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NOVEMBER/DECEMBER 1991
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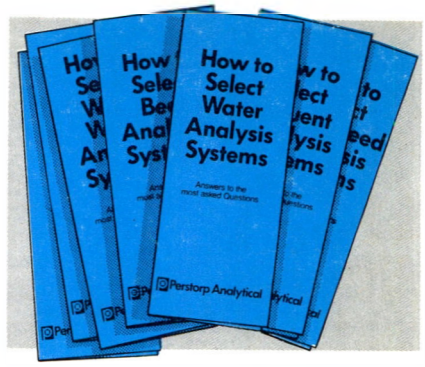

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Books in Brief

Chromatography Today. By C.F. Poole and S.K. Poole. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1991. 1026 pp. Price: U.S. $147.50/Dfl. 295.00. ISBN 0-444-89161-7.

Chromatography Today provides a comprehensive coverage of various separation methods: gas, liquid, thin-layer, and supercritical fluid-chromatography, and capillary electrophoresis. Particular attention is paid to the optimization of these techniques in terms of kinetic parameters and retention mechanisms. When these facts are understood, method selection and optimization becomes a more logical process. Sample preparation methods are treated fully as they frequently represent an integral part of the total analytical method. Also described are preparative-scale separations used for isolating significant amounts of product which are generally achieved under conditions that are not identical to those used for analytical separations. The most common hyphenated methods used for sample identification are discussed from the perspective of the information they yield and the requirements of common interfaces.

Improving Safety in the Chemical Laboratory: A Practical Guide, 2nd edition. Edited by Jay A. Young. Published by John Wiley & Sons, Inc., 1 Wiley Dr., Somerset, NJ 08875-1272, 1991. 406 pp. Price: $75.00. ISBN 0471-53036-0.

The work of accident prevention in the laboratory begins with foresight. Discerning "close calls"—near accidents—early enough prevents them from turning into full-fledged mishaps, mishaps that cost time and money, and which could result in injury. *Improving Safety in the Chemical Laboratory* is an accident prevention handbook for the professional in the laboratory that shows how to detect and eliminate the causes of dangerous mishaps—and virtually "hazard proof" any laboratory environment. *Improving Safety in the Chemical Laboratories*, 2nd edition, offers detailed procedures—from precautionary labeling to simulated drills, safety inspections, and the preparation of a chemical hygiene plan—for the development of a safety-enhanced workplace. Reflecting, in part, the upgraded procedures now mandated by the OSHA Laboratory Standard in the United States, as well as the WHMIS regulations in Canada and the COSHH regulations in the United Kingdom, this newest edition offers unparalleled and up-to-date guidance on the fine points of hazard control, with new added material on managing and handling especially hazardous substances and personal protective equipment.

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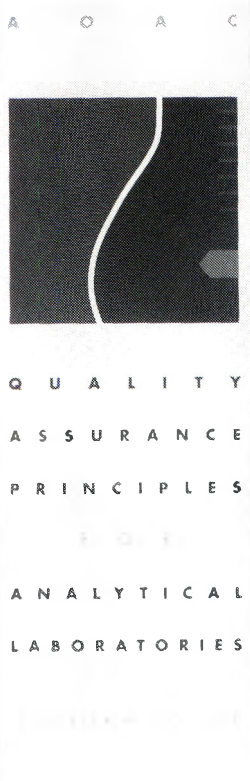

*E*ssential for any lab wanting to improve or initiate a quality assurance (QA) program. Material was extensively revised for this new edition which features new information and concepts developed since the handbook was last published. An important addition is a chapter on utilizing statistical applications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

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

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

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

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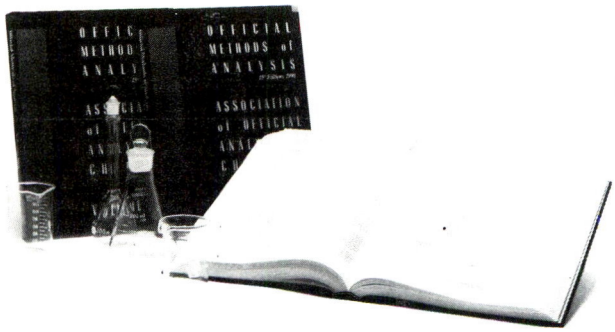

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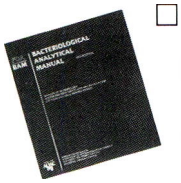

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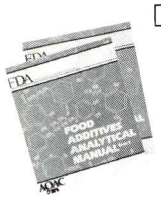

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For Your Information

Meetings

January 16–17, 1992: Pacific Southwest Regional Section Meeting, Irvine, CA. Contact: Richard Jacobs, USFDA, 50 United Nations Plaza, San Francisco, CA 94102, telephone 415/556-1463.

February 2–4, 1992: Southwest Regional Section Meeting, Dallas, TX. Contact: Donna Montague, 211 S. Johnson St, Little Rock, AR 72205, telephone 501/686-5140.

February 4–6, 1992: Southeast Regional Section Meeting, Atlanta, GA. Contact: James Hess, USDA AMS CSSD, Eastern Laboratory, 645 Cox Rd, Gastonia, NC 28054, telephone 704/867-3873.

May 1992: Northeast Regional Section Meeting, Nova Scotia, Canada. Contact: P. Christopher Ellis, Rhode Island Department of Health, 50 Orms St, Providence, RI 02904, telephone 401/274-1011.

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

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

Elkins Elected 1991–1992 AOAC President

Edgar R. Elkins of the National Food Processors Association, Washington, DC, became president of AOAC for 1991–1992 following the business meeting at the AOAC annual meeting in Phoenix, AZ, on August 15, 1991.

Elkins has been an active member of AOAC, serving as a member of Committee E (Residues) between 1986 and 1988 and as an Associate Referee for Apple Juice Adulteration, Sodium, and Tin (Metals). He was a leading participant in the Sulfite Task Force meeting at AOAC annual meetings and also served on the International Coordination Committee (1977–1986), the Steering Committee for Laboratory Accreditation (1982–1983), the Ad Hoc Committee on Laboratory Accreditation (1982–1984), the Centennial Committee (1983–1985), and the Governance Council (1988–1989). A member of the Board of Directors since September 1988, Elkins served as the Secretary/Treasurer and as Chairman of the Finance Committee for the past 2 years. In recognition of at least 10 years of meritorious service, Elkins was named Fellow of the AOAC in 1988 at the 102nd AOAC annual meeting.

Elkins has been with the National Food Processors Association (NFPA), the principal scientific and technical trade association for the food processing industry, since 1961, when he was initially involved with the determination of pesticide residues in food products. Since that time, he has been involved in all phases of analytical food chemistry, including environmental contaminants, heavy metals, nutrient composition, product/container interactions, and problems in food quality. He currently is heavily involved with pesticides and the risk assessment process. He also heads an industry project on the characterization of authentic commercially produced apple juice concentrate.

Elkins was named assistant head of the division of chemistry at the NFPA Washington Laboratory in 1967, becoming head in 1972 and director in January 1976. He continued in that position until 1990 when he was made Principal Research Scientist.

When asked about his plans for the Association, Elkins responded: "Goals for the coming year, and several years to come, have already been delineated. Focus group sessions have identified some important issues that need to be diligently addressed. The most important one to me is 'making the methods validation process more responsive to user needs.'

"I do not believe that this issue can be solved to everyones' satisfaction by the end of my term as president, but it will be my top priority," he emphasized.

"Increasing membership and member services is certainly a priority, and now that we are AOAC International, we need to improve our relations internationally. In this respect, I plan to visit The Netherlands in November and attend the second International Symposium, 'Protection of Public Health: A Challenge for Food and Environmental Analysts,' organized by the AOAC Europe Regional Section.

"AOAC continues to grow and evolve as the main source of collaborative studied analytical methods, truly it should be the association of analytical excellence internationally."

Elkins holds a B.S. degree from East Tennessee State University, and after graduation, spent the first 18 months of his career with the Kroger Food Foundation in Cincinnati, OH, before going to NFPA. He is a member of the American Chemical Society, and author and co-author of numerous publications in the field of analytical food chemistry.

AOAC Board Slate Elected, Propositions Approved

A new name, new mission statement, and a slightly bigger Board with staggered terms for its members are among the items approved by a recent vote of AOAC membership.

The new name of the Association is "AOAC International"—the scientific association dedicated to analytical excellence. The change recognizes the Association's already expanded scien-

tific base and confirms the geographic area the Association operates in.

This refinement of the name of the Association was approved, together with three other propositions, by mail ballots sent to all AOAC members this past June. From the 865 valid ballots received, the other propositions amended the AOAC Bylaws and Articles of Incorporation as follows:

- The Association's purpose statements (in the Articles of Incorporation and the Bylaws) were revised by simplifying, updating, and making them identical in each AOAC document. The new mission statement reads: "The primary purpose of the Association is to promote methods validation and quality measurements in the analytical sciences." Also, a more comprehensive IRS statement concerning exempt status compliance was incorporated into the Articles of Incorporation.
- The number of Directors was increased from five to six, and staggered 3-year terms for the Directors were adopted. This will increase the size of the Board from nine to ten members.
- The term of service for the Wiley Award Committee members was amended from 2-year to 3-year terms to bring it into conformance with the terms of other Association committees.

The following slate of officers, which the Nominating Committee proposed, was elected for the 1991–1992 year:

President-Elect: Henry B.S. Conacher, Health Protection Branch, Ottawa, ON, Canada

Secretary-Treasurer: Arvid Munson, Phoenix Regulatory Associates, Ltd., Sterling, VA

Directors: Alan R. Hanks, Office of the Indiana State Chemist, West Lafayette, IN; Nicole Hardin, Food and Drug Administration, New Orleans, LA; Albert E. Pohland, Food and Drug Administration, Washington, DC; P. Frank Ross, U.S. Department of Agriculture, Ames, IA; and Alex Williams, Laboratory of the Government Chemist, retired, U.K.

Albert E. Pohland, Associate Director for Research, Division of Contaminants Chemistry, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC, is the new Board member. Active in AOAC since 1969, when he was appointed Associate Referee on Confirmative Methods, Pohland is presently the section editor of the *Journal of the AOAC* for the Food Contaminants and Biological Methods area. In addition, Pohland has served on the Committee on Meetings, Symposia, and Educational Programs, the Constitution (now Bylaws) Committee, and has been a member of the Joint Mycotoxin Committee.

Edgar R. Elkins, having been previously elected, will serve on the 1991–1992 Board as President, as will H. Michael Wehr who will serve as Immediate Past President.

1991 Fellows of the AOAC

In recognition of at least 10 years of meritorious service to the Association, the following individuals received honors as 1991 Fellows of the AOAC at the 105th AOAC Annual International Meeting and Exposition in Phoenix, AZ:

Ruth Bandler, FDA. Associate Referee (1 method adopted): 1979–1987, baseline mold counts by blending; 1981–present, chemical method for detecting mold; 1981–1985, becol sterols; 1990–present, mold by chemical detection (with George Ziobro). General Referee (5 methods approved): 1988–present, analytical mycology and microscopy of foods. Member, 1988–present, Methods Committee F.

Reginald W. Bennett, FDA. Associate Referee (2 methods adopted): 1971–1987, *Staphylococcal* enterotoxin; 1977–present, *Bacillus cereus* toxin. 1991 Wiley Award.

Stephen G. Capar, FDA. Associate Referee: 1976–present, hydride generating techniques. General Referee (5 methods approved): 1986–present, metals and other elements. Liaison Officer between AOAC and International Dairy Federation.

Lester Hankin, Connecticut Agricultural Experiment Station. General Referee (1 method approved): 1969–1982, enzymes. Member, 1974–1980, Committee on State Participation.

Mary Lee Hasselberger, Nebraska Department of Agriculture. Associate Referee: 1979–1983, zinc in fertilizers; 1980–present, screening procedures for antibiotics in feeds; 1988–present, oxytetracycline in feeds by chemical methods; 1988–present, oxytetracycline in feeds by microbiological methods. General Referee: 1983–1986, drugs in feeds. Organized 1989 and 1991 Workshops on Antibiotics and Drugs in Feeds.

Dean Kassera, McLaughlin Gormley King Co. Associate Referee (2 methods adopted): 1964–present, allethrin; 1979–present, piperonyl butoxide and pyrethrins. Member, 1985–present, Committee on Membership. Member, 1991–present, Bylaws Committee. Service in Copper in Fertilizers.

James F. Lawrence, Health and Welfare Canada. Associate Referee: 1981–present, brominated oils; 1977–1981, S-triazine; 1978–1981, anilazine. Member, 1988–present, Governance Council. Member, 1987, Chairman, 1988–present, Committee on Membership. Member, 1988–present, Methods Committee C. Journal Section Editor. Organized 1987 Spring Training Workshop.

John O'Rangers, FDA. General Referee: 1978–present, biochemical methods. Member, 1986–present, Methods Committee B. Member, 1982–present, Interlaboratory Studies Committee.

Nominations for these awards were made by AOAC members, were reviewed and recommended by the Committee on Fellows, and finally were approved by the Board of Directors.

General Referee Award to Andrews

The 1991 winner of the General Referee Award, granted by the Official Methods Board in recognition of outstanding leadership and substantial contribution to method development, is Wallace H. Andrews, General Referee for Food Microbiology—Nondairy. In that capacity, Wallace oversees method development in some 24 topic areas.

Wallace is employed by the U.S. Food and Drug Administration, Washington, DC. He has been actively involved with AOAC since 1983, serving "a pivotal role in the emerging area of AOAC adoption of test kit-based methods," according to Donald A. Mastrorocco, chairman of the Methods Committee on Microbiology and Extraneous Materials.

Methods Committee Associate Referee Awards for 1991

Created in 1986, the Methods Committee Associate Referee Awards recognize the best Associate Referee in a committee for a given year. Those named for 1991 are: Eileen Bargo, Committee on Drugs and Related Topics; Sungsoo Lee, Committee on Foods II; Phyllis Entis, Committee on Microbiology and Extraneous Materials; Michael Ogden, Committee on Feeds, Fertilizers, and Related Materials; and Kenneth Edgell, Committee on Environmental Quality.

Collaborative Study of the Year Award–1991

The Official Methods Board named Jeanne Joseph the winner of the Collaborative Study of the Year Award for 1991 for "Fatty Acids in Encapsulated Fish Oils and Fish Oil Methyl and Ethyl Esters, Gas Chromatographic Method." Joseph works for the National Marine Fisheries Service in Charleston, SC.

The award recognizes the collaborative study judged to be best for the year as determined by scientific innovation and soundness of design, implementation, and reporting.

Wiley Scholarship Award to Student from Ball State University

The Scholarship Committee named Pamela Lynn Cooper of Ball State University, Muncie, IN, as the recipient of the 1991 AOAC Harvey W. Wiley Scholarship Award. Cooper is a chemistry major and was recommended by John H. Meiser, professor of physical chemistry at Ball State University. Professor Mesier indicated, "From her transcript, it is very clear that Pamela is a superior student. What should be emphasized is that she is also a superior person." Cooper is active in a number of campus organizations and has been on the Deans Honor List every semester.

Highlights of the AOAC Annual International Meeting

The 1991 AOAC Annual International Meeting, August 12–15, successfully combined the old and the new, reflecting both AOAC tradition and future directions that have ensured the success of the Association for more than 100 years. "This is one of the best organized meetings I have ever attended," remarked Jerry Burke of FDA's Office of Physical Sciences.

New directions for AOAC were evident in the session on the AOAC Test Kit Reviewed and Recognized Program, the name change to AOAC International, which became effective at the end of the Business Meeting, and the streamlined AOAC mission statement. The meeting also featured a new look from beginning to end by presenting all awards during the Opening Session and completely revising the format of the Business Meeting to include a luncheon and discussion session with the Board of Directors and the membership.

Social events provided the traditional opportunities for fun and relaxation and a chance to renew old acquaintances. But the new look was evident from the "Margaritas and Mariachi" theme of the President's Reception on Sunday evening to the featured event on Tuesday evening when country-western performer Shelly West entertained attendees after the Cowboy Cookout and dancers ran the gamut from toddlers to Board members.

Gene Klesta of Chemical Waste Management enthused, "The facilities were excellent from the standpoint of space. The poster sessions had plenty of room, and it was easy to walk around and talk to colleagues and presenters. This meeting allowed for a great deal of interaction, not only in the poster sessions but at committee meetings and other functions, as well." More than 1,000 attendees and guests were present.

By "Executive Order" from outgoing AOAC President H. Michael Wehr, everyone from attendees to staff dressed for comfort. Bermuda shorts and golf shirts were seen everywhere, and after the Opening Session on Monday, there were no ties in evidence.

The tradition of providing quality scientific programs was reflected in six symposia, ten poster sessions, two workshops, and a roundtable with topics that ranged from biotechnology and microbiology to natural toxicants and supercritical fluid extraction. Poster sessions and the Workshop on Antibiotics in Drugs and Feeds emphasized AOAC methods development and approval. The more than 300 presentations by scientists from all over the world offered information on a wide range of scientific challenges. Bruce Ames, Director of the National Institute of Environmental Health Sciences Center at the University of California, Berkeley, launched the meeting with his keynote address, "Understanding the Causes of Cancer and Aging," at the Opening Session.

"We tried to combine good science with good fun and casual comfort," stated Marge Ridgell, AOAC Director of Administration and Meetings. "It appears we succeeded!"

Ecology and Management of Food-Industry Pests

AOAC International announces the publication of a new book, *Ecology and Management of Food-Industry Pests*, J. Richard Gorham, Editor. This 595-page, fully illustrated hardcover book is an essential reference manual for food industry professionals responsible for food sanitation, pest control, and quality assurance, working for food processors, retailers, wholesalers, storage facilities, importers and exporters, restaurants and other food services, food banks, educational institutions with departments of food science, agriculture, and entomology, and regulatory agencies.

Prepared under the direction of the U.S. Food and Drug Administration, it brings together the knowledge of more than 50 scientists who provide the most current data on techniques of controlling pest populations attacking our food supply as it moves from the farmer to the consumer. Topics include ecology, prevention, survey and control, health considerations, legal and regulatory aspects, and general management principles such as education strategies for food-pest managers, to name a few.

Since food is a value-added product following harvest, the food industry places its greatest efforts—as reflected in the contents of this book—on pest management during storage, processing, and distribution. The authors seek to provide the reader with sufficient in-

formation to make responsible and informed decisions regarding the management of pest populations and to transform knowledge into action. They emphasize that only through diversity in pest control approaches will a quality food supply become a reality.

Edouard Saouma, Director General, FAO, Rome, comments in the Foreword to the book, "We live in a ...world in which a large proportion of agricultural production is lost between the farmer and the consumer, while millions of people suffer from hunger and malnutrition, and...simply starve to death.... Given these circumstances, the need for expertise in the ecology and management of stored-food pests and microorganisms becomes obvious.... This manual, *Ecology and Management of Food-Industry Pests*...is designed to advance our knowledge in this vital field.... I heartily recommend it to your attention."

This new hardbound 595-page text can be ordered from AOAC International by sending the following: purchaser's name and address, a check (U.S. funds on U.S. banks only) or MasterCard, VISA, or Diners credit card information (name of card, card number, and expiration date) and card holder's signature to AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201. Price is $121.00 in the U.S., $130.00 outside the U.S. for AOAC International members, $135.00 in the U.S., $145.00 outside the U.S. for non-members. Credit card orders may be placed by mail, telephone 703/522-3032, or fax 703/522-5468.

Methods Adopted First Action

As directed by the Board of Directors, the Official Methods Board is responsible for consideration of methods for first action approval. The following methods were adopted first action at the Official Methods Board meeting August 10 and 15, 1991, in Phoenix, AZ, and became official at that time. These methods will be published in the third supplement (1992) to the 15th edition (1990) of *Official Methods of Analysis*.

■ *Pesticide Formulations and Disinfectants*: Testing Disinfectants against *Salmonella choleraesuis, Staphylococcus aureus,* and *Pseudomonas aeruginosa*, Hard Surface Carrier Test Method.

■ *Foods I:* Total Aflatoxin Levels in Peanut Butter, Enzyme-Linked Immunosorbent Assay Method (Biokits).

Ochratoxin A in Corn, Barley, and Kidney, Rapid Solvent-Efficient Liquid Chromatographic Method.

■ *Foods II:* Insoluble Dietary Fiber in Food and Food Products, Enzymatic-Gravimetric Method (Phosphate Buffer).

Total, Insoluble, and Soluble Dietary Fiber in Foods, Enzymatic-Gravimetric Method (MES-TRIS Buffer).

Glycerol in Wine and Grape Juice, Liquid Chromatographic Method.

■ *Microbiology and Extraneous Materials*: *Salmonella* in Foods, Colorimetric DNA Hybridization Method—Modification of Method **990.13**.

■ *Feeds, Fertilizers, and Related Materials*: Nicotine in Environmental Tobacco Smoke, Gas Chromatographic Method—Modification of Method **990.01**.

COMING IN THE NEXT ISSUE

DAIRY PRODUCTS

■ Quantitative Analysis of Agricultural and Food Products: A New Instrumental and Computerized Approach—B.S. Lanher

DRUGS IN FEEDS

■ CI–906 and CI–925 Cyclization in Rodent Chow Using Liquid Chromatography for Detection and Assay—C.H. Spurlock

MICROBIOLOGICAL METHODS

■ Evaluation of Methods for Detection of *Listeria monocytogenes* in Foods: NMKL Collaborative Study—A. Westöö and M. Peterz

and

TRANSACTIONS OF THE 105TH AOAC ANNUAL INTERNATIONAL MEETING AND EXPOSITION

KEYNOTE ADDRESS

■ Pollution, Pesticides, and Cancer—B.N. Ames

WILEY AWARD ADDRESS

■ Biomolecular Temperament of *Staphylococcal* Enterotoxin in Thermally Processed Foods—R.W. Bennett

PRESIDENT'S ADDRESS

■ Risking for Success—H.M. Wehr

The JOURNAL OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

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The *Journal of the AOAC* is the official bimonthly journal of the Association of Official Analytical Chemists (AOAC). Published for analytical scientists who develop and use analytical methodology, it is used by its subscribers in their day-to-day work in the fields of food composition and contamination, feeds, pharmaceuticals, cosmetics, agricultural and household chemicals, water analysis, and environmental control. Users include analytical chemists, biologists, microbiologists, biochemists, toxicologists, spectroscopists, statisticians, forensic and other scientists in laboratory, administrative, and top management positions in industry, government, and universities in the United States and more than 90 other countries.

The *Journal of the AOAC* is a principal forum for exchange of information among methods researchers in the regulatory agencies and regulated industries.

The *Journal of the AOAC* has an average paid circulation of about 4,000 subscribers and a referenced readership of more than 25,000 purchasers and specifiers of laboratory equipment and supplies, testing apparatus, instrumentation, and related products and services. Subscribers order the *Journal* because it is professionally necessary to do so.

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DISCUSSIONS IN ANALYTICAL CHEMISTRY

Thoughts on the Use of Immunoassay Techniques for Pesticide Residue Analysis

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Nearly a decade ago, enzyme-linked immunosorbent assay (ELISA) was developed for determining trace pesticides. The method seemed to be fast, simple, highly sensitive, and selective. Students of analytical chemistry and life science were fascinated with its potential as an analytical tool. ELISA seemed to be a panacea for residue chemists.

However, developing ELISA for pesticide residue analysis had several difficulties, such as making the enzyme system selectively recognize a small organic molecule the size of 300 daltons rather than 100 000 daltons, and producing uniform and stable antibody. A decade of diligent work by many scientists has resulted in producing several commercial ELISA kits for pesticide residue.

ELISA is fast: One can perform concurrent analyses using 96-well plates. It is simple to do for certain matrixes using one of the commercial test kits. It is highly sensitive; in some cases, it can easily detect levels less than parts per billion. But it is not the panacea we once thought.

ELISA works well for simple matrixes, such as drinking or ground water. For a difficult matrix such as fresh produce or tissue, the sample must be vigorously cleaned up before the assay can be performed. Without any sample cleanup, results can be highly misleading. A good example of misrepresentation using ELISA occurred in a recent incident with table grapes. A misinformed individual used commercial ELISA test kits designed for ground water analysis to analyze table grapes without any sample cleanup or appropriate control samples in front of an unsuspecting television audience. Needless to say, he claimed that the grapes were full of pesticide residue, which others using more conventional analytical techniques determined was not correct.

ELISA is selective but certainly not as definitive as analytical chemists require. ELISA often shows cross reactivities to structurally related compounds. For example, ELISA for atrazine herbicide will exhibit reactivities to simazine and the other triazine herbicides. Unless one has prior knowledge of the chemicals in a given sample, the chemist must further characterize the sample by other methods.

ELISA is sensitive, yet prone to give false positives. Any false positive analysis is extremely difficult to contradict because ELISA can detect levels that other techniques can not easily verify. The assay, especially as a commercial kit, is best designed for a compound-matrix pair analysis. ELISA can not detect and identify multiple pesticides in a sample. For example, if one needs to determine atrazine and captan, 2 different ELISA kits must be used.

ELISA, however, is a valid analytical technique for pesticide residues. Panaceas exist only in one's dreams! At present, ELISA is very well suited for some selected analytical applications. ELISA can be transported to the field more easily than analyses. It is also helpful in regulatory work as a screening technique followed by conventional analysis of a small number of samples that show ELISA positives.

However, more development in several areas is needed to make the immunoassay more applicable to pesticide residue analysis. More ELISA systems should be developed for pesticides that present conventional methods can not determine well. There is no further need for demonstrating the abilities of ELISA with easy-to-analyze pesticides. Affinity solid-phase extraction columns could be developed for better sample cleanup using antibodies already developed for commercial ELISA kits. Extremely simple sample preparation could then be coupled with established conventional analytical methods.

Most of all, we all need to be more familiar not only with ELISA, but also with other immunological and bioanalytical techniques and concepts. The future indeed belongs to biotechnology and related sciences.

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CHEMICAL CONTAMINANTS MONITORING

Limited Survey of Residual Tetracyclines in Tissues Collected from Diseased Animals in Aichi Prefecture, Japan

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Tissues were collected to survey the actual conditions of tetracycline antibiotics (TCs) residues in slaughtered animals that did not pass inspection at slaughterhouses in Aichi Prefecture, Japan, because of the presence of disease symptoms. Tissues were analyzed by liquid chromatography. Among 271 samples, 49 (18.1%) were positive for oxytetracycline (OTC), 5 (1.8%) for chlortetracycline (CTC), and 5 (1.8%) for doxycycline (DC), respectively. One sample (cattle kidney) was positive for both OTC and DC. However, tetracycline was not detected in any samples. Percentage frequencies of TCs residues were 29.1% (37/127) and 15.2% (22/144) for cattle and hogs, respectively. Kidney samples showed higher incidence of TCs residues and 1.5–7 times higher residual concentrations than liver and miscellaneous samples.

Tetracycline antibiotics (TCs), which represent oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC), are commonly used all over the world as veterinary medicines and feed additives (1, 2). In Japan, over 60% of all antibiotics used for animals are TCs (3), and residual TCs have been found in organs and muscles of slaughtered animals (4, 5). Therefore, the survey of such residues in slaughtered animal tissues is one of the most important duties for a public health agency.

Microbiological assay is used mainly for analysis of residual TCs in slaughtered animal tissues because of the method's good sensitivity. However, because it can not identify certain TCs and can not determine TCs precisely, an adequate survey of residual TCs in slaughtered animal tissues has not been reported so far. In a previous report (6), we described a practical and simple analytical method for residual TCs in foods, including slaughtered animal tissues, using liquid chromatography (LC). In this paper, we apply this method for survey of residual TCs in slaughtered animal tissues and describe the actual conditions of residual TCs in these tissues.

Experimental

Reagents

(a) *Solvents.*—Methanol and acetonitrile (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

(b) *Chemicals.*—Disodium ethylenediaminetetraacetate (Na_2EDTA), oxalic acid, citric acid, and disodium hydrogen phosphate (Wako).

(c) *Water.*—Distilled and deionized.

(d) *C18 cartridge.*—Baker 10 C18 (7020–3, J.T. Baker Inc., Phillipsburg, NJ 08865).

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(e) *LC mobile phase.*—Methanol–acetonitrile–0.01M aqueous oxalic acid solution (pH 2.0) (1 + 1.5 + 2.5).

(f) *Extraction solvent.*—0.1M Na_2EDTA-McIlvain buffer, pH 4.0 (33.62 g Na_2EDTA + 614.5 mL 0.1M citric acid + 385.5 mL 0.2M disodium hydrogen phosphate).

(g) *Elution solvent.*—From C18 cartridge, 0.01M methanolic oxalic acid solution.

(h) *TCs standard solutions.*—Mixed TCs. 0.5 μg/mL. All in 0.01M methanolic oxalic acid solution. Prepare daily.

Apparatus

(a) *High-speed blender.*—Ultra-Turrax TP/18N (IKA-Works, Inc., Cincinnati, OH 45240).

(b) *LC column.*—150 × 4.0 mm id stainless steel column packed with 10 μm LiChrosorb RP-18 (E. Merck, Darmstadt, Germany).

(c) *Liquid chromatograph.*—Model 6A series equipped with constant flow pump and variable wavelength UV detector (Shimadzu, Kyoto, Japan). Operating conditions: eluant at room temperature; flow rate, 2 mL/min; injection volume, 100 μL; detection wavelength, 350 nm.

Sample Collection

Sixty-two cattle kidneys, 45 cattle livers, 20 cattle miscellaneous tissues, 66 hog kidneys, 55 hog livers, and 23 hog miscellaneous tissues were collected as samples. Samples were taken from 64 cattle and 68 hogs of 1358 diseased animals (192 cattle and 1166 hogs; during survey period, 39 317 cattle and 1 152 207 hogs were slaughtered) that did not pass inspection at the slaughterhouse in Aichi Prefecture in Japan from April, 1985 to March, 1987.

Extraction, Cleanup, and Analysis

The method used in the present study was described in detail in the previous paper (6); therefore, a brief statement is given here. A 5 g sample was blended 3 times with 20, 20, and 10 mL extraction solvent, using a high-speed blender. The sample was then centrifuged. Supernatant was applied on C18 cartridge and cartridge was washed with 20 mL water. TCs were eluted with 10 mL elution solvent and collected in 10 mL volumetric flask. For determination of TCs, each sample and the standard solution (100 μL) were injected into the LC system.

Evaluation of Method Using Spiked Samples

According to the previous method (6), TCs in various tissues spiked at the 1.0 ppm level were determined and recoveries were calculated. As shown in Table 1, good recov-

Table 1. Recovery of tetracyclines from animal tissues (6 samples each)

Sample	Rec., % (CV, %)			
	OTC	TC	CTC	DC
Cattle liver	87.7 (2.4)	87.5 (1.0)	79.6 (2.9)	67.5 (2.0)
Hog liver	91.0 (1.1)	88.1 (1.6)	88.5 (2.6)	68.9 (3.3)
Cattle kidney	90.7 (2.7)	85.9 (1.0)	92.3 (2.6)	81.4 (2.9)
Hog kidney	89.1 (1.2)	86.3 (2.3)	86.2 (2.1)	83.2 (2.2)
Cattle muscle	91.6 (2.5)	87.5 (2.7)	93.0 (2.1)	85.4 (3.5)
Hog muscle	92.8 (1.4)	90.2 (1.6)	94.9 (1.4)	85.5 (2.2)

eries and coefficients of variation (CVs) were obtained, and detection limits were 0.05 ppm for OTC and TC, and 0.1 ppm for CTC and DC, respectively.

Results and Discussion

Residual TCs in tissues obtained from 64 cattle and 68 hogs of 1358 sick animals were analyzed by our established LC method (6). Table 2 shows the frequency of TCs residue occurrence. Percentage frequencies of TCs residues were 29.1% (37/127) and 15.2% (22/144) for cattle and hogs, respectively. TCs residues in cattle are more frequent than those in hogs. Among 271 samples, 49 (18.1%) were positive for OTC, 5 (1.8%) for CTC, and 5 (1.8%) for DC, respectively. One sample (cattle kidney) was positive for both OTC and DC. However, no TC was detected in any samples. The residual TCs found most often were OTC, which showed 10 times higher incidence than those of CTC and DC. We concluded that this higher incidence of OTC residue is due to a greater use of OTC (3).

Of the 128 kidney samples (62 cattle and 66 hogs), 22.6% (19 cattle and 10 hogs) were positive for TCs (cattle, 30.6%; hog, 15.1%). Fifteen percent (cattle, 22.2%; hog, 9.0%) of the 100 liver samples (45 cattle and 55 hogs) contained TCs. Among these TCs positive samples, 20 kidney and liver pairs were obtained from the sample animal bodies, and 15 pairs (10 cattle and 5 hogs) contained TCs in both kidneys and livers. In the remaining 5 cattle pairs, only 1 kidney was positive for TCs and none of the livers was positive for TCs. Thus, kidney tissue had the greatest rate of TCs incidence.

Averages and ranges of the concentration of residual TCs in the positive samples are shown in Table 3. The highest residue was 33.61 ppm OTC in a hog kidney, and the lowest was 0.05 ppm OTC in a cattle kidney. Average concentrations of residual OTC were 1.62 ppm in cattle kidneys, 0.67 ppm in cattle livers, 0.29 ppm in cattle miscellaneous tissues, 12.37 ppm in hog kidneys, 3.41 ppm in hog livers, and 3.56 ppm in hog miscellaneous tissues. However, we could not collect statistics for the concentration of residual CTC and DC, because the number of positive samples was not sufficient. For both cattle and hogs, kidney samples showed the highest average concentration of residual TCs, and liver samples contained TCs at higher concentration than miscellaneous tissue samples. As described above, 20 kidney and liver pairs were obtained from the same animal bodies and the level of concentration of residual OTC and their concentration ratios (kidney/liver) are shown in Table 4. Only Cattle A showed a DC residue in both kidney (4.18 ppm) and

Table 2. Frequency of tetracycline residues in sick animals

Sample	No. of samples	No. of positive samples			
		OTC	TC	CTC	DC
Cattle					
Kidney	62	18	0	1	1
Liver	45	9	0	0	1
Miscellaneous	20	3	0	1	3
Hog					
Kidney	66	8	0	2	0
Liver	55	4	0	1	0
Miscellaneous	23	7	0	0	0
Total	271	49	0	5	5

Table 3. Averages and ranges of concentration of tetracyclines in various tissues of sick animals

Sample	OTC			CTC			DC		
	No.	Av.	Range	No.	Av.	Range	No.	Av.	Range
Cattle									
Kidney	19	1.62	0.05–11.04	1	4.80	—	1	4.18	—
Liver	9	0.67	0.06–4.05	1	0.84	—	1	1.22	—
Miscellaneous	3	0.29	0.12–0.53	—	—	—	3	0.55	0.53–0.57
Hog									
Kidney	8	12.37	0.22–33.61	2	0.29	0.15–0.43	—	—	—
Liver	4	3.41	0.45–6.51	1	0.15	—	—	—	—
Miscellaneous	7	3.56	0.24–6.05	—	—	—	—	—	—

Table 4. Level of concentration of residual oxytetracycline in kidney and liver obtained from the same animals, and their concentration ratios

	OTC		
	Concentration ratio	Residue, ppm	
Sample	Kidney/liver	Kidney	Liver
Cattle A	∞	0.15	ND[a]
Cattle B	1.5	1.01	0.67
Cattle C	2.9	0.50	0.17
Cattle D	0.9	0.12	0.14
Cattle E	4.1	0.69	0.17
Cattle F	11.0	1.32	0.12
Cattle G	∞	0.48	ND
Cattle H	∞	0.18	ND
Cattle I	∞	0.36	ND
Cattle J	2.9	0.29	0.10
Cattle K	∞	0.05	ND
Cattle L	2.0	0.12	0.06
Cattle M	∞	0.07	ND
Cattle N	4.4	2.58	0.59
Cattle O	2.0	9.10	4.05
Hog A	—	ND	ND
Hog B	2.6	1.36	0.53
Hog C	6.18	2.78	0.45
Hog D	4.1	26.98	6.51
Hog E	3.4	21.09	6.14

[a] ND = not detected.

liver (1.22 ppm) tissue; and only Hog A showed a CTC residue level in both kidney (0.43 ppm) and liver (0.15 ppm) tissue. Kidney samples showed 1.5–7 times higher concentration of residual TCs than liver samples, except for Cattle D. Honikel et al. also reported that concentrations of residual TCs in kidney are 4–6 times higher than those in muscle (7). Therefore, we concluded that inspection of slaughtered animal's kidneys for residual TCs is the most effective means of monitoring safety of foods.

The present survey revealed that diseased animals that did not pass inspection had a high incidence of residues (cattle, 29.1%; hogs, 15.2%). The animals also had high concentrations of TCs (OTC, 0.05–33.61 ppm; CTC, 0.15–4.80 ppm; and DC, 0.53–4.18 ppm). Although this survey has not included the meat sample for food, these results may indicate that our safety is threatened by antibiotics like TCs residues in food. Therefore, a survey of residual antibiotics for meat will be needed in the near future.

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An Analytical Survey of Aflatoxins in Tissues from Swine Grown in Regions Reporting 1988 Aflatoxin-Contaminated Corn[1]

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A joint project was undertaken by the Food Safety and Inspection Service (FSIS) and the Agriculture Research Service branches of the U.S. Department of Agriculture to determine the presence of aflatoxins in the U.S. meat supply during a drought year. In 1988, high incidences of aflatoxins occurred in corn grown in regions of the Midwest, Southeast, and South. Six states were identified as having serious aflatoxin contamination in their corn crop: Virginia, North and South Carolina, Texas, Iowa, and Illinois. Swine liver and pillars of diaphragm (muscle) tissues were sampled by federal FSIS inspectors in plants located in these states. A worst-case sampling plan was conducted. Samples were taken in January 1989 from hogs fed corn soon after harvest and in April 1989 from hogs fed corn originally stored and then fed in the spring. A modification of the official AOAC method for the thin-layer chromatography (TLC) determination of aflatoxins in animal tissue was used to permit quantitation by LC with fluorescence detection. The official AOAC TLC confirmation of identity method was used to confirm all positive samples with B_1 concentrations >0.04 ppb and M_1 concentrations >0.1 ppb. Sixty samples in the January group and 100 samples in the April group were assayed. Concentrations of aflatoxins B_1 and M_1 in the first group of pig livers ranged from 0.04 to 0.06 ppb. The identity of aflatoxin B_1 was confirmed in all positive samples. Aflatoxin M_1 could not be confirmed in any of the positive liver samples because the method was insufficiently sensitive for this aflatoxin. No positive muscle samples were found. In the second set, 9 positive livers were determined with B_1 concentrations from 0.01 to 0.24 ppb and M_1 concentrations from 0.03 to 0.44 ppb. Two samples contained M_1 only. None of the corresponding muscle samples contained aflatoxins. Of the 12 positive samples, 5 were from Iowa, 4 from South Carolina, 2 from North Carolina, and 1 from Illinois. Aflatoxins were not detected in any of the samples from Texas or Virginia. One sample from North Carolina contained more than 0.5 ppb total aflatoxins. Blind recovery studies were conducted by including an artificially contaminated liver sample for every 6 samples assayed, and 1 uncontaminated liver sample for every 18 samples. Recoveries of aflatoxins B_1, G_1, and M_1 were 71.8%, 73.2%, and 69.8% respectively.

In 1988, numerous incidences of high aflatoxin concentrations occurred in corn grown in certain regions of the Midwest, Southeast, and South (1). A joint project was undertaken by the Food Safety and Inspection Service (FSIS) and the Agriculture Research Service (ARS) branches of the U.S. Department of Agriculture (USDA) to determine the presence of aflatoxins in the U.S. meat supply in a drought year. FSIS has regulatory authority for the U.S. meat supply in federally inspected plants and is responsible for determining environmental contaminants present in tissues. When compounds have no established tolerances, such as aflatoxins in tissues, FSIS may undertake exploratory studies to ascertain if a problem exists. This study was undertaken for that purpose. The Residue Evaluation and Planning Division of FSIS arranged for the sample selection from federal inspection plants located in the regions of specific interest, and the Mycotoxin Analytical Research group at the ARS' National Center for Agricultural Utilization Research (NCAUR) conducted the analysis of the tissues.

A sampling plan was designed and conducted by FSIS (2). Six states were identified by FSIS as having significant aflatoxin contamination in their 1988 corn crop: Virginia, North and South Carolina, Texas, Iowa, and Illinois. Swine were chosen as the test animals because they concentrate aflatoxins more than other animals (3). Of the edible tissues, the liver contains the highest aflatoxin concentration (4). Pillar of diaphragm (muscle) tissue was sampled simultaneously to determine if contaminated liver samples had correspondingly contaminated muscle tissue. Samples were taken twice: in January 1989, from hogs fed corn soon after harvest; and in April 1989, from hogs fed corn stored originally and then fed in the spring.

Results of the analysis and confirmation of identity of the positive samples found in this study are described. Statistical results of a simultaneously conducted quality assurance study are also included.

Experimental

Sampling and Sample Preparation

The sampling plan was designed by FSIS and conducted by their federal meat inspectors (2). Samples were shipped frozen to NCAUR, where they were recorded, assigned NCAUR numbers, and stored at 0°C. Before analysis, samples were allowed to thaw at room temperature overnight. Tissue samples were ground with a commercial meat grinder and stored in a covered plastic freezer container at either 40°C, if analyzed within 24 h, or 0°C, if analyzed later.

Methods, Reagents, and Apparatus

The official AOAC method for determining aflatoxins in meat tissues (5) was used for all assays; however, it was modified to permit analysis by liquid chromatography (LC) with fluorescent detection. The official AOAC thin-layer chromatography (TLC) method for the confirmation of identity (6) was used for all positive samples. Good laboratory practice

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[1]Presented at the Annual Meeting of the Midwest Association of Official Analytical Chemists' Regional Section, June 19–21, 1990, in Topeka, KS. 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.

[2]Formerly of Food Safety and Inspection Service, now at Food and Drug Administration, Rockville, MD 20857.

[3]Formerly of National Center for Agricultural Utilization Research, now retired.

Table 1. Summary of analysis of contaminated pig livers from states with aflatoxins in 1988 corn[a]

Sample state	Aflatoxin concn		Confirmation[b]	
	B_1, ng/g	M_1, ng/g	B_1	M_1
Winter pigs[c]				
Iowa	0.05	ND	Y	—
South Carolina	0.06	0.04	Y	NC
South Carolina	0.05	0.04	Y	NC
Spring pigs[c]				
Iowa	0.02	0.03	NC	NC
Iowa	0.04	0.04	Y	NC
Iowa	0.04	0.05	Y	NC
Iowa	ND	0.06	—	NC
Illinois	0.01	0.03	NC	NC
North Carolina	ND	0.28	—	Y
North Carolina	0.10	0.44	Y	Y
South Carolina	0.11	0.22	Y	Y
South Carolina	0.24	0.20	Y	Y

[a] As determined by modification of official AOAC Method **982.24** described in manuscript.
[b] AOAC Method **982.25**. Y = confirmed, NC = not confirmed (minimum detection limits were B_1 = 0.04 ppb and M_1 = 0.1 ppb).
[c] Pigs were slaughtered in either January (winter) or April (spring) 1989.

was incorporated in this research. All reagents and apparatus were as specified in references 5 and 6 except for TLC descriptions in ref 5. In addition, the following were added:

(a) *Meat grinder.*—Hobart model 4162 equipped with extruder plate containing 1/8 in. diameter holes, or equivalent.

(b) *LC system.*—SP8800 solvent delivery system (Spectra-Physics, Inc., San Jose, CA 93412); 7125 injector (Rheodyne, Inc., Cotati, CA 94931); Zorbax ODS C18 column (4.6 mm × 25 cm) (DuPont Co., Wilmington, DE 19898); RP18 (15 × 3.2 cm, 7 μm) guard column (Brownlee Labs, Inc., Santa Clara, CA 95050); FS980 programmable fluorescence detector (excitation, 366 nm; emission, >418 nm) (Kratos Analytical, Ramsey, NJ 07446); and SP4270 recording integrator (Spectra-Physics).

(c) *Mobile phase.*—Mix 250 mL each isopropyl alcohol and acetonitrile in 2000 mL graduate. Add water to 2 L mark to give solvent ratio of 12.5:12.5:75. Degas solution by either sonication or helium purge.

(d) *Injection solvent.*—Same as (c) except prepare ratio of 5:15:80.

(e) *Silylated vials.*—See **986.16** B(f) and **986.16** C(i) (7).

(f) *Standard aflatoxins solution.*—Prepare solution containing 0.5 μg B_1, G_1, and M_1/mL, and 0.1 μg B_2 and G_2/mL, in acetonitrile–benzene (1 + 9). Store in sealed glass ampules at 0°C until needed. After opening, store in 1 dram vials fitted with Teflon-lined screwcaps. Keep in freezer when not in use.

