard preparation. Relative standard deviations were: clidinium bromide (single), 0.28%; clidinium bromide (combination), 0.34%; and chlordiazepoxide hydrochloride, 0.20%.

We compared assay results for clidinium bromide capsules using the current compendial method and the proposed LC method (Table 2). In each case, results agreed to within 2%. Assay results for 2 commercial samples of the combination products are summarized in Table 3. Assays by the proposed method were performed in duplicate with results agreeing to within 2% in each case.

Conclusion

The present method was developed to simultaneously determine clidinium bromide and chlordiazepoxide hydrochloride in capsules. The method is simple and specific, requiring minimal sample and standard preparation, and has routinely been applied to assay and content uniformity analysis of both the single ingredient and combination product. The method is also useful as a screening procedure for CBO, which might be present in the combination product. Levels of this impurity that would be significant (ca 3%) are easily detected in the course of normal sample preparation injections.

Table 3. Assay of clidinium bromide-chlordiazepoxide hydrochloride capsules by LC method

Manufacturer	Declared[a]	Amt declared, mg	Percent of declared
A	CB	2.5	102.0
	CP	5.0	100.6
B	CB	2.5	101.1
	CP	5.0	97.6

[a] CB = clidinium bromide; CP = chlordiazepoxide hydrochloride.

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DRUGS IN FEEDS

Turbidimetric Assay for Tetracyclines in Feeds Using a Microtiter Plate System

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The microtiter plate system for turbidimetric assay of chlortetracycline (CTC) and oxytetracycline (OTC) levels in feeds uses a 96 well microtiter plate, a multichannel pipette, and an ELISA reader to measure turbidity. Feeds are extracted for both tetracyclines using AOAC extraction systems. For CTC, the range of the standard curve is 0.001–0.005 μg CTC/mL; for OTC, the range is 0.004–0.016 μg OTC/mL. Repeatability of CTC assays, as shown by the coefficient of variation (CV), ranged from 0.54 to 5.65% for same-day assays and from 2.01 to 9.39% for assays on different days. For OTC, CVs ranged from 2.69 to 10.01% for same-day assays and 3.24 to 9.08% for different-day assays. Average recoveries for CTC were 108.7% for same-day assays and 106.8% for different-day assays; for OTC, average recoveries were 112.4% and 106.5% for same-day and different-day assays, respectively.

Turbidimetric analysis of antibiotics in feeds offers many advantages to the analyst; speed and accuracy are among the most important. There is an AOAC official method (1) for turbidimetric assay of chlortetracycline (CTC) in feeds (**42.236–42.241**) and for turbidimetric assay of monensin in feeds (**42.271–42.275**). Kavanaugh (2) and Hewitt and Vincent (3) discuss turbidimetric procedures and their applicability to analysis of antibiotics in a wide variety of matrixes.

The turbidimetric method using the autoturb system requires a large investment in equipment that must be dedicated to these analyses. Partially manual systems using segments of the autoturb system (such as diluters) also require a heavy investment in equipment that will be used in a limited fashion. Completely manual systems are relatively labor intensive.

The present paper describes a method that uses general laboratory equipment: an ELISA reader, not dedicated solely to a single type of analysis; microtiter plates, which eliminate the need for large water baths; and multichannel pipettes to minimize the labor of duplicates. The procedure removes the necessity to terminate organism growth and has the ability to measure turbidity at a suitable wavelength within 15 s and, through properly designed programs, to computerize calculations.

Experimental

Antibiotic Standard Solutions

(a) *Chlortetracycline (CTC).*—100 μg/mL. Dissolve chlortetracycline hydrochloride standard in 10 mL 0.1N HCl and dilute to 100 mL with potassium phosphate buffer, pH 4.5.

(b) *Oxytetracycline (OTC).*—100 μg/mL. Dissolve oxytetracycline hydrochloride standard in 10 mL 0.1N HCl and dilute to 100 mL with potassium phosphate buffer, pH 4.5.

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Nutrient Media, Buffer, Extraction Solvents

(a) *Maintenance medium.*—Tryptic soy agar (TSA) (Difco) for maintenance of *Staphylococcus aureus* ATCC 9144.

(b) *Inoculation broth.*—Tryptic soy broth (TSB) (Difco) for growing 16–18 h broth culture of *S. aureus* and for 9 mL dilution tubes.

(c) *Buffer.*—Phosphate buffer, pH 4.5, contains 13.6 g monopotassium phosphate, dissolved and diluted to 1 L with deionized water [**42.204(g)**].

(d) *Extraction solvents.*—For chlortetracycline, use acid-acetone-water [**42.204(j)**]; for oxytetracycline, use acid-methanol [**42.204(k)**].

(e) *Assay microorganism.*—Prepare broth culture of *S. aureus* ATCC 9144 by inoculating 25 mL sterile TSB from a slant and incubating 16–18 h at 37°C with shaking.

Feeds

Three types of feeds were used. Poultry feed was an Agway unmedicated chick starter containing 18% protein, 3% fat, and 4% fiber. Hog feed, Rutgers Ration 10, was blended at our own university facility and contained 1642 lbs yellow corn, 282 lbs of 48% soybean meal, and 76 lbs of vitamins and minerals. Dairy feed was manufactured by Agway and contained 23% protein, 4% fat, and 8% fiber. All feeds were unmedicated.

Preparation of Standard Curves

Chlortetracycline.—Dilute acid-acetone-water extract of blank feeds 1:2500 by diluting 1 mL in 50 mL pH 4.5 buffer and diluting that solution again 1:50 in pH 4.5 buffer. Add twice-diluted extract to each CTC standard at a rate of 1 mL diluted extract/50 mL standard before bringing standards to volume with buffer. Standards are 0.001, 0.002, 0.003, 0.004, and 0.005 μg CTC/mL buffer.

Oxytetracycline.—Dilute acid-methanol extract 2:50 in pH 4.5 buffer. Add diluted extract to each OTC standard at a ratio of 2 mL extract/50 mL standard before bringing to volume with pH 4.5 buffer. Standards are 0.004, 0.008, 0.010, 0.012, and 0.016 μg OTC/mL.

Extraction of Feeds and Supplementation of Extracts

Extract blank feed samples by shaking 10 g feed for 30 min with 100 mL extracting solvent and allowing feed to settle for 1 h. Decant solvent and filter through a rapid filter paper such as H. Reeve Angel No. 802. Seal extract tightly to prevent evaporation and store.

Extracts are supplemented to contain the following levels: 20 μg/mL for the 200 μg/g feed; 10 μg/mL for the 100 μg/g feed; 5 μg/mL for the 50 μg/g feed, and 2.5 μg/mL for the 25 μg/g feed.

Table 1. Repeatability of recoveries for CTC content in different feeds—same day

	Theoretical, μg/g							
	25	50	100	200	25	50	100	200
Assay	Found, μg/g				Recovery, %			
				Hog feed				
1	27.5	52.5	105.0	220.0	110.0	105.0	105.0	110.0
2	24.3	52.5	108.9	222.9	97.1	105.1	108.9	111.5
3	27.5	52.5	107.5	220.0	110.0	105.0	107.5	110.0
4	29.0	52.4	102.8	213.2	116.0	104.9	102.8	106.6
5	29.9	53.1	109.9	219.5	119.5	106.2	109.9	109.8
Mean	27.64	52.60	106.82	219.12	110.52	105.24	106.82	109.58
Std. dev.	2.13	0.28	2.90	3.57				
CV, %	7.71	0.54	2.72	1.63				
95% Conf. limits	27.64	52.60	106.82	219.12				
Plus/minus	2.64	0.35	3.60	4.42				
				Poultry feed				
1	31.0	62.0	116.0	216.0	124.0	124.0	116.0	108.0
2	30.0	62.0	108.0	208.0	120.0	124.0	108.0	104.0
3	27.0	54.0	100.0	232.0	108.0	108.0	100.0	116.0
4	31.0	60.0	108.0	216.0	124.0	120.0	108.0	108.0
5	29.0	60.0	104.0	200.0	116.0	120.0	104.0	100.0
Mean	29.60	59.60	107.20	214.40	118.40	119.20	107.20	107.20
Std. dev.	1.67	3.29	5.93	11.87				
CV, %	5.65	5.51	5.53	5.53				
95% Conf. limits	29.60	59.60	107.20	214.40				
Plus/minus	2.07	4.07	7.35	14.72				
				Dairy feed				
1	26.3	57.5	115.0	210.0	105.1	115.0	115.0	105.0
2	25.2	52.5	102.5	200.0	100.9	105.0	102.5	100.0
3	25.5	52.5	107.5	220.0	102.0	105.0	107.5	110.0
4	26.3	51.3	105.0	205.0	105.3	102.5	105.0	102.5
5	24.8	51.3	105.0	210.0	99.0	102.5	105.0	105.0
Mean	25.62	53.02	107.00	209.00	102.46	106.00	107.00	104.50
Std. dev.	0.67	2.58	4.81	7.42				
CV, %	2.61	4.86	4.49	3.55				
95% Conf. limits	25.62	53.02	107.00	209.00				
Plus/minus	0.83	3.10	5.96	8.98				

Experimental Design

Dilute a 16–18 h TSB culture of *S. aureus* with sterile TSB to an absorbance of 0.250 measured at 620 nm. Dilute the organism preparation 1:10 in TSB. Add diluted organism suspension to a sterile multichannel solution basin (PGC Scientifics). Add a volume of 125 μL to each of 60 inside wells (exclude wells along each edge) of a sterile 96 well, flat-bottomed covered microtiter plate (Corning 25861) using a multichannel pipette (Flow Laboratories) fitted with disposable sterile tips.

Pour the zero standard of the antibiotic being assayed into a disposable multichannel solution basin. Using the multichannel pipette, add 125 μL of antibiotic standard to each of the 6 wells of the first row of wells containing the seeded broth. Keeping pipet tips in wells, draw mixture up and expel 4 times to mix thoroughly, ending with the expulsion of all the liquid into the wells. Set aside the remaining zero standard and the basin. Repeat with each of the standards in order of increasing concentration in consecutive rows of wells, using fresh sterile tips and basins for each. Pipet the 4 supplemented feed extract dilutions in increasing order from 25 to 200 μg/g supplementations into the next 4 rows of wells.

Measure the zero time absorbance of the filled wells of the plate at 620 nm using a microtiter plate reader such as Easy Reader ATC (SLT Labinstruments) attached to a printer such as the Citizen 120D. Place covered microtiter plate on a platform shaker in a 37°C incubator and shake-incubate at 150 rpm for 5 h. After 5 h, place plate in microtiter plate reader, shake for 5 s, and measure absorbance of wells at 620 nm.

Measurement of Potencies

Calculate average absorbances of wells at each concentration of standards and samples and determine a standard curve by use of a linear regression calculation of absorbance vs concentration. Determine concentrations of the supplemented feed extracts by comparison with the standard response line.

Results and Discussion

The principles of the turbidimetric assay system were not

Table 2. Repeatability of recoveries for CTC content in different feeds—different days

	Theoretical, μg/g							
	25	50	100	200	25	50	100	200
Day	Found, μg/g				Recovery, %			
				Hog feed				
1	26.0	49.8	109.9	242.0	104.0	99.7	109.9	121.0
2	26.7	52.5	105.0	220.0	106.8	105.0	105.0	110.0
3	27.5	50.9	101.7	220.7	110.0	101.8	101.7	110.4
4	27.0	52.5	94.5	195.1	107.9	104.9	94.5	97.5
5	23.2	55.1	100.3	219.0	93.0	110.2	100.3	109.5
Mean	26.08	52.16	102.28	219.36	104.34	104.32	102.28	109.68
Std. dev.	1.70	2.00	5.71	16.61				
CV, %	6.52	3.84	5.58	7.57				
95% Conf. limits	26.08	52.16	102.28	219.36				
Plus/minus	2.11	2.48	7.08	20.59				
				Poultry feed				
1	27.0	60.0	100.0	216.0	108.0	120.0	100.0	108.0
2	23.5	56.0	100.0	208.0	94.0	112.0	100.0	104.0
3	27.7	51.4	114.9	219.2	110.9	102.9	114.9	109.6
4	26.7	51.3	104.7	214.2	106.9	102.5	104.7	107.1
5	26.7	50.7	104.4	211.3	106.8	101.3	104.4	105.6
Mean	26.32	53.88	104.80	213.74	105.32	107.74	104.80	106.86
Std. dev.	1.63	4.03	6.09	4.30				
CV, %	6.19	7.47	5.81	2.01				
95% Conf. limits	26.32	53.88	104.80	213.74				
Plus/minus	2.02	5.04	7.55	5.33				
				Dairy feed				
1	29.3	49.9	104.3	230.9	117.1	99.8	104.3	115.5
2	31.2	55.0	112.3	245.0	124.8	110.0	112.3	122.5
3	27.5	52.5	102.5	200.0	110.0	105.0	102.5	100.0
4	28.5	52.6	102.3	230.6	114.0	105.3	102.3	115.3
5	27.6	43.7	122.5	198.3	110.6	87.3	122.5	99.2
Mean	28.82	50.74	108.78	220.95	115.30	101.48	108.78	110.50
Std. dev.	1.52	4.33	8.69	20.75				
CV, %	5.27	8.53	7.99	9.39				
95% Conf. limits	28.82	50.74	108.78	220.95				
Plus/minus	1.88	5.37	10.78	25.73				

altered by adapting the microtiter plate system coupled with a microtiter plate reader to analysis of CTC and OTC in feeds. Absorbance, the measured response, depended on the number of organisms in the wells, which in turn was a function of the concentration of the inhibitor. Time and temperature of incubation were constant factors. The differences were the use of multichannel pipettes; 96-well sterile, covered microtiter plates; a standard laboratory reciprocating shaker; and a standard incubator. There was no need to quench the growth of the organisms in the wells of the microtiter plate because the absorbances of the various wells were measured within 10 s and the data recorded. Because this laboratory equipment is not dedicated to turbidimetric assay and because it would normally be available in a reasonably equipped laboratory, the procedure offers many laboratories not equipped with automated equipment for turbidimetric assay the opportunity to use the relatively rapid turbidimetric assay system. In addition, it eliminates the need for tiresome absorbance measurements and the bulky water baths and diluters that define systems such as the Autoturb system.

The present method uses supplemented extracts of unmedicated feeds. This was to avoid the potential for overformulation that can exist in premix concentrations as well as the uncertainty of degree of uniformity of a blended feed. Extraction efficiency was not in question, because AOAC feed extraction systems [**42.204(j,k)**] are generally accepted as quantitative.

The standard response concentrations for CTC are approximately 10-fold less than for the plate procedure and the manual version of the turbidimetric assay, and 100-fold less than for the official automated turbidimetric assay (**42.236–42.241**). This means that the final assay pH of 4.5 is essentially assured. When extracting feed, the ratio of solvent volume to grams of feed is 10:1. For the nominal 200 μg/g feed supplementation, the extract contains 20 μg CTC/mL. For the assay, the target concentration is 0.004 μg CTC/mL. Coupled with the 10:1 extraction ratio, the dilution factor is 50 000. For the 100 and 50 μg CTC/g supplementations, the factors are 2500 and 1250, respectively (25 000 and 12 500, using the feed-to-solvent ratio). The dilution factor for the 25 μg CTC/g feed is 1250 from the extract and 12 500 from the

Table 3. Repeatability of recoveries for OTC content in different feeds—same day

	Theoretical, μg/g							
	25	50	100	200	25	50	100	200
Assay	Found, μg/g				Recovery, %			
Hog feed								
1	28.2	52.7	94.6	258.5	112.7	105.5	94.6	129.2
2	28.2	51.2	85.7	251.8	112.7	102.4	85.7	125.9
3	27.5	51.1	95.2	249.8	110.1	102.3	95.2	124.9
4	27.1	57.5	103.1	266.6	108.5	115.1	103.1	133.3
5	26.2	53.9	97.5	261.6	104.7	107.7	97.5	130.8
Mean	27.44	53.28	95.22	257.66	109.74	106.60	95.22	128.82
Std. dev.	0.84	2.63	6.29	6.93				
CV, %	3.06	4.93	6.61	2.69				
95% Conf. limits	27.44	53.28	95.22	257.66				
Plus/minus	1.04	3.26	7.80	8.59				
Poultry feed								
1	37.4	57.5	120.1	225.0	149.4	115.0	120.1	112.5
2	36.4	50.6	118.0	255.0	145.5	101.2	118.0	127.5
3	33.7	48.1	112.9	224.5	134.9	96.2	112.9	112.3
4	32.4	60.0	114.4	245.0	129.4	120.0	114.4	122.5
5	28.9	49.4	99.9	212.5	115.6	98.8	99.9	106.3
Mean	33.76	53.10	113.06	232.40	134.96	106.24	113.06	116.22
Std. dev.	3.38	5.30	7.89	17.20				
CV, %	10.01	9.96	6.98	7.39				
95% Conf. limits	33.76	53.10	113.06	232.40				
Plus/minus	4.19	6.57	9.78	21.33				
Dairy feed								
1	24.4	52.0	109.1	205.6	97.5	104.0	109.1	102.8
2	24.4	58.5	117.7	192.1	97.5	117.0	117.7	96.0
3	27.5	59.8	110.1	179.0	110.0	119.6	110.1	89.5
4	29.4	61.4	111.8	213.0	117.5	122.8	111.8	106.5
5	29.4	60.0	113.6	216.5	117.5	120.0	113.6	108.2
Mean	27.02	58.34	112.46	201.24	108.00	116.68	112.46	100.60
Std. dev.	2.51	3.69	3.39	15.56				
CV, %	9.30	6.33	3.02	7.73				
95% Conf. limits	27.02	58.34	112.46	201.24				
Plus/minus	3.11	4.58	4.20	19.29				

feed, because the assay target concentration is 0.002 μg CTC/mL. Adjustment of the pH of the feed extract or the final dilution has been found to be unnecessary.

There is no official turbidimetric assay system for oxytetracycline; concentrations for the standard response line were 12.5 to 50-fold less than for the AOAC official plate assay (**42.293**). Concentrations used for the standard response line were determined empirically, but were considerably less than the 0.192–0.300 μg OTC/mL used for oxytetracycline listed by Hewitt and Vincent for tube assays (3) or the 0.154 to 0.375 μg OTC/mL listed by Kavanaugh (2).

Dilution patterns for OTC were similar to those used for the CTC assay. Conditions of similarity were obtained by addition of the appropriate blank feed extract to the standards at the 50 μg/g supplementation level. If desired, standards can be supplemented with extract at each concentration level used in the assay.

The organism level in the assay medium approximates 1×10^6 cells/mL and can be standardized by dilution from an overnight culture. Slight variations in the inoculum do not affect the overall assay. However, for the purpose of laboratory quality control of the assay, it is best to use an 18 h culture of *S. aureus* ATCC 9144 adjusted to an absorbance of 0.250 at 620 nm and diluted 10-fold to yield approximately 1×10^6 organisms/mL.

Rack design for tube assays provides for a "randomized" placement of replicate tubes. This placement aids in the elimination of the effects of temperature, seeding variability, quenching of growth and any time variations in measurement of growth in the tubes. The randomized filling of wells did not result in any measurable differences when compared to the linear placement recommended. The only wells that should not be used are the outer wells. The edge effect that has been noted in immunoassays can be seen in absorbance readings, which are usually noticeably greater at the edges than the average of the inner wells. For this reason, it is recommended that outer wells of the 96 well plate not be used for assay.

A number of parameters of the assay were varied to determine optimum conditions. No differences in accuracy and precision were noted when well volumes were between 200 and 250 μL; less than 200 μL/well produced more variable results. The number of wells used for each concentration of standard or extract was varied from 5 to 8 wells/concentration with no differences in recoveries calculated, except that

Table 4. Repeatability of recoveries for OTC content in different feeds—different days

	Theoretical, μg/g							
	25	50	100	200	25	50	100	200
Day	Found, μg/g				Recovery, %			
				Hog feed				
1	34.8	56.3	95.0	172.3	139.1	112.7	95.0	86.2
2	34.4	56.2	98.8	172.9	137.6	112.4	98.8	86.4
3	34.0	54.5	86.5	172.5	136.0	108.9	86.5	86.2
4	31.5	58.2	101.2	201.2	125.9	116.5	101.2	100.6
5	38.0	59.2	110.8	200.1	151.9	118.4	110.8	100.1
Mean	34.54	56.88	98.46	183.80	138.10	113.78	98.46	91.90
Std. dev.	2.32	1.84	8.87	15.39				
CV, %	6.73	3.24	9.01	8.37				
95% Conf. limits	34.54	56.88	98.46	183.80				
Plus/minus	2.88	2.28	10.99	19.08				
				Poultry feed				
1	24.6	51.4	102.0	212.8	98.4	102.7	102.0	106.4
2	25.3	57.5	113.7	225.0	101.2	115.0	113.7	112.5
3	22.2	49.8	97.2	204.9	88.2	99.6	97.2	102.5
4	23.5	59.5	94.0	202.7	94.1	119.0	94.0	101.4
5	26.7	51.2	104.3	201.6	106.6	102.4	104.3	100.8
Mean	24.46	53.88	102.24	209.40	97.70	107.74	102.24	104.72
Std. dev.	1.72	4.32	7.57	9.76				
CV, %	7.01	8.02	7.40	4.66				
95% Conf. limits	24.46	53.88	102.24	209.40				
Plus/minus	2.13	5.36	9.39	12.10				
				Dairy feed				
1	28.2	51.4	110.3	211.2	112.6	102.8	110.3	105.6
2	25.6	58.1	94.1	210.0	102.5	116.3	94.1	105.0
3	28.5	50.3	103.8	199.6	113.8	100.5	103.8	99.8
4	28.9	53.0	117.0	197.5	115.6	103.8	117.0	98.8
5	25.6	57.0	96.7	197.3	102.5	117.0	96.7	98.7
Mean	27.36	53.96	104.38	203.12	109.40	108.08	104.38	101.58
Std. dev.	1.63	3.44	9.48	6.90				
CV, %	5.94	6.37	9.08	3.40				
95% Conf. limits	27.36	53.96	104.38	203.12				
Plus/minus	2.02	4.27	11.75	8.56				

the use of 8 wells/row sometimes exhibited an edge effect. Absorbances read at 620 or 660 nm yielded equal recoveries. Media other than TSB were used, as well as different pH buffers; however, TSB and pH 4.5 buffer were found to produce the best results. Increasing incubation time from 5 to 6 h produced no improvement in recoveries, while a 4 h incubation yielded poor results. Concentrations determined using 5 h absorbance readings were equivalent to those using the difference in absorbance at time zero and 5 h; for this reason, 5 h absorbances were used for calculations and zero-time readings were taken only as a quality control measure, to assure that initial absorbances were within the normal range of approximately 0.050–0.060.

Table 1 shows repeatability for CTC using supplemented extracts of 3 different feed types. The data indicate that the assay has good same-day repeatability over a range of antibiotic concentrations. With the exception of the hog feed extract supplemented at 25 μg/g, coefficients of variation (CVs) for assays range from 0.54 to 5.65 (for 25 μg/g hog feed, the CV was 7.71%).

Table 2 shows repeatability of recoveries for CTC in assays performed on different days using supplemented extracts of the 3 blank feeds. CVs for different-day assays are somewhat higher than for same-day assays, ranging from 2.01 to 9.39%, but still demonstrate good repeatability over the range of concentrations.

Repeatability for OTC in the 3 feed types on the same day is shown in Table 3. CVs range from 2.69 to 10.01%. For OTC assays on different days, CVs range from 3.24 to 9.08% (Table 4). Same-day and different-day results show very similar indices of repeatability. Recovery of OTC in both hog and poultry feeds shows a high bias. This was attributed to supplementation rather than interferences because it was exhibited more noticeably in same-day repeatabilities. In general, results for OTC were somewhat less repeatable than those noted for CTC, but CVs are still reasonable.

To determine the degree of variability associated with the absorbance readings measured by the microtiter plate reader, representative assay plates for both OTC and CTC were measured 5 times over a period of 10 min. Consistency of assays for OTC in dairy feed and CTC in poultry feed for repetitive assays is shown in Table 5. CVs are quite good,

Table 5. Repeatability of recoveries in repetitive readings of same plate

Assay reading	Theoretical, μg/g							
	25	50	100	200	25	50	100	200
	Found, μg/g				Recovery, %			
	OTC in dairy feed							
1	28.2	51.4	110.3	211.2	112.6	102.8	110.3	105.6
2	27.3	51.1	107.9	202.6	109.2	102.1	107.9	101.3
3	28.4	51.5	108.1	201.8	113.4	103.0	108.1	100.9
4	27.3	51.1	108.3	199.6	109.4	102.2	108.3	99.8
5	27.4	51.1	107.9	199.7	109.4	102.2	107.9	99.9
Mean	27.72	51.24	108.50	202.98	110.80	102.46	108.50	101.50
Std. dev.	0.54	0.19	1.02	4.78				
CV, %	1.93	0.38	0.94	2.35				
	CTC in poultry feed							
1	28.9	51.2	114.5	218.1	115.5	102.5	114.5	109.1
2	28.1	51.1	114.1	209.9	112.3	102.3	114.1	105.0
3	27.7	51.4	114.9	219.2	110.9	102.9	114.9	109.6
4	28.6	51.7	114.8	219.1	114.2	103.4	114.8	109.5
5	28.2	51.4	114.6	217.9	112.6	102.9	114.6	108.9
Mean	28.30	51.36	114.58	216.84	113.10	102.80	114.58	108.42
Std. dev.	0.46	0.23	0.31	3.92				
CV, %	1.64	0.45	0.27	1.81				

ranging from 0.27 to 2.35%, demonstrating both consistency of the assay system and allowance for small deviations in time of incubation.

Average recovery for all assays on the same day for CTC in all feed types was 108.7%; on different days, average recovery was 106.8%. For OTC, average same-day recovery of all feed types was 112.4%; different-day assays averaged 106.5%. There is a slightly high positive bias associated with the assay. It should be noted that an error of 0.0001 μg/mL will cause a 5 μg/g error in the assay of CTC at the 200 μg/g supplementation level, a 2.5 μg/g error at the 100 μg/g level, and proportionally less for the 50 and 25 μg/g supplementations. Errors for OTC can be estimated in the same fashion. The theoretical error of 0.0001 μg CTC/mL represents an error of 2.5% in assay concentration.

Conclusion

The microtiter plate system offers a reasonable approach to assay of both CTC and OTC in feeds. It offers the reasonably-equipped laboratory versatility and the ability to do turbidimetric assays without the need for dedicated equipment. The system eliminates the labor intensiveness of filling test tubes with seeded medium and reading individual tubes as well as the need for rack designs and problems of stopping organism growth. In addition, accuracy and precision are not inconsistent with other approaches for assay of these antibiotics in feeds.

References

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(2) *Analytical Microbiology* (1972) F. Kavanaugh (Ed.), Vol. II, Academic Press, Inc., New York, pp. 43–121, 133

(3) Hewitt, W. and Vincent, S. (1989) *Theory and Application of Microbiological Assay*, Academic Press, Inc., San Diego, CA, pp. 80–108, 264

New Microbiological Method for Determining Spectinomycin in Pelleted and Meal Feeds Using Trifluoroacetic Acid as Primary Extractant

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A new microbiological method, identified as the spectinomycin trifluoroacetic (SPE-TFA) method, was compared with the current AOAC method for analyzing spectinomycin in meal and pelleted feeds fortified with LS-20™ premix. Feeds containing 3 concentrations of drugs and a zero level were tested in a correlation study. The data showed no significant differences in the percent of theory assayed between meal and pelleted samples using the SPE-TFA method, but the percent of theory found using the AOAC method was significantly lower for the pelleted samples than for the meal samples. The within-sample variation of the AOAC assay was also not the same for all samples; the SPE-TFA assay variation was relatively constant for all samples. The SPE-TFA method produced an overall average recovery of 98% with a range of 89–109% compared with an 85% recovery ranging from 64 to 102% for the AOAC method. In addition to producing better recoveries, the SPE-TFA method features a more sensitive response line, and final test solutions have viscosities and clarity more comparable to the standard solutions than those produced by the current AOAC method.

The antibiotics spectinomycin and lincomycin have been combined in a 1:1 ratio for effective control of *Mycoplasma pneumonia*, or swine dysentery. A microbiological assay method for determining spectinomycin in complete meal feed was collaboratively studied and published (1), but pelleted feed has been found difficult to assay by this current AOAC method. The published method for lincomycin (2) successfully assays the antibiotic in both pelleted and meal feed. Neither antibiotic interferes with the other (Stahl, G.L., unpublished data). Successful preliminary and experimental work with a new microbiological method, identified as the spectinomycin-trifluoroacetic acid (SPE-TFA) method, for analyzing spectinomycin in meal and pelleted feeds fortified with LS-20™ premix warranted a validation study. AOAC guidelines were followed to facilitate worldwide recognition and acceptance.

Validation Study

Feeds containing 0, 10, 20, and 40 g spectinomycin/ton were tested. Distribution of sample concentrations covered a range of complete feeds likely to be encountered in practice. Feeds were prepared using formulation S-850, a standard pig starter composed of 1/3 commercial base and 2/3 corn and soymeal. LS-20 premix (Lot No. 3550 B), containing spectinomycin and lincomycin at 10 g/lb of free base equivalents of each antibiotic (assayed biologically at 99% of label for spectinomycin and 101% of label for lincomycin), was used to fortify the feeds. The premix was blended with 25 kg portions of the diet for each concentration. Approximately 1/2 of the preparation for each concentration was pelleted by a California pellet Mill Master Model 40 at ca 160°F. Samples were then separated with a sample divider to ca 1 kg each and ground through a Wiley mill to pass a 2 mm screen.

An assay laboratory received 8 coded samples. Samples of both pelleted and meal feeds at each of 4 concentration levels were assayed on one of 5 days in a randomized block design (block = day). Two samples of each form of feed at each level were assayed on each day. There were 2 sizes of experimental unit in the study: a set of 2 samples on a given day and a single sample. Analysis of variance was used to compare complete meal vs pelleted feeds at each level. For complete meal and pelleted feeds at each level, a 95% confidence interval around the mean assay amount was calculated.

The following parameters were examined: precision, accuracy, linearity, repeatability, replicate analyses, and reproducibility. Both the current AOAC and the SPE-TFA methods were tested, and the standard deviations for both methods were compared for method correlation. Reproducibility was examined by the performance of the assays at a second laboratory The Upjohn Co., Dept 9760, Laboratory 519).

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METHOD

Apparatus

See **957.23c (a), (b),** and **(c)** (3), plus the following:

(a) *Shaker, mechanical.*—NBS gyrotory, or equivalent.

(b) *pH meter.*

(c) *Ultrasonic water bath.*—Ultrasonics Inc., or equivalent.

(d) *Centrifuge.*—IEC, or equivalent.

Reagents

(a) *Tris buffer.*—pH 8.0, 0.05M. Dissolve 6.05 g Tris (hydroxymethyl aminomethane) (Trizma base, available from Sigma Chemical Co.) in 1000 mL water and adjust pH to 8.0 with concentrated hydrochloric acid.

(b) *TFA solution.*—0.15 M. Pipet 11.4 mL trifluoroacetic acid (TFA) (available from Aldrich Chemical Co., Inc.) into 1 L beaker containing water and 0.5 g ethylenediaminetetraacetic acid, disodium salt (EDTA). Mix, and dilute to 1 L.

(c) *Extractant.*—TFA solution–methanol (6 + 4).

(d) *Test diluent.*—Extractant–Tris buffer (1 + 1). Mix, and adjust combined solution to pH 8.0.

Assay Organism

Escherichia coli.—UC 527 (ATCC 2998) maintained as frozen suspension over liquid nitrogen (ca 1.0×10^{10} viable cells/mL), or **957.23D(h)** (3).

Assay Medium

Antibiotic Medium No. 5 (Difco Laboratories, Detroit, MI), supplemented with either 3.7 mL 4.0 N sodium hydroxide/L or 5.0 g agar/L.

Table 1. Summary of percent recovery of spectinomycin by SPE-TFA assay method in fortified feeds

Sample code	Meal or pellet	Spectinomycin, label concn.	Recovery Day 1 g/ton	Day 1 %	Day 2 g/ton	Day 2 %	Day 3 g/ton	Day 3 %	Day 4 g/ton	Day 4 %	Day 5 g/ton	Day 5 %	Mean % of theory[a]	95% confidence intervals
1A	M	20.0	18.4	92	18.9	94	20.6	103	19.4	97	20.4	102	98	94.07
1B			20.0	100	18.0	90	19.8	99	19.4	97	20.9	104		101.73
2A	P	20.0	19.0	95	19.3	97	18.1	91	19.8	99	20.0	100	97	93.16
2B			18.8	94	20.0	100	19.3	97	19.3	97	19.4	97		99.84
3A	M	40.0	38.9	97	38.7	97	41.1	103	44.4	111	42.9	107	104	99.95
3B			40.0	100	40.4	101	40.4	101	45.0	112	43.3	108		107.60
4A	P	10.0	8.9	89	9.9	99	8.9	89	9.6	96	8.3	83	91	87.26
4B			8.9	89	9.0	90	8.9	89	9.6	96	8.6	86		93.94
5A	P	40.0	38.0	95[b]	42.8	107	44.4	111	44.1	110	44.4	111	109	105.10
5B			40.0	100	43.3	108	43.3	108	44.1	110	43.3	108		112.56
6A	M	10.0	8.7	87	9.1	91	9.0	90	8.6	86	8.5	85	89	85.57
6B			8.7	88	8.8	88	9.2	92	9.7	97	9.0	90		93.23
7A	M	0.0	0.0		0.0		0.0		0.0		0.0			
7B			0.0		0.0		0.0		0.0		0.0			
8A	P	0.0	0.0		0.0		0.0		0.0		0.0			
8B			0.0		0.0		0.0		0.0		0.0			

[a] Least squares means.
[b] Value deemed an outlier and discarded.

Inoculum

Use inoculum of 0.08%, which will produce zones of inhibition from ca 9.0–11.0 mm at the 0.8 μg/mL concentration.

Standard Solutions

(a) *Spectinomycin stock solution.*–1000 μg spectinomycin base/mL. Weigh sufficient amount of reference standard spectinomycin·HCl·5 H_2O (available from Agricultural Division, The Upjohn Co., Kalamazoo, MI 49001) and dissolve in water to give a concentration of 1000 μg spectinomycin base/mL. Store this stock solution in the dark up to ≤30 days at 2–10°C.

(b) *Standard response line.*—Dilute stock solution with (a) test diluent for concentration of 100 μg/mL. Dilute this solution with test diluent for concentrations of 0.8, 1.2, 1.8, 2.7, and 4.05 μg/mL spectinomycin. Prepare daily. Reference concentration is 1.8 μg/mL.

Assay Solution

Weigh 18 g sample into 250 mL centrifuge bottle and add 60 mL extractant (**f**). Sonicate 15 min with no heat. Add additional 50 mL extractant and shake extract 30 min on gyrotory shaker. Centrifuge 3–5 min at 2000–2500 rpm. Transfer 10 mL aliquot to 25 mL mixing cylinder, and add 10 mL Tris buffer pH 8.0. Mix, and adjust pH to 8.0. Shake briefly and let precipitate 15 min. Centrifuge 3–5 min at 2000–2500 rpm. Test at 1.8 μg/mL for 20 g/ton concentration.

Conduct of Assay

Prepare 2 plates for each sample and 2 plates for each standard concentration. Distribute agar to plates in 6 mL monolayer. Fill each cylinder with 250 μL of standard or sample (0.25 mL) and incubate 48–72 h at room temperature. Read zone sizes, and calculate as usual for a 5-point

Table 2. Summary of percent recovery of spectinomycin by AOAC assay method in fortified feeds

Sample code	Meal or pellet	Spectinomycin, label concn.	Recovery Day 1 g/ton	Day 1 %	Day 2 g/ton	Day 2 %	Day 3 g/ton	Day 3 %	Day 4 g/ton	Day 4 %	Day 5 g/ton	Day 5 %	Mean % of theory[a]	95% confidence intervals
1A	M	20.0	19.0	95	17.7	89	17.6	88	17.0	85	19.1	96	92	90.79
1B			18.0	90	19.1	95	18.6	93	20.0	100	18.4	92		93.71
2A	P	20.0	16.7	83	15.9	80	16.8	84	16.4	82	16.8	84	83	82.01
2B			16.7	83	17.7	89	16.4	82	16.8	84	15.9	80		84.09
3A	M	40.0	40.4	101	40.9	102	36.4	91	43.6	109	44.8	112	102	92.17
3B			40.9	102	40.0	100	35.4	89	44.1	110	43.3	108		112.73
4A	P	10.0	6.3	63	7.0	70	5.7	57	<C[c]	<50	6.2	62	64[b]	56.83
4B			6.9	69	6.6	66	5.9	59	<C[c]	<50	6.4	64		70.67
5A	P	40.0	36.8	92	37.7	94	32.3	81	34.5	86	40.0	100	93	83.45
5B			36.8	92	40.0	100	34.5	86	37.0	92	41.7	104		101.70
6A	M	10.0	8.5	85	9.7	97	7.7	77	<C[c]	<50	8.2	82	77	59.42
6B			8.8	88	8.4	84	7.6	76	5.4	54	7.3	73		94.58

[a] Least square means.
[b] Two values could not be used because they were less than the range of the standard curve.
[c] Activity is present but less than standard curve or 5.0 g/ton.

Table 3. Analysis of variance for percent of theory recovered by the SPE-TFA assays and AOAC assays

Source	DF	Mean square (probability of chance) SPE-TFA	AOAC (weighted)
Day	4	22.34	0.846
Sample	5	249.36	66.900
Form (meal vs pellet)	1	17.37 (.26)	14.864 (<.01)
Level	2	611.79 (<.01)	21.640 (<.01)
Form × level	2	23.00 (.20)	0.184 (.84)
Error	19	12.91	1.032

standard response line as indicated in **957.23E** and **957.23F** (3) using appropriate adjustments for 2 plates per sample and standard concentrations.

Results and Discussion

Results of the SPE-TFA assays and AOAC assays shown in Tables 1 and 2 reflect the order in which feeds were coded and assayed on a blind basis. For the SPE-TFA assays (Table 1), results indicate no difference in recovery for meal and pelleted feeds. The fact that recoveries for the 10 g/ton feeds were lower than those for either 20 or 40 g/ton feeds may be partially explained by the quantity of sample assayed, which was the same for all concentrations because of the blind nature of the study. This locates the results of the 10 g/ton feeds at the lower end of the standard curve where a low bias in recoveries is more inherent in the microbiological cylinder plate testing system. The same type of influence occurs at the upper end of the curve for the 40 g/ton sample causing an apparent high bias (4). Although recoveries were higher for the 40 g/ton feeds than the 20 g/ton feeds, results for both feeds fall well within accepted limits of the antibiotics label compliance range (±25%, Canadian Health Protection Bureau).

The AOAC assay results for method correlation show a lower percent recovery for pelleted feeds than meal feeds at all concentrations. Recoveries progressively declined as the concentration in the feeds declined. In addition, as the concentration increased for the meal feeds, results became closer to target concentrations. In contrast to the SPE-TFA assays, nearly all results of the AOAC assays were below the targeted 100% of theory expected with the possible exception of the 40 g/ton meal feeds (Table 2).

For the SPE-TFA assays, the percent of theory for one of the 40 g/ton pelleted samples (day 1) was found to be abnormally low, 95% of theory compared to the overall average for the 5 assays of 107%, and was not used in the analyses. No other outliers were found for either assay (5). Because of heterogeneity of sample variances for the AOAC assays, sample variances were used to calculate the confidence intervals for the outlier, and a weighted analysis of variance (with weights equal to the inverse of the sample variances) was used to test for form and level effects. No other heterogeneity of variance was detected (6).

Table 4. Least-squares mean differences for percent of theory recovered by SPE-TFA assays and AOAC assays

Form	Level, g/ton	No. of samples	Mean difference, %	Mean % of theory SPE-TFA	AOAC
Meal	10	5	12.40	89	77
Meal	20	5	5.65	98	92
Meal	40	5	1.33	104	102
Pellet	10	4	27.09	91	64
Pellet	20	5	13.45	97	83
Pellet	40	4	15.03	109	93

Table 5. Reproducibility comparison of SPE-TFA assay method

Sample code	Meal or pellet	Spectinomycin, label concn	Recovery 519 (R-1)[a] g/T	%	519 (R-2)[a] g/T	%	Assay 6 g/T	%
1A	M	20.0	19.9	100	21.1	106	20.8	104
1B			21.0	105	21.0	105	20.8	104
2A	P	20.0	20.0	100	20.0	100	20.0	100
2B			19.3	96	20.4	102	20.0	100
3A	M	40.0	44.0	110	52.0	130	44.0	110
3B			41.1	103	<C[b]		45.3	113
4A	P	10.0	8.7	87	9.2	92	9.1	91
4B			9.1	91	9.7	97	9.3	93
5A	P	40.0	44.1	110	49.3	123	46.5	116
5B			44.0	110	48.0	120	42.4	106
6A	M	10.0	9.7	97	9.7	97	9.5	95
6B			8.2	82	9.1	91	9.3	93
7A	M	0.0	0.0		0.0		0.0	
7B			0.0		0.0		0.0	
8A	P	0.0	0.0		0.0		0.0	
8B			0.0		0.0		0.0	

[a] Laboratory 519 reproducibility tests, days 1 and 2.
[b] Result greater than the curve.

For the AOAC assays, results of weighted analysis of variance show that mean percent of theory for meal samples is greater than mean percent of theory for the pelleted samples. For the SPE-TFA assays, there is no difference (significance = 0.05) in the recovery between pelleted and meal samples (Table 3).

The estimated standard deviation between replicates (repeatability) for the SPE-TFA method is 3.15 g/ton for the meal samples and 2.47 g/ton for the pelleted samples. The estimated standard deviation between replicates for the AOAC assay is 4.84 g/ton for the meal samples and 3.22 g/ton for the pelleted samples (7).

Analysis of variance of the differences in results between the 2 assays (Table 4) shows that the results for the SPE-TFA assays are consistently higher than those for the AOAC assays. The difference between the assays is smaller for meal than for pelleted samples, and is also greater for the 10 g/ton samples than for either the 20 or 40 g/ton samples.

To test for reproducibility of the new assay, all samples were assayed using the SPE-TFA method at a second laboratory on each of 2 days. Results from this laboratory are similar to those from the first laboratory. However, an isolated problem causing aberrations on the agar surface of 2 plates produced asymmetrical zones of inhibition greater than the standard curve and were not used. To cover any questions regarding timing, assay performance, or inherent possible bias, a final SPE-TFA assay (Assay 6) was performed at the end of the study. Results of this assay correlated very well with the previous 5 SPE-TFA assays as well as the assays from the second laboratory irrespective of timing differences (Table 5). There is no statistically significant

Table 6. Analysis of data from second laboratory

Source		DF	Mean squares (probability of chance) percent of theory
Day		1	215.689
Form (meal vs pellet)		1	13.814 (.54)
Level		2	648.884 (<.01)
Form × level		2	4.275 (.88)
Error		5	32.608
			g/ton %
Means:	Meal		10 g/ton 91.75
	Meal		20 g/ton 103.75
	Meal		40 g/ton 118.19
	Pellet		10 g/ton 91.75
	Pellet		20 g/ton 99.63
	Pellet		40 g/ton 115.88

difference (significance = 0.05) in the recovery between meal and pelleted samples. The percent of theory for the 40 g/ton samples is greater than the percent of theory for either the 20 or 10 g/ton samples (which are not significantly different from each other). Between-replicate standard deviation (repeatability) is 6.02 g/ton for the meal samples and 2.38 g/ton for the pelleted samples for the second laboratory (Table 6).

The consistent linearity of response is shown in Figure 1. The slopes from all 5 assays were almost identical, showing an average response of 8.7 to 21.0 mm across the 5 standard

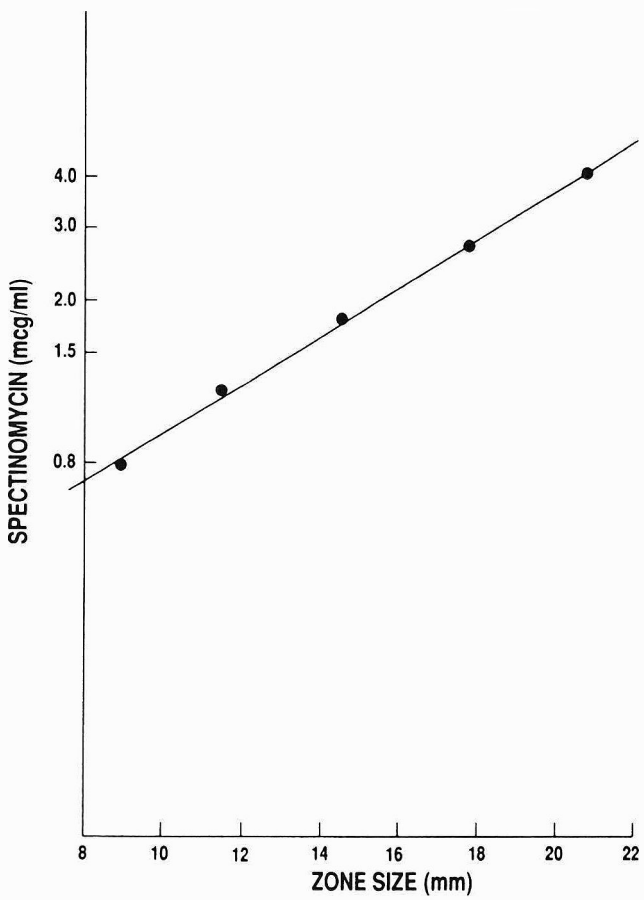

Figure 1. Standard response for SPE-TFA assay (day 4).

Table 7. Assay results of lincomycin in validation feeds

Sample code	Meal or pellet	Lincomycin, label concn	Recovery g/ton	Recovery %
1A	M	20.0	20.4	102
1B		20.0	20.4	102
2A	P	20.0	19.4	97
2B		20.0	21.4	107
3A	M	40.0	40.4	101
3B		40.0	41.9	105
4A	P	10.0	9.4	94
4B		10.0	10.0	100
5A	P	40.0	40.9	102
5B		40.0	39.6	99
6A	M	10.0	9.6	96
6B		10.0	9.8	98
7A	M	0.0	0.0	
7B		0.0	0.0	
8A	P	0.0	0.0	
8B		0.0	0.0	

increments. Results of the day 4 assay show a low response of 8.7 mm and high response of 20.6 mm.

An additional check on test performance was done by assaying the lincomycin, which is also present in the feed at concentrations equal to spectinomycin. Overall results averaged 100% with a range of 94–107% (Table 7).

The SPE-TFA assay features the use of 40% methanol in the extractant, which provides maximum efficiency in wetting the dry feed without agitation at the initial sonication step. The addition of another 50 mL extractant for the second-phase shake extraction ensures complete solubility of the spectinomycin. Use of the chelating agent, EDTA, helps prevent metallic ion interference that may occur from various feed matrixes. The combination of the sonication step followed by the shake extraction step is in effect a double extraction method with no analyte transfer. This allows for a simplified system with aliquot removal and subsequent 2-fold dilution to a 20% methanol assay solution. The test organism has been stress tested for compatibility with the system. Use of the test diluent as described in *Reagents* equilibrates the comparative solvent matrix for both standards and samples. The TFA solution at 0.15 M has been optimized for extractability without hydrolyzing the spectinomycin.

The new SPE-TFA method produced an overall average recovery of 98% with a range of 89–109% compared with an 85% recovery with a range of 64–102% for the current method. In addition to this better performance, the SPE-TFA method eliminates the concentration step and much of the handling required in the AOAC method. The SPE-TFA method produces a 3.5 times more sensitive response line, and final test solutions have viscosities and clarity more comparable to the standard solutions than those produced by the AOAC method.

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DRUG PACKAGING

Capillary Gas Chromatographic Determination of Cyclohexanone and 2-Ethyl-1-Hexanol Leached from Solution Administration Sets

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A capillary gas chromatographic method is described for the determination of cyclohexanone and 2-ethyl-1-hexanol leached from solution administration sets. A preliminary study was made of compounds leached from solution administration sets by 5% sodium bicarbonate solution (pH 8.1), 0.9% sodium chloride solution (pH 6.8), and water. Water was selected as the leaching solvent because similar quantities of the compounds were leached into water and into both types of parenteral solutions. The correlation coefficients were 0.99977 for cyclohexanone and 0.99974 for 2-ethyl-1-hexanol, and recoveries were good (93–94%). Five administration sets from each of 2 manufacturers were analyzed by this method. The amounts of cyclohexanone that were leached from the individual sets varied considerably; however, similar quantities were leached from sets of both manufacturers. 2-Ethyl-1-hexanol was also found in extracts from each of the sets analyzed.

Previous analyses have shown that various quantities of chemicals leach from rubber stoppers (1) and from plastic containers (2) into parenteral solutions. Polyvinyl chloride plastics used for medical devices can consist of up to 40%, by weight, of di(2-ethylhexyl) phthalate (DEHP), which is lipophilic (3). Bhujle et al. (4) and Piechocki and Purdy (5) found DEHP in blood stored in flexible polyvinyl chloride bags. Cyclohexanone and 2-ethyl-1-hexanol were reported to leach from polyvinyl chloride (2, 3). Needham and Jones (6) reported that when solutions such as alcohol, physiological saline, and dextrose were drained through administration sets for 6 h, DEHP could not be detected. Ulsaker and Hoem (7) reported a method to determine 2-ethyl-1-hexanol after the hydrolysis of DEHP. Cyclohexanone, an industrial solvent, is used in the production of cellulose acetate natural resins, vinyl resins, rubber, and waxes and as a solvent cement for polyvinyl chloride in medical devices (8). Ulsaker and Korsnes (9) found cyclohexanone in intravenous solutions stored in plastic bags. Cyclohexanone at 20 μL/L was listed as mutagenic in a microsomal assay for *Salmonella typhimurium*; at 5 μg/L, it was mutagenic for human lymphocytes in a cytogenetic assay (10). In a study by the National Cancer Institute (11), cyclohexanone had only a weak carcinogenic effect in $B6C3F_1$ mice.

The purpose of this study was to develop a quantitative capillary gas chromatographic procedure to determine cyclohexanone and 2-ethyl-1-hexanol leached from parenteral solution administration sets.

METHOD

Apparatus and Materials

(a) *Gas chromatograph.*—Hewlett-Packard Model HP 5790A with flame ionization detector and Shimadzu Model C-R3A recording integrator. Operating conditions: on-column 1 μL injection; initial oven temperature 30°C; no initial hold time; final oven temperature 275°, programmed at 6°/min; no final hold time; detector 350°C; hydrogen carrier gas; flow rate 4 mL/min at 60°C.

(b) *Chromatographic column.*—Capillary, 30 m × 0.32 mm, J&W DB-5 column (J & W Scientific, Folsom, CA 95630).

(c) *Concentrator.*—Kuderna-Danish evaporative concentrator.

(d) *Adapter.*—1/4 in. od × 3/4 in. brass compression ring adapter obtained through a local hardware supplier.

(e) *Solution administration sets.*—Venoset, Y-type plastic sets, 86 in. (No. 1) (Abbott Laboratories, North Chicago, IL). Plexitron sets, 70 in. (No. 2) and solution sets with injection site, 70 in. (No. 3) (Travenol Laboratories, Morton Grove, IL).

(f) *Containers.*—1 L; Wheaton bottles with Teflon-lined caps (Scientific Products, Minneapolis, MN 55441).

Reagents and Standards

(a) *Sodium bicarbonate.*—J. T. Baker Chemical Co., Phillipsburg, NJ 08865.

(b) *Disodium edetate.*—Eastman Kodak Co., Rochester, NY 14650.

(c) *Sodium chloride.*—Sigma Chemical Co., St. Louis, MO 63178.

(d) *Distilled water.*—Purified with 4-module Nanopure II water filter (0.2 μm) with an organic-free cartridge (Barnstead Co., Boston, MA).

(e) *Methylene chloride, chromatographic grade.*—Burdick & Jackson Laboratories, Muskegon, MI 49442.

(f) *Cyclohexanone.*—Aldrich Chemical Co., Milwaukee, WI 53201.

(g) *2-Ethyl-1-hexanol.*—Eastman Kodak Co.

Leaching Procedure

Place leaching solvent (filtered, distilled water) in 1 L Wheaton bottle. Drill 5/8 in. hole through top of Teflon-lined cap. Put Teflon washer on both sides of cap. Place through cap a 1/4 in. × 3/4 in. brass adapter (threaded on both ends) with 1/4 in. compression ring. Tighten nut on inside of cap to obtain water-tight seal. Place spike (1/4 in. diameter with airway) of administration set in compression ring and tighten nut. Invert bottle and place in ring stand. Adjust solvent flow with clamp to drain 1 L through administration set in 24 h.

Extraction

Place 100 mL drained solvent in separatory funnel. Adjust pH to 2.0 or less with 1N HCl. Extract solvent 4 times with 25 mL portions of methylene chloride; shake solution ca 1 min each time. Filter methylene chloride extract through 2 g layer of anhydrous sodium sulfate supported by glass wool plug. Concentrate filtrate to ca 3 mL by using Kuderna-

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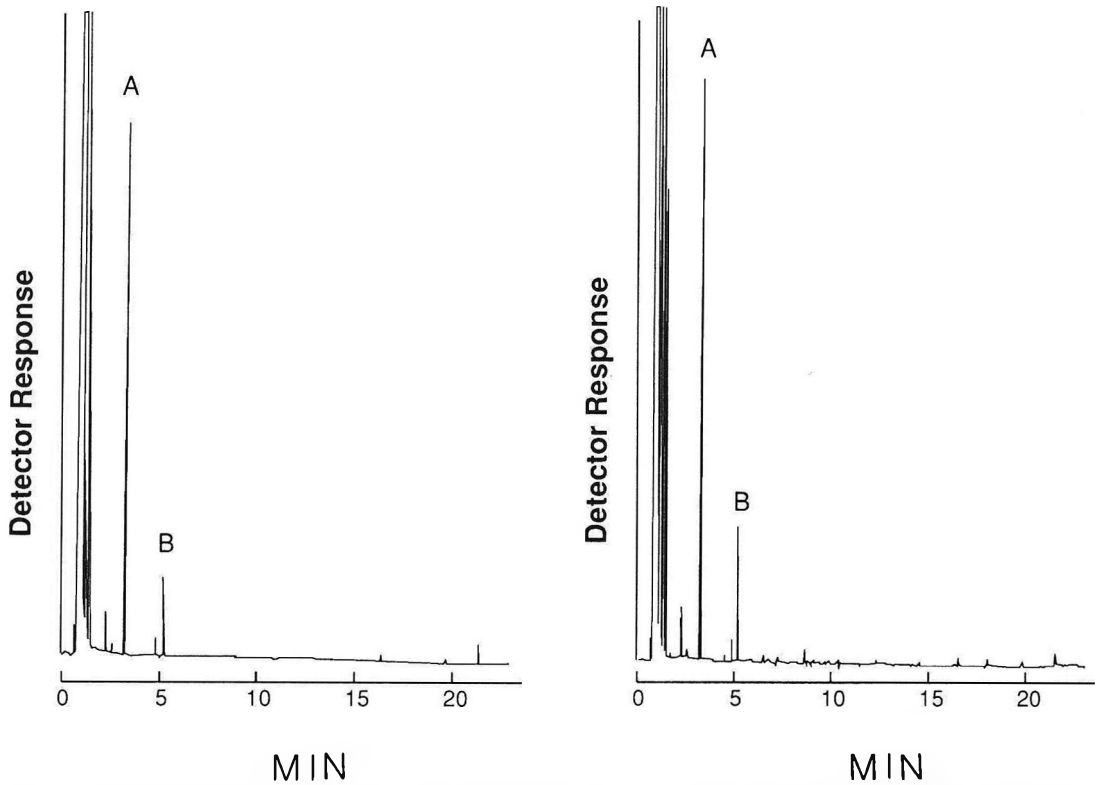

Figure 1. Capillary gas chromatogram of cyclohexanone (A) and 2-ethyl-1-hexanol (B) leached from representative solution administration sets (No. 1, left; No. 3, right).

Danish concentrator; then evaporate with flow of nitrogen to 2 mL.

Determination

Inject 1 μL extract into gas chromatograph. Calculate quantities of leached components by comparing responses to the standard curves. Perform duplicate determinations.

Standard Curves

Prepare stock solution of 100 ppm cyclohexanone and 100 ppm 2-ethyl-1-hexanol in methylene chloride and dilute appropriate aliquots to obtain final concentrations of 1, 5, 10, 15, 25, and 50 ppm. Construct standard curves under chromatographic conditions used for analyses. Use 2 injections for each concentration and average responses to obtain a curve. Calculate correlation coefficient for each set of data by linear regression analysis.

Quantitation Limit

Determine lower limits of quantitation based on 3 standard deviations of line of regression from area of concentration injected into gas chromatograph (12).

Preliminary Study

In a preliminary study, 3 solution administration sets (No. 2) were tested for chemical leaching with an aqueous solution of 5% sodium bicarbonate containing 0.009% disodium edetate (pH 8.1), an aqueous solution of 0.9% sodium chloride (pH 6.8), and filtered distilled water to determine a suitable leaching solvent. A total of 500 mL of each solution was extracted with four 25 mL portions of methylene chloride. The 4 extracts were combined and concentrated to 1 mL. Aliquots of 1 μL were injected into the gas chromatograph, and the responses were compared to standard curves.

Identification and Quantitation of Leached Compounds

Ten solution administration sets (5 of No. 1, Lot number 03 006 DT 01; 5 of No. 3, Lot number 2C 5419 S) were extracted with methylene chloride to identify and quantify compounds leached during 24 h by 1 L filtered distilled water. For a control, 100 mL filtered distilled water was extracted with four 25 mL portions of methylene chloride. The combined methylene chloride extracts were evaporated to 2 mL, and a 1 μL aliquot was injected into the gas chromatograph. Gas chromatographic peaks were identified by comparison with chromatograms of standard solutions of cyclohexanone and 2-ethyl-1-hexanol. To confirm compound identity, 2 separate determinations were made; the temperature was programmed at 6°C/min and at 12°C/min.

The quantities of cyclohexanone and 2-ethyl-1-hexanol that leached from solution administration sets were determined. During 24 h, similar quantities of these components leached into 1 L of each of the leaching solutions tested: an aqueous solution of 5% sodium bicarbonate, an aqueous solution of 0.9% sodium chloride, and filtered distilled water.

Results and Discussion

The correlation coefficients were 0.99977 for cyclohexanone and 0.99974 for 2-ethyl-1-hexanol. The lower limits of quantitation, found by using capillary gas chromatography, were 1.9 ng cyclohexanone and 2.5 ng 2-ethyl-1-hexanol.

Chromatograms of cyclohexanone and 2-ethyl-1-hexanol leached from representative solution administration sets for each manufacturer are shown in Figure 1. The quantities of these components leached during a 24 h period are shown in Tables 1 and 2. The quantities of leached components per set ranged from 72 to 174 μg/mL for cyclohexanone and from 22 to 41 μg/mL for 2-ethyl-1-hexanol. The means and standard deviations of the quantities of these components are

Table 1. Compounds leached from Venoset solution administration sets[a]

Administration set	Cyclohexanone		2-Ethyl-1-hexanol	
	μg/set	ppm	μg/set	ppm
1	174	0.17	30	0.03
2	88	0.09	27	0.03
3	112	0.11	28	0.03
4	166	0.17	31	0.03
5	72	0.07	22	0.02
$\bar{X}$	122	0.12	28	0.03
SD	46	0.05	3.5	0.004

[a] Into 1 L filtered distilled water drained through each set over 24 h.

Table 2. Compounds leached from solution administration sets with injection site[a]

Administration set	Cyclohexanone		2-Ethyl-1-hexanol	
	μg/set	ppm	μg/set	ppm
1	163	0.16	41	0.04
2	101	0.10	29	0.03
3	151	0.15	41	0.04
4	72	0.07	28	0.03
5	114	0.11	30	0.03
$\bar{X}$	120	0.12	34	0.03
SD	37	0.04	6.6	0.005

[a] Into 1 L filtered distilled water drained through each set over 24 h.

given in Tables 1 and 2. Corrections for recovery were not made.

Cyclohexanone and 2-ethyl-1-hexanol were added to 100 mL water in quantities similar to those found in solution administration sets and taken through the method. The same procedures were followed for recovery and sample analyses. Recoveries of the standard compounds added to water were as follows: cyclohexanone, 94% for 5 μg/mL, 94% for 10 μg/mL; 2-ethyl-1-hexanol, 93% for 3 μg/mL. Similar recoveries were found by Snell (2) when these compounds were extracted from water with methylene chloride. No interfering peaks were found in chromatograms obtained for the controls; however, a small peak for DEHP (retention time 22.0 min) was noted. DEHP peaks in chromatograms obtained for the sample were similar in size to those obtained for the control; therefore, it was concluded that DEHP was not leached from the administration sets.

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DRUG RESIDUES IN ANIMAL TISSUES

Quantitation and Confirmation of Sulfamethazine Residues in Swine Muscle and Liver by LC and GC/MS

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The present paper describes a liquid chromatographic (LC) method for purification of crude swine tissue extracts before gas chromatographic/mass spectrometric (GC/MS) quantitation and confirmation of sulfamethazine at low ppb levels. Fractions corresponding to sulfamethazine were collected, evaporated to dryness, *N*-methylated with diazomethane, concentrated, and analyzed by GC/MS. A mass spectrometer was set to selected ion monitoring (SIM) mode. Ions 233, 227, 228, and 92 *m/z* were detected. Ratio 227/233 *m/z* (sulfamethazine/internal standard, [phenyl $^{13}C_6$] sulfamethazine) was used for quantitation, while ratios 228/227 and 92/227 *m/z*, respectively, were used for confirmation. Quantitation in spiked blank muscle tissue was tested from 100 to 1 ppb and found acceptable at all concentrations studied; coefficients of variation ranged from 4.9 to 14.4%. Similar results were obtained for liver tissue from 5 to 20 ppb; coefficients of variation ranged from 1.2 to 9.1%.

Sulfamethazine (SMZ) is widely used as an antimicrobial agent and as a growth promoter for swine, cattle, turkeys, and chickens. The present U.S. tolerance of 0.1 ppm in edible animal tissue (1) is being reassessed after results by the National Center for Toxicological Research (NCTR) showed that sulfamethazine may be a carcinogen in test animals (2).

Sulfamethazine residues have been determined using several methods, including colorimetry (3, 4), gas chromatography (GC) (5, 6), gas chromatography/mass spectrometry (GC/MS) (7–9), liquid chromatography (LC) (10–12), LC/MS (13), immunoassay (14), and thin layer chromatography (TLC) (15); however, no method offered the advantage of simultaneous quantitation and confirmation in the low ppb concentration range.

A method for confirming the presence of SMZ residues in milk at trace levels (≈5 ppb) was developed in our laboratory (16). The present work was undertaken to see whether this method could also be applied to swine tissue. The present paper describes an LC method combined with a GC/MS method for simultaneous quantitation and confirmation of sulfamethazine residue in swine muscle and liver tissue. Purification of crude sulfamethazine extract is achieved by LC before GC/MS quantitation and confirmation.

METHODS

Reagents

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

(a) *Solvents.*—Acetonitrile and methanol (LC grade, J.T. Baker Inc., Phillipsburg, NJ 08665); *n*-hexane (distilled in glass, Caledon Laboratories Ltd, Georgetown, Ontario, Canada, L9H 4R9); diethyl ether anhydrous (Mallinckrodt Inc., Paris, KY 40361).

(b) *Mobile phase.*—Mix 600 mL methanol with 1400 mL water. Filter through 0.2 μm membrane (Ultipor $N_{66}R$, Pall Trinity Micro Corp., Cortland, NY).

(c) *n-Hexane purified.*—Stir 500 mL *n*-hexane overnight with 100 mL concentrated sulfuric acid. Wash *n*-hexane 3 times with 100 mL water.

(d) *Diazomethane saturated solution.*—Prepare a saturated diethyl ether solution from 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, WI 53233) using diazomethane generation apparatus (17).

(e) *Standard solutions.*—Prepare stock solutions of sulfamethazine (Sigma Chemical Co., St. Louis, MO 63178) and internal standard, ^{13}C-sulfamethazine [phenyl-$^{13}C_6$, 90%] (ICN Biochemicals Inc., Cambridge, MA 02142) at 10 μg/mL in methanol and store in refrigerator. Before use, dilute stock solution with water to appropriate concentrations.

(f) *Fortification solution.*—Dilute 1 mL sulfamethazine stock solution to 10 mL with water.

Apparatus

(a) *LC pump.*—Spectroflow 400 (Kratos Analytical, Ramsey, NJ 07446). Set flow rate at 1.5 mL/min.

(b) *Columns.*—250 × 4.6 mm id, 5 μm, Supelcosil LC-18-DB column (Supelco Inc., Bellefonte, PA 16823) and 15 × 3.2 mm id, RP-18 NewGuard column (Brownlee Labs Inc., Santa Clara, CA 95050).

(c) *Autosampler.*—Model SP8770XR (Spectra-Physics, San Jose, CA 95134) equipped with 2 mL sample loop and operated in "fixed loop mode" at 400 μL. This combination permits injection of ca 850 μL.

(d) *Detector.*—Spectroflow 783 (Kratos Analytical, Ramsey, NJ 07446) variable UV detector set at 265 nm.

(e) *Integrator.*—Computerized data acquisition and control system (Isaac, 42A, Cyborg Corp., Newton, MA 02158).

(f) *Software.*—Appligration II (Dynamic Solutions Corp., Pasadena, CA 91101).

(g) *Switching valve.*—HPSC system (Autochrom Inc., Milford, MA 01757).

(h) *Fraction collector.*—Model 10447U Universal fraction collector (Eldex Laboratories Inc., Menlo Park, CA 94025) with remote control vial advance.

(i) *Homogenizer.*—Sorvall Omni-Mixer 17105 (Dupont Instruments Products, Newtown, CT 06470) with 250 mL sample bucket.

(j) *Vortex-evaporator.*—Model 4322000 (Haake Buchler Instruments Inc., Saddle Brook, NJ 07662).

(k) *Centrifuge.*—Sorvall Model RC-5B, with rotor No. HS-4 and four 24-place swing-out test tube holders (Dupont Instruments Products, Newtown, CT 06470).

(l) *Gas chromatograph/mass spectrometer.*—HP 5985A

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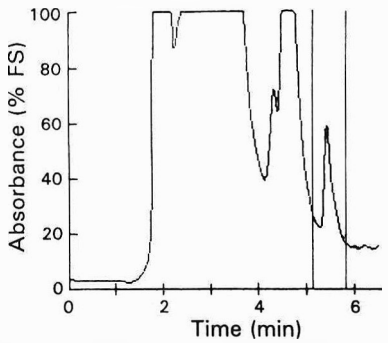

Figure 1. LC chromatogram of incurred muscle sample extract. Retention time of sulfamethazine = 5.4 min. The window between 5.1 and 5.8 min. (vertical lines) represents the collection of the fraction. Estimated concentration = 13.2 ppb sulfamethazine and 67 ppb internal standard (sulfamethazine [phenyl $^{13}C_6$]). Chromatographic conditions are described in text.