(g) *Trifluoroacetic acid (TFA) derivative formation.*—Add equal 200 μL aliquots of hexane and TFA to vials containing either dry standard aflatoxins (50 μL) (use silylated vial) or the extract residue. Cap vials with Teflon-lined screwcaps, shake vials with mixer (Vortex), and heat vials 10 min at 40°C. Evaporate to dryness under nitrogen. Save for LC determination.

Table 2. Statistical data for artificial contamination of swine liver[a]

Statistic	B_1	G_1	M_1
Mean, ng/g	0.359	0.366	0.349
SD	0.057	0.056	0.056
CV, %	15.9	15.3	16.0
Range, high	0.510	0.480	0.490
Range, low	0.270	0.290	0.280
Theoretical, ng/g	0.50	0.50	0.50
Rec. %	71.8	73.2	69.8
N	28	14	28

[a] As determined by method in text.

(h) *Confirmation.*—Use **982.25** (TLC hexane–TFA spray method). Method is sensitive for B_1 and M_1 at >0.04 ppb B_1 and 0.1 ppb M_1.

LC Quantitation

Redissolve dry sample TFA derivative mixture in 200 μL injection solvent, and dissolve dry standard TFA derivatives in 1000 μL injection solvent. Mix with vortex mixer to ensure complete solubilization. Adjust flow rate of mobile phase to 1.0 mL/min. Adjust fluorometer sensitivity to produce B_1 peak height of ca 60–70% full scale for 1.25 ng B_{2a} (B_1 TFA derivative) injection. After LC is adjusted, inject 3 × 50 μL standard aliquots and 50 μL sample aliquot with precision syringes. Retention times will be 4–5 min and 6–7 min for M_1 and B_1 (TFA derivatives), respectively. Average peak areas for standards and calculate aflatoxin concentrations from the following formula:

$$C = A_{SL}/A_{SD} \times I_{SD}/I_{SL} \times S \times V/W$$

where C = concentration of sample in ppb; A_{SL} = peak area of sample; A_{SD} = peak area of standard; I_{SL} = injection volume of sample in μL; I_{SD} = injection volume of standard in μL; S = concentration of standard in ng/μL; V = final volume (including dilutions) in μL; and W = weight sample in g.

Quality Assurance Procedure

To ensure and measure assay reliability, an artificially contaminated liver sample was included in every set of 6 sample assays, and a blank liver sample was included for every 3 sets (18 assays). Analysts were not informed of sample identity; therefore, all samples were treated identically. This blind analysis gave a quality control assurance to the assay, and a measure of the recovery of aflatoxins added to the livers.

Results and Discussion

The FSIS sampling plan was designed to obtain meat samples in regions known to have serious aflatoxin contamination in their 1988 corn crop (2). The plan ensured the greatest probability of finding contaminated commercial meat when present. Samples were collected during January and April 1989. The aflatoxin contamination problem in corn was attributed to the major drought incurred over much of the United States in 1988 (1). Consequently, during the January sampling period, liver and muscle were collected from swine

that were fed corn immediately following harvest; in the April period, samples were collected from the livestock fed corn that was initially stored and then fed in the spring.

A modification of the official AOAC method for the determination of aflatoxins in tissue was used for the assay to include the improved sensitivity of LC with fluorescence detection. The official method uses 2-dimensional TLC (2D-TLC). Although aflatoxins are resolved by 2D-TLC, 0.1 ppb concentrations are detected as very faint fluorescent zones. Swine tissue extracts contain many interfering contaminants that make identification of low level concentrations difficult. LC permits detection of aflatoxins at the low ppt level and can be automated, while TLC cannot. Many modifications were tried to permit substitution of LC for TLC. The simplest and most efficient change was the use of less original extract (10 mL/5 g).

The official TLC spray method for confirmation of identity (TFA derivative) was used to confirm all positive samples. LC positive samples were re-extracted and subjected to TLC confirmation. B_1 was confirmed at levels greater than 0.04 ppb. However, 0.1 ppb M_1 was necessary before it was confirmed. Aflatoxin M_1 does not react completely with TFA under the conditions in the official method. Tandem mass spectrometry studies (R. Plattner, private communication) will not confirm either aflatoxin below 0.1 ppb; therefore, it was not used.

Results of the assays are given in Table 1. Of the 60 samples from the January period, 3 were positive, with concentrations of B_1 and M_1 of 0.04–0.06 ppb. The identity of B_1 was confirmed in all positive samples. Aflatoxin M_1 was not confirmed in any of the LC-positive samples because the AOAC method lacked sufficient sensitivity. No positive muscle samples were found.

Aflatoxins were detected in 9 of 100 livers from the second sampling period. B_1 concentrations ranged from 0.01 to 0.24 ppb, and M_1 levels ranged from 0.03 to 0.44 ppb. Two samples contained M_1 only. All samples containing concentrations of >0.04 and >0.1 ppb for B_1 and M_1, respectively, were confirmed. One sample from North Carolina contained 0.54 ppb total aflatoxins (B_1 + M_1), and another sample (South Carolina) had a total of 0.44 ppb aflatoxins. No legal federal guidelines or tolerance levels exist for total aflatoxin concentration in meat tissue; however, these 2 samples are near the federal guidelines for milk, 0.5 ppb. Of the 12 positive samples, 5 were from Iowa, 4 from South Carolina, 2 from North Carolina, and 1 from Illinois. Aflatoxins were not detected in any of the 35 Texas samples or any of the 25 Virginia samples. All corresponding muscle samples were negative. Although none of the liver samples presented a human health hazard in either sampling period, the data indicate that perhaps more stored contaminated corn was fed, because both the incidence and levels were higher in the spring sampling period.

Recovery of better than 60% was established as mandatory, or the entire 6 sample set was reassayed. Recoveries less than 60% were obtained twice, and both were attributed to improper artificial contamination. Satisfactory results were obtained when the analyses were repeated.

Statistical data for the quality assurance study are presented in Table 2. Recoveries for B_1, G_1, and M_1 were similar, 71.8%, 73.2%, and 69.8%, respectively. G_1 was removed from the spiking standard after sufficient samples were assayed and it was not found. Therefore, only 14 data points were available for G_1, but its recovery is nearly identical to that of B_1 and M_1. These data compare favorably with data obtained in the international collaborative study on the validation of the method (8). The coefficient of variation is less than the international study, but that is normal because within-laboratory variation is always lower than between-laboratory variations. These data support the conclusion that the use of LC quantitation with fluorescent detection for the determination of aflatoxins in meat tissue can be substituted for TLC quantitation.

The study results support the goal of the FSIS that only quality meat is approved for the commercial marketplace. Even in the worst-case scenario, a drought year, and in regions of the United States with known heavy aflatoxin contamination (up to 400 ppb) in the corn crop, just 7.5% of the edible swine samples had aflatoxins present. Only 3 of the 12 positive samples had 0.1 ppb total aflatoxins or more; the remaining 9 positive samples had 0.05–0.1 ppb total aflatoxins.

Acknowledgments

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DRUGS

Polyvinyl Chloride Matrix Membrane Electrodes for Manual and Flow Injection Analysis of Chloroquine in Pharmaceutical Preparations

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Two types of polyvinyl chloride (PVC) matrix membrane electrodes responsive to the antimalarial drug chloroquine have been constructed, electrochemically evaluated, compared, and used in pharmaceutical analysis. Type 1 is the classic PVC model with chloroquine–tetraphenylborate (TPB) sensor; Type 2 is a coated silver disk without internal filling solution. Both electrode types exhibited rapid linear potentiometric response to the logarithmic concentration of diprotonated chloroquine cation in the 10^{-1}–10^{-6}M range with calibration slopes 28–30 mV/concentration decade over the pH range 1.8–6.2. These electrodes were sensitive enough to permit determination of chloroquine phosphate at concentrations as low as 5 μg/mL with good accuracy and precision. Determination of chloroquine in various pharmaceutical preparations using direct potentiometry and potentiometric titration with NaTPB gave an average recovery of 98.8% of the nominal values (SD 0.5%). The Type 2 electrode was also assessed in a flow-through sandwich cell for flow injection analysis. Results were compared with data obtained by the U.S. Pharmacopeia method.

Chloroquine [7-chloro-4-(4-diethylamine-1-methylbutylamino)-quinoline] is the most widely used antimalarial drug. Determination of this drug by British (1) and U.S. Pharmacopoeia (USP) (2) methods includes extraction followed by nonaqueous titrimetric and spectrophotometric measurements, respectively. Other spectrophotometric methods have also been described based on reactions with fast green and orange II (3), bromophenol blue (4), quinones (5, 6), iodine (7), ammonium molybdate (8), *N*-bromosuccinimide–acetic anhydride (9), tungstophosphate and tetraphenylborate (TPB) (10), amine tetrathiocyanatochromate (11), and cobalt thiocyanate (12). Because of the nonspecificity of most of these reactions, prior extraction of chloroquine is commonly involved in the assay methods (2, 4, 6, 7, 10, 12). Fluorimetric (13) and liquid chromatographic (14, 15) methods for determination of chloroquine have been reported to be more selective than the assay methods. Gravimetry (16), direct titrimetry with sodium dioctylsulfosuccinate (17), and indirect complexometry, argentimetry, and redox titrimetry after prior formation of chloroquine–tetrathiocyanatochromate complex (11) have been suggested. These methods are not sensitive enough to permit measurement of low concentrations of chloroquine; moreover, they suffer from severe interferences by organic bases.

Ion selective membrane electrodes, however, are finding considerable use for monitoring various drugs (18, 19), and they may have various applications in pharmaceutical analysis. The only electrode system described for determination of chloroquine was based on the use of chloroquine–dinonylnaphthalene sulfonate ion-pair complex in polyvinyl chloride (PVC) matrix membrane (20). This paper presents the response characteristics and applications of 2 types of chloroquine–TPB PVC membrane electrode systems. Type 1 is similar to the classic PVC membrane model with internal reference solution. In Type 2, the sensor material is applied to the outer surface of a silver disk, providing an all-solid-state electrode system without internal reference solution, similar in principle of the coated-wire electrodes. This type is incorporated in a flow-through potentiometric cell and lends itself nicely to the dynamic feature of flow-injection analysis (FIA). These electrode systems were evaluated manually and with FIA for determination of chloroquine in various pharmaceutical preparations.

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Experimental

Apparatus

(a) *Microprocessor meter.*—Microprocessor Ionalyzer 901 meter (Orion Research Inc., Cambridge, MA 02139). Used to make all electrode measurements at 25 ±1°C.

(b) *Sensing chloroquine electrodes.*—Type 1 and Type 2.

(c) *Double junction Ag–AgCl reference electrode.*—Model 90–02 (Orion). Used in conjunction with sensing chloroquine electrodes. With 10% m/v KNO3 as outer chamber filling solution.

(d) *Ag–Ag2S solid state membrane electrode.*—Model 94–16 (Orion). Used to standardize NaTPB with silver nitrate.

(e) *Combination glass electrode.*—Model 91–01 (Orion). Used to adjust pH.

(f) *Flow-through sandwich cell.*—Fabricated in this laboratory, as described by Machado (21). Electrode was a silver disk coated with chloroquine–TPB PVC membrane. Cell was incorporated in manifold system with injection valve (Omnifit, Cambridge, UK) and peristaltic pump (AutoAnalyzer proportionating pump, Technicon, Tarrytown, NY 10591). Potential response was measured at room temperature with the microprocessor.

(g) *Strip-chart recorder.*—Linear 1200 or Heath-Schlumberger SR-210. Used to receive output from flow-through sandwich cell.

Reagents

(All reagents were prepared from reagent grade chemicals unless specified otherwise. Doubly distilled deionized water was used throughout.)

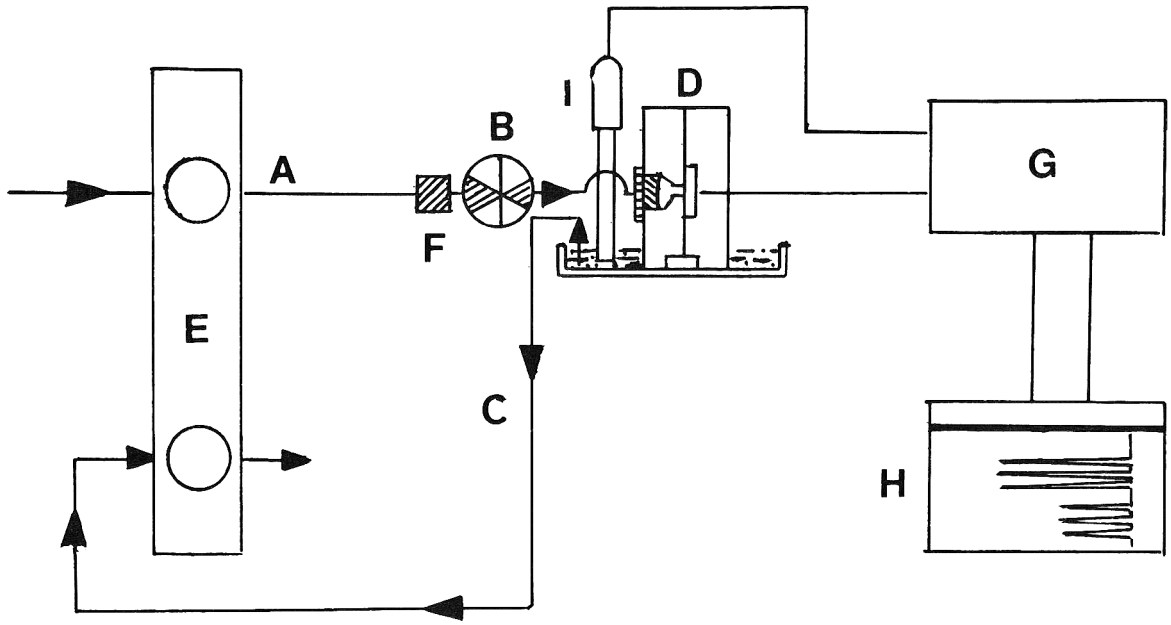

Figure 1. Manifold of single line FIA used for determination of chloroquine: (A) Carrier phosphate buffer, (B) sample valve, (C) waste, (D) potentiometric cell, (E) peristaltic pump, (F) pulse damper, (G) Orion Microprocessor Ionalyzer, (H) recorder, and (I) reference electrode.

(a) *Sodium tetraphenylborate (III) (NaTPB).*—0.01M in water. Prepared, filtered twice, and standardized by potentiometric titration with standard 0.01M silver nitrate solution, using Ag–Ag_2S membrane electrode (d) in conjunction with double junction electrode (c).

(b) *Polyvinyl chloride, dioctylphthalate (DOP), tetrahydrofuran (THF).*—Aldrich Chemical Co., Milwaukee, WI 53233.

(c) *Phosphate buffer.*—0.01M, pH 4.5 background. Prepared fresh and used as carrier in FIA.

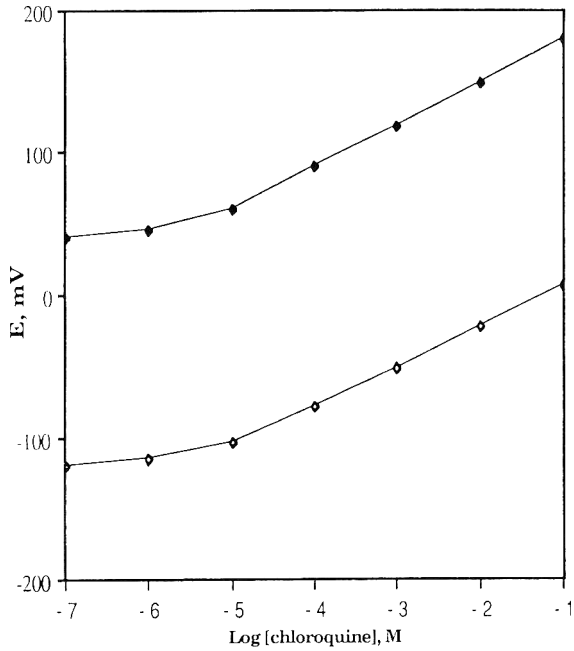

Figure 2. Typical calibration graphs for: (◆) Type 1, and (◊) Type 2 chloroquine–TPB PVC matrix membrane electrodes.

(d) *Chloroquine phosphate standards.*—10^{-1}–10^{-7}M, prepared in 0.01M phosphate buffer.

Chloroquine–TPB Ion-Pair

A 50 mL aliquot of 0.01M aqueous chloroquine phosphate was mixed with 50 mL 0.01M NaTPB. The resulting precipitate was filtered, washed well with twice-distilled water, and dried under vacuum at 50°C. Elemental analysis data of the precipitate agreed with the formation of chloroquine–TPB complex (1 + 2).

Chloroquine–PVC Membrane Electrode (Type 1)

A 10 mg portion of dried chloroquine–TPB complex was thoroughly shaken with 352 mg DOP plasticizer and mixed with 187 mg PVC powder and 7 mL THF. This cocktail was used to prepare the master membrane (5 cm diameter). Disks of the membrane were used to assemble the electrode in the conventional mode by the general procedure previously described (22, 23). Internal reference solution was 0.01M chloroquine phosphate–0.01M KCl (1 + 1).

Chloroquine–PVC Membrane Electrode (Type 2)

A small ring of polyethylene tubing (1.0 cm od and 0.5 cm long) was fixed with araldite on the inner surface of a Perspex tube (1.0 cm id and 10 cm long). A distance of 0.4 cm was left between the ring and the end of the tube. A clean silver disk (0.9 cm od and 0.5 mm thick) was fixed with araldite to the polyethylene tubing to form a cylindrical cavity. The inner surface of the disk was soldered to a copper wire of a coaxial cable, thereby dispensing with the internal reference solution. The tube was inverted, and the disk was polished with sandpaper and coated with the sensor cocktail in THF, using a dropping pipet. The cocktail was added in the cavity and the

solvent was allowed to evaporate at room temperature to give a first layer of the coat. This operation was repeated several times until the membrane filled the cavity. The membrane was left to dry at room temperature for 2 days.

The silver disk incorporated in the flow-through sandwich cell used in FIA was coated by a similar procedure. When the membrane deteriorated, it was removed from the cavity, the surface of the silver disk was cleaned with emery paper, and a new sensor layer was applied by the same procedure.

Electrode Conditioning and Calibration

Electrodes were preconditioned after preparation by soaking for at least 1 day in 0.01M aqueous chloroquine phosphate and stored in the same solution when not in use. Electrodes were thoroughly washed with deionized water before use and between measurements, and they were calibrated as follows: Aliquots of 0.1–10^{-7}M chloroquine standard were transferred to 100 mL beakers. Chloroquine membrane electrodes (Type 1 or 2), in conjunction with the double junction Ag–AgCl reference electrode, were immersed in solutions. Potentials were recorded when stable readings to ±0.2 mV were obtained. Potential vs logarithmic concentration of chloroquine phosphate was plotted and used for subsequent measurement of unknown chloroquine concentrations.

Determination of Chloroquine in Drugs

Sample preparation.—Five tablets were weighed and finely powdered in a small dish. An accurately weighed portion of powder equivalent to 1 tablet was dissolved in a minimum of 0.01M phosphate buffer solution, pH 4.5, and filtered into a 500 mL calibration flask. The filtrate was diluted to the mark with buffer solution and shaken. Contents of 5 ampoules were mixed and shaken, and a 1.0 mL aliquot of the mixture was transferred to a 250 mL calibration flask, diluted to the mark with buffer solution, and shaken. Chloroquine phosphate in these dilute test solutions was determined as described below.

Direct potentiometric measurements.—Chloroquine electrodes (Type 1 and 2) and the double junction Ag–AgCl reference electrode were immersed in a 50 mL aliquot of chloroquine drug test solution contained in a 100 mL beaker. Electrodes were allowed to equilibrate with stirring and the emf values were recorded and compared with the calibration plot. As an alternative, the standard addition (spiking) technique (18) was used by adding 1.00 mL 0.01M standard chloroquine phosphate to the dilute test drug solution, recording the change in the electrode potential readings, and calculating the original concentration of chloroquine in the drug test solution.

Potentiometric titration.—Aliquots (5–30 mL) of chloroquine phosphate test solutions (ca 0.001M) were pipetted into a 100 mL beaker. The chloroquine selective electrode (Type 1 or 2) and a double junction reference electrode were immersed in the solution. The solution was stirred and titrated slowly with standard 0.01M NaTPB. The inflection point of the titration curve corresponds to a 1 + 2 chloroquine–TPB reaction (1.0 mL 0.01M NaTPB ≡ 2.579 mg chloroquine phosphate).

Flow injection analysis.—The single stream FIA system shown in Figure 1 was used. The peristaltic pump was used to deliver 0.01M pH 4.5 phosphate buffer solution at a flow rate of 5 mL/min. PTFE tubing (0.6 mm id) was used for the flow lines. The length of the tubing between the sampling valve and the sensing cell was 20 cm; between the pump and the injection valve, the length was 30 cm. The sample loop (300 μL) of the injection valve was filled and the valve was rotated to allow the sample to be carried out by the buffer stream to the flow-through cell. The potential response was measured with an Orion Microprocessor Ionalyzer. A strip chart recorder (Linear 1200 or Heath-Schlumberger SR-210) was used to record signals. The average peak height of 5 replicate runs on each sample was measured and compared with a calibration plot prepared under identical conditions.

Table 1. Response characteristics for Type 1 and Type 2 chloroquine–TPB PVC matrix membrane electrodes

Parameter	Type 1	Type 2
Slope, mV/decade	29.9 ± 0.7	28.1 ± 0.5
Intercept, mV	209.7 ± 0.8	34.3 ± 0.7
Correlation coefficient, r	0.998	0.999
Lower limit of linear range, M	10^{-5}	10^{-5}
Detection limit, M	3.2×10^{-6}	3.0×10^{-6}
Dynamic response time for 0.001M, s	20	15
Working pH range	1.8–6.2	1.9–6.1
Lifetime, days	30	40

Results and Discussion

Response Characteristics of the Electrode Systems

Two types of PVC matrix membrane electrodes responsive to chloroquine were prepared, evaluated according to the International Union of Pure and Applied Chemistry recommendations (24), and compared. Both electrode systems were fabricated from a sensor cocktail consisting of PVC, chloroquine–TPB, and DOP plasticizer (34 + 2 + 64, m/m). The first electrode model (Type 1) was made as previously described for the classic PVC electrodes (22, 23). Type 2 was constructed in the form of a coated silver disk (1 cm diameter) without internal filling solution. The Type 2 electrode system is structurally similar to that previously described (25, 26), but a silver disk is used as a membrane base instead of a blob of silver-conductive epoxy resin. The dependence of the potentials of Type 1 and 2 electrodes on the concentrations of chloroquine was tested in freshly prepared 10^{-1}–10^{-7}M pH 4.5 chloroquine phosphate solutions. Results are shown in Figure 2.

The mean slope of the calibration plot of the Type 1 electrode over the 10^{-1}–10^{-6}M concentration range was typically 29.9 mV/decade (SD 0.7 mV/decade, n = 5). The corresponding mean slope for the Type 2 model was 28.1 mV/decade (SD 0.5 mV/decade, n = 5). Least squares treatment of the sets of data gives the expressions:

$$E_1 = (29.9 \pm 0.7) \log (\text{chloroquine}) + (209.7 \pm 0.8), \text{ and}$$

$$E_2 = (28.1 \pm 0.5) \log (\text{chloroquine}) + (34.3 \pm 0.7)$$

for electrodes of Types 1 and 2, respectively. The general response characteristics of the electrodes, which are compiled in Table 1, show that the performances of both electrodes are

generally similar. The lower limit of detection is better than 1.7 μg chloroquine phosphate/mL. Both electrode types obtain stable potential response for 0.001M chloroquine in less than 20 s. Ageing of the membranes for up to 5 weeks did not influence the potential response.

Effect of pH on Performance of Electrodes

Upon protonation, chloroquine gives mono- and dications depending on the pH of the solution. On the basis of the dissociation constants of chloroquine, the formation of the dibasic ion is most favorable at pH <8, and a calibration plot with Nernstian slope corresponding to the doubly charged cation (approximately 29 mV/concentration decade) is obtained.

$$\text{Chloroquine} - H_2^{2+} \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} \text{Chloroquine} - H^+ \quad (pKa_1\ 8.4)$$

$$\text{Chloroquine} - H^+ \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} \text{Chloroquine} \quad (pKa_2\ 10.8)$$

Responses of Type 1 and of Type 2 PVC matrix chloroquine membrane electrodes for 0.01 and 0.001M chloroquine phosphate solutions were investigated at different pH values. From the potential-pH profiles (Figure 3), it is apparent that the potential is invariant in the range of pH 2–6; over this range, the predominant species is the diprotonated chloroquine. As the pH increases above 7, the potential response increases due to the formation of monobasic species. Chloroquine in 0.01M pH 8–9 phosphate buffer displays a calibration plot with a slope of 40–50 mV/concentration decade, but the membrane response deteriorates rapidly.

Potentiometric Selectivity of Chloroquine PVC Membrane

Responses of Type 1 and of Type 2 electrodes were examined in the presence of a number of organic and inorganic cations. Selectivity coefficient ($K^{pot}_{ch,B}$) data with fresh electrodes in parallel experiments were calculated by the separate solution method (18) at the 0.01M level for both chloroquine phosphate and the interferents. Results presented in Table 2 are averages of 3 determinations. The 2 electrodes exhibit similar response as expected, because both are based on the same sensor cocktail. Pharmaceutical excipients and diluents commonly used in the formulation of chloroquine drugs (e.g., lactose, starch, talc, and magnesium stearate) at concentrations as high as 1000-fold excess over chloroquine have no adverse effect on the response of the electrodes ($K^{pot}_{ch,B} < 10^{-4}$).

Manual Determination of Chloroquine

Preliminary work has shown the feasibility of the known addition (spiking) technique (18) for determination of chloroquine phosphate in pharmaceutical powders at levels of 5 μg/mL to 1 mg/mL using the PVC membrane electrodes of Types 1 and 2. Over this concentration range, the average

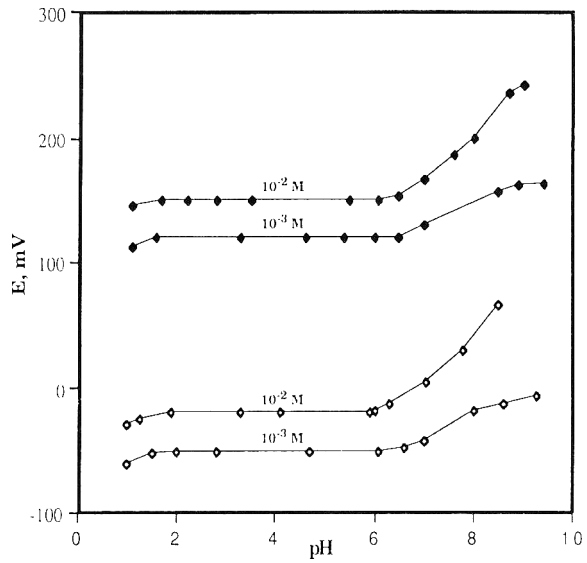

Figure 3. Effect of pH on the response of: (◆) Type 1, and (◊)Type 2 chloroquine–TPB PVC matrix membrane electrodes.

recovery is 98.9% and the mean standard deviation is 0.6% (n = 20). Chloroquine was also determined by potentiometric titration with standard NaTPB solution using electrodes of Type 1 or 2 as the indicator electrode. The 2 electrode types yield almost identical titration curves, which display sharp inflection breaks (about 150 mV) at points equivalent to 1 + 2 chloroquine–TPB reaction (Figure 4). Results obtained by titrating approximately 10 mL sample containing 0.2–5.0 mg chloroquine phosphate/mL show an average recovery of 99.7% and a mean standard deviation of 0.2% (n = 20). These results demonstrate that on the basis of its high accuracy and precision, the proposed electrode method could readily be applied to the determination of chloroquine in pharmaceutical preparations.

Table 2. Potentiometric selectivity coefficients for Type 1 and Type 2 chloroquine–TPB PVC matrix membrane electrodes

	$K^{pot}_{ch,B}$	
Interference, B	Type 1	Type 2
Glycine	1.5×10^{-3}	2.3×10^{-3}
Alanine	4.7×10^{-3}	3.1×10^{-3}
Glutamic acid	2.5×10^{-3}	6.1×10^{-3}
Ethylamine	1.2×10^{-3}	3.4×10^{-3}
Butylamine	3.2×10^{-3}	1.9×10^{-3}
Urea	2.5×10^{-3}	2.0×10^{-3}
m-Aminophenol	5.4×10^{-3}	5.2×10^{-3}
p-Aminobenzoic acid	2.3×10^{-3}	3.2×10^{-3}
Maltose	7.4×10^{-4}	7.1×10^{-4}
Glucose	9.3×10^{-4}	5.1×10^{-4}
K^+	8.6×10^{-3}	2.2×10^{-3}
Na^+	6.3×10^{-3}	3.6×10^{-3}
Mg^{2+}	1.0×10^{-3}	2.5×10^{-3}
Ca^{2+}	1.1×10^{-3}	2.4×10^{-3}
NH_4+	7.4×10^{-3}	4.4×10^{-3}

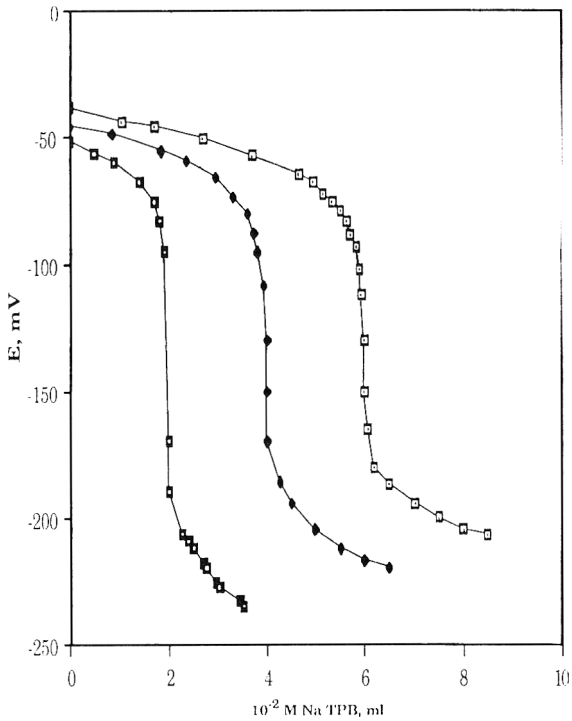

Figure 4. Typical potentiometric titration curves of: (□) 10 mL, (◆) 20 mL, and (▣) 30 mL aliquots of 0.001M chloroquine phosphate with 0.01M NaTPB using Type 2 chloroquine–TPB PVC matrix membrane electrode.

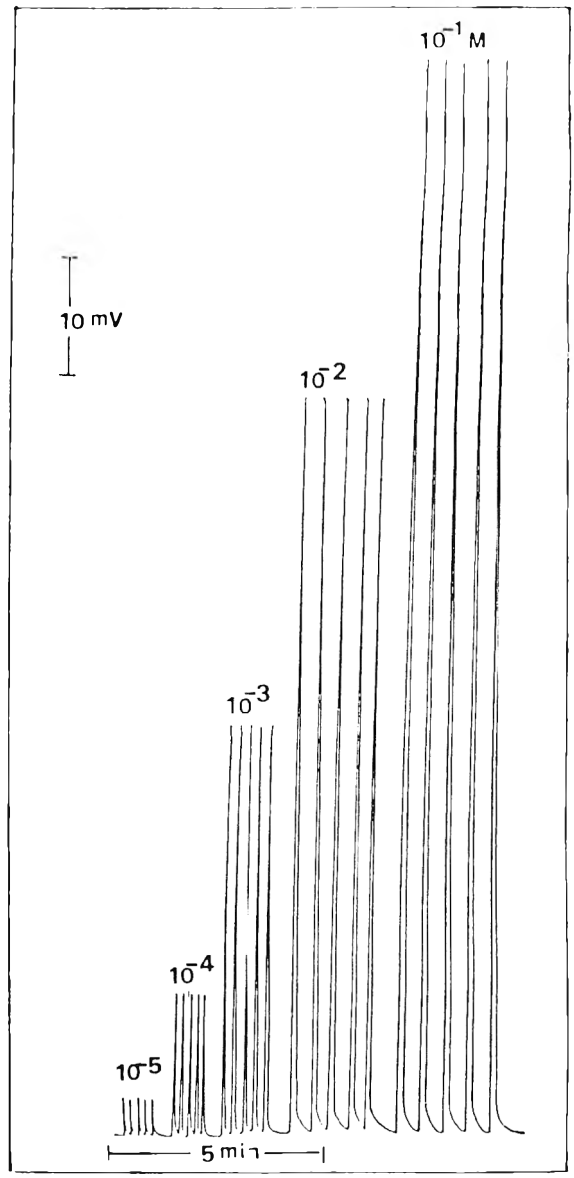

Figure 5. Typical FIA recorder peaks produced by injection of standard chloroquine phosphate solutions.

Flow Injection Analysis of Chloroquine

The high sensitivity, fast response, good selectivity, and reasonable stability offered by the coated silver disk PVC membrane electrode (Type 2) suggested incorporation of this sensor in a flow-through detector to be used for flow injection analysis of chloroquine. A flow-through sandwich potentiometric detector, similar to that described by Machado (21), was fabricated in our laboratory and used in a single stream flow injection system (Figure 1). Standard chloroquine phosphate solutions (5 replicates of each concentration) were injected in the cell through the injection valve. A typical chart recorder trace is shown in Figure 5. The mean standard deviation of the peak heights does not exceed ±0.7%.

Determination of Chloroquine in Pharmaceutical Preparations

Table 3 presents results obtained by direct potentiometry and potentiometric titration of chloroquine in pharmaceutical tablets and injections (ampules) using the Type 2 electrode. Average recoveries of 98.8% (SD 0.5%; n = 45) and 98.9% (SD 0.2%; n = 45) of the nominal values are obtained for direct potentiometry (standard addition method) and potentiometric titration with NaTPB, respectively. Results obtained for determination of chloroquine in some pharmaceutical preparations by FIA show an average recovery of 98.7% and a mean standard deviation of 0.5% (n = 45).

These data were evaluated by comparison with results obtained by using the standard USP method (2), which involves treatment of the drug test solution with alkali, extraction of chloroquine base with chloroform, and spectrophotometric measurement at 343 nm. The *t*-test reveals that the means of 45 runs obtained by direct potentiometry using the chloroquine electrode, when compared to the USP method, are not different at the 0.05 significance level. The F-test reveals no significant difference between means and variances of results obtained by direct potentiometry, potentiometric titration, FIA, and USP methods (Table 3). Results obtained with the USP method (2) are less precise; the mean standard deviation is ±0.8% (n = 45), compared to ±0.2–0.5% (n = 45) obtained with the proposed electrode methods. Electrode systems based on the use of chloroquine–TPB as the electroactive material have shorter response times and longer life times

Table 3. Determination of chloroquine in some pharmaceutical preparations using chloroquine–TPB PVC matrix membrane electrode (Type 2) and USP methods

Pharmaceutical product and source	Chloroquine phosphate, % of nominal[a,b]			
	FIA	Direct potentiometry	Potentiometric titration	USP method
Dagrinol, tablets (Alex. Pharm. Co., Egypt)	98.7 ± 0.5	98.8 ± 0.6	99.0 ± 0.2	98.5 ± 0.8
Dagrinol, ampoules (Alex. Pharm. Co., Egypt)	99.6 ± 0.6	99.8 ± 0.4	100.0 ± 0.3	99.8 ± 0.7
Cidoquine, tablets (Cid Pharm. Co., Egypt)	97.1 ± 0.6	97.0 ± 0.4	97.1 ± 0.3	96.8 ± 0.7
Resochin, tablets (Bayer, W. Germany)	99.0 ± 0.5	98.7 ± 0.4	98.8 ± 0.1	98.7 ± 0.6
Resochin, ampoules (Bayer, W. Germany)	98.8 ± 0.5	99.1 ± 0.6	99.0 ± 0.3	98.9 ± 0.6
Malarochin, tablets (El-Nasr Pharm. Co., Egypt)	98.0 ± 0.6	98.2 ± 0.5	98.3 ± 0.3	98.1 ± 0.6
Chlodichin, tablets (Misr Pharm. Co., Egypt)	97.3 ± 0.7	97.4 ± 0.7	97.9 ± 0.3	98.3 ± 1.1
Delagil, ampoules (Medimpex, Hungary)	98.9 ± 0.4	99.1 ± 0.5	99.2 ± 0.2	98.8 ± 0.6

[a] The nominal content of chloroquine in all drugs was 250 mg/tablet or ampoule except chlodichin (80 mg/tablet).
[b] Average of 5 measurements.

compared to those with an electrode system incorporating chloroquine–dinonylnaphthalene sulfonate (20).

In conclusion, the present electrode methods offer several advantages. Beside the omission of the time-consuming extraction step, the electrode methods are more precise, directly applicable to automated systems, and not liable to interferences by the active ingredients, excipients, and diluents commonly used in chloroquine drug formulations.

Acknowledgment

The authors are grateful to El-Nasr Pharmaceutical Co. (Egypt) for supporting this work. The methods described have been used satisfactorily during 1990 in the Quality Control Department of this company.

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DRUG RESIDUES IN ANIMAL TISSUES

Assay of Oxolinic Acid Residues in Salmon Muscle by Liquid Chromatography with Fluorescence Detection: Interlaboratory Study

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A previously developed method that uses a simplified sample preparation and fluorometric detection of liquid chromatographic eluates for the determination of oxolinic acid in salmon muscle has been collaboratively studied. Five laboratories participated in the study to analyze, in quintuplicate, blank salmon muscle fortified at 10, 20, 50, and 100 μg/kg (ppb), and 2 incurred samples from salmon given feed with medicated oxolinic acid. The tissue, 2 g mixed with 2 g Na_2SO_4, is extracted with ethyl acetate and centrifuged, and the solvent is evaporated. The residue is partitioned in a mixture of hexane and 0.01M oxalic acid, and the aqueous phase is chromatographed using fluorescence detection at 327 nm excitation and 369 nm emission. Mean recoveries ranged from 77.2 to 84.5% in spiked samples with reproducibility relative standard deviation (RSD_R) ranging from 11.5 to 18.3%. Treated salmon were found to contain 8.71 and 53.8 μg/kg with RSD_R of 18.6 and 16.7%, respectively. The corresponding repeatability relative standard deviations (RSD_r) were 5.8–12.2%, and 7.7 and 6.2%. The method is recommended for regulatory purposes in Canada.

The rapid growth of aquaculture in Canada and in other countries has stimulated the need for better regulation of drug residues in edible tissues of fish. Oxolinic acid, a powerful antibacterial agent, is used in aquaculture in Canada. It is especially potent in curing or preventing diseases caused by certain species of *Yersinia, Aeromonas*, and *Vibrio*. The drug is administered orally, mixed in the feed at a recommended dose level of 12 mg/kg per day (Austin Laboratories, Canada). Very little is known about its elimination rate, especially at different temperatures, and no tolerance level has been established yet in Canada for residues.

A potentially suitable method for the determination of oxolinic acid residues in salmon at the 10–2000 μg/kg (ppb) level has been developed and evaluated in our laboratory. It uses a simplified extraction based on a method by Ikai et al. (1) with detection by fluorescence (2). The results have been fully discussed previously (3). The present paper describes the results of an interlaboratory collaborative study designed to validate the method in the 10–100 μg/kg (ppb) range.

Collaborative Study

Five laboratories received a copy of the method and 2 sets of incurred salmon muscle (five 2 g preweighed samples each). Salmons, 1–2.5 kg, were fed pellets containing 0.1% oxolinic acid for 10 days, at the rate of 1% body weight per day. After a withdrawal period of 3–5 days, the fish were slaughtered. Incurred sample sets were prepared by using both filets from a single fish ground together and carefully mixed to assure uniformity. Enough blank salmon muscle was also provided to perform practice runs and recovery studies at 4 different concentrations (10, 20, 50, and 100 μg/kg). Collaborators were instructed to perform all determinations in quintuplicate. Oxolinic acid standard material was also supplied (Cat No. O-0877, lot 47F-0594, Sigma Chemical Co., St. Louis, MO 63178). Standard solutions for constructing calibration curves and preparation of spiked tissue for recovery studies were the responsibility of the participating laboratory. No special instructions were given to the individual collaborators.

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METHOD

Method Performance:

Recovery studies:
10 μg/kg level: Av. rec. = 84.5%
s_r = 9.2; s_R = 15.5; RSD_r = 10.9%; RSD_R = 18.3%
20 μg/kg level: Av. rec. = 84.0%
s_r = 6.1; s_R = 9.6; RSD_r = 7.3%; RSD_R = 11.5%
50 μg/kg level: Av. rec. = 80.0%
s_r = 6.6; s_R = 10.6; RSD_r = 8.2%; RSD_R = 13.2%
100 μg/kg level: Av. rec. = 77.2%
s_r = 4.4; s_R = 9.1; RSD_r = 5.7%; RSD_R = 11.8%
Incurred salmon muscle #1: Av. = 8.71 μg/kg
s_r = 0.67; s_R = 1.62; RSD_r = 7.67%; RSD_R = 18.62%
Incurred salmon muscle #2: Av. = 53.80 μg/kg
s_r = 3.32; s_R = 8.96; RSD_r = 6.18%; RSD_R = 16.66%

Principle

Oxolinic acid is extracted from tissue by homogenization with dry ethyl acetate. The organic phase is evaporated and residue is partitioned between aqueous oxalic acid and hexane. The aqueous phase is chromatographed with fluorometric detection. Extraction and the liquid chromatography (LC) conditions are based on method described by Ikai et al. (1), with modifications to increase sensitivity and throughput. Fluorometric detection, described by Horie et al. (2), is optimized for mobile phase.

Apparatus

(a) *Liquid chromatograph.*—Isocratic pump system, injector, fluorometric detector capable of monitoring emission at 369 nm and excitation at 327 nm, recorder, and integrator. Detector should be optimized for both emission and excitation wavelength and should give a signal:noise ratio of ≥5

Table 1. Recovery of oxolinic acid in spiked blank and concentration found in incurred salmon muscle by collaborating laboratories

	Rec., %				Concn found, μg/kg	
Coll.	10 μg/kg[a]	20 μg/kg[a]	50 μg/kg[a]	100 μg/kg[a]	Incurred No. 1	Incurred No. 2
A	102.8	87.6	91.5	81.3	15.60[b]	61.83
	104.8	92.8	76.6	86.3	8.16	53.13
	96.8	102.8	85.2	76.4	7.70	63.02
	96.8	84.9	75.2	80.3	8.19	53.44
	94.3	—[c]	89.8	81.3	8.54	56.77
B	65.0	75.2	75.6	75.9	10.07	44.41
	68.3	80.5	51.1	67.3	7.91	50.00
	75.1	79.2	64.1	65.1	7.74	49.18
	80.6	63.0	73.0	71.4	8.61	52.52
	84.9	70.9	71.0	70.0	9.08	52.42
C	92.0	78.3	86.4	86.6	9.04	59.31
	78.2	84.6	86.6	76.3	9.65	60.77
	81.8	86.4	82.2	85.1	10.07	53.83
	81.8	86.7	86.9	85.6	10.27	60.95
	84.9	78.9	82.4	83.7	9.60	60.06
D	56.7	—[c]	—[c]	59.7	6.50	40.74
	70.4	—[c]	—[c]	66.9	5.45	38.77
	79.7	—[c]	—[c]	73.1	5.62	38.98
	60.0	—[c]	—[c]	62.0	—[d]	43.47
	—[c]	—[c]	—[c]	—[c]	7.29	45.87
E	82.3	91.0	83.9	84.5	10.49	61.47
	118.6	80.3	78.5	79.4	9.94	—[d]
	95.8	93.1	86.0	90.1	10.85	65.37
	88.6	86.8	85.9	79.0	9.59	62.36
	86.9	92.2	88.9	83.4	9.93	62.43
Mean	84.5	84.0	80.0	77.2	8.71	53.80
s_r[e]	9.2	6.1	6.6	4.4	0.67	3.32
s_R	15.5	9.6	10.6	9.1	1.62	8.96
RSD_r, %[f]	10.9	7.3	8.2	5.7	7.67	6.18
RSD_R, %	18.3	11.5	13.2	11.8	18.62	16.66

[a] Spiked blank salmon muscle at the indicated concentration.
[b] Outlier as determined by Grubbs test (6, 7). Not included in calculation.
[c] Not available.
[d] Experimental errors as notified by collaborators.
[e] s_r and s_R are the repeatability and reproducibility standard deviations, respectively.
[f] RSD_r and RSD_R are the repeatability and reproducibility relative standard deviations, respectively.

when 10 ng/mL standard aliquot is injected. Detector should also have a linear dynamic range of ≥200 ng/mL.