GC/MS system (Hewlett-Packard, Palo Alto, CA 94304), equipped with a DB-5, 15N, 0.25 mm id, 0.25 μm thickness, 15 m long, capillary column (J & W Scientific, Folsom, CA 95630). Set helium carrier gas flow to linear velocity of 30 cm/min. Set injection port to 250°C. Close split/splitless valve of injection chamber, then inject 1 μL aliquot. After 0.5 min, open split/splitless valve. Maintain column oven temperature at 220°C for 1 min, then raise at 10°C/min to 270°C. Set auxiliary transfer line to 275°C. Chromatograph sample for a total of 9 min. Ion source temperature is 200°C and electron multiplier voltage, 2800 V. Use MS in electron impact ionization mode with 70 eV ionization potential and 300 μA ion currents. Use autotune program to calibrate all other parameters. Operate mass spectrometer in selected ion monitoring mode (SIM) tuned to 233, 227, 228, and 92 *m/z* with a dwell time of 300 μs for each ion.

Sample Preparation

Weigh 15 g frozen, ground tissue into 250 mL homogenizer cup. Let sample partially thaw. Add 100 μL internal standard solution and 150 mL acetonitrile. Homogenize for 3 min at full speed. Add 25 g anhydrous sodium sulfate and homogenize again for 1 min. Then let stand for 30 min. Filter through an acetonitrile-washed, Whatman No. 1 fluted filter paper and collect filtrate into 250 mL round bottom flask containing 5 mL 0.1M potassium dihydrogen phosphate. Using rotary evaporator at 35°C, evaporate all acetonitrile, leaving 0.5 to 1 mL aqueous solution. Add 3 mL water and 15 mL *n*-hexane to flask and shake 3 times for 1 min each, over a 15 min period on wrist-action shaker (12). Filter lower aqueous layer with some of the hexane through 0.2 μm membrane Millex-HV filter into 12 × 75 mm test tubes. Centrifuge at 4500 rpm for 15 min at 4°C. Transfer lower layer into autosampler vials. Chromatograph in 3–4 portions of ca 850 μL each and collect fraction corresponding to SMZ (Figure 1). Evaporate pooled fractions on vortex evaporator at 40°C.

Methylation

Add 100 μL methanol and 1.5 mL diazomethane saturated ether solution to dry residue. Allow to react for 30 min. Shake 3 times during this period, release pressure after each shaking. Transfer ethereal solution in 2 portions to 1 mL Reacti-Vial (Pierce, Rockford, IL 61105) and evaporate to dryness at 35°C, using a gentle stream of nitrogen. Redissolve residue in 10 μL methanol.

Analysis

Inject 1 μL aliquot of methylated sample into GC/MS system. Monitor ions 233, 227, 228, and 92 *m/z*. Measure peak area of each at the retention time window corresponding to sulfamethazine and calculate the following ions ratios: 227/233, 228/227, and 92/227.

Standard Curve

Add 0, 15, 30, 75, 150, 375, 750, and 1500 μL sulfamethazine fortification solution to separate 15 g samples of blank muscle tissue (equivalent to 0, 1, 2, 5, 10, 25, 50, and 100 ppb, respectively). Proceed as described in *Sample Preparation*. Measure peak area for ions 233, 227, 228, and 92 *m/z*. Calculate correlation coefficient and linear regression (*r*, slope, and Y-intercept) of peak area ratio against sulfamethazine concentration (ppb). Also calculate peak area ratios of ions 228/227 and 92/227.

Calculation

Using standard curve parameters, calculate sulfamethazine concentration in samples using the following:

$$\text{Concn, ppb} = \frac{R(A227/A233) - b}{s}$$

where b = Y-intercept, s = slope, and $R\,(A227/A233)$ = peak area ratio of 227 to 233 *m/z*.

Results and Discussion

The electron impact (EI) fragmentation pattern of sulfonamides has been discussed by Davis et al. (18). In the case of N^1-methyl-sulfamethazine, the molecular ion was not detected; however, major fragments were found to be M^+-65 ($-HSO_2$), M^+-64 ($-SO_2$), $[C_6H_7N_2]H^+$, and $[C_6H_6N]^+$ corresponding to 227, 228, 108, and 92 *m/z*, respectively. The internal standard (sulfamethazine [phenyl $^{13}C_6$]) had

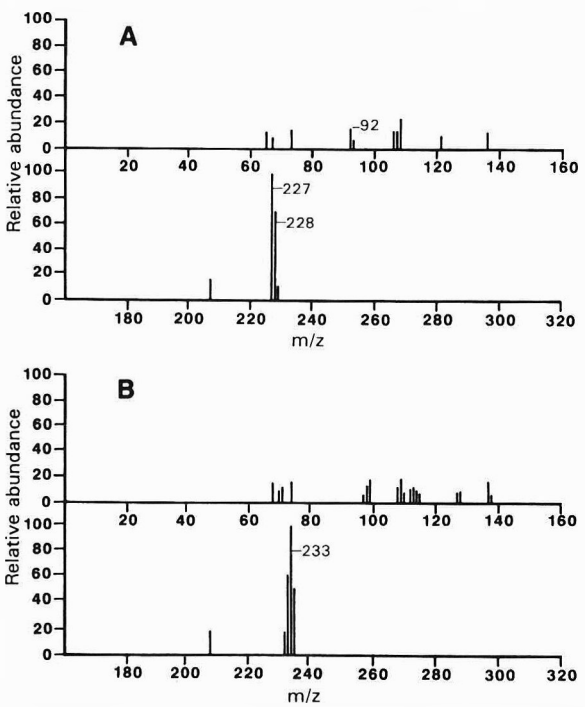

Figure 2. Electron impact mass spectra of (A) N^1-methyl-sulfamethazine and (B) N^1-methyl-sulfamethazine [phenyl $^{13}C_6$].

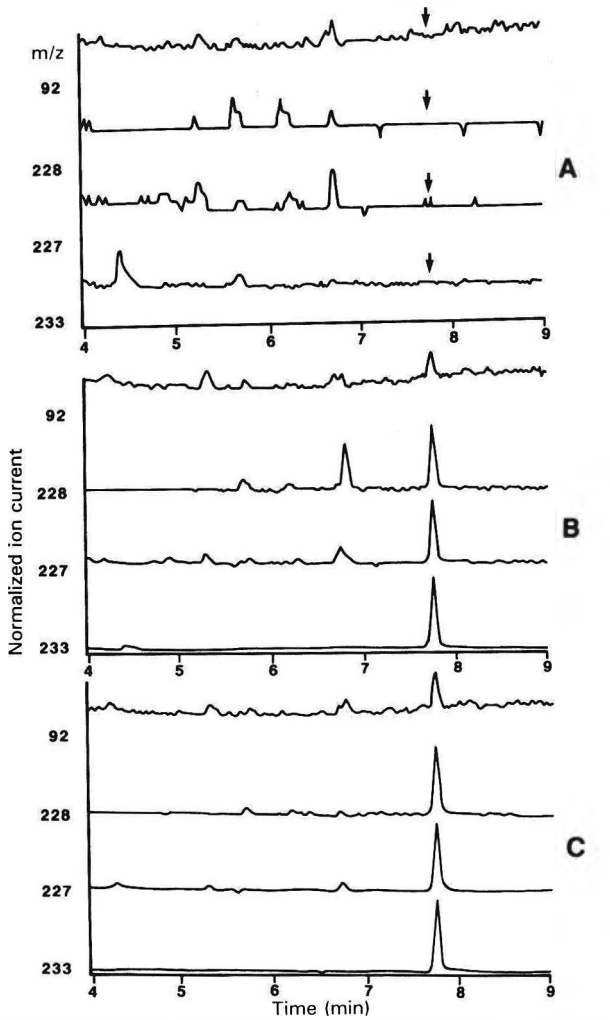

Figure 3. GC/MS chromatograms with detector set at 233, 227, 228, and 92 *m/z*. (A) Blank swine muscle tissue, (B) blank swine muscle tissue spiked with 10 ppb sulfamethazine, (C) incurred swine muscle tissue. Estimated concentration = 13.2 ppb. Arrow indicates GC retention time of methylated sulfamethazine (7.8 min.).

the corresponding ions 233, 234, 108, and 98 *m/z* (Figure 2).

Specific requirements for confirmation of drug residues by MS have been studied by Sphon (19). A minimum of 3 structurally significant ions eluting at the right retention time is necessary to provide proof of presence, with recommended intensity variation for ion abundance ratios within 5%, when compared with a reference standard analyzed under identical conditions. For these reasons, the apparatus was operated in SIM mode set at 233, 227, 228, and 92 *m/z* and the ion ratio 227/233, calculated for quantitation needs, and ion ratios 228/227 and 92/227 for confirmation purposes.

Under LC conditions described above, sulfamethazine eluted at approximately 5.4 min. with a 0.7 min peak width at base (Figure 1). It is important that all acetonitrile in the tissue extract be evaporated from the sample. A small residual amount of organic solvent will decrease sulfamethazine retention time, thus interfering with fraction collection.

The LC column was cleaned at regular intervals after every 50 samples, under reversed flow, by passing 75 mL of each solvent in the following order: methanol, tetrahydrofuran, chloroform, *n*-hexane, chloroform, tetrahydrofuran, and methanol. The guard column was replaced as needed.

Table 1. Standard curve for SMZ in spiked swine muscle tissue

Amount added, ppb	*N*	Ratio ion areas 227/233 $\bar{X}$	Ratio ion areas 227/233 CV, %	Ratio ion areas 228/227 $\bar{X}$	Ratio ion areas 228/227 CV, %	Ratio ion areas 92/227 $\bar{X}$	Ratio ion areas 92/227 CV, %
0	12	—	—	—	—	—	—
1.0	10	0.0158	14.4	0.73	32	0.61	40
2.0	8	0.0253	8.3	0.77	10	0.61	17
5.0	16	0.0681	10.0	0.65	12	0.44	48
10.0	15	0.1370	10.6	0.70	10	0.30	28
25.0	10	0.3323	4.9	0.66	11	0.28	14
50.0	9	0.6692	6.6	0.68	5	0.24	6
100.0	9	1.2968	7.2	0.67	8	0.22	6

Mean CV, % 8.9
Slope = 0.01295 ± 0.00013
Constant = 0.0049 ± 0.0349
Correlation coeff. = 0.9964

Figure 3 shows GC chromatograms of blank, spiked (10 ppb), and incurred (estimated concentration, 13.2 ppb) muscle tissue. Blank muscle and liver background determinations at the retention time of SMZ were minimal and allowed accurate quantitations within the concentration ranges studied.

Tables 1 and 2 show results obtained from spiked muscle and liver tissues. Linearities of standard curves are excellent (r = 0.9964 and 0.9955); intercepts are essentially 0, and coefficients of variation (CV) are acceptable to levels of 1 and 5 ppb, respectively.

CVs for the ion ratios 228/227 and 92/227 are in the range recommended by the U.S. Food and Drug Administration (≤20%) (20) (i.e., down to 50 ppb in muscle and 20 ppb in liver). Somewhat higher CVs were obtained for lower concentrations, but should still be considered good proof of identity when retention times, both in LC and GC, are taken into consideration.

Because the internal standard, an isotope labelled SMZ, was added at the beginning of sample preparation, the ratio peak area of unknown to peak area of the internal standard internally corrects for recovery. However, recovery for the extraction step, determined by LC using UV detection, was found to be about 50% for spiked samples at 100 ppb. Under these conditions, the recoveries found at the concentrations used appear to be acceptable.

Reproducibility and linearity of the standard curve in

Table 2. Standard curve for SMZ in spiked swine liver tissue

Amount added, ppb	*N*	Ratio ion areas 227/233 $\bar{X}$	Ratio ion areas 227/233 CV, %	Ratio ion areas 228/227 $\bar{X}$	Ratio ion areas 228/227 CV, %	Ratio ion areas 92/227 $\bar{X}$	Ratio ion areas 92/227 CV, %
5.0	4	0.0693	6.2	0.78	6	0.30	13
10.0	4	0.1222	9.1	0.79	8	0.31	9
20.0	4	0.2546	1.2	0.77	5	0.25	5

Mean CV, % 5.5
Slope = 0.01243 ± 0.00037
Constant = 0.0032 ± 0.0081
Correlation coeff. = 0.9955

Table 3. Sulfamethazine residues in incurred swine muscle and liver tissues

	LC GC/MS assay			Ratio ion areas		TLC[a]
Sample	$\bar{X}$, ppb	CV, %	*N*	228/227	92/227	assay, ppb
Muscle						
A	9.6	6.7	4	0.80	0.39	10
B	23.5	3.2	4	0.75	0.24	20
C	13.2	3.3	7	0.73	0.24	10
Liver						
D	27.0	3.8	4	0.77	0.25	20

[a] Results supplied by Agriculture Canada (15).

swine muscle tissue (Table 1) indicate good reliability of the method. Quantitation was tested to 1 ppb and found acceptable at all concentrations. A similar standard curve in swine liver tissue tested to 5 ppb (Table 2) does not show any significant difference.

Incurred samples were previously analyzed and kindly supplied by Health of Animals Laboratory, Agriculture Canada, Saskatoon using a thin-layer chromatographic/densitometric technique (15). There was good agreement between both methods (Table 3) in the 3 muscle and 1 liver samples supplied.

In conclusion, this method combines the power of LC purification with the selectivity of GC/MS to produce simultaneous quantitation and confirmation of sulfamethazine residue down to 1 ppb with excellent reproducibility. It is also simple and fast. Using an automated LC, coupled to an autosampler and fraction collector, an experienced analyst could easily analyze 12 samples every 2 days.

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Liquid Chromatographic Determination of Chloramphenicol in Calf Tissues: Studies of Stability in Muscle, Kidney, and Liver

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A liquid chromatographic method has been developed for the measurement of chloramphenicol (CAP) in muscle, liver, and kidney. The mean recovery levels were 82.6, 75.3, and 79.2% in muscle, liver, and kidney, respectively. The method was repeatable and reproducible for CAP measurement in muscle, with a detection limit of 1 μg/kg. Investigation of CAP stability in muscle, liver, and kidney showed that CAP stability in muscle was good at −20°C; for spiked liver and kidney, degradation of CAP was observed, and the use of piperonyl butoxide (PB) for metabolism inhibition was recommended for recovery and linearity studies. However, PB was unnecessary for preservation of treated animal tissues if samples were cut into cubes and cooled at −20°C, just after slaughter, pending analysis. With these limitations, CAP can be measured in liver and kidney.

Chloramphenicol (CAP) is a bacteriostatic, broad spectrum antibiotic that is often used in veterinary practice for treating various infectious diseases. Nevertheless, adverse reactions and side effects in humans have been extensively demonstrated. For these reasons, within the European Economic Community, a maximum residue level of 10 μg/kg has been proposed for meat (1). During the last few years, several methods were proposed for the determination of CAP in meat (2–4). However, it must be emphasized that liver and kidney cortex were recognized as unsuitable target tissues for monitoring CAP residues, because of the extensive postmortem degradation in these organs (5, 6).

Chloramphenicol was metabolized in liver with a phase I metabolism involving oxidation, catalyzed by cytochrome P-450 followed by phase II, glucuronic acid conjugation (7). Recently, the stability of CAP in liver was examined and it was suggested that piperonyl butoxide (PB), a potent cytochrome P-450 inhibitor, could be used to inhibit CAP degradation by enzymes (6, 8).

In this study, CAP degradation was investigated in muscle, liver, and kidney with spiked samples and tissues from treated animals. The intralaboratory repeatability and reproducibility for the assay of CAP in muscle were determined and the stability of CAP in the different tissues was studied.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Pump, Model 5500 with Rheodyne injector with 200 μL loop, UV-200 variable wavelength detector, and VISTA 402 calculator integrator (Varian, Les Ullis, France).

(b) *Liquid chromatography column.*—Novapack C18, 4.6 mm × 15 cm long (Waters Chromatography) with a guard column C8 4 × 4 mm.

(c) *Centrifuge.*—Jouan GR 4.11 (Jouan, St Herblain, France).

(d) *Kitchen mixer.*—Moulinex Moulinette.

(e) *Rotary evaporator.*—Rotavapor Büchi.

(f) *Stirrer.*—Heidolf Reax 2.

(g) *Rotary stirrer.*—Vortex.

(h) *Water purification kit.*—Reagent grade waters system MilliRo, MilliQ (Millipore, St Quentin Yvelines, France).

Reagents

(a) *Solvents.*—Analytical grade ethyl acetate, chloroform, hexane, methanol, acetonitrile (Merck, Darmstadt, Germany).

(b) *Ultrapure water.*—Obtained from reagent grade waters system.

(c) *Ethylacetate (LC grade).*—Distilled in rotary evaporator before use (Merck).

(d) *Piperonyl butoxide suspension.*—Add 2 mL concentrated piperonyl butoxide (Virbac, Batch 147-1; purity 84.9%) to 50 mL water and ultrasonicate for 10 min. Let stand for 15 min before using. PB concentration, 0.78 mg/mL.

(e) *Chloramphenicol analytical standards.*—Chloramphenicol base (Batch 4216025, purity 99.7%, Lepetit, Milan, Italy). (*1*) *Stock solution.*—Weigh 50 mg in a 100 mL volumetric flask, and dissolve with 2 mL methanol. Dilute to 100 mL with water. (*2*) *Working solution.*—Dilute 1 mL stock solution to 100 mL with water in a volumetric flask (CAP concentration, 5 μg/mL). Pipet 1, 0.75, 0.5, and 0.25 mL of this solution into 50 mL volumetric flasks and dilute to volume with water. These solutions contain 25, 50, 75, and 100 μg chloramphenicol/L, respectively. Prepare solutions daily.

(f) *CAP metabolites.*—Nitrobase (Batch 4540015, Lepetit, Milan, Italy), CAP Glucuronide (Batch C9899, Sigma Chemical Co., St Louis, MO), Nitroso-CAP (Batch HS 294140), Dehydro-CAP (Batch BM 311054), Dehydro-CAP-Base chlorhydrate (Batch BM 311053) (Boehringer-Mannheim, Mannheim, Germany).

(g) *Metabolite solution.*—Weigh 10 mg of each metabolite and CAP in 100 mL volumetric flasks. Dissolve in 2 mL methanol and dilute to 100 mL with water.

(h) *Diammonium buffer (pH 7.9).*—Dissolve 0.66 g diammonium hydrogenophosphate (Merck) in 1 L ultrapure water.

(i) *Diammonium buffer (pH 8.3).*—Adjust diammonium buffer (pH 7.9) at pH 8.3 with ammonia solution at 25%.

(j) *Mobile phase.*—Diammonium buffer (pH 7.9)-acetonitrile (v/v, 71 + 29) for muscle analysis. Diammonium buffer (pH 7.9)-acetonitrile (v/v, 82 + 18) for liver analysis. Diammonium buffer (pH 8.3)-acetonitrile (v/v, 71 + 29) for kidney analysis.

(k) *Sulfuric acid.*—0.1%. Dilute 0.1 mL sulfuric acid (Merck) in 100 mL water.

Sample Preparation

Grind fresh or partially thawed muscle, liver, or kidney for

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1 min with a kitchen mixer. Weigh 5 g tissue, thawed at room temperature, into a 15 mL glass stoppered centrifuge tube. Add 2 mL water. Stir with a vortex stirrer for 1 min at maximal speed. Let stand 10 min.

Extraction of CAP from Sample

Add 6 mL ethyl acetate to the sample, cap, and stir with a vortex stirrer 1 min at maximal speed. Centrifuge for 5 min at 4000 rpm. Three layers will separate: the remaining tissue at the bottom, the aqueous layer in the middle, and the organic layer at the top. Pipet 4.2 mL of the organic layer and transfer into a 20 mL round-bottom flask. Evaporate to dryness at 30°C. Make sure no ethyl acetate remains but only an oily residue. Suspend the residue in 1.4 mL of a mixture of hexane-chloroform (v/v, 1 + 1). Add 0.7 mL water, and stir with a Heidolf stirrer at 35 rpm for 5 min. Transfer into a tube. Centrifuge for 10 min at 4000 rpm and repeat if the partitioning is not complete. Inject 200 μL supernatant into the LC system.

Liquid Chromatographic Determination

Set column temperature at room temperature. Set the wavelength at 278 nm, the flow rate at 1.0 mL/min, and the UV detector at 0.002 absorbance unit full scale. Alternatively, inject 200 μL working standard solution and an extract of sample. Calculate CAP levels (Y) by comparing the sample peak area with the external standard peak area with the following formula:

$$Y = \frac{P1}{P2} \times \frac{CS}{CF}$$

where CF is a concentration factor (CF = 5), P1 is the sample peak area, P2 is the standard peak area, and CS is the standard concentration. Flush system daily with 0.1% sulfuric acid-acetonitrile (v/v, 82 + 18).

Experimental

Linearity of the UV detector response for CAP was determined from a set of 4 working standards ranging in concentration from 0.025 to 0.1 μg/mL. A single calibration curve was prepared by plotting the peak area response vs the amount expected on the column from a 200 μL injection.

CAP was determined in muscle, kidney, and liver samples from calves that had not been treated with CAP. The effect of using piperonyl butoxide in the preparation of kidney and liver samples on the precision and recovery of the method was studied.

For recovery studies without PB, 5 g ground blank samples of calf muscle or liver were added to 1 mL water and 1 mL standard CAP solutions ranging from 0.025 to 0.1 μg/mL to obtain fortified samples ranging from 0.005 to 0.020 μg/g. Tubes were capped and stirred for 1 min at maximal speed and submitted to the analytical procedure.

For recovery studies with PB, 5 g ground blank samples of calf liver or kidney were added to 1 mL of one of the standard solutions as above. Tubes were capped and stirred for 30 s, and 1 mL fresh piperonyl butoxide suspension was added to each. Tubes were capped and stirred for 30 s and then submitted to the analytical procedure.

All recoveries were determined by comparing the amount of drug recovered from tissue (Y) to the amount of drug spiked onto tissue (C). Working standard solution and extracted samples were injected alternately. To calculate recovery (R%), the following formula was used:

$$R\% = \frac{Y}{C} \times 100$$

Intralaboratory repeatability and reproducibility for LC determination of CAP in muscle.—To determine the intralaboratory repeatability and reproducibility, muscle samples were spiked at 4 levels (0, 6, 11, and 17 μg/kg). They were randomly coded and undistinguishable. Three technicians using 3 different LC systems received 12 samples (3 blanks and 9 positives) and a detailed description of the method. Repeatability and reproducibility were calculated as described in ISO recommendations (9).

Specificity for LC determination of CAP in muscle.—200 μL metabolite solution was injected into the LC system. To obtain fortified tissue at 20 μg/kg for each metabolite, 1 mL of the metabolite solution was added to 5 g ground muscle. Fortified tissue was submitted to analysis.

Stability in muscle.—The investigation of in vitro CAP stability during storage at −20°C in calf muscle was carried out on a sample fortified at 10 μg/kg. Recovery was measured on 6 replicates at 1, 15, 30, 60, and 90 days.

Stability in liver and kidney.—The in vitro stability of CAP in liver was investigated with samples fortified at 20 μg/kg. The stability was studied at different temperatures (room temperature, 4°C, and 1°C) and at several time intervals (5 min, 10 min, 30 min, 1 h, and 2 h) on 4 replicates with and without using PB in the preparation of fortified tissues.

To study the in vivo stability of CAP in liver and kidney of treated animals, the following procedure was used: A 50 kg calf received CAP at 20 mg/kg by the intravenous route. The calf was sacrificed 4 h after injection, and the liver and kidney were immediately collected and prepared as described above. CAP concentrations in tissues were immediately measured with and without the use of piperonyl butoxide in sample preparation. Four different preparations of samples were applied: (*1*) Tissue was ground after sampling and frozen at −20°C. (*2*) Tissue was ground after sampling; then 1 mL PB was added to 5 g tissue, and the ground material was frozen at −20°C. (*3*) Tissue was cut into cubes (8 cm^3) and frozen. (*4*) Tissue was cut into cubes and frozen, and PB was used in the sample preparation. The analysis schedule was 1, 2, 7, 30, and 85 days post-sampling.

Results and Discussion

Linearity of the LC UV detector response for CAP in the working standard solution was satisfactory (r = 0.9983). Average recoveries of CAP in muscle ranged from 80.15 to 86.64% as shown in Table 1, with a mean coefficient of variation (CV) of 4.84%. Recoveries were quite good and reproducible for each concentration. For muscle, stability in fortified samples stored at −20°C was evaluated. It was accurate for a period of 180 days (Table 2). The mean intralaboratory repeatability for the 3 levels was good, with r = 1.366 μg/kg (Table 3). No value was questionable or statistically erratic. No apparent relationship was observed between r and the mean (m), whereas reproducibility (R) increased in relation to m in the range of concentrations assayed (Table 3). For this method, with our LC materials, the limit of detection, which was 3 times the background noise, was 1 μg/kg. The analytical process was specific to chloramphenicol. No interfering peaks were observed in chromatograms of fortified samples with CAP or its metabolites. With metabolite solutions, nitroso-CAP was observed

Table 1. Recovery levels for spiked samples of calf muscle, kidney, and liver with and without use of piperonyl butoxide (PB)

Concn added, μg/kg	No. measurements	Mean concn observed, μg/kg	SD	CV, %	Mean rec., %
		Muscle without PB			
5	10	4.332	0.28	6.67	86.64
10	10	8.174	0.60	7.35	82.15
15	10	12.01	0.69	5.76	80.15
20	10	16.26	0.30	1.87	81.47
		Liver without PB			
5	20	2.82	0.38	13.46	56.52
10	20	4.53	0.72	16.06	45.39
15	17	6.44	0.86	13.46	42.97
20	20	11.76	1.12	9.53	58.83
		Liver with PB			
5	12	3.85	0.25	6.63	77.17
10	12	7.52	0.47	6.29	75.28
15	11	11.06	0.33	3.04	73.79
20	12	14.98	0.43	2.88	74.90
		Kidney with PB			
5	12	3.86	0.22	5.71	77.26
10	12	7.85	0.54	5.78	78.59
15	12	12.10	0.75	6.24	80.72
20	12	16.08	0.81	5.06	80.40

with a retention time near that of CAP. The time required for 24 analyses is 1 day. This method seemed a good analytical system and was useful for evaluating an interlaboratory study (10).

For CAP determination in liver and kidney, the method was used to investigate the stability of spiked samples and recovery levels. With liver, stability of fortified samples was a function of time and temperature. At room temperature, recovery was 62.6% after 5 min and 9% after 30 min (Table 4). At 4°C, the recovery attained 40% after 1 h and 44% if the temperature was 1°C (Table 4). The same results were obtained with kidney (Table 4). If metabolism was reduced, it was not inhibited by cold. Therefore, it is not possible to slow it sufficiently so as to attain working conditions suitable to fortification. To solve this problem, Parker and Shaw (6) proposed the use of piperonyl butoxide to inhibit the enzyme system during the determination of CAP. At room temperature, the use of piperonyl butoxide allowed a high enough recovery percentage to be maintained for 30 min (Table 4). Piperonyl butoxide is not water-soluble, however, and the use of a fresh suspension of PB in water was necessary.

Table 2. Study of stability of chloramphenicol in spiked muscle (10 μg/kg)

Time, days	No. measurements	Mean rec., %	SD
0	6	84.9	2.4
1	6	84.3	0.5
15	6	84.5	0.6
30	6	89.0	2.0
60	6	87.2	1.1
90	6	88.5	2.0
180	6	84.5	1.1

Table 3. Intralaboratory repeatability (r) and reproducibility (R) for 3 levels of spiked muscle by 3 analysts using different liquid chromatography systems[a]

	Level, μg/kg		
System	6	11	17
T1	5.72	10.46	15.99
	5.72	10.44	16.83
	5.72	10.64	15.99
Mean, μg/kg	5.72	10.51	16.27
SD	0	0.11	0.48
T2	5.68	9.58	17.47
	6.27	11.03	17.33
	7.09	10.94	17.76
Mean, μg/kg	6.35	10.51	17.52
SD	0.70	0.81	0.22
T3	6.22	11.63	16.63
	6.12	10.75	17.27
	6.41	10.75	—
Mean, μg/kg	6.25	11.18	16.95
SD	0.15	0.44	0.45
Global mean	6.1	10.73	16.9
r, μg/kg	1.17	1.52	1.4
R, μg/kg	1.35	1.65	2.2

[a] Global mean, repeatability, and reproducibility were calculated as described in ISO 5725.

For spiked samples of kidney or liver, the activity of PB on mixed function oxidase is useful for good reproducibility of standard curves (Table 1). With our method, the peak area was linear against the dose added in tissue (r = 0.9968 for liver and 0.9921 for kidney). The mean recovery levels for liver when PB was used for the fortification process ranged from 73.79 to 77.17%, which were higher than those obtained without use of PB (range, 42.97–58.53%) (Table 1). Moreover, CVs when PB was used ranged from 2.88 to 6.63, which were lower than those without PB use (range, 9.53–16.06%; Table 1). Mean recovery levels and CVs for kidney were similar to those for liver (Table 1). Use of PB in fortification of these tissues permitted a correct construction of standard curves.

Parker and Shaw (6) recommended that samples taken for analysis be frozen immediately after excision and homogenized in water containing 2.5% m/V of PB before assay. In order to assess this recommendation, different treatments of dosed animal tissues were studied. After sampling, analysis of fresh tissue permitted us to determine that a higher concentration of CAP may be detected if piperonyl butoxide is added (Table 5). However, these concentrations were lower than those measured on meats cut into cubes and frozen from day 1 to day 85 (C & D, Table 5). Performing grinding, extraction, and centrifugation on liver that is still warm may account for this phenomenon.

In the same manner, if grinding is carried out before freezing, metabolism may still degrade a great part of CAP (A & B, Table 5). By adding PB to ground liver before freezing, a higher concentration could be obtained (A & B, Table 5). However, this difference in PB activity was not established for tissues frozen as cubes (C & D, Table 5), since treatment was carried out on thawing tissues at low temperature. Hence, enzymatic activity of cytochrome P-450 was low and addition of PB had no effect (D vs C, Table 5). In addition,

Table 4. Influence of length of time, temperature, and use of piperonyl butoxide (PB) on stability of chloramphenicol in spiked liver (20 ng/g) and spiked kidney (20 ng/g)

Time, min	Temp., °C		Liver		Kidney	
			Without PB	With PB	Without PB	With PB
0	20	N[a]	6	4	3	4
		mean	15.52	14.82	17.80	15.87
		SD	0.23	0.64	1.56	0.49
		R[b]	77.6	74.1	89	79.3
5	20	N	4	4		4
		mean	12.44	15.46	NA[c]	15.62
		SD	0.25	1.75		0.46
		R	62.2	77.3		78.1
10	20	N	5	3	3	4
		mean	8.1	14.88	13.11	15.18
		SD	0.68	1.16	1.43	0.88
		R	40.5	72.7	65.5	75.9
30	20	N	6	4	3	4
		mean	1.79	14.20	5.2	14.23
		SD	0.49	1.05	0.3	1.20
		R	9	71.0	26.0	71.1
60	20	N	6	4	4	4
		mean	1.9	12.67	2.37	14.28
		SD	0.89	0.38	0.42	0.95
		R	9.5	63.3	12.0	71.0
60	4	N	4		4	
		mean	7.99	NA	9.29	NA
		SD	0.80		0.42	
		R	40.0		46.5	
60	1	N	4		6	
		mean	8.86	NA	10.71	NA
		SD	0.23		1.14	
		R	44.0		53.5	

[a] N = number of measurements.
[b] R = % recovery.
[c] NA = not analyzed.

Table 5. Study of chloramphenicol stability at −20°C in liver and kidney of calf slaughtered 4 h after 20 mg/kg intravenous injection of CAP; influence of storage time and sample preparation before analysis[a]

	Storage time, days					
	0	1	2	7	30	85
	CAP concentration observed (μg/kg) in liver					
A	6.42	1.57	1.54	1.18	0.99	1.49
	6.52	1.58	1.57	1.19	0.98	1.48
B	8.74	4.10	3.67	3.13	2.86	3.46
	8.80	4.22	3.76	3.30	2.94	3.47
C		12.03	11.55	12.01	16.06	10.70
		12.10	11.62	11.85	15.63	11.43
D		12.00	11.98	11.34	14.62	10.10
		12.06	11.79	11.35	15.83	10.00
	CAP concentration observed (μg/kg) in kidney					
A	5.00	0.18	0.09	0.09	0.12	0.06
	5.11	0.12	0.09	0.09	0.12	
B	5.93	0.55	0.40	0.38	0.36	0.40
	5.98	0.54	0.41	0.40	0.38	0.42
C		4.45	5.42	6.12	5.69	3.88
		4.54	5.42	6.15	5.83	3.89
D		4.33	5.55	6.05	5.84	3.68
		4.38	5.72	6.15	5.88	3.67

[a] Sample preparations:
A: Tissue was ground after sampling and frozen at −20°C.
B: Tissue was ground after sampling and then 1 mL of PB was added to 5 g tissue and frozen at −20°C.
C: Tissue was cut into cubes (8 cm^3) and frozen.
D: Tissue was cut into cubes and frozen, and PB was used in the sample assay.

grinding the sample destroyed cells and increased contact between active enzymes and CAP. If thin slices of liver were added to 1 mL buffer solution with CAP for the stability assay, CAP stability was quite good and no degradation was observed (10).

This study shows the difficulty of the recovery concept in analysis for elimination organs such as liver and kidney. Their enzymatic material is important and must be studied by the analyst for the concept of techniques using spiked samples for concentration measurement.

To analyze kidney or liver with our method, information on recovery, linearity, and variability may be obtained by using PB for fortification of samples. For routine analysis of treated animal liver or kidney, samples may be stored as tissue cubes at −20°C, and PB need not be used to measure CAP concentrations.

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Liquid Chromatographic Method for Multiresidue Determination of Benzimidazoles in Beef Liver and Muscle: Collaborative Study

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A liquid chromatographic method for determination of thiabendazole, 5-hydroxythiabendazole, oxfendazole, mebendazole (MBZ), and fenbendazole (FBZ) in cattle liver and muscle was collaboratively studied in 7 laboratories in 1986. For blind fortified samples containing 800 ppb FBZ, average recovery and relative standard deviations for repeatability and reproducibility (RSD_r and RSD_R) based on results from 6 of the participating laboratories were 83%, 12.7%, and 14.0%, respectively. Recoveries of FBZ from incurred liver samples were more variable. Recoveries of MBZ from livers fortified at the 100 ppb level were encouraging; however, the drug levels were too low in the incurred samples used for MBZ studies. Except for FBZ and MBZ in liver, the study data were not satisfactory. The method has been adopted official first action by AOAC for determination of 800–1600 ppb fenbendazole in liver. The analysis should be repeated using a smaller sample size when initial analyses show levels greater than 1600 ppb FBZ.

Local veterinarians claim that benzimidazoles are widely used as anthelmintics in horses, sheep, cattle, and swine. The U.S. Food and Drug Administration, which funded this work, is interested in the development of multiresidue methods for separation and determination of these drugs in animal tissues. Such procedures would be useful for monitoring misuse of these substances. Techniques that can be used to determine individual benzimidazoles include thin-layer chromatography (1), fluorometry (2), and gas chromatography (3). Bogan and Marriner (4) demonstrated that several of the benzimidazole drugs in body fluids could be resolved by reverse-phase liquid chromatography (LC).

LeVan developed an LC method for determination of fenbendazole (FBZ), oxfendazole (OFZ), thiabendazole (TBZ), 5-hydroxythiabendazole (5-OH-TBZ), and mebendazole (MBZ) in cattle liver and muscle. The method exploits the weakly basic nature of the target benzimidazoles for effective extraction and cleanup. Tissues are made basic with Na_2CO_3, and then are homogenized in ethyl acetate. The ethyl acetate is evaporated to dryness and the residue is partitioned between phosphoric acid and hexane. The acid fraction containing the benzimidazoles is neutralized and then extracted with ethyl acetate. The ethyl acetate is evaporated and the residue is dissolved in methylene chloride and applied to a silica microcolumn. The benzimidazoles are eluted with methanol–methylene chloride (1 + 3). The eluate is evaporated and the residue is dissolved in mobile phase for injection onto the LC column. The target benzimidazoles are separated and quantitated by absorbance at 298 nm.

The present report describes a collaborative study of the method. The target sensitivity was 100 ppb for all of the drugs except FBZ, for which sensitivity was 800 ppb. A modification of the method for determination of 10–30 ppb of several benzimidazoles in milk was reported earlier (5).

Preliminary Studies

Exhaustive-Extraction Study

Exhaustive extraction experiments were performed prior to the collaborative study with liver samples from the animals dosed with TBZ and FBZ; data were obtained for TBZ, OFZ, and FBZ for these tissues. The liver samples were homogenized and extracted in the usual way (2 successive portions of ethyl acetate). The combined first and second extracts were cleaned up and analyzed by liquid chromatography. For exhaustive extraction, the liver homogenates were extracted with 2 additional portions of ethyl acetate (i.e., third and fourth extracts). The third and fourth extracts were combined, cleaned up, and analyzed in the same manner as the combined first and second extracts.

Data obtained from the study on TBZ-dosed liver indicate that the combined first and second extracts contained 0.77 ppm TBZ (based on original tissue), and the combined third and fourth extracts contained <0.02 ppm (based on original tissue). Thus, more than 97% of the extractable TBZ residue was obtained in the first and second ethyl acetate extracts. Similar data were obtained for OFZ and FBZ extracted from the FBZ-dosed liver. For OFZ, 0.74 ppm was obtained in the combined first and second extracts; the combined third and fourth extracts contained <0.02 ppm. For FBZ, 0.72 ppm (tissue basis) was obtained in the first 2 extracts; the third and fourth extracts contained <0.02 ppm.

Potential Drug Interferences

The following drugs were studied to determine the extent to which they might interfere in the multiresidue procedure: amprolium, chloramphenicol, chlortetracycline, erythromycin, levamisole, morantel, oxytetracycline, phenothiazine, sulfadimethoxine, sulfamethazine, and sulfaquinoxaline. LC analysis of the drug standards indicated that only chloram-

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Table 1. Composition of fortification solutions in methanol

Drug	Concentration, μg/mL A	B	C
5-Hydroxythiabendazole	1.0	2.5	0.5
Thiabendazole	0.5	1.0	2.5
Oxfendazole	0.5	2.5	1.0
Mebendazole	1.0	0.5	2.5
Fenbendazole	8.0	4.0	16.0

phenicol and phenothiazine were chromatographically similar to the target benzimidazoles. Chloramphenicol eluted just before 5-OH-TBZ, and the retention time for phenothiazine is similar to that of FBZ. When added to liver, only chloramphenicol appeared in the final chromatogram. The resolution of chloramphenicol from 5-OH-TBZ is such that it does not represent a serious interference.

Collaborative Study

Seven laboratories participated in the collaborative study. Each laboratory was asked to analyze 36 blind samples that consisted of triplicates of 12 different samples: 9 fortified muscle, 9 fortified liver, 9 incurred liver, 6 incurred muscle, and 3 control muscle samples. To evaluate interferences in control liver, the results from selected incurred samples were examined. For example, the results from the analyses of samples containing incurred TBZ and 5-OH-TBZ were examined as controls for OFZ and FBZ.

To preserve the "blind" nature of the study, control, fortified, and incurred samples were made indistinguishable. Tissue samples containing incurred drug residues at up to 4 times the legal tolerance levels were used "as is," rather than blended with control tissues to provide samples with drug residues at tolerance levels. Hazleton Laboratories America, Inc., supplied preweighted 10 g samples in glass scintillation vials, which were labeled only with code numbers. A companion vial with the same code number containing 1 mL fortification solution was supplied with each tissue sample. The Teflon-lined screw-cap vial contained methanol for control and incurred samples, and drugs dissolved in methanol for fortified samples. To eliminate concern over the instability of fortified samples during shipment or storage, collaborators were asked to add the contents of the companion vial to the sample at the beginning of the analysis. Table 1 lists the drug levels in the vials for the fortified samples.

Note: Results returned by the collaborators for determination of the benzimidazoles in liver and muscle tissues indicated that the method did not perform as well in their laboratories as it did earlier in LeVan's laboratory. The collaborative study data showed satisfactory quantitation of ≥800 ppb FBZ in cattle liver and acceptable recoveries of MBZ from liver samples fortified at 100 ppb. The method was adopted only for determination of 800–1600 ppb FBZ in liver. The *additional* materials and procedures used to perform the multiresidue determination during the study follow the adopted method.

991.17 Fenbendazole in Beef Liver

Liquid Chromatographic Method

First Action 1991

(Applicable to determination of 800–1600 ppb fenbendazole in beef liver)

Method Performance:

800 ppb fenbendazole

s_r = 84.00; s_R = 92.47; RSD_r = 12.68%; RSD_R = 13.95%

A. Principle

Tissues are made basic with Na_2CO_3 and homogenized in ethyl acetate. Ethyl acetate is evaporated to dryness and residue is partitioned between H_3PO_4 and hexane. Acid fraction, which contains fenbendazole, is neutralized and extracted with ethyl acetate. Ethyl acetate is evaporated and residue is dissolved in CH_2Cl_2 and passed through a silica microcolumn with CH_3OH–CH_2Cl_2 as mobile phase. Eluate is evaporated and residue is dissolved in mobile phase for injection onto LC column. Fenbendazole is quantitated at 298 nm.

B. Apparatus

(a) *Liquid chromatograph.*—With automatic sample injector using 50 μL sample loop, variable wavelength UV detector capable of operation at 298 nm, and dual pen recorder operated at 10 mV and 20 mV full scale and 0.2 cm/min chart speed [Perkin-Elmer Corp. Series 10 pump and Model ISS-100 injector and Kratos Analytical Instruments (Ramsey, NJ 07446) Model SF773 UV detector meet these specifications].

(b) *Column.*—25 × 0.46 cm id, C-18, 5 μm (Alltech Associates meets these specifications).

(c) *Guard column.*—3 × 0.46 cm id [Brownlee RP-18 Spheri-10 MPLC guard cartridge (Anspec Co., Ann Arbor, MI 48107) meets this specification].

(d) *Disposable silica columns.*—2.8 mL. 500 mg, 40 μm particle size, and 60 angstrom pore size [Bond Elute®, No. 1210-2037 (Analytichem International, Harbor City, CA 90710) meets these specifications].

(e) *Filters.*—0.2 μm [Acrodisc® CR No. 4405 (Gelman Scientific, Inc.) meets this specification].

(f) *Rotary evaporator.*—With water bath set at 30–35° (Buchi Rotavapor R-110 meets these specifications).

(g) *Blender.*—With explosion-proof motor (Waring® laboratory blender with explosion-proof motor meets this specification).

(h) *Blender cup.*—Glass, 500 mL capacity.

(i) *pH meter.*—Capable of ±1400 mV with accuracy of ±7 mV (Beckman Zeromatic® IV meets these specifications).

(j) *Vortex mixer.*

C. Reagents

(a) *Solvents.*—Ethyl acetate, hexane, methylene chloride, and methanol (distilled in glass); anhydrous ethyl alcohol, USP grade; and dimethyl sulfoxide.

(b) *Fenbendazole standard.*—Analytical reference grade.

(c) *Butylated hydroxytoluene (BHT) solution.*—10 mg/mL. Dissolve 1 g BHT (Sigma Chemical Co. or equivalent) in 100 mL ethyl acetate.

(d) *Sodium sulfate.*—Anhydrous, granular, reagent grade.

(e) *Sodium carbonate solution.*—1M. Dissolve 106 g anhydrous reagent grade Na_2CO_3 in 1 L H_2O.

(f) *Phosphoric acid solution.*—1M. Dilute 68.3 mL reagent grade H_3PO_4 to 1 L with water.

(g) *Potassium hydroxide solution.*—10N. Dissolve 560 g reagent grade KOH pellets in water and dilute to 1 L. (*Cau-*

tion: See safety note on potassium hydroxide.) Keep solution tightly closed.

(**h**) *LC mobile phase buffer.*—0.01M, pH 7.0. Dissolve 1.15 g reagent grade ammonium phosphate monobasic in ca 950 mL H_2O, adjust pH to 7.0 with dilute NH_4OH, and dilute to final volume of 1 L. Prepare just before use.

(**i**) *LC mobile phase.*—Methanol–ammonium phosphate buffer prepared in 2 different proportions (70 + 30, 53 + 47) from appropriate volumes of CH_3OH and pH 7 buffer. Pass through 0.45 μm filter before use.

D. Preparation of Standard Solutions

(**a**) *Stock solution.*—1 mg/mL. Prepare by dissolving 10 mg fenbendazole, **C(b)**, in 10 mL dimethyl sulfoxide.

(**b**) *Intermediate solution.*—10 μg/mL. Dilute 1 mL stock solution to 100 mL with methanol.

(**c**) *Working solutions.*—0.25, 0.5, 1.0, 2.0, and 4.0 μg/mL. Add 0.25, 0.5, 1.0, 2.0, and 4.0 mL portions of intermediate solution to separate 10 mL volumetric flasks. Evaporate to dryness under nitrogen; then dilute to volume with mobile phase (53 + 47). Solutions were equivalent to 25, 50, 100, 200, and 400 ppb and in 10 g tissue. Inject 50 μL or other selected volume of working solutions and, by regression analysis, establish relationship between concentration of solution injected, μg/mL and peak heights or areas for each solution.

E. Sample Homogenization and Extraction

Weigh 10 g portion of representative minced tissue into blender cup. Add 5 mL 1M Na_2CO_3 and 150 mL ethyl acetate. Add 1 mL BHT solution (10 mg). Blend 5 min at high speed, add 80 g anhydrous Na_2SO_4, and blend 1 min at low speed. Decant ethyl acetate into 500 mL round-bottom flask through funnel fitted with No. 41 filter paper. Let ground tissue and Na_2SO_4 settle briefly before pouring, in order not to plug filter paper. Use metal spatula to break up any tissue collar in blender cup. Add 150 mL ethyl acetate to tissue in blender cup and blend 2–3 min at low speed. Filter ethyl acetate solution into round-bottom flask, rinse filter paper with ca 10 mL absolute ethanol, and evaporate to dryness on rotary evaporator. (*Caution:* Watch for bumping.)

F. Solvent Partitioning Cleanup

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

Drain lower aqueous phase into 100 mL beaker. Adjust pH to 8.5 ± 1.0 by slowly adding, with stirring, ca 9 mL 10N KOH. Keep beaker in ice or cold water bath during neutralization. Check pH with meter or indicator paper. Transfer beaker contents to 250 mL separatory funnel and rinse beaker with total of 50 mL ethyl acetate, adding rinse to separatory funnel. Extract by shaking vigorously 2 min; let phases separate. Then drain lower aqueous layer into 100 mL beaker. Plug neck of a funnel with glass wool and add ca 40 g Na_2SO_4. Drain ethyl acetate through glass funnel and into 250 mL round-bottom flask. Pour beaker contents back into separatory funnel and re-extract with another 50 mL ethyl acetate. Discard lower aqueous layer and drain ethyl acetate through funnel containing Na_2SO_4 into same 250 mL round-bottom flask. Rinse Na_2SO_4 with 25 mL ethyl acetate, and add rinse to round-bottom flask. Add 0.2 mL BHT solution (2 mg) to round-bottom flask and evaporate to dryness on rotary evaporator. (*Caution:* Watch for bumping.)

G. Silica Column Cleanup

Add 3 mL CH_2Cl_2 to round-bottom sample flask and swirl to dissolve residue. Condition disposable silica column, **B(d)**, with 2 mL CH_2Cl_2. Perform following operations by gravity flow. Apply sample to conditioned column. Rinse sample flask twice with 3 mL portions of CH_2Cl_2, and apply each rinse to column. Wash column with 5 mL CH_2Cl_2, and discard wash. Elute fenbendazole with 5 mL 25% methanol in CH_2Cl_2, and collect eluate in 10 or 15 mL conical tube. Evaporate eluate to dryness under nitrogen, and reconstitute in 1.0 mL mobile phase. Mix on vortex mixer to dissolve residue; then filter through 0.2 μm filter into LC injection vial.

H. Determination

With detector set at 298 nm and 0.02 AFS sensitivity, inject 50 μL sample into LC system, using 70 + 30 mobile phase, **C(i)**, at 1 mL/min flow rate. Approximate retention volume of fenbendazole is 14 mL.

Calculate μg fenbendazole/mL in LC sample from equation developed with working standard solutions. For 10 g initial sample and 1 mL final sample volume:

$$\text{Fenbendazole (ppb) in tissue} = 100 \times \text{fenbendazole } (\mu g/mL) \text{ in LC sample}$$

Ref.: JAOAC **74,** May/June issue (1991).

CAS-43210-67-9 (fenbendazole)

Multiresidue Determination

In the study that resulted in adoption of the LC method only for determination of FBZ residues in liver, the collaborators used the following reagents and procedures in addition to those in the adopted method.

Solvent.—Acetone (distilled in glass).

Drug standards.—USP Reference Standards Mebendazole and Thiabendazole (U.S. Pharmacopeial Convention, Rockville, MD 20852); fenbendazole (gift of Hoechst-Roussel Pharmaceuticals, Inc.; Somerville, NJ 08876); oxfendazole (gift of Syntex Laboratories, Palo Alto, CA 94304); and 5-OH-thiabendazole (gift of Merck and Co., Inc., Rahway, NJ 07065).

LC mobile phase.—Methanol–ammonium phosphate buffer (40 + 60).

Standard solutions.—Individual 1 mg/mL stock solutions were prepared using 10 mg drug standard in 10 mL dimethyl sulfoxide for MBZ and OFZ and in 10 mL methanol for TBZ and 5-OH-TBZ.

Individual 10 μg/mL intermediate solutions were prepared by diluting 1 mL stock solution to 100 mL with methanol. A mixed standard intermediate solution containing 5-OH-TBZ, TBZ, OFZ, and MBZ was prepared at 10 μg/mL

Table 2. Collaborative results for LC determination of fenbendazole in liver (ppb)

Sample	Laboratory 1	2	3	4	5	6	7
Control liver[a]	29	21	(156)	0	4	24	0
Fortified liver							
400 ppb added	377	348	(41)	297	307	392	(325)
	375	348	(80)	304	356	327	(455)
	391	360	(226)	296	341	397	(165)
Lab. average	381	352	(116)	299	335	372	(315)
Overall average	310 ppb; CV = 33%						
Overall average[b]	348 ppb; CV = 10%						
800 ppb added	761	598	(76)	529	670	701	(631)
	614	631	(144)	566	695	677	(599)
	702	694	(28)	548	676	704	(932)
Lab. average	692	641	(83)	548	680	694	(721)
Overall average	580 ppb; CV = 39%						
Overall average[b]	663 ppb; CV = 14%						
	s_r = 84.00; s_R = 92.47;						
	RSD_r = 12.68%; RSD_R = 13.95%						
1600 ppb added	1248	654	(210)	1174	1286	1062	(722)
	1138	1368	(140)	1147	378	1072	—
	2075	1040	(199)	1133	1230	1056	(670)
Lab. average	1487	1021	(183)	1151	1258	1063	(696)
Overall average	950 ppb; CV = 50%						
Overall average[b]	1137 ppb; CV = 32%						
Dosed liver	771	728	(0)	574	712	674	(674)
	748	707	(72)	446	562	765	(740)
	665	724	(83)	540	771	755	(0)
Lab. average	728	720	(52)	520	682	731	(471)
Overall average	558 ppb; CV = 49%						
Overall average[b]	676 ppb; CV = 15%						

[a] From animals dosed with TBZ or MBZ.
[b] Values in parentheses not included.

Table 3. Collaborative results for LC determination of mebendazole in liver (ppb)

Sample	Laboratory 1	2	3	4	5	6	7
Control[a]	12	0	23	12	0	4	3
Overall average	8 ppb						
Fortified liver							
50 ppb added	32	44	(0)	34	39	45	(27)
	66	36	(12)	42	43	42	(22)
	48	43	(41)	37	44	49	(33)
Lab. average	49	41	(18)	38	42	45	(27)
Overall average	37 ppb; CV = 37%						
Overall average[b]	43 ppb; CV = 19%						
100 ppb added	99	84	(0)	68	85	117	(58)
	94	72	(17)	78	86	84	(57)
	82	86	(0)	71	86	89	(64)
Lab. average	92	81	(6)	72	86	97	(60)
Overall average	70 ppb; CV = 43%						
Overall average[b]	85 ppb; CV = 14%						
250 ppb added	205	186	(0)	193	198	169	(11)
	173	187	(17)	183	—	158	—
	204	178	(32)	182	191	179	(185)
Lab. average	194	184	(16)	186	194	168	(98)
Overall average	149 ppb; CV = 48%						
Overall average[b]	185 ppb; CV = 7%						
Dosed liver	34	36	(0)	30	—	32	(36)
	40	30	(51)	27	34	—	—
	30	29	(51)	26	33	30	(36)
Lab. average	35	31	(34)	29	33	31	(36)
Overall average	32 ppb; CV = 33%						
Overall average[b]	32 ppb; CV = 12%						

[a] From animals dosed with TBZ or FBZ.
[b] Values in parentheses not included.

by combining 1 mL of each stock solution in a 100 mL volumetric flask and diluting to volume with methanol.

Mixed standard working solutions containing 5-OH-TBZ, TBZ, OFZ, and MBZ were prepared as described for FBZ working solutions (Method, **D**(c)), and the concentration–response relationship for each standard was established as for FBZ.

Homogenization and extraction.—Muscle tissue was prepared as described for liver, except acetone was used for the second tissue extraction step.

Silica column cleanup.—All benzimidazoles are eluted by the 5 mL 25% methanol in CH_2Cl_2 eluant.

Determination.—Because of the wide polarity range of the benzimidazoles, it is not practical to determine all of the target compounds in a single LC run. The scheme outlined provides a rapid means of screening sample extracts by using different isocratic mobile phases in succession as an alternative to gradient elution. Fenbendazole is determined first with the strongest mobile phase (70% methanol); the remaining compounds are determined in a subsequent run with a weaker mobile phase (53% methanol). Even with the latter, 5-OH-TBZ elutes near the solvent front and it may be necessary to use a still weaker mobile phase (40% methanol) to obtain adequate resolution of 5-OH-TBZ from background peaks.

5-OH-TBZ, OFZ, TBZ, and MBZ: Inject 50 μL of sample into the LC system, using the 53 + 47 mobile phase at 1 mL/min flow rate. Set the detector at 298 nm and 0.02 AFS sensitivity. Approximate retention volumes are 5-OH-TBZ, 5 mL; OFZ, 8.5 mL; TBZ, 10 mL; and MBZ, 20 mL.

Supplemental determination of 5-OH-TBZ: If the peak tentatively identified as 5-OH-TBZ is insufficiently resolved from the background, perform a supplemental determination by reinjecting 50 μL of sample into the LC system, using the 40 + 60 mobile phase at 1 mL/min. Change the detector wavelength to 318 nm.

Calculation.—Calculate each benzimidazole, μg/mL, as for FBZ calculation (Method, **H**).

Results

Detailed recovery data are presented in Tables 2 and 3 for FBZ and MBZ, respectively, in cattle liver. Table 4 contains summary data for recovery of FBZ from muscle, OFZ from muscle and liver, TBZ from liver and muscle, and 5-OH-TBZ from liver and muscle. The results in the tables are average results for each laboratory for each fortification level or incurred drug level. Overall averages for each sample are also shown. Estimates of the interlaboratory percent coefficients of variation (CV) were calculated by simply using all of the recovery data from the various laboratories. Values shown in parentheses were calculated after eliminating the individual recovery values (not laboratory averages) that are in parentheses.

Two of the 7 laboratories frequently obtained poorer results than did the other 5 laboratories. Overall averages and CV values frequently improved when data from laboratories 3 and 7 were not included. For recovery of fenbendazole

Table 4. Collaborative results for LC determination of benzimidazole in tissues (ppb)

Sample	Laboratory 1	2	3	4	5	6	7
FBZ in muscle							
Control[a]	53	12	168	5	0	1	63
Fortified							
400 ppb added	294	335	(208)	274	269	301	(17)
Overall av.	241 ppb; CV = 46%						
Overall av.[b]	296 ppb; CV = 10%						
800 ppb added	728	595	(96)	620	582	644	(173)
Overall av.	491 ppb; CV = 51%						
Overall av.[b]	634 ppb; CV = 13%						
1600 ppb added	901	680	(34)	1021	849	988	—
Overall av.	721 ppb; CV = 54%						
Overall av.[b]	888 ppb; CV = 24%						
Dosed	195	181	(238)	174	162	198	(0)
Overall av.	164 ppb; CV = 53%						
Overall av.[b]	182 ppb; CV = 16%						
MBZ in muscle							
Controls	3	0	42	0	0	0	0
Fortified							
50 ppb added	43	43	(53)	81	39	38	(13)
Overall av.	42 ppb; CV = 59%						
Overall av.[b]	46 ppb; CV = 46%						
100 ppb added	77	84	(26)	86	78	85	(46)
Overall av.	69 ppb; CV = 35%						
Overall av.[b]	82 ppb; CV = 12%						
250 ppb added	164	158	(12)	156	137	184	(115)
Overall av.	130 ppb; CV = 44%						
Overall av.[b]	158 ppb; CV = 13%						
TBZ in liver							
Controls	33	9	11	30	10	0	0
Fortified							
50 ppb added	47	46	(0)	28	48	50	(28)
Overall av.	35 ppb; CV = 52%						
Overall av.[b]	43 ppb; CV = 25%						
100 ppb added	97	82	(6)	67	85	86	(73)
Overall av.	71 ppb; CV = 43%						
Overall av.[b]	84 ppb; CV = 16%						
250 ppb added	196	210	(25)	174	190	170	—
Overall av.	159 ppb; CV = 42%						
Overall av.[b]	188 ppb; CV = 10%						
Dosed	164	177	(32)	118	157	200	(101)
Overall av.	139 ppb; CV = 46%						
Overall av.[b]	163 ppb; CV = 24%						
TBZ in muscle							
Control	17	0	62	1	0	1	0
Overall av.	12 ppb						
Fortified							
50 ppb added	51	49	(66)	48	38	37	(19)
Overall av.	44 ppb; CV = 59%						
Overall av.[b]	44 ppb; CV = 19%						
100 ppb added	84	52	(63)	81	60	82	(24)
Overall av.	63 ppb; CV = 48%						
Overall av.[b]	71 ppb; CV = 32%						
250 ppb added	182	204	(33)	158	110	138	(106)
Overall av.	134 ppb; CV = 44%						
Overall av.[b]	162 ppb; CV = 24%						
Dosed	27	0	21	7	0	3	0

Sample	Laboratory 1	2	3	4	5	6	7
5-OH-TBZ in liver							
Control	0	10	21	33	4	10	43
Fortified							
50 ppb added	40	16	(42)	26	38	32	(70)
Overall av.	36 ppb; CV = 68%						
Overall av.[b]	30 ppb; CV = 50%						
100 ppb added	86	13	(0)	129	78	88	(37)
Overall av.	62 ppb; CV = 80%						
Overall av.[b]	79 ppb; CV = 59%						
250 ppb added	235	15	(19)	162	183	206	(139)
Overall av.	137 ppb; CV = 62%						
Overall av.[b]	160 ppb; CV = 51%						
Dosed	734	497	(0)	679	829	590	(8)
Overall av.	495 ppb; CV = 67%						
Overall av.[b]	671 ppb; CV = 23%						
5-OH-TBZ in muscle							
Control	12	0	45	3	0	2	0
Fortified							
50 ppb added	40	(10)	76	50	35	10	(83)
Overall av.[b]	31 ppb; CV = 73%						
100 ppb added	80	33	(3810)	101	53	60	(105)
Overall av.[b]	65 ppb; CV = 51%						
250 ppb added	185	128	(19)	164	100	195	(61)
Overall av.[b]	61 ppb; CV = 29%						
Dosed	107	76	(45)	72	62	76	(33)
Overall av.[b]	76 ppb; CV = 32%						
OFZ in muscle							
Control	0	2	80	0	0	0	65
Fortified							
50 ppb added	64	74	68	65	72	88	(4)
Overall av.	58 ppb; CV = 64%						
100 ppb added	91	108	10	79	82	149	(8)
Overall av.	79 ppb; CV = 72%						
Overall av.[b]	103 ppb; CV = 43%						
250 ppb added	196	193	(78)	225	173	296	(25)
Overall av.	164 ppb; CV = 50%						
Overall av.[b]	213 ppb; CV = 18%						
Dosed	313	232	87	253	249	317	148
Overall av.	232 ppb; CV = 37%						
Overall av.[b]	273 ppb; CV = 17%						
OFZ in liver							
Control	50	0	48	16	4	0	55
Fortified							
50 ppb added	56	52	(160)	38	52	60	(29)
Overall av.	64 ppb; CV = 87%						
Overall av.[b]	51 ppb; CV = 21%						
100 ppb added	107	(86)	145	84	94	87	(25)
Overall av.	90 ppb; CV = 60%						
Overall av.[b]	87 ppb; CV = 22%						
250 ppb added	254	198	49	165	208	254	(104)
Overall av.	176 ppb; CV = 43%						
Overall av.[b]	216 ppb; CV = 20%						
Dosed	138	131	65	60	92	160	(23)
Overall av.	96 ppb; CV = 55%						
Overall av.[b]	116 ppb; CV = 33%						

[a] From animals dosed with TBZ or FBZ.
[b] Values in parentheses not included.

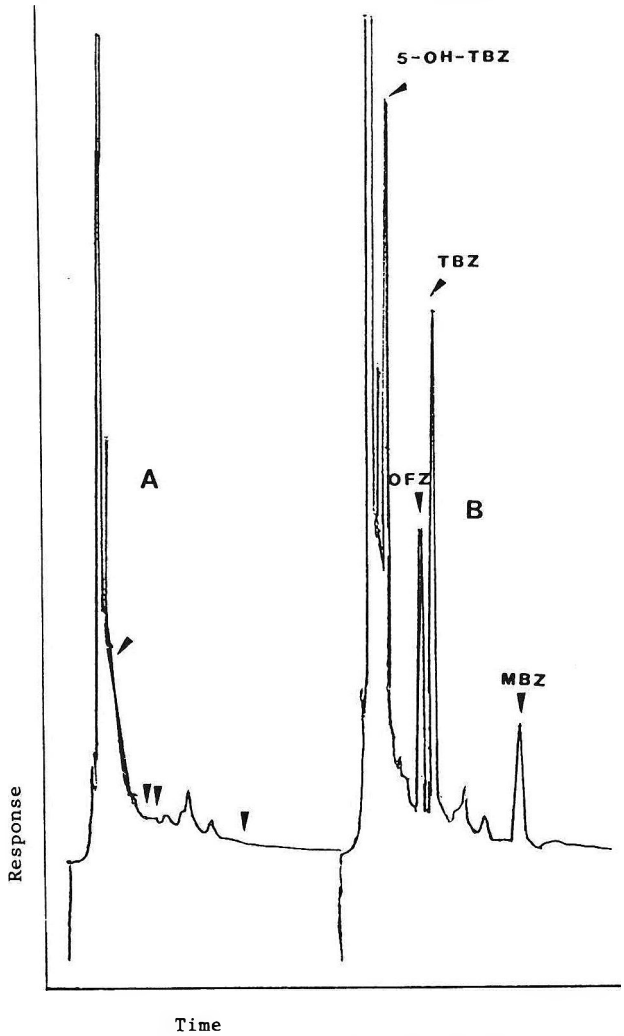

Figure 1. LC chromatograms obtained using 53 + 47 mobile phase. (A) control swine liver and (B) liver fortified with 100 ppb FBZ, OFZ, TBZ, 5-OH-TBZ, and MBZ.

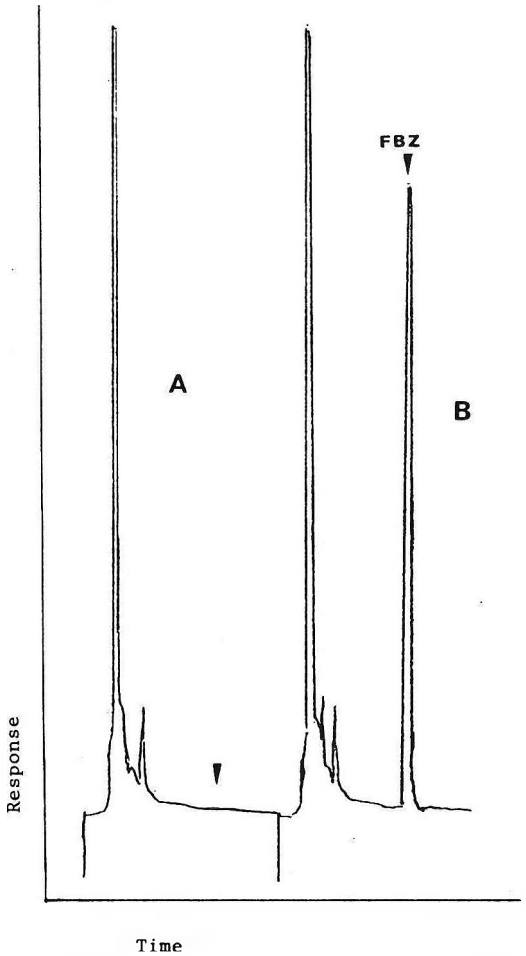

Figure 2. LC chromatograms obtained using 70 + 30 mobile phase. (A) control swine liver and (B) liver fortified with 800 ppb FBZ.

added at the 800 ppb level to liver, application of the Dixon outlier test (6) indicated that data from laboratory 3 could be eliminated (A. B. Nevius, Statistician, Center for Veterinary Medicine, Food and Drug Administration). The R value, 0.7296, was greater than 0.680; therefore, the risk of unjust rejection is less than 0.005 or 0.5%. Excluding data from laboratory 3, s_r, s_R, RSD_r, and RSD_R in Table 2 were calculated. Similar values for the data in Tables 3 and 4 were not calculated.

Recoveries of MBZ from liver fortified at 100 ppb look promising, provided data from laboratories 3 and 7 are eliminated. Because the levels of MBZ in the incurred samples were substantially below the 100 ppb target sensitivity, we do not recommend this method for determination of MBZ in liver. The method did not perform well at the target sensitivities for OFZ, TBZ, and 5-OH-TBZ in cattle muscle or liver, or for FBZ in cattle muscle.

Occurrences of missing data were generally caused by investigators reporting that the recorders reached a maximum or that some of the solvent had leaked from the fortification vials. If the leaking solvent contained no drug, as was the case for control and incurred tissues, we retained the value.

Recovery values obtained by the collaborators for incurred samples of 5-OH-TBZ in liver, OFZ and FBZ in liver, and FBZ in liver and muscle differed substantially from the values obtained before the samples were distributed to the laboratories. We accepted the average values from the collaborative study as the best estimates of the true drug levels in the incurred samples.

Figure 1 shows chromatograms generated by LeVan for controls and controls fortified at the 100 ppb level with benzimidazoles. The 53 + 47 mobile phase was used. Figure 2 shows similar chromatograms for FBZ with the 70 + 30 mobile phase.

Recommendation

Recoveries of FBZ from liver fortified at the 800 ppb level average 83% if the results from laboratory 3 are omitted. The General Referee recommends that this method be adopted official first action for determining FBZ in cattle liver at 800 ppb and above. The FDA established tolerance for parent FBZ in cattle liver is 800 ppm.

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Matrix Solid Phase Dispersion (MSPD) Extraction and Gas Chromatographic Screening of Nine Chlorinated Pesticides in Beef Fat

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A multiresidue technique is presented for the extraction and quantitative gas chromatographic screening of 9 insecticides (lindane, heptachlor, aldrin, heptachlor epoxide, *p,p'*-DDE, dieldrin, endrin, *p,p'*-TDE, and *p,p'*-DDT) as residues in beef fat. Beef fat was fortified by adding the 9 insecticides, plus dibutyl chlorendate as internal standard, to 0.5 g portions of beef fat and blending with 2 g C_{18} (octadecylsilyl)-derivatized silica. The C_{18}/fat matrix blend was fashioned into a column by adding the blend to a 10 mL syringe barrel containing 2 g activated Florisil. The insecticides were then eluted from the column with 8 mL acetonitrile, and a 2 μL portion of the acetonitrile eluate was then directly analyzed by gas chromatography with electron capture detection. Unfortified blank controls were treated similarly. The acetonitrile eluate contained all of the pesticide analytes (31.25–500 ng/g) and was free of interfering co-extractants. Correlation coefficients for the 9 extracted pesticide standard curves (linear regression analysis, n = 5) ranged from 0.9969 (±0.0021) to 0.9999 (±0.0001). Average relative percentage recoveries (85 ± 3.4% to 102 ± 5.0%, n = 25 for each insecticide), inter-assay variability (6.0 ± 1.0% to 14.0 ± 6.7%, n = 25 for each insecticide), and intra-assay variability (2.5–5.1%, n = 5 for each insecticide) indicated that the methodology is acceptable for the extraction, determination, and screening of these residues in beef fat.

Present-day agricultural practices require the use of many chemicals to increase crop yields. For example, pesticides are used routinely in integrated farm management programs to reduce possible losses due to insects. Unfortunately, the excessive use of chlorinated pesticides can have a negative impact on the environment because of their tendency to bioaccumulate. For this reason, many chlorinated pesticides, including (but not limited to) DDT, have been banned from use, or their use has been severely restricted in the United States. The presence of high levels of chlorinated pesticides in the food supply may have human health implications and has prompted the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA/FSIS) to include aldrin, endrin, dieldrin, heptachlor, heptachlor epoxide, TDE, DDE, DDT, and lindane, as well as other pesticides, in the Compound Evaluation and Analytical Capability National Program Plan of 1990 (1). Determination of chlorinated hydrocarbons is included for all domestic and imported species and production classes in this program plan.

Analytical methods used in pesticide residue monitoring programs (1) should be capable of detecting the residue below the action level (300 ng/g fat for lindane, heptachlor, heptachlor epoxide, dieldrin, and endrin; 7 μg/g fat for TDE, DDE, and DDT). Present instrumental detection capability is sufficient to meet these requirements. However, a major limiting factor in residue analysis is not the detection of the compounds of concern but the techniques used to extract the residues from a complex biological matrix such as fat. The extraction techniques used and mandated by governmental agencies (2, 3) can require large samples and large volumes of extracting solvents, back-washing and re-extraction, solvent evaporations, and residue enrichment by gel permeation chromatography to provide an extract containing pesticide residues suitable for analysis. Such multistep extraction procedures are time consuming and tedious to perform, which limits their usefulness for residue screening, and have gener-

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ally not kept pace with advances in analytical technology. The development of rapid and efficient extraction techniques that minimize time and expendable materials, especially solvents, could enhance residue monitoring protocols. Such rapid extraction techniques, which provide extracts suitable for pesticide screening by appropriate analytical instrumentation, could greatly reduce the number of samples actually subjected to official confirmational extraction procedures. By screening samples in this manner, regulatory agencies could test more food samples for more pesticide residues.

Recently, we have developed an extraction technique, namely, matrix solid phase dispersion (MSPD), that simplifies the isolation of drug and chemical residues from biological matrixes (4–10). We report here the first use of the MSPD technique for the extraction of 9 chlorinated pesticides from beef fat followed by screening of the extracts using gas chromatography with electron capture detection.

Experimental

Reagents and Expendable Materials

(a) *Solvents.*—Obtained from Mallinckrodt, Inc., Paris, KY; ChromAR LC grade. Used without further purification.

(b) *Insecticides.*—Standard insecticides (lindane (58-89-9), heptachlor (76-44-8), aldrin (309-00-2), heptachlor epoxide (1024-57-3), *p,p'*-DDE (72-55-9), dieldrin (60-57-1), endrin (72-20-8), *p,p'*-TDE (72-54-8), and *p,p'*-DDT (50-29-3)) obtained from Supelco Inc., Bellefonte, PA (purity 99%).

(c) *Dibutyl chlorendate.*—QAT 798 02 02; supplied by the Environmental Protection Agency Quality Assurance Materials Bank.

(d) *Bulk C_{18} column packing.*—Octadecylsilyl-derivatized silica, 40 μm, 18% load, encapped (obtained from Analytichem Int., Harbor City, CA). The C_{18} was cleaned by taking a 50 mL ChemElut 1010 column that had been emptied and washed with hot soapy water, rinsed with distilled water, and air dried (Analytichem Int.), filling it with 22 g bulk C_{18}, and sequentially washing it with 2 column volumes each of hexane, dichloromethane (DCM), and methanol by vacuum aspiration until dry. The material was then stored in a brown glass bottle sealed with a screw cap.

(e) *Stock pesticide and internal standard solutions.*—1000 μg/mL. The solutions were prepared by dissolving pure pesticide standards in acetonitrile and diluting with acetonitrile to the appropriate concentration in μg/mL, except dibutyl chlorendate internal standard stock solution (1000 μg/mL), which was prepared by dissolving pure standard in acetonitrile. The stock solutions were admixed and then serially diluted with acetonitrile to make 3.13, 6.25, 12.5, 25, and 50 μg/mL mixed pesticide solutions containing 25 μg internal standard dibutyl chlorendate/mL in each mixture.

(f) *Florisil.*—PR 60/100 (Alltech Associates, Deerfield, IL), activated (1250°F) at the factory and stored in a sealed amber bottle until used.

(g) *Syringe barrels as extraction columns.*—10 mL syringe barrels (obtained from Becton Dickinson, Rutherford, NJ) were thoroughly washed with hot soapy water, rinsed with double distilled water, and air dried before use as columns for sample extraction. The syringe barrel bottom was plugged with a filter paper disc, (Whatman No. 1, 1.5 cm) and 2 g activated Florisil was placed into the column; the column head was covered with a filter paper disc, and the column contents were compressed to 3.5 mL.

(h) *Beef fat.*—The fat tissues were a composite from scrap beef fat trimmed from market meat. The exact nature and anatomical origin were not determined.

Gas Chromatography Apparatus

Gas chromatograph.—Varian Vista 6000 equipped with a DB-5 column, 25 m × 0.25 mm id, 0.2 mm coating (J & W Scientific). Column temperature program: 120°C for 2 min, increased at 10° min to 290°C, and held for 5 min. Splitless injection with purge function activated at 0.75 min post-injection was used. Injection port temperature, 200°C. Detector: electron capture, 300°C, −0.24 mV at 32 attenuation and 10 range sensitivity. Carrier gas: ultra-high purity nitrogen at a linear flow rate of 14 cm/s.

Preparation of Sample Extracts

Two g C_{18} packing was placed in a glass mortar, and 0.5 g of beef fat was put on top of the C_{18}. Standard pesticide mixtures (5 μL aliquots of 3.12, 6.25, 12.5, 25, and 50 μg/mL stock solutions containing dibutyl chlorendate internal standard, 25.0 μg/mL) were injected randomly into the fat, and the fortified samples were allowed to stand for 2 min. This fortification level resulted in a final concentration of 31.3, 62.5, 125, 250, and 500 ng of each pesticide/g fat. Blank control fat was prepared similarly, except that 5 μL acetonitrile containing no insecticides was injected into the fat. The fat was then gently blended into the C_{18} material with a glass pestle until a homogeneous mixture was observed (30 s). Alternatively, a fat sample was placed in the mortar, spiked, and allowed to equilibrate, and the C_{18} was added and then blended to give equivalent results.

The resultant homogeneous C_{18}/fat matrix blend was transferred into a previously prepared (10 mL syringe barrel that contained 2 g activated Florisil. Two Whatman No. 1 filter paper discs 1.5 cm in diameter were placed on the column head and the column was compressed to 7.5 mL with a syringe plunger from which the rubber end and pointed plastic portion had been removed. (The column cannot be over-compressed.) The tip of a 100 μL plastic pipet was placed on the column outlet to increase the residence time of the eluting solvents on the column.

Pesticides were eluted with 8 mL acetonitrile into a 10 mL conical screwthread disposable glass centrifuge tube (Kimble, Vineland, NJ). A steady flow of acetonitrile through the column was initiated by applying positive pressure to the column head with a pipet bulb. When the flow ceased, any acetonitrile remaining on the column was removed by applying positive pressure from the pipet bulb to the column head and collecting the acetonitrile in the centrifuge tube. A final extract volume of 5 mL was obtained. The tube was tightly capped, and the tube contents were thoroughly mixed by inverting the tube 3 times. A 2 μL portion of the extract was then directly analyzed by gas chromatography with electron capture detection.

Data Analysis

Standard curves were generated by plotting peak area ratios (PAR) of standard pesticides at each concentration vs internal standard dibutyl chlorendate in fortified sample extracts for the concentrations examined. A comparison of extracted fortified sample pesticide PAR's to PAR's of pure pesticide standards analyzed under identical conditions gave percentage recoveries. Inter-assay variability was determined in the following manner: The PAR's for 5 replicates of each

Table 1. Percentage recoveries, average percentage recovery, intra-(IAV) and inter-assay variability (IRV) percentages for lindane, heptachlor, aldrin, heptachlor epoxide, *p,p'*-DDE, dieldrin, endrin, *p,p'*-TDE, and *p,p'*-DDT isolated from fortified beef fat (concentration 31.3–500 ng/g)

	Recovery, %								
Concn, ng/g	Lindane	Heptachlor	Aldrin	Hept. Epoxide	*p,p'*-DDE	Dieldrin	Endrin	*p,p'*-TDE	*p,p'*-DDT
31.3	89 + 16	84 + 14	71 + 18	87 + 16	85 + 13	94 + 13	94 + 10	95 + 6	110 + 19
62.5	81 + 13	87 + 10	94 + 15	80 + 10	89 + 10	93 + 8	103 + 12	96 + 6	101 + 5
125	84 + 10	80 + 10	91 + 10	81 + 9	98 + 10	98 + 10	97 + 9	92 + 4	97 + 6
250	87 + 4	87 + 8	99 + 9	89 + 9	99 + 11	91 + 7	96 + 8	99 + 7	99 + 6
500	82 + 7	94 + 9	104 + 9	94 + 11	97 + 5	98 + 6	96 + 3	105 + 5	102 + 3
Av. % rec.	85 + 3	86 + 5	92 + 13	86 + 6	94 + 6	95 + 3	97 + 3	97 + 5	102 + 5
IAV %	4.1	5	5.1	4.8	3.7	3.1	3.6	2.5	2.5
IRV %	11 + 5	12 + 3	14 + 7	12 + 3	10 + 4	9 + 3	9 + 3	6 + 1	7 + 6
r[a]	0.9986 + 0.0014	0.9989 + 0.0011	0.9986 + 0.0014	0.9999 + 0.0001	0.9983 + 0.0012	0.9969 + 0.0021	0.9969 + 0.0028	0.9987 + 0.0009	0.9994 + 0.0006

[a] r = correlation coefficient from linear regression analysis.

concentration (31.3, 62.5, 125, 250, and 500 ng/mL, 2 μL injection volume) were averaged. From the resulting means ± standard deviations (SD), coefficients of variation (CV) were calculated, and the average standard deviation of the CVs for each concentration were determined as the inter-assay variability. Intra-assay variability was defined as the CV for the mean of 5 replicates of the same sample and represents the variability associated with the analytical instrumentation used.

Results and Discussion

The isolation of drug and chemical residues from a complex biological matrix such as fat can be tedious to perform. Multistep extraction procedures are often required to fractionate the residue(s) of interest away from the many cellular constituents that are present. Classical extraction methods (2, 3) consist of blending the sample with extracting solvents and sodium sulfate, centrifugations, back-washing of the extract, evaporation of relatively large volumes of solvents, gel permeation chromatography, and Florisil residue enrichment to isolate and purify pesticides present in a sample of fat. Such extraction protocols provide extracts suitable for analytical determinations but are time consuming to perform, making them unsuitable for routine screening purposes. Improved residue extraction techniques that are rapid, extract the target residue(s) with high recoveries, and are suitable for direct screening are ideal for the enhancement of residue monitoring programs. Samples obtained in this manner and screened by appropriate analytical techniques could allow regulatory agencies to test more foods for more residues and also reduce the number of samples that require the use of "official" extraction methods for confirmational purposes.

Methodology using the MSPD extraction and gas chromatography-electron capture detector screening of the 9 chlorinated pesticides examined in this report meet the requirements for such a rapid screening technique. In the MSPD approach, the fat sample is dispersed over a C_{18} covalently modified silica. The bound C_{18} acts as a fat dispersant due to its hydrophobic characteristics. The resulting dispersion of fat (0.5 g) over a large surface area (1000 m^2/2 g C_{18} theoretical) results in an efficient extraction (Table 1) of the compounds examined here when acetonitrile is used as the extracting solvent. A column fashioned from the C_{18}/tissue matrix blend in combination with a co-column of activated Florisil and eluted with acetonitrile produces an extract that has minimal interferences, as can be seen in GC chromatograms of blank control extracted with acetonitrile (Figure 1A) and acetonitrile-extracted fat samples fortified with pesticides (Figure 1B). The average relative percentage recoveries (85 ± 3.4 to 102 ± 5.0%) and intra-assay (2.5–5.1%) and interassay variability percent (6 ± 1.0 to 14 ± 6.7%) are indicative of a suitable method for the determination and screening of these 9 pesticides in beef fat (Table 1). Linear regression analyses and corresponding correlation coefficients indicate that the extraction technique is linear with respect to increasing concentrations for the pesticides examined when an electron capture detector is used.

Many of the theoretical aspects of the MSPD technique have previously been published (4–10). However, we propose that the absence of interfering peaks in the acetonitrile eluate is due to the combined effect of the MSPD extraction and the combination Florisil column used. The acetonitrile eluting solvent extracts pesticides from the dispersed fat without extracting large quantities of observable lipids, as determined from GC examinations of the extracts using a flame ionization detector (FID, data not shown). Elution of the MSPD column, prepared as described, with either hexane or ethyl acetate rather than acetonitrile produced large quantities of fats, as evidenced by the quantity of residue observed after solvent evaporation and by the number and intensity of peaks recorded by GC/FID analysis of the extracts. The acetonitrile eluate, however, contained very little residue after solvent evaporation. Furthermore, the contribution to interference by co-elution of lipids as well as other tissue components is reduced by the direct analysis of the sample in its 8 mL elution volume and the specificity of the EC detector. Nevertheless, the fat has a limited solubility in acetonitrile, and a greater affinity for the covalently bound hydrophobic C_{18}. Thus, the pesticides are partitioned out of the lipid without eluting the majority of the lipids from the column. The activated Florisil functions as an additional cleanup of the eluting acetonitrile. As the acetonitrile elutes from the C_{18}/fat blend portion of the column and passes through the activated Florisil, the activated Florisil removes water and residual fat present in the acetonitrile eluate. A 2 μL portion of the resultant clear acetonitrile eluate (31.3 ng of each pesticide/g tissue, diluted to a final volume of 5 mL) can then be directly analyzed without any additional concentration of the final extract volume, resulting in a minimal

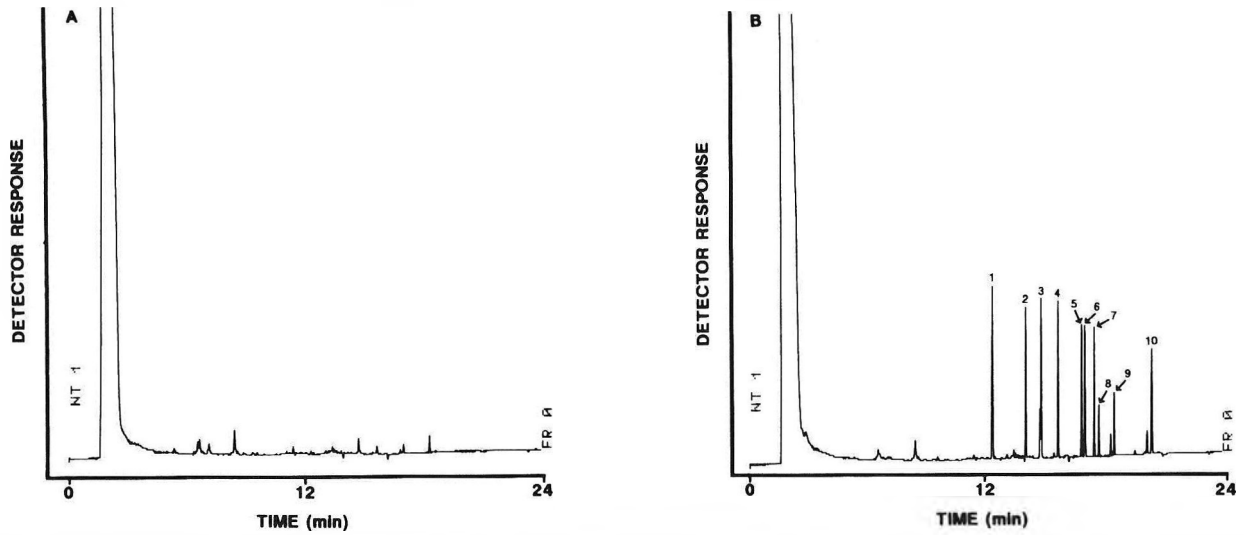

Figure 1. Representative gas chromatograms obtained from the electron capture detector analysis of the acetonitrile extract of blank control (A) and beef fat fortified with pesticide (B; 250 ng/mL, 2 μL injection volume). Peak identities are lindane (1), heptachlor (2), aldrin (3), heptachlor epoxide (4), *p,p'*-DDE (5), dieldrin (6), endrin (7), *p,p'*-TDE (8), *p,p'*-DDT (9), and dibutyl chlorendate (10).

detectable limit of 12.5 pg on-column. Because the extract is free from interferences, a high detector sensitivity can be used and pesticide levels (31.3 ng/g) well below the action levels are easily detected. The acetonitrile extracts are sufficiently clean that more than 100 injections have been made without observing any deterioration in sensitivity or chromatography, and without the need to change the injection port liner.

The results presented here are based on fortified samples, such as would be required and obtained for the preparation of standard curves or for conducting recovery studies for the qualitative analysis of pesticide residues in biological samples. Although the examination of beef fat tissues from incurred samples would be ideal, such samples were not available to us during this study, and this is outside the scope of the present methods development research.

The MSPD method outlined overcomes many of the complications associated with traditional pesticide isolation techniques because it uses small samples and small volumes of solvent, involves few steps, eliminates gel permeation chromatography procedures, and requires no complex chemical manipulations of the sample. The savings in terms of time, solvent requirements, and disposal costs and the minimal utilization of expendable materials make this method attractive compared to classical pesticide extraction techniques. The method as outlined may serve as a rapid screening technique for the 9 pesticides examined in beef fat. This approach has the potential for use in screening pesticides or other chemicals in different biological matrixes and may be useful in environmental biomonitoring programs as well.

Acknowledgments

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Determination of Penicillin G Residues in Edible Animal Tissues by Liquid Chromatography

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An improved method has been developed for the determination of benzyl penicillin in animal tissues. Tissues are fortified with a known amount of penicillin V (internal standard) and extracted with water. The extract is deproteinized with sulfuric acid and sodium tungstate, filtered, and concentrated on a conditioned C18 solid phase extraction column. Penicillin V and benzyl penicillin are then eluted from the column with 1 mL 60% acetonitrile–35% water–5% 0.2M phosphate buffer solution and derivatized with 1 mL 1,2,4-triazole-mercuric chloride solution at 65°C for 30 min. An aliquot of this sample is analyzed by reverse phase liquid chromatography with UV detection at 325 nm. The limit of detection is 5 μg/kg (ppb) penicillin G (8.4 IU/kg) in liver, kidney, and muscle tissues.

Penicillin G (benzyl penicillin), a β-lactam antibiotic usually administered in salt form with counter-ions of procaine, potassium, or sodium (Figure 1), is frequently used in veterinary medicine for the treatment of microbial infections. Because of the hypersensitivity of some individuals to penicillin G (1), a zero tolerance has been established in Canada for penicillin residues in edible animal tissues (2) to protect the consumer and maintain confidence in the meat and meat products of the Canadian livestock industry.

Tissue samples from suspect and positive Swab Test On Premises (STOP) (3) animals from Canadian abattoirs are tested for antibiotic residues by thin layer chromatography bioautography (TLCB) (4). This testing program has indicated that penicillins are the antibiotics most frequently detected. Although several bioassays have been developed for the detection of penicillin residues (5–8), it has been observed that TLCB, our laboratory confirmatory method for penicillin residues in tissues, does not distinguish among β-lactams (4).

Moats (9) reported a liquid chromatographic (LC) method for the determination of penicillin G in animal tissues which included a lengthy and cumbersome extraction procedure. Terada et al. (10) described an on-line trace enrichment LC method which required specialized chromatographic equipment. Both methods necessitated extensive sample cleanup because penicillin G was detected in the low UV wavelength range (208–220 nm). Precolumn derivatization reactions have been described for the LC analysis of penicillins in serum (11, 12), plasma (13), milk (14), and lymph (15) by which the mercuric mercaptides of penicillin can be detected at higher wavelengths (325–331 nm), where tissue co-extractives are more transparent.

This paper describes an improved method for the determination of penicillin G in animal tissues which combines a modified extraction procedure based on the method described by Terada et al. (10) with the precolumn derivatization reaction described by Haginaka and Wakai (11). This modified method uses phenoxymethyl penicillinic acid (penicillin V) as internal standard (Figure 1) and has a detection limit of 5 μg/kg. Data are presented in this paper to demonstrate the suitability of this method for routine diagnostic analyses. The method was applied to the determination of penicillin G residues in edible tissues of a Holstein steer calf that was injected with 15 000 IU procaine penicillin G/kg of body weight.

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METHOD

Apparatus

(a) *Polytron homogenizer.*—With 20 mm probe (Brinkmann Instruments, (Canada) Ltd., Rexdale, Ontario).

(b) *Filter papers.*—GF/B, 5.5 cm (Whatman Inc., Clifton, NJ) and a 5.8 cm Buchner funnel.

(c) *Solid phase extraction cartridges.*—6 mL (500 mg) BondElut C18 (Analytichem International Inc., Harbor City, CA).

(d) *Mechanical shaker.*—Eberbach flat bed.

(e) *Liquid chromatograph.*—Waters 501 pump, 712 autosampler, and Nova-Pak C18 column (4 μm; 3.9 × 150 mm) (Waters Chromatography Division, Ontario, Canada); Kratos 783 variable wavelength UV detector (Kratos Analytical, Ramsey, NJ). Data acquisition, the operation of the pump, and injector were controlled by a Waters 820 Chromatography Workstation.

Reagents

(a) *Water.*—Obtained from a Barnstead RO/Nanopure ultrafiltration unit.

(b) *Derivatizing reagent.*—2M 1,2,4-triazole containing 0.001M mercuric chloride. Weigh 34.45 g 1,2,4-triazole (Aldrich, Milwaukee, WI) into a 400 mL beaker, add 150 mL water, and stir with a magnetic spinbar to dissolve. Add 25

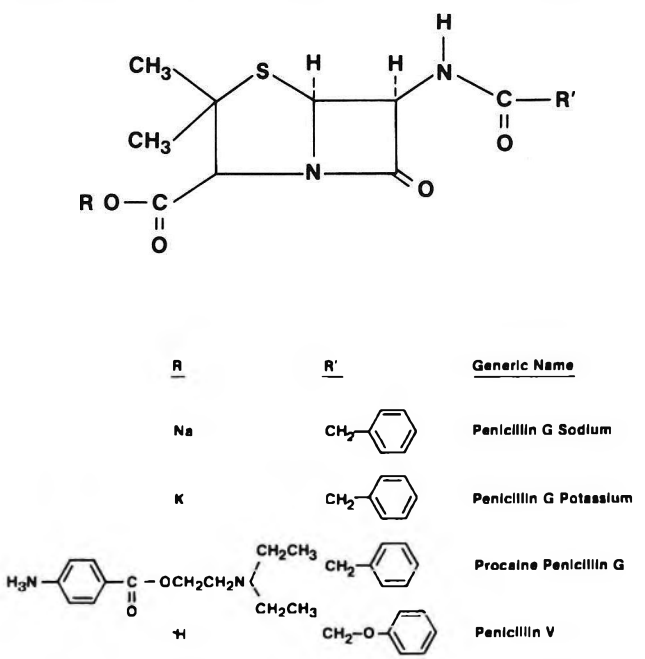

Figure 1. Chemical structures of benzyl penicillins and penicillin V.

mL 0.01M mercuric chloride solution, mix, and adjust the pH to 9.0 ± 0.5 with 5M NaOH. Transfer quantitatively into a 250 mL volumetric flask and dilute to volume with water.

(c) *Phosphate buffer.*—0.2M. Weigh 0.994 g dibasic sodium phosphate (anhydrous) and 1.794 g monobasic sodium phosphate (monohydrate), dissolve in 80 mL water, and dilute to 100 mL in a volumetric flask (pH = 6.5).

(d) *Elution solution.*—Measure 60 mL acetonitrile (LC grade) and 5 mL 0.2 M phosphate buffer into a 100 mL volumetric flask and dilute to volume with water.

(e) *Phosphate buffer.*—0.1M containing 0.0157M thiosulfate. Weigh 4.969 g anhydrous dibasic sodium phosphate, 8.969 g monobasic sodium phosphate monohydrate, and 2.482 g anhydrous sodium thiosulfate. Dissolve in 800 mL water and dilute to volume in a 1 L volumetric flask.

(f) *Mobile phase.*—Measure 750 mL 0.1M phosphate buffer containing 0.0157M thiosulfate into a 1 L volumetric flask. Dilute to volume with acetonitrile. Mix thoroughly and filter through a 0.45 μm unit under vacuum.

(g) *Procaine penicillin G.*—Sigma Chemical Co., St. Louis, MO.

(h) *Phenoxymethyl penicillinic acid.*—Sigma.

(i) *Ethacilin.*—Sterile injectable formulation of procaine penicillin G (Rogar/STB Inc., London, Ontario, Canada).

Preparation of Solutions

(a) *Stock standard solutions.*—(1) *Penicillin G.*—1000 IU/mL. Weight 0.100 g procaine penicillin G salt (1000 IU/mg), dissolve in 10 mL methanol, and dilute to volume with water in a 100 mL volumetric flask. (2) *Penicillin V.*—1000 μg/mL. Weight 0.100 g phenoxymethyl penicillinic acid (1696 IU/mg), dissolve in 80 mL methanol-water (1 + 1), and dilute to volume in a 100 mL volumetric flask with methanol–water (1 + 1). (*Note:* prepare fresh stock solutions every 2 months and keep frozen at −20°C.)

(b) *Working standard solutions.*—(1) *Penicillin G.*—20 IU/mL. Pipet 200 μL penicillin G stock solution into a 10 mL volumetric flask and dilute to volume with water. (2) *Penicillin V.*—20 μg/mL. Pipet 200 μL penicillin V stock solution into a 10 mL volumetric flask and dilute to volume with water. (*Note:* Prepare fresh working solutions every fortnight and keep frozen in 2 mL aliquots.)

Animals

Two Holstein steer calves (approximate weight, 100 kg) that had not been administered any antibiotics were held on pasture for 3 weeks. One of the animals was injected with 15 000 IU of an injectable formulation of procaine penicillin G/kg body weight (Ethacilin) and the other animal served as a control. The animals were sacrificed 24 h after injection. Muscle, liver, and kidneys from both animals were collected for analysis.

Tissue Samples

Edible tissues (control and incurred) from the 2 slaughtered animals were stored at −20°C until assayed.

Sample Preparation

Accurately weigh 5.0 g partially thawed, blended control tissue into each of five 50 mL disposable polypropylene centrifuge tubes. Fortify each tissue with 0, 12.5, 25, 50, and 100 μL portions of penicillin G standard solution, 20 IU/mL, and 75 μL penicillin V working standard solution, 20 μg/mL, to produce samples with tissue equivalencies of 0, 50, 100, 200, and 400 IU/kg penicillin G, respectively, and 300 μg/kg penicillin V as internal standard. Accurately weigh 5.0 g partially thawed, incurred tissue into a 50 mL centrifuge tube, and fortify with 75 μL penicillin V working standard solution, 20 μg/mL. Add 20 mL water to each sample, and homogenize. Rinse the homogenizer probe with water into the centrifuge tube to the 35 mL mark. Cap the centrifuge tube and mix on the mechanical shaker for 5 min. Add 5 mL 0.17M sulfuric acid and 5 mL 5% sodium tungstate to the homogenate, cap, stir by vortexing for ca 20 s, and centrifuge at 2200 *g* for 10 min. Transfer the supernatant to a clean 50 mL centrifuge tube. Add 15 mL water to the residual plug, shake for 5 min, and centrifuge at 2200 *g* for 10 min. Add the supernatant from this extraction to the one from the previous extraction and filter by vacuum through a GF/B filter paper into a 125 mL filter flask. Add 10 mL 20% sodium chloride solution to the filtrate in the 125 mL flask and mix thoroughly.

Table 1. Tissue levels of penicillin G found in a Holstein steer calf injected with 15 000 IU procaine penicillin G/kg body weight and slaughtered 24 h post-treatment[a]

Tissue	Concn, IU/kg, of penicillin G detected in Holstein steer tissue (Mean ± SD)
Bovine muscle	51 ± 5
Bovine kidney	23 ± 5
Bovine liver	229 ± 9

[a] Five replicate analyses were conducted for each tissue matrix.

C18 Column Cleanup

Mount a 75 mL solvent reservoir onto the C18 cartridge with an adapter and place on a solid-phase extraction vacuum manifold. Wash the C18 cartridge with 20 mL methanol followed by 20 mL water and 10 mL 2% sodium chloride solution, and discard washes. (*Note:* It is very important not to allow the cartridge to run dry at this stage.) Pour the solution from the 125 mL filter flask into the reservoir. Pull the sample through the C18 cartridge with vacuum at a flow rate of ca 3 mL/min. Wash the column with 10 mL 2% sodium chloride followed by 10 mL water, and draw air through the cartridge for 5 min. Discard washes.

Remove the adapter and reservoir from the cartridge, place a clean 10 mL glass centrifuge tube under the C18 cartridge, and elute the sample immediately with 1 mL Elution solution.

Precolumn Derivatization

Add 1.0 mL derivatizing reagent to the eluate in the 10 mL centrifuge tube, cap, stir by vortexing, and allow to react in a 65°C water bath for 30 min. Remove the centrifuge tube from the water bath and quickly cool to room temperature by immersing the tubes into a beaker of water. Stir samples by vortexing after cooling, and filter through a 0.45 μm Acro filter into LC sample vials. Inject a 50–100 μL sample into the LC operated in an isocratic mode with a mobile phase flow rate of 0.8 mL/min (the optimized flow rate for this column). Measure the peak heights of penicillin G and penicillin V peaks detected at 325 nm at 0.005 AUFS.

Intralaboratory Study

Three analysts in our laboratory who had familiarized themselves with the method were given blended incurred

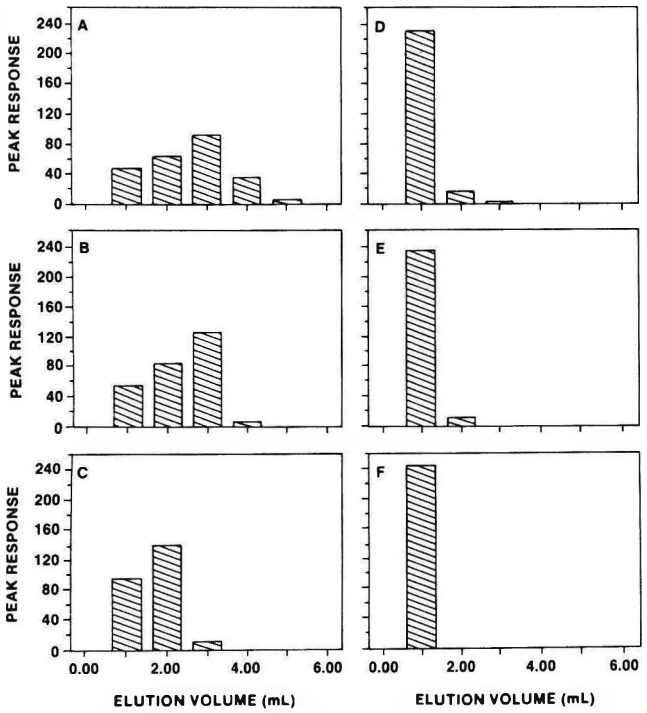

Figure 2. Elution profile of penicillin G charged onto a C18 BondElut cartridge with various compositions of elution solutions. The elution solutions contained a fixed composition of 0.2M phosphate buffer (5%) and varying amounts of acetonitrile or methanol and water. A. 45% methanol–50% water; B. 55% methanol–40% water; C. 60% methanol–35% water; D. 45% ACN–50% water; E. 55% ACN–40% water; F. 60% ACN–35% water.

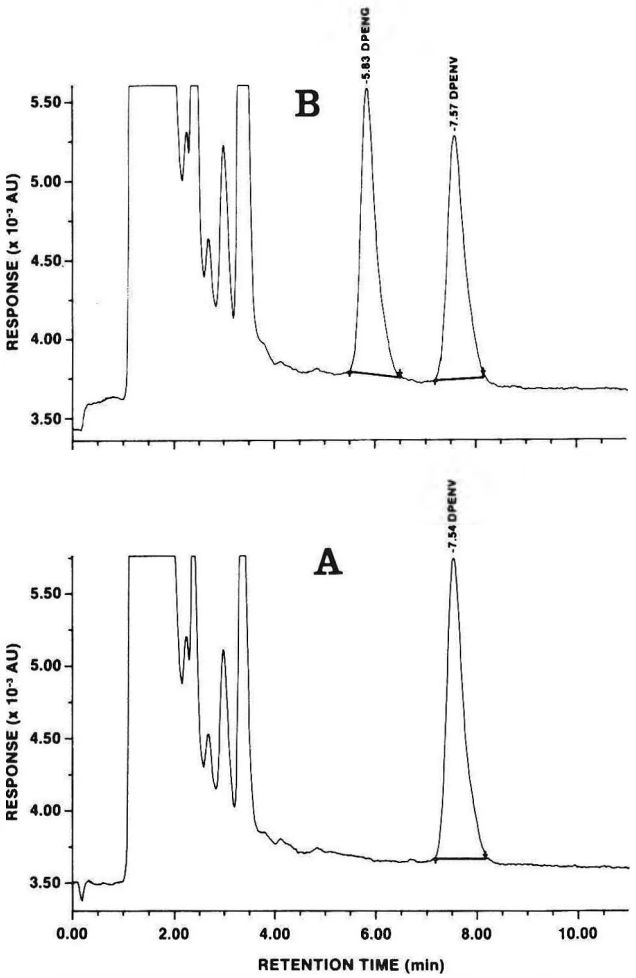

Figure 3. Chromatogram of a 50 μL injection of a derivatized extract from a control muscle tissue. (A), a control muscle tissue fortified with procaine penicillin G at 200 IU/kg and (B), a constant amount of penicillin V (300 μg/kg). Peaks represent the mercury mercaptide complexes of penicillin G (DPENG) and penicillin V (DPENV) detected at 325 nm at 0.005 AUFS.

tissues (bovine muscle and kidney and porcine muscle and kidney) to analyze. The results of this intralaboratory study are presented in Table 1.

Results and Discussion

Method Modification and Refinement

In the original method reported by Terada et al. (10), 10 g homogenized tissue is extracted with 40 mL water, 20 mL 5% sodium tungstate, and 20 mL 0.17M sulfuric acid and then centrifuged. The supernatant (pH = 2.4) is passed through a glass column packed with 18 g basic aluminum oxide and allowed to drain by gravity.

In our method, 5 g homogenized tissue is taken and the penicillin G is extracted with 20 mL water. Then 5 mL 0.17M sulfuric acid and 5 mL 5% sodium tungstate solution are added for deproteinization. The residual plug of tissue is extracted a second time with 15 mL water. We found it unnecessary to use the alumina column cleanup incorporated in the original method to remove the yellow pigmentation and presumably to adjust the tissue extract to about pH 5 before subsequent cleanup on the C18 cartridge because our method gives a combined extract whose pH is 5.6. The elimination of the basic alumina cleanup step in our method represents a substantial saving of almost 2 h in sample preparation time that, in our opinion, is more beneficial than the removal of the yellow pigmentation from the tissue extract as explained by Terada et al. (10).

Although 19 mL water is used in the original method to elute penicillin G charged onto a Waters Sep-Pak C18 cartridge, we elute penicillin G charged onto a C18 BondElut cartridge with only 1 mL elution solution (Figure 2) whose composition is described earlier. This modification provides an eluate from the C18 cartridge that is 20 times as concentrated as that obtained in the previous method (10). BondElut cartridges were used in our method instead of Sep-Paks because we found that tissue extracts obtained from them were cleaner with respect to contaminants eluting in the region of interest. It is recommended that the flow rate of ≈3 mL/min be followed closely when the sample extract is being charged onto the preconditioned BondElut cartridge. It is also recommended that once the C18 cartridge has been loaded with sample and washed and air has been drawn through it for 5 min, the penicillins should be extracted immediately. Failure to observe these recommendations will result in lower recoveries of the analytes.

A 30 min derivatization step has been included in our method to form the mercuric mercaptide penicillin complexes. These derivatives allow the wavelength for maximum UV absorption to be shifted from below 230 nm to above 325 nm, where interference from endogenous tissue contaminants is minimized.

Penicillin V was selected as an internal standard in this

Table 2. Estimation of the precision and accuracy of the developed analytical methodology using fortified tissue samples obtained from an antibiotic-free Holstein steer calf[a]

Tissue	Level of fortification, IU/kg	Concentration found (Mean ± SD), IU/kg	Deviation, %
Kidney	50.0[b]	46 ± 2(4)	−8
	120.0	117 ± 9(4)	−3
	240.0	235 ± 12(4)	−2
Muscle	50.0	47 ± 3(4)	−6
	120.0	121 ± 6(4)	+1
	240.0	239 ± 9(4)	0
Liver	50.0	48 ± 3(4)	−4
	120.0	118 ± 14(4)	−2
	240.0	243 ± 16(4)	+1

[a] Numbers in brackets represent the number of replicate determinations conducted at each level of fortification.
[b] This represents 29.3 μg/kg (ppb) of penicillin G in tissue.

method because it is efficiently recovered by the extraction procedure described, forms a mercaptide complex which exhibits a suitable UV absorbance at 325 nm, is chromatographically resolved from penicillin G (Figure 3), and is not registered in Canada for use in food-producing animals (16). An internal standard of 300 μg/kg is recommended for use in samples (e.g., muscle, kidney) in which the penicillin G residue concentration is generally below 800 μg/kg. In cases such as injection sites, livers, and samples from extra-label drug use, in which the penicillin G residue levels may exceed 800 μg/kg, it is recommended either to use 900 μg/kg penicillin V as internal standard or to start with a smaller sample, e.g., about 2 g. In countries other than Canada, where penicillin V is approved for use in food-producing animals, the use of an external penicillin G standard would be the method of choice for quantitative analysis.

Evaluation of the Revised Method

Figure 3 shows the chromatogram of a control muscle tissue and a control muscle tissue fortified with penicillin G. The capacity factors for penicillin G and penicillin V on this column operated at room temperature are 4.0 and 5.5, respectively, at retention times of 5.8 and 7.6 min.

Using control tissues fortified with penicillin G at various concentration levels, several analytical parameters for the modified method were determined (Tables 2 and 3). The

Table 3. The recovery of penicillin G from fortified control muscle tissue[a]

Concn of penicillin G		
Spiked, IU/kg	Recovered (Mean ± SD), IU/kg	Recovery; %, ± SD[b]
25	23 ± 2	92 ± 9
50	47 ± 3	94 ± 6
250	213 ± 12	85 ± 5
500	439 ± 36	88 ± 7
1000	910 ± 19	91 ± 2

[a] Five replicate determinations were conducted at each level of fortification.
[b] Recoveries from fortified tissues were calculated based on external penicillin G standards.

Table 4. Typical calibration parameters for the analysis of penicillin G residues in edible tissues[a]

Level of fortification, IU/kg	Mean response ratio[b] ± SD
50.0	0.25 ± 0.02 (4)
100.0	0.54 ± 0.04 (4)
200.0	1.11 ± 0.07 (4)
400.0	2.27 ± 0.12 (4)

[a] Linear regression equation: Y = (0.005768 ± 0.000014) X − 0.03913, correlation coefficient = 0.9999, where X is the concentration in IU/kg and Y is the response ratio.
Responses were obtained on extracts from blank muscle tissues spiked at the indicated levels of penicillin G and a constant amount of penicillin V (300 μg/kg) and taken through the extraction-derivatization method.
[b] Response ratio is calculated as response of penicillin G/response of penicillin V (300 μg/kg) detected at 325 nm.

method is accurate and reproducible with coefficients of variation of ≤12%, and yields recoveries of ≥80% for the determination of penicillin G in edible tissue. Calibration curves generated from plots of response ratios (peak height of penicillin G/peak height of penicillin V) vs concentration of penicillin G in fortified control samples are linear in the 10–1000 IU/kg concentration range with correlation coefficients of ≥0.995 (Table 4). The method has a detection limit of 8.4 IU/kg (5 μg/kg) (S/N = 5) which is tenfold lower than that reported in the original method (10).

Table 1 illustrates the tissue levels of penicillin G determined for the Holstein steer calf that was injected with 15 000 IU procaine penicillin G/kg body weight and sacrificed 24 h post-treatment. The tissue levels of penicillin G in this Holstein steer calf administered twice the recommended dose of penicillin G indicate that the method is capable of detecting penicillin G residues in incurred tissues.

The results of an intralaboratory study to demonstrate whether the method is suitable for routine laboratory use are shown in Table 5. Except for the low result obtained for porcine kidney (an obvious outlier) and the high value for bovine kidney obtained by analyst A, the results of the intralaboratory analyses indicate that the method can be reproduced and is not analyst-dependent.

Ampicillin, a synthetic β-lactam, and chloramphenicol, a veterinary drug banned for use in food-producing animals, are both extracted from tissues with recoveries of ≥80% (unpublished data) by our method. With retention times of 3.8 and 4.6 min for ampicillin and chloramphenicol, respectively, they are resolved completely from penicillin G and penicillin V and therefore do not interfere with the chromato-

Table 5. Intralaboratory study to determine the concentrations of penicillin G residues in incurred porcine and bovine tissues[a]

Sample	Concn, IU/kg, of penicillin G[b] in tissues determined by			Mean concn of analysts' results, IU/kg
	Analyst A	Analyst B	Analyst C	
Bovine muscle	50 ± 3(5)	51(2)	49 ± 2(5)	50 ± 1
Bovine kidney	92 ± 6(5)	77(2)	72 ± 4(5)	80 ± 10
Porcine muscle	127 ± 14(5)	134(2)	144 ± 4(5)	135 ± 9
Porcine kidney	366 ± 23(5)	610(2)	571 ± 35(4)	516 ± 131

[a] Numbers in brackets represent the number of replicate analyses conducted on each sample by each analyst.
[b] Mean ± SD.

graphic analysis. The recovery of these veterinary drugs from tissue by this method offers a potentially useful route for the development of a multiresidue LC method for β-lactams and chloramphenicol. We have recently developed a thermospray/LC/MS method (unpublished paper) that provides simultaneous detection and confirmation of the analytes presented in this paper and greatly increases the usefulness of the method for regulatory monitoring of these residues in tissues and biological fluids of food-producing animals.

Conclusion

A simpler, more sensitive, and more reproducible method for the determination of penicillin G residues in edible tissues has been developed. This method has been shown to be suitable for the routine diagnostic analysis of penicillin G in tissues of food-producing animals and is now being used routinely in our laboratory for the determination of benzyl penicillin residues in submissions of suspect and STOP-positive tissues of edible animals from federally inspected abattoirs across Canada.

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FOOD ADDITIVES

Enzyme Immunoassay for Hen Egg White Lysozyme Used as a Food Additive

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An indirect competitive enzyme immunoassay for hen egg white lysozyme (HEL) used as a food additive was investigated. Anti-HEL antibodies were obtained from B10A mouse ascites immunized by intraperitoneal injection of HEL. HEL samples to be assayed were extracted from foods with 1% gelatin in borate buffer. Goat anti-mouse IgG (H+L)-peroxidase complex was used as a second antibody, and 3,3′,5,5′-tetramethylbenzidine was used as a substrate for the peroxidase. The working range for quantitative analysis was 1–50 ng/mL, because in this range the binding inhibition curve of anti-HEL antibodies to HEL-coated plates by HEL was linear. Even after losing the lysozyme activity by heat treatment, HEL could be detected by indirect competitive enzyme immunoassay. Recoveries of HEL by this assay were >85% for Japanese noodles and Japanese traditional-style confectioneries, 53—95% for miso and cooked beans, and 30—85% for fried fish pastes. HEL contents of 55 commercial foods were determined; HEL was detected in 19 samples in the range 25–20 000 ng/g. HEL as a food additive was detected more frequently in plant-derived foods than in foods of animal origin.

Lysozyme is widespread in animals and plants. Hen egg white contains a large amount of lysozyme, and it has been added to various foods as a food preservative (1, 2). Lysozyme hydrolyzes mucopolysaccharides in the cell wall of certain microorganisms such as *Micrococcus lysodeikticus*, and then causes the lysis of microorganisms (3).

For the analysis of hen egg white lysozyme added externally to foodstuffs, liquid chromatography or an assay system of lytic activity has been used thus far (4). These methods, however, have the disadvantage of lack of specificity for the detection of a particular lysozyme, e.g., hen egg white lysozyme (3) or human urine lysozyme (5); they do not detect the origin-specific lysozyme protein but rather detect the common lysozyme activity.

On the other hand, enzyme immunoassy (EIA) has both high specificity and very low detection limits, so that complicated cleanup procedures are not required (6). We attempted to apply EIA to the determination of hen egg white lysozyme which was present in commercial foods as a food additive.

Experimental

Materials

Hen egg white lysozyme (HEL) and complete Freund's adjuvant were purchased from Wako Pure Chemical Industries. Goat anti-mouse IgG (H+L)-peroxidase was obtained from Jackson Immno Research Lab. 3,3′,5,5′-Tetramethylbenzidine (TMBZ) was purchased from Dojin Laboratories.

Substrate solution was prepared, just before the assay, by dissolving both 100 μL TMBZ solution (10 mg TMBZ in 1 mL dimethylformamide) and 1.5 μL 30% hydrogen peroxide in 9.9 mL 0.1M acetate buffer, pH 5.5.

Preparation of Anti-HEL Antibodies

HEL aqueous solution was used as the immunogen by emulsification with 9 volumes of complete Freund's adjuvant. Female B10A mice were immunized by intraperitoneal injection of this emulsion (HEL 100 μg/mouse) every 4 weeks; ascites was collected and centrifuged at 10 000 rpm for 10 min, and the supernatant was used for EIA.

EIA Protocols

(a) *Preparation of plates.*—Microtiter plates (Nunc Immno Module, Maxisorp F16, 96 wells) were coated with 100 μL aliquots of HEL solution (100 μL/mL borate buffer (BBS, pH 8.0) at room temperature for 1 h. Plates were washed 3 times with BBS, then blocked with 250 μL 1% gelatin in BBS at room temperature for 1 h. These plates were stored in a refrigerator until required.

(b) *Indirect EIA.*—Indirect EIA was used to examine the dilution ratio of anti-HEL ascites, as follows: 100 μL diluted ascites was added to the appropriate wells of the HEL-coated plates. Plates were incubated 1 h at room temperature and washed 3 times with BBS; then 100 μL goat anti-mouse IgG (H+L)-peroxidase (diluted 1/5000 (v/v) in 1% gelatin in BBS) were added. Plates were incubated 1 h at room temperature and washed 5 times with BBS; then 100 μL substrate solution were added and the plates were incubated for 30 min at room temperature. The reaction was stopped with 100 μL 1N-H_2SO_4 and absorbance was measured at 450 nm. The absorbance at 450 nm was corrected by subtracting the absorbance obtained by a non-specific binding of both anti-HEL antibodies and the second antibody to the plate. To estimate this latter absorbance, the entire procedures were carried out in parallel using plates which were not coated with HEL solution but only blocked with 1% gelatin in BBS.

(c) *Indirect competitive EIA.*— This assay was done to estimate the amount of HEL in foods. HEL-coated plates were prepared as described. Diluted ascites (6/10 000 (v/v) in 1% gelatin in BBS) was mixed with the same volume of HEL solution (dissolved with 1% gelatin in BBS) as standard or sample extract, and incubated 1 h at room temperature. Then 100 μL portions of these mixtures were added to HEL-coated plates and/or HEL-non-coated plates, and incubated 1 h at room temperature. The determination was completed as described above for EIA.

Preparation of Sample Extract

Samples (3–5 g) were weighed into a 50 mL centrifuge tube, and 40 mL 1% gelatin in BBS was added. The mixtures were homogenized, diluted to 50 mL with 1% gelatin in BBS, and centrifuged at 10 000 rpm for 10 min. The supernatant was used as the EIA sample.

Assay System of Lytic Activity

Lysozyme activity was determined with a modified version

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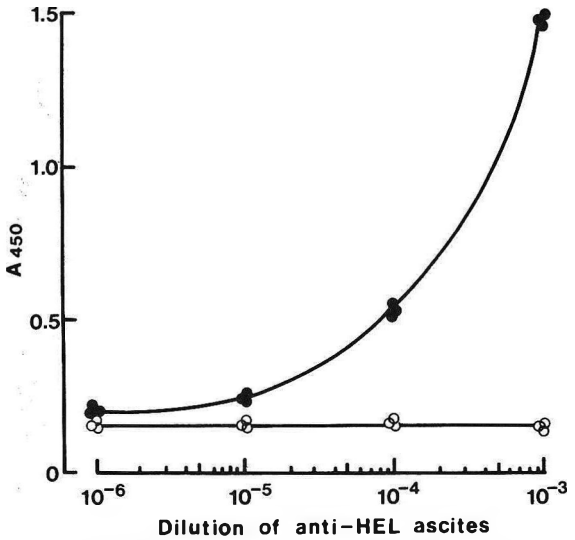

Figure 1. Binding curves of anti-HEL antibodies in mouse ascites to HEL-coated (-●-) and HEL-non-coated (-○-) plates.

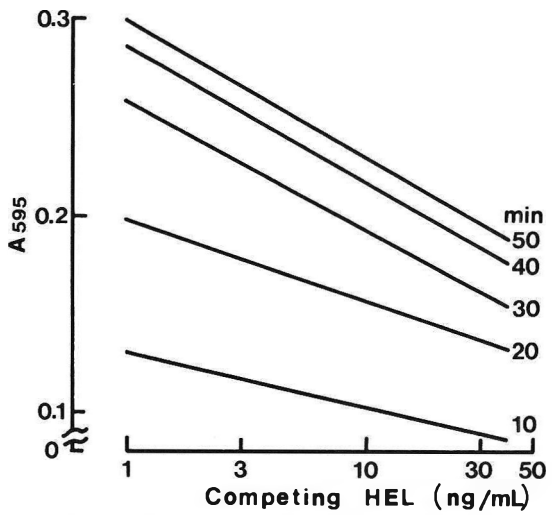

Figure 3. Effect of reaction time with the substrate on binding inhibition curve of anti-HEL antibodies in mouse ascites to HEL-coated plates by HEL.

of the method used by Shugar (7) in which 9 mg dried *Micrococcus luteus* cells were suspended in 30 mL 0.1M phosphate buffer (pH 7.0), 2.9 mL of the suspension was pipetted into a cuvette, and absorbance was measured at 450 nm. Then 0.1 mL standard HEL solution or sample extract was added to the cuvette and incubated for 10 min at 22°C. The change of absorbance at 450 nm (A_{450}) was recorded, and the content of lysozyme in samples was estimated from the change of A_{450}, using a separately prepared dose-absorbance curve. The result was shown as an HEL equivalent.

Results and Discussion

Specific anti-HEL antibodies were prepared by injecting HEL into mice. The anti-HEL titers were monitored by a gel precipitation test with 0.9% agarose in BBS.

The dilution ratio of the anti-HEL ascites for indirect competitive EIA was determined. Figure 1 shows a binding curve of the antibody to HEL-coated plates. In indirect competitive EIA, the most appropriate absorbance is approximately 1.0 when competing HEL is not added. From this binding curve, the working dilution ratio was selected to be $3/10^4$. A_{450} by the nonspecific binding was about 0.15 at the dilution ratio of $1/10^3$ to $1/10^6$.

Dynamic range for the determination of HEL by indirect competitive EIA was estimated (Figure 2). A_{450} was monitored while the concentrations of competing HEL were varied from 1 to 1000 ng/mL (final concentration). Linearity of the binding inhibition was observed from 1 to 50 ng/mL, and the working range for the quantitative analysis of HEL in foods was selected to be 1–50 ng/mL.

The optimum concentration of the peroxidase-conjugated second antibody was determined by testing 3 dilution ratios of 1/5000, 1/10 000, and 1/20 000 of the commercially obtained preparation. The absorbances by the nonspecific binding were approximately the same for these 3 second antibody concentrations, and the highest A_{450} obtained by indirect EIA was achieved at 1/5000. Then the working dilution ratio of the second antibody was selected to be 1/5000.

Figure 3 shows the effect of reaction time with the substrate on binding inhibition curves of anti-HEL antibodies to HEL-coated plates by HEL. Absorbances at 595 nm were monitored before the reaction was stopped with H_2SO_4. The slope of the curve increased when the incubation was extended to an incubation time of 30 min. After 30 min, the binding curves were parallel; hence, the reaction time was selected to be 30 min.

One of the problems in detection of HEL by the lytic assay is cross reactivity (6, 8) because lysozyme has a widespread distribution in many kinds of foodstuffs such as meats, vegetables, fish, and shellfish (4, 5). On the other hand, it was also reported that the content in vegetables and fruits was relatively low (1). Further, the structures of lysozymes in vegetables and fruits are considered to be different from HEL. Consequently, the cross reactivity in the case of EIA between lysozymes of vegetables or fruits and HEL was presumed to be low. Practically, this was shown by the "None Detected" (ND) data of some Japanese pickles or boiled vegetables in Table 1. Shellfish was reported to have high lysozyme activity (9). Therefore, we further tested the cross reactivity between HEL and corb shell lysozyme in our system. Lysozyme content of the corb shell was also determined by the lytic assay and found to be 201 μg/g as an HEL equivalent. However, HEL was not detected by our indirect competitive

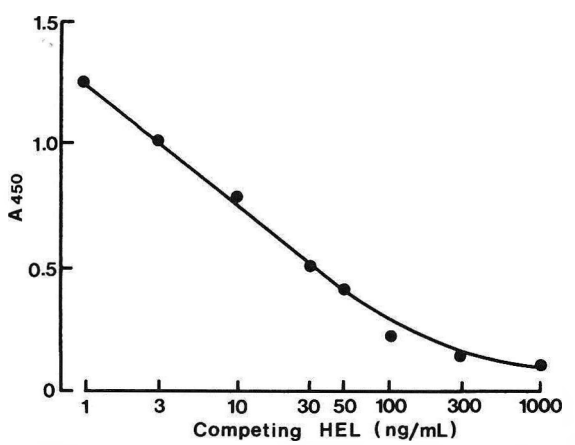

Figure 2. Binding inhibition curve of anti-HEL antibodies in mouse ascites to HEL-coated plates by HEL.

Table 1. Determination of HEL in commercial foods

Foods	HEL, ng/g[a]	Foods	HEL, ng/g[a]
Japanese	9800	Japanese pickles	
traditional-	5800	Japanese radish	160
style	2400	Eggplant	ND (1)
confectioneries	590	Chinese cabbage	ND (1)
	520	Nozawa leaves	ND (1)
	ND (9)[b]	Boiled octopus	ND (3)
Japanese	20000	Spanish mackerel,	
noodles	3800	Miso	ND (1)
	3300	Ham	
	ND (1)	Pork ham	ND (3)
Biscuit	1100	Fish ham	ND (2)
	190	Jam	
Cracker	4800	Strawberry jam	360
Miso	250	Orange marmalade	70
	ND (2)	Boiled vegetables	
Tofu	ND (2)	Bracken	ND (1)
Cooked beans		Mushroom	ND (1)
Kintoki-beans	280	Taros	ND (1)
	ND (1)	Fried fish pastes	170
Uzura-beans	ND (1)		ND (4)
		Boiled and semi-dried	34
		whitebait	ND (2)

[a] Overall detection limits in the original sample are 50 ng/g in biscuit and in fried fish pastes, or 20 ng/g in others.
[b] Number of samples below detection limits.

EIA, and the 2 were completely differentiated. Further investigations of the lysozymes from meat and fish are now underway.

It is often desirable for food additives to be determined after heat treatment. Determination of heat-denatured HEL was carried out by both assay systems, i.e., lytic activity and EIA. HEL solution (50 μg/mL) was heated at 100°C for 30 min and for 40 min. The results (Figure 4) showed that HEL could be detected by EIA but not by the lytic assay. The discrepancies seemed to arise from the different heat stability of the sites having lytic activity and immunogenicity in HEL as reported by Kenett et al. (10). HEL is used as a food additive under more moderate heating conditions (1), and in many cases, HEL activity remains more or less the same. Those conditions are covered in the heating conditions of Figure 4, indicating the superior sensitivity of EIA to the lytic assay.

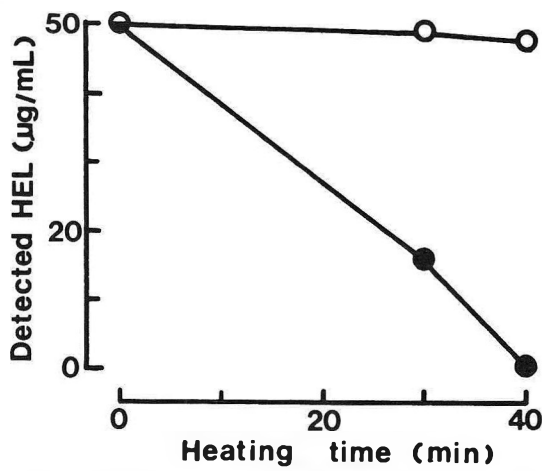

Figure 4. Detection of HEL heat-treated at 100°C by EIA (-○-) and the lytic assay (-●-).

Table 2. Recovery tests of HEL in foods

Foods	Added, ng/g	Found, ng/g	Rec.,[a] %
Japanese noodles	0	0	—
	100	93.5	93.5
	1000	1180	118
	10000	12000	120
Japanese	0	0	—
traditional-style	100	84.8	84.8
confectioneries	1000	920	92.0
	10000	11500	115
Fried fish pastes	0	0	—
	100	29.5	29.5
	500	425	85.0
Miso	0	0	—
	100	98.0	98.0
	500	360	72.0
Cooked beans	0	0	—
	100	52.5	52.5
	500	475	95.0

[a] Average of 3 examinations.

Extraction of HEL from foods was tested. To avoid binding of HEL at the surface of the centrifuge tube, 1% gelatin in BBS was used as the extraction solvent.

Table 2 shows the results of recovery tests in 5 kinds of commercial foods, i.e., Japanese noodles, Japanese traditional-style confectioneries, cooked beans, fried fish pastes, and Miso. Recoveries from Japanese noodles and confectioneries were satisfactory (85–120%). In Miso and cooked beans, recoveries were relatively good. In fried fish pastes, however, low recoveries were obtained, especially when the amount of HEL was relatively small. It is inferred that the lipid from the samples might interrupt binding of anti-HEL antibodies to HEL-coated plates. A cleanup procedure to remove the lipid from lipid-rich samples is being investigated.

Table 1 shows the HEL content of 55 commercial foods determined by our indirect competitive EIA. HEL used as a food additive was detected more frequently in plant-derived foods than in foods of animal origin. HEL at 500–10 000 ng/g was detected in 5 Japanese traditional-style confectioneries among 14 samples. In the case of Japanese noodles, 3 of 4 samples contained HEL at 3300 to 20 000 ng/g. In Japan, hydrogen peroxide had been used for preserving Japanese noodles until 1977, when hydrogen peroxide was banned because of its carcinogenicity. Thereafter, HEL has been used for preserving noodles as a substitute for hydrogen peroxide. HEL has several advantages as a food additive, e.g., it is highly soluble in water and is relatively tolerant to heat, especially under acidic conditions. Futhermore, HEL can be broken down in the human stomach after digestion (1, 2).

Thus, it is likely that HEL is added to many kinds of foods as a safe food preservative. Further investigation of the determination of HEL in many kinds of foods is underway.

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FOOD PACKAGING

Determination of Food Contamination by Mineral Oil from Jute Sacks Using Coupled LC-GC

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Jute fibers are treated with about 5–7% of a high boiling mineral oil fraction ("batching oil") to render them flexible for making fabrics. Foods transported in jute bags are contaminated by this batching oil. A method involving automated on-line LC-GC is described for determining these hydrocarbons in various foods. Complete transfer of the LC fraction to GC is presupposed for obtaining the required sensitivity. Results are given for nuts, coffee, cocoa products, and rice. Contamination ranged between about 5 and 500 ppm.

During experiments for determining irradiation of foods (1), involving on-line LC-GC analysis of alkanes and alkenes as radiolysis products from fat, we observed numerous GC peaks on an unresolved background ("hump") in the extracts of hazelnuts. These peaks were typically eluted in the range of the alkanes from C_{14} to C_{22}. In most samples, not only the corresponding *n*-alkanes were detected, but also the branched pristane and phytane, which are not naturally present in hazelnuts. The latter 2 components, also found in sediments of rivers or lakes and other environmental samples, are often used as markers for the presence of mineral oils; although the straight chain alkanes rapidly disappear because of digestion by microorganisms, pristane and phytane persist.

The suspicion that the alkanes found in hazelnuts are due to contamination with mineral oil was confirmed by the absence of such peaks in hazelnuts from our own garden. Concentrations of summed alkanes were between about 5 and 500 ppm, thus in a range corresponding to severe food contamination. The source of the contamination was identified as the "batching oil" applied to jute fibers used for jute bags. Once this was known, many other foods were found to be contaminated by the same hydrocarbons: cocoa beans, chocolate, coffee, rice, etc. Batching oil also contains aromatics (15–30%), but these compounds were not detected by the method applied here.

Jute Sacks

Jute sacks are primarily produced in Southeast Asia; Bangladesh and Thailand are the most important exporters. Jute fibers obtained from the jute plant are brittle and dusty. Therefore, fibers are processed with batching oil before spinning and weaving. Commonly about 7% batching oil is added to the fibers (2). At the end of the manufacturing process, bags are aired for months to remove the most volatile part of the batching oil (primarily to avoid the strong smell of mineral oil). According to the producer's information, bags are sold with a maximum content of 5–5.5% batching oil. Most of the batching oil for the Bangladesh jute is produced by refineries in Dhaka. It seems to be a straight mineral oil fraction, distillation being characterized by a 50% recovery at 348°C and 90% recovery at 395°C, thus resembling gas oil. However, GC analyses of batching oils extracted from jute sacks suggest that batching oils are not of uniform quality.

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Coupled LC-GC

Determinations were carried out by automated on-line coupled LC-GC (3) involving transfer of whole LC fractions to GC. This saved considerable time for sample preparation and resulted in high sensitivity; (detection limits were achieved only with difficulty when an off-line procedure was used; see *Discussion*). Finally the on-line system avoided contamination of the samples with the hydrocarbons present in many kinds of laboratory equipment.

Transfer in presently used on-line coupled LC-GC may be carried out with the 2 eluant evaporation techniques summarized by Munari and Grob (4). The first step of developing an LC-GC method involves the choice of the evaporation technique. Concurrent evaporation, i.e., complete evaporation during transfer, is preferred whenever possible. However, substances that are eluted at up to 50–100° above the column temperature during transfer (which must be above the pressure-corrected boiling point) form broad peaks and/or are at least partially lost through an early vapor exit. Thus, concurrent evaporation is suitable for applications involving elution temperatures exceeding about 120–170°C (depending on the eluant, for example).

For GC analysis with lower elution temperatures, the retention gap techniques must be used. Solvent trapping is exploited for retaining volatile components in the flooded uncoated pre-column up to the end of eluant evaporation. However, this presupposes the use of uncoated pre-columns of a size that allows the retention of flooding eluant. Up to about 100 μL can be retained in a pre-column 10 m × 0.53 mm id. If larger fractions are to be transferred, a substantial proportion of the eluant is evaporated during transfer to reduce the volume of flooding liquid that must be retained by the uncoated pre-column (partially concurrent eluant evaporation).

Experimental

Apparatus

LC-GC was carried out on the prototype of the Carlo Erba Dualchrom 3000 instrument (5, 6). The apparatus consisted of an LC system with a syringe pump Phoenix 20, an autosampler, an injection valve with a 10 or 50 μL loop, and a 10-port backflush valve containing a 1 mL wash loop slowly filled with wash solvent from a pressurized flask. The loop-type interface was used with pressure/flow-regulated carrier gas supply (7) and an early vapor exit (8). The whole system was controlled from the GC.

Sample Preparation

Nuts.—A 100 g sample of nuts was ground and immersed in redistilled *n*-pentane overnight. The extract was decanted, the solvent was evaporated, and 1 g of the residue, consisting

primarily of oil, was dissolved in 10 mL *n*-pentane (10% solutions). Then, 20 μL of a 100 ppm solution of *n*-eicosane or *n*-tridecane in hexane was added as internal standard (= 2 ppm). Assuming a fat content of 50%, the 2 ppm equivalent in the oil residue corresponded to 1 ppm in the nuts.

Cocoa beans.—Shells and kernels of the cocoa beans were separated. Kernels were ground and extracted like the nuts, and 2 g shells were ground. Then, 2 μL internal standard solution (100 ppm *n*-tridecane in hexane) was added (1 ppm for the shells). The mixture was extracted overnight with 20 mL *n*-pentane, centrifuged if turbid, and determined by LC-GC. Rice, coffee beans, instant coffee, cocoa powder, cocoa beverages, and chocolate were extracted as described for the shells of the cocoa beans; sesame and cheese as for the nuts.