(b) *Chromatographic column.*—Reversed-phase, octadecyl-bonded silica, 5 μm spheres, 4.6 × 150 mm id (Keystone Scientific Inc., Bellefonte, PA 16823, or equivalent column that meets system suitability requirements). Operating conditions: Set flow rate 1.0 mL/min and flush system ≥1 h before injecting standards. Flow rate for runs is 2.0 mL/min at ambient temperature. Equilibrate system 1 min between runs. Oxolinic acid elutes at 3–4 min. As part of system shut-down at end of day, pump mobile phase through column at least 15 min, then rinse with methanol 15 min at 2 mL/min.

(c) *Tissue homogenizer.*—Polytron Model Pt-10/35 with Pt-10ST probe (diameter 11 mm, Brinkmann Instruments, Inc., Westbury, NY 11590).

Reagents

(All reagents used are reagent grade, or LC grade when available, unless otherwise specified.)

(a) *Oxolinic acid.*—(Sigma, Cat. No. O-0877.)

(b) *Purified water.*—Distilled, deionized (4 bowls Milli-Q-System, Millipore Corp., Bedford, MA 01730).

(c) *Dried ethyl acetate.*—Add 50 g anhydrous sodium sulfate to 1 L ethyl acetate, stir, and let settle. Use clear supernatant only. (Untreated ethyl acetate gives emulsion in the final partition step.)

(d) *Oxalic acid solution, 0.01M.*

(e) *Oxalic acid solution, 0.01M (pH 3.00).*—Adjust pH of 0.01M oxalic acid to pH 3.00 with 3N NaOH.

(f) *Mobile phase.*—Mix acetonitrile–redistilled methanol (3 + 1) with 0.01M oxalic acid at ratio of 4:6 (adjust ratio of organic modifiers and oxalic acid according to *Chromatog-*

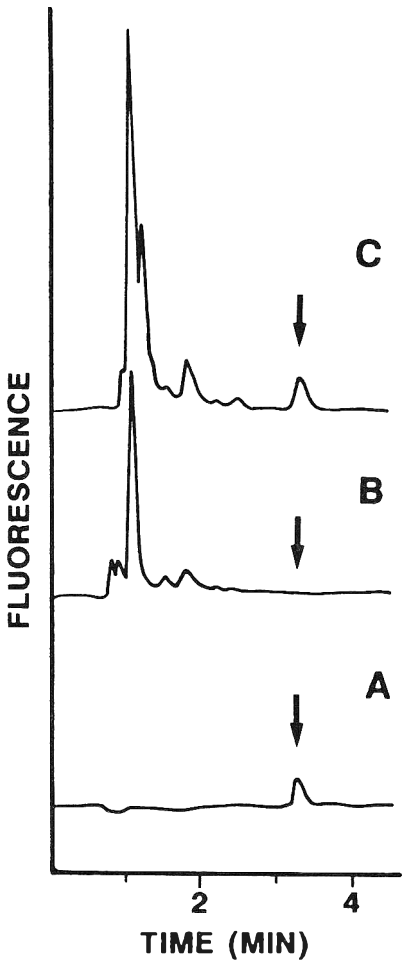

Figure 1. Liquid chromatograms of: (A) 10 ng/mL standard solution; (B) blank salmon muscle extract; (C) incurred sample (estimated concentration of 10.49 μg/kg, not corrected for recovery). Retention time of oxolinic acid, 3.2 min (arrows). Conditions: Ultrasphere ODS, 5 μm, 4.6 × 150 mm id; mobile phase, acetonitrile–methanol (3 + 1) mixture with 0.01M oxalic acid solution (3.5 + 6.5); flow, 2.0 mL/min.

raphy Suitability Test, below). Filter through 0.22 μm membrane. Prepare daily and store in glass container.

(g) *Standard solutions.*— Store all standard solutions below 10°C. Stock solution is stable at least 3 months, but diluted solution should be kept no longer than 2 weeks. (*1*) *Stock solution.*—100 μg/mL. Accurately weigh ca 10 mg oxolinic acid at room temperature and transfer to 100.0 mL volumetric flask. Add ca 10 mL dimethyl sulfoxide and swirl until completely dissolved. Dilute to 100.0 mL with acetonitrile and mix. (*2*) *Oxolinic acid solution.*—10 μg/mL. Measure 10.0 mL stock solution with 10.0 mL volumetric pipet into 100 mL volumetric flask. Dilute to volume with acetonitrile and mix. (*3*) *Fortification solution.*—1 μg/mL. Pipet 10.0 mL 10 μg/mL oxolinic acid solution into 100 mL volumetric flask with 10.0 mL volumetric pipet. Dilute to volume with 0.01M oxalic acid (pH 3.00) and mix.

(h) *Solutions for standard curve.*—(*1*) *200, 100, 50 ng/mL.*—Dilute 2.0, 1.0, and 0.5 mL, respectively, of 1 μg/mL solution to 10.0 mL with 0.01M oxalic acid solution (pH 3.00). (*2*) *20, 10 ng/mL.*—Dilute 1.0 mL of 200 ng/mL and 100 ng/mL solutions, respectively, to 10.0 mL with 0.01M oxalic acid solution (pH 3.00).

Preparation of Sample

Add 2.0 ±0.02 g thawed muscle tissue, 12.0 mL dried ethyl acetate, and 2.0 g sodium sulfate to 50 mL disposable centrifuge tube.

Spike samples at this point for recovery studies. For 10 or 20 μg/kg spiked samples, add 20 or 40 μL fortification solution, respectively. For 50 or 100 μg/kg spiked samples, add 10 or 20 μL of 10 μg/mL oxolinic acid.

Homogenize contents of centrifuge tube with probe at medium speed for ca 1 min or until well homogenized. Centrifuge 10 min at 1500 rpm (530 × *g*).

Rinse homogenizer probe with 12 mL dried ethyl acetate in 20 × 150 mm disposable culture tube, and reserve rinse for second sample extraction. Wash probe with distilled water. Discard water and wipe probe thoroughly before next sample is homogenized. Carefully decant supernatant from centrifugation into 16 × 125 mm culture tube, and place tube into well of dry bath at 50–55°C. (For better heat transfer, well may contain few drops of water.) Rinse gas discharge nozzles with small amount of methanol and wipe them clean. Place them above surface of liquid in tubes. Use slow gas flow to avoid splashing. Evaporate until oily liquid film remains.

Rehomogenize sediment left in centrifuge tube, using ethyl acetate rinse from above. Rinse probe with distilled water and wipe thoroughly between samples. Centrifuge tubes again for 10 min at 1500 rpm (530 × *g*).

Transfer top layer into appropriate tube that was used previously to evaporate first extraction. Evaporate until oily liquid film remains.

Redissolve residue in 16 × 125 mm culture tube, using mixture of 2.0 mL 0.01M oxalic acid (pH 3.00) and 2.0 mL hexane. Cap tube and swirl liquid after each addition (vigorous shaking may cause emulsion), ensuring that walls of tube are thoroughly rinsed.

Centrifuge at 2600 rpm (870 × *g*) for 10 min. Discard upper layer and transfer only clear aqueous liquid into tightly capped vial (LC autosampler vial, if one is used). Sample preparations are stable 2–3 days in refrigerator.

Chromatography Suitability Test

Inject 200 μL 0, 10, 20, 50, and 100 ng/mL oxolinic acid standards (0 is control consisting of 0.01M oxalic acid, pH 3.00). Retention time for oxolinic acid should be 3.5 ±0.5 min. Run 5 sets and calculate relative standard deviation (RSD) at each concentration (RSD should not exceed 20%).

Although RSD of 5–20% is theoretically acceptable, it may indicate instrumental problems. Standard curve with peak height (y) vs concentration (x) should have overall R^2 ≥0.98. Control injection should give no visible peak with retention time same as, or close to, that of oxolinic acid.

Chromatography of Unknowns

Inject 200 μL aliquots of standard solutions and sample preparations. Chromatograph, using one injection of each oxolinic acid standard solution equivalent to 0, 10, 20, 50,

and 100 ng/mL at beginning, and one at end of each run. Calculate linear regression, $y = b_0 + b_1x$, using peak heights (y, dependent variable) vs concentration (x, independent variable). From y-intercept (b_0, constant) and (b_1, coefficient) of calibration curve, calculate concentration of unknowns, using the formula:

$$x = \frac{Y_{obs} - b_0}{b_1}$$

Results and Discussion

All collaborators were able to meet the system suitability criteria for resolution, reproducibility, and coefficient of correlation. Two of the 5 laboratories reported slightly longer retention times. One laboratory (D) was using a column of the same type as the recommended column, except that it was longer (250 mm), and was also pumping at half the suggested flow rate, resulting in a longer retention time without significantly changing the k'. This laboratory also used peak areas instead of peak height, as recommended. When used in a consistent manner, these 2 methods are generally considered to be equivalent. The second laboratory (B) used a slightly weaker mobile phase (less organic mixture) to avoid a problem with peak symmetry.

Although the instructions given to the collaborators specified that all extracts were to be chromatographed in duplicate, some of the laboratories submitted single injections only. Some official methods recommend duplicate injections on each extract as an instrumental check, whereas others require single readings only. Thus, for consistency, only the first value of any duplicate submitted was taken into consideration for the calculations.

Data from collaborators were statistically evaluated according to AOAC guidelines (4). Thus, Youden and Steiner's formulas (5) were used to calculate the repeatability (within-laboratory precision, sr) and the reproducibility (among-laboratory precision, sR). Outliers were determined by using the Grubbs test (6, 7). Table 1 shows the results from all collaborators for the recovery study and the assays of the incurred sample, as well as their statistical evaluation. The mean recoveries ranged from 77.2 to 84.5%, with repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) ranging from 5.7 to 10.9%, and 11.5 to 18.3%, respectively. The overall mean recovery for the entire range was 81.4%, with RSD_r of 8.0% and RSD_R of 13.7%. All within-laboratory mean values are within the recommended limits of 60–110% for recovery, and they are under 20% for within-laboratory RSD at levels less than 100 μg/kg (8). One laboratory (D) reported values for both incurred samples that were significantly lower than those of the others. However, inclusion of these results did not increase the RSD_r and RSD_R beyond the acceptable limits.

Figure 1 shows typical chromatograms from one collaborator. Oxolinic acid eluted as a symmetrical peak with a retention time of 3.2 min. The salmon muscle extract did not reveal any interference.

Collaborators' Comments

All collaborators commented favorably on the method. Most of them found it straightforward and easy to use.

The suggestion of collaborator D to clarify the residue redissolution step was accepted. The last sentence of the paragraph was changed to read: "Cap the tube and swirl the liquid after each addition...."

The fish samples were packed in dry ice and sent by air, to be delivered the day after or, in the case of Japan, the second day after packing. All samples were still frozen when they arrived at their destinations.

No optimization of excitation and emission wavelengths was required according to the original instructions. Nevertheless, 3 of the 5 laboratories did so because of the large variety of available fluorometer types, and this requirement should be included in the method.

One laboratory commented that the method meets the Center of Veterinary Medicine (CVM) guidelines for recovery and precision for residue levels at 10–100 μg/kg.

Recommendation

We recommend that the LC/fluorometric detection method described in this report for the determination of oxolinic acid residue in salmon muscle be acceptable for regulatory purposes in Canada.

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Bacillus stearothermophilus Disk Assay for Determining Ampicillin Residues in Fish Muscle

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The *Bacillus stearothermophilus* disk assay for penicillin in milk (AOAC official method) was adapted for the determination of ampicillin in fish muscle. The method was evaluated in 2 species of cultured fish: channel catfish and striped bass. Recoveries of ampicillin ranged from 99 to 104% when muscle specimens from both species were spiked at concentrations of 0.025–1.00 μg/g. The lower limit of determination (LOD) was 0.025 μg/g. The assay was applied to monitor the elimination of ampicillin from the muscle of striped bass after intravascular administration (dosage of 10 mg/kg body weight). The mean concentrations in the muscle declined from 1.160 μg/g at 2 h to 0.063 μg/g at 18 h. The half-life of ampicillin in the muscle was 3.6 h. Ampicillin concentrations were below LOD at 24 h. No inhibitory activity was observed in the muscle of control fish.

Several microbiological methods have been developed for detection of drug residues in foods, most of which are based on bacterial growth inhibition. The *Bacillus stearothermophilus* disk assay has been used extensively to determine the presence of penicillin residues in milk. Although relatively unsophisticated compared with chemical methods of analysis such as liquid chromatography (LC), this assay is rapid and sensitive enough (to about 0.010 μg/mL in milk) for residue monitoring. The AOAC method incorporates a confirmatory step (β-lactamase treatment), which offers a degree of specificity to this assay (1).

Penicillins are not presently approved by the U.S. Food and Drug Administration for use in aquatic food species. In Japan, however, ampicillin is used to treat pseudotuberculosis (caused by *Pasteurella piscicida*) in cultured food fish (2). This disease has caused massive mortalities of both feral (3) and cultured (4) populations of striped bass in the United States; therefore, ampicillin may have similar therapeutic applications in this country. The approval and use of drugs in food fish necessitates the development of methods for residue analysis and depletion.

The present study evaluates the *B. stearothermophilus* disk assay for the quantitative analysis of ampicillin in the muscle of channel catfish and striped bass (1). Sensitivity of the assay and extraction efficiency were determined in spiked muscle from both species. The method was further evaluated for the quantitation of incurred residues of ampicillin in the muscle of striped bass.

Experimental

Apparatus and Reagents

Apparatus and reagents were the same as described in the quantitative disk method for penicillin in milk in *Official Methods of Analysis* (1), with the following modifications.

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(a) *Ampicillin (sodium salt).*—Used in preparation of standard curves and dosing of fish (Sigma Chemical Co., St. Louis, MO 63178). Solutions were prepared fresh daily.

(b) *Phosphate buffer (pH 6.0).*—Used in preparation of standard curves and in muscle extraction. Prepared according to AOAC method (1).

(c) *Fish anesthetic (tricaine methanesulfonate).*—(Sigma.)

(d) *Homogenizer.*—Fish muscle was homogenized with a Polytron PT 10/35 homogenizer with PTA 10S generator (Brinkmann Instruments, Inc., Westbury, NY 11590).

Animal Treatment

Striped bass (*Morone saxatilis*) and channel catfish (*Ictalurus punctatus*) were supplied by Southeastern Fish Cultural Laboratory, U.S. Fish and Wildlife Service, Marion, AL. These fish were not previously treated with antibiotics. They were transferred to the laboratory and acclimated in 90 L aquaria at 22°C.

Channel catfish were not treated with ampicillin. Several fish were euthanized, and muscle specimens were collected for use in spiking studies.

Eighteen striped bass, mean body weight 570 ± 90 g, were given 10 mg/kg ampicillin. Ampicillin dosing solution (20 mg/mL) was prepared in 0.85% NaCl. Fish were anesthetized in a tricaine methanesulfonate (0.15 g/L) bath and weighed before ampicillin administration. The dosing solution was administered (0.5 mL/kg body weight) intravascularly by syringe at the caudal peduncle. Control fish were given the saline solution without ampicillin. Three fish were euthanized at 2, 4, 8, 12, 18, and 24 h. Muscle specimens were collected and frozen until analysis (within 3 days). Muscle elimination data (2–18 h) for striped bass were adequately described by a monoexponential equation, using PCNONLIN software (Statistical Consultants Inc., Lexington, KY).

Extraction

A 5 g portion of fish muscle was weighed, chopped into 0.5 g portions, and homogenized (speed setting of 6 for 1 min) in 20 mL phosphate buffer. The homogenate was centrifuged for 15 min at ca 2000 × *g*. A 2 mL aliquot of supernatant was heated at 82°C for 2 min. Solution was recentrifuged and 90 μL aliquots were analyzed according to the AOAC method, including the confirmatory β-lactamase step (1). Supernatant was diluted with buffer if zone of inhibition exceeded range of standard curve.

Standard Curve and Recovery of Ampicillin

Ampicillin stock solution (1 mg/mL) was prepared and diluted in phosphate buffer. Control muscle specimens (5.0 g) were spiked to yield 0.025, 0.050, 0.100, 0.250, 0.500, and 1.00 μg/g. Ampicillin solutions used in spiking were dispersed over chopped muscle specimens before homogeni-

Table 1. Recovery of ampicillin from spiked fish muscle (μg/g)

Ampicillin added	Ampicillin rec. (mean ± SD, n = 4)
0.025	0.026 ± 0.001
0.050	0.051 ± 0.001
0.100	0.100 ± 0.001
0.250	0.252 ± 0.002
0.500	0.512 ± 0.002
1.000	1.012 ± 0.006

zation. The lowest concentration gave a mean zone diameter of 16.0 mm, which was considered the lower limit of determination. Ampicillin recoveries from spiked muscle were calculated by comparing zone diameters with those of corresponding concentrations of ampicillin in buffer.

Results and Discussion

Ampicillin was completely recoverable (99–104%) from spiked muscle specimens of channel catfish and striped bass. A linear relationship was found between zone diameter and the log concentration. Comparison of regression lines by covariance analysis (5) revealed no significant differences (P > 0.05) between fish species; therefore, the recovery data were combined (Table 1). Data for zone diameter (mm) vs log concentration (μg/g) were well described by the following equation:

$$\text{zone diameter} = 7.95\ (\log \text{concentration}) + 28.8$$

The limit of determination was 0.025 μg/g. A limit of detection of 0.03 μg/g in fish tissue has been determined by LC (6).

The inhibitory activities of extracts from spiked muscle (and from the muscle of treated fish) were completely eliminated by β-lactamase treatment. None of the muscle specimens from control fish (striped bass or channel catfish) produced zones of inhibition. A limited market survey of farm-raised channel catfish (n = 89) also failed to reveal any inhibitory activity in the edible portions. The assay is considerably more sensitive to β-lactam drugs than to other antibiotics. For example, equivalent zones of inhibition were produced when the concentrations of tetracycline were 50- to 100-fold greater than those of ampicillin (7).

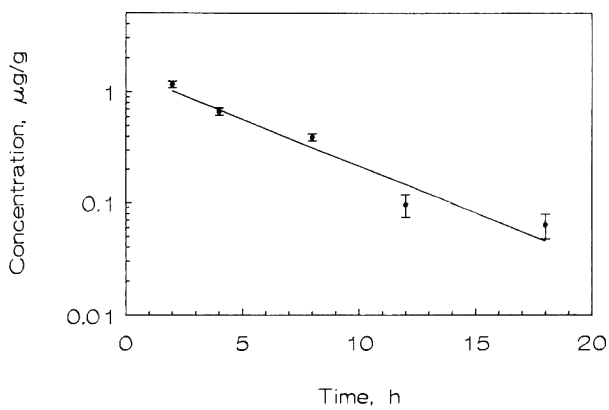

Figure 1. Elimination of ampicillin from muscle of striped bass after intravascular administration (10 mg/kg). Values are mean ±SD of 3 fish/interval.

Ampicillin was rapidly eliminated from the muscle of striped bass after intravascular administration (Figure 1). Mean concentrations (± standard deviation) declined from 1.160 ±0.04 μg/g at 2 h to 0.063 ±0.016 μg/g at 18 h. At 24 h, muscle concentrations were below the limit of determination. Muscle elimination data were fitted to the following monoexponential equation:

$$C_t = 1.50e^{-0.195t}$$

where C_t is the muscle concentration (μg/g), and t is the time after dosing (h). The elimination half-life based on this equation was 3.6 h. In cows (8) and in humans (9), the half-life of ampicillin in the plasma is about 1.2 h.

Dosage administered in the present study was within the effective therapeutic range for treatment of pasteurellosis in fish, although the drug is typically administered orally (10). Rate and extent of drug absorption (i.e., bioavailability) would affect the concentrations in the tissues; however, elimination of a drug is assumed to be the same for oral and intravascular routes of administration (11). The oral bioavailability of ampicillin in striped bass has not been established. In humans, the value is about 62% (9).

Drugs and other xenobiotics are often highly concentrated in the bile. Examination of this excretory fluid may be useful for assessing exposure of a food animal to a given chemical. In preliminary studies, the disk assay was evaluated for the quantitation of biliary concentrations of ampicillin. Analysis of undiluted bile specimens from control fish revealed strong inhibitory activity that was not eliminated by the heating or enzymatic treatment steps. In the striped bass study, dilution (1:10 000) of bile with phosphate buffer eliminated the inhibitory activity in control fish. Strong inhibitory activity remained in bile of treated fish, however, suggesting significant biliary excretion of ampicillin. Because of the nonspecific inhibitors in control bile, this assay may have limited application for use with this fluid.

The present study demonstrates the applicability of the *B. stearothermophilus* disk assay for the determination of ampicillin and its persistence in fish muscle. The method is sensitive and capable of quantitating active compound. It may prove useful for the rapid screening of aquaculture products for penicillin drug residues and for ensuring sufficient withdrawal times after disease treatment.

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Comparison of Automated Liquid Chromatographic and Bioassay Methods for Determining Spiramycin Concentration in Bovine Plasma

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The performance of a liquid chromatographic (LC) method for spiramycin measurement in bovine plasma has been compared with that of a microbiological method. Plasma samples were obtained from cattle administered spiramycin intravenously. Comparison tests used were intraclass correlation (r_i), correlation (r), and Student's paired t-test. For concentrations lower than 2.5 IU/mL, microbiological values were higher than LC values. This difference in results modified pharmacokinetic interpretation and might be explained by the presence of microbiologically active metabolites.

Spiramycin is a macrolide antibiotic used in veterinary practice for the prevention and treatment of animal diseases caused by Gram-positive bacteria and some mycoplasma (1). Plasma was assayed by microbiological techniques for pharmacokinetic studies (1, 2). An automated liquid chromatographic (LC) determination of spiramycin by direct injection of plasma, using column switching for sample cleanup, was developed and used for the determination of spiramycin I in human plasma with a sensitivity of 50 ng/mL (3).

This LC technique was modified by the use of an automated LC sample preparation/injection system. The present study was undertaken to obtain pharmacokinetic data from treated cattle and to compare results obtained by the LC method with those obtained by bioassays.

Liquid Chromatographic Method

Apparatus

(a) *Liquid chromatograph.*—Advanced automated sample processor (AASP™ module) with 50 µL injection loop (AASP-LC module); pneumatic processing station (PrepStation); AASP cassette C2; Model 5020 liquid chromatograph; Model 2050 UV wavelength detector set at 231 nm (Varian Instrument Group, Sunnyvale, CA 94034).

(b) *LC column.*—LiChrospher-100 Rp8 column, 12.5 × 4 mm, 5 µm particle size, 4 × 4 mm guard column containing the same sorbent (E. Merck, Darmstadt, Germany).

(c) *Stirrer.*—Heidolf Reax 2.

(d) *Rotary stirrer.*—Vortex.

(e) *Centrifuge.*—Model GR4.11 (JOUAN S.A., St. Herblain, France).

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

Reagents

(a) *Solvents.*—Tetrahydrofuran, analytical grade (Merck); acetonitrile (Carlo-Erba/Fisons, Valencia, CA 91355).

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

(c) *Sulfuric acid.*—0.5%. Dilute 0.5 mL sulfuric acid (Merck) to 100 mL with water.

(d) *Spiramycin analytical standard.*—Free base (4133 IU/mg) (Batch CA8408700, Rhône-Mérieux).

(e) *Spiramycin stock solution for LC.*—2066.5 IU/mL. Weigh 50 mg spiramycin into 100 mL volumetric flask, dissolve with 5 mL methanol, and dilute to 100 mL with water.

(f) *Spiramycin working solutions.*—Pipet 0.1, 0.5, 1, 2.5, and 5 mL aliquots of stock solution into separate 50 mL volumetric flasks and dilute to volume with water. These solutions contain 4.133, 20.66, 41.33, 206.6, and 413.3 IU spiramycin/mL, respectively. Store in a cold, dark place when not in use.

(g) *Spiked plasmas.*—Add 1 mL of each working solution to 9 mL antibiotic-free bovine plasma, and mix gently with a stirrer at 45 rpm for 30 min. These spiked plasmas contain 0.4133, 2.066, 4.133, 20.66, and 41.33 IU spiramycin/mL. Freeze at −20°C pending analysis.

(h) *Mobile phase.*—0.5% Sulfuric acid–acetonitrile (79 + 21, v/v).

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Determination

Extraction.—Thaw plasma samples and centrifuge at 5900 × *g* for 5 min. Load cassette C2 on PrepStation, apply 0.5 mL 4% acetonitrile to each cartridge, and add 0.5 mL plasma and 0.5 mL 4% acetonitrile. Drive samples through sorbent with pressure manifold (Nitrogen 100 kPa). Wash each cartridge of the cassette with 1 mL 4% acetonitrile and store in refrigerator (4°C) pending analysis.

Injection.—Load cassette into AASP-LC module, programmed as follows: Purge before injection with four 25 μL portions of water, purge after injection with twenty 25 μL portions; injection valve in "inject position" (valve reset): 1 min.

Cassette cleanup.—Before first use, wash each cassette cartridge successively with 1 mL tetrahydrofuran, 1 mL acetonitrile, and 1.5 mL water. Between each use, wash cassette cartridges with 1.5 mL 0.5% sulfuric acid–acetonitrile (2 + 1, v/v).

Calculations.—Calculate amount of spiramycin from ratio of peak area observed for sample to peak area of spiked plasma prepared and applied in the first 2 cartridges of each cassette.

Microbiological Method

Apparatus and Reagents

(a) *Bioassay medium.*—Antibiotic medium 11 (Difco Laboratories, Detroit, MI 48232).

(b) *Bioassay plates.*—140 mm diameter (C.A. Greiner & Sohne, Labortechnik, Frickenhausen, Germany).

(c) *Steel cylinder.*—6 mm id × 8 mm od × 10 mm height.

(d) *Vision system for diameter measurement.*—AMS 40–10 (Système Analytique) with PC 110 computer (Hermes).

(e) *Bioassay standards.*—Prepare spiramycin stock solution, 2000 IU/mL, with 100 mL water–methanol (95 + 5). Dilute stock solution with pH 8 phosphate buffer to obtain concentrations of 2, 4, 8, 16, 32, and 25, 50, 100, 200, and 400 IU/mL. Further dilute these solutions in antibiotic-free bovine plasma to obtain standards.

(f) *Bacterial spores.*—Suspend *Sarcinea lutea*, ATCC 9341, in 9 °/oo saline water to obtain 0.5 optical density at 546 nm.

(g) *Plates.*—For the first standard curves (0.2, 0.4, 0.8, 1.6, and 3.2 IU/mL), inoculate agar medium No. 11 with 1 mL bacteria suspension for 100 mL medium. Pour 20 mL inoculated agar into each petri dish and let the agar layer harden on a level surface (Method 1). For the second standard curve (2.5, 5, 10, 20, and 40 IU/mL), inoculate agar medium with 5 mL bacteria suspension for 100 mL medium. Pour 40 mL inoculated agar into each petri dish (Method 2).

Assay

Place 0.2 mL standard test solution in each steel cylinder. For the first standard curve, allow to prediffuse 2 h at 20°C. Incubate overnight at 37°C. Use first standard concentrations for samples expected to be lower than 3.2 IU/mL, and second concentrations for samples higher than 2.5 IU/mL. Test sample undiluted. If concentration is expected or found to be greater than highest concentration of standard, dilute sample with antibiotic-free bovine plasma to an estimated concentration close to reference concentrations. Proceed as for standard, filling 5 alternate cylinders with reference concentrations and 4 cylinders with samples. Use 5 plates for each sample. Read inhibition diameter with vision system, and calculate corresponding concentration. When sample is diluted, multiply by dilution factor to obtain final concentration.

Experimental

Recovery of spiramycin I from plasma by LC.—For recovery study, 9 cassettes with spiked plasmas (2 cartridges per concentration) were prepared and injected on separate days. Before each cassette analysis, 50 μL working solution (41.33 IU/mL) was injected into loop of the LC system, and peak area, AA, was measured. For each concentration C_i, peak area A_i was measured. Concentration Y_i was calculated by the following formula:

$$\text{Spiramycin } Y_i \text{ (IU/mL)} = [(A_i \times 50)/(AA \times 500)] \times 41.33$$

To calculate recovery ($R\%$), the following formula was used:

$$R\% = 100 \times (Y_i/C_i)$$

Linearity of LC method.—A plot of concentration of spiramycin found (Y_i) against the concentration of spiramycin 1 added (C_i) was used to calculate the linear regression equation.

Accuracy and precision of the microbiological method.—Method precision and method accuracy were evaluated with 2 seeded control samples for each standard curve (0.5 and 3 IU/mL for the first standard curve, and 3 and 16 IU/mL for the second standard curve). Each control sample was determined after 4 separate runs of 10 determinations.

Comparison of LC and bioassay methods.—Three bullocks, each weighing 207 ±20 kg, were used. Spiramycin (Suanovil 20, Rhône-Mérieux) at concentrations of 100 000 and 50 000 IU/kg was injected intravenously, with a 3 weeks interval between treatments. Blood samples were collected at regular intervals after administration. Each plasma sample was assayed twice by each technique. Mean estimations by microbiological methods (MB_1 for Method 1 or MB_2 for Method 2) and by the LC method (MH) were calculated for each plasma sample. Statistical evaluation of agreement between LC and bioassay results for spiramycin concentration in plasma of treated cattle was determined by calculating intraclass correlation (r_1) (4), correlation (r), and Student's paired t-test.

Differences between mean concentrations obtained by microbiological methods (MB_1 or MB_2) and LC method (MH) were calculated for each sample. The mean spiramycin concentration for each plasma sample from the treated animals was evaluated as follows:

$$\text{Mean} = [(MB_1 \text{ or } MB_2) + MH]/2$$

The coefficient of variation (CV) for each value obtained by LC and by microbiological assay was calculated as follows:

$$CV = 100 \times [(Y_1 - Y_2)/\sqrt{2}]/[(Y_1 + Y_2)/2]$$

where Y_1 and Y_2 are values obtained for each plasma sample of treated animals by each method (Bioassay Method 1 or 2 and LC method).

Table 1. Determination of spiramycin in spiked bovine plasma by liquid chromatographic method[a]

Concn in spiked plasma, IU/mL	N	Mean concn found, IU/mL	Rec., %[b]	CV %
0.41	14	0.331	80.32	17.0
2.06	16	1.698	82.17	5.1
4.13	18	3.438	83.19	4.7
20.66	18	17.961	86.91	6.4
41.33	18	36.602	87.17	7.6

[a] N = number of measurements.
[b] Mean recovery = 84.17 ± 7.6%.

Results and Discussion

LC Method

Absorbance was measured at 231 nm as suggested by Dow et al. (3), who reported that using 4% acetonitrile will allow spiramycin to be stabilized in plasma. Although the cycle of rinsing with water after injection was carried out, no interference was observed, probably because of the use of AASP, which allows the sample purification to take place on the cartridge. The cartridge is moistened by the water used at the beginning of the cycle before the eluant passes. This procedure permits assays to be performed over a long period without the cartridge drying out. As the technique is automated, the apparatus can remain in continuous activity. Also, preparation on a cassette lengthens the life span of the guard column.

Recovery of Spiramycin by LC

Recovery from spiked plasmas ranges from 80.32 to 87.17%, depending on the concentration (Table 1). This rise in the recovery level was observed by Dow et al. (3), who used an internal standard, whereas we used spiramycin I as external standard. Only spiramycin I needs to be assayed in plasma, because the drug formulation administered to animals contains chiefly this form of spiramycin (5).

Linearity and Precision

The regression line of concentrations observed (Y_i) plotted against the concentrations added (C_i) follows the equation:

$$Y_i = (0.8742 \times C_i) - 0.1074$$

The regression coefficient is $r = 0.9952$.

Table 2. Determination of spiramycin in spiked bovine plasma by 2 microbiological methods (Method 1, low concentrations; Method 2, high concentrations)[a]

Measurement	Method 1		Method 2	
Concn in spiked plasma, IU/mL	0.5	3	3	16
Mean concn found, IU/mL	0.51	3.25	3.13	15 67
Rec., %	101.30	108.42	104.31	97.98
CV, %	7.5	7.8	6.00	5.40

[a] 40 measurements by concentration.

Mean concentrations and their CVs are given in Table 1. The highest CV, 17%, is observed with the lowest concentration, 0.4 IU/mL. For other concentrations, CVs do not exceed 8%.

Microbiological Assay

Recovery was between 90 and 110% for each microbiological method. Precision of the microbiological assay was good; the CV was less than 10% at each control concentration (Table 2).

Comparison of Techniques

The correlation between mean concentrations of spiramycin in plasma of treated cattle determined by LC and those obtained by microbiological assay is described in Figure 1. A logarithmic scale was used to estimate measurement dispersion over the entire assay area. The correlation straight lines of microbiological values (Y) against the LC values (X) were calculated for each microbiological method, as well as intraclass correlation (r_1) and Student's paired t-test.

Assay ranges for concentrations <2.5 IU/mL (Method 1).—The regression line was as follows:

$$Y = 0.0823 + 1.156 \times X \qquad d.f. = 67$$

The slope does not significantly differ from 1. The intercept does not significantly differ from 0. Intraclass correlation, however, was 0.91352, with the r_1 interval ranging from 0.5070 to 0.9711. Significant differences are observed by variance analysis and the Student's paired t-test. Microbiological assays give significantly higher concentrations than the LC method for the low concentration of spiramycin in plasma of treated cattle.

Assay ranges for concentrations >2.5 IU/mL (Method 2).—The regression line was as follows:

$$Y = 0.375 + 1.006 \times X \qquad d.f. = 161$$

The slope is not significantly different from 1. The intercept significantly differs from 0 ($p<0.001$; Student's t-test). A constant error is detected by this statistical method. The intraclass correlation, however, was 0.9804; the lower limit of r_1 was 0.9679. The 2 techniques show good agreement for high spiramycin concentrations in plasma of treated cattle, and results were not significantly different for the paired Student's t-test.

A plot method (Figure 2A, B) showing the difference of spiramycin concentration in plasma of treated animals ($y_2 - y_1$) vs mean $(y_1 + y_2)/2$, where y_1 = mean result of LC analysis, and y_2 = mean result of bioassay analysis. For high concentrations under the microbiological standard curve (>40 IU/mL), large differences between techniques were observed. Such differences can be explained by the dilution of plasma greater than 40 IU/mL for bioassay or LC in the range of standard curves. For low standard concentration curves (0.2–3 IU/mL), bioassay Method 1 gives higher results than the LC method.

The divergence between correlation (r) and the intraclass correlation test were explained as follows: Product-moment correlation r measures the relationship or trend between 2 quantitative variables; intraclass correlation is an index measurement of the agreement of results obtained with different techniques (4). If high variability in the measurement was

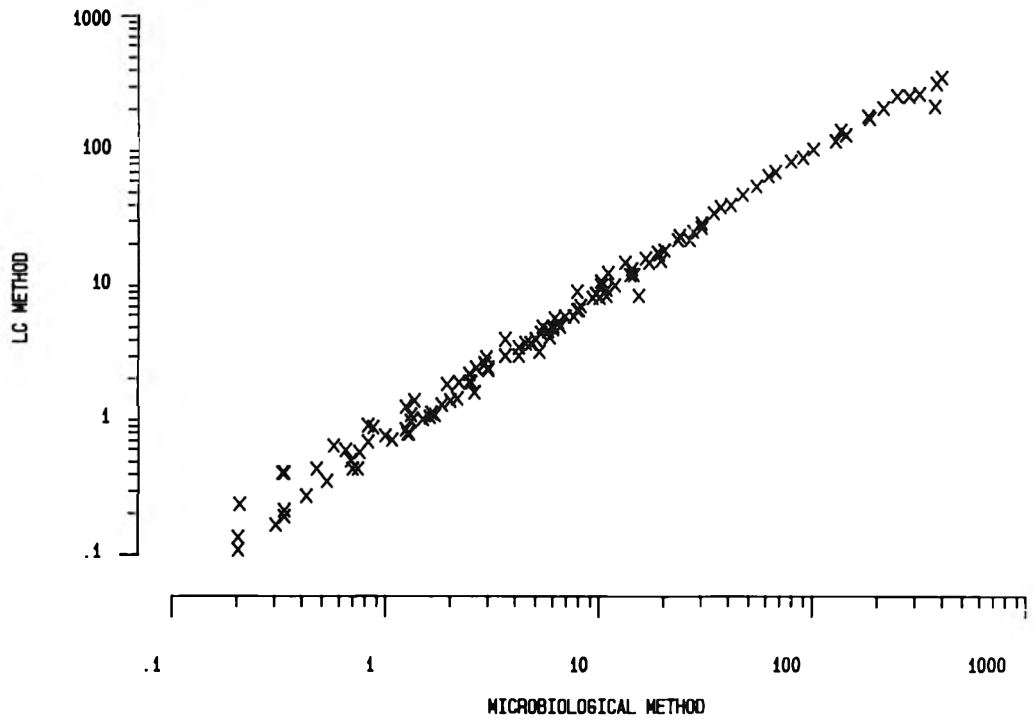

Figure 1. Mean plasma concentration assayed by microbiological methods (X-axis) plotted against mean plasma concentrations determined by LC method (Y-axis).

observed for a method, differences between intercept and zero or slope and 1 cannot be significant, although differences by pairs were significant. Intraclass correlation was an interesting test for comparison of analytical methods. In our study, this test detects a difference between bioassay and the LC method for low spiramycin concentrations in plasma. A similar difference between LC and microbiological measurements of spiramycin was observed with spiramycin in human serum by Harf et al. (6). In this study, results were 5-fold higher by microbiological measurement than by LC for spiramycin concentrations lower than 3 IU/mL.

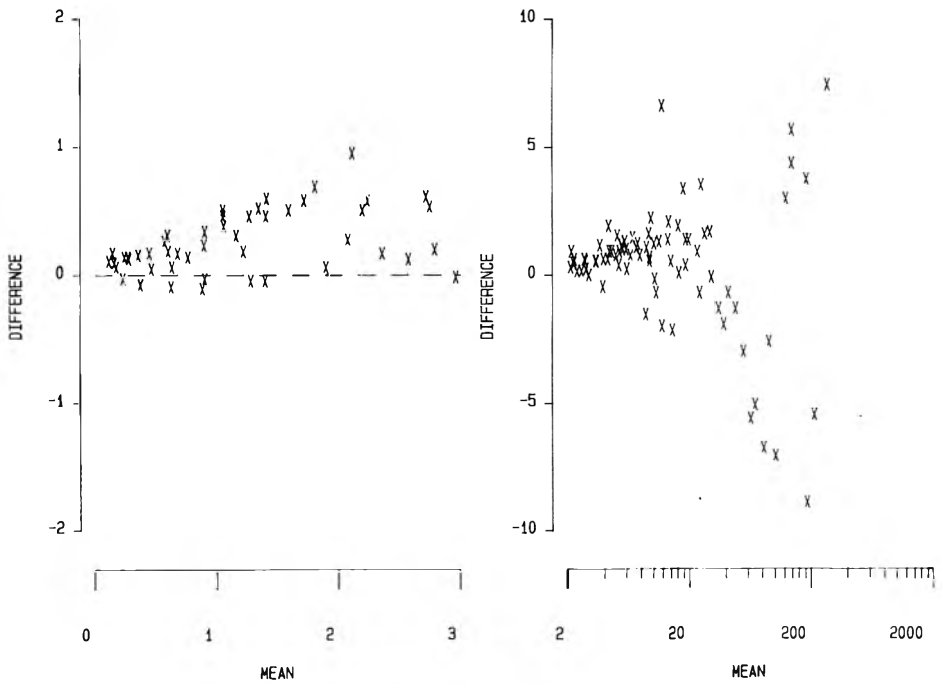

Figure 2. (A) Relationship between difference ($MB_1 - MH$) and mean [($MB_1 + MH$)/2] spiramycin concentrations in treated cattle plasma, obtained by bioassay with low standard curve (Method 1) and those obtained by LC. (B) Relationship between difference ($MB_2 - MH$) and mean [($MB_2 + MH$)/2] spiramycin concentrations in treated cattle plasma, obtained by bioassay with high standard curve (MB_2) and those obtained by LC.

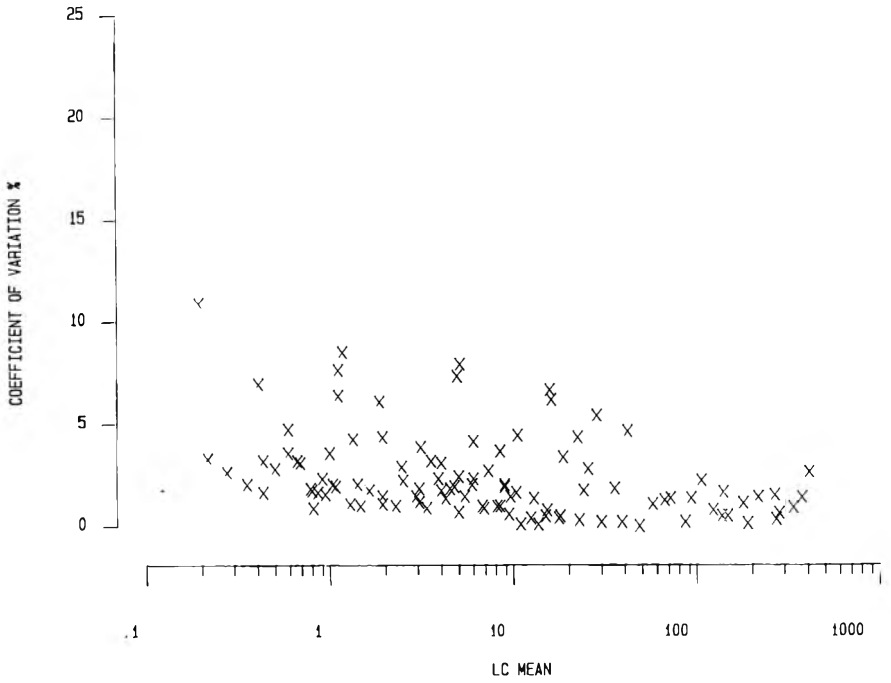

Figure 3. Distribution of CV against mean plasma concentration of spiramycin in treated cattle plasma, determined by LC.

Figures 3 and 4 show a plot of distribution of the CV for each measurement of spiramycin concentration in plasma of treated cattle obtained by LC and by microbiological assay.

LC determinations are less subject to variation than those obtained by microbiological assay, because 59.4% of the assays have a CV less than 2%, whereas this percentage is less than 25% for microbiological assays. The LC method was more precise than bioassay methods.

Pharmacokinetics

The curve of concentrations obtained by each technique against time for 1 animal is presented in Figure 5. The areas under the curve (AUC) were calculated from the results by LC and the microbiological assay. The ratio of AUC estimated by each technique should be close to 1 if there is no interfering bias. The mean ratio calculated for 3 animals and

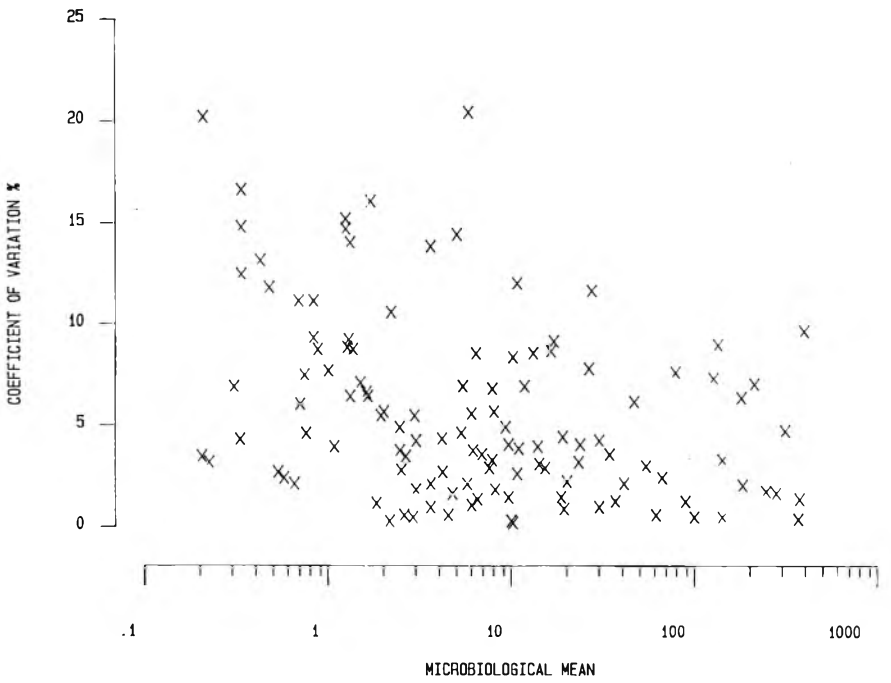

Figure 4. Distribution of CV against mean plasma concentration of spiramycin in treated cattle plasma, determined by microbiological assay.