Jute bags.—A 1 g sample of a jute bag was extracted with 10 mL *n*-pentane, and 10 μL of a 500 ppm solution of *n*-tridecane was added as internal standard; the resulting solution was diluted 1 + 50 and determined by LC-GC.

LC-Preseparation

The alkanes were separated from the rest of the sample on an LC column 10 cm × 2 mm id packed with silica gel Spherisorb S-5-W and eluted with redistilled *n*-pentane at 200 μL/min. The fraction window of the paraffins was determined by injecting an oil similar to the batching oil into the LC and transferring 50 μL volumes at various LC retention times to GC. The paraffin fraction started at the dead time of the column (1.25 min) and reached a retention time of 2 min, at which no more *n*-alkanes were eluted. The methylnaphthalenes, the most volatile aromatics of batching oils, were eluted clearly later. The transferred fraction had a volume of 150 μL. The LC column allowed introduction of up to 5 mg of fat in up to about 50 μL of solvent. Larger amounts can be introduced, provided larger bore LC columns are used.

On-line Transfer to GC

Partially concurrent eluant evaporation.—For the first part of the experiments, because the most volatile components to be determined were not known, transfer occurred by the retention gap technique. Using an uncoated pre-column 10 m × 0.53 mm id with a capacity of retaining up to some 100 μL liquid, at least 50 μL of the LC fraction had to be evaporated during transfer (partially concurrent eluant evaporation). However, when the standard pre-column system with early vapor exit for partially concurrent eluant evaporation is applied (9), the minimum required eluant evaporation rate of 67 μL/min is easily exceeded.

The uncoated fused silica pre-column, 10 m × 0.53 mm id, was deactivated by phenyldimethyl silylation. It was followed by a retaining pre-column, 3 m × 0.32 mm id, taken from the separation column, and the early vapor exit. Connections between the pre-columns and to the vapor exit T-piece were of press-fit type (10). The separation column, attached to the vapor exit T-piece, was a glass capillary column, 25 m × 0.32 mm id, coated with PS-255 (a methyl silicone) of 0.6 μm film thickness. Hydrogen was used as carrier gas at 0.8 bar inlet pressure. Transfer occurred at 48°C column temperature. The evaporation rate, determined at the vapor exit as described previously (9), was 125 μL/min. Thus, only some 55 μL of condensed eluant flooded the uncoated pre-column at the end of the transfer.

Because partially concurrent eluant evaporation requires the use of the on-column interface, the uncoated pre-column was installed in the on-column injector, and the transfer line, a 0.17 mm od fused silica capillary, was inserted into its inlet. The LC-GC procedure involved the steps and the timetable summarized in Table 1.

Table 1. Timetable of the LC-GC transfer by partially concurrent eluant evaporation with the on-column interface

Time elapsed	Activity
0:00	GC oven thermostated at 48°C; LC syringe pump (automatically) refilled when required for the run.
0:00	Automatic injection of 50 μL of the sample.
1:15	On-column transfer valve switched to transfer; vapor exit opened.
2:00	Transfer valve returned to stand-by.
2:10	LC column switched to backflush, the wash loop containing 1 mL methyl *tert*-butyl ether (MTBE).
2:20	Closure of the vapor exit (switched to a restriction of 1 m × 75 μm id).
2:30	GC oven temperature increased at 15°/min to 130°C and at 5°/min to 250–330°C.
12:10	Backflush valve returned to stand-by position.

Concurrent eluant evaporation.—Because the first eluted alkane was never smaller than tetradecane, concurrent evaporation was applicable and a majority of samples were analyzed this way. In fact, all of the chromatograms shown, except those for the hazelnuts, were obtained by the concurrent evaporation technique. *n*-Tridecane (the most commonly used internal standard) formed an obviously broadened peak, indicating that the sample was a borderline case for concurrent evaporation. Broadening is the result of the first transferred solute material being flushed excessively far into the retaining pre-column by the eluant vapors. However, the tridecane peak was not broadened enough to cause loss of material through the vapor exit. As it was a critical case, application of concurrent evaporation presupposed a fairly long separation column of somewhat elevated film thickness (which increased the elution temperature), a carefully selected transfer temperature, and the transfer only of restricted fraction volumes.

Concurrent evaporation requires an oven temperature during transfer that is at least slightly above the eluant's boiling point at the inlet pressure. When the standard loop-type interface (7) is used, the inlet pressure during transfer corresponds to the pressure determined by the pressure regulator installed upstream to the flow regulator. As this pressure was

Table 2. Timetable of the LC-GC transfer using concurrent eluant evaporation with the loop-type interface

Time elapsed	Activity
0:00	GC oven temperature adjusted to 58°C and LC-pump refilled when required.
0:00	Autosampler injection of 50 μL.
2:00	Transfer and carrier gas valves switching to transfer; solvent vapor exit opened.
2:10	Column switched to backflush with MTBE.
x:xx	Automatic return of sample and carrier gas valves to stand-by upon drop of inlet pressure (threshold 10 kPa).
x:xx + 30 s	Closure of the vapor exit.
5:00	Temperature increase in the GC oven by 15°C to 130°C, followed by 5°C to 300°C.
12:10	LC column returned to normal flow direction.

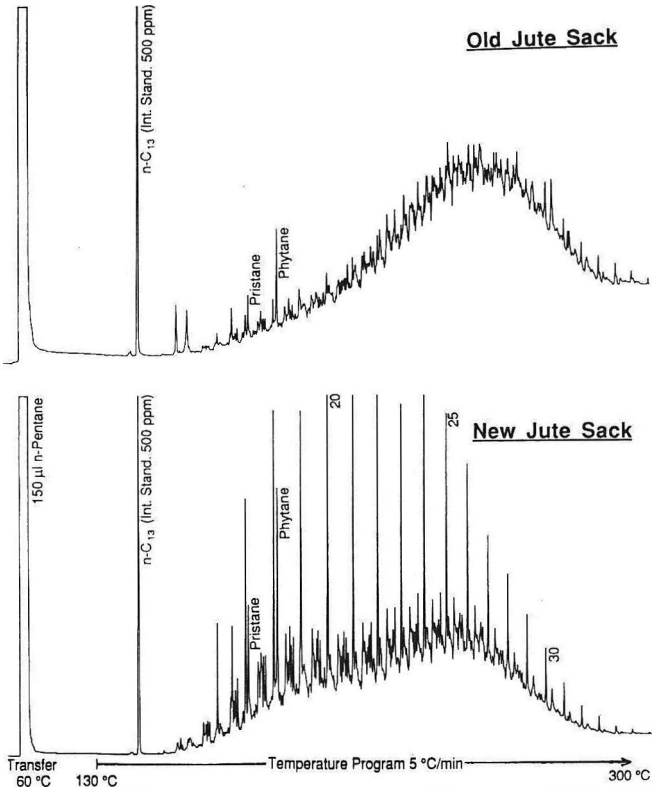

Figure 1. Batching oils from jute sacks. Mixture with large *n*-alkane peaks from a new-looking sack (left); strongly degraded oil from an old-looking sack (right). Pristane and phytane are persistent branched alkanes.

1 bar (hydrogen), a transfer temperature of 58°C was used (11). The flow regulator, Porter DFC 1400 with the laminar element A-10, was set at 40. At 1 bar pressure behind the flow regulator, this resulted in a flow rate of some 2.5 mL/min.

A sample loop of 150 μL internal volume (75 cm × 0.5 mm id; check volume—tubing is not always accurate!) was installed in the sample valve. An uncoated fused silica pre-column, 2.5 m × 0.32 mm id, was combined with a glass capillary retaining pre-column, 3 mm × 0.32 mm id, (from the separation column), the vapor exit T-piece, and the separation column mentioned above.

The steps and the timetable of the LC-GC transfer are listed in Table 2. Because the end of the transfer was automatically detected by the drop of the inlet pressure upon disappearance of the eluant plug in the inlet of the pre-column (time xx:x), synchronisms controlled the return of the sample and the carrier gas valve as well as closure of the vapor exit.

Quantitation

Accurate quantitation of a broad "hump," i.e., of an unresolved zone (see chromatograms below), was difficult. Usually most of the peak area was in the "hump," i.e., integrated peak areas represented only a small fraction of the total material present. The area of the unresolved background was determined manually by the following procedure: The baseline, as repeatedly determined by blanks, was transferred to the chromatograms of the sample. Then, the area of the "hump" was determined by approximation with simple geometrical forms and compared with the area of the internal standard, also measured in terms of mm^2 (calculated as half height width multiplied by peak height).

Reasonably accurate determinations presuppose a stable baseline (i.e., moderately high attenuation) and introduction of amounts creating rather large "humps." Both requirements called for high overall sensitivity. The detection limit was around 0.5 ppm; reasonably accurate quantitation was possible above 2–5 ppm.

Results

Jute Bags

Figure 1 shows 2 strongly differing paraffin mixtures extracted from jute sacks used for transporting hazelnuts. They give an idea about the expected fingerprints of the contamination by jute sacks. The left chromatogram is dominated by *n*-alkane peaks (on a broad unresolved background). From the other jute sack (right), *n*-alkanes had disappeared almost completely (probably through degradation) while the isoalkanes, which formed the "hump," were still present. The isoalkanes are dominated by pristane and phytane, 2 branched hydrocarbons known to strongly resist microbial degradation. Furthermore, the shape of the "hump" indicates that a large part of the more volatile alkanes was lost by evaporation into air or transfer to the nuts. The first sack looked rather new while the second was dirty and worn-out. However, it is questionable whether the sack was really old, because importers claim that sacks are only used once.

The 2 batching oils shown in Figure 1 are of similar molecular weight distribution: C_{16}–C_{28}. Some oils (from the approximately 30 jute bags analyzed) were of a more narrow range of molecular weights: C_{21}–C_{29} in one case, C_{17}–C_{25} in another. The broadest range was C_{16}–C_{31}. Keeping this background in mind, we should expect that food contamination

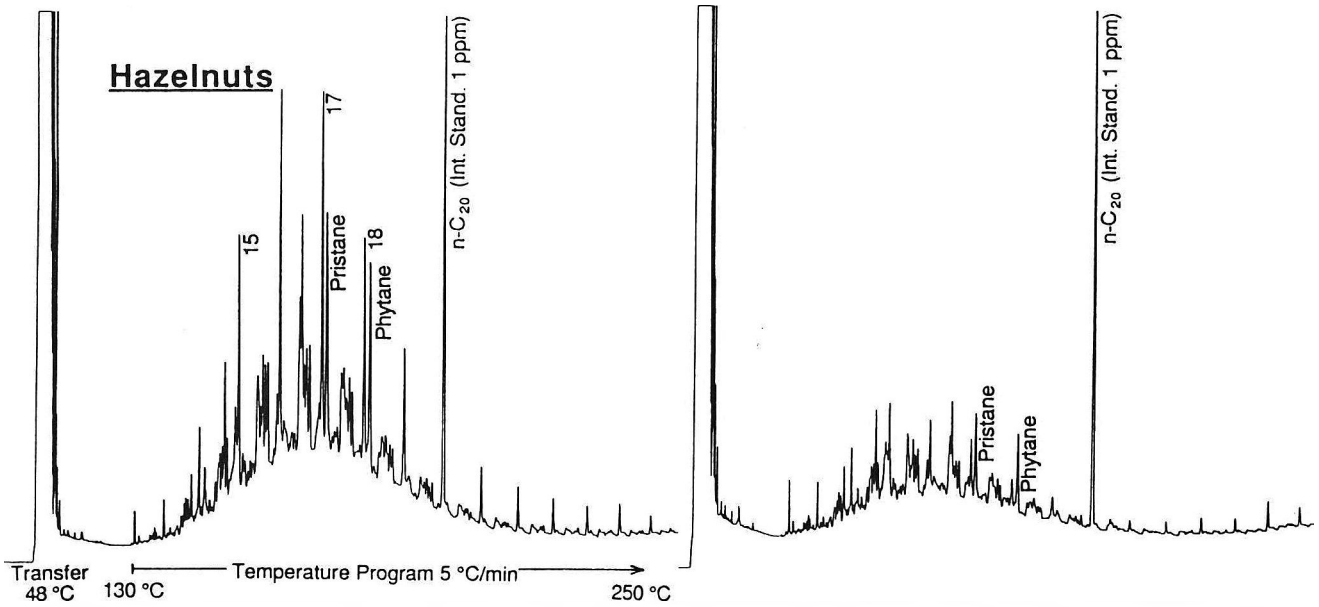

Figure 2. Paraffins on hazelnuts: At left, nuts from a jute bag with dominating *n*-alkanes (indicated by numbers of carbon atoms); at right, from a bag with degraded *n*-alkanes. Transfer from LC to GC by partially concurrent eluant evaporation (all other chromatograms shown were obtained by concurrent evaporation).

should also show different pictures: varying contents of *n*-alkanes and varying molecular weight distributions.

Concentrations of batching oil found in the fabrics of jute sacks varied between 1.4 and 4.1%, mostly ranging between 2.5 and 3.5%. As an average sack for transporting, say, 50 kg of nuts has a weight slightly exceeding 1 kg, each sack contains about 30 g batching oil. Hypothetical complete transfer to the content of the sack would result in a contamination by nearly 1000 ppm.

A small bag used for selling a 1 kg portion of rice (with an internal plastic bag) contained only 0.6% batching oil. Obviously, jute can be treated with less batching oil than standard, although the fabric felt somewhat harder than that of normal sacks with more batching oil.

Nuts

Hazelnuts.—Figure 2 shows 2 chromatograms of paraffins extracted from hazelnuts. With 50 and 15 ppm, paraffin concentrations were intermediate. In the left chromatogram, *n*-alkanes predominate, while the right chromatogram indicates contamination with partially degraded batching oil; *n*-alkanes have largely disappeared, leaving the isoalkanes behind. Pristane and phytane now become predominant peaks (see also Figure 1). In cases in which both the jute bags and the packed hazelnuts were available, presence or absence of *n*-alkanes usually agreed, suggesting that degradation of the *n*-alkanes occurred before the hazelnuts were packed into the bag. In a single case, contamination of hazelnuts included rather large *n*-alkane peaks, while few *n*-alkanes were detected on the jute sack, indicating that degradation of the *n*-alkanes may also occur at a late stage, considerably after the nuts were packed.

Contaminants found on hazelnuts correspond to the most volatile part of the batching oil. Apparently transfer occurs primarily through the gas phase; thus, it depends on vapor pressure. Furthermore, diffusion into the nuts occurs at a rate that decreases rapidly with increasing molecular weight, with the result that amounts of high boiling material found at the surface far exceed those in the center of the nuts. Only a small proportion of the hazelnuts is in direct contact with the jute sack.

Figure 3 shows concentrations of paraffins found in hazelnuts. Some 50 samples were randomly taken from food stores and from the chocolate industry. Another 20 samples, with usually low paraffin contents, were from experiments designed to provide evidence of the source of the problem, i.e., contamination by the jute bags. The paraffin concentrations found most frequently were between 10 and 50 ppm; in 20% of the samples, contamination exceeded 100 ppm. The maximum concentration found was 500 ppm.

There was no significant difference whether nuts were imported from Italy or Turkey. The strong variation of the concentrations could be explained by factors such as the duration of storage in the jute sacks, the temperature, and the proportion of volatiles in the batching oil on the sack. Furthermore, a single sack stored at a well-aired place might contaminate its content far less than a sack stacked in a large pile in a closed storehouse.

In general, hazelnuts are stored in shells. Shells are re-

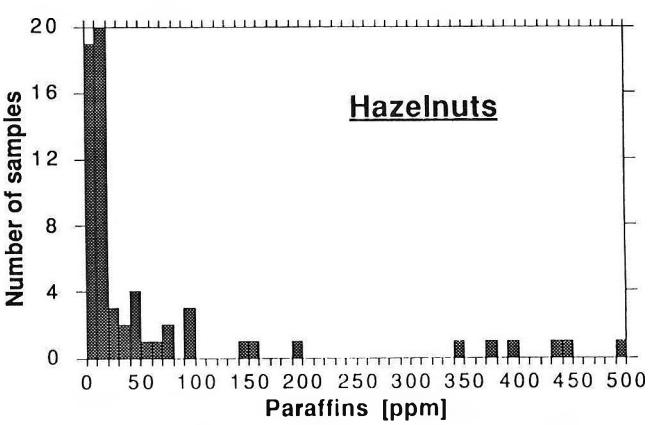

Figure 3. Paraffin concentrations found in hazelnuts.

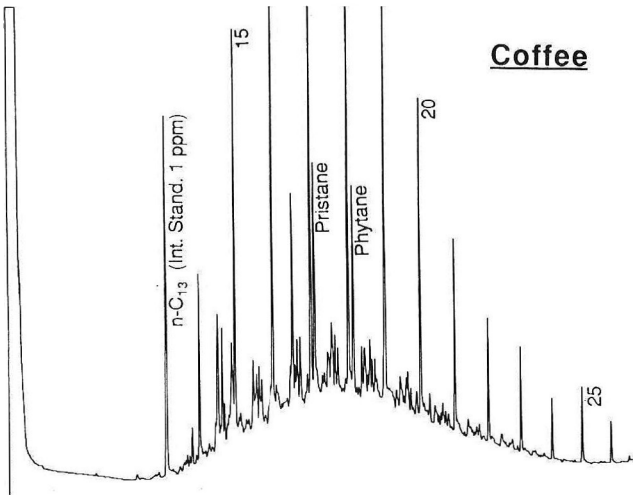

Figure 4. Paraffins from roasted coffee beans.

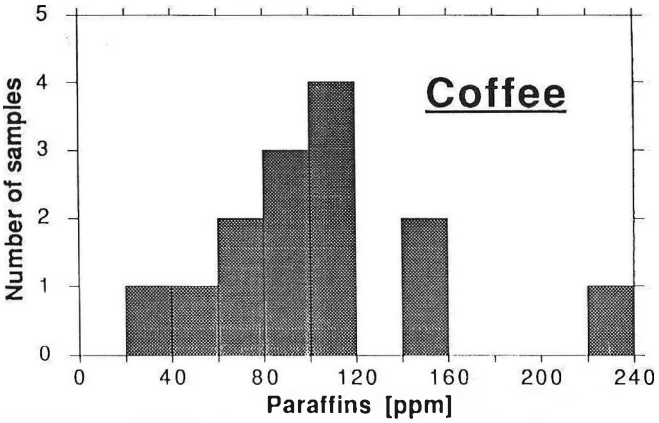

Figure 5. Paraffin concentrations found in 14 coffee samples.

moved in the country of origin shortly before shipping. Also, because hazelnuts are seldom stored over extended periods of time by the buyers, hazelnuts should not stay in jute bags for more than a few months. However, because of the high content of fat, nuts are prone to pick up large amounts of apolar material.

Walnuts, almonds.—The 4 samples of open walnuts analyzed contained no paraffin contamination. Probably walnuts are too fragile to be transported in jute bags. Eight out of 10 samples of almonds were contaminated, with paraffin concentrations ranging between 10 and 200 ppm (average of 80 ppm, clearly more than found in hazelnuts). The 2 uncontaminated samples were shipped in cardboard boxes.

Internal paper bags.—Some foods are packed in jute sacks with an internal paper bag, presumably to avoid contamination with dust from the jute. In a sample of almonds packed in a double sack, 60 ppm paraffins was found. Hence, the paper bag did not prevent severe contamination.

Hazelnuts free of batching oil material were split into 2 portions: One part was directly packed into a jute sack, the other into a jute sack with an internal paper bag. After 3 weeks, the first portion of hazelnuts contained 7 ppm of contaminants, while the nuts protected by the paper bag only contained some 2 ppm batching oil material. This experiment, involving little contamination primarily because of short-term packing, suggests that the paper bag provides some protection, probably by slowed exchange of air and vapors of batching oil material.

Coffee

Many other foods are also stored and transported in jute sacks (sometimes also in sisal bags). Not surprisingly, they were all contaminated with batching oil. Figure 4 shows the paraffins recovered from roasted and ground coffee beans (estimated total concentration, 100 ppm). The composition of the paraffins does not differ significantly from paraffins typically found in hazelnuts.

Concentrations found in 14 samples of coffee from the market are shown in Figure 5. Contamination of the roasted coffee reached 150 ppm; 230 ppm paraffins was determined in the single sample of raw coffee beans analyzed. The average contamination was about 100 ppm, clearly higher than for the hazelnuts. This is most likely due to considerably longer storage in jute bags.

Four samples of instant coffee contained up to 2 ppm paraffins. As they were produced in Switzerland, it must be assumed that the coffee beans used for their production were also imported in jute bags and, thus, contained far higher concentrations of batching oil components than those found in the instant coffee. Apparently extraction of the beans leaves the paraffins in the residue.

Cocoa Beans and Chocolate

Most cocoa beans are stored and transported in jute bags, where they become contaminated by batching oil. Of 2 lots of roasted beans, shells were analyzed separately from the kernels. In one case, shells contained 110 ppm aliphatic contaminants and the kernel 8 ppm; in the other case, the contents were 120 and 13 ppm, respectively. Thus, concentrations of paraffins in the shells were some 10 times higher than in the kernels, but the shells did not fully protect the kernel from contamination. Only a limited proportion of the shells is processed for recovering the relatively small amount of cocoa butter. However, cocoa butter from shells, which carries along practically all the paraffins, is expected to be contaminated at extremely high levels.

The above results for cocoa bean kernels appear to be low in terms of cocoa products. Figure 6 shows the alkane fraction of a black chocolate with an unusually broad "hump." The concentration of the contaminants was estimated as 160 ppm. Of 10 chocolates analyzed, 2 contained paraffins at 160 and 270 ppm; the others contained 5–15 ppm. Cocoa powders and cocoa-containing beverages contained 10–135 ppm of aliphatic contaminants (5 samples).

For chocolate to be contaminated at a level of 270 ppm

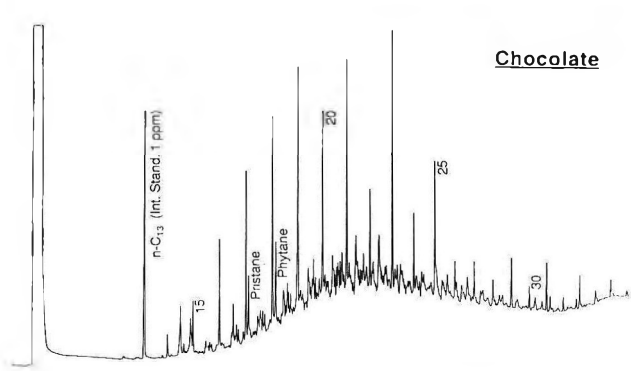

Figure 6. Paraffins from a black chocolate.

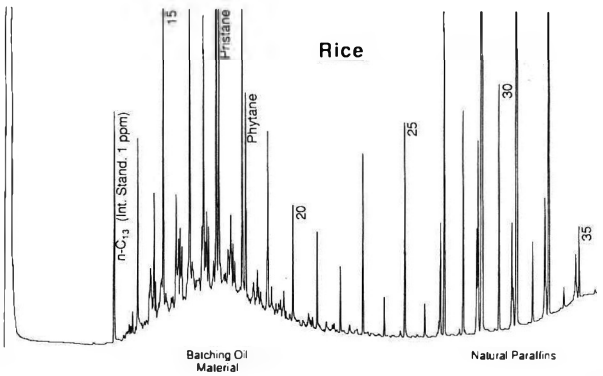

Figure 7. Batching oil material on rice together with natural hydrocarbons from rice in the rear part of the chromatogram (C_{25} to C_{33}).

through the cocoa beans, these beans would have had to contain about 900 ppm of paraffins (30% cocoa butter in the chocolate), which exceeds the level of contamination observed for cocoa beans. The peak pattern shown in Figure 6 does not fully correspond to that expected; it attains rather high molecular weights. It will be subject to further investigation to check whether this contamination is due exclusively to jute sacks.

Rice

Some rice, primarily Asian rice, is imported to Switzerland in jute bags. Rice differs from nuts, for example, by a far lower fat content. If fat absorbs the hydrocarbons, contamination of rice should be lower. Furthermore, air exchange, transporting the hydrocarbons from the jute into the center of the bag, should be slower through densely packed rice than through a packing of nuts or beans. However, such expectations were wrong: In 6 samples, 20–160 ppm paraffins were found, averaging just over 100 ppm.

Figure 7 shows rice containing some 100 ppm batching oil material. Contaminants, ranging from C_{14} to C_{20}, correspond to the volatile end of batching oil. In addition to the alkanes from the jute bags, alkanes and alkenes from rice are visible in the second half of the chromatogram. These natural hydrocarbons found in many plants, are easily distinguished from those of the jute bags: odd-numbered *n*-alkanes strongly predominate over the even-numbered, and no isoalkanes form an unresolved background. Smaller peaks of natural hydrocarbons are also seen in Figure 6.

Discussion

Coupled LC-GC vs More Conventional Methods

Of course, hydrocarbons can also be determined by conventional methods. Any silica or aluminum oxide column will separate the paraffins from the bulk of the sample (primarily fat), and high separation efficiency is not of first importance. However, there might be a simple reason why food contamination by hydrocarbons from jute sacks was not detected previously: lack of sensitivity. For the same reason, several laboratories, trying to reproduce our results without on-line LC-GC, had great difficulty in detecting at least 10 ppm batching oil. However, detection at concentrations down to 1 ppm would be desirable.

On-line coupled LC-GC provides much higher sensitivity than off-line methods, as can be explained by the following estimations. Usually the GC-FID determination of a single component at a concentration of 1 ppm is simple. However, detection and quantitation of a material primarily forming a "hump" requires far higher sensitivity. If the "hump" is conceived to be composed of 200 fused peaks, a concentration of 5 ppb must be visible to allow the detection of 1 ppm of the total material. Moreover, even this estimation is optimistic because a "hump" is more difficult to detect than a sharp peak. A relatively high "hump" is required on a fairly straight baseline (elevated attenuation) for a safe detection and reasonably accurate quantitation.

In on-line LC-GC, 50 μL of a 10% fat solution (e.g., from hazelnuts) was injected, i.e., 5 mg fat or an equivalent of about 10 mg hazelnut was analyzed. Because of the complete transfer of the LC fraction to GC, all contained paraffin material reached the FID. If a conventional LC column is applied, 100 mg fat might be chromatographed on 5–10 g silica gel, 10 mL of which is perhaps collected. When 1 μL of this solution is injected into the GC by a splitless technique, an equivalent of 0.01 mg fat or 0.02 mg hazelnut is introduced, i.e., only $^1/_{500}$ of that with on-line LC-GC. To achieve the same sensitivity, the solution must be reconcentrated to 20 μL before injection into GC. This is difficult and probably is accompanied by considerable losses of the more volatile components (12). The analyst may try to improve the method by scaling up. To reconcentrate to a final volume of 200 μL, for example, 1 g fat might be chromatographed on a 50–100 g silica gel column, ending up with a fraction of 100 mL. Thus, sample preparation really becomes what is often called "wet chemistry."

The conventional method not only creates problems about the detection limit and the amounts of solvent and adsorbent. It is also laborious (LC columns of such size must be packed in the laboratory) and introduces problems of sample contamination. Since hydrocarbons are present all over the laboratory (plastic parts, grease, etc.), problems with blanks are considerable.

Jute Sacks for Storage and Transportation of Foods?

Batching oils on jute sacks essentially are raw mineral oil fractions resembling industrial heating oil or (after normal paraffins are degraded) hydraulic oils. Contamination of foods by such material at concentrations of up to several hundred ppm certainly cannot be tolerated. In cases of food transport by ships or trucks previously used for carrying such commodities as heating oil, contamination at far lower levels is sufficient reason to consign foods to municipal incinerators.

The limits for migration of materials from plastics into foods are well-defined; however, there is no corresponding legislation for jute bags yet. Such limits are determined primarily from 2 aspects: toxicity of the contaminants, and, in the case of low toxicity, food contamination considered as unavoidable. Toxicity depends on the composition of the batching oil. Paraffins are not considered highly toxic; in fact, "clean" paraffins are accepted as a skin around certain cheeses as well as for pharmaceutical applications. However, batching oil cannot be considered a "clean" paraffin. Preliminary analyses revealed 20–30% aromatics of molecular weights ranging between those of fluorene and benzpyrene (13).

Transport and (short-term) storage of hazelnuts occurs nearly exclusively in jute bags. Apparently, there is no equivalent alternative, primarily because controlled exchange of

air and humidity is important to prevent excessive loss of water, but also to avoid recondensation of water and formation of mold upon decrease of temperature outside the sack. Finally jute bags are mechanically stable and do not present problems because of the high fat content of nuts, as does cardboard, for example. Finally, heavy jute bags are used because nuts are usually sold as gross for net.

As shown above, the use of internal sacks of porous paper prevents contamination by jute dust rather than that by batching oil. On the other hand, air-tight internal sacks, which effectively stop food contamination by batching oil, cannot be used for the reasons mentioned above, just as simple plastic sacks cannot replace the jute sacks.

The use of jute sacks also is widely supported because jute is a typical third world product; it is, for instance, the most important export article of Bangladesh. In fact, there is a broad consensus that the problem of food contamination with batching oil should not be solved by replacing jute sacks. However, a more acceptable replacement for the presently used batching oil is urgently needed. Even in view of these considerations, contamination at the extent shown above cannot be tolerated for long.

Many other sources of food contamination with hydrocarbons are currently under investigation. Numerous food samples were found to contain paraffins at concentrations similar to or even exceeding those observed for foods contaminated with batching oil. For instance, more than 1000 ppm paraffins were found in rice samples that had never been in a jute sack. Probably purified paraffins were involved, but food contamination by hydrocarbons obviously is not under control.

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FRUITS

Quantitative Analysis of Limonin in Grapefruit Juice Using an Enzyme-Linked Immunoassay: Interlaboratory Study

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Eleven laboratories participated in an interlaboratory study of an enzyme-linked immunoassay for limonin in grapefruit juice. Participating laboratories received training and practice samples until familiar with the procedure. Laboratories then received 8 sample pairs of grapefruit juice as blind duplicates. Samples were refrigerated and analyzed within 4 days of receipt. Results from 1 laboratory and 6 individual samples were excluded from the statistical analysis. Method performance was measured by the one-way component of variance analysis. Repeatability relative standard deviations (RSD_r) ranged from 7.0 to 15.6%. Reproducibility relative standard deviations (RSD_R) ranged from 12.9 to 29.4%.

Limonin, a triterpenoid dilactone, is an intensely bitter compound present in the fruit (and extracted juice) of many citrus cultivars. The average taste threshold for limonin has been reported at one part per million (ppm) in water and 3 ppm in orange juice (1). The higher threshold in orange juice was reportedly due to component interactions that suppress or interfere with bitterness perception. Guadagni et al. (2) reported an additive effect on bitterness perception when limonin and the bitter flavonoid naringin were both present at subthreshold levels.

Grapefruit juice usually contains limonin and naringin at concentrations above their taste thresholds. An analysis of various quality factors and preference from a survey of commercial grapefruit juices over 3 seasons exhibited an inverse correlation between preference and bitterness (3). Limonin concentrations were directly proportional to perceived bitterness in the juice and naringin concentrations were inversely proportional to bitterness. This suggests that limonin is the more important contributor to bitterness perception at the levels in which these 2 constituents are found in grapefruit juice. Therefore, limonin levels in grapefruit juice need to be monitored to ensure product quality and acceptability.

The enzyme-linked immunoassay (4) was chosen for interlaboratory study because of its rapid analysis time, low capital outlay, and its specificity for limonin. Other methods of limonin determination require subjective evaluation (5, 6), require extensive sample preparation (7), are susceptible to interferences with closely eluting compounds (8, 9), or require radiosynthesis and costly counting equipment (10, 11).

Eleven laboratories participated in the collaborative study. Analysts participating in the study received training on the procedure. They were provided with all necessary equipment in their laboratories and received practice samples until each was familiar with the method. The 11 analysts then received blind duplicates of 8 commercial grapefruit juice samples. Analysts were asked to keep samples refrigerated and perform the analysis within 4 days of receiving samples.

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METHOD

Principle

An IgG antibody fraction specific for limonin is produced from rabbit serum and coated onto a microwell surface. Juice or standard mixed with a tracer solution containing alkaline-phosphatase limonin oxime (APLO) tracer is placed in the antibody coated well. The APLO tracer and limonin compete for antibody binding sites. Unbound limonin and APLO tracer are washed away. *p*-Nitrophenyl phosphate (PNPP) is added as substrate for the alkaline-phosphatase portion of bound tracer with subsequent color development. Color intensity is proportional to amount of APLO tracer present and inversely proportional to amount of limonin in the juice or standard. Color intensity is measured by absorbance at 405 nm after stopping the phosphatase reaction by raising the solution pH.

Apparatus

(a) *Single channel vertical light path photometer.*—With 405 nm filter (Idetek, San Bruno, CA; or Flow Laboratories, McLean, VA).

(b) *Microplate incubator.*—(Idetek, Flow Laboratories) or equivalent, capable of 37 ± 1C temperature regulation.

(c) *Pipet.*—Eight channel, 200 μL fixed volume multichannel pipet (Flow Laboratories).

(d) *Limonin starter kit.*—No. 8011 (Idetek), or equivalent. Consists of micronic test tubes, racks, tube caps, strip sealers, and reagent reservoirs.

Reagents

All reagents are available in Bitterdetek Limonin Kit No. 8009 (Idetek) and should be stored at 2 to 4C. *Caution:* Some reagents contain sodium azide and may form explosive compounds that can accumulate when poured into a sink drain as a means of disposal. These reagents should be disposed of in a proper manner.

(a) *8-Well antibody-coated reaction wells.*

(b) *Limonin tracer.*—17 mg, lyophilized alkaline phosphatase conjugate limonin oxime.

(c) *Limonin tracer diluent.*—17 mL, 0.1M phosphate buffered saline with 0.1% gelatin and 0.1% sodium azide, pH 7.4.

(d) *Enzyme substrate.*—10 mg, *p*-nitrophenyl phosphate.

(e) *Enzyme substrate diluent.*—10 mL, diethanolamine with 0.1% sodium azide, pH 9.8.

(f) *Wash solution.*—50 mL, 0.9% physiological saline with 0.5% Tween-20 and 0.1% sodium azide.

(g) *Limonin standards.*—4 0.3 mL, clarified orange juice

with 0.1% sodium azide spiked to contain 2, 4, 8, and 16 ppm limonin.

Preparation of Working Solutions

Limonin tracer solution.—1 mg/mL. Add 1 mL limonin tracer diluent (bottle A) to lyophilized limonin tracer (bottle B) and mix. When completely dissolved, transfer all contents of bottle A into bottle B and mix. Rinse bottle B 3 times with solution in bottle A, returning rinse to bottle A. Tracer solution is stable for 7 days at 2 to 4C.

Substrate reagent.—1 mg/mL. Add two 5 mg PNPP tablets to 10 mL substrate diluent (bottle C) and mix 5 to 10 min until dissolved. Substrate reagent is stable for 8 h at 2 to 4C.

Preparation of Samples

Filter or centrifuge juice to remove pulp. If excessive limonin is suspected, dilute juice sample 1:1 with distilled water. If testing fresh nonpasteurized juice, deactivate enzymes and convert all limonin A-ring lactone (limonin precursor) to limonin by heating juice just to boiling; allow to cool.

Enzyme Immunoassay

Due to small quantities used, visually check fluid level in pipet tips to ensure accurate volumes. Avoid contact with bottom of reaction wells; fingerprints or scratches may cause increased absorption readings on photometer.

Turn on incubator and photometer and allow a minimum of 15 min for instruments to stabilize. Be sure incubator temperature is 37 ± 1C. Pour tracer solution into tracer reservoir and pipet 400 μL of tracer into each micronic test tube (8 at a time) by delivering 200 μL twice with multichannel pipet. Pipet 25 μL sample or standard in duplicate to test tubes using clean pipet tip for each sample or standard. Cap each tube and mix.

Using multichannel pipet with clean tips, add 200 μL of tracer–sample or tracer–standard from test tubes to reaction wells. Seal reaction wells with strip sealers and incubate 30 min at 37C.

Remove reaction wells from incubator, discard strip sealers, and shake contents thoroughly into waste container. Pour wash solution into wash reservoir. Wash each reaction well 3 times with 200 μL wash solution each time using multichannel pipet. Discard wash into waste container.

Pour substrate reagent into substrate reservoir and add 200 μL substrate reagent to each reaction well using multichannel pipet. Seal reaction wells with strip sealers and incubate 30 min at 37C. Remove reaction wells from incubator. Read absorbance at 405 nm of lowest limonin standard. If less than 1.5 (*Note:* newer Bitterdetek kits now specify the absorbance minimum at 1.0), incubate wells longer (not to exceed an additional 30 min) until absorbance is 1.5 or greater.

Discard strip sealers, add 1 drop of stopping reagent to each reaction well, and wait 5 min. Read and record absorbance of wells in photometer.

Calculations

Determine the average absorbance of duplicate samples and standards. Plot absorbance (ordinate) vs concentration of standards (abscissa) on semilog graph paper and draw best fit line through 4 standard points. Determine limonin concentration by plotting average absorbance of samples on standard curve; read corresponding concentration in ppm.

Alternatively, limonin values can be determined by calculator or computer capable of performing statistical best fit curves.

Results and Discussion

Eleven laboratories completed analysis of the 8 sample pairs in duplicate. Six collaborators reported a need to extend the second incubation time by 4 to 10 min. Results shown in Table 1 were those submitted by each collaborator with the exception of collaborators 7 and 11. Collaborators 7 and 11 submitted values based on the average of 2 concentrations. Results were recalculated using average absorbance, as specified in the instructions, and are the values reported. Corrected values for collaborators 7 and 11 differed slightly from those submitted, lowering the relative standard deviations by 0.4% or less.

From the results in Table 1, it is evident that collaborator 4 experienced difficulty with reproducibility, a problem also encountered with this collaborator when practice samples were analyzed. Data from collaborator 4 were eliminated from the statistical analysis. Two other samples, 3 and 6 for collaborator 2, also needed to be eliminated from the statistical analysis as suspect data. Examination of data for collaborator 2 indicated sample 3 (yielding 0 ppm) and sample 6 (yielding 18.2 ppm) were tested in adjacent reaction wells. Sample 3 was most likely added to sample 6.

The valid data were subjected to analysis by the Cochran

Table 1. Results for collaborative study of enzyme immunoassay for limonin in grapefruit juice (ppm)

Laboratory	Sample							
	1	2	3	4	5	6	7	8
1	6.1	5.4	6.3	7.0	6.9	10.5	11.3	12.0
	6.5	6.0	7.2	7.3	7.7	9.7	12.4	13.9
2	5.8	7.5	0.0[a]	7.5	7.7	18.2[a]	11.9	15.0
	6.7	6.5	7.0	8.4	9.0	10.8	11.2	13.3
3	7.3	5.4	6.7	6.0	8.0	9.5	9.6	13.2
	4.2	6.9	6.3	6.7	7.1	7.7	10.6	11.6
4[a]	1.2	1.9	3.5	3.0	2.5	6.0	5.7	7.0
	5.2	5.1	6.0	1.9	15.0	2.5	4.9	12.8
5	4.8	6.0	7.0	6.5	7.9	8.4	9.7	12.1
	5.5	5.7	6.9	7.4	6.7	10.0	11.8	11.0
6	4.8	5.8	6.1	7.3	7.4	9.4	10.9	12.6
	4.2	4.8	5.7	7.8	6.9	9.0	11.7	12.5
7[b]	7.0	5.7	6.8	7.7	6.8	12.2	11.0	14.2
	5.0	7.0	8.2	5.9	8.5	9.1	11.9	11.2
8	10.2	9.5[c]	13.8	10.9	10.8	12.7	15.3	16.0
	8.7	12.3[c]	9.9	11.3	11.5	14.6	14.4	14.6
9	8.1	8.4	8.9	9.2	8.6	10.5	14.9	14.5
	8.0	7.5	9.5	8.4	10.1	9.7	14.2	13.8
10	5.8	7.0	4.5	6.6	7.8	9.0	11.2	12.8
	5.5	6.4	5.9	7.0	5.9	6.8	10.8	9.7
11[b]	5.6	6.2	5.6	6.1	7.8	12.0	13.0	16.0
	4.6	4.8	7.4	7.3	9.4	8.7	11.0	11.6
Mean	6.2	6.3	7.4	7.6	8.1	10.0	11.9	13.1
S_r	0.97	0.73	1.15	0.64	0.92	1.41	0.84	1.59
S_R	1.63	0.98	2.17	1.49	1.46	1.92	1.66	1.69
RSD_r	15.6	11.6	15.6	8.4	11.2	14.2	7.0	12.2
RSD_R	26.2	15.6	29.4	19.5	18.0	19.3	13.9	12.9

[a] Validity of data is suspect. Indicated sample pairs and collaborator omitted from calculations.

[b] Values submitted by collaborators 7 and 11 were recalculated and reported.

[c] Outlier by Grubbs test. Omitted from calculations.

and Grubbs tests for outlier detection (12). One Grubbs outlier was detected (sample 2 for collaborator 8) and was eliminated from the statistical analysis. Both collaborator 8 and 9 reported values consistently higher than the mean. The reason for this could not be determined and repeatability statistics for these 2 collaborators were acceptable.

Statistical analysis was performed on the data by the one-way component of variance analysis. Results appear at the bottom of Table 1. The method has sufficient sensitivity and appears to have sufficient repeatability for screening limonin in grapefruit juice. This is necessary to determine whether limonin levels need to be lowered for product quality improvement. It should be noted that the sample analyses reported here were analyzed with 1 test kit/collaborator. Repeatability standard deviations do not include variance between test kits and may in fact be larger than indicated. Reproducibility standard deviations reflect variation between test kits as well as interlaboratory variance. With the large reproducibility standard deviations found, use of this method would be difficult for regulatory purposes. Samples with limonin values exceeding the allowable limits would have to be reanalyzed, probably in triplicate, to more precisely determine values. Subsequent correspondence with the test kit supplier indicated there were slight problems with uniformly coating the reaction wells used in this study. This manufacturing problem has, presumably, been solved through more rigorous quality control.

Pipetting errors are another source of error. Inexact volumes of sample and reagents can be major problems unless special attention is paid to this step. Analysts need to be particularly careful to exclude air bubbles during pipetting. Pipetting such small volumes (25–200 μL) requires close attention and accurate, well-cared-for pipets. Further testing would be needed to determine how much the improvement in manufacturing quality control (i.e., improved reaction well uniformity) has increased the precision of this method for determination of limonin in grapefruit juice. However, because of the considerable costs in personnel and reagents, no additional tests are contemplated.

Acknowledgments

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METALS AND OTHER ELEMENTS

Continuous Flow Vapor Generation for Inductively Coupled Argon Plasma Spectrometric Analysis. Part 2. Arsenic

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Total arsenic is determined by inductively coupled plasma atomic emission using hydride vapor generation. A 1 g sample is wet ashed in a 16 × 150 mm 10 mL volumetric test tube on a programmed heating block with nitric, sulfuric, and perchloric acids at up to 310°C. After treatment with hydrochloric acid and potassium iodide, arsenic is reduced by sodium borohydride to arsine in a simplified continuous flow manifold. A standard pneumatic nebulizer effects the gas-liquid separation of AsH_3, which is quantified by ICP atomic emission at 193.756 nm. The instrument detection limit for the method has been determined to be 0.4 μg/L. For a 10:1 dilution of a nominal 1 g sample, the detection limit is 4 μg/kg and the linear range is up to 4 mg/kg. Recoveries from 3 matrixes were 99–104%, with a typical RSD of 2%. The method has demonstrated statistical control for samples of biological interest and is especially well suited to analysis of small samples.

Analytical technique for arsenic is generally considered to be complementary to that for selenium. Most of the analytical concerns in determination of arsenic are similar to those for selenium described in Part 1 of this series (1). The accurate and precise determination of total arsenic in samples of biological interest has been complicated by the natural partitioning of this metalloid into organic and inorganic forms. These forms, which include arsenate, arsenite, and alkylarsenic compounds, all maintain a distinct set of physical and chemical properties that preclude a casual approach to analysis.

Arsenic in biological and environmental matrixes has been routinely determined by several methods, the most popular being hydride generation atomic absorption spectrophotometry (HG-AAS) (2, 3) and graphite furnace atomic absorption spectrophotometry (GF-AAS) (3-6). HG-AAS has been highly successful in many applications when care has been given to sample preparation and management of matrix interferences (7-9). Analysis for arsenic by HG-AAS suffers from a narrow working concentration range, which can require dilution and re-analysis for some samples.

Arsenic determination by inductively coupled argon plasma (ICP) with atomic emission spectrometry (AES) (10, 11) or mass spectrometric detection (MS) (12) has attracted attention in the literature because of the wide dynamic range and high sensitivity of these techniques. Sample introduction via both pneumatic nebulization and vapor generation (13-17) has been explored. The gain in analytical sensitivity using hydride vapor generation with ICP-AES has made it a most desirable technique; however, the literature usually specifies specialized apparatus (18-20).

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The general sequence of events for arsine generation methods are to (a) release the arsenic from the matrix and oxidize it to arsenate, (b) reduce the arsenate to arsenite, (c) reduce the arsenite to arsine, and (d) separate the AsH_3 vapor for quantitation (2, 3). Kaiser et al. (21) and Welz et al. (22, 23) have shown that a high temperature digestion with nitric, sulfuric, and perchloric acids completely destroys the organic matrix and liberates arsenic, mercury, and selenium from even the most resistant complexes. We developed modifications to allow for small sample analysis, sample matrix variation, and automation of sample preparation and analysis. The present paper describes a simple approach to continuous arsine vapor generation in which a standard pneumatic nebulizer becomes the gas-liquid separator.

METHOD

The method used in determination of arsenic by vapor generation ICP (VGICP) is derived from the selenium procedure described in detail in Part 1 of this series (1). The apparatus and procedure will be described briefly here along with the specific conditions needed for As determination.

Instrumentation and Apparatus

The ICP is an Applied Research Laboratories Model 3520 with a Meinhard concentric nebulizer operated under the standard conditions recommended by the manufacturer. The vapor generation manifold consists of a peristaltic pump with 3 channels to mix the sample first with 12M HCl, then with 0.6% $NaBH_4$. The manifold connects directly to the nebulizer. One gram samples are digested in tall 10 mL volumetric test tubes on a programmable heating block.

Instrument Operating Parameters

(a) *ICP operating conditions.*—Pre-integration time, 60 s; 3 integrations for 10 s each; sampler rinse time, 15 s; wavelength, 193.756 nm; off-peak background correction at +0.050 nm; photomultiplier bias, 110; RF forward power 1150 W at 27 MHz; reflected power <10 W.

(b) *Calibration points.*—0, 10, and 300 μg/L arsenic; blank subtraction was not used.

(c) *Mixing manifold.*—Mixing ratios are set by the diameters of the pump tubing. Use 1.4 mm id tubing for sample and 0.79 mm id tubing for $NaBH_4$ and HCl solutions. Sample uptake rate is 2.5-3.0 mL/min.

Reagents

(a) *Stock arsenic standard.*—1000 mg/L As as As_2O_3 in 10% nitric acid (Fisher Scientific, Fair Lawn, NJ).

(b) *KI solution.*—Dissolve 10 g KI in 100 mL water (Mallinckrodt, AR grade, Paris, KY).

(c) *Al, Fe, Cu, Pb, and Hg stock solutions.*—1000 mg/L atomic absorption standards (EM Science, Cherry Hill, NJ).

(d) *Nitric, perchloric, and hydrochloric acids.*—(Instra-Analyzed, J.T. Baker, St. Louis, MO)

(e) *Sulfuric acid.*—Ultrapure (Seastar Chemicals, Seattle, WA)

(f) *Sodium borohydride solution.*—0.6%. Dissolve four 0.3 g pellets of $NaBH_4$ (98%, pellet, Alfa/Ventron, Danvers, MA) in 200 mL of 0.5% (w/v) sodium hydroxide. Prepare fresh solution daily.

(g) *Reference materials.*—(*1*) Bovine Liver SRM-1577a [National Institute of Standards and Technology (NIST), Gaithersburg, MD]. (*2*) Trace elements in water SRM-1643b (NIST). (*3*) Rice flour SRM-1568 (NIST). (*4*) Dogfish muscle reference material for trace metals DORM-1 [National Research Council Canada (NRCC), Ottawa, Canada]. (*5*) Dogfish liver reference material for trace metals DOLT-1 (NRCC). (*6*) Lobster hepatopancreas marine reference material for trace metals and other elements TORT-1 (NRCC).

(h) *Standards.*—Intermediate standards are 1 mg/L and 10 mg/L As in 1% HNO_3. Prepare calibration standards in 10 mL digestion tubes by pipetting 0.100 mL of 1 mg/L intermediate for the 10 μg/L standard and 0.300 mL of 10 mg/L intermediate for the 300 μg/L standard. Also prepare a blank. Digest blanks and standards in the same way as samples.

Sample Preparation

Weigh 1.0–1.5 g wet tissue, or weigh 0.25–0.50 g desiccated tissue, or pipet 1 mL blood into 10 mL digestion tubes. For desiccated tissue samples, add about 0.5 mL water and vortex. Add 3 mL concentrated HNO_3 and one PTFE boiling stone to each tube. Dissolve sample first at room temperature, then on the digester ramped up to 175°C and hold for 90 min. Cool to room temperature. Add 1 mL $HClO_4$ and 1 mL H_2SO_4, then mix gently. Complete digestion as shown below:

Temperature, °C	*Ramp, min*	*Hold, min*
190	30	60
210	10	20
250	20	20
310	20	25

The procedure can be interrupted at this stage for up to 3 days without adverse effects. Cool to room temperature, then slowly add 7 mL of 5M hydrochloric acid. The first few drops of 5M hydrochloric acid should be added very slowly with swirling to prevent spattering. Add 1 mL 10% KI solution. Vortex mix solution, then return tubes to digester to reduce at 95°C for 15 min. Cool to room temperature and dilute to 10 mL mark with deionized water. Vortex mix tubes thoroughly. Analyze samples the same day.

Modifications to the procedure for other matrixes have been discussed in Part 1 of this series (1). The same modifications can be applied to determination of As. For water, add 1 mL H_2SO_4 to 10 mL sample, then heat sample at 105°C until volume is reduced to 2–3 mL. Then add 1 mL $HClO_4$ and continue digestion beginning at 190°C.

The high temperature perchloric acid digestion described here is potentially dangerous. Excessive amounts of organic matter can react violently with hot perchloric acid. *Note:* Do not exceed 0.5 g of organic material/digestion. For samples with a high fat content, use no more than 0.5 g of sample. Bumping during digestion is likely when a copious precipitate forms; therefore, we recommend against highly calcareous samples (e.g., bone or shell) and soils with this procedure. Of course, a properly functioning perchloric acid fume hood and personal safety equipment must be used at all times.

Sample Analysis

Connect hydride generation manifold with concentrated hydrochloric acid introduced at the first mixing tee and sodium borohydride solution introduced at the second tee. Ignite torch and then start manifold pump. Adjust reflected power to below 10 W. Let system warm up and stabilize for 20 min.

Calibrate instrument with 0, 10, and 300 μg/L As standards at the beginning of each run. The root-mean-square nonlinearity of the calibration line should be less than 4% for an acceptable calibration. Analyze a 10 μg/L As standard after every 20 samples to verify that drift is within acceptable limits.

Interference Study

Prepare a set of 100 μg/L As solutions by pipetting 0.100 mL of 10 mg/L As intermediate standard into a series of 10 mL digestion tubes. Pipet in the appropriate volumes of 1000 mg/L stock metal solution to yield the desired concentrations of interfering ion. Add 1 mL of $HClO_4$ and 1 mL of H_2SO_4 to each. Continue with sample preparation procedure beginning with the 190°C digestion. Prepare blanks, controls, and calibration standards in the same way. The metals tested in this way were Al and Fe up to 150 mg/L, Cu up to 50 mg/L, and Pb and Hg up to 5 mg/L.

Spike Recovery Studies

We spiked 3 matrixes (tap water, SRM 1568 rice flour, and SRM 1577a bovine liver) with arsenic to produce prepared solutions with arsenic concentrations from 5 to 300 μg/L. Each spike was prepared in duplicate. A theoretical arsenic concentration for each spiked sample was computed using the actual weight of matrix and the certified concentrations for SRM 1568 and SRM 1577a, and the average of 10 determinations for tap water. To 10 mL of tap water, ca 0.3 g of SRM 1568, or ca 0.3 g of SRM 1577a, we added 0, 50, 100, 500, 1000, 2000, or 3000 ng of As and then prepared each to a final volume of 10 mL.

We used the AOAC recommended statistical treatment in Wernimont (24) to evaluate the data. This treatment breaks the systematic and random error into constant and proportional terms by calculating a linear regression of recovered As against theoretical As.

Results and Discussion

The instrument detection limit is calculated as 3× SD of the intensity of the blank standard (which is integrated thrice) times the slope of the calibration line. In practice, this value is 0.4 to 0.6 μg/L As. Replicate analysis of unknown samples in each batch is used as a routine quality control measure to evaluate repeatability of the procedure. Relative standard deviations for 27 pairs of water samples determined over a period of 6 months gave a log-normal distribution where 90% of the pairs have <12% RSD and the geometric mean was 1.2% RSD. Twelve pairs of liver samples had <12% RSD at the 90th percentile and a geometric mean of 2% RSD. These statistics include the uncertainties of both sampling and determination.

We selected Fe, Al, Cu, Pb, and Hg as the interferents most likely to be present in samples submitted to our laboratory. No chemical interferences were observed in the hydride generation process by iron or aluminum up to 150 mg/L,

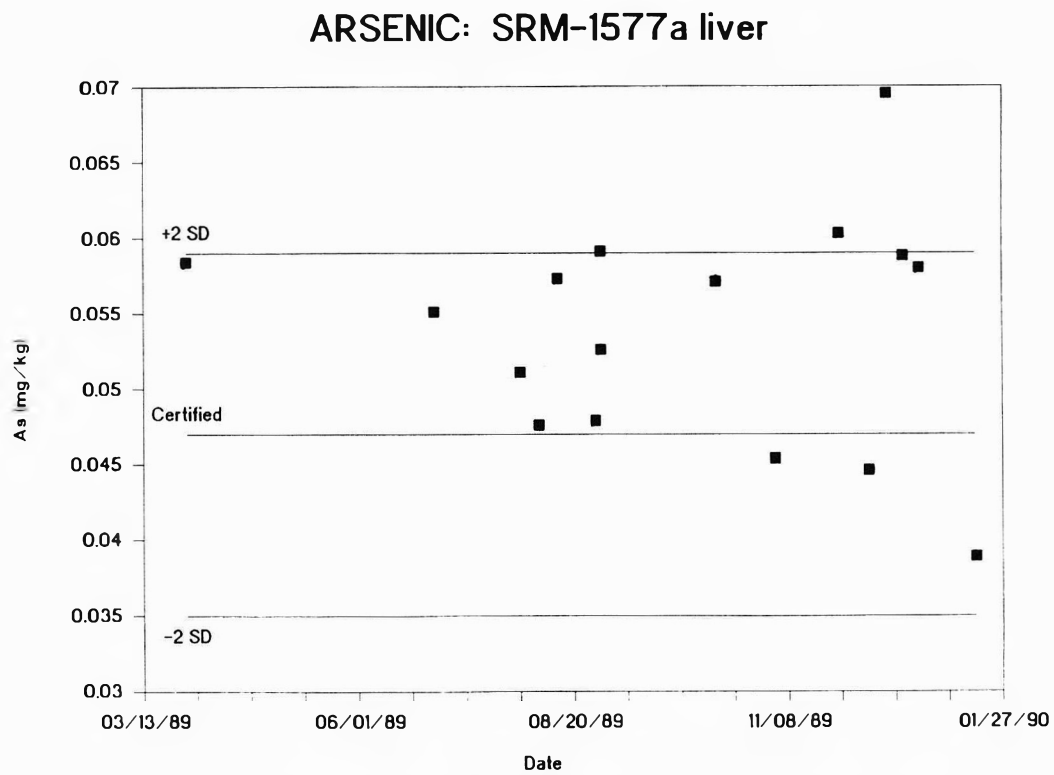

Figure 1. NIST Bovine Liver 1577a control chart for arsenic VGICP method.

copper up to 50 mg/L, or lead up to 5 mg/L, which were the highest levels tested. Pyen & Browner (14) studied these and other interfering ions in a substantially similar system, and our findings are in agreement with theirs. Mercury did not cause any effect at up to 5 mg/L. In contrast, in studies on selenium, selenium was sensitive to mercury interference (1). The off-peak background correction point at +050 nm was selected after scanning a prepared As standard under VGICP conditions. Aluminum caused a small increase in the off-peak background correction, but did not affect the overall measurement. We attribute the smallness of spectral effect to the relative enrichment of volatile AsH_3 versus the nonvolatile metals in the plasma.

As with the analogous selenium procedure, a significant

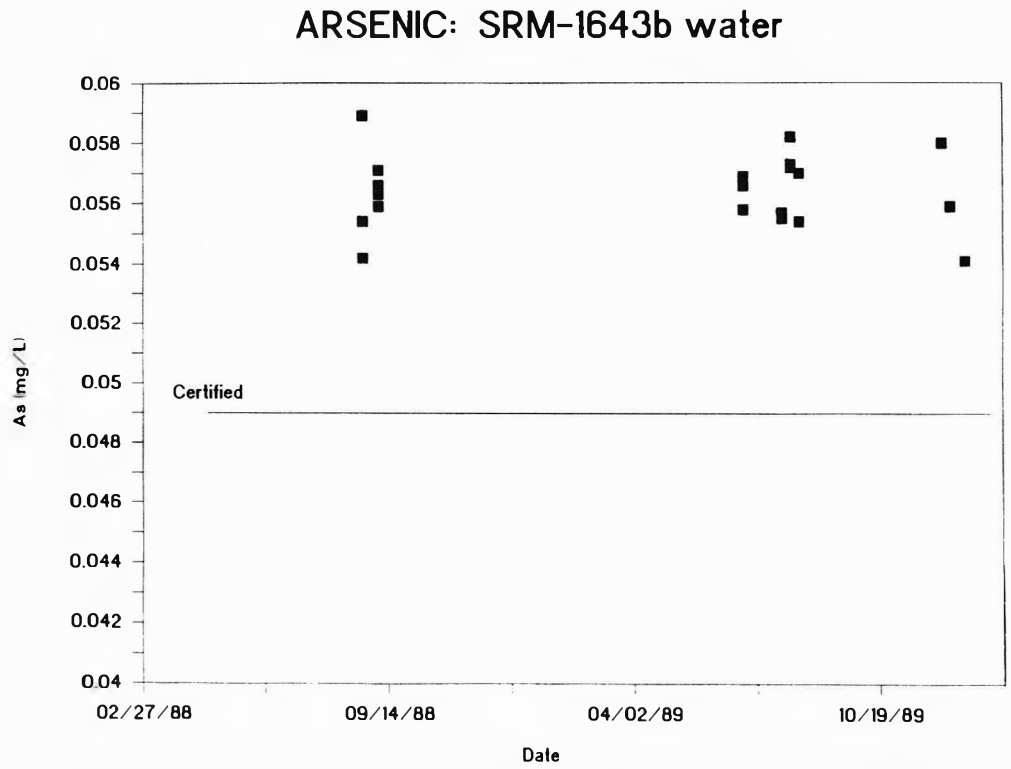

Figure 2. NIST Trace Elements in Water 1643b control chart for arsenic VGICP method.

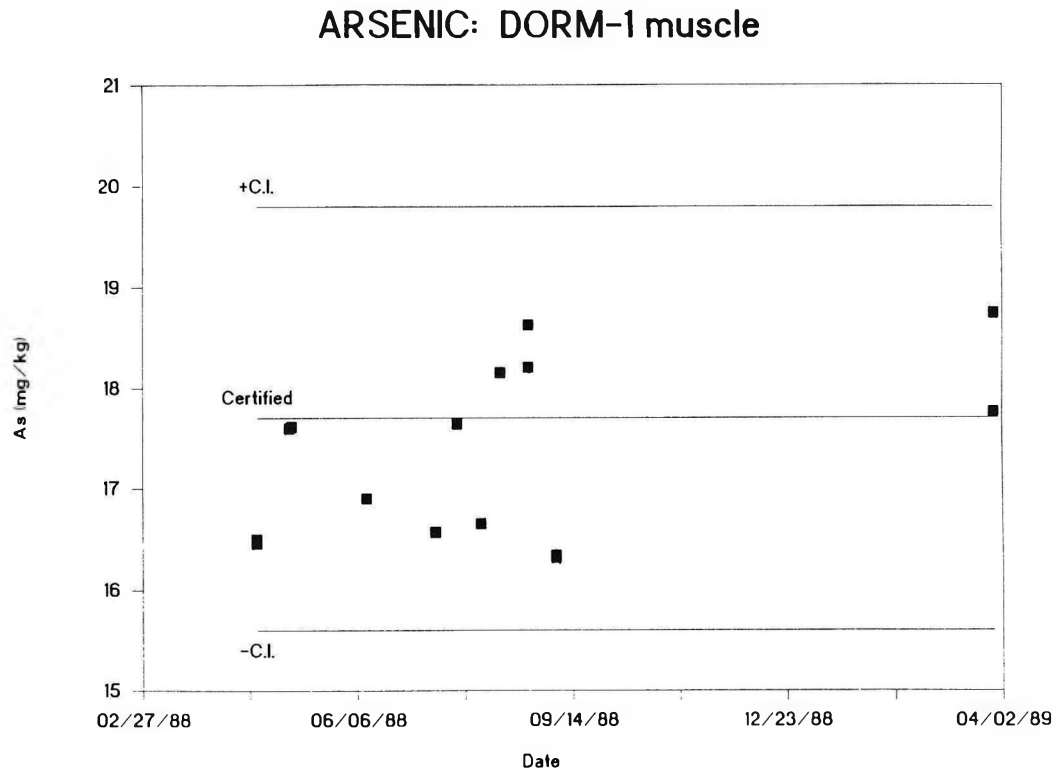

Figure 3. NRCC DORM-1 control chart for arsenic VGICP method.

amount of boron contamination in the torch and waste trap occurs due to the $NaBH_4$ solution, which can cause blanking problems with a subsequent boron determination. Also, iodine vapor forms in the waste trap, which causes problems with iodine determinations. Iodine also has a spectral overlap with a commonly used phosphorus emission at 178.287 nm. For these reasons, the waste trap and torch need to be thoroughly cleaned or changed before attempting ICP determination of B, I, or P.

The ruggedness of the VGICP arsenic method in routine analysis was tested by analysis of numerous standard reference materials over an 18 month period. These included SRM 1577a bovine liver, SRM 1643b trace elements in water, DORM-1 dogfish muscle, DOLT-1 dogfish liver, and

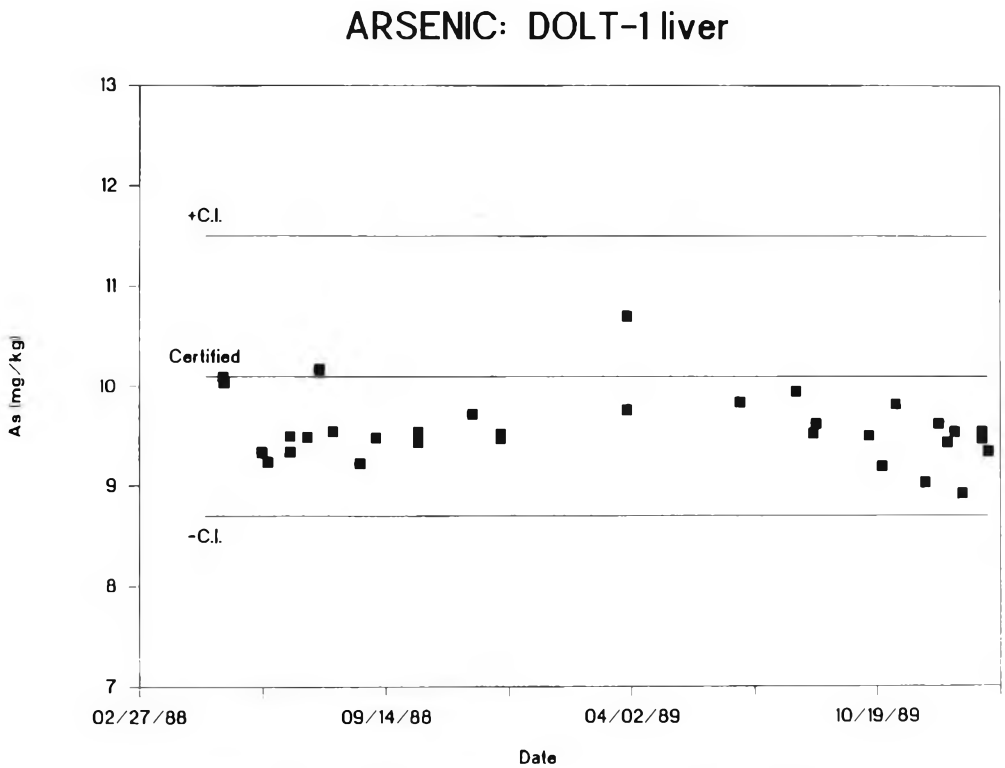

Figure 4. NRCC DOLT-1 control chart for arsenic VGICP method.

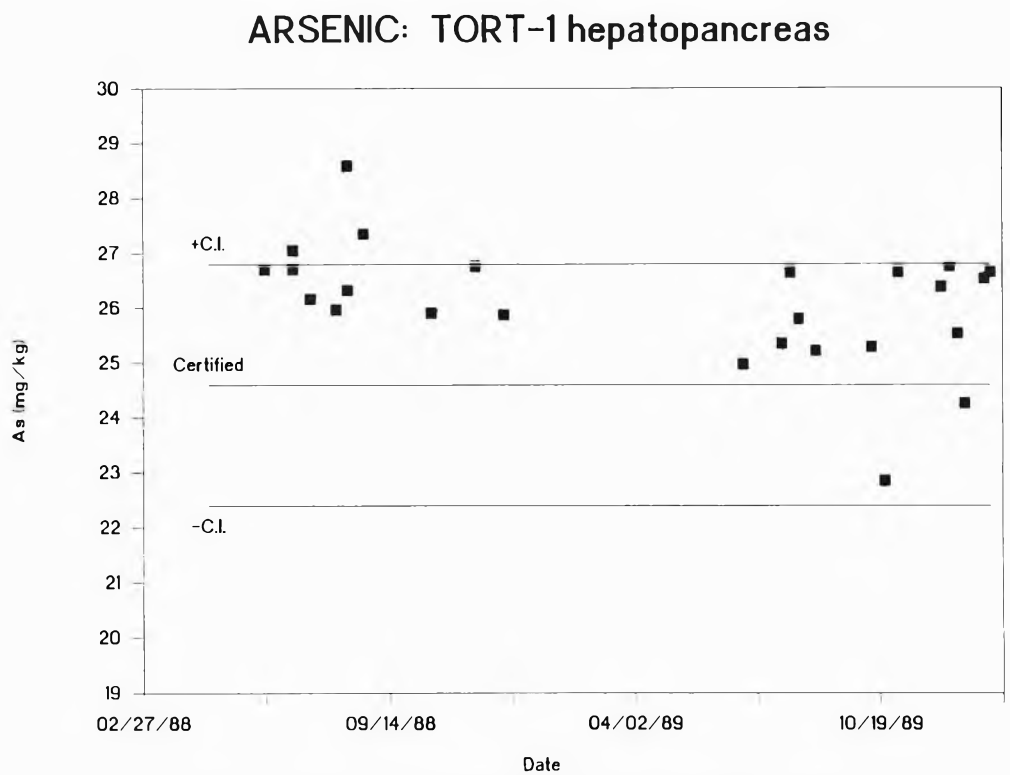

Figure 5. NRCC TORT-1 control chart for arsenic VGICP method.

TORT-1 lobster hepatopancreas. Results of the analyses over the study time period are presented in Figures 1 through 5. Mean values and standard deviations obtained are listed in Table 1. Excellent correlation with published values is found for DORM-1, DOLT-1, and TORT-1. Recovery for SRM 1577a is high (130%) and the RSD is high (26%). This is due, in part, to the low concentration, (<4 times the method detection limit for a 0.3 g sample); however, there may also be some method bias.

Recoveries of spikes from tap water, bovine liver, and rice flour are shown in Table 2. We selected these matrixes as being representative of typical samples submitted to our laboratory. After regression analysis, tap water had an offset of 0.6 ± 3 μg/L and a percent recovery of $103 \pm 1\%$. The bovine liver had an offset of 1.6 ± 4 μg/L and a percent recovery of $104 \pm 1\%$. Rice flour had an offset of -0.6 ± 3 μg/L and a percent recovery of $99 \pm 1\%$. Within the precision of the experiments, none of the offset errors were significantly different from zero. Precision of the regression lines was not significantly different than precision of the replicates; therefore, recovery of As is linear over the tested range.

Our laboratory participates in interlaboratory reference material exchanges sponsored by the Department of Land, Air, and Water Resources of the University of California, Davis. Participants include the federal government, state government, and academic and commercial laboratories in the western United States. The choice of analytical technique is left to the individual laboratories. In 1989, we analyzed

Table 1. Reference material arsenic concentrations (mg/kg) by VGICP

Reference material	Reference value	Vapor generation ICP value	Count
NIST			
Bovine liver 1577a	0.047 ± 0.006[a]	0.061 ± 0.016[a]	20
Trace elements in water 1643b	0.049[b]	0.058 ± 0.005[a]	23
NRCC			
DORM-1	17.7 ± 2.1[c]	17.3 ± 0.4[c]	15
DOLT-1	10.1 ± 1.4[c]	9.3 ± 0.5[c]	34
TORT-1	24.6 ± 2.2[c]	26.1 ± 0.4[c]	25

[a] Standard deviation.
[b] Listed but not certified.
[c] 95% confidence interval.

Table 2. Recovery of arsenic from tap water, bovine liver, and rice flour[a]

As added, μg/L	Tap water		Bovine liver		Rice flour	
	Theor.	Found	Theor.	Found	Theor.	Found
0	—[b]	2.9	1.5	2.9	13.7	11.7
0	—[b]	2.9	1.4	2.7	14.4	13.2
5	7.9	7.6	6.4	7.1	17.8	17.1
5	7.9	7.8	6.4	7.8	17.0	16.1
10	12.9	12.8	11.5	12.2	23.0	20.7
10	12.9	12.9	11.5	13	22.6	20.8
50	52.9	55.2	51.4	54.3	62.7	61.8
50	52.9	55.7	51.5	54.3	63.7	63.2
100	102.9	106.0	101.4	119.2	114.1	114.4
100	102.9	108.5	101.5	107.3	112.9	117.4
200	202.9	212.8	201.5	209.5	212.0	204.0
200	202.9	209.9	201.5	207.4	212.8	211.2
300	302.9	305.2	301.4	313.7	312.9	311.4
300	302.9	313.9	301.4	319.6	313.4	309.8
		Regression analysis				
Slope:	1.026		1.043		0.993	
Intercept:	0.6		1.6		−0.6	
$s_{y \cdot x}$:	2.7		4.0		2.6	
		Duplicates				
Pooled s:	2.8		3.6		2.2	

[a] Concentrations are for prepared solutions as analyzed in μg/L.
[b] Concentration of As was not known in advance.

naturally occurring vegetation, sea water, surface water, and drainage water for arsenic and other elements (25). In 1988, we analyzed certified reference materials of animal tissue and water (25). We did not analyze soil or sediment matrixes that were offered. In both instances, the sponsor judged the performance of the VGICP method for arsenic acceptable.

In principle, the VGICP process can be applied to any elements that can be converted to a volatile form. The practical difficulties come in the preparation of the sample and formation of the volatile species. ICP also allows the possibility of multi-element analysis if suitable conditions can be found. On the basis of 1 experiment, we believe that using KBr or HBr in place of KI may allow simultaneous measurement of selenium, arsenic, and some other hydride-forming elements. There is work in progress in our laboratory on determination of mercury by VGICP that will be reported when final.

Conclusion

The VGICP method for arsenic determination demonstrates the statistical control, accuracy, and precision required for routine analysis of biological materials. Digestion and analysis protocols are fully amenable to programmed operation and autosampling. Analysis requires minimal sample, a strong consideration in biomedical analysis. The thousand-fold linear range and the substantial freedom from spectral and chemical interference make VGICP a rigorous method for arsenic analysis.

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MICROBIOLOGICAL METHODS

A Resuscitation/Selection System for Rapid Determination of *Salmonella* in Foods

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A resuscitation medium was developed consisting of a trypticase soy broth base supplemented with 0.5% yeast extract, 0.25% sodium pyruvate, 0.01% sodium thioglycollate, and 0.1% chicken fat. After a resuscitation period of 4 h, the medium was made selective by addition of either sodium thiosulfate, bile salts and iodine, or sodium selenite and L-cystine. The now selective medium was incubated for 16 h. The presence or absence of *Salmonella* was determined by the *Salmonella*-Tek antibody-based detection kit. The present system was compared with a method of the *Bacteriological Analytical Manual* (BAM) for naturally contaminated foods. Nineteen egg products were screened; 3/19 were positive using the BAM method, 9/19 were positive using the present system. Seventeen chicken samples were assayed; 10/17 were positive using the BAM method; 13/17 were positive using the present system. Of 8 pepper samples, 4/8 were positive using the BAM method; 6/8 were positive using the present system. Of 8 spice samples, 6/8 were positive using the BAM method, 7/8 were positive using the present system. Of 6 onion products sampled, 5/6 were positive using the BAM method; 6/6 were positive using the present system.

Roberts reported that *Salmonella* infections caused 66–86% of total food poisoning cases reported in England and the United States (1). The most common vectors of salmonellosis in man are poultry, eggs, beef, egg products, and milk (2–8). Between 53.1 and 61.9% of inspected broiler carcasses were reported contaminated with *salmonellae* (1, 8). Between 1976 and 1986, *Salmonella enteritidis* infections associated with egg-containing products increased some 6-fold (9).

The development of rapid methods for detection of pathogens in foods has been emphasized in recent years because the standard microbial methods for detection of pathogens in foods require at least 3 days to obtain results (2, 6, 10–15). In the past few years, there has been a proliferation of rapid detection systems. One of the most successful approaches has been the enzyme immunoassay or enzyme immunosorbent assay (11, 16–20). These methods use antibodies to determine the presence of a specific pathogen. While the aforementioned techniques perform well, the systems use conventional enrichment and selection systems. Hence, these methods improve detection; unfortunately, the speed of the assay remains a problem.

The present paper reports the development of a resuscitation medium that enhances recovery of injured pathogens within a 4 h period before making the medium selective for the improved detection of *salmonellae*. The resuscitation medium consists of a trypticase soy broth (TSB) as the liquid base with the following ingredients added: 0.5% yeast extract (as a protein source) to enhance recovery of injured cells under anaerobic conditions; 0.25% sodium pyruvate to neutralize the effects of H_2O_2 produced by injured cells; 0.01% sodium thioglycollate to produce an anaerobic environment, inhibit the production of H_2O_2 and stabilize the oxidation-reduction potential of the medium; and 0.1% chicken fat as a source of saturated and unsaturated fatty acids needed for membrane repair (21). Glucose, the energy source, is supplied from the TSB base. Use of the resuscitation medium enhanced recovery of *salmonellae* and shortened the time necessary for detection to 20–24 h.

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METHOD

Resuscitation Medium

The medium consists of a TSB base (Difco Laboratories or BBL) reconstituted as described and supplemented with 0.5% yeast extract (Difco or BBL), 5.0 g/L; 0.25% sodium pyruvate (Sigma Chemical Co.), 2.5 g/L; 0.01% sodium thioglycollate (Sigma), 0.10 g/L; and 0.1% chicken fat (Capital City Products Co.), 1.0 g/L. The chicken fat was mixed in Tween 80 at a ratio of 3 parts Tween (3 g) to 1 part chicken fat (1 g) in 100 mL water before addition to 1 L of the medium.

Selective Medium

To make the resuscitation medium selective for *salmonellae*, supplement TSB with 8.0 g sodium selenite and 0.02 g L-cystine/L or a 2× quantity of *m*-tetrathionate broth (Difco) containing 60 g sodium thiosulfate and 2 g bile salts/L. After the resuscitation period, add an equal volume of TSB containing the 2× concn of selective agents to the resuscitation medium. Immediately after adding the selective agents for tetrathionate, add 2 mL of a stock iodine solution (containing 6 g iodine crystals and 5 g potassium iodide/20 mL deionized water) per 100 mL of the resuscitation medium. Regardless of the selective medium ingredients added, all incubations in these selective media are for a minimum of 16 h at 35°C.

Production of Heat and Freeze-Injured Cells

The 4 pathogenic organisms used in these studies are *Salmonella bonn*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. Cultures of these organisms were grown to the stationary phase overnight at 35°C in TSB.

Before preparing injured *Salmonella* cells, centrifuge cells grown to stationary phase, wash pellet once in sterile water, and resuspend in 10 mL of 1/10th concn TSB. Prepare freeze- and heat-injured cells by the method of Ray (22). Prepare freeze-injured *Salmonella* cells by placing in a blast freezer at −30°C for 24 h. Remove cells and hold at 6°C for 30 min before use. Prepare heat-injured cells by heating washed cells to 52°C for 30 min and cooling to room temperature.

Determination of Incidence of Cell Injury

Remove samples from injured-cell preparation and spread-plate onto both trypticase soy agar plates containing

1% sodium pyruvate and trypticase soy agar plates containing 2% NaCl (22). Incubate plates at 35°C for 16 h. Count the number of colony forming units (CFUs) growing on the trypticase soy agar with 1% sodium pyruvate. These CFUs represent both injured and noninjured cells. Count CFUs growing on the salt-containing plates; these represent noninjured cells. Determine recovery incidence from the difference in numbers on both plates.

Analysis of Food Samples

Place a 75 g sample of the food to be assayed into a sterile blender jar containing 375 mL sterile water. Blend food samples and divide the sample blend in thirds by placing into three 500 mL sterile erlenmeyer flasks. For the method found in the *Bacteriological Analytical Manual* (BAM) (23), add 125 mL double strength sterile lactose broth into a sample flask. Mix and proceed as described in the BAM procedure.

Add 125 mL sterile double strength resuscitation medium to each of the remaining sample blends. Mix each sample blend and incubate for 4 h at 35°C. At the end of the 4 h incubation, make the medium selective through the addition of TSB supplemented with 2X concn selenite-cystine or tetrathionate broth. Incubate samples at 35°C for an additional 4 h. After second incubation, remove a subsample from each flask and assay for the presence of *salmonellae* using the *Salmonella*-Tek ELISA test system (Organon-Teknika, Durham, NC). Remove sample for analysis after 20–24 h and again screen for the presence of *salmonellae* using *Salmonella*-Tek ELISA kit (24). Confirm positives by streaking from selective medium onto plates containing the selective medium used in the BAM procedure. Select typical colonies and confirm/identify *Salmonella*. We used the API 20E system (Analytab Products, Plainview, NY).

Detection of Salmonellae in Spices

The previously described method for detection of *salmonellae* was modified because of the inhibitory nature of spice extractives. Incubate samples of spices in resuscitation medium for 24 h. Remove a 10 mL subsample and transfer to 90 mL of a selective medium (normal concn) and incubate as described. After the 8 h and 20 h incubations at 35°C, remove subsamples and assay for the presence of *salmonellae*, as described previously.

Results and Discussion

Rapid methods for detection of pathogens in food are needed because current microbial methods require at least 3 days to obtain results. Antibody-based kits for detection of *salmonellae* enhance detection of healthy and uninjured cells from selective media. Antibody-based test kits require the pathogen population to reach at least 10^4 to 10^5 cells/mL before detection is possible.

Injured microorganisms occur in many food products. These microorganisms suffer nonlethal injury from stresses due to heat, freezing, low pH, sanitizers, and low Aw (25–31). Such injuries can cause a loss of cellular membrane integrity, degradation of ribosomal ribonucleic acid (31–34), reduced enzyme activity, and changes in transport kinetics (22, 35, 36). The major characteristic of an injured cell is the cell's inability to tolerate the conditions and growth media in which normal cells would survive and grow (8, 37).

In the present paper, only the data using heat-injured *Salmonella bonn* are presented in the interest of brevity. Results obtained using freeze-injured cells were superimposable with results from heat-injured cells. About 90% of *Salmonella bonn* cells prepared as injured cells (either heat-injured or freeze-injured) were determined to be injured.

Resuscitation of Injured Salmonella in a Mixed Population

A factor that must be addressed is the presence of other organisms or pathogens that compete with *salmonellae* for nutrients. In many instances, *salmonellae* are present in relatively low numbers, while other organisms such as *E. coli* can be present in relatively large numbers (1, 8, 13–15, 38).

Before determining the presence of *salmonellae* in foods, it was necessary to determine the ability of injured cells to be resuscitated in the presence of other organisms and to grow after the medium was made selective. Equivalent numbers of injured cells of *Salmonella bonn* (approximately 10 cells/mL) were placed into the resuscitation medium with equivalent numbers of uninjured *E. coli*, *S. aureus*, and *Listeria monocytogenes* and resuscitated for a period of 4 h before being made selective through the addition of the selective agents in selenite-cystine or tetrathionate media. An equal portion of the same mixture was placed directly into the BAM pre-enrichment medium (lactose broth) without the resuscitation step. After 8 and 20 h incubation periods, broths were sampled, diluted serially, and spread-plated onto selective media for *Salmonella* (XLD agar), *E. coli* (EMB agar), *S. aureus* (VJ agar) and *Listeria monocytogenes* (phenylethanol agar). Plates were incubated at 35°C for 48 h. CFUs were counted and competitive growth of the resuscitated, heat-injured *Salmonella bonn* was determined. Figure 1 shows that the injured *Salmonella bonn* cells that have been resuscitated grow well in the presence of other microorganisms when placed into the selective medium. At the end of an 8 h period, the numbers of organisms were 100-fold greater than all the other added microorganisms, although their initial numbers were essentially identical. After 20 h, the numbers of *Salmonella bonn* were 10^3-fold greater than

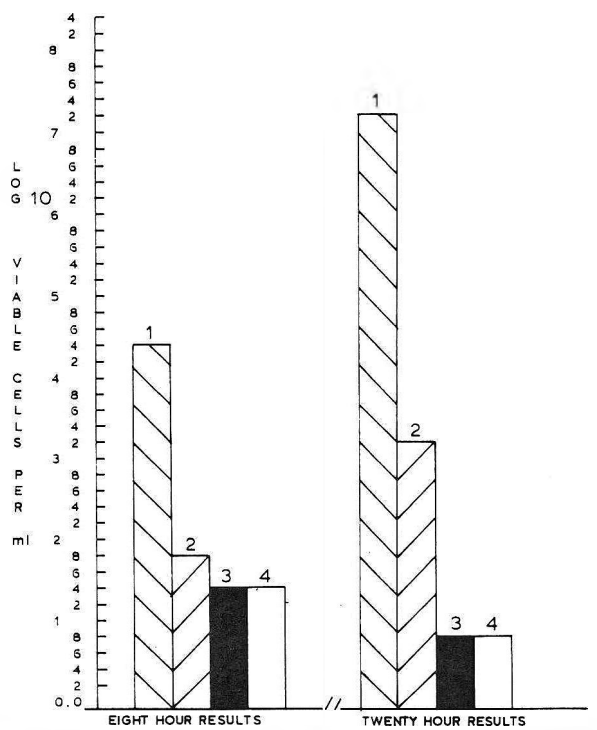

Figure 1. Competitive growth of bacteria in the resuscitation/selective medium for *salmonellae*; 1-*Salmonella bonn*, 2-*Escherichia coli*, 3-*Staphylococcus aureus*, and 4-*Listeria monocytogenes*.

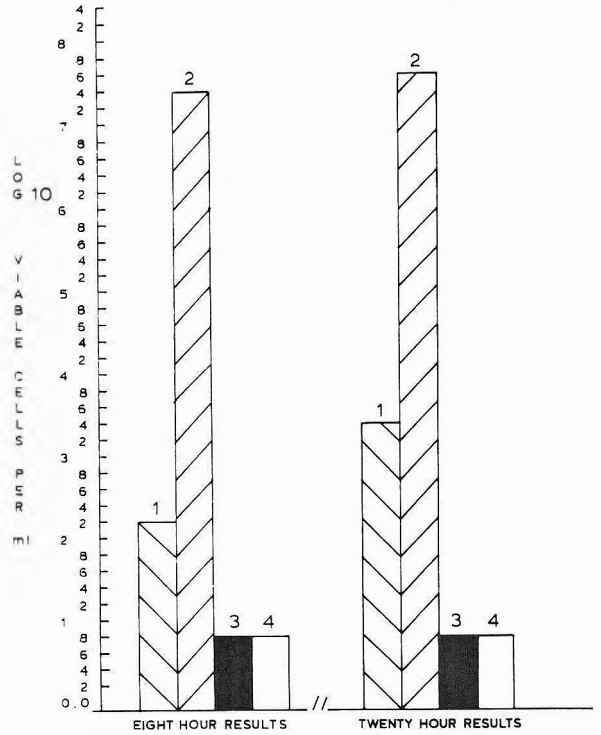

Figure 2. Competitive growth of bacteria in the BAM pre-enrichment lactose broth for isolation of *salmonellae*; 1-*Salmonella bonn*, 2-*Escherichia coli*, 3-*Staphylococcus aureus*, and 4-*Listeria monocytogenes*.

those of *E. coli* and 10^6-fold greater than those of *S. aureus* and *Listeria monocytogenes*. Figure 2 shows results when injured *Salmonella bonn* were grown in the BAM pre-enrichment medium for isolation of *salmonellae*. The *Salmonella bonn* cells were at a distinct competitive disadvantage in this medium because they cannot use lactose. The numbers of *Salmonella bonn* rose 10-fold in 8 h; by comparison, *E. coli* grew from approximately 10 cells/mL to approximately 7.5×10^7 cells/mL. At the end of 24 h, the cell numbers of *Salmonella bonn* rose to approximately 10^4 cells/mL; the numbers of *E. coli* remained relatively constant at approximately 10^8 cells/mL.

The growth of the competitive organisms studied is enhanced in lactose broth, making development of a rapid system for detection of *salmonellae* virtually impossible. Such competitive growth increases the possibility that low levels of *salmonellae* could go undetected simply because the medium was unsuitable. By comparison, the resuscitation step allows damaged cells to be repaired within 4 h. Use of the selective growth media allowed the numbers of *Salmonella bonn* cells to increase from 10 organisms/mL to ca 1.4×10^4/mL after 8 h and to ca 2×10^7/mL after 20 h (Figure 1). Thus, the numbers of organisms have increased to the levels in which the presence of *salmonellae* can be determined using antibody-based kits. The numbers of a competitive organism, such as *E. coli*, did not rise significantly above 1.2×10^3 organisms/mL during this period.

Comparison with BAM Procedures

Five different food types were analyzed for the presence of *salmonellae* using the BAM procedure (23) and the present rapid resuscitation system. Table 1 shows results when procedures were compared on the same samples. The BAM procedure confirmed the presence of *salmonellae* in 3 samples of the egg product; the resuscitation system confirmed the presence of salmonellae in 9 samples. Egg samples were treated with hydrogen peroxide during manufacture, which undoubtedly damaged any contaminating bacterial cells. The usefulness of the resuscitation medium was demonstrated by the fact that 3 times as many samples were found to contain *salmonellae* as were found by the BAM procedure. A similar pattern was found in the analysis of chicken products. The BAM procedure found 10 of 17 samples positive; the resuscitation system found 13 samples positive. Even in products in which one could hypothesize a higher potential for having contaminated samples, the resuscitation procedure yielded a higher incidence of contamination than the BAM procedure.

Table 1. Comparison of results for *Salmonella* in naturally contaminated food samples

Food type	No. of samples	BAM positive samples	Resuscitation methods: Positive samples,[a] 8 h	Resuscitation methods: Positive samples, 20 h
Egg product	19	3	3	9
Chicken	17	10	6	13
Pepper	8	4	3	6
Spices	8	6	4	7
Onions	6	5	2	6

[a] Selenite-cystine and tetrathionate as the selective reagents resulted in identical results.

Pepper, spices, and onions required an alternative procedure. When all these products were pooled under the general category of spices, the resuscitation procedure indicated that 19 of 22 samples were contaminated; the BAM procedure found 15 of 22 samples contaminated. Again, even in products that exude inhibitory extractives, the resuscitation system yielded a greater frequency of positive samples. Of 58 samples assayed, the resuscitation procedure found 41 to be contaminated; the BAM procedure found 28. All positives found using the antibody-based test kit were confirmed/identified after isolation of typical colonies and use of the API 20E system.

The concept of a resuscitation period for injured cells is not new; many reports can be found in the literature (6, 8–10, 12, 14, 15, 17, 18, 22, 25, 28, 32, 38, 39). However, the problem of other organisms competing with *salmonellae* and outgrowing them was not addressed in such a fashion as to minimize the problem.

One other factor associated with recovery of *salmonellae* from foods using the BAM procedure is the subsampling procedure. If the numbers of *salmonellae* are low, placing an aliquot of the sample in a selective medium might very well result in a failure to detect the pathogen. Unlike true solutions, bacterial suspensions are not uniform distributions of organisms. If the subsample contains too few organisms, results may not indicate presence of the pathogen. With the exception of procedural modification used for analysis of spices, the resuscitation procedure minimizes this possible source of error by eliminating the need to transfer aliquots of pre-enrichment medium to a selective medium.

The use of a 4 h resuscitation followed by making the medium selective is both a cost-effective and time-effective approach to screening food samples for the presence of *salmonellae*. Detection of *salmonellae* using antibody test sys-

tems uses these systems to their fullest, because the steps that precede use of the antibody systems are the time-consuming aspects of the assay. For those who prefer final confirmations using classical approaches, the resuscitation/selection assay system offers the advantage of a speedier confirmation.

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Defined Substrate Technology Method for Rapid and Specific Simultaneous Enumeration of Total Coliforms and *Escherichia coli* from Water: Collaborative Study

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The defined substrate technology (DST) method is a reagent system designed to enumerate specific target microbes(s) from a mixture of bacteria. The system simultaneously enumerates total coliforms and *Escherichia coli* directly from a water sample. The reagent contains *o*-nitrophenyl-β-D-galactopyranoside (ONPG), which is hydrolyzed by total coliforms to produce a yellow chromogen, and 4-methylumbelliferyl-β-D-glucuronide (MUG), which is hydrolyzed and fluoresces when *E. coli* organisms grow. Noncoliform bacteria are suppressed and cannot metabolize the indicator nutrients. Nine laboratories participated in a field evaluation of the method, which covered a wide range of surface and subsurface water sources and water-processing modalities, including the examination of natural samples. The DST system was compared to multiple-tube fermentation (MTF) (quantitative) and presence-absence (P-A) (qualitative) *Standard Methods* formats. Comparison of water samples from natural sources by using the most probable number (MPN) procedure showed that the DST test was equivalent to the currently used MTF test. Results from the DST and the qualitative P-A procedure showed that these tests agreed with each other in 94% of the water samples analyzed. Specificity of the DST method was established by subculturing a species consistent with a total coliform or *E. coli* from each positive tube. Eight laboratories participated in a collaborative study of the method. Each laboratory received 3 concentrations of *E. coli* (organisms/100 mL): 10 (low); 60 (medium); and 120 (high). The DST test was inoculated from a split sample of each bacterial density in parallel with *Standard Methods* brilliant green lactose broth. Statistical analyses for repeatability and reproducibility showed the DST system to be equivalent to currently used standard methods. The DST method has been adopted official first action by AOAC for detection and enumeration of total coliforms and *E. coli* in water.

The 2 standard formats for enumeration of coliforms in water are the multiple-tube fermentation (MTF) and the membrane filter (MF) techniques. Both methods require several sequential levels of analysis, the entire examination requires between 2 and 6 days, and these tests detect only 1 class of indicator bacteria, the total coliforms.

The MTF and MF methods are subject to false-positive and false-negative results. False-negative tests are most commonly caused by suppression of total coliforms by noncoliform heterotrophic bacteria (1, 2), inability to recover chlorine-damaged bacteria (3, 4), and difficulty in recognizing sheen colonies in the laboratory (2). False-positive results can occur because of the ability of certain species of noncoliform heterotrophs, most notably *Aeromonas* spp., to yield gas in the confirmation step (2) and the synergistic production of gas from lactose by noncoliforms (5, 6).

The defined substrate technology (DST) system utilizes an enzyme substrate as a nutrient source, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) for total coliforms and 4-methylumbelliferyl-β-D-glucuronide (MUG) for *Escherichia coli* (approximately 40% of *Shigella* and 10% of *Salmonella* also have this enzyme). ONPG is hydrolyzed by total coliforms to produce a yellow chromogen and MUG is hydrolyzed by growth of *E. coli* and fluoresces. Direction of the metabolic activity of the target microbe toward the substrate is a distinctive feature. Microbes other than target organisms usually cannot appreciably grow or metabolize in the DST reagent system and, therefore, do not affect the test. Noncoliform bacteria are suppressed and cannot appreciably metabolize the indicator nutrients (7).

The DST system is a primary water test. The hydrolyzable substrates are used in a much different way than in traditional bacterial identification tests. In the DST system, the substrates support bacterial growth; transferring heavy inoculum could cause nonspecific reactions with either substrate. Therefore, the DST test is not recommended as a confirmatory test from other media, but rather as a primary method for enumerating and detecting coliforms and *E. coli* in water samples. The MPN format can be performed as either a 5-tube or 10-tube analysis. The P-A format detects presence or absence in 100 mL of sample.

Andrews stated "the ideal would be to use naturally contaminated food" in the evaluation of a new method for microbiological analysis (8). Accordingly, an extensive precollaborative study to meet U.S. Environmental Protection Agency (EPA) guidelines to establish equivalency between methods was conducted to verify the performance of the DST under a wide range of geologically and hydrologically diverse natural field conditions (9, 10). Comparisons were conducted on both a quantitative (MPN) and qualitative (MF) basis. Results showed the DST method to be equivalent to EPA accepted methods (1, 9, 11) in both formats (12, 13). At 2 of the 5 sites, the DST method was more precise (i.e., less variation); for the other 3, performance was equal. Results

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The report was evaluated and approved by the General Referee, the Committee Statistician, and the Committee on Microbiology and Extraneous Materials. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 10–13, 1990, at New Orleans, LA. Association actions were published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal Chem.* **74**, January/February issue.

from the DST and the qualitative procedure showed that these tests agreed with each other in 94% of the water samples analyzed. Specificity of the DST method was established by subculturing a species consistent with a total coliform or *E. coli* from each positive tube.

Another, independent precollaborative study conducted by the EPA itself also showed equivalent results in both formats (14). Using the extensive precollaborative studies as a base, a collaborative study was conducted according to the protocol of Miescier et al. (11).

Collaborative Study

In accordance with AOAC guidelines, the DST test was evaluated at 8 laboratories. A widely used quality control strain of *E. coli* (ATCC 25922) was used as the test isolate to verify the biochemical mechanisms for both the total coliform and *E. coli* parts of the DST method.

Preparation of Isolate

E. coli ATCC 25922 was grown in trypticase soy broth to a density of approximately 10^{10} bacteria/mL. It was diluted in sterile distilled water containing 0.01% nutrient broth to concentrations of 10/100 mL, 60/100 mL, and 120/100 mL. This broth has been used in previous work to dilute and stabilize bacterial suspensions (11). The bacterial suspensions were kept at 4°C and delivered within 4 h to the collaborating laboratories. Each preparation was divided in 2 aliquots to be run in parallel.

Instructions to Collaborators

Each collaborator received the prepared test isolate, a box of 200 MPN tubes to receive 10 mL of sample, and a supply of P-A tests to receive 100 mL. Additional tubes or P-A tests were supplied if requested. Other vessels, pipets, etc., were supplied by the collaborators.

On receipt, each laboratory inoculated a 5-tube MPN analysis using each of the 3 concentrations of test *E. coli*. Each tube received 10 mL inoculum. For the P-A format, each tube received 100 mL inoculum. Multiple-tube fermentation broths consisted of double strength BGLB. Large laboratory test tubes were used for the 10 mL MPN comparison and milk bottles for the P-A test. BGLB and DST tubes were inoculated in parallel; both volumes were processed in the same manner. The DST system was incubated at 35 ± 1.0°C. Tubes in which the sample became yellow within 24 h were considered positive for the total coliform part of the test. The yellow tubes were exposed to 366 nm ultraviolet light, 4 w, at a distance of approximately 2 in. Tubes in which the sample fluoresced were considered positive for *E. coli*. Tubes in which the sample remained colorless for up to 28 h were considered negative. The BGLB broths were incubated at 35 ± 1.0°C. Tubes showing gas were considered positive.

Results of analyses by both the proposed and the EPA approved methods were statistically analyzed for repeatability and reproducibility by AOAC approved procedures (15).

991.15 Total Coliforms and *Escherichia coli* in Water

Defined Substrate Technology (Colilert) Method

First Action 1991

Method Performance:

6.4 bacteria/100 mL (geom. mean 21.88; log geom. mean 1.34)
$s_r = 0.27$; $s_R = 0.35$; $RSD_r = 20.15\%$;
$RSD_R = 26.12\%$

39 bacteria/100 mL (geom. mean 93.33; log geom. mean 1.97)
$s_r = 0.32$; $s_R = 0.37$; $RSD_r = 16.24\%$;
$RSD_R = 18.78\%$

81 bacteria/100 mL (geom. mean 154.88; log geom. mean 2.19)
$s_r = 0.20$; $s_R = 0.39$; $RSD_r = 9.13\%$;
$RSD_R = 17.81\%$

A. Principle

Defined substrate technology (DST) reagent system simultaneously enumerates total coliforms and *E. coli* directly and separately from a water sample. Reagent contains *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG). After inoculation of DST test, a clear solution results. Only total coliforms can hydrolyze ONPG to produce yellow chromogen. Same test tube or vessel contains MUG, which is hydrolyzed and fluoresces when *E. coli* grow. β-Glucuronidase has been found specific to the genus *Escherichia* (*Escherichia* and *Shigella*) and *Salmonella*. Practically, from water samples, only *E. coli* yield a positive result. Metabolism of ONPG by β-D-galactosidase system of enteric bacteria is specific for total coliform group. Composition of inorganic salts in DST reagent does not support growth of nonenteric bacteria. Assay may be performed in most probable number (MPN) format or as presence-absence (P-A) test.

B. Apparatus

(a) *Tubes.*—Glass. 12 mL. Sterile, free of microbial inhibitors (e.g., residual detergent), and nonfluorescent at 366 nm.

(b) *Vessels.*—Glass. 120 mL. Sterile, free of microbial inhibitors (e.g., residual detergent), and nonfluorescent at 366 nm.

(c) *Long-wave ultraviolet light source.*—366 nm. 4 watt. Hand-held lamp (UVP, Inc., San Gabriel, CA; or equivalent).

C. Reagent

For each 1000 mL of sample, completely mix the following: ammonium sulfate, 5 g; manganese sulfate, 50 mg; zinc sulfate, 50 mg; magnesium sulfate, 100 mg; sodium chloride, 5 g; calcium chloride, 50 mg; potassium dihydrogen phosphate, 900 mg; disodium hydrogen phosphate, 6.2 g; sodium sulfate, 40 mg; amphotericin B, 1 mg; *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 500 mg; 4-methylumbelliferyl-β-D-glucuronide (MUG), 75 mg; and solanium, 500 mg. (Solanium is available from Access Analytical Systems, Inc., 21 Business Park Dr, Branford, CT 06405. Other ingredients are available from Sigma Chemical Co. and other sources. Prepared reagent mixture is available from Access Analytical Systems, Inc.) When mixture is dissolved in 1 L H_2O, pH should be 7.2 ± 0.1. Powdered formula is stable up to 1 year when kept dry and stored between 4 and 30°.

D. Enumeration

For MPN format, use sufficient reagent mixture (*see* C) in each tube to accept 10 mL sample; for P-A format, use 10 times that amount in each vessel. If laboratory-prepared

Table 1. Most probable number (MPN) estimates by DST method, using 3 levels of *E. coli* densities[a]

Coll.	Level 1, 6.4/100 mL		Level 2, 39/100 mL		Level 3, 81/100 mL	
1	5	23	31	79	70	70
2	13	7	23	33	70	33
3	33	49	49	350	170	240
4	23	49	170	350	540	170
5	7	13	49	170	350	170
6	49	49	110	240	240	350
7	49	42	130	49	240	540
8	13	49	79	130	49	70
Geometric mean[b]	21.88 (1.34)		93.33 (1.97)		154.88 (2.19)	
s_r	0.27		0.32		0.20	
s_R	0.35		0.37		0.39	
RSD_r, %	20.15		16.24		9.13	
RSD_R, %	26.12		18.78		17.81	

[a] Results for duplicate samples at each level at each site.
[b] Numbers in parentheses are log of geometric mean.

Table 2. Most probable number (MPN) estimates by multiple-tube fermentation method using 3 levels of *E. coli* densities

Coll.	Level 1, 6.4/100 mL		Level 2, 39/100 mL		Level 3, 81/100 mL	
1	5	17	49	130	79	49
2	9	23	33	49	240	94
3	22	130	130	540	79	920
4	33	70	540	350	540	130
5	11	33	170	110	170	920
6	70	33	79	110	350	350
7	46	49	70	33	350	350
8	9	49	49	130	540	140
Geometric mean	27.54 (1.44)		107.15 (2.03)		204.17 (2.31)	
s_r	0.36		0.25		0.44	
s_R	0.39		0.39		0.44	
RSD_r, %	25.00		12.32		19.05	
RSD_R, %	27.08		19.21		19.05	

[a] Results for duplicate samples at each level at each site.
[b] Numbers in parentheses are log of geometric mean.

reagent is used, add powder to labeled tube, **B(a)**, or vessel, **B(b)**, containing sample. Or, add well mixed water sample to labeled tube or vessel containing predispensed reagent. Combine sample and reagent aseptically, cap container tightly, and mix vigorously to dissolve reagent. Resulting solution is colorless. Incubate samples for 24 h at 35 ± 1.0°. Yellow color in MPN tube or P-A vessel after incubation denotes presence of total coliforms. Expose positive total coliform tubes or vessel to hand-held 366 nm lamp, **B(c)**. Fluorescence denotes presence of *E. coli*.

Calculate separate MPN values for total coliforms and *E. coli* from standard MPN tables [e.g., 1.1, 6.9, etc. (*Standard Methods for the Examination of Water and Wastewater* (1985) 16th Ed., APHA, Washington, DC)].

E. Quality Control

Perform quality control as follows: (*1*) Reconstitute reagent in each of 3 tubes or vessels with appropriate volume of sterile, distilled water and mix thoroughly to aid dissolution.

(*2*) Label tubes "*Escherichia coli*," "*Klebsiella pneumoniae*," and "*Pseudomonas aeruginosa*."

(*3*) Touch sterile inoculating loop or needle to an 18–24 h pure culture slant of each of the 3 bacteria (alternatively, a "Bactrol," or equivalent, disk of 3 respective bacteria may be used directly).

(*4*) Transfer each bacterial inoculum to appropriately labeled tube or vessel.

(*5*) Incubate inoculated tube or vessel for 24 h at 35 ± 1.0°. Results should be: *E. coli*, yellow and fluorescent; *K. pneumoniae*, yellow only; and *Pseudomonas aeruginosa*, no color, no fluorescence.

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Results

Collaborative Study

Data.—The data for the duplicate 5-tube MPN analyses for the defined substrate technology and multiple-tube fermentation methods are presented in Tables 1 and 2, respectively. Numbers of tubes positive have been transformed in bacterial concentrations by using standard MPN tables (9).

Repeatability and reproducibility and analysis of variance.—The residuals from the analysis of variance of the log transformed data are normally distributed and have equal variance among laboratories and samples. There were no statistically significant differences between the 2 methods. For example, the *F*-statistic comparing repeatability between DST and BGLB is $0.82^2/0.61^2 = 1.8$, with 24 and 24 degrees of freedom, not significant at $P = 0.05$.

Analysis-of-variance models.—The same data used to evaluate the precision of the methods was used in this analysis. For a given comparison, the *F* data for the DST method were combined with the data for the companion method, and an analysis-of-variance model was developed to assess the effects of laboratory, sample, and method. The full models were simplified by removing the interaction terms, none of which was significant at $P = 0.5$. The variance ratios (*F*-statistics) for comparing DST to MTF were 2.34 and 1.43; neither is significant at $P = 0.05$. Thus, it is concluded that there is no statistical evidence of disagreement in mean (log) MPN between DST and the standard method.

Discussion

The DST method is extremely easy to perform. All ingredients are in the test tube or vessel and it is necessary only to add the proper amount of sample, 10 mL per tube for the quantitative MPN and 100 mL per vessel for the qualitative P-A formats. Unlike methods that use conventional media, no confirmatory tests are required. The test is completed within 24 h after inoculation by observation of color. All ingredients are dry and mix with the water sample within seconds to produce a colorless solution. After mixing, the tubes are placed in an incubator at 35 ± 1.0°C and are examined at a maximum of 24 h. The production of a stable yellow color is characteristic for total coliforms; no confirmatory or completed tests are needed. The positive tubes are exposed to a long-wave (not dangerous) ultraviolet lamp (366 nm) to determine if *E. coli* is present. A strong blue-white fluorescence is specific for this species.

The DST test has fulfilled the required EPA parameters of sensitivity and specificity. The study demonstrated the ability of the system to detect 1 total coliform or *E. coli* per 100

mL water sample. The DST test was able to simultaneously, specifically enumerate both *E. coli* and total coliforms. A separate quantitative value was obtained for each. The yellow chromogen is specific for total coliforms and fluorescence-specific for *E. coli*. No additional reagents or confirmatory tests are needed. Subcultures of positive DST tubes were identified to species to ensure that a member of the total coliform group was present and that false-positive analyses were not occurring.

The collaborative study demonstrated the repeatability and reproducibility of the DST system compared to *Standard Methods* testing. By all statistical parameters, including analysis of variances, the 2 methods demonstrated comparability and were indistinguishable in their ability to enumerate the *Escherichia coli* test strain.

Conclusions and Recommendation

The defined substrate technology reagent system simultaneously detects total coliforms and *E. coli* from a water sample. Precollaborative studies on the quantitative (MPN) and qualitative (P-A) formats for analyzing water samples in geographically diverse sites showed equivalency with EPA certified methods. The present collaborative study among 8 laboratories shows equivalent repeatability and reproducibility between the DST and the standard multiple-tube fermentation method.

The Associate Referee recommends that the defined substrate technology (Colilert) method for the rapid, specific simultaneous quantitative (MPN) and qualitative (P-A) enumeration of both total coliforms and *Escherichia coli* from water be adopted official first action.

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MYCOTOXINS

Rapid Immunochemical Screening Method for Aflatoxin B_1 in Human and Animal Urine

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A method has been developed to determine the presence of aflatoxin B_1 in the urine of animals (including humans) by utilizing commercial immunochemical kits that can be used in the field. Urine is treated with diatomaceous earth and filtered to clarify the sample; 2–3 ppb aflatoxin B_1, corresponding to about 300 ppb in the ingested feed/food, can be detected in the filtered urine without futher purification. To improve sensitivity, the urine filtrate is passed through a C_{18} solid phase column to extract the aflatoxin. The column is washed with acetonitrile-water (15 + 85) and water, aflatoxin B_1 is eluted with methanol-water (7 + 3), and water is added to the eluate, which is then tested for aflatoxin with the test kit. The limit of detection is 0.2 ppb, reflecting consumption of 40 ppb or more aflatoxin in the feed/food. When the initial sample volume is adequate, purification through the C_{18} column step is usually sufficient. For limited sample volumes, the eluate from the C_{18} column is mixed with water, added to an immunosorbent affinity column, and washed with water to remove excess sample matrix and impurities. Aflatoxin B_1 is eluted with acetonitrile. The extract is evaporated under nitrogen and the residue is redissolved in methanol-water (25 + 75). At this purification stage, the limit of detection is reduced to 0.05 ppb.

The aflatoxins are highly toxic and carcinogenic compounds found in food supplies grown in many areas of the world (1, 2). Aflatoxins have been found in mold-damaged foods, such as cereal grains, nuts and nut products, and edible oilseed products, and in milk and other dairy products. Although aflatoxin contamination is greatest in Asian and African countries, it is an annual problem to some degree in areas of the U.S. southeast, southern Midwest, and southwest. On occasion (e.g., 1983 and 1988), heavy contamination occurs in the midwestern corn-growing states. Animal feeding studies (beef, swine, poultry) have shown that edible meat becomes contaminated with aflatoxin when animals are fed contaminated corn (3–9).

Numerous methods that can be used to screen and/or quantitatively determine aflatoxins in many food matrixes have been developed, collaboratively tested, and adopted by AOAC (10); however, no fast screening method is available for detecting aflatoxin in animal tissues. Federal guidelines permit feeding animals aflatoxin-contaminated grain at levels of less than 300 ppb (11). Therefore, producers and regulatory agencies have an imperative need for a screening method that can quickly and accurately identify contaminated edible meat to keep it from reaching the marketplace. An accurate, sensitive, quantitative method for determining aflatoxin in animal tissue is available (12); however, it cannot be conveniently adapted to a qualitative method, nor is it a practical method for use as a screening procedure because it requires considerable time to complete.

An initial project to develop a screening method for the detection of aflatoxin residues in animal tissues that could be used by the Food Safety and Inspection Service (FSIS)/USDA was unsuccessful. An alternative approach involving development of a method to detect aflatoxin in animal urine was evaluated. Such a method would permit sample analysis that should have a high degree of correlation with tissue contamination (based on feeding studies) and that could be performed without sacrificing the animal. This paper describes a method for detecting aflatoxin in the urine of humans and other animals that permits the selection of any of 3 different minimum detection limits: 2–3, 0.2, or 0.05 ppb aflatoxin B_1.

METHOD

Equipment

(a) *Solid phase extraction (SPE) columns.*—Bond Elut C_{18} cartridge, 6 mL (Cat. No. 607306, Analytichem International, Harbor City, CA, or equivalent).

(b) *SPE column adapter.*—Bond Elut adapter to join syringes to SPE columns (Cat. No. 636001, Analytichem International, or equivalent).

(c) *Affinity column (AF column).*—Aflatest P (VICAM, Somerville, MA, or equivalent).

(d) *Filter paper.*—S&S No. 588, fast flow, high wet strength, 12.5 cm, or equivalent.

(e) *Vacuum manifold.*—Supelco vacuum manifold, or equivalent.

(f) *Aflatoxin test kit.*—EZ-Screen test kit, 3.3 ppb sensitivity (Environmental Diagnostics, Inc., Burlington, NC) or Afla-20 Cup Test Kit (International Diagnostics Inc., St. Joseph, MI). Follow instructions included in each kit. Briefly, sample extracts are added to the card containing a glass fiber filter with aflatoxin antibody immobilized on it. An enzyme conjugate (horseradish peroxidase-aflatoxin B_1) is added, followed by a water wash. A substrate (color reagent) is added and the card is read for the presence (no color) or absence (color) of aflatoxin B_1.

(g) *Syringes.*—1, 10, and 30 mL disposable Luer-Lok syringes.

(h) *Pipetter.*—50 μL automatic pipet (Gilson, or equivalent).

Reagents

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

(b) *Diatomaceous earth.*—Hyflo Super-Cel.

(c) *Aflatoxin B_1 and M_1 standards.*—Standard solutions containing 0.5 μg/mL aflatoxin B_1 or M_1 in acetonitrile were

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

prepared from concentrated solutions of crystalline aflatoxins.

(**d**) *Artificially contaminated urine.*—Standard aflatoxin B_1 solution was added by syringe to aflatoxin-free urine to obtain samples containing desired toxin levels.

Sample Treatment

Measure 60 mL bovine, porcine, or human urine into 125 mL glass-stoppered Erlenmeyer flask and add 4 g diatomaceous earth. Shake vigorously 30 s, filter through paper into a graduated cylinder, and collect 40 mL. Save for SPE extraction step or test for aflatoxin B_1 with test kit. Minimum detection limit is 2–3 ppb.

SPE Extraction

(*Note*: The SPE extraction and the affinity column chromatography steps may be performed without a vacuum manifold by applying pressure with a syringe and plunger. *Never* pull the plunger up without disconnecting the syringe from the column. Drawing solvents or air upward may ruin the test results.)

Equip C_{18} cartridge with adapter and attach 30 mL syringe as sample reservoir. Attach cartridge to vacuum manifold and adjust vacuum to ca 10 in. Hg. Pretreat cartridge with 5 ml methanol followed by 5 ml H_2O. Do not allow cartridge to run completely dry hereafter unless so specified. Add 40 mL clarified urine sample from graduated cylinder to syringe barrel and draw solution through cartridge at flow rate of ca 20 mL/min. Rinse sample graduate with 3 mL H_2O and pass rinse through cartridge. Rinse cartridge with 5 mL acetonitrile-H_2O (15 + 85), ca 1 drop/s, and discard eluate. Wash cartridge with 5 mL H_2O (fast drops), and allow cartridge to run dry. Remove cartridge from manifold. If aflatoxin B_1 is to be assayed after elution from SPE cartridge, elute aflatoxin into 1 dram vial with 1.5 mL MeOH-H_2O (7 + 3), using syringe. Add 2 mL H_2O to eluate and measure with test kit. Minimum detection limit is 0.2 ppb. If urine extract from SPE cartridge is to be further purified by affinity column chromatography, elute aflatoxins from SPE cartridge with 2 mL MeOH-H_2O (7 + 3), and mix with 12 mL H_2O.

Affinity Column Chromatography

Remove cap from column top and clip off end to fit Luer tip of 10 mL syringe. Replace cap on column. Remove bottom cap and attach column to vacuum manifold. Add SPE cartridge extract (after mixing with 12 mL H_2O) and pull solution through affinity column (10 mL/min). Rinse sample container with 2 mL H_2O and pass rinse through column. Pull column dry for ca 5 s to remove excess H_2O. Elute aflatoxins from affinity column with 1 mL acetonitrile (use 1 or 2 mL syringe) into 1 dram vial, discarding first 3 drops. Evaporate eluate to dryness under N_2 at 40°C. Redissolve residue in 200 μL (0.2 mL) MeOH-H_2O (1 + 3), cap vial, and mix vigorously for ca 1 min, preferably on a Vortex mixer. Save for aflatoxin test kit. Minimum detection limit is 0.05 ppb.

Results and Discussion

The results of the screening tests for the presence of aflatoxin B_1 in human, bovine, and porcine urine are given in Table 1. These data were obtained with the EZ Screen Card test manufactured by Environmental Diagnostics Inc., Burlington, NC. Other kits that should perform the screening tests satisfactorily are available, e.g., Afla-20 cup test (International Diagnostics Inc., St. Joseph, MI) and the CITE Probe test (IDEXX Corp., Portland, ME). Aflatoxin B_1 at the 1.5 ppb level in most raw clarified urine samples gave positive test results. Two bovine and 2 porcine urine samples tested negative at this concentration.

In a study of populations in the People's Republic of China, where a high incidence of liver cancer has been reported (2), between 1.23 and 2.18% of dietary aflatoxin B_1 was found to be excreted as aflatoxin M_1 in human urine. This corresponds to a similar ratio of M_1 found in milk of dairy cows fed aflatoxin B_1 (3). Less than 0.5% of the total B_1 intake was excreted as B_1; therefore, a factor of about 200 is indicative of the transmission differential between the aflatoxin B_1 intake in food/feed and is excretion in urine. The relationship between the B_1 intake and M_1 excretion in animals (6, 7) is similar to the human values published by Zhu et al. (2); therefore, it is likely that a similar B_1 excretion relationship also exists. Based on these transmission data, a mammal would need to consume a food source contaminated with 300 ppb aflatoxin B_1 before the toxin could be detected in raw, clarified urine. This may be satisfactory for some animals, since the FDA will allow aflatoxin levels of 300 ppb in corn to be fed to feedlot cattle (11).

Since a higher percentage of B_1 intake is excreted as M_1 than as B_1, a test kit for aflatoxin M_1 would be useful in this application because sensitivity would be increased 2- to 4-fold. However, at the time of this work, no rapid test kits were available for M_1. We evaluated the 2 test kits used in this study for cross activity to aflatoxin M_1 and found none with 40 ng of pure standard. When M_1 kits become available, it is highly probable that they can be substituted for the B_1 kits. This would significantly increase the sensitivity of the method.

An improvement in sensitivity can be obtained with a rapid cleanup step. After the various clarified urine specimens were passed through the SPE C_{18} column and the adsorbed aflatoxin eluted from the column, the extracts produced positive tests at the 0.2 ppb level for nearly all urine specimens. The negative results shown in Table 1 (0.1 ppb bovine and porcine; 0.2 ppb porcine) occurred early in our experiments. Personal communications with Environmental Diagnostics International (EDI) revealed that the test kits are designed to give uniformly negative results at the level of 1.65 ppb B_1 and

Table 1. Results of screening artificially contaminated urine of humans and other animals for aflatoxin[a]

Urine sample type	Positive aflatoxin B_1 tests (No. positive/No. total tests)							
	Clarified raw urine		SPE C_{18} column extract			Affinity column extract		
	1.25 ppb	1.50 ppb	0.05 ppb	0.10 ppb	0.20 ppb	0.010 ppb	0.020 ppb	0.050 ppb
Human	1/8	8/8	0/12	6/6	———	1/6	6/6	6/6
Bovine	0/10	8/10	0/10	7/10	20/20	5/15	15/15	15/15
Porcine	———	11/13	0/10	7/10	19/20	6/12	10/13	15/15

[a] As determined with EZ Screen Card Text (Environmental Diagnostics Inc., Burlington, NC).

uniformly positive results at the level of 3.3 ppb. All test lots meet this criterion or "window," but some lots will have a crossover point (change from negative to positive) nearer 3.3 ppb, while other lots will have a crossover nearer 1.65 ppb.

After we obtained fresh test kits, all results were positive. Obviously, the negative results mentioned above were obtained with kits that had a crossover point near 3.3 ppb, while the crossover point of new kits was nearer to 1.65 ppb. EDI indicated that their experiments have shown that there are no matrix effects with the kits; therefore, this window will exist with urine samples as well. In a test with the ability to detect 0.2 ppb aflatoxin B_1 in mammalian urine, an ingested food sample containing 40 ppb aflatoxin would produce a positive test for aflatoxin in urine. This presents a method with a satisfactory sensitivity, since the FDA guideline for aflatoxin in animal feeds ranges from 20 to 300 ppb.

If the volume of the urine sample is limited or if a lower detection limit is desired, an affinity column cleanup step may be added to the sample preparation. The data (Table 1) indicate that a sensitivity of 0.05 ppb aflatoxin in the urine of all 3 species can be achieved by the addition of this purification step. If the urine volume specified in the procedure (40 mL) is used, a concentration of 10 ppb aflatoxin B_1 in feed would cause a positive urine test with the immunochemical kits; however, a sensitivity of that magnitude is unnecessary. If only 4 mL of urine was available for analysis, however, this method would still detect B_1 in urine from an animal consuming food containing 100 ppb or more of aflatoxin B_1.

Several important and useful objectives are achieved with this method. First, it can be performed in the field; a syringe and plunger can be used to carry out the various column cleanup steps, and no expensive or complicated equipment is needed to complete the immunochemical tests. Second, the collection of the urine sample is noninvasive; a test specimen can be obtained without the need to puncture or surgically invade the body. Third, the test procedure is relatively inexpensive. No special equipment is needed and very little time is required to make the determination. For most tests, less than 30 min will be needed to clarify the urine, clean it up with a SPE C_{18} column, and run the test to determine if aflatoxin is present. Finally, this method could easily be used by regulatory agencies to determine if animals had eaten aflatoxin-contaminated feed.

Acknowledgments

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OILS AND FATS

Quantitative Determination of Mono- and Diglycerides with and without Derivatization by Capillary Supercritical Fluid Chromatography

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Mono- and diglycerides, as well as commercial monoglyceride emulsifiers, were analyzed by capillary supercritical fluid chromatography (SFC). Carbon dioxide without modifier was used as the mobile phase in the chromatography. Samples were prepared by simply being dissolved in solvents or by propionyl ester derivatization. A capillary SFC methyl silicone column (SB-methyl 100, 100 μm × 10 m, 0.5 μm film thickness, Lee Scientific) was used for the separation, and a flame ionization detector was used for the detection. A calibration standard containing known concentrations of monomyristin, monopalmitin, monostearin, dimyristin, dipalmitin, and distearin were used for the determination of the response factor (unit weight per peak area) of each analyte. The response factors were used in the quantitation of the analytes in the test samples from their respective integrated peak areas. Monomyristin was used as an internal standard for the quantitation of monoglycerides in commercial emulsifiers. The accuracy of the methods was demonstrated by comparing the percent area (%A) determined by the chromatography with the percent weight (%W) of each of the components in the standard mixture, and the precision of the methods was indicated by the relative standard deviation (RSD). The mean ratio and RSD of %A/%W were 0.98 ± 0.09 (<5%) and 1.01 ± 0.03 (<2.5%) for underivatized and derivatized samples, respectively. Two lots of commercial monoglyceride emulsifiers were analyzed with and without derivatization, and the results were compared to those obtained by gas chromatography (GC). *T*-tests comparing the method means did not indicate any significant mean differences (95% confidence level) between the GC and SFC methods. Nor did the variance ratio F-test indicate that the overall variances of the methods were significantly different. However, the error variance of the SFC-underivatized method was significantly higher than that of the GC and SFC-derivatized methods. These results demonstrated the feasibility of applying the SFC technology in the analysis of mono- and diglycerides with and without derivatization.

The determination of mono- and diglycerides by gas chromatography (GC) and liquid chromatography (LC) has been reported in the literature (1–5). Because of the low volatility of the underivatized glycerides, derivatization was necessary to facilitate the elution of the components in gas chromatography. High oven temperature and long analysis time are necessary for the elution of diglycerides of long-chain fatty acids. For glycerides of long-chain polyunsaturated fatty acids, the high temperature also causes thermal degradation, resulting in poor recoveries (2). Mono- and diglycerides can be separated by LC without derivatization. Both normal phase and reverse phase LC have been reported in the analyses of mixed glycerides (6, 7). However, because of the lack of functional groups for sensitive detection and the significant differences in response factors for the saturated and unsaturated species at low wavelength ultraviolet absorbance detection (190–210 nm), the quantitative analysis of this group of compounds remains a challenge.

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Supercritical fluid chromatography (SFC), which couples the low mobile phase viscosity and high solute diffusivity of supercritical carbon dioxide, offers the advantages of higher chromatography efficiency and shorter analysis time (8). The availability of a broad range of stationary phases in both GC and LC areas, and the compatibility with universal detectors such as flame ionization, may provide an alternative tool for overcoming the difficulties encountered by traditional GC and LC methodologies (9–13). In this study, we explore the application of SFC technology in the determination of mono- and diglycerides with and without derivatization.

METHOD

Apparatus

All labware that contacts solvents and reagents should be glass or Teflon-lined. All labware that contacts propionic anhydride must be dry.

(a) *Supercritical fluid chromatograph.*—Equipped with density and temperature programming, auto sampler, time-split injector, and flame ionization detector (Lee Scientific Model 602, or equivalent).

(b) *Peak area integrator.*—Hewlett-Packard 3396A, or equivalent.

(c) *SFC column.*—Capillary, methyl silicone, 10 m × 100 μm id, 0.5 μm film thickness (SB-Methyl-100, Lee Scientific, or equivalent).

(d) *SFC column restrictor.*—Frit restrictor, 100 μm (Cat. No. 010354, Lee Scientific, or equivalent).

(e) *Gas chromatograph.*—Equipped with flame ionization detector and column temperature programming (Hewlett-Packard 5890A, or equivalent).

(f) *GC column.*—Packed: glass, 6 ft × 2 mm id, packed with 3% OV-1 on 100–120 mesh support (Supelco Inc., or equivalent). Capillary: fused silica, methyl silicone gum, 5 m × 0.53 mm id, film thickness 2.65 μm (HP-1 Hewlett-Packard, or equivalent).

(g) *Block heater.*—With blocks of hole size to fit test tubes 16 or 25 mm od (Labline Multi-Blok Heater, or equivalent).

(h) *Analytical evaporator.*—12–24 positions with Teflon-covered needles (N-EVAP, Organomation Associates Inc., or equivalent).

Reagents

(a) *Glycerides.*—1(3)-Monomyristin (MG14), 1(3)-monopalmitin (MG16), 1(3)-monostearin (MG18), 1,3-dimyristin (DG14), 1,3-dipalmitin (DG16), 1,3-distearin

(DG18), 99% minimum purity (NuChek Prep., Inc., or equivalent).

(b) *Carbon dioxide.*—SFC grade (Scott Specialty Gas, or equivalent).

(c) *Helium.*—Carrier grade 99.999% (Columbus Oxygen Co., or equivalent).

(d) *Nitrogen, hydrogen, and air.*—Prepurified and free from organic impurities.

(e) *Methylene chloride and methanol.*—Distilled in glass (Burdick and Jackson, or equivalent).

(f) *Pyridine.*—CAS No. 110-86-1, ACS reagent grade (Kodak 116-7022, or equivalent).

(g) *Propionic anhydride.*—CAS No. 123-62-6, 99% minimum (Kodak 112-6515, or equivalent).

Sample Preparation

(a) *Propionyl ester derivatization.*—The detailed procedure has been described in the previous publication (1). Weigh ca 10 mg sample into screw-cap test tube, and add 2 mL propionic anhydride-pyridine reagent (2 + 1, v/v). Cap tightly and heat 35 min at 75 ± 3°C. Evaporate reaction mixture to dryness at ca 70°C with gentle flow of nitrogen. Dissolve derivatized sample, i.e., propionyl esters of monoglycerides and diglycerides, in 10 mL methylene chloride and analyze by GC. Derivatives may be dissolved in 20 mL methylene chloride and analyzed by SFC. If samples are dissolved in solvents and added as an aliquot, the solvents should be evaporated completely before derivatization reagents are added.

(b) *Underivatized samples.*—Weigh ca 10 mg sample into screw-cap test tube. Dissolve in 20 mL methylene chloride-methanol (1 + 1, v/v). Use mild warming and sonication, if desired, to aid the dissolution of dipalmitin and distearin. (*Note:* Underivatized samples were analyzed by SFC only.)

Chromatography

(a) *Supercritical fluid chromatography.*—Use equipment described in *Apparatus* (a)-(d) under following conditions: Injector kept at 25°C by circulating water around the injector jacket connected to a water bath; injector loop volume 200 nL, time-split 0.5 s; detector temperature 350°C. Density program: initial density 0.2127 g/mL, initial time 8 min, ramp rate 0.02 g/mL/min, final density 0.6957 g/mL, final time 10 min; oven temperature 120°C isothermal. Column flow rate ca 1.5 mL/min at initial density and temperature. Solvent methylene chloride-methanol (1 + 1, v/v) was used as the unretained species for the calculation of column flow rate.

(b) *Gas chromatography conditions.*—(*1*) *For packed column.*—Same as described in (1). Monoglycerides: injector temperature 280°C; detector temperature 280°C; oven initial temperature 260°C; initial time 3 min; program rate 2°C/min; final temperature 280°C; final time 2 min. Diglycerides: injector temperature 310°C; detector temperature 330°C; oven initial temperature 260°C; initial time 3 min; program rate 3°C/min; final temperature 300°C; final time 10 min. (*2*) *For capillary column.*—Column flow ca 15 mL/min; split ratio 1/10; injector temperature 290°C; detector temperature 295°C; oven initial temperature 195°C; initial time 5 min; program rate 3°C/min; final temperature 290°C; final time 10 min for monoglycerides and 300 min for diglycerides.

Determination of Response Factors and Quantitation of Monoglycerides in Commercial Emulsifiers by Internal Standard Method

Two calibration standards containing known concentrations of derivatized and underivatized MG14, MG16, MG18, DG14, DG16, and DG18 were analyzed by SFC as described above. A response factor (RF), amount of analyte per unit peak area, was determined for each component. A known amount of internal standard monomyristin (MG14) was spiked into each sample before the derivatization. The weight of the analyte of interest in the test sample was calculated as follows:

$$\%W_u = 100 \times (A_u \times RF_u \times W_{istd})/(A_{istd} \times RF_{istd} \times W_s)$$

where $\%W_u$ = g unknown/100 g test sample; A_u = peak area of unknown; RF_u = response factor of unknown; A_{istd} = peak area of internal standard; RF_{istd} = response factor of internal standard; W_{istd} = weight of internal standard spiked; and W_s = weight of test sample.

Results

SFC Analysis of Underivatized Mono- and Diglyceride Standards

The glycerides standard mixture consisted of mono- and dimyristin, palmitin, and stearin. Each glyceride was weighed accurately, dissolved in solvents, and analyzed by SFC as described above. A typical SFC chromatogram of underivatized glycerides is shown in Figure 1. Baseline resolutions were achieved for all components. These standards were prepared on 3 different days and each preparation was analyzed by SFC on multiple (3-4) days. The results are

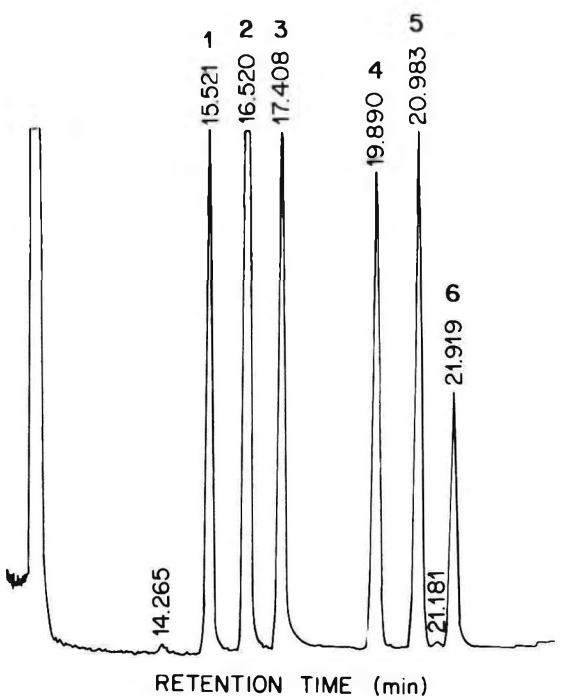

Figure 1. Typical SFC chromatogram of underivatized mono- and diglycerides. Chromatography parameters: initial density 0.2127 g/mL, initial time 8 min, ramp rate 0.02 g/mL, final density 0.6957 g/mL, final time 10 min, oven temperature 120°C isothermal. Peak identification: (1) MG14; (2) MG16; (3) MG18; (4) DG14; (5) DG16; and (6) DG18.

Table 1. Quantitative analysis of underivatized mono- and diglyceride standards by supercritical fluid chromatography

Standard	Parameter	Prep 1	Prep 2	Prep 3
MG14	% Weight	34.21	34.36	33.44
	% Area Av.	32.94	36.55	35.04
	% RSD (n)	1.37(4)	2.41(4)	1.12(4)
	Ratio (%A/%W)	0.96	1.06	1.05
MG16	% Weight	32.76	32.18	35.28
	% Area Av.	33.12	33.58	36.02
	% RSD (n)	0.63(4)	1.21(4)	0.76(4)
	Ratio (%A/%W)	1.01	1.04	1.02
MG18	% Weight	33.03	33.46	31.29
	% Area Av.	33.94	29.86	28.94
	% RSD (n)	1.92(4)	4.20(4)	2.12(4)
	Ratio (%A/%W)	1.03	0.89	0.92
DG14	% Weight	41.63	39.36	39.86
	% Area Av.	40.19	38.72	41.09
	% RSD (n)	0.46(4)	0.78(4)	0.57(4)
	Ratio (%A/%W)	0.97	0.98	1.03
DG16	% Weight	38.84	39.87	40.58
	% Area Av.	39.92	40.50	41.37
	% RSD (n)	0.19(4)	0.20(4)	0.11(4)
	Ratio (%A/%W)	1.03	1.02	1.02
DG18	% Weight	19.53	20.77	19.57
	% Area Av.	19.91	20.78	17.54
	% RSD (n)	1.04(4)	1.10(4)	1.40(4)
	Ratio (%A/%W)	1.02	1.00	0.90

Table 2. Quantitative analysis of derivatized mono- and diglyceride standards by supercritical fluid chromatography

Standard	Parameter	Prep 1	Prep 2	Prep 3
MG14	% Weight	34.21	34.60	36.56
	% Area Av.	33.71	33.78	36.47
	% RSD (n)	0.53(6)	1.19(4)	0.56(4)
	Ratio (%A/%W)	0.99	0.98	1.00
MG16	% Weight	32.76	32.18	30.51
	% Area Av.	32.78	31.90	30.66
	% RSD (n)	0.32(6)	1.25(4)	0.12(4)
	Ratio (%A/%W)	1.00	0.99	1.00
MG18	% Weight	33.03	33.46	32.93
	% Area Av.	33.50	34.32	32.87
	% RSD (n)	0.74(6)	1.91(4)	0.58(4)
	Ratio (%A/%W)	1.01	1.03	1.00
DG14	% Weight	41.63	39.36	40.00
	% Area Av.	41.06	38.64	39.24
	% RSD (n)	1.31(6)	0.67(4)	0.94(4)
	Ratio (%A/%W)	0.99	0.98	0.98
DG16	% Weight	38.84	39.87	40.38
	% Area Av.	39.68	40.41	40.97
	% RSD (n)	0.43(6)	0.24(4)	0.32(4)
	Ratio (%A/%W)	1.02	1.01	1.01
DG18	% Weight	19.53	20.77	19.62
	% Area Av.	19.23	20.95	19.79
	% RSD (n)	2.28(6)	1.55(4)	1.29(4)
	Ratio (%A/%W)	0.98	1.01	1.01

summarized in Table 1. The accuracy of the quantitation was estimated by comparing the percent area (%A) as determined by the chromatography with the percent weight (%W) of each of the components in the standard mixture. The ratio, %A/%W, which ranged from 0.89 to 1.06, demonstrated a consistent FID response for the glycerides tested. The relative standard deviation (%RSD) as a measure of precision was less than 5% throughout the study.