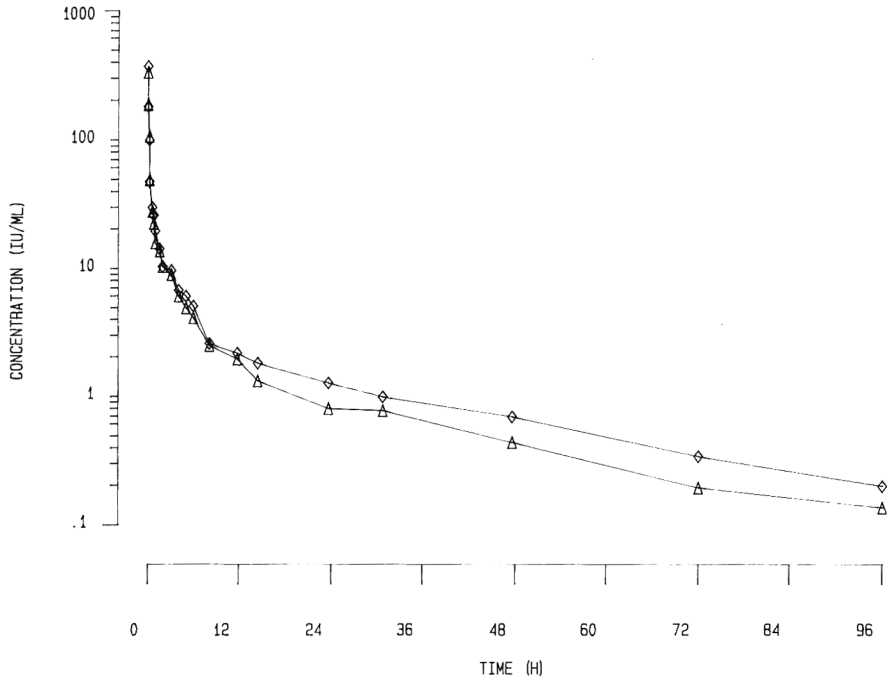

Figure 5. Comparison of plasma concentration time curves of spiramycin I determined by LC method (Δ) and microbiological methods (◊) in a representative bullock after intravenous injection of Suanovil 20 (100 000 IU/kg).

2 trials with different dosages (50 000 and 100 000 IU/kg) was 1.167. This value is significantly different from 1 (t = 4.51, $d.f.$ = 5, $p<0.01$). The evidence for bias during the correlation study influences the value of AUC and the total body clearance, which is normal, because Cl = Dose/AUC. No differences were found either in the estimation of the final slope or in the estimation of the distribution volumes.

The origin of bias cannot be explained only by the use of dilutions when assaying high concentrations, because variation was symmetric around 0 (Figure 2b). Bioassay measures potency of plasma for inhibition of bacterial growth; LC measures only spiramycin I concentration. If another substance, such as metabolite of spiramycin, has microbiological activity, its presence in plasma may explain the difference.

Conclusion

Recovery by the LC method was greater than 75% with a relative standard deviation less than 10%. The linear range of calibration curves was large enough to encompass pharmacokinetic study. The recommended precision of determination should generally be less than 10%. In addition, the LC method was simple, and sample preparation time was less than that for the bioassay method. Agreement between the 2 techniques was poor for low concentrations and had an effect on determination of pharmacokinetic parameters. However, bioassay estimates the kinetics of microbiological effect, whereas LC estimates the kinetics of the spiramycin I molecule. Each method provides information that is different and useful for both the pharmacokinetician and the clinician. The present LC method should be appropriate for the measurement of spiramycin I in plasma because it meets several conditions for analytical methods that are intended to suit the pharmacokinetician's needs (7). Clinicians, whose concern is the pharmacodynamic effect of the drug, will be more interested in the information given by the microbiological method.

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FEEDS

Analytical Evaluation of the Globulin Proteins of Cottonseed Meals

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A method for the analytical evaluation of the globulin (salt-soluble) fraction of cottonseed meals has been developed. Cottonseed of 14% moisture content is stored for 6 and 9 weeks at 50°C. Meals obtained after seeds are defatted with pet ether are analyzed for nitrogen content, and then extracted with water. Residues are then extracted with 10% NaCl to isolate the globulin fraction. This fraction is characterized by liquid chromatography (LC) and gel electrophoresis (LDS-PAGE). Kjeldahl analyses showed no loss of nitrogen in meals from stored seed compared with those from unstored seeds. However, a large decrease in NaCl extractable nitrogen was noted in meals from stored cottonseed. LC and LDS-PAGE showed that an alteration of the globulin proteins occurred during storage, and the method demonstrated a progressive change in the 11S and 7S components of the globulin fraction.

Cotton is grown principally for its fiber, but the seed is an important by-product. In 1989, 5.8 million tons of cottonseed were produced in the United States, and 3.4 million tons were crushed to yield vegetable oil and meal, with economic values of $270 and $290 million, respectively (1). Although the latter is used principally as a feed for ruminant animals, it can serve as an important protein source in rations for nonruminants, and it has potential as a source of nutrients for human consumption (2). When freshly harvested cottonseeds are processed, both oil and meal are of high quality; however, most of the seed must be stored for weeks or months before it can be processed. During the storage period, biological processes in the seed are not dormant, and breakdown of seed structures occurs that causes deterioration (3). This deterioration could result in adverse changes in oil and meal quality. A recent research program at Southern Regional Research Center was directed toward preventing this deterioration. Decreasing oil quality can be measured easily by noting increases in the free fatty acid (FFA) content and/or refining loss of the oil. Meal quality measurement, however, is based only on protein content as determined by a modified Kjeldahl analysis (4) that measures total nitrogen (%N). Protein is estimated by multiplying %N by the factor 6.25. This method measures total nitrogen, i.e., protein and nonprotein nitrogen; it does not define changes in the protein quality of the meal that may occur as the seed deteriorates. Olcott and Fontaine (5) indicated that more than 80% of the nitrogen, designated by classical nomenclature as globulin proteins, was soluble in sodium chloride. They also determined that there was a correlation between the amount of these proteins and the nutritive quality of the meal. Later studies were directed toward the evaluation of nonstorage and storage proteins (water and alkali soluble nitrogen fractions) (6). The principal goal of these studies was the preparation of protein isolates having specific functional and nutritional properties for use in foods. The objective of the work reported here was to develop a precise, rapid method to evaluate changes in the protein quality of cottonseed meal that occurs during storage of the seed. Using the work of Olcott and Fontaine (5) as a guide, the meals were extracted with water, and then with sodium chloride. The globulin fraction was analyzed by liquid chromatography (LC) and gel electrophoresis (LDS-PAGE).

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Experimental

Preparation of Cottonseed Meal Samples

Whole fuzzy cottonseed (200 g) was equilibrated to 14% moisture. Sample was divided into 3 equal portions. One portion was reserved for the control (0 week storage). The other 2 were placed in closed glass jars in a cabinet equipped with a forced air blower and thermostatically controlled to maintain a temperature of 50 ±2°C. One jar was removed for analysis after 6 weeks, and the other was removed after 9 weeks. If not processed immediately, samples were stored at 0°C until analyzed.

Preparation of Cottonseed Meal

Each sample of whole fuzzy cottonseed was frozen with liquid N_2 and placed in a blender. Contents were given a 2 s burst at full power to separate the fuzzy seed into linters, hulls, and kernels.

Cottonseed kernels were defatted with pet ether (40:1, solvent:kernels) by blending for 2 min in a Stomacher Lab Blender 400 (Tekmar Co., Cincinnati, OH 45222). Contents of the Stomacher bag were filtered to separate oil from meal. Residual meal was washed with 15 mL pet ether that was added to the filtrate. After evaporation of the solvent, oil was analyzed for FFA content. Meal was air dried overnight in the laboratory exhaust hood at ambient temperature (22°C).

Preparation of Protein Extracts

Cottonseed meal samples were ground with a Tekmar Mill, Model A-10 for 2 min. A consistency similar to that of a flour was obtained.

Samples were extracted with distilled water (50:1, solvent:meal) in 50 mL centrifuge tubes by continuous stirring for 30 min at ambient temperature. Contents were centrifuged for 30 min at 12 000 × *g* at 20°C. Supernatant was discarded. Pellets were extracted in the centrifuge tubes with 10% NaCl (25:1, solvent:meal) by continuous stirring for 30

Table 1. Effect of storage of high moisture content cottonseed at 50°C on nitrogen content (%) of defatted meal

	Storage, weeks		
	0	6	9
Total	8.63	8.20	8.58
Soluble[a]	51.88	10.93	3.49

[a] Percent of total nitrogen soluble in 10% NaCl.

min at ambient temperature. Contents were centrifuged for 30 min at 12 000 × *g* at 20°C. Residue was discarded. Supernatants were filtered immediately, using 0.45 and 0.22 μm Millipore syringe filters arranged in sequence. Portions of 100 μL of each extract were diluted with LDS-PAGE sample buffer (1:3 dilution for the 0 week sample, 1:2 for the 6 and 9 week sample), placed in a boiling water bath for 2 min, cooled, stoppered, and frozen until analyzed by LDS-PAGE. Remaining portion of each extract was frozen until analyzed by LC.

Analyses

Free fatty acids.—FFA content of oil was determined according to Official American Oil Chemists Society (AOCS) Method Ca 5a-40 (7).

Kjeldahl analyses.—Kjeldahl analyses were performed on meals and protein extracts according to AOCS Modified Kjeldahl Method Ba 4b-87 (7).

Electrophoresis (LDS-PAGE).—Gel electrophoresis was a modification of Laemmli (8) with lithium dodecyl sulfate (LDS) substituted for sodium dodecyl sulfate (SDS). Electrophoresis was performed in a Hoeffer SE 600 series vertical slab unit with power supplied by a PS 2500 DC Power Supply (Hoeffer Scientific Instruments, San Francisco, CA 94107). Conditions for electrophoresis were constant 20 mA per slab with a running time of 7 h. Gel formulation was 10% acrylamide with crosslinker concentration of 2.7% *bis*-acrylamide. Gels were stained with a 0.25% Coomassie Brilliant Blue R, 40% methanol, and 7% acetic acid solution.

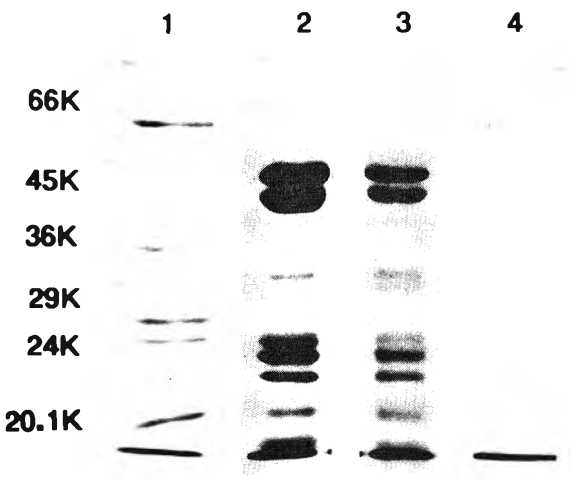

Figure 1. Comparison of electrophoretic patterns of salt-soluble globulin proteins extracted from meals prepared from unstored cottonseed and cottonseed stored at 50°C. (1) Molecular weight standard, (2) 0 weeks storage, (3) 6 weeks storage, and (4) 9 weeks storage.

Liquid chromatography.—Aliquots of the extracts (100 μL) were analyzed, with no further dilution, using a Pharmacia Superose 12 column, 30 cm × 10 mm id (Pharmacia LKB Biotechnology, Piscataway, NJ 08854). Eluant was 10% NaCl, pH 7.0, with a 0.5 mL/min flow rate. System was monitored at 280 nm using a Kratos 770R variable wavelength detector (Applied Biosystems, Inc., Foster City, CA 94404). A 286 PC programmed with Lab Calc software was used for data acquisition and processing (Galactic Industries Corp., Salem, NH 03079).

Results and Discussion

Oils extracted from unstored cottonseed and seed stored for 6 and 9 weeks had FFA contents of 0.8, 2.7, and 3.1%, respectively. This increase in FFA indicated that seed had undergone deterioration during storage.

The total nitrogen content of the 3 cottonseed meals measured by the Kjeldahl method showed no differences (Table 1). Conversely, after cottonseeds were stored for 6 and 9 weeks, there was a dramatic decrease in the amount of nitrogen extracted from the meal with 10% NaCl.

Earlier work has shown that proteins from cottonseed meals consist of 3 components, 11S, 7S, and 2S, as determined by sedimentation coefficients (9). Of these, the 11S and 7S components represent the primary globulin protein fraction (10). Previous investigations with SDS-PAGE showed 3 principal protein subunits of the 11S component. These have molecular weights of 22, 20, and 15K (11, 12). The 7S component has 2 principal subunits of 52 and 46K (11–13).

Results of LDS-PAGE of the globulin fractions extracted from meal prepared from seed stored for 0, 6, and 9 weeks are presented in Figure 1. Using the method of Laemmli (8), electrophoresis results in denaturation and reduction of proteins to their individual polypeptide subunits. Therefore, for the electrophoresis of cottonseed globulins, protein bands in the range of 52 and 46K should appear for the 7S component, and bands in the range of 22, 20, and 15K should be present for the 11S component. Allowing for the possible inherent error (±5%) in LDS-PAGE, these bands are readily apparent in the extract of meal from cottonseed stored for 0 weeks (Lane 2). Lane 3, the 6 week storage sample, shows some disappearance of the 7S component and considerable loss of the 11S component. The sample stored for 9 weeks (Lane 4) shows a complete absence of protein bands. However, the Coomassie Blue used for staining may not detect low levels of protein present in the latter sample.

LC (13–15) has been used for isolation and fractionation of the principal globulins of cottonseed. The elution profile obtained for the 0 week sample in the experiments reported here (Figure 2) does not differ significantly from those previously documented (13–15). Peak I corresponds to an aggregate of large molecular weight proteins, with Peaks II and III corresponding to the 11S and 7S components of the globulins, respectively. Kjeldahl analyses of the NaCl extracts indicate that during storage the amount of extractable globulins decreased dramatically: 1.78 mg N/mL, 0 weeks; 0.36 mg N/mL, 6 weeks; and 0.12 mg N/mL, 9 weeks. There was also a marked decrease in the 11S and 7S components from the samples stored for 6 and 9 weeks.

Table 2. Effect of storage of high moisture cottonseed at 50°C on nitrogen content (%) in water- and salt-soluble and insoluble fractions of cottonseed meals

	Storage, weeks		
Fraction	0	6	9
Water extract	26	9	8
10% NaCl extract	52	11	4
Residue	5	55	71

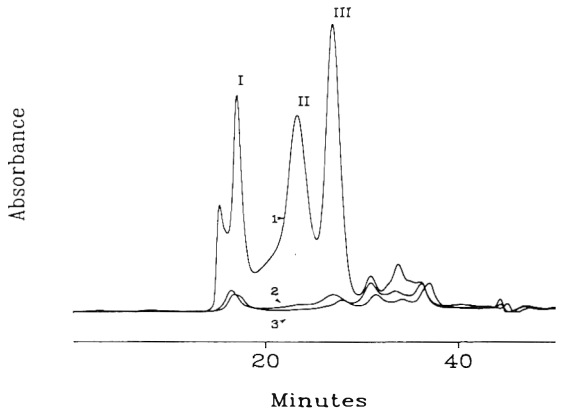

Figure 2. Comparison of LC patterns of salt-soluble globulin proteins extracted from meals prepared from unstored cottonseed and cottonseed stored at 50°C. (1) 0 weeks storage, (2) 6 weeks storage, (3) 9 weeks storage, (I) aggregate of large molecular weight proteins, (II) 11S component, and (III) 7S component.

Because there was no change in total nitrogen content of the meals prepared from unstored cottonseed compared with meals from stored cottonseed, but there was a significant reduction in the globulin fraction of these meals, an attempt was made to determine how the solubility of the globulin fraction had been changed during storage. A second series of samples was extracted, as described in *Experimental*, but the supernatant from the water extraction and residues from the salt extraction were collected rather than discarded. Nitrogen analyses were performed on all fractions. Because of the manipulations and transfers involved, complete recovery of the nitrogen was not expected. However, 73% or more of the total nitrogen was accounted for in each of the samples (Table 2). These data also indicate a significant change in the solubility of the proteins from unstored cottonseed compared with that from stored seed. Initially, 78% of the protein was soluble; however, after storage for 6 and 9 weeks, the total soluble protein was 20 and 12%, respectively. Apparently, as the seed deteriorates, the proteins aggregate together to form nitrogenous compounds insoluble in water and salt rather than being hydrolyzed into small peptides or amino acids.

The reason for the reduced salt solubility of the globular proteins of meals from deteriorated cottonseed and potential effects on the actual nutritive quality of the meal cannot be determined from these data, and this information is beyond the scope of this study. The results indicate, however, that a simple determination of the salt soluble nitrogen content of cottonseed meal could be used to indicate deterioration of the proteins of cottonseed during storage.

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FOOD ADDITIVES

Enzymatic Determination of Free Glutamic Acid in Dried Soups and in Minced Sausages: NMKL[1] Collaborative Study

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An enzymatic method for the determination of free glutamic acid in meat products and dried soups was collaboratively studied in 11 laboratories. In the presence of the enzyme glutamate dehydrogenase, L-glutamic acid is oxidatively deaminated by nicotinamide adenine dinucleotide (NAD) to 2-oxoglutarate. In a reaction catalyzed by diaphorase, the NADH thus formed converts 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride to a formazan, which is measured in the visible range at 492 nm. Fourteen samples (7 samples of minced sausage and 7 samples of dried cauliflower soup) with glutamate contents varying between 0.4 and 16 g/kg were included in the study. Materials were distributed to participants as blind duplicates and as split level pairs. The mean relative standard deviation (RSD_R) for reproducibility for the dried soup material containing glutamate between 7 and 16 g/kg was 4.6%. RSD_R values for samples of minced sausage containing glutamate at lower levels (0.4–1.3 g/kg) were between 12 and 16%.

Glutamic acid and its salts are commonly used as flavor enhancers in many prepared foods. Skurray and Pucar (1) determined the glutamic acid contents of 23 fresh and 39 processed foods. Concentrations of glutamic acid ranged from 0.5 g/kg in cottage cheese to 68 g/kg in beef stock cubes. The maximum content of glutamic acid and its salts in food products is regulated in most countries; the limit in Finland is 2 g/kg. This limit refers to the total content of glutamic acid and glutamates, both naturally occurring and amounts added in the final foodstuff. To control this limit, regulatory and food industry laboratories need reliable analytical methods.

The determination of glutamic acid using an amino acid analyzer is rather expensive and time consuming. The need for simpler and cheaper methods for this purpose was recognized by the Nordic Committee on Food Analysis. The suggestion was made that a suitable enzymatic method be tested for determination of free glutamic acid in meat products and in dried soups (2).

Collaborative Study

Minced sausage and dried cauliflower soup were selected as food types for the collaborative study. The following 7 samples were included in the study: (*1*) unspiked, homogenized material containing only the naturally occurring glutamate of the sample type; (*2*) 2 pairs of blind duplicates (unspiked materials with glutamate added at 3 levels); and (*3*) a pair of split level samples (i.e., 2 nearly identical materials that differ only slightly in analyte concentration) of unspiked materials with glutamate added in similar amounts.

Commercial sausage was chopped and homogenized with a Moulinette kitchen homogenizer. Glutamate concentration of the minced material was analyzed by the method under investigation and found to be 0.42 g/kg. After addition of amounts of monosodium glutamate (MSG) as specified in Table 1, 5 g samples were weighed into plastic bags. The bags were then frozen.

Commercial dried cauliflower soup was ground with a Bamix kitchen homogenizer using blades intended for spices. Glutamate concentration of the homogenate was found to be 7.17 g/kg using the method under investigation. Samples of 3 g were weighed into plastic bags and MSG was added to each bag in the amounts listed in Table 1. The bags were then frozen.

The 14 samples were mailed to each of the 11 participating laboratories by express delivery. The laboratories were requested to store the samples frozen before performing single determinations of L-glutamic acid according to the method under investigation. The method instructions were sent to the laboratories well in advance to allow them to practice the method.

METHOD

Principle

In the presence of the enzyme glutamate dehydrogenase (GlDH), L-glutamic acid is oxidatively deaminated by nicotinamide adenine dinucleotide (NAD) to 2-oxoglutarate *(1)*. In the reaction catalyzed by diaphorase *(2)*, the NADH formed reduces 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to a formazan that is spectrophotometrically measured in the visible range at 492 nm.

$$\text{L-glutamic acid} + NAD^+ + H_2O \overset{GlDH}{\rightleftharpoons} \text{2-oxoglutarate} + NADH + NH_4^+ \quad (1)$$

$$NADH + INT + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan} \quad (2)$$

The equilibrium of the first reaction lies far to the side of glutamate. By trapping the NADH formed *(1)* with INT *(2)*, the equilibrium is displaced in favor of 2-oxoglutarate.

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This method was accepted as an official NMKL method at the 44th Annual Meeting of the Nordic Committee on Food Analysis, 1990.

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Table 1. Collaborative results for enzymatic determination of L-glutamic acid (g/kg)

		Spiked materials					
		Level 1		Level 2		Level 3	
Collaborator	Unspiked material	A	B	A	B	A	B
		Minced sausage					
1	0.45	0.63	0.74	0.84	0.95	1.27	1.43
2	0.40	0.67	0.68	0.96	1.00	1.44	1.49
3	0.37	0.58	0.68	0.72	0.76	1.30	1.30
4	0.47	0.80	0.81	0.99	1.06	1.58	1.58
5	0.30	0.43	0.50	0.56	0.84	1.12	1.26
6	0.47	0.72	0.79	0.95	0.99	1.40	1.45
7	0.34	0.51	0.54	0.75	0.66	0.97	1.07
8	0.43	0.70	0.73	0.90	0.93	1.38	1.40
9	0.43	0.64	0.64	0.82	0.83	1.25	1.32
10	0.44	0.65	0.66	0.80	0.77	1.22	1.27
11	0.42	0.57	0.60	1.10	0.75	1.40	1.44
Added analyte concn	0.0	0.33	0.33	0.53	0.57	1.11	1.11
		Dried cauliflower soup					
1	6.98	10.62	10.93	14.96	12.32[a]	14.24	14.77
2	7.70	11.50	11.70	14.10	14.30	16.50	17.10
3	6.90	10.80	11.10	13.10	13.50	15.80	15.90
4	7.70	11.50	11.70	13.70	14.80	15.40	16.20
5	7.34	11.78	11.89	13.80	13.70	15.88	15.07
6	7.15	11.57	11.61	14.52	14.56	16.65	16.50
7	7.36	11.29	11.83	13.74	13.97	16.46	16.98
8	7.08	11.32	11.78	13.70	13.80	15.73	15.82
9	7.35	11.50	12.70	14.20	14.40	16.20	16.20
10	7.07	10.60	10.67	12.26	12.79	13.24[b]	10.43[c]
11	7.66	11.70	11.80	14.20	14.50	17.00	17.40
Added analyte concn	0.0	4.35	4.35	6.96	6.96	9.14	9.57

[a] Outlying result, Cochran test at $p < 0.01$.
[b] Outlying result, excluded as being paired to a single Grubb's outlier.
[c] Outlying result, single Grubb's test at $p < 0.01$.

Apparatus and Reagents

All chemicals must be of analytical grade. The water used must be double-distilled or demineralized and distilled in all-glass apparatus.

(a) *Meat chopper.*—Must have a perforated plate and openings of not more than 4 mm.

(b) *Homogenizers.*—Moulinette kitchen homogenizer for sausage; Bamix kitchen homogenizer with spices' blade for dried cauliflower soup.

(c) *Spectrophotometer.*—Cuvets of 1 cm path length. Cuvets must be of a material suitable for measurement at 492 nm.

(d) *Perchloric acid.*—1M: Dilute 8.6 mL 70% (w/v) perchloric acid to 100 mL with water.

(e) *Potassium hydroxide.*—2M: Dissolve 56.1 g potassium hydroxide in water and dilute to 500 mL.

(f) *Buffer.*—(Triethanolamine, 0.2M; potassium phosphate, 0.025M; pH 8.6): Dissolve 3.75 g triethanolamine hydrochloride, 1.25 mL Triton X-100, and 0.44 g KH_2PO_4 in 80 mL water. Adjust to pH 8.6 with potassium hydroxide (e) and dilute to 100 mL with water. Buffer is stable for 2 months at room temperature.

(g) *NAD.*—6.7mM: Dissolve 60 mg NAD in 12 mL water. Solution is stable for 4 weeks at room temperature.

(h) *INT.*—1.19mM: Dissolve 30 mg INT in 50 mL water. Solution is stable for 4 weeks at 4°C in the dark. INT is sensitive to light.

(i) *Diaphorase (EC 1.6.4.3).*—1 mg/mL: Dissolve 9 mg lyophilisate (3 mg enzyme protein, specific activity 80 U/mg protein) in 3 mL water. Solution is stable for 4 weeks at 4°C.

(j) *GlDH (EC 1.4.1.2).*—10 mg/L: Use GlDH solution in 50 % (v/v) glycerol (specific activity 120 U/mg protein). Solution is stable for 1 year at 4°C. A weak opalescence or slight turbidity has no influence on enzyme activity.

NOTE: Reagents (f)—(j) are commercially available as a test kit for L-glutamic acid, Boehringer Mannheim GmBH, Mannheim, Germany.

(k) *L-(+)-glutamic acid standard.*—0.136mM: Dissolve 50 mg glutamic acid in ca 25 mL water. Adjust to pH 7.0 with potassium hydroxide (e) and dilute to 50 mL with water. Dilute 1.0 mL of this solution to 50.0 mL with water. Solution must be prepared fresh before use.

Sample Preparation and Procedure

The amount of L-glutamic acid in the cuvets should be in the 0.4–14 μg range. Therefore, the glutamic acid concentration in diluted samples must not exceed 0.07 g/L.

(a) *Liquid foods.*—Filter turbid solutions. Dilute with water if necessary. Colored solutions need not be decolorized.

(b) *Solid foods.*—Homogenize solid and semisolid samples using apparatus (a) or (b). Extract (see Example 2) or dissolve, respectively, with water. Filter if necessary. Extract fatty samples with water and allow to cool to obtain separation of fat. Filter. Treat samples containing protein as described in Example 1.

(c) *Example 1.*—Determination of L-glutamic acid in meat and fish products is as follows: Warm perchloric acid, reagent (d), in a water bath at 70°C for 15 min. Homogenize 5.00 g sample with 15 mL warm perchloric acid for 5 min. Mix homogenate with 25 mL perchloric acid and incubate in 70°C water bath for 15 min. Centrifuge (10 min., 5000 × *g*), collect supernatant, and filter. Discard first 5 mL filtrate and pipet 5.0 mL into 20 mL volumetric flask. Adjust to pH 10.0 with ca 3 mL potassium hydroxide, reagent (e). Dilute to mark with water. To obtain quantitative precipitation of potassium perchlorate, place flask in ice bath or refrigerator for 20 min. Filter cold solution at 4°C. Use clear solution for determination; dilute if necessary. When estimating dilution factor, take into account water content of sample.

(d) *Example 2.*—Determination of L-glutamic acid in soups and in bouillon cubes is as follows: Dissolve 2.00 g homogenized sample in ca 100 mL warm water, heat at ca 70°C for 10 min, and allow to cool to room temperature. Transfer solution to 200 mL volumetric flask and dilute to mark with water. Shake well and filter to separate fat. In case of residual turbidity, filter again through the same filter paper. Use clear solution for determination; dilute if necessary.

Preparation of Assay Solutions

(a) *Blank.*—Pipet the following into cuvet: 1.00 mL buffer, reagent (f); 0.20 mL NAD, reagent (g); 0.20 mL INT, reagent (h); 0.050 mL diaphorase, reagent (i); and 1.50 mL water.

(b) *Sample.*—Pipet the following into cuvet: 1.00 mL buffer, reagent (f); 0.20 mL NAD, reagent (g); 0.20 mL INT, reagent (h); 0.050 mL diaphorase, reagent (i); 0.20 mL sample; and 1.30 mL water.

When analyzing samples containing low amounts of L-glutamic acid, use larger sample volumes (up to 1.0 mL). Decrease water volume accordingly to result in a total volume of 3.00 mL in cuvet.

Mix carefully and read absorbances at 492 nm against air after 4 min. Record the absorbances as A_1, absorbance of solution in sample cuvet; and A_{1B}, absorbance of solution in blank cuvet.

Add 0.050 mL GlDH, reagent (j), to both cuvets. Mix and allow to stand 10–15 min. Read absorbances. Read absorbance every 2 min until a constant increase is obtained. Plot absorbances against time and extrapolate absorbance values to the start of the reaction. The constant increase in absorbance (side reaction) is subtracted from the total absorbance in this manner. Alternatively, subtract the increase caused by the side reaction from the absorbance at a given time. Record these extrapolated absorbances as A_2, absorbance of solution in sample cuvet; and A_{2B}, absorbance of solution in blank cuvet.

Calculation

Calculate differences in absorbance for sample (ΔA_S) *and blank* (ΔA_B) *as:*

$$\Delta A_S = A_2 - A_1$$

$$\Delta A_B = A_{2B} - A_{1B}$$

Calculate ΔA *by subtracting* ΔA_B *from* ΔA_S*:*

$$\Delta A = \Delta A_S - \Delta A_B$$

The general formula for calculating concentrations is:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \quad \textit{(g/L sample solution)}$$

where V = final volume (mL) in cuvet (3.00 mL); v = sample volume (mL) in cuvet (0.20 mL); MW = molecular weight of the analyte; d = cuvet path length (cm); and ε = absorption coefficient of formazan at 492 nm (19.9 L/mmol cm^{-1}).

Before analyzing samples, check apparatus and reagents by analyzing standard solution, reagent (k), of L-glutamic acid. Result should be 20 ±1 mg/L.

Calculate the concentration of L-glutamic acid in sample (g/L) (sample volume = 0.20 mL) as:

$$c = \frac{3.00 \times 147.13}{19.9 \times 1 \times 0.2 \times 1000} \times \Delta A \ \text{g/L}$$

$$c = 0.1109 \times \Delta A \ \text{g/L}$$

Calculate concentration of L-glutamic acid in sample (g/kg) by multiplying concentration, (c), by the dilution factor corresponding to the performed sample pretreatment (3).

Results and Discussion

Results received from all 11 participating laboratories are listed in Table 1. Some laboratories reported results calculated on a dry weight basis, and these results were recalculated for fresh weight.

Statistical evaluation of the collaborative study data was carried out according to the International Union of Pure and Applied Chemistry (IUPAC) 1987 Harmonized Protocol (4). Precision estimates were first calculated using all results received, with no outliers removed. The estimates are presented in Table 2.

Application of the Cochran maximum variance test at $p < 0.01$ revealed 1 outlying result (collaborator No. 1) for the pair of blind duplicates of dried cauliflower soup at the 14 g/kg level. The single Grubb's test at $p < 0.01$ flagged 1 laboratory (collaborator No. 10) as outlying for a split level sample of dried soup at the 16 g/kg level. The other half of the pair was designated as outlying. No other outlying results for dried soup were detected. No outliers were detected among the results for minced sausage. The proportion of outlying results, 9%, is well below the recommendation of the IUPAC 1987 Harmonized Protocol, which sets the maximum at 22%. After removal of the outliers, the precision parameters were recalculated as earlier. The results are presented in Table 2.

Reproducibility relative standard deviations (RSD_R), which reflect the variation between laboratories as calculated from the minced sausage material, were between 12 and 16%. For the concentration level in question, 0.4–1.3 g/kg, these fig-

Table 2. Statistical analysis of the collaborative study data

	Unspiked material	Level 1	Level 2	Level 3
		Minced sausage[a]		
Av., g/kg	0.41	0.65	0.85 0.87[b]	1.33
"True" SD_L among labs	0.054	0.09	0.09	0.15
s_R	0.054	0.10	0.14	0.16
s_r	—	0.04	0.10	0.06
RSD_R	13.2	15.7	15.9	11.7
RSD_r	—	6.2	12.0	4.2
No. of labs	11	11	11	11
		Dried cauliflower soup[a]		
Av., g/kg	7.30	11.45	13.88	15.99 16.19[b]
"True" SD_L among labs	0.29	0.40	0.56	0.74
s_R	0.29	0.51	0.64	0.82
s_r	—	0.32	0.31	0.35
RSD_R	4.0	4.5	4.6	5.1
RSD_r	—	2.8	2.2	2.2
No. of labs	11	11	10	10
		Dried cauliflower soup[c]		
Av., g/kg	—	—	13.86	15.74 15.67[b]
"True" SD_L among labs	—	—	0.37	1.40
s_R	—	—	0.73	1.56
s_r	—	—	0.63	0.68
RSD_R	—	—	5.3	9.9
RSD_r	—	—	4.6	4.4
No. of labs	—	—	11	11

[a] Outliers excluded.
[b] Av. obtained for split level pairs (nearly identical materials that differ only slightly in analyte concentration).
[c] Outliers included.

ures are rather high. According to the Harmonized Protocol of an IUPAC workshop (5) dealing with the acceptability of analytical methods, an RSD_R value below 11.2% would be acceptable for analyte concentrations of approximately 0.1%. Repeatability relative standard deviations (RSD_r), which are measures of the within-laboratory variation, were good for 2 levels (4–6%), but high for 1 level (12%).

As expected, the precision parameters of the method calculated from results for the samples of dried soup were better than those calculated from the minced sausage material. RSD_R at glutamate levels of 0.7–1.6% were between 4.0 and 5.1%. For these analyte concentrations, the precision obtained is in agreement with those derived from statistical analysis of results from collaborative studies representing a wide range of analytes, matrixes, and measurement techniques (6). For an analyte concentration of 1%, the average figure is 4%. RSD_r for dried soups were <3% at all levels.

The precision parameters of this enzymatic method are comparable to those found in a collaborative study of a method using automatic amino acid analyzers (7). In this study, the amino acid compositions of 7 food proteins were determined in 7 laboratories. RSD_R for glutamic acid varied between 8.3 and 12.8%. RSD_r was < 2.2%.

The analysis of glutamic acid differs from the determination of most other food additives because glutamate is a naturally occurring component of biological materials. Foods also contain enzymes that are capable of both degrading and producing glutamate. Glutamate concentrations in foods may, therefore, undergo substantial changes in the span of time between sample preparation and analysis. Bearing this in mind, the glutamate recoveries and the method precision were expected to be poorer for the "biological" samples of minced sausage than for the more stable samples of dried soup. The results confirmed this assumption. Originally, samples of fish products were also planned to be included in the study. However, shipping of such samples to the study participants in a reasonably unchanged state was difficult. Because fish is biologically similar to meat, the precision parameters obtained for the meat product samples may also be applicable to fish products.

Collaborators' Comments and Remarks

One collaborator suggested that the time for homogenization be shortened from 10 to 5 min. The authors are in compliance with this suggestion, and the method has been amended accordingly.

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Sensitive and Rapid Reversed-Phase Liquid Chromatography–Fluorescence Method for Determining Bisphenol A Diglycidyl Ether in Aqueous–Based Food Simulants

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A method has been developed for determination of bisphenol A diglycidyl ether (BADGE) in 3 aqueous-based food simulants: water, 15% (v/v) ethanol, and 3% (w/v) acetic acid. BADGE is extracted with C18 cartridges and the extract is concentrated under a stream of nitrogen. BADGE is quantitated by reversed-phase liquid chromatography with fluorescence detection. Relative precision at 200 μg/L was 3.4%, the detection limit of the method was 0.1 μg/L, and recoveries of spiking concentrations from 1 to 8 μg/L were nearly 100%. Relative standard deviations for the method ranged from 3.5 to 5.9%, depending on the identity of the spiked aqueous–based food simulant.

Starting materials for cold-cured epoxy resins are mainly low molecular mass epoxy resins of the bisphenol A type, which are cured by aliphatic polyamines or by polyamine adducts. With room-temperature curing, starting materials are converted into a hard product within a few hours. However, the fully cured resin often contains some unreacted starting material (1).

Epoxy-based solution coatings are used as maintenance and product finishes, marine finishes, masonry finishes, structural steel coatings, tank coatings, aircraft finishes, appliance primers, automotive primers, can and drum linings, furniture finishes, collapsible-tube coatings, concrete-floor paints, gym and floor varnishes, spar varnishes, etc. Epoxy resins are also used in decorative floor applications; as chemically resistant mortars and floor topping compounds; in printing inks; in fabric-treating applications; in dental, surgical, and prosthetic applications; for breaking petroleum emulsions; and for lightweight, chemically resistant foams. The epoxy resins are used as additives for a variety of other plastic materials, such as vinyl and acrylic resins and natural and synthetic rubbers (2).

Because of its use in epoxy formulations for lacquer coatings on food cans and food storage vessels, bisphenol A diglycidyl ether (BADGE, an oligomer of relative molecular mass 340) is contained in list 7 of the Scientific Committee for Food's 19th report (3), including substances for which some toxicological data exist, but for which an acceptable daily intake or tolerable daily intake could not be established. Additional specified information should be furnished. The Committee recognizes that priorities will have to be set because of the large number of substances mentioned. Criteria for setting these priorities should include availability of analytical methods, data on exposure (e.g., usage and extent of migration), hydrolysis data, etc.

A study of the chemical stability of the monomer in food simulants showed that it is readily hydrolyzed, with a half-life of less than 2 days at 40°C (4, 5). By then, monomer residues are difficult to detect in the 3 aqueous-based food simulants under test conditions of 10 days at 40°C. Thus, methods for quantitating BADGE at low ppb levels must be established.

As a specific migration limit in food or in food simulant for this monomer, the Commission Directive of the European Communities of February 23, 1990 (6) established a non-detectable value when the detection limit of the method is 0.020 mg/kg (analytical tolerance included).

The American Society for Testing and Materials provided test methods that cover quantitative determination of epoxy

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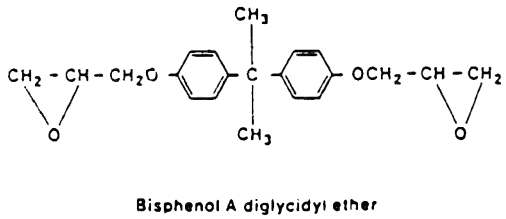

Figure 1. Molecular structure of bisphenol A diglycidyl ether (BADGE).

content of epoxy resins (7). However, gel permeation chromatography already permits coarse separation of epoxidic oligomers (8–12), although the best resolution was achieved with reversed-phase partition techniques (13–17). UV detection in reversed-phase liquid chromatography (RPLC) is the best solution for quality control of epoxy resins because of its low selectivity. Crathorne et al. (18) published a remarkable UV/LC method for determination of BADGE residues in water. According to this publication, epoxy resins based on either BADGE or bisphenol F diglycidyl ether were used for the renovation of water pipes in the United Kingdom; traces of these compounds in water appear to be due to incomplete polymerization (18).

In this work we studied the efficiency of methanol in eluting the unreacted oligomer 340, which migrated into aqueous food simulating solvents, from Sep–Pak C18 cartridges. This efficiency is evaluated by RPLC with fluorescence detection. The limit of detection was 0.1 μg/L. Hydrolysis reaction products are more polar with the opening of the epoxidic rings, and they do not interfere with measurement (19).

Experimental

Apparatus

(a) *LC system.*—Consisting of extended range SP8700 XR LC pump, SP8750 organizer, SP4290 integrator with SP WINNER software V 4.00 (Spectra-Physics, Inc., San Jose, CA 93412); fluorescence detector, PE LS 40 (Perkin-Elmer Corp., Norwalk, CT 06859).

(b) *Chromatographic conditions.*—Flow: 1.5 mL/min. Elution: 5 min linear gradient from acetonitrile–water (50 + 50) to 60% acetonitrile, 5 min isocratic elution at 60% acetonitrile, 5 min linear gradient to 100% acetonitrile, and 5 min more at 100% acetonitrile; return to initial conditions in ca 10 min. Injection: 50 μL loop in Rheodyne valve, using a Hamilton syringe. Column: 15 cm × 5 mm id, stainless steel, packed with 5 μm Pecosphere CRT C18 RC. Guard columns: C18, used to protect packing in analytical columns. Detection: attenuation factor, 16, with auto-zero; response, 4 (equivalent RC, 98% FS; response time, 2.8 s); excitation wavelength, 275 nm; emission wavelength, 300 nm; photomultiplier voltage, 750 V. Integrator attenuation: 4.

Reagents

(a) *Cartridges.*—Sep-Pak C18, No. 51910 (Millipore Corp., Bedford, MA 01730).

(b) *Helium and nitrogen.*—N-48 (SEO); He used for degassing mobile phase, N_2 for concentration to dryness.

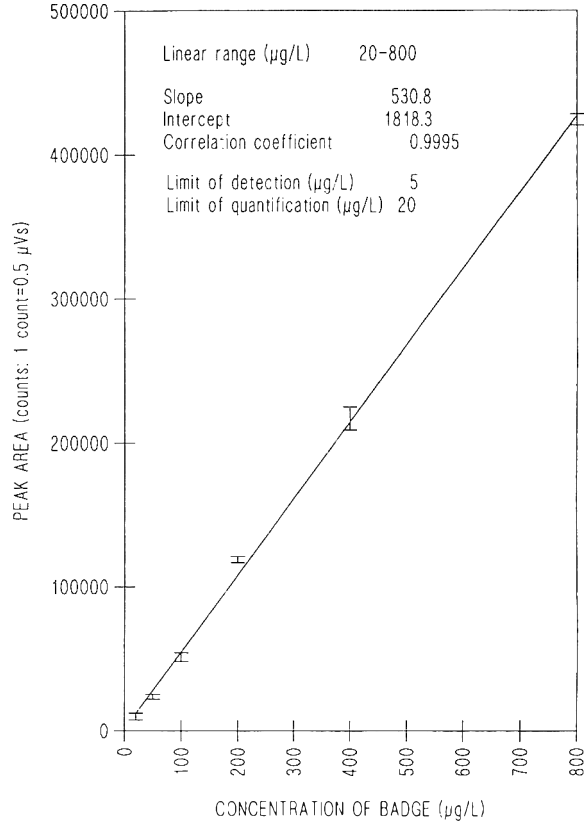

Figure 2. Calibration graph with study of the linearity of response.

(c) *Acetonitrile RS.*—LC grade (Carlo Erba/Fisons, Valencia, CA 91355).

(d) *Water.*—Demineralized, Milli-Q quality, used as food simulant (Millipore).

(e) *Solvents.*—Tetrahydrofuran and methanol (Merck & Co., Rahway, NJ 07065). Methanol was purified by distillation. (Note: Tetrahydrofuran is highly irritating and must be handled carefully.)

(f) *BADGE.*—CAS No. 001675-54-3 (Epikote 828, S.P.E. Shell). See Figure 1 for molecular structure. Purified (>99%) by Shell Laboratories for the Gairesa industry. A product of similar purity can be obtained according to Paz Abuin et al. (19). BADGE was used to prepare a stock solution containing exactly 1% (w/v) BADGE in tetrahydrofuran. It was stored in the dark in a refrigerator. Dilutions in methanol from this stock solution were prepared daily if needed.

(g) *Food simulants.*—Ethanol and acetic acid (Merck).

Calibration Curve

A 1 mL aliquot of 1% (w/v) stock solution of BADGE in tetrahydrofuran was diluted to 100 mL with methanol. A 1 mL aliquot of this solution was further diluted to 100 mL with methanol, resulting in a 1 μg/mL solution of BADGE in methanol.