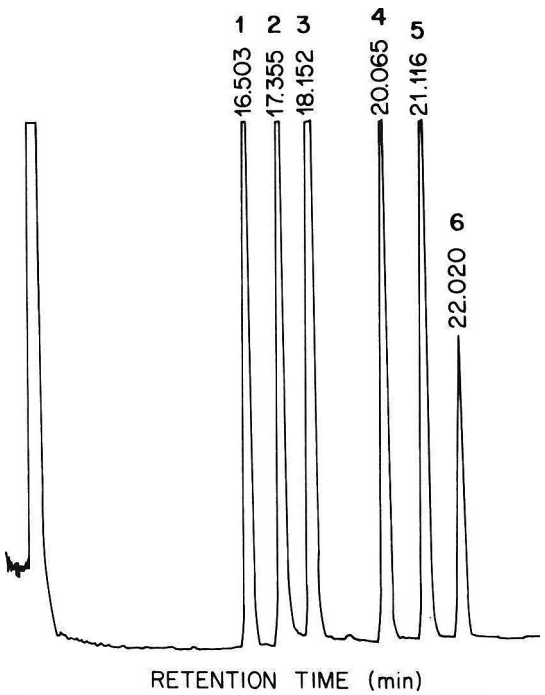

Figure 2. Typical SFC chromatogram of derivatized mono- and diglycerides. Chromatography parameters same as described in Figure 1. Peak identification: propionyl esters of (1) MG14; (2) MG16; (3) MG18; (4) DG14; (5) DG16; and (6) DG18.

SFC Analysis of Derivatized Mono- and Diglyceride Standards

Propionyl esters of the mono- and diglycerides were prepared and analyzed by SFC. Figure 2 showed a typical SFC chromatogram of the propionyl esters of the glycerides. The experimental design and treatment of the data are the same as described above. A summary of the results is presented in Table 2. The accuracy of the procedure as shown by the ratio,

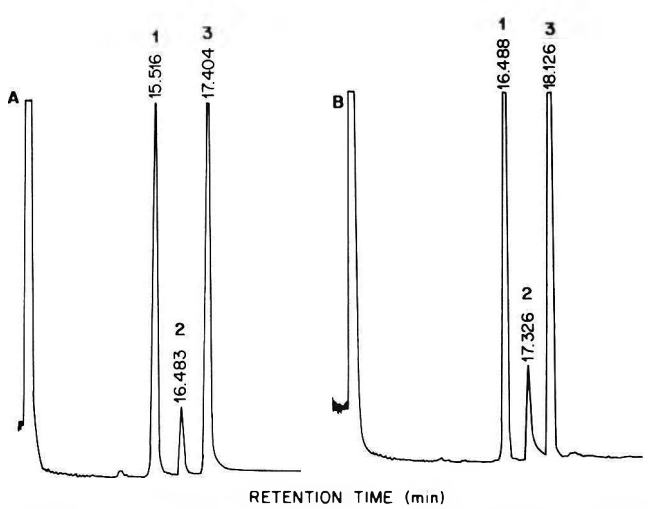

Figure 3. Typical SFC chromatogram of commercial emulsifier. (A) underivatized; (B) derivatized. Chromatography parameters same as described in Figure 1. Peak identification: (1) MG14 (ISTD); (2) MG16; and (3) MG18.

Table 3. Quantitative determination of total monoglycerides in commercial emulsifier by gas chromatography and supercritical fluid chromatography

Parameter	Total monoglycerides, g/100 g			
	Prep 1	Prep 2	Prep 3	Mean[a]
Lot 1840				
SFC/underivatized				
Mean	90.53	94.40	96.94	93.43
%RSD (n)	2.60(4)	3.95(3)	0.71(4)	3.50(11)
SFC/derivatized				
Mean	91.89	89.73	90.32	90.85
%RSD (n)	1.77(6)	2.72(4)	1.93(4)	2.24(14)
GC/derivatized				
Mean	93.31	91.35		92.33
%RSD (n)	0.57(2)	0.29(2)		1.29(4)
Lot 6022				
SFC/underivatized				
Mean	92.53	97.83	97.61	95.93
%RSD (n)	3.32(4)	2.41(4)	1.00(4)	3.38(12)
SFC/derivatized				
Mean	95.13	91.76	91.81	93.22
%RSD (n)	1.59(6)	2.79(4)	0.33(4)	2.49(14)
GC/derivatized				
Mean	95.41	92.56		93.99
%RSD (n)	0.66(2)	0.17(2)		1.79(4)

[a] Method means not significantly different for either lot.

Table 4. Analysis of variance—Comparison of GC-derivatized, SFC-derivatized, and SFC-underivatized

Parameter	Total monoglycerides, g/100 g		
	GC-Derivatized	SFC-Derivatized	SFC-Underivatized
Mean[a]	93.16	92.22	94.6719
Total variance	3.0802	5.3179	13.0784
Prep	2.8744(2)[b]	0.9623(4)	8.4135(4)
Day	—[c]	4.1020(10)	1.9483(6)
Error	0.2048(4)	0.2536(12)	2.7166[d](11)
Total std	1.7550	2.3061	3.6164
RSD, %	1.88%	2.50%	3.98%

[a] Method mean averaged across lots.
[b] Degrees of freedom are in parentheses.
[c] For GC, one prep was done on each of 2 days for each lot; thus Prep and Day variance are inseparable.
[d] Significantly larger (p-value < 0.05) than GC- and SFC-derivatized methods.

%A/%W, ranged from 0.98 to 1.03. The relative standard deviation was less than 2.5% throughout the study.

Comparison of Analyses of Monoglycerides in Commercial Emulsifiers by GC and SFC

Two lots of commercial monoglyceride emulsifier were analyzed in a 3-day study as follows: derivatized/GC, derivatized/SFC, and underivatized/SFC. The procedure for the derivatized/GC analyses was the same as described previously (1). The procedures for the derivatized/SFC and underivatized/SFC analyses were the same as described above for the glyceride standards. Figures 3A and 3B show a typical SFC chromatogram of the derivatized and underivatized commercial monoglyceride emulsifier. The data and results of a variance component analysis are summarized in Tables 3 and 4 and Figure 4. The *t*-tests comparing the method means did not indicate any significant mean differences (95% confidence level). Nor did the variance ratio F-test indicate that the overall variances of the methods were significantly different (all pairwise p-values > 0.05). However, the error variance of the underivatized SFC method was significantly higher than that of the derivatized/GC and derivatized/SFC methods.

The feasibility of using supercritical fluid chromatography in the analysis of mono- and diglycerides has been demonstrated in this study. The combination of supercritical carbon dioxide mobile phase and the flame ionization detector offers an alternative means of maintaining the advantages and circumventing the difficulties of traditional GC and LC methodologies. The supercritical carbon dioxide is higher in solute

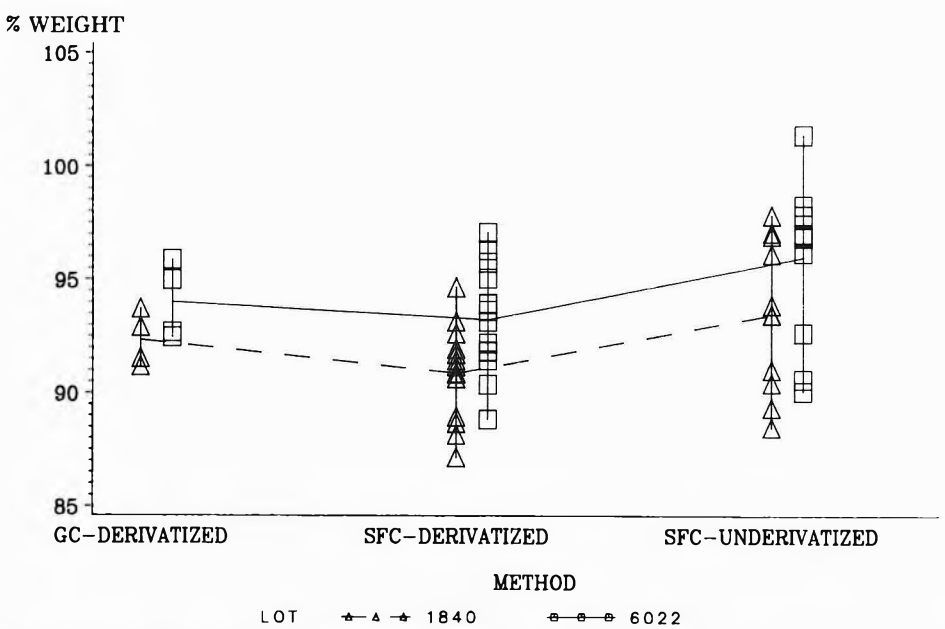

Figure 4. Quantitative determination of total monoglyceride in commercial emulsifiers by gas chromatography and supercritical fluid chromatography.

diffusivity compared to the inert carrier gas used in gas chromatography, and has lower viscosity than the liquid solvents used in liquid chromatography. Thus, the glycerides, with and without derivatization, can be separated and eluted at a lower temperature and shorter analysis time. Compared to UV detectors, the flame ionization detector responded quite uniformly across a broad range of analytes. This allows the quantitation of unknown components even when the detailed structure of the analytes is not known. However, if precise quantitation is desired, a response factor for each component should be determined as shown in this study.

During this study, the variations between injections were found to be quite large. The variation may be due to a variety of factors, i.e., nonreproducible time-split injector, sample precipitate in the injection loop, etc. Thus, we have limited our applications to relative percentage determinations, and the internal standard method was used for absolute quantity determinations. The choice of optimum solvent for the analytes and the warming of the injector are essential to minimize the precipitation of the analytes in the injector loop. The elimination of the derivatization procedure would provide savings in time and labor cost. The overall means and variances of the 3 methods were not significantly different. However, the error variance of the underivatized method was higher. Depending on the requirement of each individual practical application, the higher error variance may be acceptable to some but not to others.

The SFC instrument is very rugged, and quite easy to operate. In this study, a methyl silicone column was used and the components were separated on the basis of their solubility in carbon dioxide, volatility, and molecular weight differences. The method reported here does not separate positional or stereo isomers, i.e., 1(3)- and 2-monoglycerides; 1,3-, 1,2-, and 2,3-diglycerides. Other stationary phases, i.e., polar or chiral phases, can be explored for the separation of glycerides with different degrees of unsaturation, as well as their positional and stereo isomers.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Monocyclic and Polycyclic Aromatic Hydrocarbons in Fish Tissue

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An analytical method is presented in which fish tissue is analyzed for neutral monocyclic and polycyclic aromatic hydrocarbons (AHs) and aromatic sulfur heterocycles (ASHs) by capillary column gas chromatography (CGC) with photoionization detection. The sample enrichment procedure includes saponification with aqueous KOH, acidification of the digestates, and extraction of the aromatic compounds into cyclopentane-dichloromethane. Adsorption chromatography on tandem segments of potassium silicate and silica gel removes 99% of the coextracted lipid. Final enrichment by gel permeation chromatography eliminates residual biogenic material and potentially interfering alkanes. Relatively volatile monoaromatics are included among the analytes by virtue of the efficiency of the complementary enrichment steps, the use of small quantities of only low-boiling solvents, and the selectivity of the detector. Most targeted compounds (AHs ranging in size from C_3-alkylbenzenes through benzo[g,h,i]perylene and ASHs within the same size range) can be determined in 5 g (wet weight) samples of fish tissue at concentrations as low as 20 ng/g. Comparisons are made of recoveries of selected AHs under ordinary and gold fluorescent lighting conditions.

Aromatic hydrocarbons (AHs) are heterogenous and ubiquitous contaminants of aquatic environments (1–3). To understand the relative toxicological importance to aquatic ecosystems of compounds or groups of compounds within the AH family, we must characterize the bioavailability of AHs to fish and other aquatic animals. Woodward and coworkers (4) found, in a laboratory exposure study, that the concentrations of unmetabolized AHs in the tissues of exposed fish were directly proportional to the toxic effects (e.g., caudal fin erosion and gill lesions) and to the concentrations of the AHs in the exposure water. In a laboratory study (5) and in field studies (6–10), lower positive correlations were observed between AH concentrations in fish and concentrations in the exposure water or pathologies in the fish.

It is generally accepted that prolonged exposure of fish to AHs induces or activates enzymes that rapidly biotransform a wide range of AHs, especially those with 4 or more aromatic rings (11). Consequently, the concentrations of the unmetabolized forms of the larger AHs in fish tissues are usually low, irrespective of exposure concentrations or pathologies in the fish. Certain primitive invertebrates of the phylla Protozoa, Porifera, Coelentera, and Mollusca have inefficient mechanisms for metabolizing AHs (12) and bioaccumulate the larger AHs (4 or more aromatic rings) to an extent not seen in fish. Because of these phenomena, certain species of bivalve mollusks have recently been nominated for use as sentinel organisms of aquatic AH contamination (13).

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[1] References to trade names or manufacturers do not imply Federal government endorsement of commercial products.

Most published methods for the determination of AHs in animal tissues target some or all of the array of AHs having 2 to 6 aromatic rings (3, 14–19). Other analytical procedures are restricted in their scope to the spectrum of more volatile AHs ranging in size from toluene to the alkylated naphthalenes (20–22). The family of AHs ranging in molecular size from toluene to coronene shows continuums of physical and chemical behavior (3, 23) that render such demarcations in analyte ranges physically and chemically arbitrary. The classical enrichment sequence of saponification, liquid-liquid partitioning, and preparative chromatography, applied to the determination of AHs with 2 to 6 rings, usually sacrifices AHs more volatile than naphthalene (14, 15, 17–19). These delimitations in analyte ranges are sometimes prompted by toxicological criteria. More often, however, the greater volatilities of the AHs of lower molecular weight than naphthalene preclude substantial recoveries of these AHs by these analytical schemes, or prevent the instrumental determination of these compounds simultaneously with the 2-ring to 6-ring aromatics. Conversely, the headspace techniques most commonly applied to the determination of monoaromatics and diaromatics in tissues are usually not amenable to determination of the larger, less volatile AHs (20–22).

We have developed an analytical method that encompasses the analyte size and volatility ranges of both of these commonly used approaches, yet does not have the low determinative sensitivity usually associated with methods of expanded scope. Monocyclic and polycyclic aromatic hydrocarbons ranging in size from C_3-benzenes through benzo[g,h,i]-perylene, and aromatic sulfur heterocycles (ASHs) within the same volatility range, can be determined in 5 g (wet weight) samples of fish tissue. The analytical method was developed and validated specifically for the determination of AHs in fish samples from chronic toxicity studies with a Wyoming crude oil (WCO), a petroleum that contains no AHs larger than C_2-phenanthrenes. The method was later applied to the determination of environmentally incurred AH and ASH residues in fish.

Presented here are the results of analyses of fish samples fortified with a suite of AHs that simulates the aromatic fraction of the WCO. Also presented are recovery data from fish samples fortified with larger AHs and ASHs and constituting part of the quality control regimen for the application of the procedure to environmental fish samples. Although validated for several species of fish, this procedure is expected to be applicable to all animal tissues, including mollusks and other invertebrates.

METHOD

Materials and Reagents

(a) *Solvents.*—Cyclopentane and dichloromethane, OmniSolv™ grade (EM Science, Cherry Hill, NJ 08034). Solvents were not further purified before use.

(b) *Concentrated HCl, KOH pellets, and $NaH_2PO_4 \cdot H_2O$.*—Reagent grade and also used as received from the manufacturers.

(c) *Acid-buffer solution.*—Mixture of ca 4.9 M HCl and ca 0.9 M NaH_2PO_4). Prepared by adding 412 mL concentrated HCl to 400 mL deionized water, adding 125 g $NaH_2PO_4 \cdot H_2O$, and diluting to 1 L with deionized water.

(d) *Sodium sulfate.*—Reagent grade, anhydrous Na_2SO_4. Heated to 450°C for 8 h, and stored in a tightly capped bottle until used.

(e) *Adsorbents.*—(*1*) Silica gel (SG60, 70–230 mesh; EM Science). Washed with methanol and dichloromethane, and stored at 190°C. (*2*) Potassium silicate (KS) containing 5 meq KOH/g. Prepared as described elsewhere (24) and stored at 130°C.

(f) *Glassware.*—Washed with detergent and water, rinsed with tap water and then with 1 M HCl, and heated to 450°C for 8 h. Calcined glassware was rinsed with dichloromethane just before use. Pasteur pipets were heated to 450°C for 8 h, and stored in sealed jars.

(g) *Lighting.*—The sample enrichment and analyses for some of the samples were performed under ordinary fluorescent light. For other samples (where specified), all handling was done under gold fluorescent lamps (Model F40GO Bug-A-Way™; Philips Lighting Corp., Bloomfield, NJ 07003).

(h) *Standard materials.*—The AHs and ASHs used to develop the procedure and generate the data contained herein were obtained from the following suppliers: Aldrich Chemical Company, Inc., Milwaukee, WI 53233; Chem Service, West Chester, PA 19381; Ultra Scientific, Hope, RI 02831; and the U.S. EPA Pesticide and Industrial Chemical Repository, Research Triangle Park, NC 27711.

Apparatus and Operating Conditions

(a) *Gel permeation chromatograph (GPC).*—Autoprep® 1001 (ABC Laboratories, Inc., Columbia, MO 65205) specially modified as follows: The original pumping unit was replaced with a high-pressure liquid chromatographic pump (Model 6000A, Waters Associates, Inc.). The 23 sample loops (5.0 mL capacity) were replaced with sample loops of 1.0 mL capacity. Tandem columns (both 1 cm id) were substituted for the original 2.5 cm id column. A UV detector (254 nm) was plumbed in-line between the exit end of the second column and the dump/collect solenoid; the detector output was monitored during all GPC calibration and sample enrichment runs by means of a strip chart recorder. The sample loading system was modified to facilitate the loading of samples of between 0.4 and 0.5 mL (plus minute rinsings of the sample test tubes) into the 1.0 mL sample loops. All Teflon tubing through which samples must pass while being loaded into the sample loops was replaced with narrower bore tubing (0.75 mm id) to reduce dead volume and mixing of the solutions during the loading process. A vertically affixed 1.0 mL syringe barrel served as a sample-loading reservoir. The syringe needle was firmly inserted into the Teflon tubing that leads through the load/run valve to the sample loops. This tubing is of known dead volume, enabling a sample of 0.4 to 0.5 mL to be transferred to the reservoir-syringe, pushed downward into the tubing, and then displaced into the sample loop with the appropriate volume of rinse solvent (mobile phase). The described procedure for loading the GPC sample loops is essentially quantitative.

Bio-Beads S-X3 GPC resin (Bio-Rad Laboratories, Richmond, CA 94804), received from the manufacturer as 200–400 mesh, were sieved, and 3.8 g portions of the 270–325 mesh fraction were used to pack each of the 1.0 cm id columns. After the Bio-Beads had swollen for 24 h in the GPC mobile phase, cyclopentane-dichloromethane (50 + 50, v/v), the columns were slurry-packed. The adjustable column plungers were inserted so that each column had a bed length of 22 cm. The GPC was operated at a flow rate of 1.3 mL/min (typical pressure, 12 psi).

(b) *GC System 1.*—Model 3700 gas chromatograph (Varian Instrument Group, Walnut Creek, CA 94598) equipped with a Varian Model 8000 autosampler and a photoionization detector (PID) operated at 270°C (Model PI-52-02; HNU Systems Inc., Newton, MA 02161). A 9.5 eV lamp (with an intensity setting of 7) was used as the ionizing source. The fused silica capillary GC column (30 m × 0.25 mm id; DB-5 bonded phase of 0.25 μm film thickness) and the uncoated, deactivated, fused silica retention gap (1.0 m × 0.32 mm) were obtained from J & W Scientific, Folsom, CA 95630. Samples were injected (1.0 or 2.0 μL; either by hand or by autosampler) by the direct (flash) method, at 260°C, into a custom-made injector liner (similar to J & W Scientific Cat. No. 210-1064, but optimized for 0.32 mm tubing). Carrier gas, H_2 at 8 psig; detector make-up gas, N_2 at 28 mL/min flow rate. Immediately upon injection, the GC oven temperature was programmed from 50°C to 295°C at a rate of 5°C/min and was held at 295°C for 10 min.

(c) *GC system 2.*—Model Sigma 2B gas chromatograph equipped with Model AS-100A autoinjector and Model PI-52-02A photoionization detector (Perkin-Elmer Corp., Norwalk CT 06859). Operating parameters were similar to those for GC system 1.

(d) *Data systems used with GC system 2.*—Nelson Analytical model 2600 and 2670 chromatography software on an IBM compatible microcomputer. Chromatographic data were collected, integrated, and archived with this system. Individual AHs and ASHs were identified by their retention indices and quantitated by using 4-level calibration curves and an instrumental internal standard. Data base management was provided by computer programs written in DSM-11 (Digital Standard MUMPS) for the PDP-11/34 minicomputer (Digital Equipment Corporation) family of computers.

Saponification and Extraction

Fish samples of 5 g (wet weight), subsamples of previously ground fish or whole small fish, were weighed into tared tissue homogenizer vessels. Sample sets contained a maximum total of 23 unknown and control samples (limited by the number of GPC sample loops). Then 15 mL 3.0 M KOH was added to the vessels, and the tissue and base were homogenized by a Brinkmann Polytron. The homogenates were poured into 50 mL centrifuge tubes. When applicable, the centrifuge tubes were fortified with AHs. The tubes were tightly sealed; the caps had Teflon liners, with latex rubber disks (ca 3 mm thick) between the caps and the Teflon. The capped tubes were shaken, and then submerged to the levels of their contents in a water bath at 90°C. The saponifications were allowed to continue for 90 min; the tubes were shaken twice during that time.

The centrifuge tubes were removed from the water bath and allowed to cool to room temperature. The digestates were acidified to pH 5.9–6.1 by adding ca 8 mL acid-buffer solution. Only 7.5 mL was added initially, and the digestates were thoroughly mixed. The pH adjustments were monitored

with a pH meter while more HCl-buffer solution was slowly (dropwise) added. After the required pHs had been attained, the digestates were again allowed to cool. Each acidified digestate was partitioned, in its centrifuge tube, with two 15 mL portions of cyclopentane-dichloromethane (80 + 20, v/v). The tubes were mechanically shaken for 5 mL, and then centrifuged for 15 min at ca 3000 rpm. Pasteur pipets were used to transfer the 2 organic phases from each digestate into 125 mL double-reservoir rotary evaporation flasks (25). The organic phases were each concentrated to 5 mL on a rotary evaporator (ambient temperature water bath; ca 500 torr vacuum).

Enrichment by Adsorption Chromatography

The concentrated organic phases from the digestates were then subjected to chromatographic enrichment on tandem segments of KS and silica gel. Apparatus for these enrichments were prepared as follows: Plugs of glass wool were tamped down in the bottom of glass columns (30 cm × 2 cm id; solvent reservoirs at the top), and Na_2SO_4 was deposited over the glass wool to segment heights of ca 1 cm; 8.0 g silica gel was deposited above the Na_2SO_4, followed by 24 g KS, and then Na_2SO_4 was deposited over the KS to segment heights of ca 1 cm. The adsorbent beds were not saturated with solvent before the chromatographic enrichments. The concentrated sample extracts were quantitatively transferred to the tops of the columns, and the adsorbents were then washed with 75 mL cyclopentane-dichloromethane (80 + 20, v/v). The effluent solutions were collected in 125 mL double-reservoir flasks. The eluates contained the AHs and the ASHs, as well as aliphatic hydrocarbons, and ca 1% (gravimetrically) of the coextracted biogenic material. About 99% of the coextracted, hydrolyzed lipid and various polar xenobiotic compounds were retained by the adsorbents.

The eluates from the adsorption enrichment were each rotary evaporated (ambient temperature water bath; ca 500 torr vacuum) to 5 mL and then filtered into graduated 15 mL culture tubes. The filtration columns consisted of Pasteur pipets into which disks of glass fiber filter (3 μm retention) had been tamped.

Gel Permeation Chromatography

Residual biogenic materials and aliphatic hydrocarbons were removed from the sample extracts by GPC. Before the solutions could be loaded into the GPC sample loops, however, their solvent compositions had to be similar to the GPC mobile phase, and their volumes had to be between 0.4 and 0.5 mL. Small stir bars were added to the culture tubes, and the solutions were gently evaporated with streams of dry nitrogen to ca 1 mL each. During this process, the solutions were stirred while the culture tubes were kept partly immersed in a bath of ambient temperature water. The stir bars were removed and all adhering liquid and residues were rinsed back into the tubes with 1 mL of CH_2Cl_2. The evaporations were then continued until the solution volumes were between 0.4 and 0.5 mL.

Before each multiple-sample enrichment procedure, the GPC was calibrated by establishing an elution profile with a calibration solution containing di-*n*-octylphthalate, 15 C_3- and C_4-alkylbenzenes, and pyrene. Three peaks appeared in that sequence. The calibration chromatograms were examined to ensure that the relative retention times (RRTs) and peak shapes were as expected. The cutoff point between the dumped (first) fractions and the collected (second) fractions for the subsequent multiple-sample enrichment run was selected in such a way that any alkylbenzenes present would start eluting at the beginning of the collected fractions and would elute entirely within the collected fractions. As a result of these calibrations, the timing functions on the GPC controller box were typically set so that the first 27 mL eluate from each individual chromatographic enrichment was dumped, and the subsequent 13 mL eluate was collected.

The second fractions of eluate from the GPC were collected in graduated culture tubes. Stir bars were added, and the solutions were gently evaporated with streams of nitrogen (ambient temperature water bath; constant stirring) to volumes appropriate for analyses. The solutions were transferred to autoinjector vials, and instrumental internal standards (see below) were added.

Results

Initial Validation of Procedure

The analytical procedure was developed and validated specifically for the determination of the aromatic components of a Wyoming crude oil (WCO) in fish samples. A chronic toxicity diluter study was to be conducted at the National Fisheries Contaminant Research Center using fathead minnows (*Pimephales promelas*) and the WCO (26). The solu-

Table 1. Results of initial method validation; recoveries of selected aromatic hydrocarbons from fortified fathead minnows (samples handled under white fluorescent light)

Compound	Mean recovery, % ± SD		
	20 ng/g Spikes[a]	100 ng/g Spikes[b]	500 ng/g Spikes[b]
C_3-alkylbenzene	60 ± 15	55 ± 9	45 ± 8
C_3-alkylbenzene	62 ± 16	59 ± 8	49 ± 9
C_3-alkylbenzene	52 ± 12	53 ± 7	45 ± 8
C_4-alkylbenzene	104 ± 21	66 ± 10	51 ± 9
C_4-alkylbenzene	65 ± 18	62 ± 9	53 ± 8
C_4-alkylbenzene	56 ± 13	58 ± 9	50 ± 8
Indan	57 ± 15	60 ± 8	53 ± 10
Indene	36 ± 6	38 ± 11	26 ± 7
C_4-alkylbenzene	59 ± 12	62 ± 8	62 ± 8
C_4-alkylbenzene	63 ± 16	———[c]	63 ± 9
C_4-alkylbenzene	62 ± 14	67 ± 9	66 ± 9
Naphthalene	202 ± 36[d]	81 ± 11	76 ± 8
2-Methylnaphthalene	131 ± 14[d]	88 ± 14	75 ± 11
1-Methylnaphthalene	82 ± 14	80 ± 10	77 ± 8
Biphenyl	67 ± 12	76 ± 9	75 ± 8
Acenaphthylene	67 ± 8	73 ± 8	74 ± 7
1,2-Dimethylnaphthalene	69 ± 8	78 ± 7	74 ± 6
Acenaphthene	64 ± 8	76 ± 5	73 ± 8
(Dibenzofuran)[e]	(100)	(100)	(100)
Fluorene	75 ± 9	87 ± 10	79 ± 10
9,10-Dihydroanthracene	57 ± 19[f]	83 ± 11	69 ± 7
1-Methylfluorene	80 ± 10	93 ± 12	78 ± 9
Phenanthrene	94 ± 10	98 ± 13	82 ± 6
Anthracene	69 ± 10	91 ± 11	79 ± 9
9,10-Dimethylanthracene	73 ± 13	85 ± 17	73 ± 10

[a] Eight validation replicates were analyzed; $n = 8$ except where otherwise noted.
[b] Seven validation replicates were analyzed; $n = 7$.
[c] Gross interference in all seven replicates.
[d] Probable positive bias from background.
[e] Dibenzofuran is the instrumental internal standard.
[f] Because of analytical interference in one replicate, $n = 7$ (for this compound only).

Table 2. Recoveries of aromatic hydrocarbons and aromatic sulfur heterocycles from fortified samples of striped bass

Compound	Spike level, ng/g	Recovery, % (mean ± SD) White light 1X Spike level[a] (n = 20)	Gold light 1X Spike level[a] (n = 3)	Gold light 2X Spike level[b] (n = 3)	Gold light 4X Spike level[c] (n = 5)
Naphthalene	252	65 ± 17	104 ± 10	85 ± 6	71 ± 9
1-Methyl naphthalene	120	——[d]	88 ± 4	77 ± 2	71 ± 6
2,3-Dimethyl naphthalene	138	——	90 ± 3	84 ± 4	74 ± 3
Acenaphthylene	250	48 ± 31	70 ± 2	74 ± 5	71 ± 5
Acenaphthene	250	——	80 ± 5	82 ± 6	76 ± 4
Fluorene	250	80 ± 6	76 ± 8	78 ± 4	73 ± 5
Dibenzothiophene	258	88 ± 5	66 ± 2	68 ± 3	71 ± 11
Phenanthrene	250	103 ± 18	70 ± 3	74 ± 5	77 ± 8
Anthracene	125	17 ± 10	62 ± 3	68 ± 5	67 ± 7
2-Methyl anthracene	121	——	101 ± 5	100 ± 6	87 ± 12
1-Methyl phenanthrene	124	——	83 ± 7	79 ± 6	76 ± 5
Fluoranthene	250	102 ± 23	118 ± 1	92 ± 18	82 ± 10
Pyrene	250	67 ± 9	117 ± 13	97 ± 8	84 ± 4
Benzo[b]naphtho[2,1-d]-thiophene	132	91 ± 14	86 ± 9	92 ± 17	83 ± 8
Benz[a]anthracene	125	50 ± 9	86 ± 4	87 ± 5	88 ± 19
Chrysene	125	72 ± 21	78 ± 3	73 ± 3	82 ± 18
Benzo[b]fluoranthene	125	——	88 ± 3	87 ± 6	87 ± 15
Benzo[k]fluoranthene	125	——	80 ± 13	76 ± 8	81 ± 14
Benzo[a]pyrene	125	3 ± 6	129 ± 9	124 ± 7	103 ± 13
Perylene	130	1 ± 1	129 ± 16	116 ± 26	107 ± 28
Indeno[1,2,3-c,d]pyrene	125	——	91 ± 10	84 ± 9	83 ± 11
Dibenz[a,h]anthracene	125	——	70 ± 10	74 ± 12	83 ± 22
Benzo[g,h,i]perylene	125	——	87 ± 6	94 ± 7	86 ± 11

[a] Control tissue spiked at the concentrations listed.
[b] Control tissue spiked at twice the concentrations listed.
[c] Control tissue spiked at 4 times the concentrations listed.
[d] Compound not spiked.

tion used to fortify samples (uncontaminated fathead minnows) for the validation of the analytical method contained equal concentrations of 30 aromatic compounds ranging in size from C_3-alkylbenzenes to 9,10-dimethylanthracene, and was a good synthetic representation of the spectrum of AHs found in the WCO. Of the 30 compounds (exclusive of indan and indene), 15 were C_3- and C_4- alkylbenzenes. We did not match specific alkylbenzenes with their resultant chromatographic peaks, but used a gas chromatograph/mass spectrometer (GC/MS) only to characterize the 30-component solution. The PID (as equipped with the 9.5 eV lamp) was not responsive enough to 6 of the 15 alkylbenzenes for their recoveries to be calculated and reported. At least 7 validation replicates were evaluated at each of 3 spike levels, 20, 100, and 500 ng/g; the recoveries are listed in Table 1. Validation replicate solutions were reduced to volumes of 0.2, 1.0, and 5.0 mL, respectively, for GC analysis of the 20, 100, and 500 ng/g spikes. Immediately before GC analyses, dibenzofuran was added to the replicate solutions in the same proportions as the fortification concentrations. The recoveries shown in Table 1 were calculated on the basis of peak height measurements; dibenzofuran was used as an instrumental internal standard. All results reported in Table 1 were obtained with samples handled under white fluorescent light and analyzed on GC system 1.

Further Characterization of the Procedure

The analytical method was later applied to the determination of AHs and ASHs ranging in size from naphthalene to benzo[g,h,i]perylene in several species of freshwater fish from various sites in the United States. Data from 2 categories of quality control samples whose analyses accompanied the analyses of the environmental fish samples are shown in Tables 2 and 3. Some of the samples in each table were subjected to the analytical procedure under white fluorescent light, and others were analyzed after the gold lights had been installed in an effort to increase recoveries [(27); see *Discussion*]. Table 2 shows the recoveries of AHs and ASHs from fortified samples of striped bass (*Roccus saxatilis*).

One 5 g aliquant of homogenized fish tissue containing environmentally incorporated AH and ASH residues was analyzed with the environmental fish samples in each 23-sample set. The aliquants were taken from a composite of brown bullheads (*Ictalurus nebulosus*) from the lower Black River, Ohio. The concentration data from these quality control samples are listed in Table 3. Targeted for determination in the bullhead tissue were all of the AHs and ASHs listed in Table 2; however, no aromatic compounds of greater molecular weight than pyrene were present at quantifiable concentrations. Of the 27 replicate samples shown in Table 3, 5 were handled under gold fluorescent lamps, and the rest were handled under white fluorescent lamps.

The analytical conditions used for the samples shown in Tables 2 and 3 were slightly different from those used for the fortified samples of fathead minnow tissue shown in Table 1. After the enriched extracts were adjusted to 1.0 mL final volumes, 1-chloronaphthalene, 9-chloroanthracene, and 9,10-diphenylanthracene were added to the solutions. The

Table 3. Concentrations (ng/g) of environmentally incurred aromatic hydrocarbons and aromatic sulfur heterocycles in aliquants of brown bullhead composite; results obtained under white fluorescent light versus those obtained under gold light

	White light (n = 22)			Gold light (n = 5)		
Compound	Mean concn	SD	RSD, %	Mean concn	SD	RSD, %
Naphthalene	190	54	29	190	7	4
1-Methyl naphthalene	90	17	20	90	5	6
2,3-Dimethyl naphthalene	140	19	14	110	4	4
Acenaphthylene	200	42	21	160	32	20
Acenaphthene	170	26	16	150	12	8
Fluorene	600	86	14	580	43	7
Dibenzothiophene	200	34	17	200	29	14
Phenanthrene	1900	320	17	1500	43	3
Anthracene	160	45	29	220	52	24
2-Methyl anthracene	60	26	44	90	15	17
1-Methyl phenanthrene	100	34	34	110	7	7
Fluoranthene	880	160	18	650	37	6
Pyrene[a]	340	70	21	300	31	10

[a] Although all of the AHs and ASHs listed in Table 2 were also targeted analytes in the brown bullhead samples, no AHs or ASHs larger or later eluting than pyrene were present at quantifiable concentrations.

peaks resulting from all 3 compounds served as references for calibration of retention indices; the 1-chloronaphthalene additionally served as an instrumental internal standard. The data in Tables 2 and 3 were obtained by using GC system 2 and the data system described above. Quantitations were performed on the basis of peak areas relative to the peak areas of the instrumental internal standard, 1-chloronaphthalene.

An additional category of quality control sample was included in each 23-sample group of environmental and quality control samples. ^{14}C-labeled dibenz[a,h]anthracene was used to spike 5 g portions of various species of environmental fish samples, each at a concentration of 90 ng/g. One or more of these spiked controls were included in each 23-sample set. The radiometric recoveries of this compound were determined by liquid scintillation counting (LSC) after the samples had undergone GPC enrichment. Of these spikes, 41 underwent enrichment and analyses before the installation of gold lights, and 7 more were processed after gold lights had been installed. The mean recoveries were 89 ± 13% and 96 ± 10%, respectively. No effort was made to ascertain whether these recoveries were positively biased by recoveries of radioactive degradation products.

The amenability of the analytical procedure to the simultaneous determination of small alkylbenzenes, ASHs, and larger (Priority Pollutant and other large) AHs is illustrated by Figure 1. The method lower limit of quantitation (MLLQ) for this procedure was conservatively and generically set at 20 ng/g. This MLLQ was established by evaluation of chromatograms of fortified and unfortified control fish tissue. The average area of the background peaks (or noise) in these chromatograms was equivalent to the area of 33 pg of an AH or ASH of average detector response. This number, 33 pg per 1 μL injection (5 g eq fish tissue; solution final volume of 0.3 mL), was then multiplied by 10 (330 pg per 17 mg eq tissue), resulting in a calculated MLLQ of about 20 ng/g.

As can be seen in Figure 1, the method lower limits of detection are much lower than 20 ng/g. The PID detector, as equipped with the 9.5 eV lamp, is insufficiently responsive to some alkylbenzenes (e.g., n-butylbenzene) for a MLLQ of 20 ng/g to be feasible, and these are among the compounds in the fortification solution for which no recovery data were reported in Table 1. As is also evidenced by Table 1, the alkylbenzene region of the chromatograms were subject to sporadic background interferences. In presenting the data in Table 1, we did not subtract background, but noted the most obvious interferences. Because the QA/QC samples described by Tables 2 and 3 were analyzed at final solution volumes of 1.0 mL, the MLLQs for these samples were proportionately higher.

Discussion

The method accommodates samples containing not more than about 0.7 g of total lipid. For fish suspected of being rich in lipid, we determined the lipid percentages to decide the maximum wet weight of tissue that should be digested per sample. When 5 g portions of lipid-rich species such as walleyes (*Stizostedion vitreum*) or fathead minnows were saponified, the enrichment capacity of the method was overloaded for a few of the samples. The eluates from the KS and silica gel adsorbents, when concentrated for their application to GPC, were sometimes too viscous to be easily loaded into single 1.0 mL sample loops. Characterization of the viscous material by solid probe GC/MS indicated that it was primarily squalene, a polyunsaturated hydrocarbon. If lipid determination indicated that a fish sample contained more than ca 13% lipid, less than 5 g (wet weight) of tissue was saponified.

To achieve tight seals of the centrifuge tube caps, we placed latex disks between the caps and their Teflon liners. The resiliency of the latex helped ensure tight seals during saponification and during the later partitioning with organic solvent. To ensure good seals, we also found it important to choose Teflon liners that were smooth, rather than striated.

All fortifications of radiolabeled or "cold" AHs and ASHs were applied to the samples after the tissue-base homogenates had been transferred to the centrifuge tubes. We found that when the tissue was spiked before homogenization, some of the spiked AHs adhered to the Polytron homogenizer. This appeared to be due to the immiscibility of the hydrophobic spiking solvent with the tissue-base homogenate.

The digestates were acidified before the partitioning step to avoid intractable emulsions caused by the presence of soap at alkaline pHs. Acidification causes high percentages of the lipid to be recovered in the organic phases, however, and this lipid must be removed in later procedural steps. One lipid-removal measure that was explored was back-partitioning of the combined organic phases with fresh aqueous KOH (18), but this too caused intractable emulsions. Because the lipid recovered in the organic phases by the partitioning of acidified digestates is primarily in hydrolyzed form, we found it to be especially susceptible to removal by KS. The acidification of the digestates and the adsorption chromatography on tandem segments of KS and silica gel were mutually complementary facets of the enrichment scheme that made possible the efficient separation of AH analytes from hydrolyzed lipids. The emulsions associated with this procedural sequence were easily overcome by centrifugation.

As implied above, free fatty acids were the main biogenic constituents recovered from fish tissue subjected to the saponification-acidification-extraction procedural sequence.

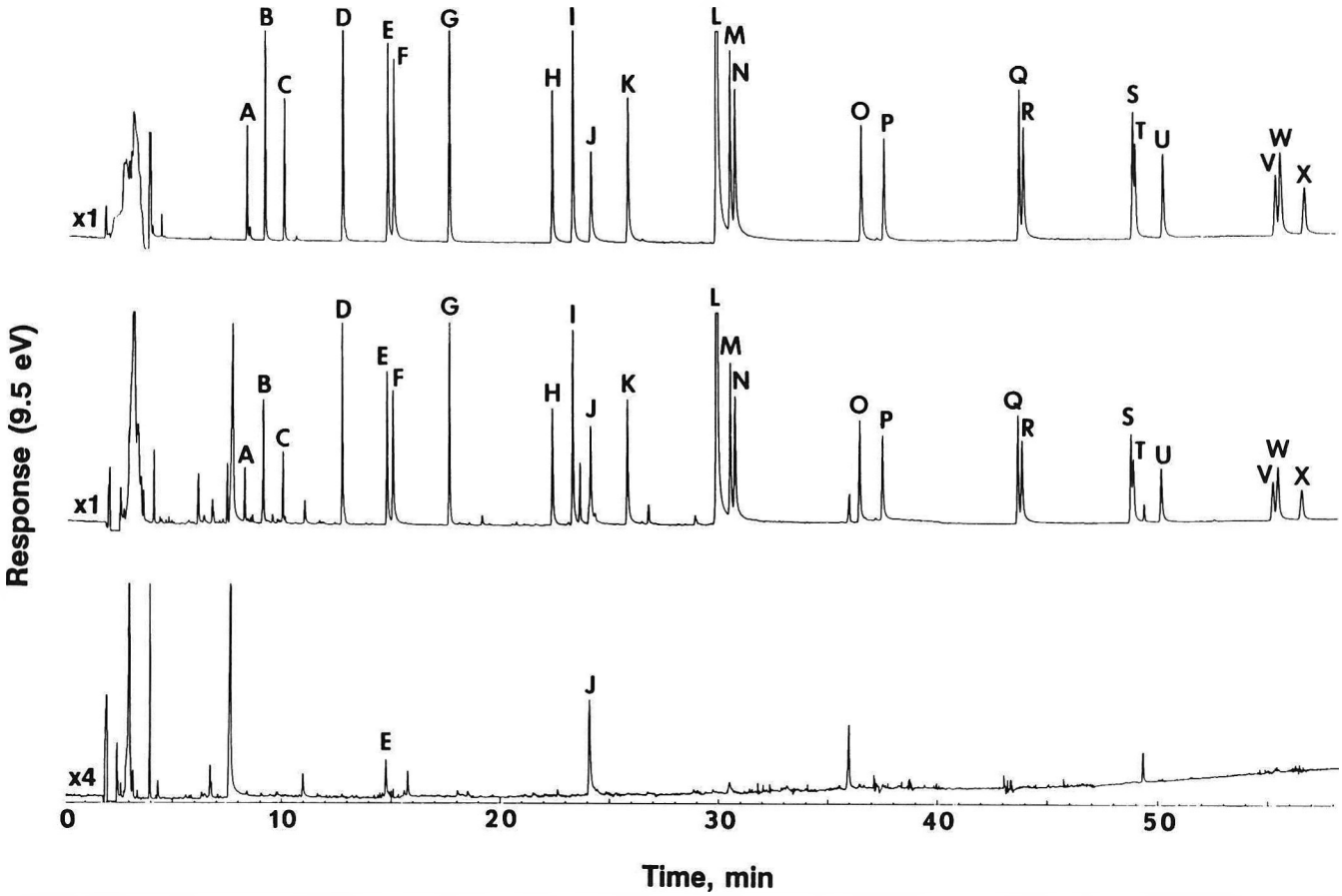

Figure 1. Normalized GC-PID chromatograms representing, from top to bottom: a standard solution containing ca 1.3 μg/mL of each of the AHs and ASHs listed below (compound L at ca 6.6 μg/mL); the same compounds as recovered from grass carp tissue fortified at concentrations of 80 ng/g (compound L fortified at 400 ng/g); an unfortified aliquant of the same tissue. Compound J (instrumental internal standard) was spiked into all 3 solutions at concentrations of ca 1.3 μg/mL, 80 ng/g, and 20 ng/g, respectively. The labeled peaks represent: (A) 4-ethyltoluene; (B) 1,2,4-trimethylbenzene; (C) 4-isopropyltoluene; (D) 1,2,4,5-tetramethylbenzene; (E) naphthalene; (F) thianaphthene; (G) pentamethylbenzene; (H) acenaphthylene; (I) acenaphthene; (J) dibenzofuran (instrumental internal standard); (K) fluorene; (L) dibenzothiophene; (M) phenanthrene; (N) anthracene; (O) fluoranthene; (P) pyrene; (Q) benz[a]anthracene; (R) chrysene; (S) benzo[b]fluoranthene; (T) benzo[k]fluoranthene; (U) benzo[a]pyrene; (V) indeno[1,2,3-c,d]pyrene; (W) dibenz[a,h]anthracene; and (X) benzo[g,h,i]perylene.

Although the acids were efficiently removed by adsorption on KS, we found that inclusion of the segment of silica gel beneath the KS improved the chromatographic enrichment. Both adsorbents were required in tandem to remove approximately 99% (gravimetrically) of the coextracted lipid.

A 90/10 (v/v) ratio of alkane to dichloromethane is more than adequately polar to easily elute all AHs and ASHs from the tandem adsorbents, but we found that the eluates resulting from this solvent mixture contained more biogenic material than when the eluant composition was the prescribed cyclopentane-dichloromethane (80+20, v/v). We were unable to explain this phenomenon.

Much of the data shown in Tables 2 and 3 had already been compiled before we installed gold lighting at our laboratory. During the validation of the procedure, we had noticed the disappearance of anthracene and 9,10-dimethylanthracene from spiking solutions containing the mixture of compounds listed in Table 1. This firsthand experience, anecdotal evidence, and publications such as those of Leversee et al. (27) and Behymer and Hites (28) led us to investigate whether gold fluorescent lamps would prevent photodegradation. The use of gold fluorescent lighting improved recoveries (27) of certain AHs, as illustrated by comparisons of recoveries (Table 2) from samples processed under both lighting conditions. Of the compounds spiked into samples handled under each type of illumination, the improvements in recoveries, by use of gold lamps, were greatest for the AHs that are especially photolabile—acenaphthylene, anthracene, and benzo[a]pyrene (28). We were unable to explain the higher recovery of phenanthrene under white lights (Table 2).

Because of a 2-month delay in the installation of the PID in GC system 2, the samples described in Table 2 suffered a lag between enrichment and chromatographic analyses. During the interim, the solutions were not consistently shielded from light (from either of the designated types of fluorescent lamps). In contrast, the fortified fathead minnow samples were analyzed within about 3 days of their final enrichments by GPC. This may explain the comparatively high recoveries of photolabile acenaphthylene and anthracene from the fathead minnows (Table 1). Determinations of recoveries of radiolabeled dibenz[a,h]anthracene (by LSC) were not delayed after completion of final enrichment, and this promptness may account for the comparatively high recoveries of this relatively photolabile (28) compound under the 2 types of illumination. The handling of samples under gold light increased the precision of the recoveries of the larger AHs. This improvement is most dramatically evidenced by Table 3.

We hypothesize that the relative effects of the 2 different lighting conditions on the recoveries of the AHs and ASHs from the brown bullhead tissue (Table 3) were lessened by the presence of extremely high total AH and ASH concentrations. The bullhead tissue contained additively high (parts-per-million) concentrations of many alkylated and non-alkylated AHs not listed in Table 3, as is typical of brown bullheads from the lower Black River, Ohio (7, 8). The enriched bullhead extracts were fluorescent orange, and the light-absorbing AH residues may have exerted a mutually protective effect, hindering the photooxidation of the especially vulnerable compounds. These protective effects probably included quenching of singlet oxygens, the putative reactive form of molecular oxygen, and competitive photon absorption by the less reactive AHs (29).

Because the evaluation of the relative effects of the 2 types of illumination on procedural recoveries was secondary to the main purposes of the studies described herein, the comparisons were performed under less than ideally controlled conditions. The comparative results were still dramatic enough for us to require gold lighting for all future analytical work targeting AHs. We have since installed gold lamps in 3 of our laboratory rooms, and we also use amber glassware whenever possible.

During the early stages of the development of this analytical procedure, we had intended to use a flame ionization detector (FID) for the analyses of the samples. Since our sample of WCO consists of about 90% aliphatic material, a procedure was required that would determine AHs from WCO while eliminating interferences from aliphatic hydrocarbons. Although the GPC removed most of the alkanes from the fathead minnow samples, the FID did not sufficiently discriminate against the remaining alkanes to prevent interferences to analyses for AHs. The FID chromatograms of fish samples and controls also had peaks resulting from background contamination. The alkylbenzene regions of these FID chromatograms contained many peaks that rendered analyses for C_3- and C_4-benzenes problematic. Most of these early eluting peaks were caused by solvent impurities.

We emulated Warner (30) in developing the miniaturized analytical procedure described here, but despite the small (about 150 mL) total volume of solvents used per 5 g sample, there were still interferences from this source. Rather than add steps to the enrichment procedure by fractionating the partially enriched sample solutions on silica gel, alumina, or Sephadex LH-20 (14, 15, 19, 30, 31), or partitioning them with dimethylsulfoxide (6, 14, 19, 32) or with dimethylformamide (15, 28), we opted to quantitate with a PID detector. When equipped with a 9.5 eV lamp, the PID is highly selective for AHs (33). For tissue samples not containing large amounts of alkanes, however, an FID can be substituted for the PID. The modified analytical procedure will be amenable to the determination of AHs (naphthalene and larger), although with decreased determinative sensitivity.

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Zinc Chloride-Diphenylamine Reagent for Thin Layer Chromatographic Detection of Some Organophosphorus and Carbamate Insecticides

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Zinc chloride-diphenylamine reagent, whose use has been reported for the detection of organochlorine insecticides by thin layer chromatography, was further studied for its ability to detect the organophosphorus insecticides phorate, phosphamidon, DDVP, and phosalone and the carbamate insecticides carbaryl and aldicarb. These insecticides give intense blue-green spots with this reagent. The procedure can be applied to the detection of the insecticides in biological materials and thus has a potential use in forensic toxicology.

Organophosphorus, organochlorine, and carbamate insecticides are increasingly being used domestically and in agriculture on a daily basis. New varieties of these insecticides are becoming available every year. Because of their easy availability, insecticides are frequently misused in poisoning episodes involving homicides and suicides. Consequently, forensic toxicologists need to be able to characterize these insecticides.

A number of spray reagents for the detection of these insecticides by thin layer chromatography (TLC) have been described in the literature. One of them, zinc chloride-diphenylamine (1-4), has commonly been used for the identification of organochlorine insecticides, which give a blue-green spot with this reagent. It has been observed, however, that some organophosphorus and carbamate insecticides also give intense blue-green spots with zinc chloride-diphenylamine reagent. Hence, it was thought worthwhile to study this reagent further for its ability to detect various insecticides in biological material.

In this paper, we describe the application of zinc chloride-diphenylamine reagent for the TLC detection of the organophosphorus insecticides phorate, phosphamidon, DDVP, and phosalone, and the carbamate insecticides carbaryl and aldicarb, all of which give intense blue-green spots with this reagent. The limit of detection is approximately 10 μg.

Experimental

Materials

(a) *Silica gel.*—Silica Gel G, particle size 75 μg (E. Merck, Darmstadt, FRG).

(b) *Insecticide standard solutions.*—Technical grade samples of the organophosphorus insecticides phorate, phosphamidon, DDVP, and phosalone, and the carbamate insecticides carbaryl and aldicarb were used as standards. A 1 mg/mL solution in ethanol of each insecticide was prepared.

(c) *Zinc chloride and diphenylamine.*—Analytical reagent grade.

(d) *Acetone and ethanol.*—Analytical reagent grade.

(e) *Chromogenic spray reagent.*—Prepared by dissolving 1.0 g anhydrous zinc chloride and 0.5 g diphenylamine in 100 mL acetone. Freshly prepared reagent should be used.

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Extraction of Insecticides from Biological Material

Portions of ca 50 g each of various types of visceral tissue (stomach, intestine, liver, spleen, and kidney) containing the above insecticides were individually minced in aqueous solution. The insecticide being sought was then extracted with diethyl ether and the solvent was evaporated at room temperature. The residue was dissolved in 1-2 mL ethanol. A known volume of the solution was spotted on an activated TLC plate together with the standard solution of the known insecticide. The plate was then developed as described under *TLC Procedure* and sprayed with zinc chloride-diphenylamine chromogenic spray reagent.

TLC Procedure

A standard glass TLC plate was coated with a slurry of Silica Gel G in water (1 + 2) to a thickness of 0.25 mm. The plate was activated at 110°C for ca 1 h. A 10 mL aliquot of each of the 1 mg/mL standard insecticide solutions was spotted on the plate. The plate was then developed in a TLC chamber previously saturated with *n*-hexane-acetone (4 + 1). After the solvent had eluted 10 cm up the plate, the plate was removed from the chamber, dried in air, sprayed with the zinc chloride-diphenylamine chromogenic spray reagent, and heated at 110°C for 10 min. Blue-green spots were formed. The R_f values for these insecticides are listed in Table 1.

Results and Discussion

Zinc chloride-diphenylamine solution, which is generally used as a TLC spray reagent for the detection of organochloride insecticides, gives dirty green spots upon being heated at 110°C for about 15 min. Faucheux (2) reported that the organophosphorus insecticides ethyl parathion, methyl parathion, and fenitrothion, all of which contain the NO_2 group, give bluish-green spots with this reagent. We observed that phorate, phosalone, phosphamidon, and DDVP (organophosphorus compounds) and carbaryl and aldicarb (carbamates) give intense blue-green spots with the reagent.

The formation of blue-green color with chlorinated pesticides and some chlorine-containing organophosphorus insecticides (phosalone, phosphamidon, and DDVP) can be explained on the basis that when organic compounds containing halogens are heated with zinc chloride, oxidative decomposition occurs and liberates free halogen. The halogen, like other strong oxidants, converts diphenylamine into the blue quinoidal compound (5). Likewise, the formation of blue color with ethyl parathion, methyl parathion, and fenitrothion, all of which contain the NO_2 group, is based on oxidation of diphenylamine to an intensely blue quinoidal compound by organic nitrates, nitrites, and nitroamines (6). Similarly, aldicarb, a C-nitro compound, acts as a hydrogen acceptor through conversion to the NHOH group, while diphenylamine is a hydrogen donor and hence oxidizes to the blue quinoidal compound (7). Phorate and carbaryl, on the other hand, give a blue-green color with this reagent; upon being heated with zinc chloride, they form degradation products which probably further oxidize the diphenylamine to the blue quinoidal compound.

Table 1. R_f values of some organophosphorus and carbamate insecticides[a]

Insecticide	Color of spot after heating	R_f value
Phorate	blue-green	0.9
Phosalone	blue-green	0.54
Phosphamidon	blue-green	0.35
DDVP	blue-green	0.22
Aldicarb	blue-green	0.4
Carbaryl	blue-green	0.38

[a] Solvent system: *n*-hexane-acetone (4 + 1).

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Determination of Oxyfluorfen Herbicide and Oxyfluorfen Amine Residues in Garbanzo Beans by Liquid Chromatography

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Oxyfluorfen and oxyfluorfen amine were determined by liquid chromatography (LC) with ultraviolet (UV) and photoconductivity detection (PCD). A simple extraction procedure acceptably recovered both analytes from garbanzo beans over a wide range of fortifications (0.05 to 20 ppm) (83 ± 4 for oxyfluorfen; 85 ± 4 for oxyfluorfen amine). Percent recoveries decreased slightly as the fortification level decreased. Both analytes could be determined simultaneously at a concentration >0.2 ppm in garbanzo beans. Detection limits were 3 ng for oxyfluorfen and 100 ng for oxyfluorfen amine using LC/UV, and 12 ng for both oxyfluorfen and oxyfluorfen amine with LC/PCD. Different knitted reaction coils and photoreactors were evaluated. Photoproduct yields and identification were determined by ion chromatography. The LC/PCD method measures oxyfluorfen and oxyfluorfen amine separately and has a shorter analysis time, while the standard method using gas chromatography measures total residues and is more sensitive.

Oxyfluorfen [Goal®, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene] is a selective contact herbicide used on a variety of tropical and subtropical crops (Figure 1). Its low mammalian toxicity [acute oral LD_{50} (lethal dose, median) for rats >5000 mg/kg] and its strong sorption to soils and subsequent minimal leaching make it a low environmental risk and attractive to users.

The current method for oxyfluorfen residues in crops is a complex procedure involving extraction, refluxing, distillation extraction, derivatization, Florisil cleanup, and gas chromatography (GC) (1–3). The refluxing apparatus (4) is a specialty item and the number of available units limits the rate of sample preparation. Some analysts have reported that this method is time consuming, with a single analysis requiring 48 h (3). One advantage of this method is that the parent herbicide (oxyfluorfen), the phenylamine (oxyfluorfen amine), and phenylamide metabolites are all reduced to the phenylamine, derivatized, and measured as total residue. Nevertheless, the parent compound is the major excretion product in rats dosed with oxyfluorfen (5) and its discrete measurement is desirable.

We have shown that several herbicides, including oxyfluorfen, can be determined selectively in crops by photoconductivity detection (PCD) after separation by reverse-phase liquid chromatography (LC) (6). Luchtefeld used a similar postcolumn photolysis unit in conjunction with fluorogenic labeling in the proposed method for phenylurea herbicides (7). Recently, we used a modified *Pesticide Analytical Manual* (PAM) method (3) to determine oxyfluorfen in garbanzo bean samples. Both the success of the LC/PCD technique and the disadvantages of the PAM method discussed above led us to investigate analysis of oxyfluorfen and oxyfluorfen amine in fortified garbanzo bean matrix by LC/PCD. Sample preparation was modified to use a less toxic solvent and employ the selectivity of PCD.

The present paper demonstrates acceptable recovery of oxyfluorfen and oxyfluorfen amine from fortified garbanzo bean samples. Comparison of PCD and UV liquid chromatographic detectors and photoreactor design also are evaluated.

Experimental

LC Apparatus

Figure 2 is a schematic of the LC/PCD system.

(a) *Gradient pump.*—With eluant degas module (Model GPM and EDM II, respectively, Dionex, Sunnyvale, CA 94086).

(b) *Injector.*—2000 psi limit with 50 μL loop (Dionex).

(c) *Analytical column.*—3 × 30 mm, 3 μm C_{18} (CR and HS) (Perkin Elmer, San Jose, CA 95131) for reverse-phase LC and 4 × 250 mm, 5 μm OmniPac PAX-500 with a 4 × 50 mm, 5 μm OmniPac PAX-500 guard column (Dionex) for ion chromatography.

(d) *Suppressor.*—Dionex AMMS cation suppressor.

(e) *Photoreactors.*—(*1*) Laboratory constructed as de-

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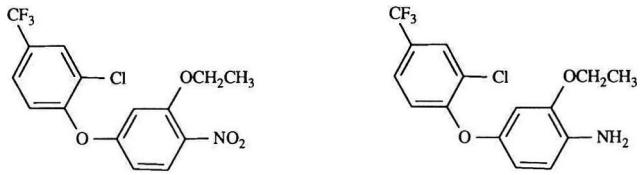

I II

Figure 1. Oxyfluorfen (I) and oxyfluorfen amine (II).

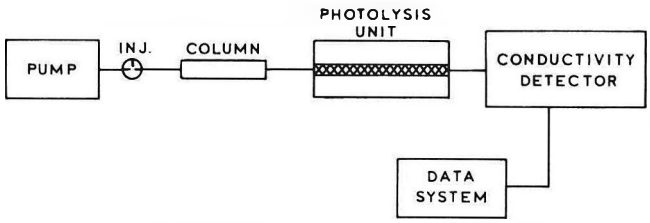

Figure 2. Schematic of LC/PCD system.

scribed in Reference 8. (*2*) Commercial photoreactor (Beam Boost, ICT Corp., Frankfurt, Germany. Available through ASTEC, Box 297, Whippany, NJ 07981).

(**f**) *Reaction coils.*—Commercial reaction coils: (*1*) 20 m × 0.4 mm id × 1.6 mm od and (*2*) 10 m × 0.4 mm id × 1.6 mm od (Beam Boost). Laboratory constructed reaction coils: (*3*) 10 m × 0.6 mm id × 1.6 mm od (ca 1.4 mL), (*4*) 5 m × 0.6 mm id × 1.6 mm od (ca 2.8 mL) TFE Teflon coils woven as (KOT-3) described by Selavka et al. (9), and (*5*) 5.8 m × 0.8 mm id × 1.6 mm od (ca 2.7 mL) TFE Teflon coil woven as described by Engelhardt and Neue (10).

(**g**) *Conductivity detector.*—Model CDM II (Dionex).

(**h**) *UV detector.*—UVIS 200 (Linear Instruments Corp., Reno, NV 89523).

(**i**) *Integrator.*—PC Integrator (PE Nelson, Cupertino, CA 95014).

(**j**) *GC apparatus.*—HP 5890A (Hewlett Packard Co., Avondale, PA 19311).

(**k**) *Electron capture detector.*—^{63}Ni 15 mCi (Hewlett Packard).

(**l**) *GC analytical column.*—25 m × 0.53 id mm, BP-5 capillary column (SGE Corp. Victoria 3134, Australia). Conditions: injector, 250°C; column, 210°C; detector, 300°C.

Reagents

(**a**) *Oxyfluorfen.*—98.9% (Rohm & Haas).

(**b**) *Oxyfluorfen amine.*—99.9% (Rohm & Haas).

(**c**) *Water.*—Obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(**d**) *Acetonitrile.*—Optima grade (Fisher Scientific, Fair Lawn, NJ 07410).

(**e**) *Suppressor reagent.*—Transfer 2.8 mL concn H_2SO_4 to 4 L deionized water and mix well (0.025N H_2SO_4).

(**f**) *Mobile phase (reverse phase).*—Combine acetonitrile–deionized water (58 + 42) (both degassed) using gradient pump controller and delivered at 1 mL/min (mobile phase A) for oxyfluorfen analysis only. Or, combine acetonitrile–deionized water (69 + 31) (mobile phase B) for both oxyfluorfen and oxyfluorfen amine analysis. Or, combine acetonitrile–deionized water (61 + 39) (mobile phase C) for LC/UV analysis.

(**g**) *Mobile phase (ion chromatography).*—Transfer 10 mL AS4A eluant concentrate (Part No. 39513, Dionex) and make up to 1 L with deionized water (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) and deliver at 2 mL/min.

(**h**) *Heptafluorobutyric anhydride (HFBA).*—(Pierce Chemical Co., Rockford, IL 61105).

General Apparatus

(**a**) *Homogenizer.*—Tissumizer (Techmar Co., Cincinnati, OH 45222).

(**b**) *Chopper.*—Hobart mill (Troy, OH 45374).

(**c**) *Centrifuge bottle.*—Teflon 20 mL capacity (Nalge Co., Rochester, NY 14602).

(**d**) *Centrifuge.*—Capable of 1500 g (Model RC2-B, Sorvell, Newtown, CT 06470).

(**e**) *Filter.*—PVDF Acrodisc, 0.45 μm pore size, 25 mm disc (Gelman Sciences, Ann Arbor, MI 48106).

(**f**) *Rotary evaporator.*—Büchi 461 (Flawil/Swithlands).

Sample Extraction

Chop garbanzo beans (dried and husked) using dry ice and a Hobart mill (ca 0.5 cm). Weigh 10 g chopped sample in Teflon centrifuge tubes, fortify with oxyfluorfen and/or oxyfluorfen amine and let stand at room temperature for 1 h before proceeding. Add 10 mL acetone, homogenize samples for 1 min, centrifuge at 700 g for 20 min, and transfer supernatant to a round bottom flask. After evaporating to dryness, reconstitute sample in 2 mL acetonitrile, add 2 mL deionized water, and filter mixture (0.45 μm) before injection onto LC system. For 0.05 ppm fortifications, double sample size and extraction solvent volume; final volume should remain unchanged.

For GC analysis, prepare samples according to PAM method (3) with minor modifications. These modifications involve rinsing Bleidner condensers with 10 mL hexane after refluxing, drying hexane with 15 g anhydrous sodium sulfate before derivatization, and adding 0.5 mL HFBA (vs 0.2 mL).

Calculations

Calculate residue levels as follows:

where PH = peak height of sample, PH' = peak height of standard, S = μg/mL standard (not corrected for purity), V = final volume [mL reconstituted solvent (acetonitrile) plus mL deionized water], W = sample dry weight (g).

Results and Discussion

The standard PAM method extraction scheme was modified to substitute a less toxic solvent (acetone vs acetonitrile). The high solubility of oxyfluorfen and oxyfluorfen amine in acetone and the compatibility of acetone with other LC solvents made it a suitable choice for extraction. The selectivity of LC/PCD suggested that cleanup could be eliminated. Column life will be adversely affected by injecting raw sample matrix; in addition, other crops may have interferences that make this approach unsuitable. This method has not been tested on weathered residues or other crop matrixes. More work is needed to use this method for routine residue analysis. The simple extraction was used to demonstrate selectivity and sensitivity of the LC/PCD method.

Using the HS reverse-phase liquid chromatographic (LC) column, oxyfluorfen had acceptable peak shape; oxyfluorfen amine had unacceptable tailing. Use of a CR (deactivated) column produced acceptable peak shape for both analytes. With a CR C_{18} column and mobile phase A, oxyfluorfen had

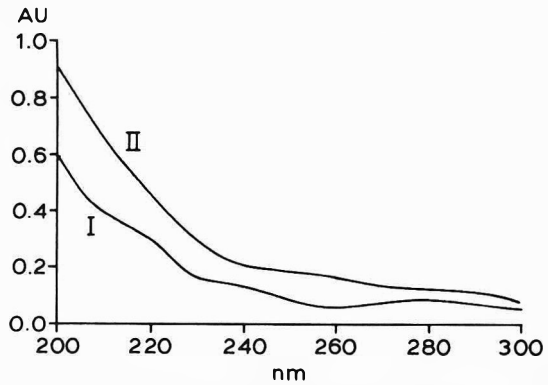

Figure 3. Ultraviolet absorption spectra of oxyfluorfen (I) and oxyfluorfen amine (II).

a capacity factor (k') of 1.2; for oxyfluorfen and oxyfluorfen amine using mobile phase B, $k' = 0.5$ and 1.3, respectively.

Both oxyfluorfen and oxyfluorfen amine were good chromophores, although the most sensitive detection occurred at wavelengths where garbanzo bean matrix obscured the analyte peaks (<220 nm). However, oxyfluorfen had a secondary absorption band at 277 nm and this wavelength offered more selectivity (Figure 3). Nevertheless, even with the optimum mobile phase (61% acetonitrile), interferences found in control garbanzo beans matrix coeluted with oxyfluorfen and oxyfluorfen amine making quantitative measurement difficult, especially with oxyfluorfen amine at a lower concentration (Figure 4). With UV detection at 277 nm, linear ranges were 3 ng [limit of detection (LOD)] to 20 μg for oxyfluorfen

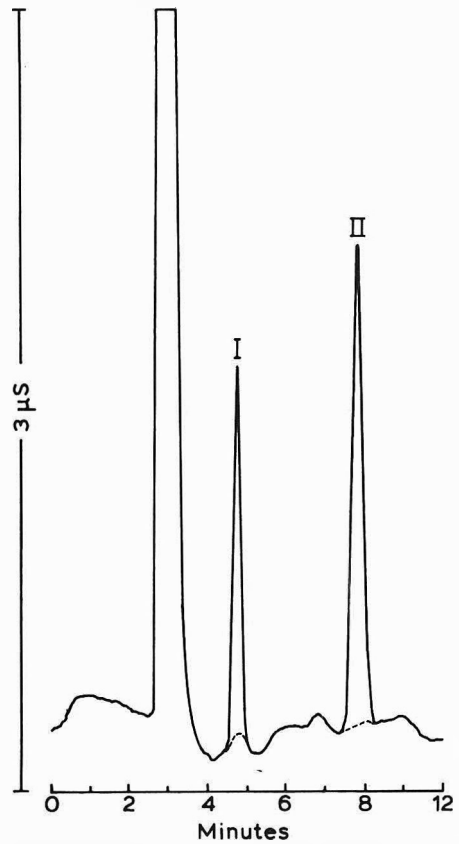

Figure 5. Chromatogram of oxyfluorfen (I) and oxyfluorfen amine (II) using LC-PCD. [Conditions are identical to those in Figure 4 except for acetonitrile–water mobile phase (69 + 31).]

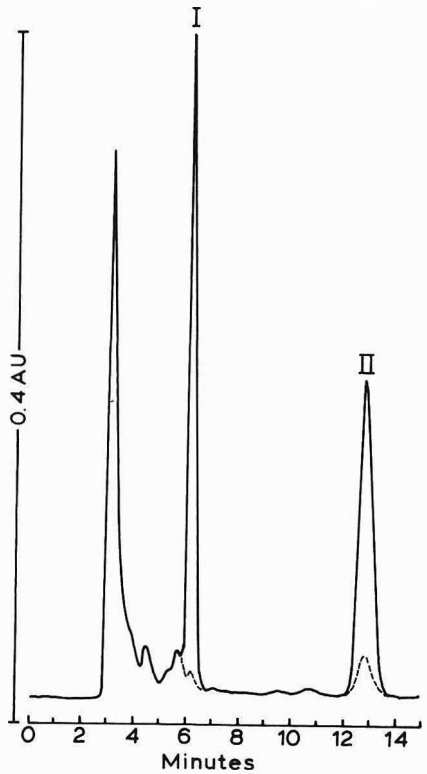

Figure 4. Chromatogram of garbanzo bean fortified with 3 ppm oxyfluorfen and oxyfluorfen amine (150 ng each) using LC-UV detection at 277 nm. Dashed lines indicate response of control. Conditions are CR-3 C_{18} (3 × 30 mm) 3 μm column with acetonitrile–water (61 + 39) delivered at 1 mL/min.

and 6 ng to 20 μg for oxyfluorfen amine. Although the method detection limit ($S/N = 3$) for oxyfluorfen was 3 ng (0.015 ppm in sample), the interference peak in garbanzo beans limited detectability of oxyfluorfen amine at only 100 ng or 0.5 ppm.

Using the PCD (photoreactor No. 1; reaction coil No. 5), oxyfluorfen could be resolved from interferences and was determined best using mobile phase A (58% acetonitrile). Optimum conditions for oxyfluorfen amine were found using mobile phase B. Under these conditions, a minor interference was observed for oxyfluorfen in the garbanzo bean matrix (Figure 5). A gradient run from 55% to 70% of acetonitrile within 15 min to delay retention time of oxyfluorfen and shorten that of oxyfluorfen amine raised the detection limit (30 ng) of oxyfluorfen. Therefore, separate chromatographic conditions were used for optimum determination of oxyfluorfen and oxyfluorfen amine. Oxyfluorfen and oxyfluorfen amine were recovered acceptably from garbanzo beans fortified from 20 ppm to 0.05 ppm (Table 1) and percent recoveries decreased as fortification level decreased.

Ion chromatographic analysis of PCD effluent showed photochemical release of chloride and fluoride ions from both oxyfluorfen and oxyfluorfen amine. Photoproduct yields were 53% chloride and 102% fluoride for oxyfluorfen, and 106% chloride and 268% fluoride for oxyfluorfen amine based on 1 precursor per molecule. Oxyfluorfen amine released almost twice the amount of fluoride and chloride ions compared to oxyfluorfen using the PCD constructed in our laboratory with coil No. 3. This difference is apparently associated with the amino group in oxyfluorfen amine. There

Table 1. Recovery of oxyfluorfen and oxyfluorfen amine from fortified garbanzo beans[a]

Spike, ppm	Oxyfluorfen Recovery (n) ± RSD, %[b]	Oxyfluorfen amine Recovery (n) ± RSD, %
20	95 (4) ± 2	97 (2) ± 3
10	93 (4) ± 5	93 (2) ± 2
5	87 (6) ± 3	88 (2) ± 1
2.5	88 (6) ± 4	89 (2) ± 2
1.0	86 (4) ± 4	89 (2) ± 4
0.5	73 (2) ± 4	81 (2) ± 6
0.2	77 (4) ± 7	79 (4) ± 3
0.1	76 (4) ± 6	78 (2) ± 5
0.05[c]	70 (4) ± 3	69 (2) ± 5

[a] Conditions are HS-3 C_{18} or CR-3 C_{18} 3 × 30 mm, 3 μm with acetonitrile–water (58:42 for oxyfluorfen and 69:31 for oxyfluorfen amine).
[b] n = number of determinations; RSD = relative standard deviation.
[c] Twenty g sample was used for 0.05 ppm fortification, while other fortified samples were based on 10 g sample weight.

Table 2. Band broadening of oxyfluorfen in different woven photolysis reaction coils

No.	Type of coil (TFE)	Approx. diameter, mm od	Approx. diameter, mm id	Length, m (vol, mL)[a]	Band broadening[b] $\sigma^2(s^2)$
1	Commercial[c]	1.6	0.4	20 (2.51)	3.5
2	Commercial	1.6	0.4	10 (1.26)	1.4
3	KOT 3[d]	1.6	0.6	10 (2.83)	16.5
4	KOT 3	1.6	0.6	5 (1.42)	14.0
5	Tool knitted[e]	1.6	0.8	5 (2.51)	45.1

[a] Determined by (retention time of analyte passing through photolysis coil only) × (flow rate).
[b] Measured by $(2.354 \times W_{0.5})^2$ in seconds.
[c] Beam Boost (ICT Corp., available through ASTEC, P.O. Box 297, Whippany, NJ 07981).
[d] Described by Selavka et al. (9).
[e] Described by Engelhardt and Neue (10).

are 2 possibilities related to the ease of releasing halides from oxyfluorfen amine. First, with certain activating groups such as amino in oxyfluorfen amine, the increased density of electronic clouds from an adjacent aromatic ring, through a cross-inductive effect, might elevate the photoreactivity of the halogenated group. Second, the amine group, unlike nitro, would preferably become a hydrogen donor responsible for formation of halogen acid ions soon after halide radicals were produced.

Peak shape was not significantly affected by the photoconductivity reaction coil because of a weaving technique that causes secondary flow patterns and reduces band broadening (9, 10). Evaluation of different woven photolysis coils showed that band broadening increased as length and inside diameter of tubing increased (Table 2). Smaller bore tubings produced larger linear velocities and thus have well developed radial mixing, which minimizes band spreading (9). It is apparent from the present study that different weaving techniques have a large influence on postcolumn band broadening. It is imperative to run a mobile phase with the UV lamp on for at least 20 h to exhaust the release of fluoride ions from newly knitted Teflon reaction coils and reduce the background conductivity of the PCD.

Photoproduct yields of oxyfluorfen amine from both photoreactors were similar to each other, but the product yield from oxyfluorfen using the commercial PCD (coil No. 2) was less than that obtained from the PCD constructed in our laboratory (coil No. 5) (ca 1:6 commercial vs lab-constructed). Apparently, the lower degree of photolysis in the commercial reaction coil was caused by either a thicker Teflon coil wall (0.6 mm) or less residence time (1.2 min.) in coil during photolysis, compared to those of our reaction coil (wall thickness, 0.4 mm; residence time, 3.0 min).

When we compared UV and PCD detection, both approaches had a nanogram sensitivity, which meets the need for residue analysis. Garbanzo bean interferences made LC/PCD a more selective method than LC/UV, especially for oxyfluorfen amine.

Using the GC method, chromatograms were relatively

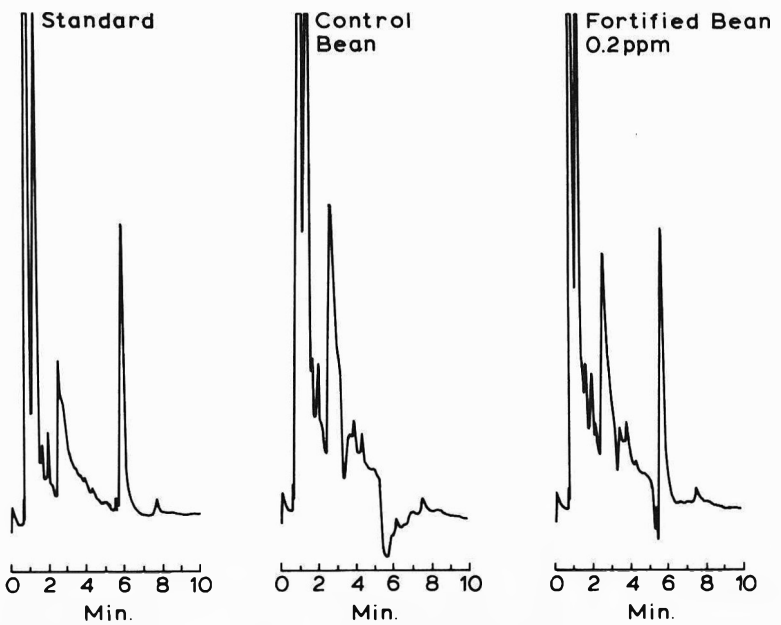

Figure 6. Chromatograms of oxyfluorfen amine (HFBA-derivative) using GC-ECD (garbanzo bean fortified with 0.2 ppm oxyfluorfen). HFBA–oxyfluorfen amine retention time = 6 min. Conditions are 25 m × 0.53 id mm BP-5 capillary column; injection, 250°C; column, 210°C; detector, 300°C.

interference-free (Figure 6). A negative peak eluting just before the analyte made measurement of small peaks difficult. Oxyfluorfen was recovered acceptably from garbanzo beans fortified from 0.02 to 20 ppm with an average of 81 ± 11%. Relative standard deviations were higher than the LC/PCD method and were probably the result of more sample handling with the GC method.

Although the GC method had better sensitivity than the LC/PCD method, the number of samples and standards that could be processed was limited to the number of Bleidner distillation units. Our 4 Bleidner units and the 16 h distillation time resulted in 4 samples/day. We could easily analyze 2–3 times more samples using the LC method. In addition, as noted before, the GC method measures total residues while LC method described here measures both oxyfluorfen and oxyfluorfen amine separately. Either situation could be advantageous to the residue chemist. Obviously, this method compliments the existing GC method.

Acknowledgments

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Determination of MCPA, Bromoxynil, 2,4-D, Trifluralin, Triallate, Picloram, and Diclofop-Methyl in Soil by GC-MS Using Selected Ion Monitoring

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A double space extraction procedure was developed to simultaneously determine neutral herbicides (trifluralin, diclofop-methyl, and triallate) and acid herbicides (MCPA, 2,4-D, bromoxynil, and picloram) in soil. The method uses capillary column gas chromatography (GC) and mass spectrometry (MS) detection with a high degree of confidence in the interpretation of herbicides in many soil types to the 0.003 ppm level. Detection limits at these low levels are possible because extracts can be concentrated by a factor of 50 with few coextractive interferences evident in the chromatography. Recovery data are excellent for both groups of herbicides (81.5–97.8%, average); relative standard deviations vary from 15–23% because of a larger response variation using GC/MS detection.

Triallate, 2,4-D, MCPA, diclofop-methyl, trifluralin, bromoxynil, and picloram are 7 of the 10 most commonly used herbicides in Alberta, Canada, according to the Alberta Department of Environment. Because ground water contamination at pesticide container sites is a concern, a method was developed for determination of these herbicides in soil at <0.01 ppm. Surface soil and soil form bore holes at various depths were analyzed to determine herbicide levels at these sites.

To successfully determine neutral and acid herbicides simultaneously, we needed a method with low detection limits and a high degree of confidence in any positive results. An organic solvent (acetone) was used for extraction of neutral herbicides; a basic aqueous solution was required for effective extraction of picloram. A mass selective detector was used to obtain the high degree of confidence in positive results.

Neutral herbicides (e.g., trifluralin and triallate) and acid herbicide esters have been analyzed in sediment according to methods by Lee and Chau (1). It has been our experience that acetone extraction is not effective for picloram extraction from clay and loam soils. Private communications (Lee Burkee, Dow Elanco, Midland MI, and Walter Breitkreitz, Alberta Agriculture, Edmonton, Alberta, Canada) confirmed that picloram can only be effectively extracted with a basic aqueous solution. In addition, use of the electron capture detector (ECD) has made positive identification of herbicides in loam soils difficult because of the presence of coextractive peaks in the chromatography. Smith (2) used acetonitrile as the extraction solvent for neutral and some acid herbicides from clay and loam soils. Although good recoveries were obtained at 0.1–0.5 ppm, identification at levels <0.1 ppm by ECD were again difficult. Picloram was not effectively extracted from loam soils using acetonitrile; and MCPA showed a poor response with ECD as well. An acid-buffer extraction used by Gurka et al. (3) (EPA Method 8150) was not tried because of the problem with picloram extraction under acidic conditions.

Double extraction ensured good recovery of neutral and

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acid herbicides, and the selective mass spectrometer detector produced a high confidence level in results. The combination of 3 ions present with the correct ion abundance ratio and favorable retention times produced a high level of confidence in assigning the identity of peaks during gas chromatography/mass spectrometry (GC/MS).

METHOD

Apparatus

(a) *Centrifuge.*—Sorvall RC2-B, 5000 rpm, refrigerated, with 250 mL polypropylene centrifuge bottles (Fisher Scientific).

(b) *Rotary evaporator.*—Buchi No. 110/RE-120 (Buchi Labortoriums-Technik AG, Flawil, Switzerland).

(c) *Nitrogen evaporator.*—Model 111 (Organomation Assoc., Berlin, MA 01503).

(d) *Sonic bath.*—Branson 3200 (Baxter/Canlab).

(e) *Gas chromatograph.*—HP 5890A (Hewlett-Packard) coupled with an HP 5970 series mass selective detector. GC/MS conditions.—Column: DB17, 30 M × 0.25 mm id and 0.25 μm film thickness. Temperature program: 100 to 280°C at 15°C/min, initial temperature hold, 0 min. Run time: 18 min. Injector temperature: 250°C splitless injection mode. GC-to-MSD interface: capillary direct interface temperature: 250°C. Temperature: 250°C. Ion source pressure: 4×10^{-5} TORR. Emission current: 1 MA. Voltage: 2200.

(f) *Automatic sampler.*—HP 7673A (Hewlett-Packard), set for 2 μL injection volume.

(g) *Gauge controller.*—HP 59822A (Hewlett-Packard).

(h) *Printer.*—HP 2934A (Hewlett-Packard).

(i) *GC/MS Chemstation.*—HP 35731B computer with HP 9133 disc drive (Hewlett-Packard).

Reagents

(a) *Standards.*—Anthracene d_{10}, MCPA, bromoxynil, 2,4-D, trifluralin, triallate, picloram, and diclofop-methyl, reported to be 98–100% pure, were obtained from EPA. Prepare individual stock solutions in toluene containing 10 mg in 10 mL (1000 ppm). Make a spiking solution of neutral and acid herbicide by taking 1 mL each stock and diluting to 10 mL in ethyl acetate (100 ppm). Prepare working standards in methyl ester form by taking 1 mL of 100 ppm mix including acid and neutral herbicides by esterifying with diazomethane (DCM) and bringing to 10 mL final volume in ethyl acetate (10 ppm). Working standards of 5 ppm and 1 ppm are prepaid by dilution of these in hexane–dichloromethane (4:1).

(b) *Solvents.*—Acetone, DCM, and hexane, distilled in glass, pesticide grade (Burdick & Jackson).

(c) *Diazomethane solution.*—Prepare in ethyl ether from *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) according to method of Stanley (4). *Caution:* It is suspected that this precursor is a carcinogen. Handle with gloves in a fumehood. Diazomethane is explosive, although no distillation is performed in the method of Stanley (4).

(d) *Acidified sodium sulfate.*—Anhydrous coarse, granular. Heat at 425°C for 4 h, then cool. Fill a 1 L flask 3/4 full with Na_2SO_4 and cover with ethyl ether containing ca 0.5 mL concentrated H_2SO_4. Then filter acidified Na_2SO_4 through a büchner funnel and dry in air tight container.

(e) *Purified water.*—Milli-Q purification system (Millipore Corp., Bedford, MA).

Extraction

Weigh 50 g wet soil (sandy clay, clay, mud, or loam) into a polypropylene centrifuge bottle and shake overnight with 120 mL acetone–water–acetic acid (80 + 19 + 1). Centrifuge sample at 4000 rpm for 3 min and decant supernatant into 250 mL erlenmeyer flask. Reextract sample by sonication using a sonic bath for 15 min with 150 mL 0.1N KOH. Centrifuge sample again and decant supernatant into a 1 L separatory funnel. Acidify KOH extract to pH <2 with 6N H_2SO_4 and combine with acetone–water extract in separatory funnel, along with 600 mL organic free water. Prepare powder funnel containing 30 g acidified Na_2SO_4 and rinse with 20 mL DCM. Extract aqueous–acetone phase with 100 mL DCM and then dry DCM through the acidified, anhydrous Na_2SO_4. Collect DCM extracts in 500 mL boiling flasks and concentrate on a rotary evaporator at 35°C to about 0.5 mL.

Derivatization

Add ca 2 mL diazomethane solution to each flask and let reaction proceed for 15 min. Add another 1–2 mL diazomethane solution and let sit for another 15 min. Leave flask in fumehood for 1 h to allow excess diazomethane to evaporate. Remove ethyl ether on a rotary evaporator or with a gentle N_2 stream in a fumehood. Quantitatively transfer sample to a 3.5 mL vial with 25% DCM–hexane, add 10 μL of a 200 ppm anthracene d_{10} internal standard and make to a final volume of 1 mL.

Ion Selection, Quantitation Limits, and Detection Limits

Ions used for analysis of the 7 target herbicides are shown in Table 1; the ion used for quantitation is underlined. The most intense ions with large m/z ratios are chosen for selected ion monitoring (SIM) of the various analytes. Anthracene d_{10} is used as an internal standard using only the intense 188 ion for quantitation because soil extracts do not interfere with this ion. Relative ion abundance (RIA) is shown in parentheses after each ion. Detection limits are in parts per million.

Method detection limits were determined by establishing positive identification with 2 ion confirmation of levels about 5 times the method noise level. The minimum detection limit was about 3 times lower than the limit of quantitation.

Calculations

$$RF = (PA_A)\,(Cr)/(C_A)\,(PA_I)$$

$$C_{A'}\,\text{ppm} = (PA_A)\,(C_I)\,(FV)/(RF)\,(PA_I)\,(A_x)$$

where RF = response factor, PA_A = peak area of analyte, C_I = concentration of internal standard, C_A = concentration of analyte, PA_I = peak area of internal standard, A_x = amount extracted in grams, and FV = final volume in milliliters .

Fortification and Recovery Data

Soil types such as clay, black loam, and sandy clay were used for fortification of herbicides. Several sources of these soil types were used for fortification with a moisture ranging from 0–30% and pH varying from 5–9. About 50 g soil was fortified with 25–150 μL herbicide mix standard, allowed to equilibrate for 1 h and then analyzed by the procedure out-

Table 1. Selected ions and detection limits in soil[a]

Compound	Ion (relative ion abundance)	LOD,[b] ppm	LOQ,[c] ppm
Internal standard	188	—	—
MCPA	214 (1.0), 141 (0.9), 155 (0.6)	0.003	0.010
Bromoxynil	293 (0.5), 291 (1.0), 289 (0.5)	0.003	0.010
2,4-D	234 (0.56), 201 (0.33) 199 (1.0)	0.003	0.010
Trifluralin	306 (1.0) 290 (0.14) 264 (0.9)	0.003	0.010
Triallate	272 (0.14), 270 (0.7) 268 (1.0)	0.003	0.010
Picloram	255 (0.17), 201 (0.21) 199 (1.0)	0.005	0.020
Diclofop-methyl	340 (0.52), 255 (0.76), 253 (1.0)	0.003	0.010

[a] Ions used for quantitation are underlined. Relative ion abundance (RIA) is shown in parentheses after each ion.
[b] LOD = limit of detection.
[c] LOQ = limit of quantitation.

lined above. Soils were not fortified lower than the stated limit of quantitation. Recovery data and spiking levels are shown in Table 2.

Results and Discussion

The chromatography of 2 ppm standards of (A) MCPA, (B) 2,4-D, and (C) trifluralin is shown in Figure 1; chromatograms of soil samples naturally contaminated with these 3 herbicides are shown in Figure 2. The trifluralin level in naturally contaminated soil (0.003 ppm) illustrates the ability of this method to detect residues below quantitation levels of 0.01 ppm.

The identification and quantitation of herbicides at 0.01 ppm in complex, organic soil matrices can be achieved by applying SIM techniques. Although some sands, sediments, and clays can be analyzed by ECD, it has been our experience that many other soil types cannot because of the many coextractive peaks in ECD chromatography. With GC/MS in the SIM, there is not only a retention time for compound identification, but for 2 or more confirmation ions and their ratios as well. Ratios of confirmation ions (RIAs) should be within 20% of the RIA for the standard compound. This 20% criteria is also used in identification of dioxins by low resolution mass spectrometry.

With these criteria, there is a high level of confidence in the identification of these herbicides in soil. No soil samples showed interferences for analysis of these herbicides by GC/MS. Also, because there are no lengthy derivitization or column cleanup steps, this approach is considerably faster than conventional ECD methods.

Table 2. Recovery data (%) for herbicide double extraction procedure

Soil type	Spiking level, ppm	MCPA	Bromox[a]	2,4-D	Trifluralin	Triallate	Picloram	Diclofop-methyl
Clay	0.010	75	83	82	96	81	68	112
Loam[b]	0.012	83	97	79	104	75	75	108
Clay	0.05	100	80	118	114	120	93	110
Clay	0.07	80	87	83	63	93	117	95
Clay	0.07	64	73	71	77	84	84	102
Clay	0.07	77	91	88	78	102	93	102
Clay	0.1	96	101	107	117	76	61	78
Clay	0.1	84	110	92	91	78	51	73
Clay	0.1	94	64	102	123	101	80	83
Clay	0.1	86	89	81	96	84	66	78
Clay	0.1	105	122	106	106	91	57	85
Clay	0.1	99	101	100	110	88	63	57
Loam[b]	0.1	89	86	100	70	80	87	110
Loam[b]	0.1	100	121	89	113	97	101	87
Loam[b]	0.1	115	131	135	131	121	92	115
Loam[b]	0.1	109	138	147	74	111	108	117
Clay	0.1	74	89	91	84	77	60	71
Clay	0.5	106	93	98	112	102	89	80
S. clay[c]	0.7	65	70	69	70	76	68	105
S. clay[c]	0.7	79	67	102	118	97	97	97
S. clay[c]	0.7	93	99	97	92	95	109	94
S. clay[c]	0.7	77	74	74	82	76	73	65
Clay	0.8	103	109	94	108	102	60	96
Loam[b]	0.8	91	105	96	93	84	87	86
Loam[b]	1.0	103	109	102	109	104	93	96
Loam[b]	1.0	99	116	105	87	74	67	74
Av. (n = 26)		91.2	96.9	97.8	96.6	92.2	81.5	89.8
RSD		±13.8	±20	±17.9	±19.1	±13.9	±18.5	±16.1
RSD, %		15	21	18	19.8	15.1	22.7	17.9
LOQ[d] = 0.01 ppm								
LOD[e] = 0.003 ppm								

[a] Bromox = bromoxynil.
[b] Black loam.
[c] S. clay = sandy clay.
[d] LOQ = limit of quantitation.
[e] LOD = limit of detection.

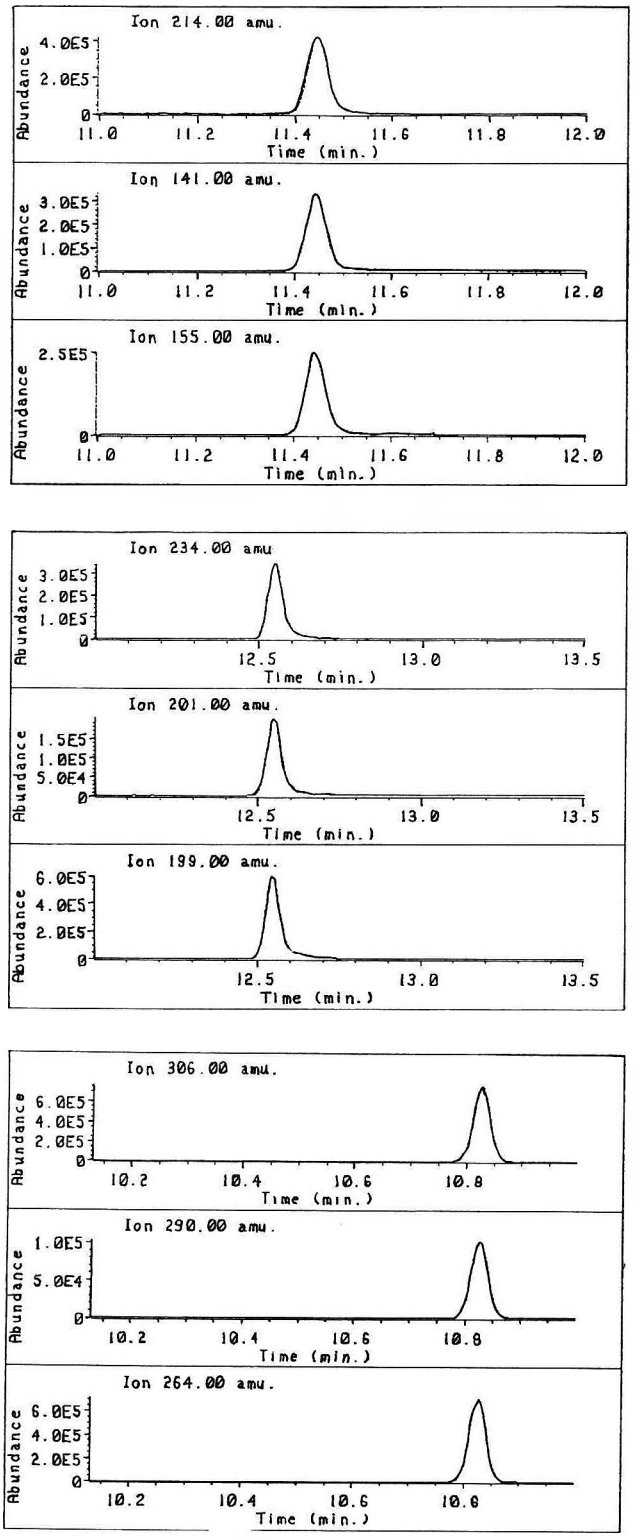

Figure 1. (A) MCPA ester, relative ion abundance (RIA): 214 (1.0), 141 (0.9), 155 (0.6); (B) 2,4-D ester, RIA: 234 (0.56), 201 (0.33), 199 (1.0); (C) trifluralin, RIA: 306 (1.0), 290 (0.14), 264 (0.9).

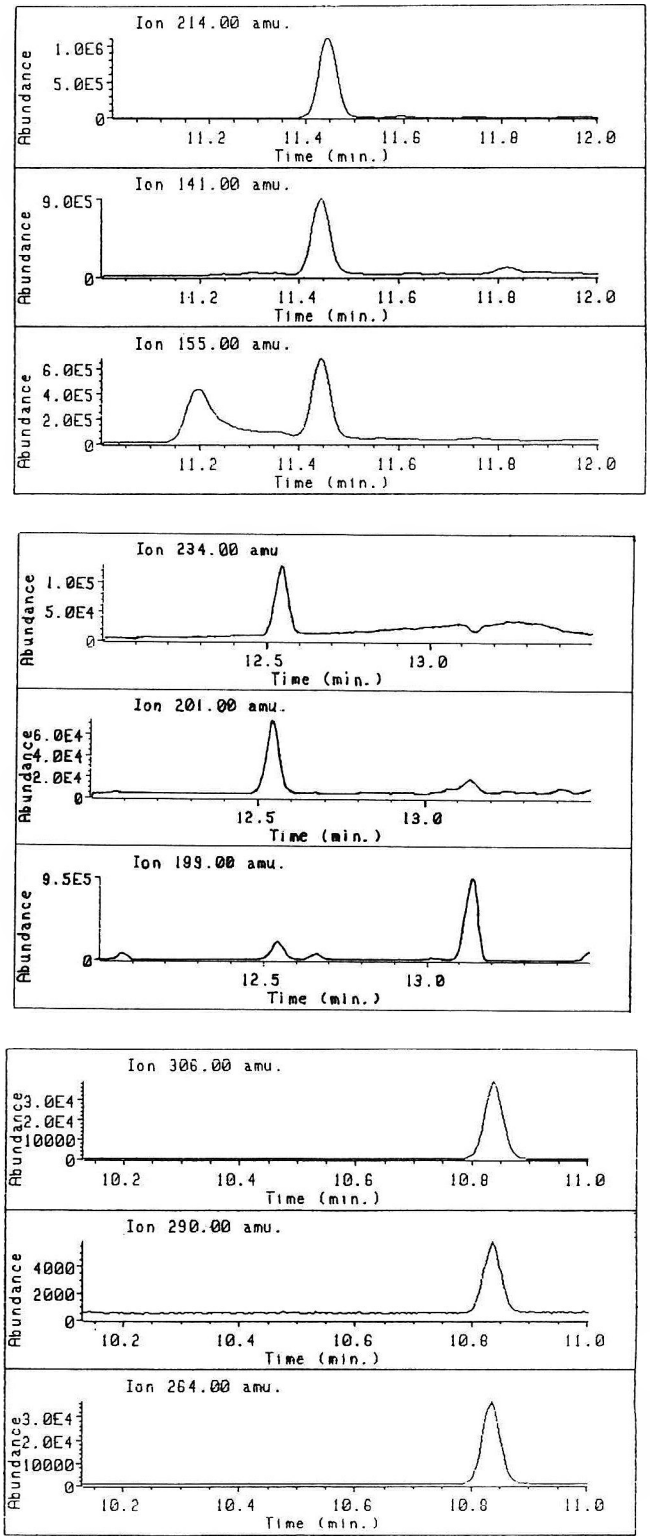

Figure 2. (A) MCPA in clay at 0.15 ppm, RIA: 214 (1.0), 141 (0.81), 155 (0.65); (B) 2,4-D in black loam at 0.019 ppm, RIA: 234 (0.61), 201 (0.33), 199 (1.0); (C) trifluralin in sandy clay at 0.003 ppm, RIA: 306 (1.0), 290 (0.13), 264 (0.9).

Some limited recovery data for other neutral and acid compounds such as pentachlorophenol, MCBA, atrazine, 2,4,5-T, and some organochlorines indicate that this procedure would be effective for a "customized" screen of many neutral and acidic compounds.

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Multiresidue Screening Method for Fresh Fruits and Vegetables with Gas Chromatographic/Mass Spectrometric Detection

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A gas chromatographic/mass spectrometric (GC/MS) screening method has been developed for the determination of pesticide residues in a variety of crop samples. Samples are extracted with acetonitrile and partitioned with sodium chloride saturated aqueous solution. Targeted pesticide analytes are separated on a short, narrow bore capillary column, identified by electron ionization MS scanned from 100 to 400 amu, and quantitated by the monitoring of base ions and internal standards. The accuracy of the quantitative determination measured in terms of average percentage recovery of 143 compounds in 13 crop samples was 92% with a relative standard deviation of 22%.

Methods for analyzing pesticide residues have been studied extensively over the years. A recent report (1) summarized the current ability of analytical technologies to detect pesticide residues in food and offered options to government regulators on how to improve their analytical capability. Maximizing the number of analytes to be determined with given resources is considered the best practical approach in a monitoring program. The most frequently used methods rely on gas chromatographic (GC) separation and detection with various selective detectors (2).

In recent years, gas chromatography/mass spectrometry (GC/MS) has become a common secondary confirmation technique supporting the multiresidue pesticide screening methods using element-specific detectors as the initial determinative step for pesticide residues on fresh produce. Typically, pesticide residues are first determined by a GC/flame photometric detector (FPD), GC/electron capture detector (ECD), or GC/electrolytic conductivity detector (ELCD); then, the GC/MS is used to confirm the positive findings by selective ion monitoring or the full scan mode, depending on the matrix or the amount of interferences. Recently, a GC/MS method incorporating the chemical ionization technique has been successfully used to determine multiresidues of pesticides in produce samples (3). The chemical ionization mass spectrometric method, however, may still require electron impact ionization data to confirm its positive findings.

The technique presented in this paper involves the use of the GC/MS as a primary screening tool rather than in the traditional secondary confirmation role. This screening technique involves establishing a data base of reference pesticide standards in the data system and automation software in the initial screening of fresh produce extracts introduced into the GC/MS system. Criteria such as relative retention time, relative intensities of primary and secondary ions, and examination of limited-scan mass spectrum (100–400 amu) of the suspected finding are all used in the algorithm for the GC/MS identification. The pesticide finding is also quantitated in the same sequence by using established parameters in the initial calibration of the GC/MS system.

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This technique is a useful tool in pesticide residue detection because it offers simultaneous detection and confirmation of any pesticide that can be volatilized. A potentially useful GC/MS method, used as a front-end screening for pesticides on produce samples, could be established by using the following technique.

Experimental

Materials

Most of the pesticide standards used in this work were obtained from the U.S. Environmental Protection Agency's (EPA) Pesticides and Industrial Chemicals Repository. Some were purchased in the highest available purity from commercial sources. They were individually tested for integrity before use.

Samples

Samples used in this study were determined to contain no detectable residues via California Department of Food and Agriculture's (CDFA) market surveillance program. Various combinations of samples were chosen from a list of 13 crops: apple, bell pepper, broccoli, carrot, cauliflower, cilantro, cucumber, grape, green bean, green onion, head lettuce, lettuce, and tomato.

Instrumentation and Calibration

Gas chromatograph-mass spectrometer.—Hewlett-Packard Model 5890 gas chromatograph coupled with Model 5988A quadrupole mass spectrometer with a GC column of cross-linked methyl silicone capillary, 12 m × 0.2 mm id HP-1 with 0.33 μm film thickness. GC operating conditions: splitless injection, injector temperature 260°C, helium carrier gas with column head pressure 7.5 psi, oven temperature from 60°C (1 min hold) to 265°C at 18°C/min, transfer line 200°C. Mass spectometer setting: electron impact ionization mode with 70 eV electron energy, scan mass range 100–400 at 0.62 sec/scan cycle, ion source temperature 200°C.

The GC/MS was tuned daily with a 100 ng decafluorotriphenylphosphine (DFTPP) injection to meet EPA ion abundance criteria (4). Initial 3-point quantitative calibration (5, 10, and 20 ng/μL) was made with 3 internal standards (anthracene-d10, pyrene-d10, and chrysene-d12 at 5 ng/μL) for each of the target compounds.

All the target compounds with their corresponding internal standards are listed in 3 groups in Table 1. The relative response factor (RRF) for each compound was measured and calculated as follows:

$$RRF = (Ax/Ais) \times (Cis/Cx)$$

where Ax = area of the base ion of the target compound, Ais = area of the base ion of the corresponding internal standard, Cis = concentration of the internal standard, Cx = concentration of the target compound. Injection volume = 1 μL. Linearity or calibration curves were established over the concentration of interest in this study. One-point recalibra-

Table 1. Target compounds analysis by gas chromatography-mass spectrometry in the electron impact ionization mode scanning *m/z* 100–400

Compound name	Relative retention time[a]	Characteristic masses *m/z*, % relative abundance[b]	Calibration	
			Mean RRF (% RSD)[c]	Mean RRF (% RSD)[d]
Anthracene-d10 (internal std)	1.000 (8.86 min)	188(100), 187(40)	1.00	1.00
Atrazine	0.940	200(100), 215(60), 173(30)	0.22(4.0)	0.22(12)
Ametryne	1.072	227(100), 212(60), 170(40)	0.29(1.4)	0.29(8.9)
Alachlor (lasso)	1.079	188(100), 224(30), 237(40)	0.27(3.2)	0.26(4.3)
Aldrin	1.134	263(100), 265(60), 293(25)	0.11(2.3)	0.11(3.5)
Aroclor 1232	—			
Aroclor 1260	—			
Bendiocarb	0.901	151(100), 126(45), 166(40)	0.61(5.2)	0.64(15)
BHC (alpha)	0.926	183(100), 181(95), 219(90)	0.23(3.0)	0.22(4.0)
BHC (beta)	0.954	109(100), 181(70), 219(60)	0.21(3.6)	0.20(5.6)
BHC (lindane)	0.970	181(100), 183(95), 219(80)	0.18(3.9)	0.18(4.3)
BHC (delta)	0.984	181(100), 183(90), 219(95)	0.11(2.5)	0.11(17)
Biphenyl[e]	0.683	154(100), 153(40), 152(20)	0.07(4.8)	0.07(13)
Bufencarb	0.978	121(100), 122(85), 164(50)		
Bromacil	1.099	205(100), 207(100)	0.20(7.9)	0.24(27)
Benthiocarb (thiobencarb)	1.117	125(100), 257(40), 259(18)	0.22(5.6)	0.24(12)
Bayleton	1.140	208(100), 181(42), 210(33)	0.15(9.7)	0.16(22)
Bromophos	1.154	331(100), 329(80), 125(65)	0.28(3.0)	0.72(21)
Fenobucarb	0.862	121(100), 150(25)	1.64(3.1)	1.60(4.0)
Cyanophos	0.976	109(100), 125(45), 243(40)	0.91(3.8)	0.88(8.2)
Chorpropham (CIPC)	0.899	127(100), 213(53), 171(42)	0.46(4.4)	0.44(3.4)
Chlordimeform	0.903	196(100), 181(85), 152(65)	0.23(5.4)	0.22(1.8)
Carbofuran	0.949	164(100), 149(62), 131(28)	0.39(9.7)	0.41(10)
Chlorothalonil (bravo)	1.001	266(100), 264(80), 268(50)	0.27(12)	0.27(7.1)
Carbaryl	1.067	144(100), 115(57), 201(8)	0.33(18)	0.37(42)
Chlordane:				
Chlordane (C10H9C15)	1.125	232(100), 230(100), 303(95)	0.02(19)	0.01(51)
Chlordane (C10H9C15)	1.131	232(100), 230(100), 303(100)	0.01(25)	0.01(36)
Chlordane (C10H8C16)	1.164	339(100), 341(95), 201(50)	0.02(10)	0.02(31)
Chlordane (C10H7C17)	1.210	375(100), 373(85), 377(45)	0.05(9.0)	0.05(18)
Chlorpyrifos (dursban)	1.132	197(100), 199(92), 314(54)	0.23(2.4)	0.22(4.2)
Captan	1.168	149(100), 117(95), 264(20)	0.08(12)	0.07(57)
Dimethachlor	1.055	134(100), 197(40)	1.06(3.8)	1.00(4.4)
Dichlorobiphenyl[e]	0.936	222(100), 224(60), 152(60)	0.22(3.2)	0.21(2.9)
Disulfoton	0.010	142(100), 186(70), 274(75)	0.14(6.2)	0.13(1.3)
Dichlorvos	0.559	109(100), 185(25), 145(7)	0.86(0.9)	0.81(13)
Dichlobenil	0.633	171(100), 173(75), 136(30)	0.92(8.7)	0.93(1.5)
Demeton-O	0.866	115(100), 171(90), 143(70)	0.18(6.9)	0.16(7.6)
Demeton-S	0.947	114(100), 170(80), 143(65)	0.15(10)	0.15(3.2)
Dicrotophos	0.900	127(100), 193(15)	0.73(3.0)	0.76(16)
Dimethoate	0.940	125(100), 143(20)	0.19(7.2)	0.18(27)
Dicloran (botran)	0.942	124(100), 176(80), 206(65)	0.88(14)	0.86(3.3)
Dioxathion (delnav)	0.974	125(100), 153(30), 270(30)	0.09(6.4)	0.09(15)
Diazinon	0.988	137(100), 179(75), 152(65)	0.39(14)	0.37(5.2)
Dicofol	1.133	139(100), 141(40), 250(15)	0.69(7.4)	0.58(18)
Dacthal (DCPA)	1.144	301(100), 299(72), 332(25)	0.31(8.1)	0.31(8.8)
DEF (degree)	1.257	169(100), 202(60), 258(25)	0.30(5.5)	0.27(6.1)
Formothion	1.022	125(100), 126(70), 170(20)	0.15(5.0)	0.14(11)
Fluchloraline (basalin)	1.016	306(100), 326(80), 264(75)	0.17(4.8)	0.16(1.0)
Fenthion (baytex)	1.125	278(100), 125(70), 169(30)	0.41(3.4)	0.39(6.2)
Folpet	1.180	104(100), 260(50), 262(35)	0.10(17)	0.09(42)
Heptachlor	1.086	272(100), 274(70), 237(35)	0.16(2.6)	0.15(7.2)
Heptachlor epoxide	1.180	353(100), 355(85), 237(35)	0.11(3.3)	0.10(8.9)
Heptenophos	0.833	124(100), 109(40), 250(7)	0.37(5.3)	0.37(5.0)
Iprobenfos	1.032	204(100), 288(18), 246(18)	0.52(4.4)	0.49(9.7)
Isozophos	1.016	119(100), 161(100), 257(40)	0.34(4.0)	0.32(4.5)

Table 1. Continued

Compound name	Relative retention time[a]	Characteristic masses *m/z*, % relative abundance[b]	Calibration: Mean RRF (% RSD)[c]	Calibration: Mean RRF (% RSD)[d]
Indalone (butopyronoxyl)	0.857	125(100), 115(90), 171(20)	0.50(3.3)	0.51(3.4)
Mevinphos-beta (phosdrin)	0.711	127(100), 192(24), 109(32)	1.03(5.2)	1.05(9.8)
Mevinphos-alpha (phosdrin)	0.718	127(100), 192(30), 109(25)	1.34(8.4)	1.23(8.1)
Mexacarbate	1.017	165(100), 150(80), 222(25)	0.24(2.1)	0.26(19)
Malathion-oxygen analog	1.062	127(100), 109(30), 195(15)	0.19(10)	0.18(31)
Methyl parathion	1.060	109(100), 125(80), 263(50)	0.46(11)	0.41(18)
Metalaxyl (ridomil)	1.081	206(100), 220(55), 249(40)	0.17(4.1)	0.16(10)
Methiocarb	1.098	168(100), 153(70), 109(40)	0.28(17)	0.32(33)
Malathion	1.114	127(100), 125(100), 173(95)	0.40(3.7)	0.38(7.0)
Methidathion (supracide)	1.198	145(100), 125(20)	0.64(7.2)	0.56(22)
Monolinuron	0.965	214(100), 126(100), 153(30)	0.14(3.7)	0.13(18)
Monochlorobiphenyl	0.782	188(100), 190(35), 152(45)	0.23(5.4)	0.26(6.0)
Propham (IPC)	0.744	137(100), 179(80), 120(80)	0.29(6.6)	0.27(5.0)
Propoxur	0.861	110(100), 152(20)	2.05(7.6)	2.03(1.0)
Phorate-oxygen analog	0.865	171(100), 111(75), 143(45)	0.26(5.6)	0.25(16)
Phorate	0.927	121(100), 260(38), 231(29)	0.46(4.1)	0.45(4.3)
Pentachloronitro-benzene	0.984	237(100), 249(90), 295(85)	0.12(4.2)	0.12(8.3)
Pronamide	1.001	173(100), 175(60), 145(40)	0.49(5.0)	0.50(3.3)
Profluralin	1.005	318(100), 330(45), 264(55)	0.15(5.3)	0.15(5.1)
Pirimicarb	1.032	166(100), 238(15)	0.85(5.0)	0.86(3.5)
Propanamide (propanil)	1.050	161(100), 163(70), 217(20)	0.42(17)	0.47(25)
Prometryne	1.080	241(100), 184(85), 226(60)	0.25(3.2)	0.28(16)
Parathion-oxygen analog	1.079	109(100), 220(20), 275(20)	0.08(14)	0.08(42)
Pirimiphos-methyl	1.105	290(100), 276(90), 305(65)	0.25(4.0)	0.24(3.8)
Parathion	1.132	109(100), 139(40), 291(35)	0.24(8.4)	0.25(14)
o-Phenylphenol	0.792	170(100), 169(80), 141(37)	0.70(7.7)	0.69(1.3)
Prothoate	1.060	115(100), 285(8), 121(18)	0.59(6.4)	0.55(5.9)
Promecarb	0.923	135(100), 150(80)	0.82(6.7)	0.82(5.8)
Quinomethionate (morestan)	1.201	206(100), 234(100), 116(80)	0.27(8.0)	0.25(15)
Sonalan	0.915	276(100), 316(80), 292(45)	0.11(9.0)	0.10(7.7)
Systox	0.945	170(100), 114(100), 143(80)	0.07(4.6)	0.07(18)
Simazine	0.953	201(100), 186(80), 173(60)	0.31(2.2)	0.35(9.3)
Safrotin	0.988	138(100), 194(30), 236(15)	0.66(5.5)	0.65(1.2)
Simetryne	1.066	213(100), 170(40), 155(40)	0.46(5.0)	0.45(9.8)
Thiometon-ethyl	0.939	125(100), 158(10)	0.19(6.1)	0.18(6.2)
Trichlorobiphenyl[e]	1.059	256(100), 258(90), 186(65)	0.13(6.5)	0.12(3.3)
Trifluralin	0.927	264(100), 306(95), 248(15)	0.36(2.9)	0.36(4.9)
Terbacil	1.005	161(100), 160(70), 117(50)	0.34(7.5)	0.33(15)
Tetrachlorvinphos	1.216	109(100), 329(60), 331(55)	0.12(2.4)	0.14(41)
Vegadex	0.933	188(100), 116(15)		
Vinclozolin (ronilan)	1.069	285(100), 212(100), 287(60)	0.09(6.0)	0.09(13)
Pyrene-d10 (internal std)	1.000 (10.72 min)	212(100), 106(40)	1.00	1.00
Bostar	1.095	156(100), 140(95), 322(90)	0.51(5.8)	0.48(6.4)
Chlorobenzilate	1.070	251(100), 139(100), 253(65)	0.50(5.1)	0.51(57)
Carbophenothin (trithion)	1.103	157(100), 121(60), 342(37)	0.37(6.5)	0.36(19)
Chlordane (C10H7C17)	1.014	375(100), 373(90), 377(45)	0.06(4.0)	0.06(4.4)
Carbetamide	0.934	119(100), 236(8)	0.38(10)	0.31(25)
CNP (chlornitrofen)	1.103	317(100), 319(100), 236(75)	0.23(3.6)	0.22(8.8)
DDE-p,p′	1.036	246(100), 248(70), 318(55)	0.33(2.1)	0.33(5.0)
Dieldrin	1.059	263(100), 265(75), 115(75)	0.07(5.2)	0.07(9.4)
DDD-p,p′	1.077	235(100), 237(65), 165(60)	0.56(3.7)	0.55(2.7)
Dimethametryn	0.965	212(100), 255(15)	1.41(1.7)	1.34(4.1)
Diclobutrazol	1.049	270(100), 271(60), 159(45)	0.41(2.4)	0.39(9.0)
Endosulfan-alpha	1.013	170(100), 237(90), 339(40)	0.03(9.0)	0.03(11)

Table 1. Continued

Compound name	Relative retention time[a]	Characteristic masses *m/z*, % relative abundance[b]	Calibration: Mean RRF (% RSD)[c]	Calibration: Mean RRF (% RSD)[d]
Ethylan	1.061	223(100), 224(25), 179(15)	1.05(2.1)	1.02(5.2)
Endrin	1.059	263(100), 265(80), 279(50)	0.07(5.2)	0.07(9.4)
Endosulfan-beta	1.063	195(100), 237(80), 339(30)	0.04(28)	0.04(20)
Ethion	1.085	231(100), 153(85), 125(80)	0.45(3.4)	0.42(7.0)
Endosulfan sulfate	1.118	272(100), 229(80), 387(30)	0.07(3.0)	0.06(25)
Etaconazole (diastereoisomer B)	1.072	173(100), 245(80), 247(55)	0.33(4.5)	0.31(10)
Etaconazole (diastereoisomer A)	1.076	173(100), 245(80), 247(55)	0.43(3.7)	0.40(10)
Ediphenophos	1.103	109(100), 173(55), 310(25)	1.23(6.3)	1.16(10)
Ethirimol	1.102	166(100), 209(18)	0.30(11)	0.30(19)
Fluorochloridone	0.932	187(100), 311(75), 174(75)	0.27(4.5)	0.26(6.9)
Fenson	0.938	141(100), 268(35)	0.98(5.0)	0.93(1.5)
Flamprop-methyl	1.041	105(100), 230(2), 276	2.63(4.0)	2.39(5.9)
Flamprop-isopropyl	1.079	105(100), 276(4)	4.14(4.6)	3.84(5.6)
Hexachlorobiphenyl[f]	1.100	360(100), 362(80), 288(35)	0.19(4.0)	0.17(9.6)
Lironion	0.928	241(100), 226(30)	0.71(4.4)	0.66(15)
Lenacil	1.104	153(100), 110(8)	2.08(7.2)	2.06(7.5)
Methfuroxam	0.937	137(100), 229(20)	2.47(4.8)	2.33(3.6)
Methoprotryne	1.042	256(100), 213(35), 271(30)	0.38(3.1)	0.35(7.1)
Metolachlor	0.932	162(100), 238(50), 211(10)	1.10(3.2)	1.08(4.1)
Nitrofen (TOK)	1.051	283(100), 285(65), 202(70)	0.16(8.1)	0.16(24)
Oxadiazon	1.040	175(100), 177(60), 258(35)	0.30(4.3)	0.29(11)
Oxyfluorfen	1.042	252(100), 300(35), 361(27)	0.20(3.8)	0.18(8.9)
Ovex (chlorfenson)	1.011	175(100), 111(100), 302(25)	0.88(1.8)	0.84(3.6)
Ofurace	1.090	132(100), 160(100), 232(65)	0.22(4.0)	0.21(8.8)
Profenofos	1.029	139(100), 208(75), 339(30)	0.15(6.6)	0.16(9.7)
Pentachlorobiphenyl[f]	1.007	326(100), 324(55), 254(45)	0.06(5.4)	0.06(2.3)
Quinalphos	0.978	146(100), 157(60), 118(35)	0.22(1.3)	0.22(4.3)
Rally (systhane)	1.033	179(100), 150(60)	0.19(8.1)	0.21(27)
Triazophos	1.085	161(100), 162(80), 257(30)	0.36(6.1)	0.32(20)
Tetrachlorobiphenyl[e]	0.946	292(100), 290(70), 220(40)	0.03(7.3)	0.03(3.9)
Tolyfluanid	0.970	137(100), 238(20)	0.33(8.2)	0.35(8.3)
Chrysene-d12 internal std)	1.000 (12.59 min)	240(100), 236(25), 118(28)	1.00	1.00
Bifenox	1.014	341(100), 343(60), 173(30)	0.08(14)	0.07(40)
Benzoylprop-ethy	0.992	105(100), 292(2), 365(2)	4.45(5.2)	4.02(5.0)
Bitertanol (diastereoisomer A)	1.116	170(100), 112(22)	1.84(5.1)	1.67(18)
Bitertanol (diastereoisomer B)	1.123	170(100), 112(22)	1.65(6.4)	1.46(14)
DDT-p,p′	0.957	235(100), 237(60), 165(45)	0.19(4.2)	0.22(36)
Fenpropathrin	1.015	181(100), 125(100), 265(50)	0.31(1.6)	0.28(9.5)
Guthion	1.026	160(100), 132(85)		
Heptachlorobiphenyl[f]	1.029	394(100), 396(95), 324(60)	0.11(4.9)	0.11(13)
Iprodione	0.991	314(100), 316(65)	0.09(12)	0.09(21)
Methoxychlor-p,p′	1.007	227(100), 228(10)	0.59(11)	0.56(33)
Mirex	1.062	272(100), 274(80), 237(55)	0.18(4.4)	0.19(14)
Nuarimol	0.966	107(100), 139(60), 314(38)	0.49(4.1)	0.46(3.4)
Propargite (omite)	0.975	135(100), 173(60), 201(30)	0.39(10)	0.41(16)
Phosmet (imidan)	0.990	160(100), 161(15)	0.38(16)	0.32(44)
Phosalone	1.034	182(100), 184(35), 121(65)	0.27(13)	0.24(31)
Permethrin-*cis*	1.124	183(100), 163(20)	0.58(9.4)	0.63(29)
Permethrin-*trans*	1.134	183(100), 163(20)	0.51(10)	0.57(32)
Piperophos	1.007	122(100), 140(80), 320(50)	0.59(5.8)	0.54(4.6)
Quizalofop-ethyl	1.222	299(100), 372(80)	0.36(3.9)	0.33(14)
Tetradifon (tedion)	1.022	159(100), 229(50), 356(40)		

[a] Ratio of retention time to the retention time of the preceding internal standard in table.

[b] The characteristic masses are usually the most abundant or the distinctive ions extracted from the limited-scan mass spectrum between 100 and 400 amu. The relative abundances are subject to variations from daily recalibration.

[c] Mean relative response factor of 5 repetitive injections at 10 ng/μl.

[d] Mean relative response factor of 3 injections at 5, 10, and 20 ng/μl.

[e] Representative major component among isomers in Aroclor 1232. RRF calculated based on total amount of Aroclor.

[f] Representative major component among isomers in Aroclor 1260. RRF calculated based on total amount of Aroclor.

tion with 10 ng/μL standards and examination of deviation from the initial calibration were made daily.

Sample Preparation

Fresh produce samples were first chopped into small pieces (<1 cm) and mixed well with a Hobart 8185 food chopper. A 50 g portion of the sample was transferred to a Mason jar. The sample was spiked with pesticide surrogates in acetone just before 100 mL acetonitrile was added. The mixture was homogenized with a Model 17150 Omni-mixer at 3500 rpm for 3–5 min and then filtered through a double-layer sharkskin filter paper. About 10 g NaCl was weighed into the filtrate; the mixture was shaken vigorously for 1 min and then centrifuged (Model CS International Centrifuge) until 2 phases were distinctly separated. A 40 mL aliquot of the acetonitrile extract (upper phase) was transferred into a 150 mL glass bottle. Toluene (0.5 mL), used as a keeper, was added to the extract, which was then passed through a column containing 5 g granular anhydrous sodium sulfate.

The solution was then concentrated to a volume of about 0.5 mL by one of the following techniques: (*a*) evaporation over a steam bath and under a gentle flow of nitrogen gas, (*b*) rotary evaporation at reduced pressure at 60°C, or (*c*) nitrogen stream evaporation at 58°C using a TurboVap Evaporation Workstation (Zymark). Complete dryness must be avoided in any step. The sample extract was filtered through a 0.2 μm nylon Acrodisc (Gelman Science) into a vial, followed by two 0.5 mL acetone rinses of the glassware. The final volume of the sample extract was reduced to 0.5 mL under a gentle stream of filtered nitrogen. The extract was spiked with 2.5 μg each of d10-anthracene, d10-pyrene, and d12-chrysene (25 μL of a 100 ppm solution) as internal standards; 1 μL portions were injected into the GC/MS and analyzed by Grob splitless technique with 0.7 min delay.

GC/MS Analysis of Spiked Samples

Recovery studies were conducted on a variety of fruit and vegetable samples. Samples of 50 g each were spiked with a standard solution containing a mixture of 20 ppm each of the pesticide compounds. Except for chlordane (1 ppm) and polychlorobiphenyls (1 ppm), each pesticide was fortified at the 0.25 ppm level. To minimize handling error, an aliquot of the spike solution was used to make up calibration standards for the GC/MS analysis.

Pesticides in the sample extracts were identified by matching their relative retention times and characteristic mass spectra with those of the standards. To speed up the raw data analysis in a timely manner, an Aquarius MS data processing software program (5) was used for the preliminary search for the presence of targeted compounds. Two criteria must be satisfied for each target compound finding: (*a*) the presence and the co-elution of all the characteristic masses (Table 1) must be within the predetermined retention time window (retention time of daily standard ±0.2 min), and (*b*) the relative ion abundance of the characteristic masses must agree within 20% as in the mass spectrum of its standard. If the criteria were met, the initial finding was examined and verified by comparing the mass spectra between the suspected component and the standard. Figure 1 illustrates a typical example of how a targeted pesticide was detected in a sample extract. The normalized single ion chromatograms of *m/e* 200, 215, and 173 (Figure 1b, c, and d) were extracted and reconstructed from the total ion profile (Figure 1a). With the assistance of computerized graphing and data manipulation, the extracted ion data showed the possible presence of atrazine: the 3 ion profiles match exactly, and their abundance ratios fall within the expected range (100:60:30). The above positive finding led to further examination of the full mass spectral data at 8.33 min. The exact matching of the 2 spectra (Figure 1e and f) provides the conclusive fingerprint evidence of the presence of atrazine in the sample extract.

Once identified, each compound was quantitated by measuring the chromatographic area of a single primary quantitation ion and response factor with respect to the internal standard (Table 1) as follows:

$$C = (Ax \times Cis)/(Ais \times RRFd \times CF)$$

where C = concentration of compound in the sample, RRFd = daily relative response factor, and CF = 40 (sample to extract dilution/concentration factor).

Results and Discussion

Method evaluations were conducted over a 1-year period. Table 2 lists recovery data for spiked samples. Method detection capabilities are in the ppb to ppm range, which is comparable to historical screening levels for most of the pesticides in fresh produce matrixes.

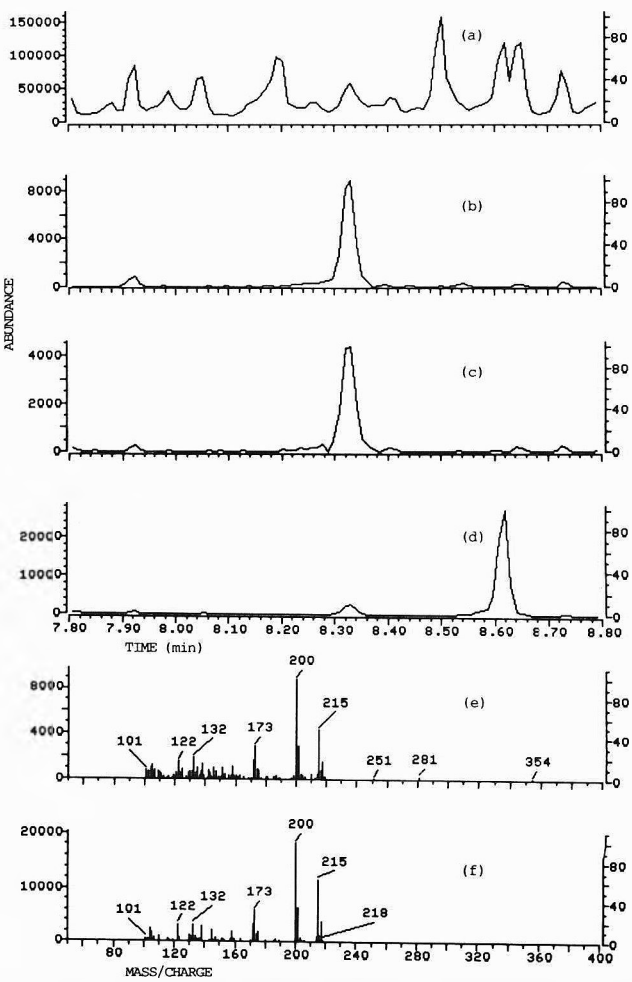

Figure 1. (a) Display of the total ion chromatogram from retention time 7.8 min to 8.8 min of a green bean sample fortified with 0.25 ppm atrazine. (b, c, d) Extracted single ion chromatograms of the most characteristic ions of atrazine. (e) Mass spectrum of the eluate at 8.33 min. (f) Reference mass spectrum of atrazine standard.