Aliquots of 2, 5, 10, 20, 40, and 80 mL of the 1 μg/mL solution were pipetted into separate 100 mL volumetric flasks and diluted to volume with methanol to give concentrations of 20 μg/L (limit of quantitation of the calibration curve) to 800 μg/L (at upper levels the detector signal is sat-

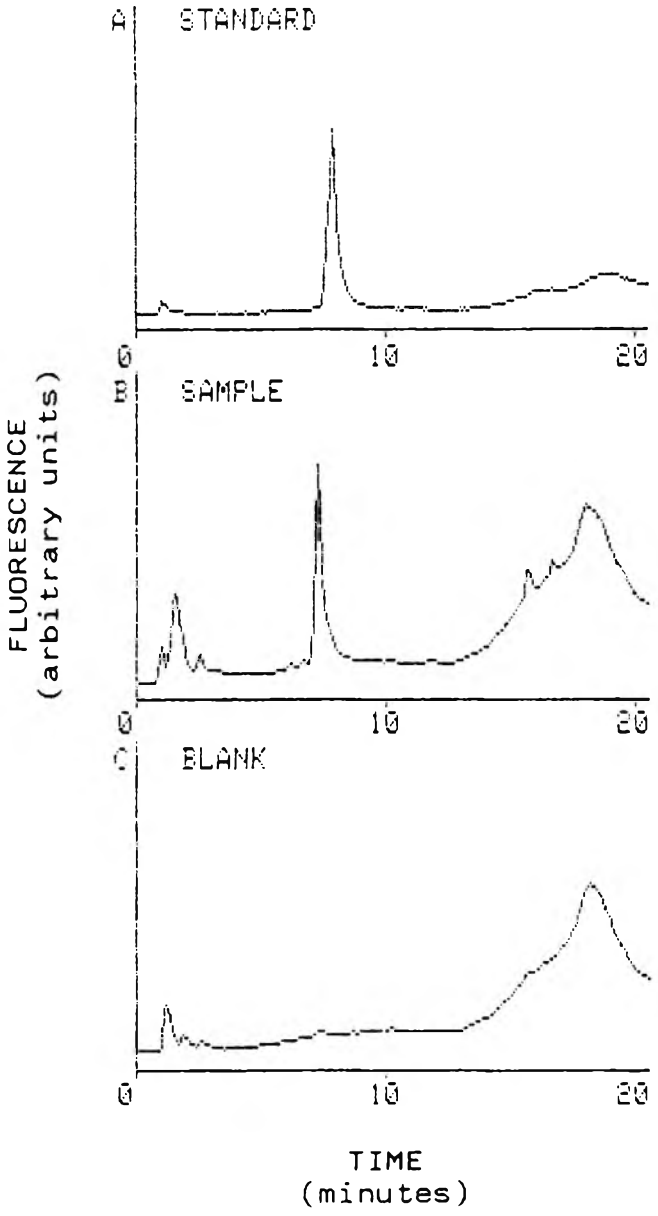

Figure 3. Chromatograms: (A) External standard of BADGE concentration at 200 μg/L; (B) spiked aqueous-based food simulant with BADGE at 4 μg/L to obtain a concentration of 200 μg/L at end of method; and (C) unspiked aqueous-based food simulant.

urated under the chromatography conditions listed above). These 6 new solutions were used to construct the calibration curve (Figure 2). The limit of detection for BADGE satisfies the following criterion (20, 21):

$$A_s - A_b \geq 3 \times S_b$$

where A_s = average of sample signal (height), A_b = average of blank signal (height), and S_b = standard deviation of blank signal (height). The limit of detection for BADGE was 5 μg/L for the calibration curve.

The limit of quantitation for the calibration curve indicated above (20 μg/L) is calculated according to the criterion (20, 21):

$$A_s - A_b \geq 10 \times S_b$$

Precision

A single specimen with a fortified BADGE concentration of 200 μg/L was chromatographed 6 times. Percent relative standard deviation for chromatography was ±3.4%.

Procedure for Low ppb Levels of BADGE in Simulants

Sample preparation.—Sep-Pak cartridges were prepared for use by elution with methanol (5 mL) and deionized double-distilled water (5 mL). Only new cartridges were used. Portions of 50 mL aqueous-based food simulant (distilled water, 15% (v/v) ethanol, or 3% (w/v) acetic acid) were eluted through a clean Sep-Pak cartridge, using a 50 mL Luer-Lok glass syringe at a flow rate of ca 3 mL/min. The cartridge was then eluted with methanol (3 mL) at a flow rate of ca 1 mL/min. This eluate was concentrated to dryness under a stream of nitrogen. The residue was then dissolved in 1 mL methanol and chromatographed. Standard, sample, and blank chromatograms are represented in Figure 3. Chromatograms of the 3 (spiked or unspiked) aqueous-based food simulants are similar.

BADGE content was calculated from the formula:

$$R = 0.02 \times C$$

where R = the BADGE content of sample in μg/L; 0.02 = the concentration factor of the Sep-Pak procedure (1 mL/50 mL = 0.02); and C = the concentration (μg/L) obtained from the calibration curve.

Recovery and precision of methodology.—Spiked solutions of BADGE in different aqueous-based food simulants at the levels of 1, 4, and 8 μg/L were prepared by diluting a 1 mL aliquot of each of the 50, 200, and 400 μg/L standard solutions, respectively, to 50 mL with the appropriate simulant. These solutions were assayed twice. Results of these experiments are presented in Table 1. Average recovery is not significantly different from 100%, and the method relative standard deviation (%) ranges from 3.5 to 5.9%, depending on the spiked aqueous-based food simulant.

Method detection limit.—If the Sep-Pak procedure concentration factor is considered (50 mL simulant concentrated to 1 mL in methanol), the limit of detection of the method is 0.1 μg/L (50 times smaller than the limit of detection of the calibration, i e., 5 μg/L). This was experimentally verified by spiking a blank at these levels with BADGE. Also with regard to that factor, the limit of quantitation of the method is 0.4 μg/L.

Discussion

Crathorne et al. (18) used 500 mL portions of water. This is slow and cumbersome. On the other hand, laboratory migration assays use smaller volumes. Moreover, fluorescence detection, because of its higher selectivity than UV detection, is a more appropriate technique for detection of low levels of BADGE. It is interesting to remark that our quantitation limit for the calibration line (Figure 2) coincides with the method detection limit required by the Commission Directive of the European Communities. Our detection and quantitation calibration limits can be decreased 50-fold by using the Sep-Pak concentration of 100% accuracy, as described in the 2-tailed test in Table 1, to test the difference between 2 means, regardless of the sign of the difference (positive or negative). As far as we know, no attempt has been reported of using RPLC to

Table 1. Recovery of BADGE in 3 aqueous-based food simulants

	Distilled water		Ethanol, 15% v/v		Acetic acid, 3% w/v	
Added, μg/L	Found, μg/L	Rec., %	Found, μg/L	Rec., %	Found, μg/L	Rec., %
8.00	7.78	97.3	7.90	98.8	7.82	99.0
	7.67	95.9	7.34	91.8	7.38	92.3
4.00	3.74	93.5	3.61	90.3	3.90	97.5
	3.96	99.0	3.86	96.5	3.72	93.0
1.00	1.03	103.0	1.07	107.0	0.98	98.0
	0.94	94.0	0.98	98.0	1.04	104.0
Av. ($\bar{X}$)		97.1	—	97.1	—	97.3
RSD, %		3.5	—	5.9	—	4.3
t^a		2.030	—	1.204	—	1.538

[a] Experimental t calculated as follows: $t = [(100 - \bar{X}) \times n^{1/2}]/RSD\ (\%)$
where $\bar{X}$ and RSD (%) are the average and relative standard deviation under each Rec. % column respectively, and n = number of observations ($n = 6$). Theoretical t ($\alpha = 0.05$; DF = 5); $t_{(1-\alpha/2)} = 2.571$ where α = confidence level and DF = degrees of freedom.

determine unreacted BADGE that has migrated from cured epoxy resins into aqueous-based food-simulating solvents.

Conclusions

The method described above is not time-consuming. It is recommended for determining BADGE in aqueous-based food simulants because of its high precision, accuracy, and very low levels of detection and quantitation.

Acknowledgments

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FOOD COMPOSITION

Potassium as an Index of Fruit Content in Baby Food Products. Part I. Banana-Containing and Apricot-Containing Products

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Percentage ingredient labeling has been proposed for baby foods. We determined whether or not the potassium content of baby foods could be used to verify the quantity of fruit when the characterizing ingredients were apricots or bananas, fruits rich in potassium. Official values for potassium in fruit (USDA Handbook No. 8–9) did not agree well with actual analyses. The potassium levels of products of known composition were accurately predicted from analyses of the actual ingredients used to make the foods. For banana-containing monofruit products of variable or unknown composition, potassium analysis led to fruit level estimates consistent with either the known composition or the label declaration. For products of unknown composition made with apricot concentrate, however, potassium analysis led to fruit level estimates lower than the probable fruit content. The quantity of fruit in baby foods made with potassium-rich fruits can be estimated from the potassium content if the potassium value for the fruit is representative of the actual ingredients used to make the product. If potassium analysis is to be used to verify compliance with percentage ingredient labeling, there must be statutory specification of the single-strength fruit level for fruit reconstituted from concentrate.

Percentage labeling of the ingredients in prepared foods has been debated for almost 15 years (1). A class of food considered especially appropriate for percentage ingredient labeling is baby food (1–3). Because most baby foods are fine purees, the amount of the characterizing ingredient is impossible to determine visually.

Discussions of percentage labeling have not addressed the critical technical issue of how to verify compliance with stated percentages of principal or characterizing ingredients. The U.S. Department of Agriculture (USDA) has authority to review formulations of products containing meat and poultry and to continuously inspect the products during production. However, the U.S. Food and Drug Administration (FDA) lacks statutory authority to inspect a manufacturer's formulations. Consequently, methods are needed to estimate amounts of characterizing ingredients in products so that ingredient declarations can be verified.

Of the several categories of baby food, "fruits" are the most popular. Some commercial baby fruit products contain sugar and starch in addition to the characterizing fruit ingredient. Consequently, neither total carbohydrate nor total soluble sugar analysis provides sufficient information to permit quantitative estimation of fruit ingredients.

Potassium may be a useful index of fruit content. Certain fruits, such as bananas and apricots, are particularly rich in potassium; nonfruit ingredients of "baby fruits," such as purified starches, sugar, citric acid, vitamin C, and water, are not. Adding potassium intentionally is unlikely. The highly acidic nature of fruits makes them easy to process (only pasteurization is required for shelf stability in a hermetically sealed container). This reduces the likelihood or necessity of adding potassium hydroxide, potassium carbonate, potassium malate, or potassium citrate, alkalizing salts that have no "telltale" inorganic anion. Adding the chloride or sulfate salts of potassium would be detectable from ash level and anion analysis.

The purpose of this work was to test the hypothesis that potassium is a reliable index of the amount of fruit in baby foods in which either bananas or apricots are the characterizing ingredient. Three questions were addressed: (*1*) Are the official published reference values for the potassium content of banana and apricots reliable (4)? (*2*) Can potassium content of commercially prepared monofruit and multifruit baby foods with known percentage composition be accurately predicted by potassium analyses? (*3*) Can potassium content of commercially prepared monofruit baby foods of unknown composition be used to make reasonable estimates of fruit levels that are consistent with labeled ingredient declarations?

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Experimental

Fresh apricots, bananas, pears, and apples were obtained from local grocery stores in New York State. Other materials were obtained from the following commercial sources: banana puree, Chiquita Brands, Inc.; apricot concentrate, Sabrosa, OR, and Rich Fruit Pack, CA; pear concentrate, 32° Brix, Sabrosa, OR; and rice flour, Rivland, TX, and Gulf Pacific, LA. Rice starch was purchased from a proprietary supplier.

Baby food products were purchased in supermarkets in New York and Missouri. Seven or more lots of each of the following products were analyzed in duplicate for potassium by atomic absorption spectroscopy: Beech-Nut Stage 1 Chiquita Bananas, Beech-Nut Stage 2 Bananas with Pears & Apples, Beech-Nut Stage 2 Apricots with Pears & Apples, Gerber Strained Bananas with Tapioca, Gerber Strained Apricots with Tapioca, Heinz Strained Bananas with Tapioca, and Heinz Strained Apricots with Tapioca.

Five lots of an additional product of known composition, Beech-Nut Stage 2 Bananas with Rice/Banana Pudding, were also analyzed for potassium to help validate the method.

Results and Discussion

Potassium Content of Fruits

The official reference for potassium content of foods, USDA Agriculture Handbook No. 8–9 (4), lists the potassium content of bananas, edible portion, as 396 mg%. Analysis of

Table 1. Potassium content (mg/100 g) of ingredients used in strained baby food made with edible portions of bananas or apricots

Constituent	Potassium, mg/100 g, edible portion			
	Official reference[a]	Fresh fruit	Puree	From concentrate[b]
Apricots	296 ± 51 (18)[c,a]	273 ± 12 (4)	—	335 ± 21 (3)
Bananas	396 ± 47 (55)[d]	346 ± 39 (10)	315 ± 16 (18)	—
Apples	113 ± 22 (91)[e]	102 ± 28 (5)	—	—
Pears	125 ± 18 (22)[d]	102 ± 23 (4)	—	159 ± 6 (3)
Rice flour	—	73 ± 18 (3)	—	—
Rice starch	—	13 ± 1 (4)	—	—

[a] USDA Agriculture Handbook No.8–9.
[b] Brix for single-strength: apricots, 14.3°; pears, 15.4°.
[c] Mean and standard deviation; number of samples in parentheses.
[d] Raw.
[e] Raw, without skin.

10 fresh fruit samples gave a mean of 346 mg%, with a range of values (288–424 mg%) above and below the official reference value (Table 1). The mean value for 18 banana puree samples of the type used in commercial baby food was 315 mg%, 20% less than the reference value.

Four samples of fresh apricots, edible portion, averaged 8% less potassium than the official reference value of 296 mg%. Puree concentrate converted to single-strength puree contained 13% more potassium than the reference (Table 1). Baby food manufacturers routinely use apricot puree concentrate in products containing apricot. Conversion of apricot puree concentrate to single-strength puree (14.3° Brix soluble solids) was based on the standard conversion prescribed in current federal regulations for other food categories (5). Industry associations recently proposed a single-strength standard for apricot puree of 14.0° Brix. Converting the concentrate according to the proposed standard would yield a potassium content of 328 mg%, 11% more than the reference value.

Potassium was determined in fresh pears, fresh apples, and pear puree concentrate, and results were compared with the official reference value for these fruits (Table 1). Pear concentrate was converted to single-strength fruit using the standard conversion of 15.4° Brix (5). Whether or not the single-strength fruit standards established in other food categories (5) for reconstituting fruit concentrates are applicable to baby food is a regulatory question that must be addressed by FDA before requiring percentage ingredient labeling.

Table 2. Potassium content of 2 banana-containing baby foods (mg/100 g)

Product	Found	Calculated
Beech-Nut Stage 2 Banana Pudding	86 ± 9 (5)[a]	86 ± 4
Bananas, 26.7%	—	84
Rice flour, 2.5%	—	2
Rice starch, 3.5%	—	< 1
Beech-Nut Stage 2 Bananas with Pears and Apples	203 ± 8 (7)	210 ± 10
Bananas, 42.1%	—	133
Pears, 31.1%[b]	—	49
Apples, 26.8%[c]	—	27

[a] Mean and standard deviation; number of samples in parentheses.
[b] From 32° Brix concentrate; single-strength is 15.4° Brix (ref. 5).
[c] From fresh apples, edible portion.

Prediction of Potassium Level from Ingredients

Banana-containing products.—The potassium content of Beech-Nut Stage 2 Banana Pudding (containing 26.3% banana) was accurately predicted from the potassium contents of its ingredients (Table 2). Stage 2 Bananas with Pears & Apples is made with 3 fruits: banana (from puree), pears (from concentrate), and fresh apples. The agreement between the potassium content calculated from the ingredients and that found on analysis was extremely good (Table 2). Thus, the determined potassium content of these 2 banana-containing products validated the ingredient percentages.

Apricot-containing products.—Beech-Nut Stage 2 Apricots with Pears & Apples is made with, on a single-strength basis, 45% apricots (from concentrate), 32% pears (from concentrate), and 32% fresh apples (edible portion). The total exceeds 100% because the product is formulated to achieve a desirable spoon-feeding consistency. The product contained 218 mg% potassium. This value agrees well with the theoretical potassium content of 234 mg% calculated from analysis of the 3 ingredients (Table 3). Thus, potassium content appears to be a valid index of fruit content for products made with "apricots from concentrate" and it can validate percentage ingredient declarations.

Estimation of Fruit Level from Potassium Content

Banana-containing products.—Beech-Nut Stage 1 Chiquita Bananas has a variable composition, with the pro-

Table 3. Potassium content of an apricot-containing baby food made with 3 fruit components (mg/100 g)

Product	Found	Calculated
Beech-Nut Stage 2 Apricots with Pears and Apples	218 ± 14 (7)[a]	234 ± 13
Apricots, 45%[b]	—	151
Pears, 32%[c]	—	51
Apples, 32%[d]	—	33

[a] Mean and standard deviation; number of samples in parentheses.
[b] From 32° Brix concentrate; single-strength is 14.3° Brix.
[c] From 32° Brix concentrate; single-strength is 15.4° Brix (ref. 5).
[d] From fresh apples, edible portion.

Table 4. Fruit content of monofruit baby foods estimated from potassium analysis

Product	Potassium content, mg/100 g	Estimated fruit content, %	
		From official reference	From raw material analysis
Banana-containing products			
Beech-Nut Stage 1 Chiquita® Bananas	237 ± 27 (13)[a]	60	75
Gerber Strained Bananas with Tapioca	144 ± 17 (7)	36	46
Heinz Strained Bananas with Tapioca	84 ± 14 (8)	21	27
Apricot-containing products			
Gerber Strained Apricots with Tapioca	108 ± 19 (7)	37	32
Heinz Strained Apricots with Tapioca	104 ± 18 (7)	35	31

[a] Mean and standard deviation; number of samples in parentheses. Chiquita is a registered trademark of Chiquita Brands, Inc.

portion of banana puree and water varied to achieve a standard consistency. No starch or sugar is added to this product. Potassium was determined in 13 individual lots containing 65–85% banana. Lots were found to contain 192–279 mg% potassium, with a mean of 237 ±27 mg%.

Banana content was calculated by dividing the mean potassium content by either the official reference potassium value or by the actual analytical mean for banana puree (Table 4). Banana content of the product calculated from banana puree potassium content was 75%, which is consistent with the actual amount of banana puree used to make the lots that were analyzed.

Proportions of banana in 2 strained bananas with tapioca baby food products of unknown composition were estimated from the assayed potassium content of banana puree (Table 4). Gerber Strained Bananas with Tapioca was estimated to contain 46% bananas, confirming the ingredient declaration, where "fully ripened bananas" is listed as the predominant ingredient.

Heinz Strained Bananas with Tapioca was estimated to contain 27% bananas. This too is consistent with the ingredient declaration, where the predominant ingredient is "water necessary for preparation" and the second ingredient is "bananas."

The mean potassium levels found by analysis were similar to the manufacturers' latest published values (Table 5).

Apricot-containing products.—Gerber Strained Apricots with Tapioca is made with "apricots from concentrate"; 7 lots contained an average of 108 mg% potassium, similar to the manufacturer's published value of 111 mg% (Table 5). On the basis of our analysis of apricot puree concentrate, this average analyzed potassium content calculates the contents to be 32% apricots (single-strength basis). This estimate seems low. A formula published in 1985 for a product similar to this manufacturer's then-produced "Apricots with Tapioca" product contained 42% apricots on a 14.3° Brix single-strength basis (6). Moreover, the label ingredient declaration lists "apricots from concentrate" as the primary ingredient, which suggests an apricot level in excess of 40%. This inexplicable discrepancy between the calculated apricot content (32%) and the expected apricot content (more than 40%) may be due to a change in apricot source (i.e., how much potassium the apricot contains) or a recent change in formulation (i.e., how much apricot the product contains). Converting apricot concentrate to puree with a single-strength standard of 14.0° Brix would alter the apricot content estimate very slightly, from 32 to 33%.

Heinz Apricots with Tapioca, made with "apricots," contained 104 mg% potassium, substantially more than the manufacturer's published value of 77 mg% (Table 5). The analyzed potassium content corresponds to 31% apricots on a single-strength basis.

Table 5. Comparison of analytical results with manufacturers' published values for potassium (mg/100 g)

Product	Published	Found
Gerber Strained[a]		
Bananas with Tapioca	150	144 ± 17
Apricots with Tapioca	111	108 ± 19
Heinz Strained[b]		
Bananas with Tapioca	80	84 ± 14
Apricots with Tapioca	77	104 ± 18

[a] Gerber Nutrient Values 1990 (55–77 Rev. 190). Gerber Products Company, Fremont, MI.
[b] Heinz Nutritional Information per 100 g (BF9509). Heinz USA, Pittsburgh, PA.

Conclusion

Fruit content of baby foods made with fruits rich in potassium, such as bananas and apricots, and labeled with ingredient percentages can be validated by potassium analysis. Potassium analysis also can be used to estimate fruit content of monofruit products. However, the potassium value used for the fruit in question must be representative of the fruit actually used in the product, and the other ingredients must be relatively free of this mineral.

Banana puree used in baby food manufacture contains 20% less potassium than the reference value for fresh fruit given in USDA Agriculture Handbook No. 8–9. The product "apricots from concentrate" contains 13% more potassium than the reference value for fresh fruit. Therefore, the kind of material actually used to make baby food should be analyzed. Furthermore, the "single-strength" fruit level for fruit reconstituted from concentrates, such as apricots and pears, must be defined.

Apples and pears contain much less potassium than bananas. Potassium analysis would be less sensitive and thus less useful for verifying percentage compositions of products made with these low-potassium fruits, especially products made with 2 or more fruits nearly equal in potassium content.

Actual potassium analyses of products, rather than a manufacturer's published values, should be used, because we found that values can differ substantially. Variations in potassium content due to soil conditions, seasonality, climate, and specific variety of a fruit are confounding variables to the method.

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A New Approach to the Study of Glucosinolates by Isocratic Liquid Chromatography. Part I. Rapid Determination of Desulfated Derivatives of Rapeseed Glucosinolates

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Liquid chromatographic (LC) analysis of desulfated derivatives of rapeseed glucosinolates has been carried out under isocratic elution conditions with different CN-bonded stationary phases. The effects of the eluant composition (water, acetonitrile, and methanol) with the stationary phase (Zorbax CN, Lichrospher CN, and Ultrasphere CN) and temperature (20 and 50°C) are described. An isocratic LC method performed at room temperature using a Lichrospher CN column and water as mobile phase is proposed. The chromatographic analysis can be done in less than 12 min, and it is easier and less expensive than the traditional gradient mode. Four commercial samples of rapeseed containing various quantities of other cruciferous seeds (wild mustard and stinkweed) as an admixture have been analyzed to determine the total glucosinolate content. Relative standard deviations of repeatability of the isocratic and gradient LC methods ranged from 0.4 to 1.7% and from 2.7 to 4.7%, respectively. Comparison of the results showed good agreement between the 2 methods (better than 98%).

Glucosinolates (GSLs) are thioglucosides whose structures vary mainly by the nature of their R substituents (Figure 1, Table 1), which can be aliphatic or aromatic. They are found in every species of the plant family Cruciferae (1), specifically in *Brassica*. Approximately 10 are found in rapeseed (*Brassica napus*). After oil is extracted, a large percentage of these compounds remains in residues, which are then used in animal feed. During food preparation or ingestion, GSLs are easily broken down, leading to compounds with detrimental and antinutritional characteristics. For this reason, the European Economic Community (EEC) rates different rapeseeds according to their high- or low-level GSL content. Hence, the amount of GSL found in rapeseed (meals and seeds) is an important qualification.

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For the past 15 years, GSLs analyses have been improved by chromatographic techniques going successively from gas chromatography (GC) of GSLs degradation products (2) to GC of desulfated and silylated derivatives (3–6), and then to liquid chromatography (LC) of intact GSLs (7, 8) or desulfated GSLs (DSGSLs) (9–12). DSGSLs are represented by the abbreviation of the concerned GSL preceded by DS. Intact GSLs have been determined after purification by ion-exchange (7, 8), and DSGSLs after enzymatic desulfation of GSLs on an ion-exchanger (5) (Figure 2).

GSLs were separated by reversed-phase LC with alkyl-bonded silica and an ion-pairing reagent chosen to increase retention and selectivity for the isocratic-mode analysis (7). Unfortunately, this is a rather inefficient system and, therefore, not frequently used. However, DSGSLs determinations on alkyl-bonded silicas (9–11) give good efficiency and selectivity results, but require a gradient elution (from 0 to 25% acetonitrile in water) to reduce the vast retention differences. Although this method was standardized by the International Organization for Standardization (ISO) and accepted by the European Communities (12), we felt the need to develop an isocratic method because it is technically and economically superior to a gradient mode for this use.

Studies of the behavior of each GSL under various isocratic conditions offer important information for developing purification and extraction methods using large quantities of GSL from different natural products. Our access to individual very pure GSLs that could enable researchers to study their physiological effects on animals and humans prompted our own work in this area. Our aim was to develop a very simple isocratic system using inexpensive, nonpolluting, and rather nontoxic eluants. This would allow laboratories with LC capabilities to analyze GSLs, without necessarily being specialized in LC. In this paper, we present an analysis by isocratic LC of DSGSLs with a CN-bonded stationary phase and a water only mobile phase. The effects of temperature and small additions of organic cosolvents were studied on 3 different CN-bonded silicas to optimize analysis times and

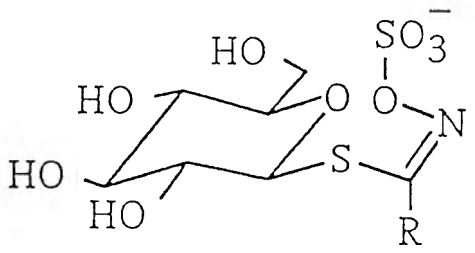

Figure 1. The general structure of glucosinolates.

selectivities. This system was evaluated by determining various GSLs in rapeseed.

Experimental

Apparatus

(a) *Apparatus for sample preparation.*—Laboratory mill (coffee grinder); water bath set at 80°C (Memmert, Schwabach, Germany); vortex stirrer for tubes (Bioblock Scientific, Illkirch, France); polypropylene tubes, 75 × 13 mm (Polylabo, Strasbourg, France); polystyrene columns, 102 × 8 mm (Cat. No. 29920, Pierce Chemical Co., Rockford, IL 61105); centrifuge, 5000 rpm, MLW T5 (Bioblock Scientific); immersible filter, PTGC 11K25 (Millipore Corp., Bedford, MA 01730); 13 mm id glass tube.

(b) LC apparatus.—High-pressure gradient LC device: 2 Model 64 pumps and dynamic mixing chamber (Knauer, Berlin, Germany); injector Model 7125 (Rheodyne Inc., Cotati, CA 94931) with 20 μL loop; column oven, Model Croco-Cil (Cluzeau Info Labo, Ste Foy la Grande, France); diode array detector, Model Polychrom 9065 (Varian Instrument Group, Sunnyvale, CA 94034); spectrophotometric UV detector with monochromator, Model 87 (Knauer); integrator-recorder C-R3A (Shimadzu, Kyoto, Japan).

(c) *Chromatographic columns.*—(*1*) Zorbax ODS, 10 μm, 250 × 21.2 mm; Zorbax CN, 5 μm, 150 × 4.6 mm (Dupont de Nemours, Wilmington, DE 19898). (*2*) Lichrospher C8 end-capped, 5 μm, 125 × 4 mm; Lichrospher CN, 5 μm, 250 × 4 mm, and Manucart fittings (E. Merck, Darmstadt, Germany). (*3*) Ultrasphere CN, 5 μm, 250 × 4.6 mm (Beckman Instruments, Inc., Fullerton, CA 92634).

Reagents

(a) *Sodium acetate, acetic acid, and formic acid.*—Normapur quality (Prolabo).

(b) *Imidazole.*—Cat. No. 26081 (Serva, Heidelberg, Germany)

(c) *Sephadex and Sepharose.*—DEAE Sephadex A25 and DEAE Sepharose CL-6B (Pharmacia, Uppsala, Sweden).

(d) *Sulfatase type H1.*—Cat. No. 9626 (Sigma Chemical Co., St. Louis, MO 63178). Purified as follows: Prepare five 102 × 8 mm icn-exchange columns, each containing 0.5 mL DEAE Sepharose Cl-6B gel. To each add 1 mL 6M imidazole formate, and rinse twice with 1 mL water. Add 0.5 mL sulfatase (10 mg/mL), and wash with 1.5 mL water. Elute sulfatase with 1.5 mL 0.2M sodium acetate and add eluates from the 5 columns to a 13 mm id tube. Suppress sodium acetate with an immersible filter linked to a vacuum source. When the eluate volume reaches 0.1 mL, add 2.5 mL water and repeat filtration. Adjust volume to 2.5 mL with water (master solution). Store at −18°C and dilute twice before use.

(e) *DSGSL standards.*—(*1*) Sinigrin (Cat. No. S330-5 Aldrich Chemical Co., Steinheim, Germany). (*2*) Glucotropaeolin (Cat. No. 24758, Merck). (*3*) Desulfogluconasturtiin (DSGST) (13) and desulfoglucobrassicin (DSGBS) (14): synthesized by M.-C. Viaud and P. Rollin (Laboratoire de Chimie Bioorganique et Analytique, Orléans, France).

(f) *LC solvents.*—Use only LC quality solvents. Acetonitrile and tetrahydrofuran (Merck); methanol (S.D.S., Peypin, France); demineralized water purified on Norganic cartridge (Cat. No. C 1512000, Millipore); and 0.1mM potassium nitrate.

Preparation of Sample

For LC of DSGSLs (12).—Extract 200 mg crushed seeds twice in a 75 × 13 mm polypropylene tube with 2 mL boiling methanol–water (7 + 3) for 5 min. (The hot solvent treatment allows GSLs to be completely extracted without hydrolysis by the endogenous myrosinase.) Centrifuge at 5000 rpm for

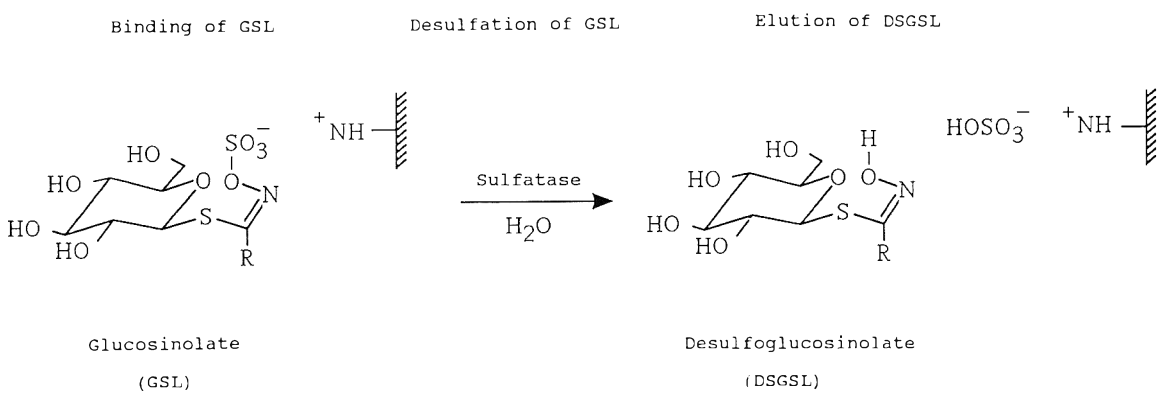

Figure 2. Enzymatic desulfation of GSL on ion-exchanger.

5 min, combine supernatants, and adjust volume to 5 mL with water. At beginning of extraction, introduce appropriate internal standard (0.2 mL of either 7.5mM sinigrin or 7.5mM glucotropaeolin; glucotropaeolin was used in this work, but the choice of internal standard depends on its presence in seeds and its LC separation from other DSGSLs). Place 1 mL extract on a 102 × 8 mm ion-exchange column containing 0.4 mL DEAE Sephadex A 25 gel previously regenerated with 1 mL 6M imidazole formate and rinsed with 4 mL water. After passage of extract, add 2 successive 1 mL portions of 0.02M, pH 4.0 sodium acetate, and apply 75 μL purified and twice diluted sulfatase solution to the resin (Figure 2). Incubate overnight and elute DSGSLs with 1.5 mL water. Reserve for LC analysis.

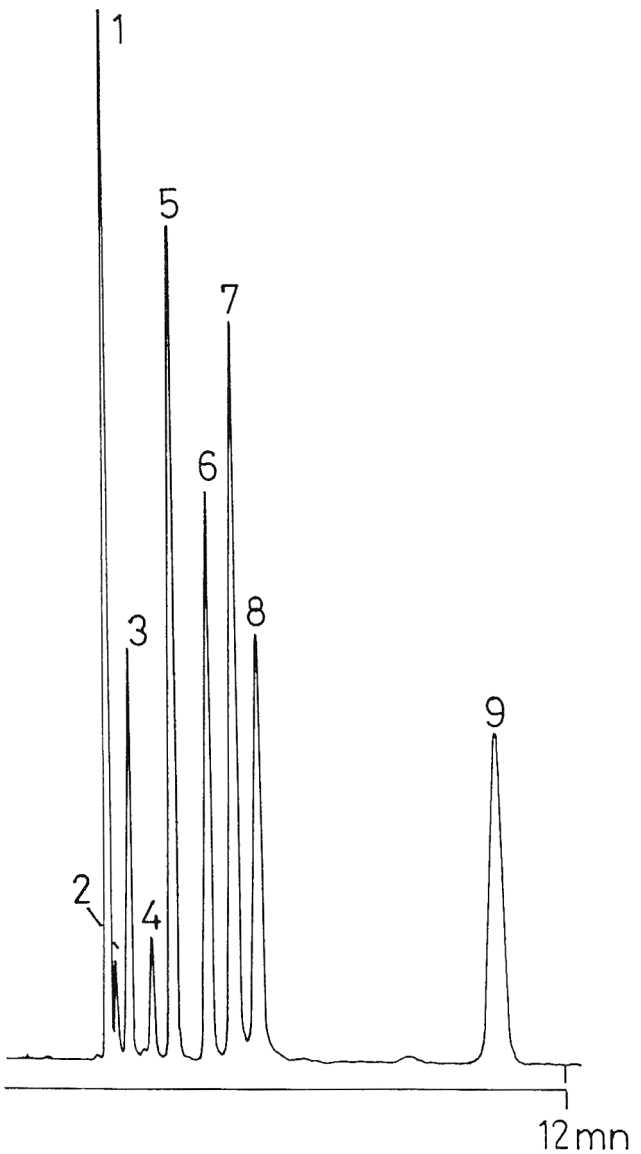

Figure 3. Chromatogram of standard solution of DSGSLs. Column: Lichrospher CN, 250 × 4 mm. Eluant, water, 1 mL/min. Temperature, 21°C. Peaks: 1, DSPRO; 2, DSGNL; 3, DSGNA; 4, DSGBN; 5, DSGTL (internal standard); 6, DS4-OHGBS; 7, DSGST; 8, DSGBS; 9, DSnGBS.

Preparation of Standard

A standard mixture containing several DSGSLs was prepared in the following way: desulfoprogoitrin (DSPRO), desulfogluconapoleiferin (DSGNL), desulfogluconapin (DSGNA), desulfo-4-OH-glucobrassicin (DS4-OHGBS), and desulfoglucobrassicanapin (DSGBN) (present in great quantities in rapeseed) were prepared from seed extracts as in *Preparation of Sample*. Desulfoglucotropaeolin (DSGTL) was prepared by enzymatic desulfation of commercial GTL. DSGST and DSGBS were synthesized by Viaud and Rollin. Desulfoneoglucobrassicin (DSnGBS) was isolated by semi-preparative LC of a DSGSLs mixture obtained by desulfating a large amount of rapeseed extract (nGBS is present in small quantities in rapeseed).

DSGSLs (ca 25 mg) containing ca 1% DSnGBS was injected into the Zorbax ODS column with the following gradient elution: 100% water to 50% acetonitrile in water in 60 min with a flow rate of 5 mL/min. The fraction containing DSnGBS was collected at a retention time of 31.8 min. After the solvent was evaporated, DSnGBS was dissolved in 1 mL water, and a 0.3mM solution was obtained.

Determination

Isocratic LC method.—Samples were injected onto the LC system with isocratic elution at 1 mL/min and at room temperature. The Lichrospher CN column was used with demineralized water as the mobile phase filtered at 0.22 μm. DSGSLs were determined by UV absorbance at 229 nm. The correction coefficients for quantitation were determined previously (11, 12), as follows: DSPRO, 1.15; DSGNL, 1.05; DSGNA, 1.17; DSGBN, 1.21; DSGTL, 1.00; DS4-OHGBS, 0.29; DSGST, 1.00; DSGBS, 0.30; DSnGBS, 0.21; DSSNB, 1.00; and DSSIN, 1.05 (Figure 3).

Gradient LC method.—EEC method (12).

Results and Discussion

Methodology

The R residues carried by GSLs (Table 1), which can be alkenyl or hydroxy-alkenyl, arylalkyl, and indolyl or hydroxy-indolyl, are distinguished by their solubility properties, polarities, hydrophilicity, and hydrophobicity. A stationary phase of intermediate polarity, such as CN-bonded silica, was used to elute the different compounds with only one mobile phase to remain in an isocratic mode.

Chromatographic behavior of bonded silicas, whatever their polarities, can differ greatly from one another in a given category, depending on the characteristics of the initial silica and the bonding techniques used for their synthesis. For this reason, we studied the properties of 3 CN-bonded silicas with spheric particles (5 μm diameter), belonging to 2 large classes (15) called monomeric (Zorbax and Ultrasphere) and polymeric (Lichrospher). None of the 3 was "end-capped," i.e., no postbonding treatment was carried out to inactivate the residual silanol functions. These CN-phases were chosen simply because of their availability in our laboratory.

When DSGSLs are prepared in solution in an aqueous phase, the mobile phases must contain high percentages of water to avoid loss of efficiency and resolution (16), peak distortions, and peak splittings due to a too great difference

Table 1. Glucosinolates in rapeseed

Class	Nature of R[a]	Glucosinolate	GSL
Alkenyl	Prop-2-enyl	Sinigrin[b,c]	SIN
	But-3-enyl	Gluconapin	GNA
	Pent-4-enyl	Glucobrassicanapin	GBN
Hydroxy alkenyl	2-Hydroxybut-3-enyl	Progoitrin	PRO
	2-Hydroxypent-4-enyl	Gluconapoleiferin	GNL
Arylalkyl	Benzyl	Glucotropaeolin[b]	GTL
	2-Phenylethyl	Gluconasturtiin	GST
	4-Hydroxybenzyl	Sinalbin[c]	SNB
Indolyl	Indol-3-ylmethyl	Glucobrassicin	GBS
	4-Hydroxyindol-3-ylmethyl	4-OH-glucobrassicin	4-OHGBS
	1-Methoxyindol-3-ylmethyl	Neoglucobrassicin	nGBS

[a] Variable part of the molecule (Figure 1).
[b] Glucosinolate absent in rapeseed, used as internal standard for chromatographic analysis.
[c] Occurs in small amounts in rapeseed but in large amounts in other cruciferous seeds that may be associated in commercial samples.

between the eluant polarities and the solvent injected. This precaution could avoid a supplementary step in preparing the sample before injection into the LC system.

Mainly for reasons of miscibility, the choice of the organic solvent is limited to polar solvents, the most common of which are methanol (MeOH), acetonitrile (ACN), and tetrahydrofuran (THF). However, the remarkable differences in selectivities that may be induced by these different organic solvents, particularly by THF (17), should be considered. As is often the case in a hydro-organic medium, the high wetting power of THF, its total miscibility, and its great affinity for water cause the THF–water eluants to lead to "induced peaks" when pure aqueous solutions are injected. These variations take place at retention times near those of the first eluted DSGSL and make the chromatogram difficult to interpret. Consequently, these studies concern only the use of MeOH and ACN as water cosolvents.

Choosing the Organic Cosolvent

Table 2 shows the effect of the organic cosolvent (MeOH or ACN) on an Ultrasphere CN column. The eluting powers of ACN and MeOH are known to be very different. For example, 10% MeOH is needed to obtain the same eluting power as only 5% ACN. Selectivity becomes excellent with MeOH to quantitate DSPRO–DSGNL, which is impossible with ACN. However, a reverse effect exists with the DSGBS–DSGST pair, which can be quantitated in an ACN eluant but not in an MeOH eluant. Because of its transparency in the UV and its reduced toxicity, ACN was chosen for further studies.

Comparison of Retentions and Selectivities with 3 CN-Type Stationary Phases

Table 3 shows the k′ capacity factor values of the DSGSLs contained in the standard solution that was chromatographed with 2 eluants at room temperature. Table 4, obtained from the k′ values of Table 3, shows the selectivities of some DSGSLs pairs having possible separation difficulties. Both Tables 3 and 4 show the advantages and disadvantages of each of these 6 systems. As in the case of apolar silicas, the use of ACN reduces retentions. With other previously mentioned organic cosolvents (MeOH and THF), the same effect on the capacity factors may be noted. To separate DSGSLs in an isocratic mode within a reasonable time span, Lichrospher (bonded polymeric phase) is more practical than both Ultrasphere and Zorbax monomeric phases, especially with indolyl DSGSLs, where "hydrophobic" interactions increase. The last 2 columns of Table 3 show the vast differences between Zorbax and the other 2 phases for solutes that were eluted last. Lichrospher and Ultrasphere phases show the smallest interactions because the k′ values are clearly smaller than those of the Zorbax phase.

Table 2. k′ Capacity factors[a] and selectivities α[b] with Ultrasphere CN at 21°C. Mobile phase CH_3CN or CH_3OH added to water

k′	DSPRO	DSGNL	DSGNA	DSGBN	DSGTL	DS4–OHGBS	DSGST	DSGBS	DSnGBS
CH_3CN, 5%	0.51	0.51	0.83	1.32	1.75	2.23	2.95	3.36	7.60
CH_3OH, 10%	0.45	0.66	0.82	1.30	1.70	2.17	3.12	3.12	7.80
CH_3OH, 5%	0.52	0.76	0.98	1.67	2.26	2.82	4.01	4.10	—

α	DSPRO, DSGNL	DSGNL, DSGNA	DS4–OHGBS, DSGBN	DS4–OHGBS, DSGTL	DSGBS, DSGST
CH_3CN, 5%	1.00	1.63	1.69	1.27	1.14
CH_3OH, 10%	1.47	1.24	1.67	1.27	1.00

[a] $k' = (V_r - V_0)/V_0$.
[b] $\alpha A,B = k'B/k'A$.

Table 3. k' Capacity factors[a] and desulfoglucosinolate elution order[b] for CN-bonded silicas, room temperature (21°C)

Stationary phase	Eluant	DSPRO	DSGNL	DSGNA	DSGBN	DSGTL	DS4–OHGBS	DSGST	DSGBS	DSnGBS
Lichrospher CN	CH_3CN, 5%[b]	1.35 (1)	1.50 (2)	1.66 (3)	1.94 (4)	2.32 (5)	2.69 (6)	3.03 (7)	3.35 (8)	5.58 (9)
Ultrasphere CN	CH_3CN, 5%[b]	0.51	0.51	0.83	1.32	1.75	2.23	2.95	3.36	7.60
Zorbax CN	CH_3CN, 5%[b]	1.00	1.60	2.42	5.37	6.73 (6)	6.12 (5)	16.2 (8)	13.5 (7)	60.3
Lichrospher CN	water	1.35	1.50	1.72	2.14	2.50	3.01	3.63	3.96	8.30
Ultrasphere CN	water	0.64	1.02	1.30	2.45	3.18	3.59	5.86 (8)	5.41 (7)	23.0
Zorbax CN	water	1.93	4.97	7.18	22.4 (5)	26.2 (6)	16.5 (4)	81.8 (8)	53.7 (7)	> 160

[a] $k' = (V_r - V_0)/V_0$.
[b] In water.

Our previous studies, aimed at classifying a great number of bonded silicas according to their hydrophobicity (18), showed that the interactions with Zorbax phases were considerably hydrophobic. Under these conditions, a Zorbax CN phase can be compared with the apolar μBondapak C18 phase (Waters) (19, 20). Among the 3 CN phases studied, the Zorbax phase showed much greater retention in an aqueous medium than in a 5% ACN medium, and weaker retention for DSGSLs having either a reduced apolar residue (DSGNA) or a hydroxyl group (DSPRO, DSGNL, DS4-OHGBS). Increasing the chain length of the residue by methylene (Table 1) considerably increases the apolar interactions, thus helping to raise the corresponding capacity factor (Table 3).

For the Lichrospher CN phase, the retention order of the 9 DSGSLs is identical with either a hydro-organic eluant or pure water. In the Ultrasphere CN phase, there is only one elution inversion between the DSGST and DSGBS when a pure aqueous medium is used. Finally, relative to these 2 phases, the Zorbax CN showed a different retention order in both a hydro-organic and pure water medium. Moreover, the hydroxylated indolyl compound (DS4-OHGBS) was more rapidly eluted.

Temperature Influence

Efficiency varies inversely with the viscosity of the eluant. Thus, lower efficiencies were observed with pure water in comparison with the water–ACN (95 + 5) mixture. To avoid this problem, analyses were performed at higher temperatures. Tables 5, 6, and 7 give details on the advantages and disadvantages of 50°C compared to 21°C. Except for analyses times practically halved on the Lichrospher or Ultrasphere columns (Table 7), the elution order remained the same (Table 5). Table 6 shows that the selectivities were slightly modified following the stationary phases and DSGSLs pairs.