Table 2. Determination of pesticides in spiked produce samples by gas chromatography-mass spectrometry

No.	Compound name	Samples[a]	Fortified level, μg/g	Mean % recovery (RSD)[b]	Estimated detection limit, μg/g
1	Atrazine	GB, L, C, BP	0.25	75(19)	0.05
2	Ametryne	GB, G, T, A, Ci	0.25	115(21)	0.05
3	Alachlor (lasso)	GB, L, C, BP	0.25	101(12)	0.10
4	Aldrin	GB, L, C, BP	0.25	85(7.6)	0.05
5	Aroclor 1232:	HL, G, B, Cu, Ca	1.00		
	Biphenyl	HL, G, B, Cu, Ca	—	108(16)	—
	Monochlorobiphenyl	HL, G, B, Cu, Ca	—	95(12, 7)	—
	Dichlorobiphenyl	HL, G, B, Cu, Ca	—	93(9.6)	—
	Trichlorobiphenyl	HL, G, B, Cu, Ca	—	91(10)	—
	Tetrachlorobiphenyl	HL, G, B, Cu, Ca	—	96(8.2)	—
6	Aroclor 1260:	HL, G, B, Cu, Ca	1.00		0.40
	Pentachlorobiphenyl	HL, G, B, Cu, Ca	—	89(4.8)	—
	Hexachlorobiphenyl	HL, G, B, Cu, Ca	—	85(7.2)	—
	Heptachlorobiphenyl	HL, G, B, Cu, Ca	—	80(8.1)	—
7	Benzoylprop-ethyl	HL, G, B, Cu, Ca	0.25	97(6.0)	0.05
8	Bendiocarb	GB, L, C, BP	0.25	103(16)	0.05
9	BHC (alpha)	GB, L, C, BP	0.25	88(15)	0.05
10	BHC (beta)	GB, L, C, BP	0.25	93(19)	0.05
11	BHC (lindane)	GB, L, C, BP	0.25	93(35)	0.05
12	BHC (delta)	GB, L, C, BP	0.25	112(37)	0.05
13	Bitertanol (diastereoisomer B)	HL, G, B, Cu, Ca	0.25	102(9.7)	0.05
14	Bitertanol (diastereoisomer A)	HL, G, B, Cu, Ca	0.25	79(5.0)	0.05
15	Bufencarb	GB, GO, T, A, Ci	0.25	94(49)	0.05
16	Bromacil	GB, GO, T, A	0.25	114(14)	0.10
17	Benthiocarb (thiobencarb)	GB, L, C, BP	0.25	96(11)	0.05
18	Bayleton	GB, L, C, BP	0.25	103(9.8)	0.05
19	Bromophos	GB, GO, T, A, Ci	0.25	70(25)	0.05
20	Bifenox	GB, L, C, BP	0.25	114(24)	0.15
21	Bolstar	HL, G, B, Cu, Ca	0.25	90(15)	0.05
22	Chlorpropham (CIPC)	GB, L, C, BP	0.25	103(12)	0.05
23	Chlordimeform	GB, GO, T, A, Ci	0.25	103(41)	0.05
24	Carbofuran	GB, L, C, BP	0.25	110(14)	0.05
25	Chlorothalonil (bravo)	GB, L, C	0.25	71(47)	0.05
26	Carbaryl	GB, L, C, BP	0.25	128(23)	0.05
27	Chlordane:	GB, L, C, BP	1.00		1.00
	Chlordane (C10H9C15)	GB, L, C, BP	—	90(28)	—
	Chlordane (C10H9C15)	GB, L, C, BP	—	92(17)	—
	Chlordane (C10H8C16)	GB, L, C, BP	—	83(10)	—
	Chlordane (C10H7C17)	GB, L, C, BP	—	96(4.1)	—
	Chlordane (C10H7C17)	GB, L, C, BP	—	66(5.2)	—
28	Chlorpyrifos (dursban)	GB, L, C, BP	0.25	107(12)	0.05
29	Captan	GB, L, C, BP	0.25	67(8.0)	0.10
30	Chlorobenzilate	GB, L, BP	0.25	102(5.5)	0.05
31	Carbophenothin (trithion)	GB, L, BP	0.25	99(5.9)	0.05
32	Cyanophos	HL, G, B, Cu, Ca	0.25	94(8.9)	0.05
33	Chlornitrofen	HL, G, B, Cu, Ca	0.25	102(11)	0.05
34	Carbetamide	HL, G, B, Cu, Ca	0.25	90(19)	0.05
35	DEF (degreen)	HL, G, B, Cu, Ca	0.25	111(15)	0.05
36	Dimethachlor	HL, G, B, Cu, Ca	0.25	100(14)	0.05
37	Dimethametryn	HL, G, B, Cu, Ca	0.25	100(6.4)	0.05
38	Diclobutrazol	HL, G, B, Cu, Ca	0.25	105(12)	0.05
39	Dichlorvos	GB, L, C, BP, A	0.25	67(20)	0.05
40	Dichlobenil	GB, GO, T, A, Ci	0.25	59(31)	0.05
41	Demeton-O	GB, L, C, BP	0.25	78(29)	0.05
42	Demeton-S	GB, L, C, BP	0.25	75(19)	0.05
43	Dicrotophos	GB, GO, T, A, Cl	0.25	73(22)	0.05
44	Dimethoate	GB, L, C, BP	0.25	82(32)	0.05
45	Dicloran (botran)	GB, L, C	0.25	122(8.3)	0.05
46	Dioxathion (delnav)	GB, L, C, BP	0.25	120(20)	0.20

Table 2. Continued

No.	Compound name	Samples[a]	Fortified level, μg/g	Mean % recovery (RSD)[b]	Estimated detection limit, μg/g
47	Diazinon	GB, L, C, BP	0.25	88(17)	0.05
48	Dicofol	GB, L, C, BP	0.25	88(41)	0.10
49	Dacthal (DCPA)	GB, L, C, BP	0.25	81(12)	0.05
50	Disulfoton	HL, G, B, Cu, Ca	0.25	84(13)	0.05
51	DDE-p,p′	GB, L, C, BP	0.25	90(12)	0.05
52	Dieldrin	GB, L, C, BP	0.25	85(10)	0.15
53	DDD-p,p′	GB, L, C, BP	0.25	119(14)	0.05
54	DDT-p,p′	L, C, T, A	0.25	64(30)	0.05
55	Endosulfan-alpha	GB, L, C, BP	0.25	85(23)	0.10
56	Endosulfan-beta	GB, L, C, BP	0.25	105(19)	0.10
57	Endosulfan sulfate	GB, L, C, BP	0.25	63(15)	0.10
58	Ethylan (perthane)	GB, L, C, BP	0.25	99(15)	0.05
59	Endrin	GB, L, C, BP	0.25	95(20)	0.15
60	Ethion	GB, L, C, BP	0.25	121(17)	0.05
61	Ethirmol	HL, G, B, Cu, Ca	0.25	82(28)	0.05
62	Etaconazole (diastereoisomer A)	HL, G, B, Cu, Ca	0.25	121(9.3)	0.05
63	Etaconazole (diastereoisomer B)	HL, G, B, Cu, Ca	0.25	96(15)	0.05
64	Ediphenophos	HL, G, B, Cu, Ca	0.25	46(36)	0.05
65	Fenobucarb	HL, G, B, Cu, Ca	0.25	108(10)	0.05
66	Fluchloralin (basalin)	GB, L, C, BP	0.25	80(11)	0.05
67	Fenthion (baytex)	GB, L, C, BP	0.25	95(13)	0.05
68	Folpet	L, C	0.25	72(5.1)	0.25
69	Formothion	HL, G, B, Cu, Ca	0.25	64(24)	0.05
70	Fluorochloridone	HL, G, B, Cu, Ca	0.25	116(3.4)	0.05
71	Fenson	HL, G, B, Cu, Ca	0.25	119(20)	0.05
72	Flamprop-methyl	HL, G, B, Cu, Ca	0.25	131(24)	0.05
73	Flamprop-isopropyl	HL, G, B, Cu, Ca	0.25	102(8.3)	0.05
74	Fenpropathrin	HL, G, B, Cu, Ca	0.25	93(7.0)	0.10
75	Guthion	GB, L, C	0.25	61(40)	0.15
76	Heptenophos	HL, G, B, Cu, Ca	0.24	74(22)	0.05
77	Heptachlor	GB, L, C, BP	0.25	83(44)	0.05
78	Heptachlor epoxide	GB, L, C, BP	0.25	96(11)	0.10
79	Indalone (butopyronoxyl)	GB, GO, T, A, Ci	0.25	96(38)	0.05
80	Iprodione (rovral)	GB, L, C, BP	0.25	150(30)	0.10
81	Isozophos	HL, G, B, Cu, Ca	0.25	74(22)	0.05
82	Iprobenfos	HL, G, B, Cu, Ca	0.25	99(11)	0.05
83	Lenacil	HL, G, B, Cu, Ca	0.25	97(11)	0.05
84	Metolachlor	HL, G, B, Cu, Ca	0.25	98(6.3)	0.05
85	Mevinphos-beta (phosdrin)	GB, GO, T, A, Ci	0.25	85(31)	0.05
86	Mevinphos-alpha (phosdrin)	GB, L, C, BP	0.25	92(11)	0.05
87	Mexacarbate	GB, L, C, BP	0.25	125(58)	0.05
88	Malathion-oxygen analog	GB, L, C,	0.25	100(9.6)	0.05
89	Methyl parathion	GB, L, C, BP	0.25	105(28)	0.05
90	Metalaxy (ridomil)	GB, L, C, BP	0.25	134(16)	0.05
91	Methiocarb	GB, L, C, BP	0.25	109(7.9)	0.05
92	Malathion	GB, L, C, BP	0.25	98(17)	0.05
93	Methidathion (supracide)	GB, L, C, BP	0.25	108(25)	0.05
94	Methoxychlor-p,p′	L, C	0.25	94(40)	0.05
95	Mirex	GB, GO, T, A, Ci	0.25	89(33)	0.05
96	Monolinuron	HL, G, B, Cu, Ca	0.25	41(24)	0.10
97	Methoprotryne	HL, G, B, Cu, Ca	0.25	96(16)	0.05
98	Nuarimol	HL, G, B, Cu, Ca	0.25	100(14)	0.05
99	Nitrofen (TOK)	GB, L, C, BP	0.25	106(6.9)	0.05
100	Oxadiazon	GB, L, C, BP	0.25	89(11)	0.05
101	Oxyfluorfen	GB, L, C, BP	0.25	127(22)	0.05
102	Ofurace	HL, G, B, Cu, Ca	0.25	98(16)	0.05
103	Ovex (chlorfenson)	HL, G, B, Cu, Ca	0.25	101(4.7)	0.05

Table 2. Continued

No.	Compound name	Samples[a]	Fortified level, μg/g	Mean % recovery (RSD)[b]	Estimated detection limit, μg/g
104	*o*-Phenylphenol	HL, G, B, Cu, Ca	0.25	108(7.1)	0.05
105	Propham (IPC)	GB, L, C, BP	0.25	93(5.5)	0.05
106	Propoxur	GB, L, C, BP	0.25	102(15)	0.05
107	Phorate-oxygen analog	GB, GO, T, A, Ci	0.25	80(12)	0.05
108	Phorate	GB, L, C, BP	0.25	96(16)	0.05
109	Pentachloronitro-benzene	GB, GO, T, A, Ci	0.25	78(24)	0.05
110	Pronamide	GB, L, C	0.25	95(14)	0.05
111	Profluralin	GB, L, C, BP	0.25	80(11)	0.05
112	Pirimicarb	GB, L, C, BP	0.25	114(13)	0.05
113	Propanamide (propanil)	GB, L, C, BP	0.25	123(19)	0.05
114	Prometryne	GB, GO, T, A, Ci	0.25	105(12)	0.05
115	Parathion-oxygen analog	GB, L, C	0.25	91(17)	0.15
116	Pirimiphos-methyl	GB, L, C, BP	0.25	98(10)	0.05
117	Parathion	GB, L, C, BP	0.25	93(21)	0.05
118	Profenofos	GB, GO, T, A, Ci	0.25	93(19)	0.05
119	Proparagite (omite)	GB, L, C, BP	0.25	96(16)	0.05
120	Phosmet (imidan)	GB, L, C, BP	0.25	59(19)	0.05
121	Phosalone	GB, L, C, BP	0.25	91(9.9)	0.05
122	Permethrin-*cis*	GB, L, BP	0.25	103(8.5)	0.05
123	Promecarb	HL, G, B, Cu, Ca	0.25	109(8.9)	0.05
124	Prothoate	HL, G, B, Cu, Ca	0.25	113(9.1)	0.05
125	Permethrin-*trans*	GB, L, BP	0.25	101(7.0)	0.05
126	Piperophos	HL, G, B, Cu, Ca	0.25	87(10)	0.05
127	Quinomethionate (morestan)	GB, L, C, BP	0.25	132(22)	0.05
128	Quinalphos	HL, G, B, Cu, Ca	0.25	96(11)	0.05
129	Quizalofop-ethyl	HL, G, B, Cu, Ca	0.25	83(7.7)	0.05
130	Rally (systhane)	GB, GO, T, A, Ci	0.25	101(38)	0.05
131	Sonalan	GB, L, C, BP	0.25	77(11)	0.05
132	Simetryne	HL, G, B, Cu, Ca	0.25	102(18)	0.05
133	Systox	GB, GO, T, A, Ci	0.25	96(34)	0.10
134	Simazine	GB, GO, T, A, Ci	0.25	60(36)	0.05
135	Safrotin	GB, L, C, BP	0.25	93(11)	0.05
136	Trifluralin	GB, GO, T, A, Ci	0.25	55(13)	0.05
137	Terbacil	GB, L, BP	0.25	99(18)	0.05
138	Tetrachlorvinphos	GB, GO, T, A, Ci	0.25	69(41)	0.05
139	Triazophos	GB, L, C, BP	0.25	134(15)	0.05
140	Thiometon-ethyl	HL, G, B, Cu, Ca	0.25	91(23)	0.05
141	Tolyfluanid	HL, G, Cu	0.25	124(11)	0.05
142	Vegadex	GB, L, C, BP	0.25	74(8.1)	0.05
143	Vinclozolin (ronilan)	GB, L, C, BP	0.25	93(8.7)	0.10
	Grand Mean			92(22)	

[a] Sample code: GB, green bean; BP, bell pepper; L, lettuce; C, carrot; HL, head lettuce; Cu, cucumber; T, tomato; A, apple; Ca, cauliflower; GO, green onion; B, broccoli; G, grape; Ci, cilantro.

[b] One spike recovery was performed on each selective produce sample. The number of determinations used to calculate the mean % recovery and the RSD is equal to the number of samples tested for each compound.

Target Compound Selection

Practically, compounds (up to several hundred) that are suitable for GC analysis can be included in the target listing for routine screening. They must be volatile and thermally stable under the applied gas chromatographic conditions. In the interest of efficiency and cost effectiveness in screening large numbers of agricultural products in a short turnaround time (usually less than 24 h), it is highly desirable to determine as many of the analytes as possible that are potentially present on food by using a single analytical procedure.

The compounds selected in this study are pesticides, including some metabolites, and other chemicals that are of concern to the safety of domestic or imported food. In general, they include organophosphorus, organohalogen, organonitrogen, carbamate, triazine, thiourea, phenolic, and aromatic hydrocarbon compounds. Other groups of pesticides are not presented in this paper because they were determined to be not suitable for GC separation. They are usually nonvolatile compounds. Alternative methods, such as liquid chromatography (LC), would be more appropriate for these compounds.

A number of analytes experienced degradation in the injector. This is most likely due to reactions with residues deposited around the GC injector liner and at the front end of

Table 3. Monitoring of possible GC degradation compounds

Parent compound	Degradation compound	Relative retention time[a]	Monitoring ions *m/z*, % relative absorbance
Bendiocarb	2,3-(Isopropylidene-dioxy)phenol	0.592	151(100), 126(100), 166(55)
Bufencarb	*m*-(1-Methylbutyl)phenol	0.706	121(100), 164(30), 107(30)
Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranol	0.616	149(100), 164(80), 131(40)
Chlorotoluron	3-Chloro-4-methyl-aniline	0.555	132(100), 167(60)
Carbaryl	1-Naphthol	0.793	144(100), 115(95), 116(40)
DDT-p,p′	DDD-p,p′	1.303	235(100), 237(65), 165(60)
Folpet	Phthalimide	0.737	147(100), 104(70), 103(30)
Methoxychlor	Methoxychlor olefin	1.372	227(100), 228(10)
Mexacarbate	4-(Dimethylamino)-3,5-dimethylphenol	0.706	165(100), 149(80), 164(60)
Methiocarb	4-(Methylthio)-3,5-dimethylphenol	0.824	168(100), 153(80), 125(30)
Metoxuron	3-Chloro-4-methoxy-aniline	0.698	168(100), 183(100), 185(30)
Propoxur	2-(1-Methylethoxy)phenol	0.496	110(100), 152(20)
Thiofanox	3,3-Dimethyl-1-methyl-thiobutanone O-oxime	0.603	115(100), 161(65)

[a] Relative retention time based on d10-anthracene.

the GC column. As the deposited residues accumulate from multiple injections of sample extract, the extent of degradation becomes more significant. It is suggested that the replacement of the injector liner (filled with silanized glass wool plug) and the removal of the first few centimeters of the GC column be performed daily as a routine practice. Nevertheless, the presence of the degradation compounds provides additional confirmation of the parent analytes. Because the retention times and mass spectra of the resulting injection degradation compounds are also specific and reproducible during the GC/MS analysis, they are included as part of the targeted compound list as shown in Table 3.

Sample Preparation

Preparing the sample extract for GC/MS analysis is critical for the purpose of screening a broad spectrum of pesticides. The acetonitrile extraction-partition procedure was used for this study because it is well-documented and widely used (6–8). However, there was no additional organic solvent partition or column (e.g., Florisil) cleanup involved. In addition, to minimize possible loss and decomposition of analytes during the concentration step, the acetonitrile extract was concentrated by one of the 3 procedures as described above. The direct steam evaporation is simple and quick. The rotary evaporation reduces the possibility of thermal decomposition, and the nitrogen steam evaporation using TorboVap is convenient and requires the least attention. The addition of toluene to the extract prevents the concentrate from going to complete dryness, and further adds to the convenience at the end of extract handling. When the results obtained from the 3 concentration procedures were compared, it was found that there were no apparent differences in recoveries of the analytes. The variation among recoveries was mainly introduced from the GC/MS instrumental measurement process.

Target Compound Identification

The target compounds are identified, if present, in the predetermined retention time windows (±0.2 min) that have the identical mass spectra as the corresponding standards. To differentiate them from the chromatographic background interferences that were co-extracted from the produce samples, it is necessary to use a high resolution capillary GC column with a fast temperature program rate. This allows better spectral enhancement through background subtraction for analyzing samples of complex matrix (e.g., Figure 2). The 12 m × 0.2 mm id polydimethylsiloxane column was selected because it gives adequate resolution and allows fast analysis (18 min).

In pesticide analysis, the mass spectrometer is used as a general purpose spectroscopic detector. It collects structural data from all types of chemicals that are traditionally obtainable by multiple injections with various element-selective detectors. This procedure requires experience and skill for the chemist to extract and sort out the most useful information from the abundant raw data and then make the best judgment in interpreting the GC/MS results. Recent developments in commercial data system software have allowed data manipulation and processing to perform more effectively and accurately.

Co-elution (but not exactly matching) is usually not a problem because each pesticide has its own characteristic ion pattern. The combined GC and MS displays allow recognition and determination of each analyte. The presence of co-extracted material in the sample contributed to a continuous background which included most of the ion signals. The background, mainly resulting from hydrocarbons and carbohydrates, consisted of intense ions at low mass ranges as clusters around masses 39, 41, 43, 55, 57, 67, 69, 73, 81, 85, 93, and 97. The intense low mass signals sometimes complicated the normalized spectrum, especially for components at trace level (<0.25 ppm). To maintain minimum background and lessen spectral interference, an optional limited scanning from mass 100 to 400 was chosen (Figure 3). Although some analytes have their base ions below 100, all the analytes produce abundant fragmentation ions in the 100–400 amu scan range that essentially provide the unique characteristics

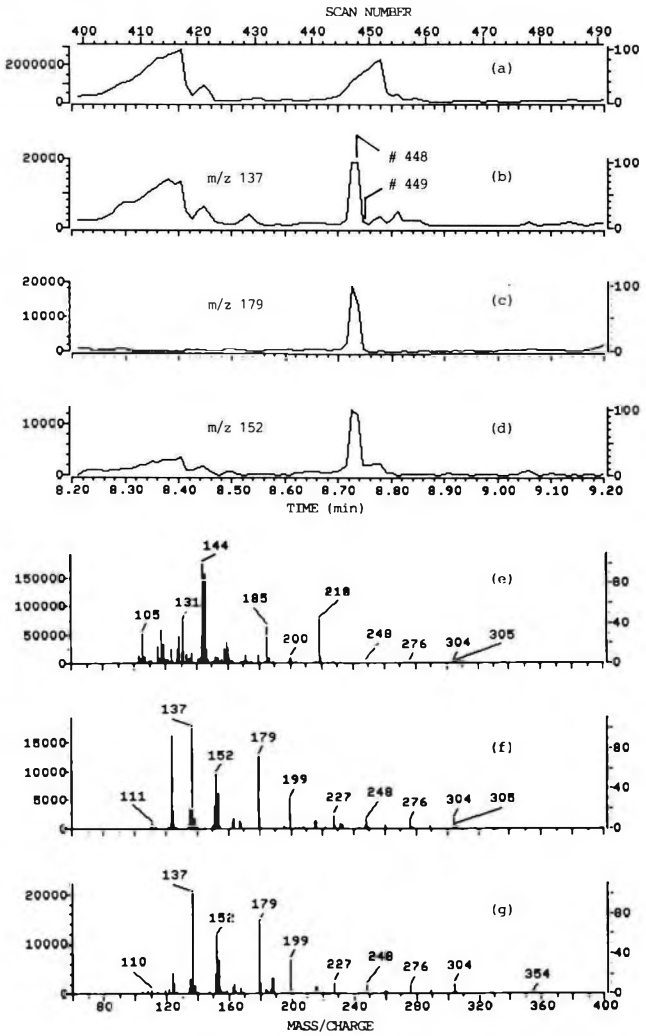

Figure 2. (a) Total ion chromatogram of a complex bell pepper sample fortified with 0.25 ppm diazinon. (b, c, d) Coelution of the characteristic ions of diazinon. (e) Mass spectrum of scan 448. (f) Enhanced spectrum of scan 448 after background subtraction by scan 449. (g) Reference spectrum of diazinon standard.

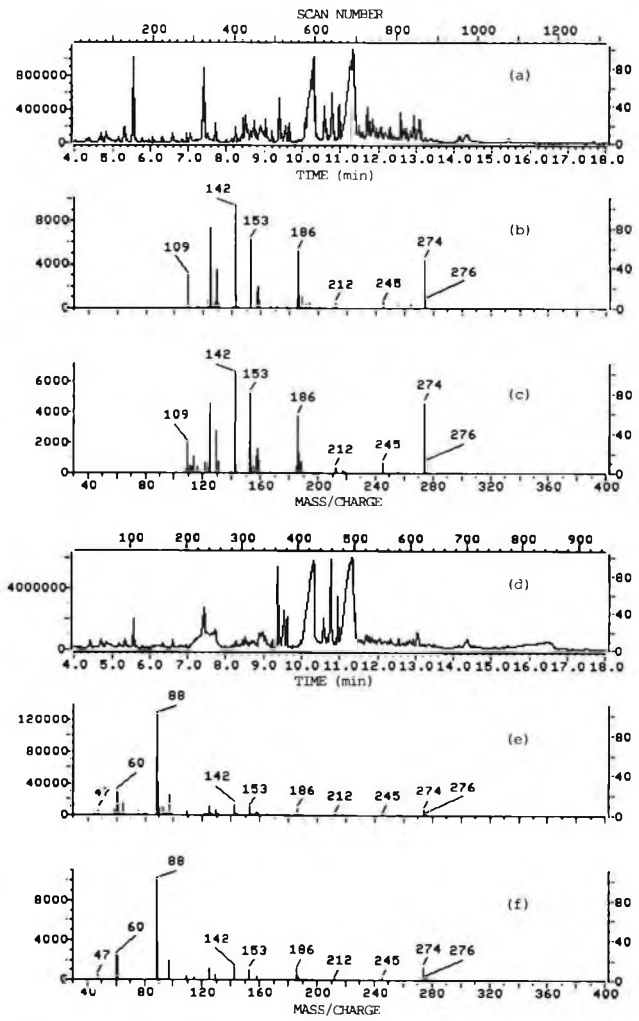

Figure 3. (a) Limited scan (100–400 amu) total ion chromatogram of a broccoli sample fortified with 0.25 ppm disulfoton. (b) Mass spectrum of disulfoton found at scan 473 versus (c) daily standard spectrum of disulfoton. (d) Full scan (35–450 amu) total ion chromatogram of the same sample extract. (e) Mass spectrum of disulfoton found at scan 338 versus (f) full scan reference spectrum of disulfoton.

necessary for decisive confirmation. Starting the mass scan at 100 amu has not limited the detection of pesticide residues in a variety of fresh produce matrixes. Compared to the full mass range scan, the limited scan appeared to result in better sensitivity and less contamination of the focusing lenses and the quadrupole. Limited scanning also yields a convenient blank background between multiple injections. Recent MS instrumentation offers a switchable scan-range capability during a GC/MS run that makes the application of the limited scan more flexible and effective. Figure 4 presents examples of total ion chromatograms of pesticide standards and grape, broccoli, and cilantro sample extracts, respectively. The normalized chromatograms show the relative ion abundances of each sample.

Analysis of pesticides in food for regulatory purposes demands unambiguous evidence, including a well-documented quality control practice, before initiating regulatory action. Whenever uncertainty is encountered (most likely, the occurrence of interference ions), it may be necessary to repeat the analysis, re-run the sample using a different GC oven temperature program, use a column of a different polarity, or analyze the sample by using a conventional elemental-specific detector.

Of the total 665 determinations of analytes in fortified crop samples in Table 2, 2.3% failed to give meaningful recoveries. This was due to severe background interference or the instability of some of the marginal analytes.

Quantitation

An initial study was made to demonstrate the reproducibility and linearity of quantitative measurement of pesticides by GC/MS. Once an analyte was identified, the base ion shown in Table 1 was selected as the quantitation ion. The relative standard deviation (RSD) of the mean responses from repetitive injections in Table 1 ranged from 0.9 to 28% with a mean of 6.5%. The RSDs from the 3-point calibration injections ranged from 1.0 to 57% with a mean of 13.4%. In general, the precision ranges for pesticide measurements agreed with typical GC/MS measurements in the electron impact ionization mode (9).

The produce samples selected for this study represented 2

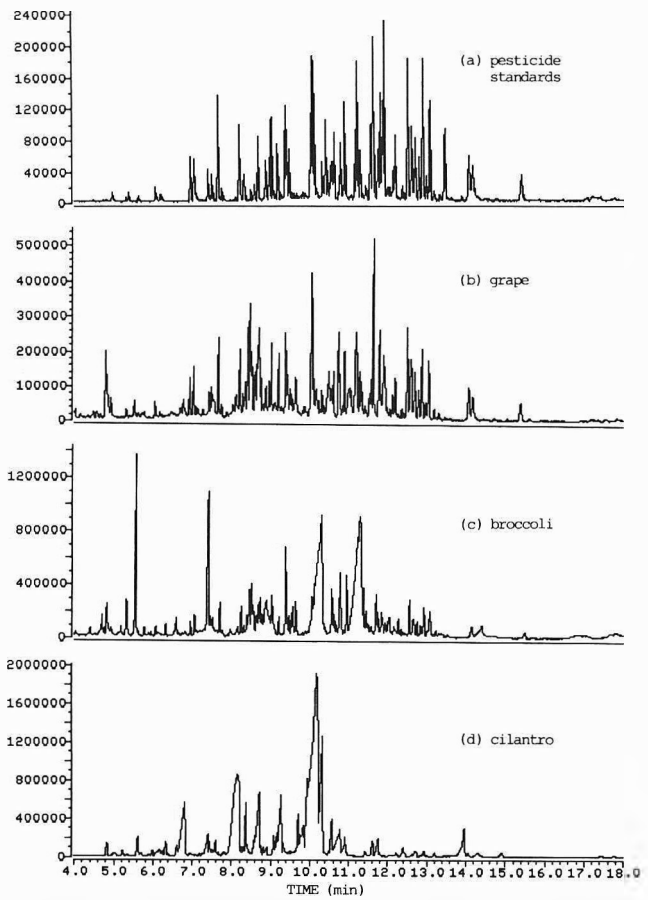

Figure 4. Total ion chromatograms of extracts of fresh produce samples spiked with pesticide standards (a) at 0.25 ppm. Examples of representative (b) clean, (c) medium, and (d) complex samples fortified with the same amount of pesticides as (a).

categories: fruits and vegetables. The current pesticide tolerances established by the Environmental Protection Agency for these crops range from no tolerance to 50 ppm. A great many of the tolerances are less than 1 ppm. The main objective of the work was to evaluate the suitability of the GC/MS method for both qualitative and quantitative determination of pesticide residues, particularly at low levels, in produce samples. Fortifications were, therefore, made at the 0.25 ppm level except for chlordane and Aroclors, which were fortified at 1 ppm. This is also consistent with the expected levels at which residue findings were frequently reported (10).

For quantitative analyses, a standard calibration was made daily. In addition, an examination of variations in response factors of the marginal compounds (folpet, DDT, carbaryl, etc.) and formations of their GC degradation compounds (Table 3) may indicate the extent of contamination at the injector port. Concentrations of analytes extracted from samples were computed by using the daily calibration response factors. Over a period of 3 months, the GC retention times remained stable (varying within 2.5% or 0.3 min), and the MS ion source did not need cleaning.

Table 2 summarizes the recovery results, including a total of 665 recoveries for 143 pesticides and groups of chemicals. The results show the precision and accuracy of the GC/MS method for each analyte while the matrix varied. The grand mean accuracy of all analytes was 92% with a mean RSD of 22%, which is comparable to the widely used GC/MS measurements in environmental sample testings (9).

The estimated method detection limit of each analyte is also shown in Table 2. It ranges from 0.05 ppm for the majority of the analytes to 1 ppm for chlordane, which has multiple components. Because the detection limit is also dependent on sample matrix, accurate determination of an individual detection limit on various types of crops would require additional studies.

Pesticide residues at much lower levels were routinely detected in our laboratories by adjusting the sample size or extract volume to yield concentrations in the desired ranges. Quantitation of residues at such low levels may not be accurate because the stability of the quantitation ion peak deteriorates. Additional measurements and/or confirmations may be necessary and can be made by re-analyzing the sample by using the GC/MS in the selective ion monitoring mode, or using appropriate element-specific detectors.

Conclusion

This 1-year study has demonstrated that the GC/MS pesticide screening technique presented here has a significant potential as one of the major analytical tools in the arsenal of multiresidue screening techniques. The current list of over 140 pesticides that potentially could be detected and quantitated with this method rivals many of the existing multiresidue methods using different element-specific detectors.

For samples with an urgent need for laboratory results such as poisoning cases, this GC/MS screening technique can offer a coverage significantly greater than the conventional scans for organophosphates and organohalogens using element-specific detectors alone. Compounds such as *o*-phenylphenol and biphenyl that do not respond well in the element-specific detectors can be included in this GC/MS screen. The potential to add new groups of pesticides or other chemicals of food safety concerns that were not previously screenable by element-specific detectors suddenly becomes feasible by this technique.

It is commonly believed that most GC/MS cannot endure the multiple injections of produce extracts without appreciable degradation in sensitivity. Keeping in mind that the produce extracts we used in this study have not been subjected to cleanup procedures, we still have the option to introduce cleaner samples into the GC/MS by using some cleanup in the sample preparation. So far, we have not found it necessary, and in this study, we have made multiple sample analyses on consecutive days on many occasions. We are encouraged by our observations that our GC/MS did not have any apparent loss of sensitivity or reproducibility. This final observation might well be the key to reversing the role of the GC/MS from a confirmational tool to a front line screening instrument in multiresidue screening for fresh fruits and vegetables.

This study has, thus, demonstrated that GC/MS is a promising analytical method for the determination of a broad spectrum of pesticide residues in fresh produce samples. A major advantage attained from using this method for analysis is that it permits rapid, routine, and confirmatory screening of hundreds of chemicals of food safety concern, provided their analytical parameters through the method have been established.

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SEAFOOD PRODUCTS

Comparison of Microwave Digestion with Conventional Wet Ashing and Dry Ashing Digestion for Analysis of Lead, Cadmium, Chromium, Copper, and Zinc in Shellfish by Flame Atomic Absorption Spectroscopy

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A closed vessel microwave digestion procedure was developed for shellfish samples. This procedure was compared with wet and dry ash procedures for levels of lead, cadmium, chromium, copper, and zinc. Results obtained for microwave and conventional wet ash digestion were comparable. The dry ashing procedure produced results consistently lower than either of the other methods. Recoveries ranged from 80–92% for microwave and wet ashing procedures and 54–72% for the dry ashing procedure. Accuracy was also determined by analyzing lobster hepatopancreas marine reference material. Values for Pb, Cd, and Cr fell within the range specified for the reference material for all 3 digestion procedures; however, values were lower for Cu and Zn. Results of this study show that microwave digestion is comparable to wet ashing.

Mollusks selectively concentrate certain trace metals from the hydrosphere they inhabit. Trace metals are concentrated in animal tissues in varying degrees, depending on the species, temperature of the hydrosphere, and concentration of the metal in the environment (1, 2). From a public health viewpoint, levels of trace metals such as cadmium, lead, copper, zinc, and chromium are useful indicators of the extent of pollution in the hydrosphere and also of the health of edible species of shellfish. Because Rhode Island is a coastal state with a substantial market for seafood, it has long been involved in monitoring levels of toxic metals in shellfish on a routine basis.

Frequent testing of large numbers of shellfish samples requires a method that is sensitive, precise, accurate, rapid, and cost effective. Determination and quantification of heavy metals in foods is most often accomplished via atomic absorption spectroscopy (AAS) (3–7). Before measuring the quantity of metal in a particular shellfish sample, tissue must be digested to free the metal under investigation. This digestion process is the limiting factor as to how much time the entire procedure takes and also as to the efficiency of recovering the actual amount of metal present in the sample. Metals can be released from the tissue by combusting the organic tissue at high temperatures (the dry ashing method of digestion), or by combusting the organic tissue using heat and concentrated acid (the wet ash method). Both techniques have certain limitations including excessive time, possible loss of metals by volatilization, excessive manipulation of sample, and incomplete digestion.

Recently, commercial microwave digestion systems have become available for laboratory use, and several studies have been published (8, 9) in which investigators have demonstrated success in digesting various types of samples for metal analysis by atomic absorption spectroscopy. Microwave heating can offer advantages over traditional wet or dry ashing techniques. Microwave heating is rapid, safe, and programmable. Programmability makes it possible to control the amount and energy intensity applied to samples.

The present study compares conventional wet ashing and dry ashing with microwave digestion for shellfish tissue in terms of quantity of metal measured, accuracy, precision, and length of time needed for each method. The metals investigated are lead, cadmium, copper, zinc, and chromium.

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Experimental

Apparatus

Wash all glassware with nitric acid (HNO_3) (50%, v/v), rinse with cold tap water followed by another rinse with deionized water.

(a) *Atomic absorption spectrometer.*—Perkin-Elmer model 303 equipped with a 4-in triple-slotted burner head and hollow-cathode lamps. Wavelengths (nm) as follows: Pb (283.3), Cd (228.8), Cu (324.8), Cr (357.9), and Zn (213.9). Slit setting is at 4; flame: air–acetylene with oxidizing (lean blue) flame for Pb, Cd, Cu, and Zn, and reducing flame (rich yellow) for Cr.

(b) *Microwave digestor.*—Commercial oven, model MDS-81D (CEM Corp., Indian Trail, NC) equipped with Teflon®-coated oven cavity, removable 12-position sample carousel, exhaust fan, hose to permit venting of fumes into fume hood, and Teflon PFA vessels, 120-mL capacity (CEM Corp.) for microwaving samples. Use capping station (CEM Corp.) to tighten digestion vessel caps to ensure reproducible torque. Before use and between sample digestions, clean vessels by adding nitric acid (50%, v/v) to each vessel and heating in microwave.

Reagents

All reagents were analytical reagent grade. Deionized, distilled water was used throughout.

(a) *Nitric acid.*—70% v/v.

(b) *Nitric acid–saline solution.*—14% v/v HNO_3 and 1.25% w/v NaCl. Prepared by adding 200 mL of 70% v/v HNO_3 to 500 mL water containing 12.5 g NaCl and diluting to 1 L.

(c) *Nitric acid.*—14% v/v. Prepared by adding 200 mL of 70% HNO_3 to 500 mL water and diluting to 1 L.

(d) *Atomic absorption reference solutions.*—1000 ppm (Fisher Scientific, Fairlawn, NJ).

Preparation of Standards

Atomic absorption working standards.—Mixed working standards of Pb, Cd, Cu, and Cr were prepared by diluting 1000 ppm stock reference solutions with nitric acid–saline solution to produce concentrations ranging from 0.05 to 4.0 ppm. A saline solution was used in the standard to correct for high salt concentration associated with shellfish [ca

1.25% (see Ref. 10)]. Zinc standards (0.05 to 1.5 ppm) were prepared in a nitric acid–dilute saline matrix (14% HNO_3 in 0.0125% NaCl). Because of high Zn content in shellfish, samples were diluted 100-fold for zinc determination; the initial salt concentration was similarly diluted from 1.25 to 0.0125% NaCl.

Sample spiking standard solutions.—Spiking standards of Pb (50 ppm), Cd and Cr (20 ppm) and Cu (250 ppm) were prepared in approximately 1M HNO_3. Zinc standard reference solution (1000 ppm) was used directly for spiking.

Analytical Procedure

Method procedure.—Each sample batch (10 batches total) was analyzed for heavy metals by 3 digestion methods: conventional wet ashing, dry ashing, and microwave digestion. Each sample batch (except batch 1 that contained 9 replicate samples and 9 spiked samples) was analyzed in replicates of 5 along with 5 replicate recovery samples and 2 control reagent blank samples. To measure the accuracy of each procedure, a standard reference material, lobster hepatopancreas marine reference material (11), was also analyzed with each of the 3 procedures. Seven trials were run for the reference material.

Sample preparation.—Composite samples of hard shell clams (*Mercenaria mercenaria*) and soft shell clams (*Mya arenaria*) were prepared in the following manner. Calcareous shells of clams were removed and meats were drained, homogenized, and divided into 10 batches of 500–700 g. Ten batches were stored at 4°C. At the time of analysis, each batch of composite shellfish was blended at high speed for 2 min using a 2-speed Waring blender with a stainless steel blade and glass container.

Wet ashing.—Modification of the wet ash method for heavy metal determination in shellfish by the National Shellfish Sanitation Program (12) was followed. A 12.5 g sample portion was weighed into a tared 250 mL pyrex beaker and 30 mL concentrated nitric acid was carefully added. The shellfish mass was broken up by mixing with a glass rod. To determine percent recoveries, 0.5 mL aliquots of sample spiking standard were added to replicate samples and mixed into the sample with a glass rod. Blank samples containing only nitric acid were run concurrently through the entire procedure. Following overnight predigestion at room temperature, samples were placed on a steam bath and allowed to simmer with frequent stirring with a glass rod until frothing subsided and digest turned a dark orange color. After gentle boiling was established, the volume was reduced to near dryness. Charring of samples was avoided to minimize possible loss of analyte. An additional 10 mL concentrated nitric acid was added to samples and blanks, and digestion was continued until approximately 5 mL solution remained. The inside of beakers was rinsed with 10–15 mL deionized water and allowed to heat for another 10 min. Samples were then removed from heat, cooled to room temperature, filtered through filter paper (Schleicher & Schuell 588 prepleated, Keene, NH) into 25 mL volumetric flask, and brought to volume using deionized water.

Dry ashing.—Modification of dry ash method for heavy metal determination in shellfish by the National Shellfish Sanitation Program (12) was followed. A sample portion (12.5 g) of blended sample was weighed into a tared Vycor crucible and placed in a low temperature oven (100°C) and heated for 4.5 h. Crucibles were transferred to a high temperature muffle furnace and ashed overnight at 450°C. The following morning, samples were removed from the muffle furnace, cooled, and dissolved in 2 mL concentrated nitric acid with warming on a hot plate. The resulting solution was filtered into separate 25 mL volumetric flasks and brought to volume with deionized water. Each batch (except batch 1 that contained 9 replicate samples and 9 replicate spiked samples) consisted of 5 replicate samples, 5 replicate spiked samples, and 2 reagent blank samples.

Microwave digestion.—A sample portion (12.5 g) was weighed into a tared PFA vessel and 9 mL concentrated nitric acid was added to the vessel. The digestion procedure was run in 2 stages. The first stage (open vessel digestion) was a predigestion. The vessel containing the sample was capped hand-tight (no pressure or disc seals) and programmed to run at reduced power in the following cycle: 30% power for 15 min, cool cycle for 10 min, and 30% power for 15 min. If no clumps of shellfish were present, then stage 2 of digestion was started. If clumps were present, the cycle calling for 30% power for 15 min was repeated. During stage 2 (closed vessel digestion), vessels were capped under pressure using the capping station and disc seals. The microwave unit was programmed to run alternating hot-cold cycles (30% power for 15 min followed by a 10–15 min cooling time). Samples and vessels were checked after each heating cycle to verify that the bottom of the vessels were hot and the top of the vessel warm to the touch and that samples were light-orange to yellow in color. If sample appearance was burnt orange, vessels were allowed to cool and vent before continuing the heating process. After digestion was completed (sample color: clear, light yellow), the acid digest was filtered into 25 mL volumetric flask and brought to volume with deionized water.

Determination.—Determination of heavy metals following digestion procedures described was performed with flame AAS. Samples analyzed for Pb, Cr, and Cd were run without further dilution. The dilution factor for Pb, Cr, and Cd was 2× (12.5 g sample in a total volume of 25 mL). Determinations for Cu and Zn required samples to be diluted 1 + 9 for Cu and 1 + 99 for Zn. All dilutions were made with 14% HNO_3 to match standards as closely as possible. Standards and samples were run under the same instrumental conditions as previously described. Standard curves for each element under investigation were run at the beginning and end of each session and an EPA-certified 1 ppm mixed standard solution in dilute nitric acid was periodically evaluated during a run of samples. The Perkin-Elmer model 303 AAS does not have a means of correcting for background interferences. However, a previous experiment in this laboratory (10) demonstrated no significant differences between a deuterium-arc background corrected signal and an uncorrected signal for the 5 heavy metals of interest in a shellfish matrix. Samples, standards, and blanks were aspirated until maximum peak heights were obtained and produced flat peaks on the chart recorder paper. Water was aspirated between each sample, blank, or standard until the chart pen returned to baseline.

Calculations.—Regression lines were determined for each element from standards run using concentrations (ppm) and peak heights (mm) for corresponding X,Y values. Metal concentrations in each sample were calculated from the corresponding regression lines and dilution factors.

Statistical analysis.—Data from the 3 digestion methods were compared using the NCSS statistical package (NCSS, Kaysville, UT) on an IBM PC-compatible computer. Using the NCSS package, data were analyzed by analysis of vari-

Table 1. Metals found (ppm) in shellfish batch samples by dry ashing (DA), wet ashing (WA), and microwave digestion (MI)

Batch No.	N[a]	Lead			Cadmium			Copper			Chromium			Zinc		
		DA	WA	MI	DA	WA	MI	DA	WA	MI	DA	WA	MI	DA	WA	MI
1	9	0.39	0.73	0.86	0.19	0.37	0.39	3.76	3.96	4.07	0.47	0.53	0.55	16.29	18.38	18.62
2	5	1.38	1.62	1.79	0.32	0.72	0.76	3.43	3.63	4.32	0.60	1.12	1.15	7.40	12.50	12.19
3	5	1.61	2.38	2.26	0.80	0.94	1.02	3.28	3.90	4.54	0.83	1.50	1.47	10.67	13.57	14.96
4	5	1.39	2.01	2.35	0.77	0.87	1.07	5.83	6.06	5.72	1.05	2.01	2.43	10.83	15.88	13.34
5	5	1.26	1.77	2.23	1.10	1.31	1.52	4.78	5.00	4.96	0.79	1.41	1.63	13.23	19.68	17.01
6	5	1.39	1.59	1.99	0.72	0.77	0.99	5.83	7.68	6.99	0.72	1.05	1.19	17.69	22.93	21.27
7	5	1.22	1.11	1.48	0.56	0.52	0.63	3.35	2.37	2.70	0.64	0.66	0.82	15.03	17.03	13.34
8	5	1.09	1.13	1.22	0.42	0.39	0.55	2.90	2.70	2.97	0.57	0.73	0.68	11.35	13.08	10.67
9	5	1.08	1.44	1.52	0.69	0.69	0.81	3.61	3.82	3.79	0.80	1.25	1.09	14.23	17.03	18.13
10	5	1.15	1.34	1.43	0.67	0.65	0.81	3.51	3.56	3.46	0.51	0.79	0.82	13.29	16.64	17.46
Av.		1.20	1.51	1.71	0.62	0.72	0.86	4.03	4.27	4.35	0.70	1.11	1.18	13.00	16.67	15.70
Max.		1.61	2.38	2.35	1.10	1.31	1.52	5.83	7.68	6.99	1.05	2.01	2.43	17.69	22.93	21.27
Min.		0.39	0.73	0.86	0.19	0.37	0.39	2.90	2.37	2.70	0.47	0.53	0.55	7.40	12.50	10.67
Range		1.22	1.65	1.49	0.91	0.94	1.13	2.93	5.31	4.29	0.58	1.48	1.88	10.29	10.43	10.60

[a] Replicates for each batch analyzed.

ance (ANOVA) and confirmed, when appropriate, by Fisher's LSD test.

Results and Discussion

The present study compares 3 different procedures for digesting shellfish samples before determining levels of Pb, Cd, Cu, Cr, and Zn. Levels present in shellfish samples after digestion by each procedure are shown in Table 1. Estimated detection limits for Pb, Cd, Cu, Cr, and Zn were 0.24 ppm, 0.06 ppm, 0.12 ppm, 0.12 ppm, and 0.12 ppm, respectively, using EPA protocol (13). All reported values were well within estimated detection limits except for batch 1 in which the dry ashing procedure produced values close to the detection limit for lead. Overall, values obtained for conventional wet ashing and microwave wet digestions were similar; digestion by dry ashing almost always resulted in lower values.

Statistical analysis of data (Table 2) showed significantly lower values for dry ashing as compared to conventional wet ashing and microwave wet digestions, with the exception of copper, which showed no significant differences among any of the digestion procedures. Values for copper, chromium, and zinc were comparable for both wet digestion procedures. Lead and cadmium showed significantly different results for all 3 procedures. However, lead and cadmium values for wet digestion procedures were most similar. Difficulties encountered in measuring lead and cadmium in shellfish have been documented by other researchers (14, 15) and emphasize the need for continued method development. Accuracy of the 3 digestion procedures under investigation was verified by recovery assays and analysis of lobster hepatopancreas marine certified reference material (11).

Recovery values for 10 batches of shellfish, spiked with varying amounts of Pb, Cd, Cu, Zn, and Cr were analyzed with unspiked samples (Table 3). Average recovery values for conventional wet and microwave digestions ranged from 80–92% and 54–72% for the dry ashing procedure. The low recovery values of the dry ashing procedure are in line with lower values obtained for shellfish as compared to conventional wet and microwave digestion results (Table 1), and indicate an overall loss or incomplete liberation of metal during the ashing process. Although Cu showed no significant differences among the 3 digestion procedures in this study, average results for dry ashing were lower than either of the other procedures (Table 1).

Analysis of lobster hepatopancreas marine reference material was conducted to provide further evidence of the accuracy of the methods (Table 4). Values obtained for the 5 metals under investigation were comparable for both conventional wet ashing and microwave digestions; both methods of digestion provided values very close to or within actual limits

Table 2. Statistical analysis of mean values of unspiked samples of shellfish analyzed by dry ashing, wet ashing, and microwave digestion

Metal	ANOVA result[a]	Fisher's LSD Test[b]		
		Dry ash	Wet ash	Microwave digestion
Lead	significant	A	B	C
Cadmium	significant	A	B	C
Copper	not significant	A	A	A
Chromium	significant	A	B	B
Zinc	significant	A	B	B

[a] Results based on alpha value of 0.05.

[b] Fisher's least significant difference (LSD) test for comparing mean values among dry ash, wet ash, and microwave digestion. For each metal, methods having the same letters can be considered equal at the alpha = 0.05 level of significance.

Table 3. Recovery values (%) for spiked samples of shellfish analyzed by dry ashing, wet ashing, and microwave digestion[a]

Method	Lead[b]	Cadmium[c]	Copper[d]	Chromium[e]	Zinc[f]
Dry ashing	59.1	70.9	71.7	54.5	57.9
Wet ashing	91.4	81.9	83.5	91.2	89.8
Microwave	91.5	91.2	89.0	84.8	79.9

[a] Results are averages of 10 batches of 5 replicates each, except for batch No. 1, which included 9 replicates.

[b] 2 ppm added

[c] 0.8 ppm added

[d] 10 ppm added

[e] 0.8 ppm added

[f] 40 ppm added

Table 4. Analysis of 5 heavy metals in 7 replicate samples of lobster hepatopancreas marine reference material by dry ashing (DA), wet ashing (WA), and microwave digestion (MI)

	Lead			Cadmium			Copper			Chromium			Zinc		
	DA	WA	MI	DA	WA	MI	DA	WA	MI	DA	WA	MI	DA	WA	MI
Actual value range, ppm	8.4–12.4			24.2–28.4			417–461			1.8–3.0			167–187		
Observed av. value, ppm	11.9	12.8	10.8	21.6	26.7	24.1	243.0	348.4	357.6	1.8	2.1	1.7	99.6	125.0	130.4
Std. dev.	1.4	2.7	1.4	1.1	2.1	0.7	8.1	18.3	9.2	0.3	0.5	0.3	7.3	5.0	4.2
CV	11.5	21.1	12.7	5.2	7.8	2.8	3.3	5.3	2.6	16.0	21.8	16.0	7.4	4.0	3.2
Standard error of the mean	0.5	1.0	0.5	0.4	0.8	0.3	3.1	6.9	4.5	0.1	0.2	0.1	2.8	1.9	1.6

set for Pb, Cd, and Cr. Copper and zinc values were low for all 3 procedures.

The lobster hepatopancreas marine reference material contained high levels of both copper and zinc, necessitating high dilution factors (1:250 or more)—a factor that could have magnified a low value resulting from an incomplete digestion. The marine reference material, as indicated by longer digestion times, was difficult to digest. Results from the microwave digestion were slightly higher for Cu and Zn, indicating a more complete digestion of the sample matrix and/or less loss of the analyte.

Precision for each digestion method was estimated from the average coefficient of variation (CV) calculated from replicate analyses of 10 batches of shellfish (Table 5). All CV values were below 10% except for the dry ashing value obtained for lead. Coefficients of variation were closest in value for the conventional wet and microwave digestion procedures indicating similar precision.

The present study has shown that, of the 3 digestion procedures investigated, microwave and conventional wet ashing both produced precise results. They exhibited similar recovery values for spiked samples and produced acceptable values of Pb, Cd, and Cr for the lobster hepatopancreas marine reference material.

Microwave digestion offers certain advantages over conventional wet ashing. Microwave digestion is faster and requires less of the analyst's time than does wet ashing; the microwave unit allows programming of time and power cycles so that once a method has been setup, sample digestion can be automated. From this study, we determined that a carousel of 6–12 samples could be digested within 1.25 h by the conditions described. If a second carousel is available, further time savings can be realized. On the other hand, wet ashing digestion takes about 4 h of almost constant attention. Vigilance is necessary to ensure that samples do not froth to the point of boiling over nor evaporate to dryness. Because heating units can develop hot and cool spots over time, no 2 samples ever progress at the same speed. Thus, the end point of digestion for individual samples occurs at different times making it very difficult to batch process samples.

Conventional wet ashing is dependent on the space available within a fume hood; the microwave unit, however, is equipped with a venting hose and can be set up outside the fume hood, freeing hood space for other purposes. Microwave digestion is convenient and easy to use. It is also safer than the conventional wet ash procedure because there is less chance of acid spillage or glass breakage because of high digestion temperatures in glass beakers.

Results of this study show that microwave digestion is comparable to the conventional wet ash method of digestion and that both microwave and wet are superior to dry ashing. The data indicate that microwave digestion is a viable alternative for digestion of shellfish.

Table 5. Coefficients of variation (%) for determination of metals by dry ashing (DA), wet ashing (WA), and microwave digestion (MI)[a]

Method	Lead	Cadmium	Chromium	Copper	Zinc
DA	14.0	8.2	5.6	6.7	8.0
WA	8.6	5.1	7.1	3.5	3.8
MI	8.2	6.6	5.4	4.1	6.9

[a] Each number represents av. value for 10 batches of shellfish. Each batch of shellfish in turn represents 5 replicates except, for batch No. 1, which consists of 9 replicates.

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TECHNICAL COMMUNICATIONS

Improved Selenium Recovery from Tissue with Modified Sample Decomposition

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The present paper describes a simple modification of a recently reported decomposition method for determination of selenium in biological tissue by hydride generation atomic absorption. The modified method yielded slightly higher selenium recoveries (3–4%) for selected reference tissues and fish tissue spiked with selenomethionine. Radiotracer experiments indicated that the addition of a small volume of hydrochloric acid to the wet digestate mixture reduced slight losses of selenium as the sample initially went to dryness before ashing. With the modified method, selenium spiked as selenomethionine behaved more like the selenium in reference tissues than did the inorganic spike forms when this digestion modification was used.

Recently, we described a simple method for determination of arsenic and selenium in fish with a combined wet chemical and dry ash digestion and subsequent analysis by hydride generation atomic absorption (1). The procedure yielded good recoveries for both elements but slight losses of selenium (up to 10%) resulted when selenomethionine was used for spiking. We have modified the procedure by adding a small amount of hydrochloric acid during the preliminary step as reported in a similar digestion by Hansson et al. (2). The present paper reports results from a comparison of selenium recoveries for our former and improved methods.

METHOD

Digestion and analysis by the present modified procedure (Method B in this discussion) are exactly as previously described in the earlier method (Method A) (1), except for the addition of 2 mL subboiled HCl to each sample at the initial digestion step.

Briefly, Method A is as follows. Add 0.5 g dry or 2 g wet tissue to a 100 mL beaker along with 10 mL nitric acid and 10 mL 40% (v/v) magnesium nitrate. Reflux mixture overnight, evaporate to dryness, ash at 500°C, boil with 20 mL 50% (v/v) hydrochloric acid, and dilute to 100 mL.

Results and Discussion

Results for determination of selenium in reference materials and spiked fish samples are compared in Table 1 for Method A (without HCl at the preliminary digestion step) and Method B (with HCl at the preliminary digestion step). Means for all 5 quality control types were significantly different between methods ($p \leq 0.01$, t-test for σ unknown and $\sigma_a \neq \sigma_b$). Plots of all data points indicate no general upward or downward trends with time that could invalidate the t-test.

Improvement in the recovery of selenium in the reference tissues is slight but real with hydrochloric acid in the digestion mixture. The magnitude of this improvement parallels that observed by Hansson et al. (2) for similarly digested flour samples. Recovery of selenomethionine spikes followed the same trend as reference tissues; however, recovery of selenate (Na_2SeO_4) spikes tended to be slightly lower with Method B.

A pilot study with a small number of fish tissues spiked with radiotracer indicated a similar, if not greater, improvement for Method B. We found the recovery of selenomethionine (labeled with Se^{75}) that was spiked at 3 levels onto a channel catfish homogenate to improve from 91.6 ± 3 percent (mean ± standard deviation) for Method A to 99.1 ± 0.9 for Method B (n = 3 for each). Any loss of selenium from selenomethionine occurred when digestates went to dryness during wet chemical digestion and not during the dry ashing step. This loss was only observed in the presence of tissue. The mechanism by which the hydrochloric acid reduces these losses is not clearly understood. Perhaps through hydrolysis, hydrochloric acid enhances migration of protein-bound selenium into the magnesium nitrate (or mixed nitrate/chloride) matrix where the selenium is thermally more stable. Kinetic factors are probably important during such a process and may depend strongly upon vessel type, heating times, and so on. Although Method B is reliably accurate and precise, further improvement by slightly different combinations of the reagents is possible. We suspect that samples with high fat content (fish vary from about 5 to 25% lipid) may still have somewhat variable selenium recovery, but correlations between spike recovery and lipid content with our existing data are not strong. In any case, addition of hydrochloric acid has improved overall recovery, reduced variability for selenomethionine spikes, and improved recoveries for reference tissues.

General trends for spike recoveries as compared to reference tissues for each method suggest that for spike forms, selenomethionine better reflects the behavior of selenium in tissue during this digestion, especially for potential losses. The tendency of selenium to accumulate in tissues through replacement of sulfur in amino acids is well documented (3). Because selenomethionine is readily available and stable in solution, we recommend that this form be used for spiking when selenium is determined in tissue.

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Table 1. Determination of selenium in reference tissues and spiked fish by method A and method B

Material	Concn, μg/g			Recovery, %		
	Method A[a]	Method B[a]	Reported[b]	Method A[a]	Method B[a]	Change, %[c]
NIST Tuna RM-50	3.59 ± 14 (77)	3.69 ± 0.17 (51)	3.6 ± 0.4	—	—	+2.8
NIST 1577 Liver	1.05 ± 0.03 (30)	1.08 ± 0.03 (35)	1.1 ± 0.1	—	—	+2.9
NFCRC Bass[d]	2.20 ± 0.12 (72)	2.30 ± 0.10 (44)	2.3 ± 0.2	—	—	+4.5
Fish spike (selenomethionine)	—	—	—	90.4 ± 8.4 (67)	94.4 ± 5.3 (64)	+4.4
Fish spike (Na_2SeO_4)	—	—	—	98.8 ± 6.2 (92)	95.7 ± 5.9 (44)	−3.1

[a] Mean ± std. dev. (number of determinations).
[b] Mean ± 95 percent confidence interval.
[c] Percent change of mean measured by Method B relative to Method A.
[d] National Fisheries Contaminant Research Center "in house" reference fish tissue (see Ref. 1).

Determination of Polydextrose (Polymer) and Residual Monomers in Polydextrose by Liquid Chromatography

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A simple, liquid chromatographic method has been developed for determination of the reduced-calorie food additive polydextrose—a water soluble, 1 kilocalorie/gram bulking agent used extensively for manufacture of low-calorie food products. The method allows rapid determination of polydextrose content as well as determination of the minor components: dextrose, sorbitol, and 2 isomeric anhydroglucoses in bulk samples of polydextrose. The proposed method is superior to current *Food Chemical Codex* (FCC) methodologies for determination of polydextrose (phenol sulfuric acid spectrophotometry) and for determination of residual monomers [gas chromatographic (GC) determination as their trimethylsilyl derivatives]. The proposed method is more precise, considerably faster, and is safer to execute. The method uses a resin-based cation exchange column, 0.001N sulfuric acid mobile phase, and differential refractive index detection. Analyte response curves were linear; and data displayed good precision with coefficients of variation ranging from 1.1 to 1.6%. The efficiency, specificity, precision, and ruggedness of the method make it amenable to implementation in a routine quality control operation.

Polydextrose is a water soluble, randomly bonded polymer of dextrose (1). It is categorized as a reduced-calorie food additive because it is only partially metabolized in the human body [1 kilocalorie/gram (kcal/g)] (2). Polydextrose was introduced as a reduced-calorie, bulking/texturizing agent for the manufacture of reduced-calorie foods. Using polydextrose, a variety of foods can be produced that simulate the hedonic attributes of their higher-calorie counterparts (3).

Polydextrose is commercially available in powder form or as a 70%, partially pH neutralized aqueous solution (pH of dilute solution, 5–6). The commercial product typically contains small quantities of residual citric acid, dextrose, sorbitol, and 2 isomeric anhydroglucoses that occur in an approximate 1:1 ratio.

The current *Food Chemical Codex* (FCC) determination of polydextrose polymer uses the classic, nonselective, colorimetric phenol–sulfuric acid reagent (4) to produce an in situ hydrolysis of the polymer. Reaction of the resulting dextrose allows the polymer to be determined spectrophotometrically vs a similarly treated dextrose reference standard. It is necessary to correct the spectrophotometric result for the positive bias generated by the content of monomeric dextrose and anhydroglucoses. FCC determination of residual dextrose, anhydroglucoses, and sorbitol uses silyl derivatization of these substances in a separate GC procedure. Both FCC assays are lengthy and exacting in their execution.

The present study describes a rapid, liquid chromatographic (LC) method that determines polydextrose, dextrose, sorbitol, and the anhydroglucoses simultaneously by direct injection of test solutions, prepared simply by dilution with mobile phase.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Solvent delivery pump operating at 0.6 mL/min (Model 590, Waters Associates, Inc., Milford, MA), Waters WISP, Model 710B automatic injector (20 mL injection), Aminex HPX-87H column, 30 cm × 7.6 mm id (Bio-Rad, Richmond, CA), Waters Model 410 differential refractive index detector, and Spectra Physics 4200 computing integrator (Spectra Physics Inc., San Jose, CA). For experiments using UV detection at 210 nm, a Model 783 variable wavelength detector (Applied Biosystems, Foster City, CA).

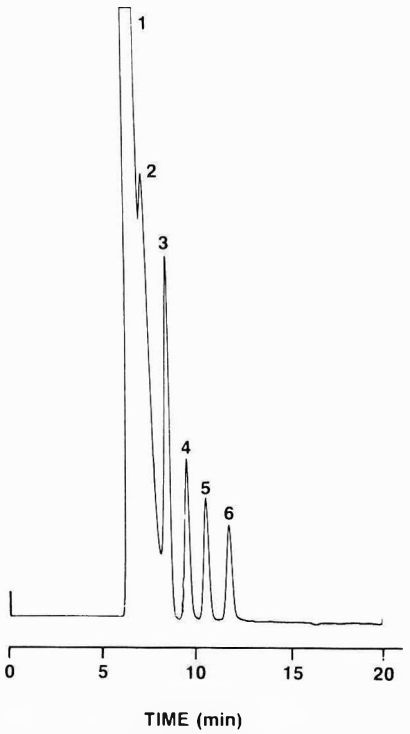

Figure 1. Typical chromatogram of polydextrose sample. Peak identities are: (1) polydextrose, (2) citric acid, (3) dextrose, (4) sorbitol, (5) anhydroglucose I, and (6) anhydroglucose II. LC conditions as described in text.

Reagents

(a) *Dextrose.*—CAS No. 50-99-7, NIST reference standard 41c.

(b) *1,6-Anhydro-β-D-glucose.*—CAS No. 498-07-7 (levoglucosan), 99% (Aldrich Chem. Co.).

(c) *Sorbitol.*—CAS No. 50-70-4, 99.4% (Pfizer quality control reference standard).

(d) *Polydextrose.*—CAS No. 68424-04-4 (Pfizer quality control reference standard, 91.5%).

(e) *Polydextrose.*—CAS No. 68424-04-4. Samples from routine production (Pfizer Inc).

(f) *Solvents.*—Sulfuric acid was analytical reagent grade; deionized water (Milli-Q, Millipore Co., Bedford, MA).

(g) *Mobile phase/sample diluent.*—0.001N sulfuric acid, prepare with deionized water.

Sample/Standard Preparation

(a) *Polydextrose sample preparation.*—Prepare sample solutions to contain ca 4 mg polydextrose/mL by dilution with mobile phase.

(b) *Polydextrose standard preparation.*—Prepare solutions of the polydextrose standard to contain ca 4 mg standard/mL by dilution with mobile phase.

(c) *Combined monomer standard.*—Prepare a standard solution containing dextrose (0.16 mg/mL), sorbitol (0.08 mg/mL), and 1,6-anhydro-β-D-glucose (0.08 mg/mL) by dilution with mobile phase.

Method Development

The Aminex series of resins has found application in a variety of carbohydrate analyses (5–7). The Aminex HPX-87H column contains an 8% cross-linked, sulfonic acid cation exchange resin that provides chromatographic resolution of polydextrose, citric acid, dextrose, sorbitol, and 2 isomeric anhydroglucoses (see Figure 1). Because of molecular size, polydextrose is essentially unretained. Retention times for the minor components were identified with authentic standards, where applicable. Thermospray liquid chromatography triple quadrupole mass spectrometry was used to assess the peak homogeneity and to confirm the identity of the chromatographic peaks. In particular, the anhydroglucose I peak, for which a reference material is not commercially available, was identified as an anhydroglucose based on a molecular weight of 162 daltons.

To analyze polydextrose samples by LC, appropriate calibration protocols for the polymer and the residual monomers are required. For residual monomers, commercially available standards of dextrose, sorbitol, and 1,6-anhydro-β-D-glucose (to quantitate both isomers of anhydroglucose) could be used, as is currently described in the FCC GC procedure (1). However, because a reference standard for polydextrose (polymer) is mandatory, and because the most reasonable approach to fulfill this need is to thoroughly characterize the content of minor components in a polydextrose reference standard, we recommend that a polydextrose reference standard be used as the calibrating standard for quantification of both polydextrose and residual monomers.

In the present study, we established a polydextrose/monomers reference standard via the following protocol. Levels of dextrose, sorbitol, and anhydroglucose of a typical polydextrose bulk were determined by the method described in the present paper, using an external standard prepared from 8 dextrose, sorbitol, and 1,6-anhydro-β-D-glucose standards (the combined monomer standard). The citric acid content was determined to be approximately 0.4% with the chromatographic method, using UV detection at 210 nm. The monomer and citric acid data, along with moisture and ash data (FCC methods), allowed the polymer content to be calculated using the following equation:

$$\text{polydextrose, \%} = 100 - [g + s + an_I + an_{II} + c + m + as]$$

where g = % glucose, s = % sorbitol, an_I = % anhydroglucose I, an_{II} = % anhydroglucose II, c = citric acid, m = % moisture (FCC), and as = % ash (FCC).

Results and Discussion

Selectivity, reproducibility, and accuracy of the assay were studied. Although peak height measurement is applicable, use of area measurement is recommended. In normal execution of the method, citric acid and dextrose appeared as shoulders, whose peak areas were tangent skimmed off the tail of the polydextrose peak (Figure 1).

Well-behaved response curves were obtained for the species studied. Polydextrose response, from 6 concentrations spanning the range from 1 mg/mL to 10 mg/mL, was linear with an observed correlation coefficient of 0.9999. Linearity of the minor component responses was studied over concentration ranges covering approximately 20–200% of the concentration levels typically expected in polydextrose. Linearity of dextrose response was demonstrated over the range corresponding to 0.8% and 8% by weight; a correlation coefficient of 0.9999 was obtained. Linearity for sorbitol response was tested from 0.4% to 4% by weight of the polydextrose sample; a correlation coefficient of 0.9997 was obtained. To obtain the response curve for anhydroglucoses, 1,6-anhydro-β-D-glucose was employed over a concentration range corresponding to 0.33% to 3.3% by weight of polydextrose sample. A linear response curve with a correlation coefficient of 0.9992 was obtained.

Table 1. System and assay precision data

Analyte	CV, %	
	Assay precision[a]	System precision[b]
Polydextrose	1.2	0.2
Glucose	1.2	0.1
Sorbitol	1.2	0.5
Anhydroglucose I	1.6	1.4
Anhydroglucose II	1.1	0.9

[a] Assay precision data (weight-to-weight precision) are derived from 5 parallel analyses of a typical polydextrose sample.

[b] System precision data (injection-to-injection precision) are derived from 5 injections of a single polydextrose solution. Anhydroglucose peaks showed greater imprecision because they were smaller and broader peaks that were more difficult to integrate. The low relative standard deviations (system precision) for the polydextrose and glucose peaks should be considered fortuitous.

Because citric acid is present in low concentrations and is not directly determined in the FCC monograph, no attempt was made to determine citric acid in routine production samples.

The data summarized in Table 1 demonstrate that the determination of polydextrose had a coefficient of variation (CV) of 1.2% for these samples. While the same precision applied to determination of glucose and sorbitol present in a sample of polydextrose, anhydroglucose I was determined with a CV corresponding to 1.6% and anhydroglucose II with a CV of 1.1%.

Table 2. Residual monomer determinations by LC and GC

	Polydextrose powder					
	Glucose		Sorbitol		Total anhydro-glucose	
Lot No.	GC	LC	GC	LC	GC	LC
1	3.6	3.2	1.7	1.7	2.9	3.0
2	3.8	3.9	1.9	1.8	3.1	3.0
3	2.9	3.1	1.7	1.7	2.4	2.5
4	3.3	3.6	1.7	1.7	2.8	3.0
5	3.7	4.0	1.7	1.8	2.6	2.8
Av.	3.5	3.6	1.7	1.7	2.8	2.9
Std dev.	0.4	0.4	0.09	0.05	0.3	0.2
	Polydextrose solution[a]					
6	3.1	3.0	1.9	1.8	2.3	2.6
7	3.0	3.2	1.9	1.8	2.5	2.6
8	3.0	3.4	1.8	1.9	2.5	2.8
9	3.3	2.9	1.9	1.8	2.6	2.6
10	3.2	3.1	1.9	1.9	2.4	2.6
Av.	3.1	3.1	1.9	1.8	2.5	2.6
Std dev.	0.1	0.2	0.04	0.05	0.1	0.09

[a] 70% solutions

Table 3. Polydextrose bulk purity determinations by phenol/sulfuric spectrophotometric and LC methodologies

Lot[a]	Polydextrose, %		
	Phenol/sulfonic[b]	LC	Difference
1	93.8	92.0	−1.8
2	92.6	93.4	+0.8
3	92.8	94.0	+1.2
4	92.5	92.7	+0.2
5	91.7	93.0	+1.3
6	95.6	92.3	−3.3
7	93.1	91.5	−1.6
8	93.3	93.2	−0.1
9	94.1	92.9	−1.2
Av.	93.3	92.8	−0.5
Std dev.	1.1	0.8	1.6

[a] Sample set = 4 powder lots and 5 solution lots.

[b] Polydextrose values are reported on an anhydrous, ash-free basis.

Comparative residual monomer data were obtained by analysis of polydextrose samples using the FCC GC method (see Table 2). These data show close agreement between the 2 procedures and confirm that the LC data are acceptable. Results of a 9-lot comparison between polydextrose content determined by phenol-sulfuric acid and LC methodologies are reported in Table 3. The data indicate that LC determination of polydextrose may tend to produce slightly lower results (ca 0.5%); however, based on a statistical paired *t* comparison (*t* observed 0.955), this cannot be stated with certainty.

The LC method described here is capable of rapidly and precisely quantifying residual minor components as well as polydextrose in commercially available polydextrose. This approach provides an efficient alternative to the 2 FCC procedures, a silylation/GC determination of residual monomers and a nonselective colorimetric assay in sulfuric acid medium for quantification of polydextrose.

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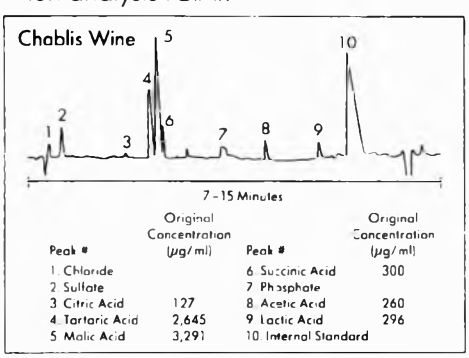
If our approach to organic acid analysis was any easier, you could call it child's play.

Introducing Waters Capillary Ion Analysis.

Measuring the organic acids in samples like wine or juice can be a complicated matter. So why bother with conventional methods when you can get good, quantitative results easily and quickly with capillary ion analysis (CIA).



Peak #	Original Concentration (µg/ml)	Peak #	Original Concentration (µg/ml)
1. Chloride		6. Succinic Acid	300
2. Sulfate		7. Phosphate	
3. Citric Acid	127	8. Acetic Acid	260
4. Tartaric Acid	2,645	9. Lactic Acid	296
5. Malic Acid	3,291	10. Internal Standard	

The Unique Selectivity of CIA.
CIA separates analytes according to molecular charge and size. In an organic acid CIA separation, the acids move at different speeds than the more neutral carbohydrates and phenolics. These interferences are left behind in the capillary and are purged automatically before the next injection. So in most cases, all you have to do to prepare a sample is dilute it in water and the unique separating power of CIA takes care of the rest.

Waters™ Quanta 4000 Capillary Electrophoresis System and NICE-Pak™ Chemistries for CIA.
Waters Quanta 4000 CE System and proprietary NICE-Pak Chemistries are unbeatable at keeping organic acid analysis simple. And, on a cost per test basis, the CIA approach is as much as 6X cheaper than HPLC and 25X cheaper than enzyme analysis.

We'd like to tell you more.
Write, fax, or call us for more information and while you're at it, send for a copy of our free videotape by clipping and returning the attached coupon.

Waters Chromatography Division,
Millipore Corporation, 34 Maple Street, Dept.-MC, Milford, MA 01757
Phone: (508) 478-2000 Ext. 2614
Facsimile: (508) 478-5839

Send me Waters videotape *The Science of Capillary Electrophoresis.*

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