When the Ultrasphere column is used, the coelution of DSGTL (internal standard) with DS4-OHGBS requires another standard. This choice may be made easily because the preparation of some 20, mostly artificial GSLs (14, 21), using different synthetic routes, has now been worked out in our laboratory.

Choice of an Optimal Method

Our preliminary study clearly shows that an effective analysis can be performed on Lichrospher CN in less than 12 min, using pure water at room temperature (Figure 3). A better analysis on Ultrasphere CN is possible; however, it requires approximately 55 min in the presence of DSnGBS, or less than 15 min in its absence.

Using pure water at 50°C and 1 mL/min on Ultrasphere CN, analyses may be obtained within 30 min (Figure 4), or 10 min if DSnGBS is not quantitated and an internal standard other than the GTL is used (Table 6). However, SIN, often used as internal standard, is not suitable for CN-phase analy-

Table 4. Compared selectivities α[a] for some desulfoglucosinolates (calculated from Table 3)

Stationary phase	Eluant	DSPRO, DSGNL	DSGNL, DSGNA	DS4–OHGBS, DSGBN	DS4–OHGBS, DSGTL	DSGBS, DSGST
Lichrospher CN	CH_3CN, 5%[b]	1.11	1.22	1.37	1.16	1.11
Ultrasphere CN	CH_3CN, 5%[b]	1.00	1.63	1.69	1.27	1.14
Zorbax CN	CH_3CN, 5%[b]	1.60	1.51	1.14	0.91 1.10[c]	0.83 1.20[c]
Lichrospher CN	water	1.11	1.15	1.41	1.20	1.09
Ultrasphere CN	water	1.59	1.27	1.46	1.13	0.92 1.08[c]
Zorbax CN	water	2.57	1.46	0.74 1.36[c]	0.63 1.59[c]	0.66 1.52[c]

[a] $\alpha\ A,B = k'B/k'A$.
[b] In water.
[c] $\alpha\ B,A = 1/\alpha\ A,B = k'A/k'B$.

Table 5. k' Capacity factors of desulfoglucosinolates at 21 and 50°C for CN-bonded silicas (Eluant: water)

Stationary phase	Temperature, °C	DSPRO	DSGNL	DSGNA	DSGBN	DSGTL	DS4–OHGBS	DSGST	DSGBS	DSnGBS
Lichrospher CN	21	1.35	1.50	1.72	2.14	2.50	3.01	3.63	3.96	8.30
	50	1.18	1.18	1.44	1.73	1.99	2.23	2.61	2.73	4.85
Ultrasphere CN	21	0.64	1.02	1.30	2.45	3.18	3.59	5.86	5.41	23.0
	50	0.64	0.89	1.09	1.85	2.30	2.30	4.04	3.80	11.4
Zorbax CN	21	1.93	4.97	7.28	22.4	26.2	16.5	81.8	53.7	—
	50	1.32	2.95	4.39	11.8	12.7	7.70	35.6	21.1	—

Table 6. Compared selectivities α^a for some desulfoglucosinolates (calculated from Table 5 — Eluant: water)

Stationary phase	Temperature, °C	DSPRO, DSGNL	DSGNL, DSGNA	DS4–OHGBS, DSGBN	DS4–OHGBS, DSGTL	DS4–OHGBS, DSGST
Lichrospher CN	21	1.11	1.15	1.41	1.20	1.09
	50	1.00	1.22	1.29	1.12	1.05
Ultrasphere CN	21	1.59	1.27	1.46	1.13	0.92 1.08[b]
	50	1.33	1.22	1.24	1.00	0.94 1.06[b]
Zorbax CN	21	2.57	1.46	0.74 1.36[b]	0.63 1.59[b]	0.66 1.52[b]
	50	2.23	1.49	0.65 1.53[b]	0.61 1.65[b]	0.59 1.69[b]

[a] α A,B = k'B/k'A.
[b] α B,A = 1/α A,B = k'A/k'B.

ses because of the insufficient selectivities among DSPRO, DSSIN, and DSGNL.

Evaluation of the Proposed Method

Results from our first study illustrate a simple isocratic method of DSGSLs analysis. The method is 2 or 3 times faster than the gradient method, it is inexpensive, and it uses a nonpolluting, nontoxic eluant.

When rapeseed samples are pure, only DSPRO, DSGNL, DSGNA, DSGBN, DS4-OHGBS, DSGBS, DSnGBS, and DSGST (Table 1) have to be determined by LC analyses (Figure 5). Unfortunately, other cruciferous seeds (e.g., *Sinapis arvensis L.* or *Thlaspi arvense L.*) may be present as admixtures in commercial samples, and their specific GSLs (SIN, SNB) (Table 1) may interfere in the chromatographic separation (Figure 6). Such samples were analyzed to evaluate our method. Only repeatability and recovery tests compared to the gradient LC method were performed. Reproducibility studies may be carried out at a later date in other laboratories to meet the particular needs of an alternative to the EEC method.

Four commercial rapeseed samples, 2 of which contained impurities, were prepared according to the method described above. Each of the resulting DSGSLs solutions was analyzed 5 times by the isocratic and gradient methods. The resulting differences were caused only by LC separation and peak measurements. The influence of the SIN and SNB presence found in seeds by the isocratic method is illustrated in the chromatogram (Figure 6). Separation of DSPRO, DSSIN, and DSGNL is incomplete and DSSNB elutes very close to DSGBN. Even DSSIN and DSSNB were identifiable, as well as the pollution in the rapeseed samples, but determination of these individual unresolved GSLs remained imprecise or impossible.

The results in Table 8 show that determination of total GSL content was not affected by isocratic elution. Indeed, the poorly separated DSGSLs have similar relative response coefficients in UV detection. Consequently, peak calculation errors do not have an important effect on the determination of total GSL content. Precision is due to very good separation of the 2 groups of unresolved DSGSLs from the internal standard (DSGTL) and from indolyl DSGSLs (DS4-OHGBS, DSGBS, DSnGBS), which have very different relative response coefficients.

Repeatability values obtained by the 2 LC methods are given in Table 8 and, as expected, the best results were obtained by the isocratic method, which generates more reproducible elution conditions than the gradient method. This is because the isocratic eluant composition is constant, thus minimizing ghost peaks, baseline instability, and UV spectrum modifications (absorption coefficient at 229 nm).

Table 7. Comparative time analysis (min) of DSGSLs solution — Mobile phase: water, 1 mL/min

	Temperature	
Column[a]	21°C	50°C
Lichrospher CN	10.9	6.8
Ultrasphere CN	52.8	27.3
Zorbax CN	>180	>125

[a] For characteristics, see *Experimental.*

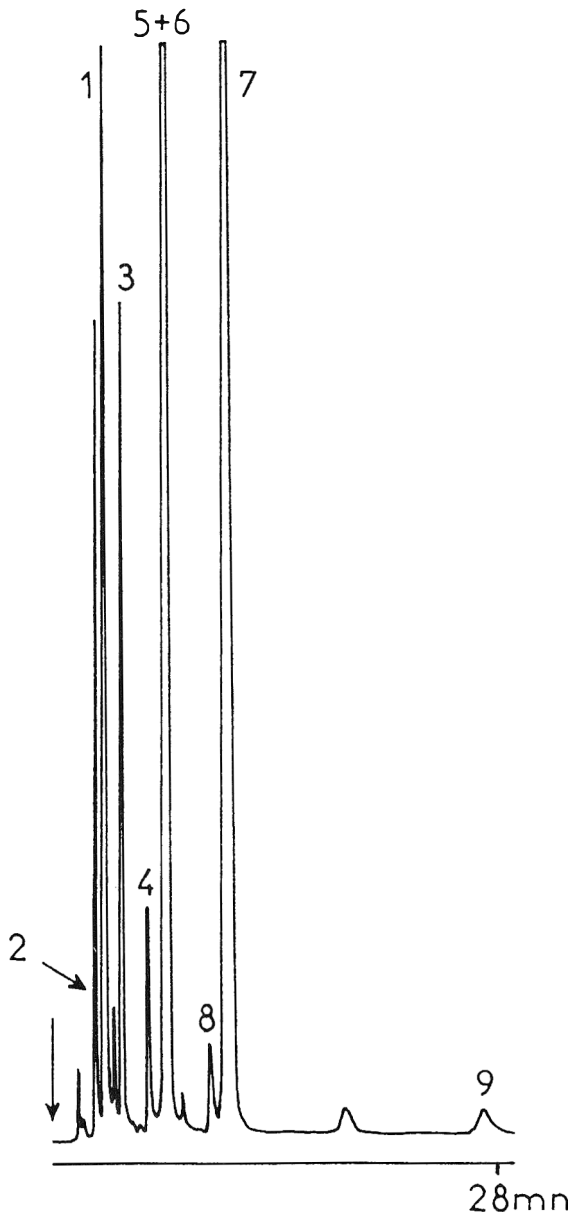

Figure 4. Chromatogram of standard solution of DSGSLs. (Standard solutions in Figures 3 and 4 are different.) Column: Ultrasphere CN, 250 × 4.6 mm. Eluant, water, 1 mL/min. Temperature, 50°C. Peaks: see Figure 3.

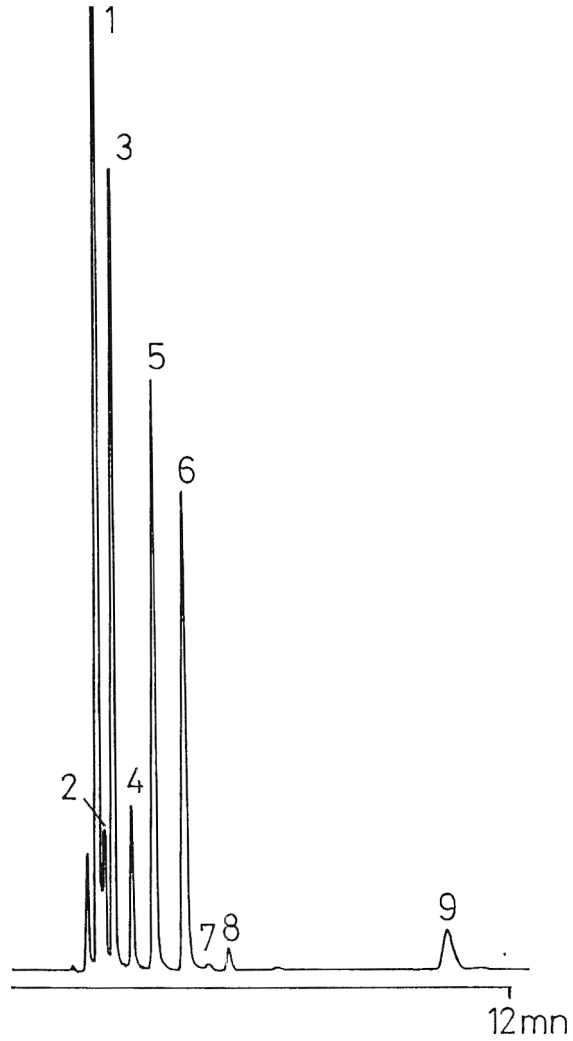

Figure 5. Chromatogram of DSGSLs solution prepared from commercial sample of pure rapeseed. Conditions and peaks: see Figure 3.

These results show that the isocratic method offers a reliable determination of total GSL content in rapeseed. Further evaluations are currently being worked out in our laboratory.

Complementary Studies

These encouraging results led us to perform tests on other weakly polar stationary phases, such as diol, phenyl, and trimethylsilyl (TMS), as well as on the more polar stationary phases, such as amino bonded and bare silicas. Diol silicas did not lead to sufficient retentions, but phenyl and TMS will require further studies because of their somewhat different selectivities. Instabilities of amino silicas, as well as certain manufacturers' advice not to use them in strongly aqueous media, discouraged our using them. Bare silicas appear to be of sufficient interest for further studies because their selectivities were rather different from those presented in this work. Consequently, tests are being undertaken on apolar, fairly polar, or polar columns, and even on bonded or unbonded polymeric supports. Hydro-organic or nonaqueous eluants should indicate the advantages and disadvantages of each system.

A preliminary study of the chromatography of DSGSLs in a supercritical phase (SFC) is currently being published (22). The pros and cons of LC in comparison with SFC will be emphasized. This work has led to a number of chromatographic data that will be further exploited by chemometric techniques such as hierarchical ascendent classification, cor-

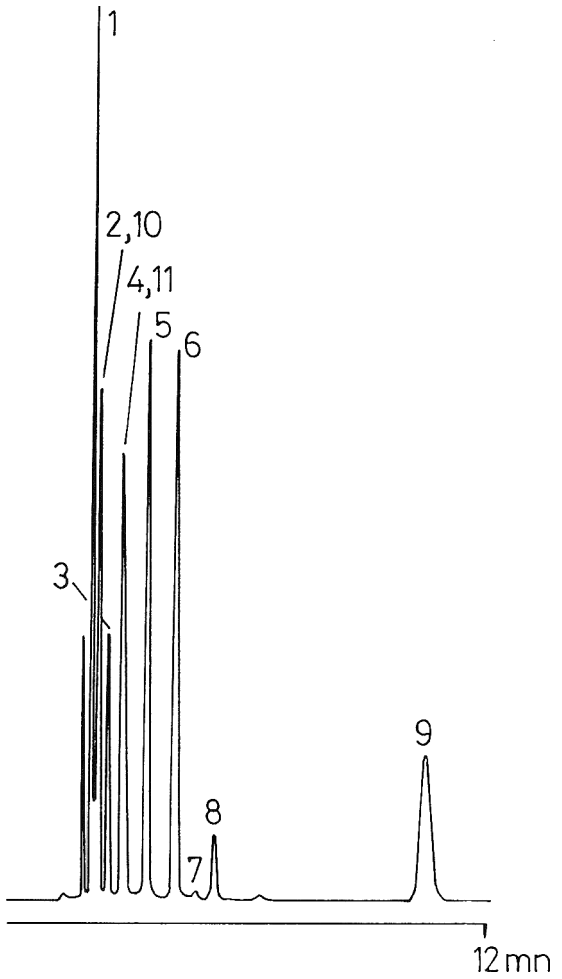

Figure 6. Chromatogram of DSGSLs solution prepared from commercial sample of rapeseed with polluting cruciferous seeds containing SIN and SNB. Peaks: 1–9, see Figure 3; 10, DSSIN; 11, DSSNB. Conditions: see Figure 3.

respondent factor analysis, and principal component analysis (18). These detailed studies could provide laboratories wishing to quantitate any GSL in any GSLs mixture with the most appropriate method in terms of time and selectivity.

Conclusion

The results of the total GSL content of commercial samples determined by our method were in good agreement with those determined by the official method. The dosage, faster than in gradient analyses, is more accessible for nonspecialists equipped with a minimum LC device, and the choice among the CN-bonded silicas of the different stationary phases is now easier. New GSLs synthesized by methods developed in our laboratory provide the ideal standard for every kind of analysis, however complex it may be. Hence, our study, using an inexpensive, nontoxic system with pure water as the eluant, opens the way to a simple analysis of GSLs.

Table 8. Comparative results for determination of total GSL content — (μmol/g) in 4 commercial rapeseed samples by isocratic and gradient LC methods

LC method		Samples			
		Rapeseed with polluting seeds		Pure rapeseed	
		A	B	C	D
Isocratic	Av.[a]	27.1	46.1	19.0	36.6
	SD[b]	0.1	0.8	0.2	0.4
Gradient	Av.[a]	27.9	46.1	19.0	36.5
	SD[b]	0.9	1.3	0.9	1.0

[a] Calculated with 5 determinations.
[b] SD = standard deviation, calculated with 5 values.

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Analysis of Trypsin Inhibitors and Lectins in White Kidney Beans (*Phaseolus vulgaris*, var. Processor) in a Combined Method

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Buffered saline extraction, affinity chromatography, and Folin–BSA protein assay were used consecutively to provide a combined method for analysis of trypsin inhibitors and lectins in white kidney beans (*Phaseolus vulgaris*, var. Processor). The method was tested by following the decrease of both antinutritional factors by germination of the beans for 7 days at 20°C. Repeatability coefficients of variation were 2–7.4% for the trypsin inhibitors and 2.2–10% for the lectins. After 7 days of germination, trypsin inhibitors and lectins were reduced by 72 and 92%, respectively.

Antinutritional factors (ANFs) are present in beans and have to be inactivated by processing (heat, germination) to make the beans more suitable for food and feed (1). In white kidney beans (*Phaseolus vulgaris*; PV) the important ANFs are trypsin inhibitors (TIs) and lectins (2), while the beans are relatively low in tannins.

For analysis of TIs in soya beans, we developed a method based on trypsin–sepharose affinity chromatography (3). The advantages of the method are increased sensitivity by concentrating TIs on the column, and increased specificity in comparison with the assay methods (4). Affinity chromatography on porcine thyroglobulin–sepharose has been used for measuring the lectin content of developing seeds in common beans (5). According to Felsted et al. (6), porcine thyroglobulin–agarose completely binds lymphocyte- and erythrocyte-subunits of lectins.

In this study, a combined method is evaluated for analysis of TIs and lectins in PV using affinity chromatography to save time for sample preparation and possibly lower cost of reagents.

Material

In cooperation with Ir. F.H.M.G. Savelkoul of the Department of Animal Nutrition of this university, white kidney beans were germinated in wet sand beds for 0.5 to 7 days at 20°C (12 h light/day at the Government Seed Testing Station). The sprouted beans were sieved, carefully washed, and water was removed by filter paper. After freezing in liquid nitrogen and homogenization in a Waring blender, the crude bean flour was freeze-dried, ground on a 1 mm sieve, stored in tightly closed plastic containers at 4°C, and analyzed for moisture content and crude protein (Kjeldahl-N × 6.25; Table 1).

METHOD

Principle

Both TIs and lectins are extracted from the sample with a phosphate buffer (pH 8.0), containing 0.5M NaCl. TIs and lectins are isolated from the extract by affinity chromatography with trypsin–sepharose 4B and porcine thyroglobulin–agarose, respectively. The amounts of protein obtained are determined by a modified method according to Lowry (7) using bovine serum albumin as a standard.

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Reagents

All solutions must be at room temperature before pH adjustment.

(a) *Monobasic potassium phosphate.*—0.05M. Dissolve 1.7 g KH_2PO_4 and 7.3 g NaCl in water and dilute to 250 mL.

(b) *Dibasic sodium phosphate.*—0.05M. Dissolve 17.9 g $Na_2HPO_4.12H_2O$ and 29.2 g NaCl in water and dilute to 1 L.

(c) *Phosphate buffer (pH 8.0).*—To 1 L (b), add sufficient (a) to adjust to pH 8.0. Keep solution refrigerated.

(d) *Acetate buffer (pH 5.2).*—0.05M, containing 0.5M NaCl. Add 2.78 mL concentrated acetic acid to 400 mL water, dissolve 29.2 g NaCl in the solution, adjust to pH 5.2 with 2M NaOH, and dilute to 1 L.

(e) *Glycine buffer (pH 3.0).*—0.05M, containing 0.5M NaCl. Dissolve 3.75 g glycine and 29.2 g NaCl in water, adjust to pH 3.0 with 2M HCl, and dilute to 1 L.

(f) *Carbonate solution.*—10% Na_2CO_3 in 0.5M NaOH. Dissolve 10 g Na_2CO_3 in 100 mL 0.5M NaOH.

(g) *Copper sulfate.*—5% $CuSO_4 \cdot 5H_2O$. Dissolve 0.5 g $CuSO_4 \cdot 5H_2O$ in 10 mL water.

(h) *Tartrate buffer.*—10% potassium sodium tartrate solution. Dissolve 1 g KNa tartrate·$4H_2O$ in 10 mL water.

(i) *Copper reagent.*—Add 0.1 mL copper sulfate (g) and 0.1 mL tartrate buffer (h) to 10 mL carbonate solution (f). Prepare daily.

(j) *Folin reagent.*—Dilute 1 mL Folin-Ciocalteu reagent (E. Merck, Darmstadt, Germany, No. 9001) with 1 mL water. Prepare daily.

(k) *Albumin stock standard.*—25 mg/25 mL. Transfer exactly 25 mg bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO 63178, No. A4378) to 25 mL volumetric flask and dissolve to volume with glycine buffer (pH 3.0). Transfer 0.6 mL aliquots to 1 mL Eppendorf cups and store at −20°C.

(l) *Intermediate albumin standard.*—50 μg/mL. Dilute 0.5 mL stock standard solution (k) to 10 mL with glycine buffer (pH 3.0) (e).

(m) *Working albumin standards.*—Dilute 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL aliquots of intermediate standard solution (l) to 1 mL with glycine buffer (pH 3.0) (e) in reagent tubes to prepare a standard curve for protein determination (5–50 μg protein).

(n) *CNBr-activated sepharose 4B.*—Pharmacia LKB Biotechnology Inc., Piscataway, NJ 08854, No. 17-0430-01.

(o) *Trypsin.*—Dissolve 375 mg trypsin (E. Merck, No. 24581) in 105 mL 0.1M sodium bicarbonate buffer (pH 8.3) containing 0.5M NaCl.

(p) *Glycine–carbonate buffer (pH 8.0).*—Containing 0.1M sodium bicarbonate, 0.2M glycine, and 0.5M NaCl.

Dissolve 8.4 g $NaHCO_3$, 15 g glycine, and 29.2 g NaCl in water, adjust to pH 8.0 with 2M NaOH, and dilute to 1 L.

(q) *Porcine thyroglobulin–agarose.*—(Sigma, No. T4398). Certified binding capacity is 0.9 mg phytohemagglutinin from *Phaseolus vulgaris* per mL gel.

Apparatus

(a) *Trypsin–sepharose 4B column.*—The coupling of trypsin to CNBr-activated sepharose 4B was performed according to the instructions of the supplier (8). A 15 g portion of sepharose (n) was dispersed in 105 mL trypsin (o). Residual active groups were removed with glycine–carbonate buffer (p). The trypsin–sepharose 4B gel obtained was suspended in glycine buffer (pH 3.0) (e) and packed into several 5 mL disposable tips (Ø 9 mm; 2 mL gels). Trypsin–sepharose 4B is also available commercially (e.g., Sigma, No. T4019).

(b) *Porcine thyroglobulin–sepharose 4B column.*—This column can be prepared as above. In this study, the commercially available porcine thyroglobulin–agarose (q) was used. The gel was also packed in 5 mL disposable tips (Ø 9 mm; 2 mL gels).

(c) *Spectrophotometer.*—DU-62, Beckman Instruments.

(d) *Centrifuge.*—Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments).

Preparation of the Sample Solution

Extract sample at a 1:50 ratio with phosphate buffer (pH 8.0) (c). Stir suspension for 2 h at room temperature (magnetic stirrer) and centrifuge suspension 15 min at 15°C at 25 000 × *g*. The supernatant is ready for immediate application on both affinity columns. No further handling is necessary, and there should be no delay.

Isolation of TIs and Lectins

Wash both types of columns with 20 mL phosphate buffer (pH 8.0) (c) before use. Apply 2–50 mL sample solution to trypsin–sepharose columns and 1–50 mL sample solution to porcine thyroglobulin–agarose columns. The volume applied depends on the amounts of TIs and lectins expected in the sample and the maximum binding capacity of the columns. Be careful not to overload columns.

Eluate both types of columns with 20 mL phosphate buffer (pH 8.0), followed by 20 mL acetate buffer (pH 5.2) for trypsin–sepharose columns only. Continue to eluate both TIs and lectins from their columns with 20 mL glycine buffer (pH 3.0) into 25 mL volumetric flasks and dilute to 25 mL with glycine buffer (pH 3.0).

Table 1. Freeze-dried samples of frozen ground white kidney beans (Savelkoul, personal communication)

Days of germination	Moisture, %	Crude protein, %	Color
0	6.9	22.4	white
0.5	7.8	22.0	white
1	8.8	22.1	white
2	9.5	22.7	white
3	9.0	22.5	gray
4	10.8	23.5	gray
5	10.1	23.9	green
6	11.1	26.7	green
7	11.2	25.6	green

Eluate Protein Determination

Add 0.2 mL copper reagent to 1 mL eluate and 1 mL working standard solutions, respectively. After 15 min, add 0.1 mL Folin reagent while stirring. After 30 min, measure absorbance (A) at 750 nm using a reagent blank. Prepare standard curve by plotting concentration (μg BSA/mL) vs A and interpolate eluates' protein concentrations (a μg/mL).

Calculation

Calculate mg (TIs or lectins)/g crude protein as follows:

$$\text{mg (TI or lectin) / g crude protein} = \frac{a \times 25 \times V_1}{1000 \times V_2 \times g \times M}$$

where a = the concentration of TIs or lectins in the eluate (μg/mL); V_1 = volume phosphate buffer (pH 8.0) used to extract the sample; V_2 = volume of extract applied to the affinity columns; g = amount of freeze-dried sample used for extraction (g); M = percent crude protein of freeze-dried sample/100.

Results and Discussion

The changes of ANF contents of white kidney beans during germination are shown in Table 2. TIs and lectins were reduced by 72 and 92%, respectively, after 7 days of germination. During the first day of germination, the ANFs increased slightly and then gradually decreased until Day 4. Subsequently, an acceleration of ANF deterioration takes place, together with a discoloration and an increase of protein and moisture content (Table 1). The loss of ANFs seems to coincide with large differences in the physiology of germinating beans (9).

The calibration equations obtained for the Folin–BSA protein assay differ between A(750) = 0.0073 μg BSA + 0.004 and A(750) = 0.0089 μg BSA + 0.007, giving 20% variation; therefore, the calibration has to be repeated for every series of samples. This is not necessary for the assay of Sedmak et al., proposed for TI–protein determination (3). However, the absorbance of lectins (glycoproteins) in the latter assay is too low for an accurate measurement. According to Hartree (10), purified glycoproteins like ovalbumin can be analyzed by the Folin–BSA protein assay, which was also adopted by Felsted et al. (6) for lectins.

Comparison of the results of the analyses of lectins in Table 2 shows that during the first days of germination, the assay method, B, gives 3 times higher values than the affinity method, A. On Days 5, 6, and 7, similar results were obtained by both methods. Using the extraction conditions for lectins proposed by Felsted et al. (6) in affinity method A for beans at 0 days, we obtained 80.4 ± 5% mg protein/g crude protein, which is not significantly different from the value presented in Table 2. Paredes-López et al. (5) reported 2% of the crude proteins as lectins in mature beans, while Sharon and Lis (11) reported up to 10% of the total proteins. This makes the data (up to 34% of the crude protein) obtained from the Department of Animal Nutrition inexplicably high [possibly

Table 2. Lectins and TIs, determined in 2 extracts of freeze-dried samples of germinated white kidney beans: by combined affinity method (A), by assay method of Department of Animal Nutrition (B), and by assay method of Hamerstrand et al. (C) (16), assuming 1 mg inhibited trypsin = 0.5 mg protein TIs, molecular weight = 12 kD (13)

	Lectins				TIs					
	(A)			(B)[a]	(A)			(C)		
Days of germination	Protein, mg/crude protein, g	No. of replicates	CV, %	Protein, mg/crude protein, g	Protein, mg/crude protein, g	No. of replicates	CV, %	Protein, mg/crude protein, g	No. of replicates	CV, %
0	82.6	7	6.1	241.5	24.9	5	6.0	28.8	2	5.3
0.5	92.7	5	8.6	220.5	27.4	5	2.5	28.4	4	7.1
1	85.5	5	4.7	340.7	26.4	6	2.9	28.3	6	11.1
2	83.7	7	8.5	196.0	26.7	6	4.3	27.8	4	6.5
3	85.3	7	10.0	165.8	25.5	5	7.4	28.4	4	10.3
4	46.4	7	4.1	127.7	20.5	4	2.2	23.4	3	7.4
5	35.4	4	7.7	24.0	16.2	6	5.4	18.0	4	9.8
6	12.0	5	5.9	8.5	10.5	5	2.0	12.8	3	5.9
7	6.7	3	2.2	8.0	7.0	3	3.3	7.7	5	5.2

[a] Method of Hamer et al. (12)

caused by aspecific interactions in the FLIA assay (12)], and brings our data (up to 9% of the crude protein) within the range found elsewhere (5, 11). In Table 2, the number of replicates is not equal because of sample availability (larger amounts of extracts were needed for getting enough purified protein from samples with lower ANF contents), and because the number of available affinity columns has been extended during the experiment. Detection limits for lectins and TIs are, respectively, 2 and 1 mg protein/g crude protein using a 50 mL extract for each column application. Lower limits might be obtained by applying more than 50 mL extract on a column; however, this is not recommended because of chances of microbial spoilage and long running times.

Overloading of the affinity column material is a main concern when these types of columns are used. During our period of analysis (6 months), bound porcine thyroglobulin kept its certified capacity of 0.9 mg lectin per mL gel. Therefore, for estimating the amount of extract to be applied on the column, one should assume that 50–75% of the certified capacity is available. The procedure used for immobilizing trypsin on sepharose delivered a gel with maximum TI binding capacity of 0.45 mg Kunitz inhibitor (soya) protein per mL gel, and 0.31 mg PV inhibitor protein per mL gel. The ratio of these capacities reflects the ratio of molecular weights: 21 kD (Kunitz inhibitor)/12 kD [PV, Wu and Whitaker (13)]. From this result, we concluded that 0.25 mg/mL gel binding capacity is a solid basis for estimating the TI application volume of the sample extract.

The method is linear for lectins in the range of 0.4–1.6 mg and for TIs in the range of 0.2–0.6 mg purified protein bound to 2 mL of affinity column material. In practice, we applied a 1–4 mL extract on 2 mL thyroglobulin–agarose column and a 2–5 mL extract on a 2 mL trypsin–sepharose column (extract prepared of native white kidney beans according to the method described).

The advantages of choosing saline phosphate buffer (pH 8) as the extractant for the ground PV beans are: (*1*) The solvent is suitable for the extraction of both lectins and TIs; (*2*) the extract can be applied on the affinity columns without further handling (3); and (*3*) both columns are eluted without a viscosity enhancing substance of PV beans that can cause blockage of the affinity columns (6).

The combined method described is only suitable for legume samples containing very low amounts of tannins. Roozen and de Groot (14) reported the use of affinity chromatography for TI analyses in different kinds of legumes. The problem with legumes rich in tannins is that their extracts turn the column material brown/black and cause it to gradually loose binding capacity. Preliminary results with acid extractants containing extraction aids like polyvinylpyrrolidone or formaldehyde look promising for getting low tannin extracts. For example, a legume rich in tannins (*Vicia faba*, var. Alfred) was ground to flour, 1 g of which was mixed with 1 mL 0.5% formaldehyde solution (15) and left overnight at room temperature. Afterward, the extraction method was used at pH 8 (basic) and pH 3 (acid), while both extracts were applied on trypsin–sepharose columns at pH 8. The amounts of TIs found were 7.6 (basic) and 7.2 (acid) mg protein/g crude protein compared to 10.3 (basic) and 9.8 (acid) mg protein/g crude protein without formaldehyde pretreatment. An improvement was obtained by adding formaldehyde to the extraction buffer (0.01%, v/v): 8.8 (basic) and 10.2 (acid) mg TI protein/g crude protein. Affinity columns used for acid or basic *Vicia faba* extracts without formaldehyde treatments were useless after 2 runs. More work is needed to evaluate the yields of TIs and lectins in acid extracts.

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Identification of Whale Species by Thin-Layer Isoelectric Focusing of Sarcoplasmic Proteins[1]

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Thin-layer isoelectric focusing was applied to the identification of whale (Cetacea) species by using water-soluble sarcoplasmic proteins of skeletal muscles. Twenty-eight samples consisting of 4 species (10 samples) of baleen whales (*Mysticeti*) and 8 species (18 samples) of toothed whales (*Odontoceti*) were analyzed. Each sample (approximately 1 g) was electrophoresed with Ampholine PAGplate, pH 3.5–9.5. The electrophoretic profiles were species-specific on the 4 toothed whale species that did not have a marked intra-species difference, and all 4 baleen whale species. However, the profiles were not specific on the 4 other dolphin species, even though they were discriminable from the other 4 toothed whale species. Numerical values of pIs and relative peak heights were obtained by densitometric analysis of the isoelectro-focused protein bands. The bands were also species-specific for the 8 toothed whale species mentioned. The values may be applicable to species identification without the need for a standard sample, which may not be readily obtainable. Experiments on test samples of minke and sei whales showed that bloodletting with ice water made the densities of isoelectro-focused bands thinner, although species identification was still possible by using the inside part of muscles. Heat treatment at below 60°C for 10 min caused little denaturation; at higher temperatures the protein bands were diminished in a temperature-dependent fashion. Therefore, the present isoelectric focusing analysis should be applicable to small samples of whale meat, excluding several species of dolphins.

Whale (Cetacea) species are generally called whales, dolphins, or porpoises according to their sizes and shapes, but are taxonomically classified into baleen whales (*Mysticeti*) and toothed whales (*Odontoceti*) (1). The chemotaxonomic identification of whale species is required because some uncertainty remains in the classification of *Odontoceti* (2, 3); and, since the International Whaling Commission decided in 1982 to prohibit commercial whaling, the conventional identification of seafood meat is required to check the use of whale meat.

Electrophoretic analysis of enzymes in meat and other tissues is a reliable method to identify whale species (4). However, this method requires standard samples of known species, which are not easily obtainable. On the other hand, isoelectric focusing is also a useful and reliable method for the identification of species of marine products (5–10). Moreover, we have established the usefulness of thin-layer isoelectric focusing, which is easy to perform and does not require species standards once the assay is performed with them. We have also established numerical values for each protein band in the identification of species in foods such as fish and mushrooms (11, 12). In this paper, we demonstrate that thin-layer isoelectric focusing of water-soluble sarcoplasmic proteins is effective for identifying whale species, excluding 4 dolphin species.

Experimental

Apparatus

(a) *Electro-focusing apparatus.*—Flat bed apparatus (Multiphor II); electrophoresis constant power supply (MacroDrive 5); thermostatic circulator MultiTemp II slab cooling system; electro-focusing strip (cat. No. 1850–911); sample application pieces (cat. No. 1850–901); cellophane preserving sheet (cat. No. 1850–221)(Pharmacia LKB Biotechnology AB, Sweden).

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Table 1. English and scientific names of whale species examined

Sample No.	English name	Scientific name	Suborder
1–2	Fin whale	*Balaenoptera physalus*	*Mysticeti*
3–5	Sei whale	*Balaenoptera borealis*	*Mysticeti*
6–7	Bryde's whale	*Balaenoptera edeni*	*Mysticeti*
8–10	Minke whale	*Balaenoptera acutostrata*	*Mysticeti*
11–12	Sperm whale	*Physeter macrocephalus*	*Odontoceti*
13	Baird's beaked whale	*Berardius bairdii*	*Odontoceti*
14–15	Short-finned pilot whale	*Globicephala macrorhynchus*	*Odontoceti*
16–17	Dall's porpoise	*Phocoenoides dalli*	*Odontoceti*
18–20	Northern right whale dolphin	*Lissodelphis borealis*	*Odontoceti*
21–23	Pacific white-sided dolphin	*Lagenorhynchus obliquidens*	*Odontoceti*
24–26	Common dolphin	*Delphinus delphis*	*Odontoceti*
27–28	Striped dolphin	*Stenella coeruleoalba*	*Odontoceti*

(b) *Densitometer.*—Dual-wavelength chromato-scanner CS-910 (Shimadzu Corp., Kyoto, Japan).

(c) *Polyacrylamide gel plate.*—Ready-made polyacrylamide gel containing Ampholine (carrier ampholytes); Ampholine PAGplate, pH 3.5–9.5 (Pharmacia LKB).

Reagents

(a) *Electrolytes.*—1M NaOH (catholyte); 1M H_3PO_4 (anolyte).

(b) *pI marker solution.*—pI Calibration Kit, pH 3–10, dissolved in 100 μL distilled water (Pharmacia LKB).

(c) *Fixing solution.*—57.5 g trichloroacetic acid and 17.25 g sulfosalicylic acid dissolved in 500 mL water.

(d) *Destaining solution.*—500 mL ethanol and 160 mL acetic acid in 2 L water.

(e) *Staining solution.*—Solution of 0.46 g Coomassie Brilliant blue R-250 and 400 mL destaining solution.

(f) *Preserving solution.*—Solution of 40 mL glycerol and 400 mL destaining solution.

Preparation of Samples

Skeletal muscles of 10 individuals of 4 species in *Mysticeti* and of 18 individuals of 8 species in *Odontoceti* were examined. English and scientific names and sources of whale species used are shown in Tables 1 and 2, respectively. The species of all samples were identified by whale morphologists upon capture. Sarcoplasmic proteins were isolated from muscles preserved at −30°C (1 g unless stated otherwise) of these 28 whales by homogenizing with 20 mL cold (4°C) distilled water. Extracts were centrifuged at 1600 × *g* for 20 min at 4°C to remove debris, and the supernatants were used for the analysis. In addition, 2 whale meats that were sold as Dall's porpoise and sei whale were examined in the same manner.

Isoelectric Focusing

PAGplates were placed on a cooling (4°C) platform of electro-focusing apparatus over a thin-layer of kerosene to ensure good thermal contact. Sample application pieces were placed midway between anode and cathode. Sample supernatants (15 μL) or pI marker solution (15 μL) was pipetted onto these pieces. Electro-focusing strips were soaked in catholyte and anolyte. Strips were applied to edges of gels to provide electrical contact with platinum wire electrodes. A power supply was connected to the electrodes and a constant power of 30 watts (maximum voltage = 1500 V, maximum current = 50 mA) was applied for 1.5 h until equilibrium focusing was attained. After the focusing, electrolytes were removed and gels were placed in fixing solution for 45 min and then in destaining solution for 5 min. Gels were stained in staining solution at 60°C for 10 min, and placed in destaining solution overnight, until backgrounds of gels became clear. Stained gels were placed in preserving solution for 1 h, dried at 50°C for 40 min, and covered with cellophane preserving sheet.

Densitometry and Numerical Expression of Isoelectric Focusing Patterns

Preserved gels were scanned at a sample wavelength of 595 nm and a reference wavelength of 700 nm in a densitometer, using the scanning mode of linear scanning in transmission mode, beam length of 1 mm, and beam width of 0.05 mm. The pH gradient was graphed from the densitometric pattern of the pI maker. Isoelectro-focused protein bands of all whale samples (samples 1–28 in Table 2) were scanned. About 10 isoelectro-focused protein bands characteristic to each whale species were selected. The chart of scanned whale protein bands was set on the gradient calibration curve for pI markers, and each pI value and relative peak height (RPH) of these bands were numerically expressed. RPH values were expressed as the ratios to that of the maximum peak, which was set at 100.

Results and Discussion

Species-Specificity of Isoelectric Focusing Patterns of Sarcoplasmic Proteins

To determine the optimum quantity of sarcoplasmic proteins to be analyzed, we chose sei whale as a test sample. Figure 1 represents isoelectric focusing patterns of water-extractable sarcoplasmic proteins of sei whale at various concentrations. When 1 g meat was extracted with 20 mL water (50 mg/mL), the major bands appeared dense enough for further densitometric analysis; the densitometric profile of this gel is shown in Figure 2. Therefore, experiments were performed under this concentration.

Figure 3 shows the isoelectric focusing patterns of water-extracted sarcoplasmic proteins of 4 baleen whale species

Table 2. Sources of the whales examined

Sample No.[a]	English name	Body length, m	Sex	Capture location		Capture date
				Latitude	Longitude	
1	Fin whale	—	—	(The coast of Iceland)		1987
2	Fin whale	—	—	(The coast of Iceland)		1987
3	Sei whale	—	—			
4	Sei whale	—	—	(The coast of Iceland)		1986
5	Sei whale	—	—	(The coast of Iceland)		1986
6	Bryde's whale	13.8	Female	11°16'S	93°34'E	1978.11.15
7	Bryde's whale	13.5	Female	11°49'S	94°25'E	1978.11.18
8	Minke whale	6.1	Female	37°12'N	141°24'E	1984.4.1
9	Minke whale	6.2	Male	37°44'N	141°30'E	1984.4.1
10	Minke whale					
11	Sperm whale	11.5	Male	31°05'N	133°03'E	1985.11.30
12	Sperm whale	11.6	Male	31°06'N	133°01'E	1985.11.30
13	Baird's beaked whale	9.6	Female	35°50'N	141°17'E	1988.11.4
14	Short-finned pilot whale	4.7	Female	38°50'N	141°54'E	1984.11.8
15	Short-finned pilot whale	6.4	Male	39°12'N	142°02'E	1984.11.9
16	Dali's porpoise (*dalli*-type)	1.88	Male	50°07'N	142°45'W	1987.9.4
17	Dali's porpoise (*dalli*-type)	1.88	Male	50°09'N	142°47'W	1987.9.4
18	Northern right whale dolphin		Male	42°16'N	154°28'E	1987.9.23
19	Northern right whale dolphin	2.20	Female	43°37'N	178°03'E	1987.9.15
20	Northern right whale dolphin	1.96	Female	42°16'N	154°28'E	1987.9.23
21	Pacific white-sided dolphin	1.74	Male	43°37'N	177°53'E	1987.9.15
22	Pacific white-sided dolphin	1.68	Female	43°37'N	177°53'E	1987.9.15
23	Pacific white-sided dolphin	1.68	Male	43°37'N	177°53'E	1987.9.15
24	Common dolphin	1.55	Female	40°33'N	149°06'E	1987.9.24
25	Common dolphin	1.72	Female	40°31'N	149°37'E	1987.9.24
26	Common dolphin	1.76	Male	40°31'N	149°37'E	1987.9.24
27	Striped dolphin	—	—	(The coast of Japan)		1980.11.27
28	Striped dolphin	—	—	(The coast of Japan)		1980.11.27

[a] Sample numbers correspond to those in Table 1.

(sample numbers correspond to those in Tables 1 and 2). The 4 species gave species-specific patterns, indicating that this assay is effective for identification of each species. With regard to intraspecific variations, no marked difference was observed, although a few bands of sei and minke whales showed different densities in each individual. Furthermore, all baleen whales had a sharp band of pI 5.60, which was not detected in any toothed whales except sperm whale (Fig-

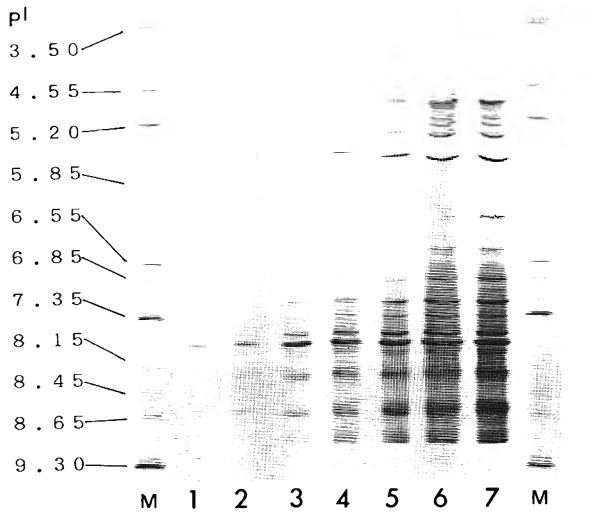

Figure 1. Isoelectric focusing patterns of various concentrations of sarcoplasmic proteins of a sei whale. Meat was extracted with 20 mL water. Concentration in g/20 mL: (1) 0.1, (2) 0.2, (3) 0.5, (4) 1.0, (5) 3.0, (6) 5.0, and (7) 7.0. M: pI marker. Sample size: 15 μL.

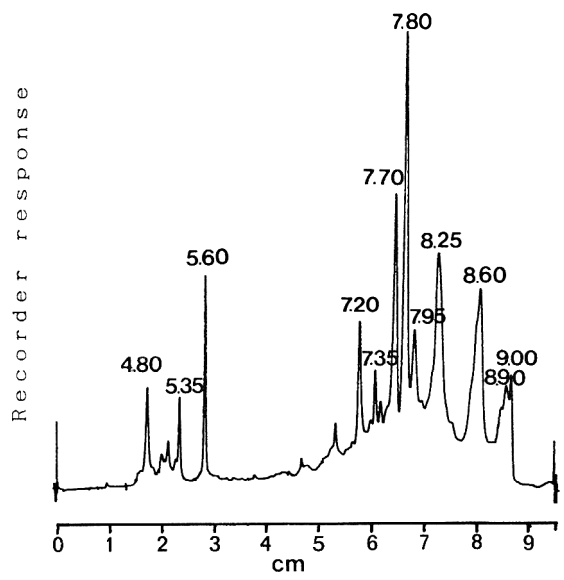

Figure 2. Densitometric pattern of isoelectric focusing of sarcoplasmic proteins shown in Figure 1, lane 4. The numbers above peaks indicate pI values.

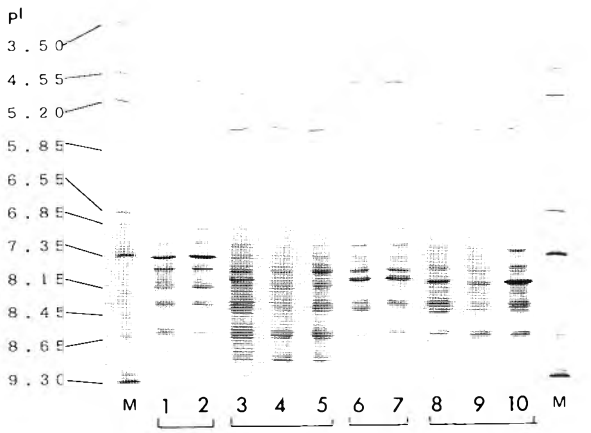

Figure 3. Isoelectric focusing patterns of sarcoplasmic proteins of 4 baleen whale species. Sample numbers correspond to those in Tables 1 and 2. M: pI marker.

ure 4). This band, therefore, may be useful for the preliminary identification of baleen whales.

Figure 4 represents protein profiles of the 8 toothed whale species, where sample numbers are the same as in Tables 1 and 2. Profiles of sperm whale, Baird's beaked whale, short-finned pilot whale, and Dall's porpoise (*dalli*-type) were species-specific and were useful for species identification. On the other hand, the profiles of dolphins, i.e., northern right whale dolphin, Pacific white-sided dolphin, common dolphin, and striped dolphin, were too similar to use for the identification of their species, even though these profiles were characteristic for toothed whales. With regard to intraspecific difference, no remarkable difference was found in any of the species, although some individual variations were found in the densities of a few bands of the short-finned pilot whale and common dolphin. Furthermore, the protein band of pI 6.05 was commonly found in toothed whales examined, with the exception of sperm and Baird's beaked whales, suggesting that the pI 6.05 band might be useful as a preliminary marker for the identification of toothed whales.

Numerical Values for Bands in Isoelectric Focusing Patterns

Because standard samples of whale meat can not be obtained at present, establishment of numerical values of pIs and densities of protein bands in isoelectric focusing patterns might be required for the practical application of this method. Table 3 indicates the numerical values of pIs and RPHs calculated from the patterns of 12 whale species shown in Figures 3 and 4. Listed numerical values of pIs and RPHs are the results of a single determination. Table 4 shows the variations in numerical values of pI and RPH for sarcoplasmic proteins of sperm whale (sample 11 in Table 2). Variations in numerical pI values were small, with relative standard deviations (CV%) below 1%. On the other hand, numerical RPH values showed higher variations. For the identification of whale species by these numerical values, the comparison of the main pI values of an unknown sample with those of an authentic sample in Table 3 may be more important than reference RPH values. Therefore, these numerical values in Table 3 may be applicable to the identification of whale species, except 4 dolphin species (samples 18–28), by the isoelectric focusing method. After obtaining the densitometric pattern of an unknown sample, the numerical values in Table 3 can be used to find out the corresponding pI value(s), RPH of which is 100 or nearly 100, and then to determine the other pI values.

Identification of Unknown Whale Species of Two Kinds of Meats on the Market

Figure 5 represents the isoelectric focusing patterns of sarcoplasmic proteins of 4 authentic baleen whale species (samples 1, 4, 6, and 8), 8 authentic toothed whale species (samples 11, 13, 14, 16, 18, 21, 24, and 27) and 2 unknown

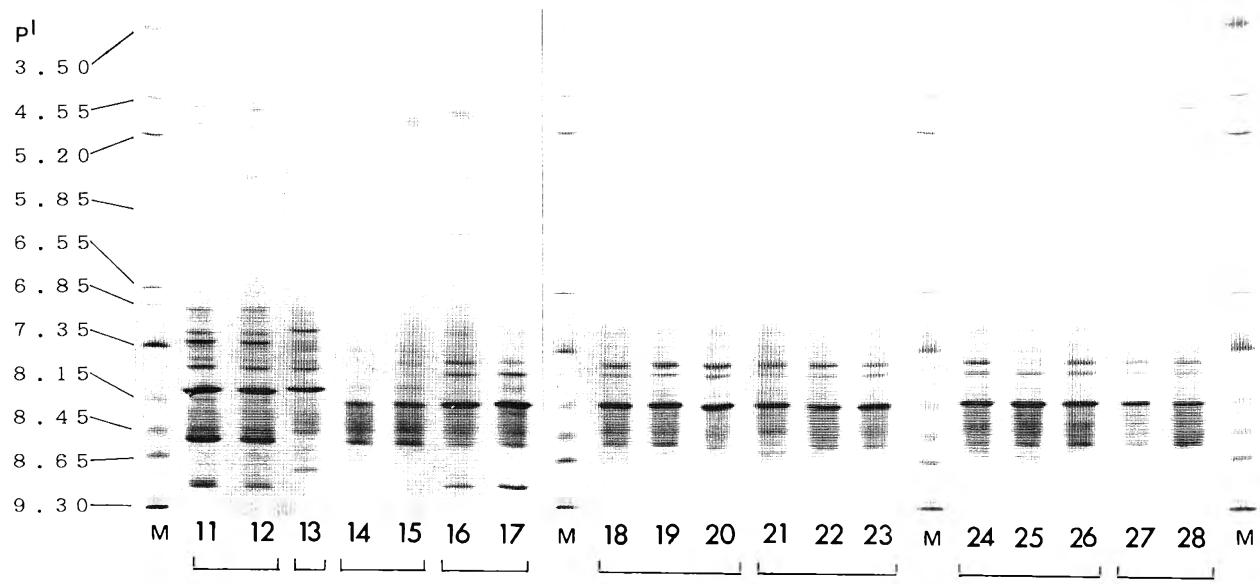

Figure 4. Isoelectric focusing patterns of sarcoplasmic proteins of 8 toothed whale species. Sample numbers correspond to those in Tables 1 and 2. M: pI marker.

Table 3. Numerical values of protein bands of isoelectro-focused sarcoplasmic proteins of 12 whale species on pH gradient (3.5–9.5) polyacrylamide gel

Sample No.	English name		Numerical values of isoelectro-focused protein bands												
1	Fin whale	pI	4.80	5.55	6.90	7.15	7.40	7.65	8.00	8.25	8.60	—	—	—	—
		RPH	39	56	13	26	100	48	38	46	34	—	—	—	—
2	Fin whale	pI	4.80	5.60	6.90	7.15	7.40	7.65	8.00	8.25	8.65	—	—	—	—
		RPH	41	28	22	23	100	34	49	28	16	—	—	—	—
3	Sei whale	pI	4.80	5.35	5.60	7.20	7.35	7.70	7.80	7.95	8.25	8.60	8.90	9.00	—
		RPH	45	43	100	43	25	82	97	25	48	52	30	36	—
4	Sei whale	pI	4.80	5.35	5.60	7.20	7.35	7.70	7.80	—	8.25	8.60	8.95	9.00	—
		RPH	41	24	100	10	12	83	37	—	27	78	44	98	—
5	Sei whale	pI	4.80	5.35	5.60	7.20	7.35	7.70	7.80	—	8.25	8.60	8.90	9.00	—
		RPH	28	55	100	18	27	75	32	—	37	52	30	58	—
6	Bryde's whale	pI	4.80	5.60	6.90	7.20	7.40	7.50	7.70	7.85	8.25	8.30	8.60	—	—
		RPH	82	61	14	56	19	26	67	100	35	53	32	—	—
7	Bryde's whale	pI	4.85	5.60	6.90	7.20	7.40	7.50	7.70	7.85	8.25	—	8.60	—	—
		RPH	76	24	16	50	18	19	66	100	44	—	34	—	—
8	Minke whale	pI	4.75	5.55	7.20	7.65	7.90	8.25	8.30	8.60	—	—	—	—	—
		RPH	28	48	25	50	100	51	41	29	—	—	—	—	—
9	Minke whale	pI	4.85	5.60	—	7.65	7.90	8.25	8.30	8.60	—	—	—	—	—
		RPH	54	100	—	35	77	29	27	73	—	—	—	—	—
10	Minke whale	pI	4.80	5.60	7.30	7.60	7.90	8.25	8.30	8.60	—	—	—	—	—
		RPH	17	46	43	35	100	31	29	34	—	—	—	—	—
11	Sperm whale	pI	4.75	5.05	5.60	6.80	7.15	7.25	7.45	7.55	8.00	8.05	8.40	8.50	8.95
		RPH	17	14	13	21	28	70	20	49	91	15	26	100	51
12	Sperm whale	pI	4.80	5.10	5.60	6.85	7.15	7.25	7.50	7.60	8.00	8.10	8.40	8.50	9.00
		RPH	16	8	11	21	29	62	14	48	93	12	32	100	38
13	Baird's beaked whale	pI	4.75	6.85	7.10	7.20	7.25	7.30	7.50	7.60	7.90	8.20	8.30	8.40	8.70
		RPH	7	10	65	12	19	23	18	54	100	14	20	21	25
14	Short-finned pilot whale	pI	5.05	6.05	7.35	7.55	—	7.90	8.15	8.20	8.40	8.50	8.65	—	—
		RPH	12	18	15	5	—	15	83	42	67	100	15	—	—
15	Short-finned pilot whale	pI	5.05	6.05	7.35	7.50	7.70	7.90	8.15	8.20	8.40	8.50	8.60	—	—
		RPH	18	16	14	17	7	24	100	20	50	78	9	—	—
16	Dall's porpoise (*dalli*-type)	pI	4.90	5.10	5.20	6.10	7.15	7.55	7.70	—	8.15	8.25	8.40	8.50	9.00
		RPH	18	7	10	17	17	62	52	—	100	24	33	32	47

Table 3. (Continued)

Sample No.	English name		Numerical values of isoelectro-focused protein bands												
17	Dall's porpoise (*dalli*-type)	pI	4.80	5.00	5.15	6.00	7.10	7.50	7.65	7.90	8.15	8.20	8.40	8.50	8.95
		RPH	11	7	10	17	10	21	42	11	100	26	34	40	63
18	Northern right whale dolphin	pI	4.85	5.05	5.15	6.10	7.10	7.55	7.70	8.15	8.20	8.40	8.50	—	—
		RPH	14	10	7	12	7	4	25	100	18	33	34	—	—
19	Northern right whale dolphin	pI	4.85	5.05	5.20	6.10	7.10	7.60	7.70	8.15	8.25	8.40	8.50	—	—
		RPH	16	8	6	15	9	47	26	100	20	34	42	—	—
20	Northern right whale dolphin	pI	4.80	5.05	5.15	6.10	7.10	7.55	7.70	8.15	8.25	8.40	8.50	—	—
		RPH	17	10	7	16	10	54	28	100	17	26	21	—	—
21	Pacific white-sided dolphin	pI	4.90	5.10	5.20	6.10	7.60	7.75	8.15	8.25	8.40	8.50	—	—	—
		RPH	8	4	5	18	46	15	100	18	37	18	—	—	—
22	Pacific white-sided dolphin	pI	4.80	5.05	5.15	6.10	7.55	7.70	8.15	8.25	8.45	8.50	—	—	—
		RPH	13	4	4	17	48	12	100	23	30	29	—	—	—
23	Pacific white-sided dolphin	pI	4.85	5.10	5.20	6.10	7.55	7.70	8.15	8.25	8.40	8.50	—	—	—
		RPH	13	9	10	15	28	21	100	18	23	28	—	—	—
24	Common dolphin	pI	4.75	5.05	5.15	6.10	7.10	7.55	7.65	8.15	8.20	8.40	8.50	—	—
		RPH	18	18	14	17	8	53	27	100	20	38	34	—	—
25	Common dolphin	pI	4.75	5.05	5.15	6.10	7.10	7.55	7.65	8.15	8.20	8.40	8.50	—	—
		RPH	18	10	9	15	7	8	28	100	30	36	46	—	—
26	Common dolphin	pI	4.75	5.10	5.20	6.10	7.10	7.50	7.65	8.15	8.20	8.35	8.50	—	—
		RPH	27	8	7	18	7	45	28	100	21	38	55	—	—
27	Striped dolphin	pI	4.80	6.10	7.50	7.70	8.15	8.20	8.35	8.45	8.50	—	—	—	—
		RPH	32	10	25	13	100	17	20	20	25	—	—	—	—
28	Striped dolphin	pI	4.80	6.10	7.50	7.70	8.15	8.20	8.35	8.45	8.50	—	—	—	—
		RPH	33	16	34	13	100	28	34	45	72	—	—	—	—

[a] Sample numbers correspond to those in Tables 1 and 2. pI: Isoelectric point. RPH: Relative peak height (maximum peak height = 100). Samples 1–10: *Mysticeti*. Samples 11–28: *Odontoceti*. Major peaks characteristic to each species are underlined.

Table 4. Variations of isoelectric points and relative peak heights[a]

n	Numerical values of isoelectric points and relative peak heights									
	a		b		c		d		e	
	pI	RPH	pI	RPH	pI	RPH	pI	RPH	pI	RPH
1	4.75	30	5.60	24	7.30	56	7.95	100	8.45	80
2	4.75	18	5.60	18	7.30	66	8.00	100	8.45	74
3	4.75	23	5.55	23	7.25	42	7.90	100	8.40	77
4	4.70	18	5.60	14	7.20	72	7.90	97	8.50	100
5	4.80	18	5.60	12	7.20	68	8.00	100	8.50	100
$\bar{x}$	4.75	21	5.60	18	7.25	61	7.95	99	8.46	86
CV,%	0.74	25	0.40	30	0.69	29	0.63	1.4	0.49	15

[a] Whale species: Sperm whale (sample 11 in Table 2). a–e: Main peaks of sarcoplasmic protein bands. pI: Isoelectric point. RPH: Relative peak height (maximum peak height = 100). $\bar{x}$: Average. CV,%: Relative standard deviation. Measuring unit: 0.05 pI.

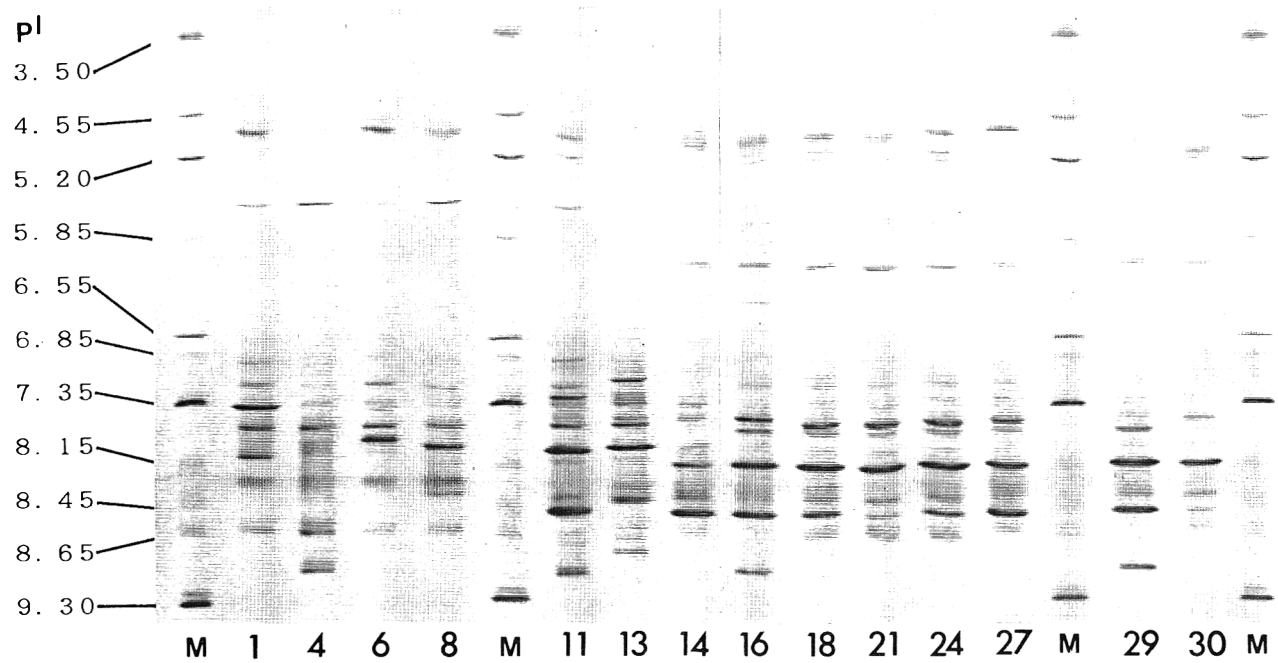

Figure 5. Isoelectric focusing patterns of sarcoplasmic proteins of 4 authentic baleen whale species, 8 authentic toothed whale species, and 2 unknown whale species. Baleen whale species: 1, 4, 6, and 8. Toothed whale species: 11, 13, 14, 16, 18, 21, 24, and 27. Sample numbers correspond to those in Table 2. Whale meat sold as Dall's porpoise: 29. Whale meat sold as sei whale: 30. M: pI marker.

Table 5. Numerical values of protein bands of isoelectro-focused sarcoplasmic proteins of 2 whale meats on the market on pH gradient (3.5–9.5) polyacrylamide gel[a]

Sample No.	Whale meat sold		Numerical values of isoelectro-focused protein bands											
29	as "Dali's porpoise"	pI	4.85	5.00	5.15	5.55	6.05	7.15	7.50	7.65	8.15	8.25	8.50	9.00
		RPH	14	8	18	12	26	7	12	41	100	16	71	45
30	as "sei whale"	pI	5.00	5.15	6.10	7.55	7.70	8.15	8.25	3.40	8.50	—	—	—
		RPH	30	25	28	30	12	100	30	24	16	—	—	—

[a] pI: Isoelectric point. RPH: Relative peak height (maximum peak height = 100).

whale species. These sample numbers correspond to those in Table 2. Sample 29, sold as the meat of Dall's porpoise, gave a focusing pattern similar to that of sample 16 (Dall's porpoise). Sample 30, however, which was sold as the meat of sei whale, was identified not as sei whale but as toothed whale because its focusing pattern did not resemble that of sample 4 (sei whale), but did resemble those of samples 18, 21, 24, and 27 (toothed whale). A good identification of

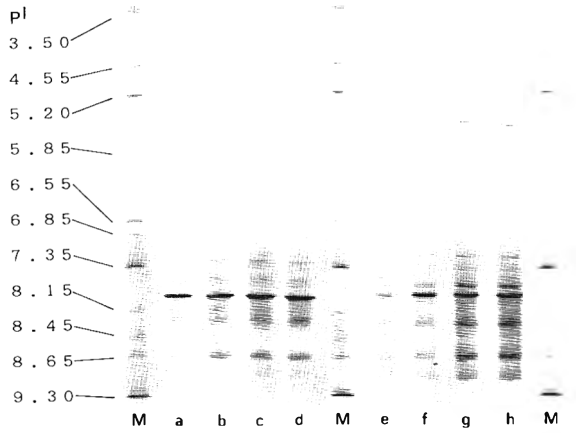

Figure 6. Isoelectric focusing patterns of sarcoplasmic proteins after bloodletting. Whale species: a–d, minke whale; e–h, sei whale. Condition of bloodletting: muscles (about 100 g) were soaked in iced water for 20 h. Positions of muscles: a and e, outside; b and f, shallow inside; c and g, deep inside; d and h, control (no bloodletting). M: pI marker.

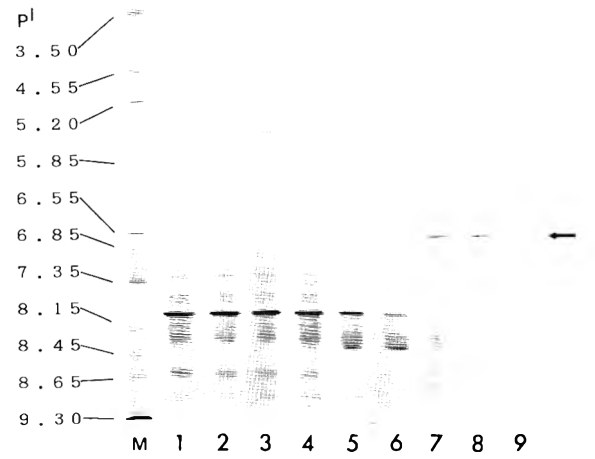

Figure 7. Isoelectric focusing patterns of sarcoplasmic proteins of a minke whale after heating for 10 min. Heating conditions: 1 = 5°C, 2 = 30°C, 3 = 40°C, 4 = 50°C, 5 = 60°C, 6 = 70°C, 7 = 80°C, 8 = 90°C, and 9 = 100°C. M: pI marker. Band-like lines indicated with an arrow are artifacts.

whale species may be possible by comparison of isoelectric focusing patterns of unknown species with those of authentic whale species on the same gel.

Table 5 indicates the numerical values of pI and RPH calculated from the isoelectric focusing patterns of samples 29 and 30 in Figure 5. Sample 29 was identified as Dall's porpoise from the numerical pI values with RPHs of 6.05 (26), 8.15 (100), and 9.00 (45) with reference to samples 16 and 17 in Table 3. Sample 30 was sold as the meat of sei whale, but it was identified as toothed whale from the numerical pI values with RPHs of 6.10 (28), 7.55 (30), 8.15 (100), 8.25 (30), etc., with reference to samples 3–5 and 18–28 in Table 3.

These results, using the numerical values, indicate that identification may be possible even when authentic samples are not available.

Effects of Bloodletting and Heat Treatment

The method may need to be applicable to samples treated with bloodletting, because whale meat is often received after bloodletting with ice water that occurs before transporting. Therefore, we examined the effect of bloodletting, using minke and sei whale samples. As shown in Figure 6, bloodletting made the bands thinner, particularly on the samples from the outer surface of muscle. However, the samples from deep inside muscle showed almost the same densities of bands as those of nontreated samples, indicating that the muscles located deep inside are valid for this assay even after bloodletting. This result also suggests that water-soluble proteins to be analyzed are, at least in part, of blood origin.

We next tested the effect of heat treatment of whale meat, using a minke whale sample, because actual samples to be assayed have sometimes been heat-treated. As shown in Figure 7, heat treatment for 10 min below 60°C had no effect on isoelectric focusing patterns; at 60°C or higher, particularly above 70°C, it caused disappearance of the bands, indicating that this method is not applicable to such high temperature-treated meats.

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MYCOTOXINS

Use of the Mycosep Multifunctional Cleanup Column for Liquid Chromatographic Determination of Aflatoxins in Agricultural Products

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A liquid chromatographic (LC) technique has been developed that uses the Mycosep™ multifunctional cleanup (MFC) column. MFC columns provide a rapid 1-step extract purification. They are designed to retain particular groups of compounds that may create interferences in analytical methods. At the same time, MFC columns allow compounds of interest to pass through. In the method presented, test samples are extracted in a blender with acetonitrile–water (9 + 1). A portion of the extract is forced through an MFC column designed especially for analysis of numerous mycotoxins. Analytical interferences are retained, while aflatoxins pass through the column. Aflatoxins B_1 and G_1 are converted to their hemiacetals by heating a mixture of purified extract and water–trifluoroacetic acid–acetic acid (7 + 2 + 1) at 65°C for 8.5 min. An aliquot of this mixture is analyzed by isocratic LC with acetonitrile–water mobile phase and fluorescence detection. A detection limit of less than 0.5 ng/g for aflatoxin B_1 was obtained. Average recoveries greater than 95% total aflatoxins (B_1, B_2, G_1, and G_2) and coefficients of variation of less than 3% were obtained. The method was successfully applied to the following commodities: corn, almonds, pistachios, walnuts, peanuts, Brazil nuts, milo, rice, cottonseed, corn meal, corn gluten meal, fig paste, and mixed feeds.

Since the discovery in the 1960s of the carcinogenic effects of aflatoxins (1), awareness of the widespread occurrence of the mycotoxin in agricultural commodities has increased. Today, there is great concern over the regulation of aflatoxins. Numerous countries have set stringent regulatory demands on the level of aflatoxin permitted in imported and traded commodities (2). Because of increased demand to lower acceptable levels of aflatoxin in foods and feeds, testing procedures must become more sensitive and more accurate.

Method development strategies have been refined as the knowledge of aflatoxins increases. Problems such as extraction efficiency and adsorption losses encountered during extraction and purification are well recognized. On the basis of this knowledge, a method was developed that eliminates many of the problems associated with older methods.

The method uses a new extract purification with a multifunctional cleanup (MFC) column. New MFC technology offers several advantages over other cleanup procedures. Advantages include speed, simplicity, solvent efficiency, and, in some cases, increased recovery and lower cost. Furthermore, the method was developed to prevent losses during cleanup and to eliminate all drydown and analyte adsorption steps, when irreversible adsorption of aflatoxin to surfaces may occur.

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Experimental

Apparatus and Reagents

(a) *Blender.*—With 250 mL blender jar and cover (Waring Products Div., New Hartford, CT 06057, No. 7011S and MC3).

(b) *Filter paper.*—25.5 cm, qualitative grade 362 (Baxter Healthcare Corp., McGraw Park, IL 60085).

(c) *Pipet.*—Capable of delivering 1–5 mL, with tips (Baxter, No. P-5055-14 and P-5055-58).

(d) *Culture tube.*—15 × 85 mm borosilicate test tube (Baxter, No. T1290-5)

(e) *Multifunctional cleanup (MFC) column.*—Mycosep™ No. 224 cleanup column (Romer Labs, Inc., Washington, MO 63090).

(f) *Pipet.*—Capable of delivering 200 µL, with tips (Baxter, No. P-5055-36A and P-5055-41).

(g) *Syringe.*—1000 µL, graduated, glass (Unimetrics Corp., Shorewood, IL 60436, No. TP5001).

(h) *Derivatization vial and cap.*—Screw cap vial, 2.0 mL capacity, Teflon lined cap (Baxter, No. B7791-2 and B7503-1).

(i) *UV spectrophotometer.*—Spectronic 21 (Bausch and Lomb Instruments).

(j) *Water bath.*—Capable of maintaining 65 ±2°C, (Sears, 5 cup, Counter Craft Hot Pot).

(k) *Sample injection system.*—Syringe loading sample injection valve with 50 µL loop (Rheodyne, Inc., Cotati, CA 94931, Model 7125).

(l) *LC pump.*—Capable of delivering 2.0 mL/min (Shimadzu Scientific Instruments, Columbia, MD 21046, LC6A).

(m) *LC column.*—10 cm × 4.6 mm id, 5 µm, RP C8 (Brownlee Labs, Inc., Santa Clara, CA 95050, No. 0711-0003 and 0715-0014).

(n) *Fluorescence detector.*—Model RF-535 (Shimadzu).

(o) *Extraction solvent.*—Mix 900 mL reagent grade acetonitrile (Baxter) with 100 mL distilled water.

(p) *Derivatization solution.*—Mix 10 mL reagent grade trifluoroacetic acid (Sigma Chemical Co., St. Louis, MO 63178) with 5 mL reagent grade glacial acetic acid (Fisher Scientific, Pittsburgh, PA 15219) and 35 mL distilled water.

(q) *LC mobile phase.*—Mix 800 mL LC grade acetonitrile (Baxter) with 3200 mL distilled water. Degas.

(r) *Crystalline aflatoxins.*—Sigma.

Preparation of Standards

Aflatoxin spiking standards were prepared from crystalline material. Two spiking standards in acetonitrile were prepared, one at 5 µg/mL (4:1:4:1 B_1:B_2:G_1:G_2), and the other at 50 µg/mL (7:1:3:1 B_1:B_2:G_1:G_2) total aflatoxins.

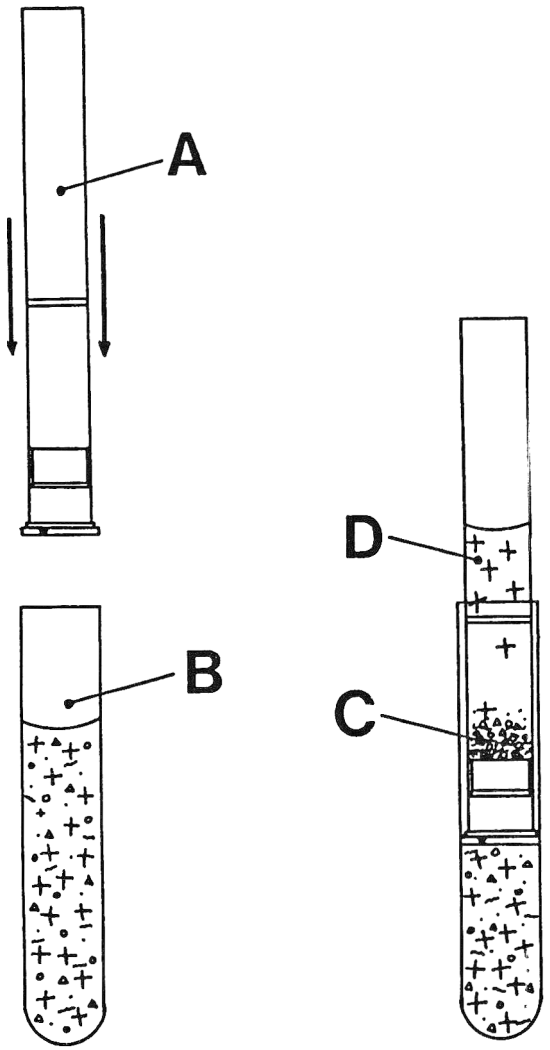

Figure 1. Schematic diagram of extract purification step. Left: Mycosep MFC cleanup column before use (A), and culture tube containing sample extract (B). Right: Column inserted into culture tube. Aflatoxin is represented by (+), all other symbols represent undesired components of extract. Undesired components are retained by packing material (C), and aflatoxin passes through and remains in purified extract (D).

Concentrations of standards were verified by UV absorption spectrophotometry as described in the AOAC official method for aflatoxin analysis (3). An aflatoxin reference standard was prepared by diluting the 50 μg/mL spiking standard to 0.036 μg/mL with acetonitrile. This reference standard was used to calibrate the LC system.

Test Samples

A 50 g test sample of each aflatoxin-free commodity was prepared. Each commodity was considered aflatoxin-free if it was found to contain less than 0.5 ng/g of any individual aflatoxin as determined by thin-layer chromatography (TLC). Both unspiked and spiked samples were tested. All spiked samples were prepared by adding the appropriate amount of spiking standard to 50 g dry commodity sample (done in triplicate) before extraction. Corn, peanuts, pistachios, almonds, and Brazil nuts were spiked at 4 ng/g total aflatoxins 4:1:4:1 B_1:B_2:G_1:G_2. Corn, milo, milled rice, corn meal, and corn gluten meal were spiked at 20 ng/g 7:1:3:1 B_1:B_2:G_1:G_2. Milo, milled rice, corn meal, and corn gluten meal were also spiked at 300 ng/g 7:1:3:1 B_1:B_2:G_1:G_2. A 500 g finely ground sample of naturally contaminated corn was mixed well and split into ten 50 g test samples.

Extraction

Weigh 50 g test sample into 250 mL blender container. Add 100 mL acetonitrile–water (9 + 1). Blend 2 min on high speed. Filter and collect 5 mL filtrate.

Extract Purification

Transfer 5 mL filtrate to 10 mL culture tube. (Optionally, transfer 1.8 mL extract to 10 mL culture tube and add 3.2 mL acetonitrile–water (9 + 1) and vortex.) Push flanged-end of Mycosep MFC column into culture tube until ca 0.5 mL extract is purified. Schematic diagram of the extract purification method is shown in Figure 1.

Derivatization

Add 200 μL purified extract and 700 μL derivatizing solution to derivatization vial. Cap and mix well. Place vial into 65°C water bath for 8.5 min with solution level in vial below water level in bath. Cool vial with cold water.

Quantitation of Aflatoxins by LC

Set flow rate at 2.0 mL/min and fluorescence detector at 360 nm excitation and 440 nm emission. Calibrate integrator with triplicate injections of 0.036 μg/mL reference standard. Set response to equivalent of 42, 6, 18, and 6 ng/g aflatoxins B_1, B_2, G_1, and G_2, respectively. Inject ca 100 μL of sample solution directly into LC system with 50 μL sample loop. Approximate retention times for aflatoxins G_1 (G_{2A}), B_1 (B_{2A}), G_2, and B_2 are 2, 2.8, 6, and 8 min, respectively.

Safety and Precautions

Protective clothing, gloves, and eye wear should be worn when performing this or any method for analysis of aflatoxins. Care should be taken when working with all reagents. Be certain to read Material Safety Data Sheets for aflatoxins and all reagents. All laboratory glassware and plasticware should be soaked in a 0.525% NaOCl (10% household bleach) before disposal. (Do not use NaOCl in areas where aflatoxin analysis is performed.)

Results and Discussion

This test procedure was designed to provide an accurate, precise, sensitive, rapid, and versatile quantitative method for the determination of individual aflatoxins. Use of an MFC column greatly simplifies extract purification for analysis of aflatoxin. The Mycosep MFC column removes analytical interferences from acetonitrile–water (9 + 1) extracts, yet it allows aflatoxins to pass through. The 1-step, 10 s process results in a purified extract with more than 90% of the ana-

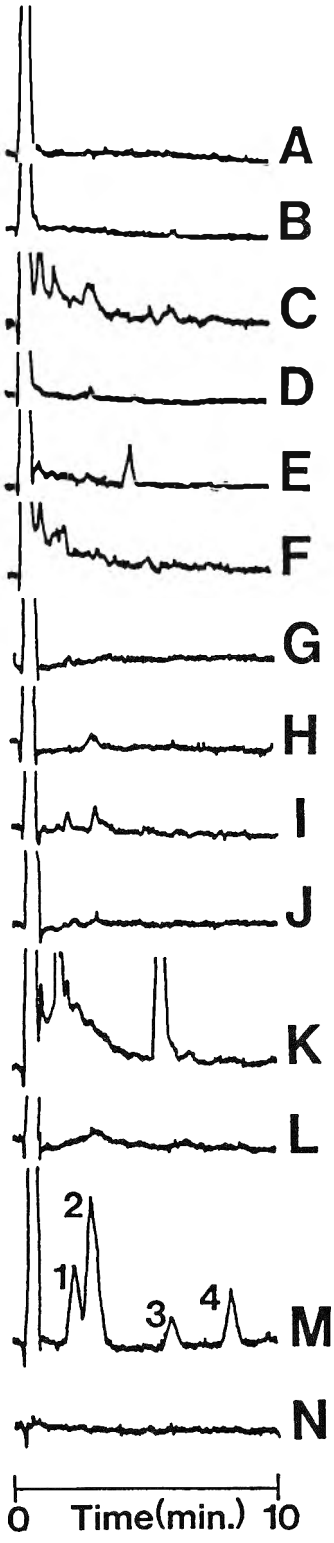

Figure 2. Chromatograms of aflatoxin-free (A) corn, (B) corn meal, (C) corn gluten meal, (D) milo, (E) cottonseed, (F) poultry feed, (G) peanuts, (H) almonds, (I) pistachios, (J) Brazil nuts, (K) fig paste, (L) milled rice, and (M) almonds spiked with 4 ng/g total aflatoxins (4:4:1:1 G_1:B_1:G_2:B_2). Peaks 1, 2, 3, and 4 are derivatized aflatoxin G_1, derivatized aflatoxin B_1, aflatoxin G_2, and aflatoxin B_2, respectively. (N) represents detector base line with no sample injected.

lytical interferences removed. Remaining interferences can easily be separated from aflatoxins by LC.

The proprietary packing material in the Mycosep MFC column contains both lipophilic (nonpolar) and charged (polar) active sites. Lipophilic sites remove fats and other nonpolar interferences such as xanthophyll pigments. Charged sites consist of both dipolar and anion-exchange sites that remove proteinaceous compounds, carbohydrates, and other polar interferences.

The packing material of the Mycosep MFC column is housed in a plastic filter tube with a rubber flange on the lower end (Figure 1). In the center of the flange is a porous frit. Above the porous frit is a 1-way valve. When the packed filter tube is pushed into a culture tube, the rubber flange creates a tight seal with the glass wall of the culture tube. As the MFC column is pushed further into the culture tube, sample extract is forced through the porous frit, through the 1-way valve, and through the packing material. The purified extract is held in the reservoir of the MFC column. The solution can then be transferred by pipet or by pouring with the MFC column still in the culture tube.

The MFC column differs from the affinity columns and solid phase extraction (SPE) columns that have been used extensively for aflatoxin extract purification (4–6). Both the affinity column and the SPE column cleanups require 3 steps for extract purification: retain aflatoxin on packing material of the column, wash away undesirable compounds, and elute compounds of interest. An MFC column cleanup requires only 1 step with no wash or elution solvents.

The Mycosep MFC column used in this method was designed for the purification of acetonitrile–water (9 + 1) extracts for the analysis of over 10 mycotoxins. The MFC column offers much more versatility than the affinity column, which is selective for aflatoxins only. SPE columns can be used for the individual analysis of several mycotoxins, but each mycotoxin requires different cleanup steps.

With MFC technology, irreversible adsorption or premature elution from the cleanup column is eliminated. Both of these phenomena may occur with SPE and affinity column cleanups. Recovery of aflatoxins from the Mycosep MFC is significantly higher than the specified and observed recovery of aflatoxins from affinity columns (6). Recovery of total aflatoxins through the Mycosep MFC column is typically above 95%.

The derivatization method presented here is a modification of a procedure described by Hutchins et al. (4), and it eliminates the drydown steps performed in ref. 4 and other methods (5). Therefore, the loss of aflatoxin from adsorption on the surface of the derivatization vial is no longer a concern. Also in the work by Hutchins et al., a drydown step is performed on the purified extract. The residue is dissolved by sonication into water–trifluoroacetic acid–acetic acid (8 + 1 + 1) and held at 55°C for 15 min. We found that, with the solvent system used in the Mycosep cleanup method, the drydown step could be eliminated and the derivatizing solution added directly to the purified extract. Initially, research was conducted to determine the reaction time necessary to insure complete derivatization at 55°C with water–trifluoroacetic acid–acetic acid (8 + 1 + 1). We found that the derivatization was complete in less than 12 min for standards containing ≤180 ppb B_1 and G_1 (1 ng of each toxin). However, we wanted to reduce the derivatization time. This was accomplished by using water–trifluoroacetic acid–acetic

Table 1. Aflatoxin content (ng/g) in free samples of corn, peanuts, pistachios, almonds, and Brazil nuts (n = 3) spiked with 4 ng/g total aflatoxins (4:1:4:1 B_1:B_2:G_1:G_2)

Commodity	Corn			Peanuts			Pistachios			Almonds			Brazil nuts		
Extract[a]	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
B_1	1.70	1.74	1.85	1.62	1.56	1.66	1.68	1.50	1.50	1.42	1.59	1.48	1.55	1.51	1.64
B_2	0.41	0.40	0.42	0.39	0.39	0.35	0.41	0.36	0.39	0.44	0.39	0.40	0.38	0.39	0.40
G_1	1.70	1.60	1.84	1.64	1.61	1.71	1.41	1.44	1.68	1.34	1.44	1.64	1.70	1.56	1.71
G_2	0.34	0.40	0.41	0.44	0.37	0.34	0.34	0.32	0.36	0.41	0.39	0.36	0.40	0.38	0.31
Total	4.15	4.14	4.52	4.09	3.93	4.06	3.84	3.62	3.93	3.61	3.81	3.88	4.03	3.84	4.06
Av. total		4.26			4.02			3.80			3.77			3.98	
SD		0.22			0.08			0.16			0.14			0.12	
CV, %		5.08			2.12			4.20			3.72			3.00	
Rec., %		106.5			100.5			95.0			94.2			99.5	

Composite statistics for the 15 extracts

Av.	3.97
SD	0.23
CV, %	5.72
Rec., %	99.2

[a] Each extract was analyzed in duplicate and the result indicated is an average of the duplicates. Av. = average; SD = standard deviation; CV = coefficient of variation; and Rec. = recovery.

acid (7 + 2 + 1) as the derivatization agent and increasing the reaction temperature to 65°C, which reduced the time of reaction to 8.5 min for the same levels. The chromatographic peaks obtained from this reaction mixture were slightly broadened. We also discovered that using water–trifluoroacetic acid–acetic acid (6 + 3 + 1) results in unacceptable peak broadening.

Versatility of the MFC method is exhibited in Figure 2. This figure contains chromatograms of 13 different aflatoxin-free commodities. Also shown is the base line for the detector with no sample injected and for an almond sample spiked at 4 ng/g total aflatoxins (4:1:4:1 B_1:B_2:G_1:G_2). Corn gluten meal and fig paste were analyzed using the optional dilution. Diluting the extract reduces the interference load on the cleanup column, which results in an improved cleanup for difficult samples. All chromatograms, except for fig paste (Figure 2K), show no significant interferences at the aflatoxin retention times. Some small peaks are present at the aflatoxin B_1 and G_1 retention times in chromatograms 2C and 2F, corn gluten meal and poultry feed, respectively. If these

Table 2. Aflatoxin content (ng/g) in free samples of corn, milo, milled rice, corn meal, and corn gluten meal[a] (n = 3) spiked with 20 ng/g total aflatoxins (7:1:3:1 B_1:B_2:G_1:G_2)

Commodity	Corn			Milo			Milled rice			Corn meal			Corn gluten meal		
Extract[b]	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
B_1	10.38	11.11	10.96	11.16	11.20	11.08	11.42	11.81	12.10	11.45	11.68	11.54	10.83	11.47	11.42
B_2	1.46	1.55	1.43	1.62	1.67	1.48	1.63	1.58	1.60	1.44	1.62	1.56	1.45	1.68	1.64
G_1	4.83	5.28	5.24	5.08	5.04	5.05	4.40	4.96	5.10	4.72	4.72	4.74	4.22	4.90	4.08
G_2	1.62	1.70	1.55	1.86	1.72	1.74	1.60	1.64	1.70	1.80	1.82	1.64	1.66	1.62	1.71
Total	18.29	19.64	19.18	19.72	19.63	19.35	19.05	19.99	20.05	19.41	19.84	19.48	18.16	19.67	18.85
Av. total		19.04			19.57			19.85			19.58			18.89	
SD		0.69			0.19			0.74			0.23			0.76	
CV, %		3.60			0.99			3.71			1.18			4.00	
Rec., %		95.2			97.8			99.2			97.9			94.5	

Composite statistics for the 15 extracts

Av.	19.35
SD	0.56
CV, %	2.92
Rec., %	96.8

[a] Corn gluten meal was analyzed using the optional dilution described in *Experimental*.
[b] Each extract was analyzed in duplicate and the result indicated is an average of the duplicates. Av. = average; SD = standard deviation; CV = coefficient of variation; Rec. = recovery.

Table 3. Aflatoxin content (ng/g) in free samples of milo, milled rice, corn meal, and corn gluten meal[a] (n = 3) spiked with 300 ng/g total aflatoxins (7:1:3:1 B_1:B_2:G_1:G_2)

Commodity	Milo			Milled rice			Corn meal			Corn gluten meal		
Extract[b]	A	B	C	A	B	C	A	B	C	A	B	C
B_1	156.5	160.8	159.8	173.4	167.7	170.8	172.2	167.8	166.7	165.6	170.0	172.8
B_2	22.8	23.2	23.1	24.7	24.3	24.6	25.3	24.2	24.3	23.7	23.5	24.1
G_1	73.2	74.9	75.6	78.8	76.6	78.7	75.4	73.1	72.4	71.8	69.8	72.6
G_2	22.2	23.3	23.4	24.4	23.9	24.4	24.8	24.2	24.1	24.0	24.3	24.1
Total	274.7	282.2	281.9	301.3	292.5	298.5	297.7	289.3	287.5	285.1	287.6	293.6
Av. total		279.6			297.4			291.5			288.8	
SD		4.25			4.50			5.44			4.37	
CV, %		1.52			1.51			1.87			1.51	
Rec., %		93.2			99.1			97.2			96.3	

Composite statistics for the 15 extracts

Av.	289.3
SD	7.80
CV, %	2.70
Rec., %	96.4

[a] Corn gluten meal was analyzed using the optional dilution described in *Experimental*.
[b] Each extract was analyzed in duplicate and the result indicated is an average of the duplicates. Av. = average; SD = standard deviation; CV = coefficient of variation; and Rec. = recovery.

peaks are integrated as either B_1 or G_1, however, they result in less than 0.5 ng/g interferences. The chromatogram for fig paste shows no significant interferences at the retention times of aflatoxins B_1 and B_2. Interferences can be separated from aflatoxins G_1 and G_2 by changing the mobile phase (acetonitrile–water) ratio from 20:80 to 17:83.

Detection limits for individual aflatoxins are at least 0.5 ng/g in all commodities tested. Results for samples spiked at 4 ng/g total aflatoxins (4:1:4:1 B_1:B_2:G_1:G_2) are shown in Table 1, and a typical chromatogram is shown in Figure 2 (spiked almonds). Individual toxin levels calculate to 1.6 ng/g for aflatoxins B_1 and G_1, and 0.4 ng/g for aflatoxins B_2 and G_2. The detection limit of aflatoxin B_1 in most commodities is estimated to be below 0.5 ng/g (Table 1). Detection limits for individual aflatoxins can be improved by altering the excitation and emission settings on the detector to be specific for the individual toxin of interest.

The accuracy of the method is established by data in Tables 1–3. Test samples spiked at 4, 20, and 300 ng/g total aflatoxins yielded an average recovery of 97.6%. Recovery varied slightly (coefficient of variation (CV), 1.19%) between commodities and spike levels. The precision for spiked samples is also documented from Tables 1–3. The average CV is 2.72%, and it is lowest for individual commodities at high levels. Figure 3 shows a standard equivalent to 20 ng/g and a sample spiked at the same level.

The linear range of the method (without the optional dilution) was investigated. We found that the response remained linear from 0.5 to 200 ng/g for the aflatoxins when they were analyzed simultaneously. This corresponds to 2.8 and 1100 pg of each toxin. Aflatoxin levels outside of this range were not tested.

Precision for analyses of naturally contaminated corn samples is documented in Table 4. CV for analyses of 10 individual extracts is 2.85 and 3.68% for aflatoxins B_1 and B_2,

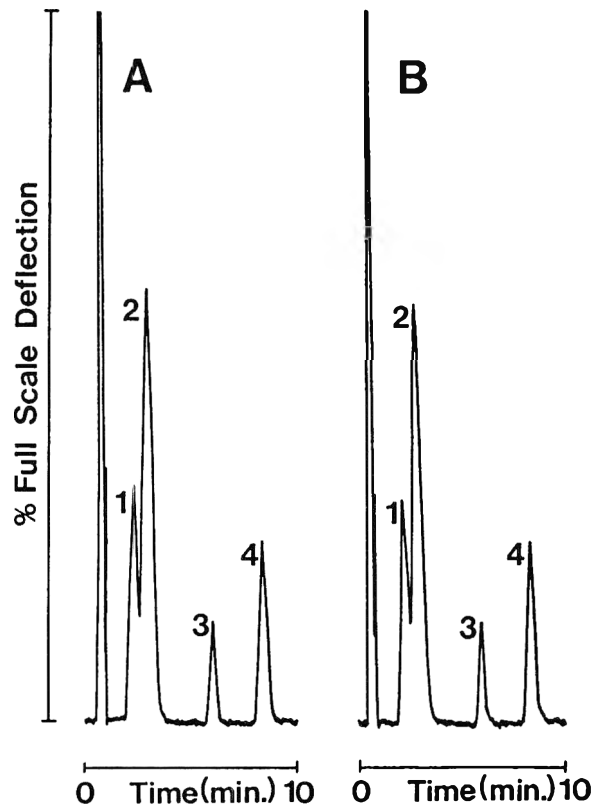

Figure 3. Chromatograms of (A) aflatoxin standard equivalent to sample containing 20 ng/g total aflatoxins, and (B) aflatoxin-free corn sample spiked with 20 ng/g total aflatoxins (4:4:1:1 G_1:B_1:G_2:B_2). Peaks 1, 2, 3, and 4 are derivatized aflatoxin G_1, derivatized aflatoxin B_1, aflatoxin G_2, and aflatoxin B_2, respectively.

Table 4. Aflatoxin content (ng/g) in naturally contaminated corn samples (n = 10)

Extract	Aflatoxin B_1	Aflatoxin B_2
1	66.9	4.29
2	65.4	4.29
3	65.6	4.39
4	63.5	4.22
5	64.9	4.47
6	62.2	4.22
7	68.3	4.74
8	63.6	4.55
9	64.4	4.46
10	63.0	4.43
Av.[a]	64.8	4.41
SD	1.85	0.16
CV, %	2.86	3.68

[a] Av. = average; SD = standard deviation; and CV = coefficient of variation.

respectively. The method proved to be rapid with complete analysis time of less than 21 min.

Other Applications of the Mycosep MFC Column

The Mycosep MFC column was successfully used in our laboratory for purification of extracts to be analyzed for deoxynivalenol, T-2 toxin, patulin, sterigmatocystin, and several other mycotoxins.

In addition, our laboratory was successful using the LC cleanup column with postcolumn bromine derivatization for analysis of aflatoxins. We have also applied the Mycosep MFC column for TLC analysis of aflatoxins (unpublished data).

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OILS AND FATS

Monitoring Aldehyde Production During Frying by Reversed-Phase Liquid Chromatography

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Acrolein (2-propenal) and other low molecular weight aldehydes (LMWAs) formed by degradation of the frying medium (triglycerides) were monitored by liquid chromatography (LC) during preparation of fried items. LMWA contents of coatings from codfish and of doughnuts and their volatiles that codistill with steam are monitored by trapping the vapors and distillate from the food matrix in a 2,4-dinitrophenylhydrazine solution. The resulting hydrazones are partitioned from the aqueous phase, first into isooctane and then into acetonitrile for LC analysis. The hydrazones are separated and quantified on a C18 reversed-phase column with acetonitrile–water as the mobile phase. LMWAs are confirmed by gas chromatography/mass spectrometry. No difference was found in LMWA content in coatings from fish fillets fried at 182 or 204°C. Cake doughnuts were higher in acrolein content than yeast-raised doughnuts prepared under similar conditions. Freshness of the frying medium, frying time, and batch size did not seem to influence LMWA production from doughnuts. Results indicated that most of the LMWAs formed codistilled with steam during frying rather than remaining with the food item.

Hydrolysis of the frying medium (triglycerides from animal fat, vegetable oils, or shortenings) with subsequent dehydration of glycerol at temperatures encountered during frying (163–200°C) produces acrolein (1) and other low molecular weight aldehydes (LMWAs). Acrolein is a mutagen, a cilioinhibitor, and a lachrymator (2). Ingredients common to commercial batter and breading systems may indirectly lead to acrolein production in fried foods. Sodium bicarbonate, a leavening component of edible coatings, may saponify part of the frying medium yielding free fatty acids and glycerol, which dehydrates to form acrolein during frying. Other components of coating systems (dextrose, whey, and dried skim milk), which are added to accelerate Maillard browning, may increase acrolein and other LMWA production during frying according to Otto and Baltes (3).

Gas chromatography (GC) was used to determine vapor-phase LMWAs in engine exhausts (4). Recently, a GC method (5) was reported for analysis of vapor-phase acrolein formed from heating of food grade fats. Selim (6) reported low level detection of propionaldehyde by liquid chromatography (LC). Others investigators have used LC as an analytical tool for separation and quantitation of LMWAs in biological samples (7) and tobacco smoke (8). Because of the potential toxicity of acrolein and other LMWAs, this investigation was undertaken to develop methodology for quantitating LMWAs associated with deep fat frying of food items.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 2010 pump with Rheodyne 2082 injector containing 20 μL loop (Varian Instrument Group, Sunnyvale, CA 94034).

(b) *Variable wavelength detector.*—Model 2050, set at 254 nm and 0.08 absorbance units full scale (Varian).

(c) *Computing integrator.*—Model 3310a (Hewlett-Packard Co., Palo Alto, CA 94303).

(d) *Chromatographic columns.*—MicroPak MCH-5-n-cap, 5 μm × 15 cm × 4 mm id reversed-phase C18 column (Varian), and 5 μm Perisorb RP 18 precolumn (Rainin Instrument Co., Inc., Woburn, MA 01801).

(e) *Gas chromatograph/mass spectrometer.*—Finnigan mass spectrometer Model 4510 equipped with INCUS data system and interfaced to Finnigan gas chromatograph (Finnigan Corp., Cincinnati, OH 45242); capillary column, DB-5, (30 m × 0.32 mm id, 1.0 μm). Temperature program: 150°C for 0.5 min, heat to 200°C at 15 mL/min, heat to 320°C at 8 mL/min, and hold for 5 min.

Reagents

(a) *Solvents.*—LC grade (Fisher Scientific, Pittsburgh, PA 15219) and deionized water (Barnstead NANOpure, Boston, MA).

(b) *Mobile phase.*—Acetonitrile–water (3 + 2, or 1 + 1). Add appropriate volumes acetonitrile and water followed by degassing and filtering.

(c) *Extracting solvents.*—Isooctane (caution: Highly flammable) and acetonitrile (caution: Toxic. Avoid contact with skin and eyes).

(d) *Extracting solution.*—0.01N HCl. Dissolve 2 mL concd HCl in 1.0 L water and adjust to pH 2.

(e) *2,4-Dinitrophenylhydrazine (DNPH).*—Dissolve 0.25 g hydrazine in 100 mL 6N HCl.

(f) *2,4-DNPH derivatives.*—According to the method of Shriner et al. (9), dissolve 0.4 g hydrazine in 2 mL concd H_2SO_4 followed by dropwise addition of water with swirling until solution is complete. Add hydrazine solution to 0.5 g acrolein or other low molecular weight aldehyde in 20 mL alcohol with swirling. Recrystallize hydrazone by dissolving in 30 mL hot alcohol and filtering.

(g) *2,4-DNPH standards.*—Weigh 0.25 g each hydrazone and dissolve in 50 mL acetonitrile. Dilute to 5 μg/mL with acetonitrile.

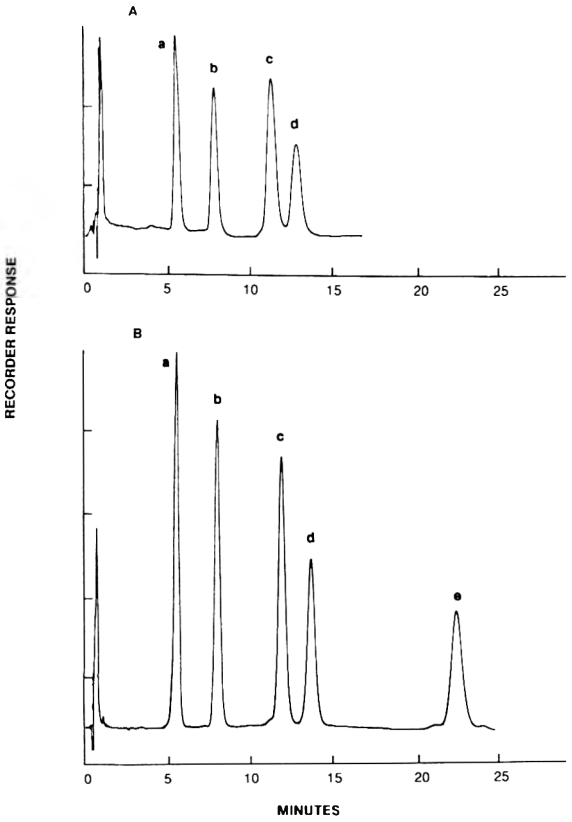

Figure 1. Chromatograms of standard 2,4-dinitrophenylhydrazones of (a) formaldehyde, (b) acetaldehyde, (c) acrolein, (d) propionaldehyde, and (e) butyraldehyde. Mobile phases: A, acetonitrile–water (3 + 2); B, acetonitrile–water (1 + 1).

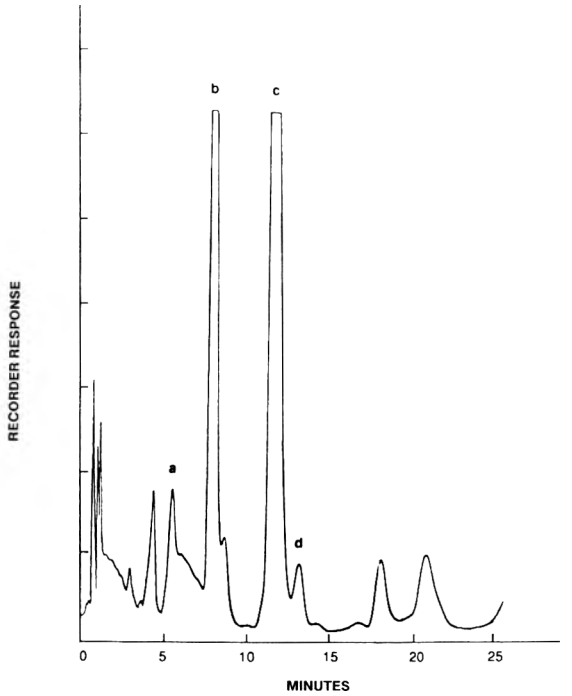

Figure 2. Chromatogram of vapor-phase components of doughnuts fried at 182°C. Mobile phase, acetonitrile–water (3 + 2). 2,4-DNPH derivatives of (a) formaldehyde, (b) acetaldehyde, (c) acrolein, and (d) propionaldehyde.

Procedure

Obtain frozen codfish fillets from a foodstore and cut into portions averaging 3.1 × 7.6 × 1.4 cm in size and weighing ca 32.3 g. Coat pieces in fish and chip batter (10) and fry 4 portions in thermostatically controlled 6 qt capacity Presto pressure cooker (Model KE03AT, Eau Claire, WI) filled with 2.5 L fresh Chef-Way Pourable Clear Fry (Riceland Foods, Inc., Stuttgart, AR) containing, according to the label, partially hydrogenated and winterized soybean oil with polyglycerol esters, and dimethylpolysiloxane added for freshness and to reduce foaming. Equilibrate fryer to 182 or 204°C at atmospheric pressure. Fry another set of 4 samples in the same kind of oil previously used for frying breaded, fresh broiler parts on 8 occasions within a 2 year period and held at 2°C. Duplicate all frying procedures.

Prepare doughnuts, both yeast-raised and cake, containing chemical leavening, according to a standard recipe for yeast-raised doughnuts (11). For chemically leavened products, substitute 11.0 g double acting baking powder containing sodium bicarbonate ($NaHCO_3$) as the base, and monocalcium phosphate monohydrate ($CaH_4(PO_4)2{\cdot}H_2O$) and sodium aluminum sulfate ($Na_2SO_4{\cdot}Al_2(SO_4)_3$) as acids for 7 g package of yeast in original recipe. Fry products in batches of 2 and 4 at 182°C for 2 and 4 min in frying apparatus described above. Replicate all frying procedures 4 times. Collect volatiles emitted during frying of yeast-raised and cake doughnuts by connecting water cooled condenser to vent opening in pressure cooker lid and submerging tip of condenser in 2,4-DNPH solution [reagent (e)].

Blend 10 g fried item in 150 mL 0.01N HCl for 3 min at high speed. Place 100 mL homogenate in 250 mL round bottom flask with ground glass fittings. Attach water cooled condenser and distill 50 mL condensate into 250 mL Erlenmeyer flask containing 25 mL trapping solution [reagent (e)] and 25 mL isooctane. Stir constantly during distillation. Continue stirring for 30 additional min after distillation is complete. Transfer mixture to 125 mL separatory funnel and allow layers to separate. Withdraw aqueous layer and extract with two 10 mL portions isooctane. Combine all isooctane extracts in 125 mL separatory funnel and extract twice with 10 mL acetonitrile. Combine acetonitrile extracts and dilute to volume in 25 mL volumetric flask. Withdraw 20 μL for LC analysis. Compute individual LMWA content as ratio of sample peak area to standard peak area. Express 2,4-DNPH derivatives as free aldehyde value by multiplying by appropriate factor: formaldehyde, 0.142; acetaldehyde, 0.196; acrolein, 0.237; and propionaldehyde, 0.244.

Results and Discussion

To simulate initial acidic conditions associated with digestion (pH 2), the fried items were blended in 0.01N HCl and distilled with steam to effect separation of LMWAs from the food matrix. Aldehydes containing up to 3 carbons were separated by this method (Figures 1 and 2). Because acrolein was the primary aldehyde of interest, we determined conditions that would maximize its recovery. Production of the 2,4-DNPH adduct of acrolein by the Selim method (6) yielded a maximum value of 91% after 30 min of stirring. Stirring partitioned the sparingly soluble acrolein adduct as it formed from the aqueous phase [reagent (e)] into isooctane. Increasing the rate of stir-

ring, the volume of trapping solution, and the reaction time did not improve the yield.

Recovery of an acrolein standard by the distillation method that was used to separate the LMWAs from the food matrix indicated that essentially all the acrolein distilled within the first 50 mL of condensate collected. Recovery of a known quantity (10.8 μg) of the acrolein standard yielded a mean value (N = 4) of 79%, whereas a doughnut homogenate spiked with the standard yielded a recovery of 90%.

An acetonitrile–water (3 + 2) mobile phase, as suggested by Selim (6), was used initially for LC separation of LMWAs. As the investigation proceeded, however, we found acetonitrile–water (1 + 1) produced greater resolution between acrolein and propionaldehyde (Figure 1), which differ by a molecular weight of 2 (m/z: CH_2:CHCH:$NNHC_6H_3(NO_2)_2$+ = 236 and CH_3CH_2CH:$NNHC_6H_3(NO_2)_2$+ = 238). This adjustment provided baseline separation between acrolein and propionaldehyde.

Quantities of residual acrolein and other LMWAs of prepared, fried items were determined by LC and confirmed by GC/MS (Tables 1 and 2 and Figure 3). Because propionaldehyde was found only in trace (<0.1 ppm) quantities in chemically leavened products (fish coatings and cake doughnuts), and was undetectable in yeast-leavened doughnuts, it was omitted from Tables 1 and 2. Table 1 contains means (N = 2) from the LMWA analyses of fish coatings. There were no differences in LMWA content (P >0.05) of coatings from breaded, fried codfish fillets when grouped by frying temperature or oil freshness (12). Freshness, as measured by free fatty acid content of old and fresh oils, was 0.55 and 0.30%, respectively. This may account for the apparent similarity in acrolein content, because the free fatty acid values serve as an indirect measure of glycerol available for dehydration to acrolein during frying.

Formaldehyde and acetaldehyde means (N = 4) of yeast-raised doughnuts were higher (P <0.01) than cake doughnuts (Table 2). This is probably a result of formaldehyde and acetaldehyde formation by yeast respiration during proofing of the dough. Mean acrolein content of doughnuts were as high as 0.9 ppm for cake doughnuts (Table 2). When grouped by type (yeast or cake), a statistical difference (P <0.01) in acrolein content was found between means for cake (0.7 ppm) and yeast-raised (0.2 ppm) doughnuts. This difference is probably a result of the saponification of neutral fat, which composed the frying medium, by $NaHCO_3$ found in the chemical leavening system of cake doughnuts. No differences (P >0.05) in acrolein production existed between type (yeast or cake) with respect to frying time or batch size. Because relatively low levels of acrolein and other LMWAs were found in prepared, fried products (Tables 1 and 2), quantitation of LMWA volatiles emitted during preparation of yeast and cake doughnuts was attempted.

During the frying of doughnuts, substantially more aldehydes codistilled with water vapor than remained with the fried items. Vapor-phase formaldehyde content was similar for both types of doughnuts, ranging up to 0.02 mg/batch. Vapor-phase acetaldehyde content was negligible (<0.01 mg/batch) with cake doughnuts, but ranged from 0.02 to 0.08 mg/batch with yeast-raised doughnuts. Vapor-phase acrolein values ranged from 0.05 to 0.32 mg/batch during frying of cake doughnuts, and from 0.02 to 0.06 mg/batch for yeast-raised doughnuts. Vapor-phase propionaldehyde content was negligible (<0.01 mg/batch) with yeast-raised doughnuts, but ranged from 0.02 to 0.07 mg/batch with cake doughnuts. Neither batch size (2 or 4 doughnuts) nor length of fry time (2 or 4 min) seemed to influence LMWA production. Moisture evaporation during frying produced vigorous bubbling at the condenser tip submerged in the trapping solution, which probably impaired quantitative trapping of the volatiles. Attempts to increase precision among replications by increasing the volume and the rate of stirring of the trapping solution as the frying operation proceeded were unsuccessful. These results, however, demonstrate that greater contact with LMWAs may be associated with individuals preparing fried items rather than those consuming them.

Table 1. Mean LMWA content (ppm) of coatings from codfish fillets fried at 182 and 204°C for 4 min in fresh and used oil

	182°C		204°C	
LMWA	Fresh	Used	Fresh	Used
Formaldehyde	0.3	0.3	0.3	0.4
Acetaldehyde	1.0	0.9	0.8	1.1
Acrolein	0.1	—[a]	—	0.1

[a] Trace = < 0.1 (lower level of detection).

Table 2. Mean LMWA content (ppm) of yeast-raised and cake doughnuts fried at 182°C

			Mean LMWA content, ppm		
Type	Fry time, min	Batch size[a]	Formaldehyde	Acetaldehyde	Acrolein
Yeast	2	2	0.2	1.1	0.1
Yeast	2	4	0.4	1.9	0.3
Yeast	4	2	0.4	1.6	0.3
Yeast	4	4	1.3	1.3	0.2
Cake	2	2	—[b]	—	0.3
Cake	2	4	—	—	0.6
Cake	4	2	—	—	0.8
Cake	4	4	0.2	0.1	0.9

[a] Number of doughnuts per batch.
[b] Trace = < 0.1 (lower level of detection).

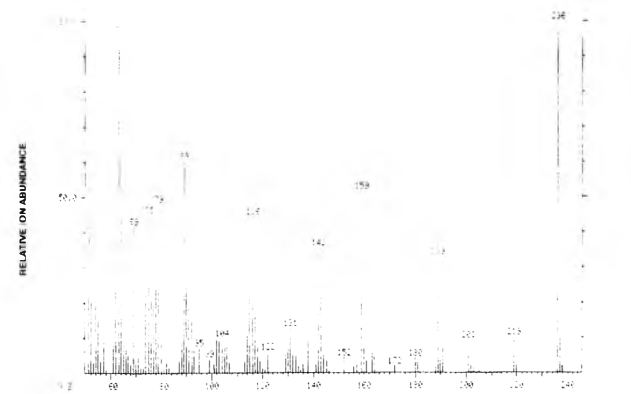

Figure 3. Mass spectrum of peak c (Figure 2) obtained from doughnuts fried at 182°C. GC/MS conditions described in text.

According to data in the Merck Index (13), exposure to vapor-phase LMWAs presents a greater risk than contact with the liquid or solid phases. Moreover, the Occupational Safety and Health Standards for air contaminants (14) specifies a time weighted average (TWA) for acrolein of 0.25 mg/m^3 and a short term exposure limit (STEL) of 0.8 mg/m^3. Ranges obtained for vapor-phase acrolein in this investigation indicate that acrolein content may approach these established limits given the much larger batch sizes associated with commercial doughnut production. These data indicate that monitoring LMWA volatiles emitted during quantity frying operations may be needed to insure the safety of those individuals employed in food service facilities.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Liquid Chromatographic Determination of Paraquat and Diquat in Crops Using a Silica Column with Aqueous Ionic Mobile Phase

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A method was developed for the determination of paraquat (PQ) and diquat (DQ) in high moisture food crops. Samples were digested with 6M HCl, and the herbicides were isolated from the digest using pH-controlled silica solid phase extraction. The analytes were then determined by ion-pairing liquid chromatography with a silica analytical column, sodium chloride as the ion-pairing reagent, and acetonitrile as the organic modifier. A diode array UV absorbance detector was used to simultaneously quantify PQ and DQ at their respective maximum absorbance wavelengths, 257 and 310 nm. Average recoveries of PQ and DQ standards from 4 different crops fortified at 0.01–0.50 ppm levels ranged from 79.3 to 104.8%.

Paraquat (PQ) and diquat (DQ) are effective general weed killers. These herbicides are registered in the United States for a variety of applications, including the control of weeds on orchard floors throughout the growing season, as preplant weed killers for many crops, as preharvest desiccants on crops such as potatoes, and for aquatic weed control (1, 2). DQ is formulated as dichloride and dibromide salts, while PQ is formulated as dimethyl sulfate or dichloride salts (2). PQ and DQ are very water soluble, nonvolatile, thermally stable, and stable in acid. DQ is known to degrade slowly at pH levels greater than 9 (3). PQ hydrolyzes at pH levels greater than 12 (1). PQ and DQ are toxic to humans and their effects from acute and chronic human exposure have been well-documented (4, 5). Most instances of poisoning involve intentional ingestion of concentrated commercial products. However, there is the potential for human exposure to PQ and DQ in the general population through residues on food crops.

Because PQ and DQ exist as doubly charged cationic species in solution, they are prone to sorptive interactions and present unique analytical challenges. PQ and DQ have been analyzed in many types of matrixes using a wide variety of analytical techniques. Gel filtration cleanup and ion-exchange liquid chromatography (LC) (6), C18 bonded silica solid phase extraction (SPE) and reversed-phase ion-pairing LC (7), and ion-pairing solvent extraction followed by pyrolysis gas chromatography (8) have been used for PQ and DQ determinations in human urine and serum. Simon and Taylor (9) isolated the herbicides from well water using silica SPE and reversed-phase ion-pairing LC with post-column reaction and UV detection. Nagayama et al. (10) used a cation ion-exchange resin to isolate the analytes from potatoes and an amino-bonded silica column for final separation. Worobey (3) used silica SPE followed by reversed-phase ion-pairing LC on a polymer-based column for potatoes. Enzyme-linked immunosorbent assays have been developed for the determination of PQ in human exposure samples (11) and in potatoes (12).

At this time, regulatory monitoring of PQ and DQ residues in food crops is not routinely performed because of the unavailability of reliable, rugged, and sensitive methodology amenable to such action. In the present study, a multiresidue procedure was sought for the determination of PQ and DQ in several crop matrixes at levels ranging from a low of 0.01 ppm (screening level) up to and including the U.S. Environmental Protection Agency (EPA) tolerance levels for the crops selected for study. An SPE cleanup using pH-controlled silica was found to be most effective for the isolation of PQ and DQ in a variety of crops because of its high sample capacity and selectivity for these quaternary ammonium compounds. The SPE cleanup technique described is an adaptation and modification of a method previously described by Worobey (3). The utility of silica for the LC analysis of amines and quaternary ammonium compounds has been discussed in numerous papers (13–18). For the LC determination of PQ and DQ, a silica column used with sodium chloride as an ion-pairing reagent in acetonitrile–water as the mobile phase provided rapid, efficient, and selective separation of these compounds in crop extracts. Simultaneous determination of PQ and DQ was achieved using a diode array UV detector.

METHOD

Reagents

(a) *Solvents.*—Methanol and acetonitrile (Omnisolv, EM Science, Gibbstown, NJ 08027).

(b) *LC grade water.*—Obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(c) *SPE solutions.*—Prepare aqueous solutions with LC grade water: *Solution A,* 1M HCl; *Solution B,* 25 mM NaOH; *Solution C,* 0.1M HCl in methanol; *Solution D,* methanol–6.5M HCl (7 + 3).

(d) *LC mobile phase.*—Dissolve 5.0 g NaCl in 600 mL LC grade water previously adjusted to pH 2.2 with 1M HCl. Add 400 mL acetonitrile, mix, and filter using Millipore type DV, 0.65 μm nylon filters. Before use, degas by sparging with helium while stirring solvent with magnetic stirrer.

(e) *Reference standards.*—PQ dichloride and DQ dibromide were obtained from EPA, Pesticides and Industrial Chemicals Repository, Research Triangle Park, NC 27711. Use plasticware or silanized glassware for preparation of standard solutions, and use methanol as diluent throughout. Prepare 100 ng/μL stock solution of each standard. Prepare mixed standard solution by serial dilution of combined aliquots of stock solutions to final concentration of 0.1 ng/μL for each compound. Store all standards under refrigeration and equilibrate at room temperature before use. Prepare

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Stephen M. Walters died in August 1991 from leukemia. This work is dedicated to his memory.

working reference standard solution by evaporating a 1 mL aliquot of mixed standard solution to dryness at 50°C under a stream of nitrogen and dissolving the residue in 1 mL LC mobile phase.

(f) *Micro laboratory cleaner.*—International Products Corp., Trenton, NJ 08601.

General Apparatus

(a) *High-speed homogenizer.*—Polytron Model PT 10/35 with generator No. PTA 20TS (Brinkman Instruments, Westbury, NY 11590).

(b) *Vortex mixer.*—Scientific Products, McGraw Park, IL 60085.

(c) *Centrifuge.*—Must accommodate Corning 50 mL centrifuge tubes; 2000 rpm minimum, with swing-out horizontal rotors.

(d) *Filter paper.*—Sharkskin analytical paper, 5.5 cm (Schleicher & Schuell, Keene, NH 03431).

(e) *Polypropylene containers.*—Corning 250 mL beakers and 50 mL centrifuge tubes with conical bottoms (Fisher Scientific, Springfield, NJ 07081).

(f) *Filters.*—For SPE, use 20 μm porous polyethylene frits (1 1/16 id, Analytichem International, Inc., Harbor City, CA 90710). For sample filtration before LC analysis, use Nylon Acrodisc 13 mm disposable filter unit, 0.45 μm pore size (Gelman Sciences, Ann Arbor, MI 48106).

(g) *pH indicators.*—ColorpHast pH indicator strips with pH range 0–14 (EM Science).

(h) *SPE apparatus.*—Visiprep SPE vacuum manifold with Teflon solvent guides (Supelco, Bellefonte, PA 16823), 75 mL polyethylene reservoirs, reservoir adapters, and 400 mg Adsorbex silica SPE cartridges (EM Science).

LC System

(a) *Pump.*—SP8700 solvent delivery system (Spectra-Physics, San Jose, CA 95134).

(b) *Injector.*—Waters U6K (Waters Chromatography Div., Millipore Corp., Milford, MA 01730).

(c) *Column.*—Du Pont Zorbax silica analytical column (25 cm × 4.6 mm id) with Du Pont reliance guard column hardware kit and Zorbax silica guard cartridge (1.25 cm × 4 mm id) (Mac-Mod Analytical, Chadds Ford, PA 19317).

(d) *Column oven.*—Model LC-100 (Perkin-Elmer Corp., Norwalk, CT 06856).

(e) *Detector.*—HP 1040M diode-array UV/VIS adsorbance detection system (Hewlett-Packard Corp., Palo Alto, CA 94303).

LC Operation

Set column oven temperature to 40°C and equilibrate system using helium sparged mobile phase at 1.0 mL/min for 15 min or until reproducible retention times for PQ and DQ are achieved (ca 5 and 5.6 min for DQ and PQ, respectively). Thoroughly rinse system with 20% acetonitrile in water before shutting down system for extended periods, e.g., overnight. Maintain slow flow (0.1–0.2 mL/min) when system is not in use for shorter periods, to prevent salt deposition. Program diode array detector for PQ and DQ quantitation at maximum absorbance wavelengths of 257 and 310 nm, respectively. Adjust sensitivity of detector so that 100 μL working standard solution (10 ng of each analyte on-column) produces 20% full scale response. (This was achieved with a setting of 0.01 AUFS using the specified system.)

Sample Preparation

Prepare sample composite using a food chopper. Weigh 10 g of sample into 100 mL Pyrex test tube (33 × 159 mm). Add 10 mL 6M HCl and homogenize with Polytron mixer (ca 0.5 min). Rinse probe into test tube with ca 10 mL water. Digest sample for 1 h in a water bath at 100°C. Cool and filter digest with suction through sharkskin paper in a 6.3 cm Buchner funnel into a 250 mL vacuum flask. Rinse test tube and funnel 3 times with a total of ca 50 mL water. Transfer sample to a 250 mL polypropylene beaker, and rinse flask with ca 10 mL water. (Acid digests may be stored under refrigeration.) Immediately before SPE cleanup, add 4 mL 50% NaOH (w/v) with swirling and adjust filtrate to pH 9 by dropwise addition of 50% NaOH and, subsequently, 10% NaOH. Transfer filtrate to two 50 mL polypropylene centrifuge tubes and centrifuge (2000 rpm, 15–20 min). (Note: After pH adjustment of samples, proceed without delay to minimize possible degradation of DQ.)

SPE

Insert a 20 μm frit into 75 mL polyethylene sample reservoir and place on top of SPE cartridge using an adapter. Condition cartridge with 20 mL water, 5 mL Solution A, 20 mL water, and 15 mL Solution B at a flow rate of ca 4 mL/min. Determine apparent surface pH of the silica sorbent by measuring pH of last ca 1 mL of Solution B eluate using ColorpHast pH indicator strip in a test tube. If eluate has not reached a pH between 9 and 10, continue to wash cartridge with 1 mL aliquots of Solution B, measuring pH as before. Do not allow silica sorbent to run dry between these washes. When apparent surface pH of the silica sorbent is between 9 and 10, transfer pH-adjusted sample supernatant to cartridge. Using a vortex mixer, thoroughly mix insoluble residue remaining in centrifuge tube with 10 mL LC grade water previously adjusted to pH 9, centrifuge for 15 min, and add supernatant to cartridge. Adjust vacuum to allow sample to elute at ca 1 drop/s. After sample elution, wash cartridge with 20 mL water, 20 mL methanol, and 5 mL Solution C. Let Solution C elute to near dryness to clear the sorbent and solvent guides of eluting crop compounds. Discard sample eluate and washes. Elute analytes with 10 mL Solution D into a clean, HCl rinsed, unsilanized 100 mL round-bottomed flask. (Note: Because of limited space in SPE apparatus, final eluate can be collected in test tubes or other appropriate containers and transferred to round-bottomed flasks, rinsing with methanol.) Evaporate to ca 1 mL using a vacuum rotary evaporator at 90°C. Evaporate remaining sample to dryness in a 100°C water bath under nitrogen. Dissolve residue in 1 mL LC mobile phase, using sonication if necessary. Filter through 0.45 μm porosity membrane.

LC Determination

Inject 100 μL sample solution into LC system. Compare chromatographic response (peak retention times, heights, and/or areas) with that of standard solution and calculate res-

idue amount. Standard peak responses established for screening analysis are equivalent to ca 0.01 ppm of PQ and DQ. If levels found in sample significantly exceed the screening level, prepare standard solutions with higher concentrations to give peak responses within 25% of those of sample. The estimated limit of quantitation is 0.005 ppm. Diode array scans of respective peaks provide qualitative information if desired.

Results and Discussion

SPE

Silica is one of the most polar sorbents available for SPE. Under aqueous conditions, however, silica exhibits primarily cation exchange selectivity. The cation exchange capacity of silica is dependent on several factors, including pH (19). In general, this capacity increases with increasing pH. For this reason, under basic conditions quaternary ammonium compounds are highly retained on silica. The most critical aspects of the SPE procedure were found to be the pH adjustment of the crop digests and the apparent surface pH of the silica sorbent before sample application. Figure 1 shows the effect of the sample pH before SPE on the recovery of PQ and DQ from fortified potato digests. Below a pH of 9, recoveries for these analytes began to decrease, particularly for DQ. In this same study, however, standards taken through the method in the absence of crop matrix gave recoveries ranging from 91 to 101% for both PQ and DQ over the entire pH range tested. The presence of crop coextractants necessitated conditions that favored increased capacity of the silica sorbent. Because DQ degrades slowly at pH >9 (10), the method was optimized using a sample solution pH of 9 and an apparent silica surface pH between 9 and 10. Recoveries for PQ and DQ were found to be somewhat low and irreproducible if the apparent silica surface pH was >10.

The SPE cartridges were easily obstructed by sample fines that were not filtered or centrifuged out of solution. This problem was alleviated by introducing the 20 μm frit into the sample reservoir. The increased surface area of this frit (ca 27 mm id) resulted in a less dense accumulation of sample fines that would otherwise plug the smaller frit (ca 9 mm id) of the SPE cartridge.

SPE methodologies generally employ preconditioning washes before sample application for the following reasons: solvation or wetting of the sorbent to ensure reproducible interaction of the sorbent with analytes (20), removal of contaminants, or preparation of the sorbent bed to accomplish specific retention mechanisms such as ion-exchange. Because the samples described in this methodology were in an aqueous solution, water was used as the initial solvating wash. Solution A (1M HCl) effectively removed contaminants from the silica sorbent before sample application, while the necessity of the basic wash has already been described. The solvated sorbent bed should not be allowed to run dry to ensure compatibility between the sorbent environment and the sample. Once the analytes of interest are retained, drying of the sorbent does not present any problems (20).

The final sample volume before SPE cleanup was approximately 100 mL. The possible effect of this volume on analyte recoveries was not studied in any detail. However, recoveries were found to be equivalent for samples that were occasionally diluted to volumes exceeding 100 mL (i.e., as much as 200 mL). The sample volume did not appear to have any significant effect on recovery; therefore, additional aqueous washings in the sample preparation step greater than 100 mL are permissible.

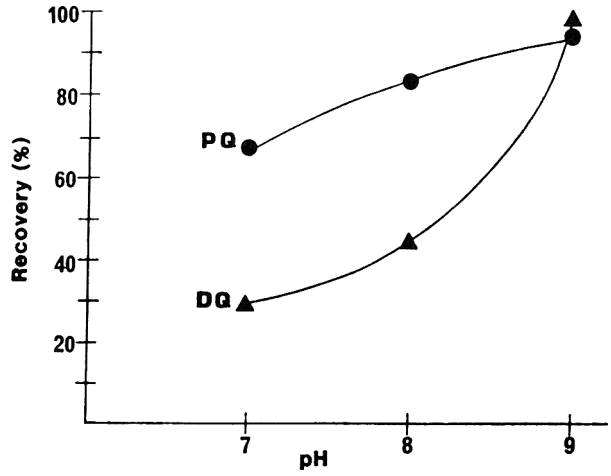

Figure 1. Effect of sample solution pH on the recovery of PQ and DQ from potato digests using silica SPE cleanup; PQ & DQ were fortified at the 0.5 and 0.1 ppm levels, respectively.

Methanol was employed to remove water from the sorbent before the application of the wash with Solution C (0.1M HCl in methanol). Final recoveries were found to be low (< 50%) and irreproducible for both PQ and DQ if residual water was not removed from the sorbent before the application of this acidic wash. Apparently, even a slight increase in water content in the methanolic acid wash was sufficient to partially elute the analytes. With the removal of residual water, the acidic wash could be applied with no detectable losses of PQ or DQ. The acidic wash effectively removed most of the sample coextractants from the sorbent before analyte elution, resulting in longer guard cartridge life and cleaner chromatograms.

Methanol was included in the final eluate to facilitate evaporation. In the final eluate (Solution D), 2M HCl was found to efficiently elute PQ and DQ. Vacuum and high temperature (90°C) were required to evaporate the eluate efficiently. Under these conditions, however, the final eluate could not be taken to total dryness because of the high (30%) aqueous concentration. Therefore, the last 1 mL was taken to dryness at 100°C under nitrogen.

Several different brands of silica cartridges were evaluated in the development of the SPE method. In general, all of the brands tested (Prep Sep, Sep-Pak, Bond Elut, and Adsorbex) gave equivalent recoveries for PQ and DQ standards alone and from fortified potato digests at the tolerance levels of 0.5 and 0.1 ppm for PQ and DQ, respectively. However, several factors should be considered in selecting a silica cartridge other than the 400 mg Adsorbex cartridge for the isolation of these analytes: The sample capacity, adjustability of apparent surface pH, and retentive characteristics of different cartridges are not necessarily equivalent under the conditions of this methodology. For instance, Prep Sep cartridges (Fisher Scientific), which contained only 300 mg of silica sorbent, showed analyte loss via breakthrough with potato digests fortified at levels exceeding the tolerances (1.0 ppm for PQ and 0.2 ppm for DQ). Bond Elut cartridges (500 mg, Analytichem International, Inc.) demonstrated

greater retention of PQ, which necessitated an increased volume (15–20 mL) of final eluate. This particular sorbent also required an increased volume of basic conditioning wash (approximately 30 mL) before sample application to adjust the apparent surface pH to the desired value. From a practical standpoint, the various SPE cartridges available may not be universally adaptable to commercially available manifolds, sample reservoirs, etc. (For example, at the time of this study, Waters Sep-Pak cartridges were not available in a configuration that was conveniently adaptable to the Supelco vacuum manifold.)

The silica SPE cartridges can be reused. Because silica is physically stable between pH 2 and 8, the cartridges should be stored with an apparent surface pH between these values. This can be accomplished by rinsing with approximately 150 mL water. A final 10 mL rinse with 10% methanol is done before storage. However, trace amounts of PQ and DQ (particularly PQ) may be retained on the sorbent even after the final elution step. Obviously, cartridges that were used for reagent blanks or crop samples in which no analyte was detected could be reused with reasonable confidence. The actual number of times that the SPE cartridges can be reused was not investigated in detail. However, in the course of this study, some cartridges were used at least twice. The reuse of cartridges should be practiced with discretion.

PQ and DQ can be adsorbed onto surfaces such as glass, particularly under alkaline conditions. This can be a troublesome source of contamination. For this reason, polypropylene containers were used when samples were in alkaline solution. Although these containers are disposable, they have been reused after soaking in a Micro solution, rinsing with dilute HCl (approximately 1M), water, and ethanol. Silanization of glassware was not incorporated into the method because of the acidic final eluate that dissolved the silanizing reagent from treated glass. Recoveries were found to be equivalent for both PQ and DQ using treated and untreated sample flasks. However, glassware (particularly the round-bottomed sample flasks) should be rinsed with water, soaked overnight with 6M HCl, and then rinsed with water and ethanol. Worobey has described a similar procedure (3). These flasks, or the plasticware, should not be scrubbed or scoured with anything that might score the surface (e.g., brushes).

Ion-pairing Chromatography

The LC system described by Nagayama et al. (10), which used an amino-bonded column, sodium chloride, and a ternary mobile phase consisting of methanol, acetonitrile, and water for the determination of PQ and DQ in several crops, was also evaluated in the course of this study. Multiple, competing retention mechanisms were apparently involved with this system, as retention times were not reproducible even after equilibrating for more than 2 h. The silica column system developed in the present study was equilibrated in approximately 15 min.

Reversed-phase ion-pairing LC is frequently employed to achieve separation of ionic compounds (21), including quaternary ammonium compounds. Typically, these systems employ ODS-bonded silica and long-chain alkylsulfonate salts as ion-pairing reagents. The ion-pairing process is dependent on many factors, including pH and ionic conditions. Reversed-phase ion-pairing LC using sodium dodecylsulfonate and an ODS column was evaluated for the determination of PQ and DQ following their isolation by the SPE procedure. Peak splitting was observed with DQ using this system. It was concluded that competing chloride ions present in the SPE residue precluded the use of reversed-phase ion-pairing LC.

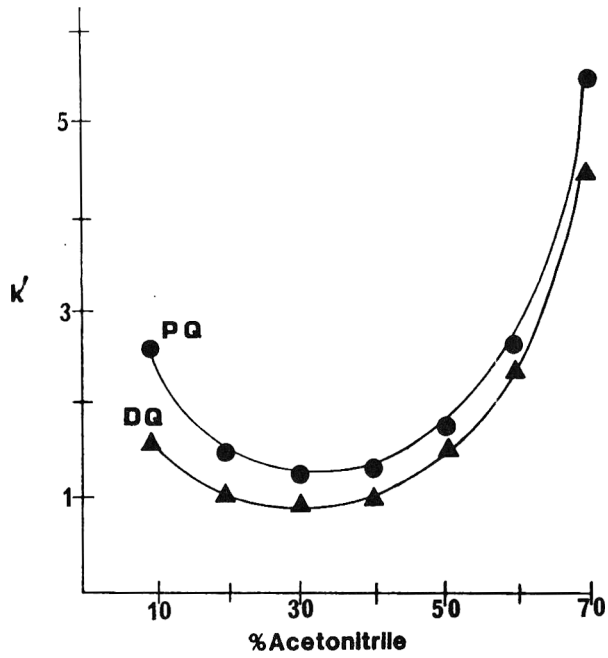

Figure 2. Effect of acetonitrile concentration in mobile phase on retention (in terms of capacity factor, k'). Mobile phase contains 42mM NaCl, and pH of water fraction is 2.2 in each case.

Greving et al. (13) demonstrated that quaternary ammonium compounds, like protonated bases, form stable ion pairs that can be chromatographed on silica using inorganic halide salts as ion-pairing reagents in a nonaqueous, methanolic mobile phase. We evaluated a similar system, using silica and nonaqueous acetonitrile with sodium perchlorate as the ion-pairing reagent. In this system, PQ and DQ were chromatographed as symmetrical, well-resolved peaks whose retention times could be controlled by the concentration of added salt. However, sample residues after SPE were difficult to dissolve in neat acetonitrile, necessitating the introduction of water into the mobile phase. In addition, the chromatography of PQ and DQ was irregular and irreproducible because of the chloride ions present in the sample residues; the chloride ions apparently competed with the perchlorate ion-pairing process. At this point, substitution of perchloric acid for HCl in the final eluate of the SPE cleanup was considered to achieve a uniform anion content. However, this was not attempted because of the unstable nature of perchloric acid.

Acetonitrile was used as the organic modifier in the proposed LC system because it demonstrated greater elution strength (22) and produced symmetrical peaks for both PQ and DQ. Methanol, on the other hand, produced relatively broad and tailing peaks for both analytes.

The effects of acetonitrile concentration on capacity factor (k') are shown in Figure 2. Concentrations between 30 and 50% afforded resolution of the analytes from early eluting

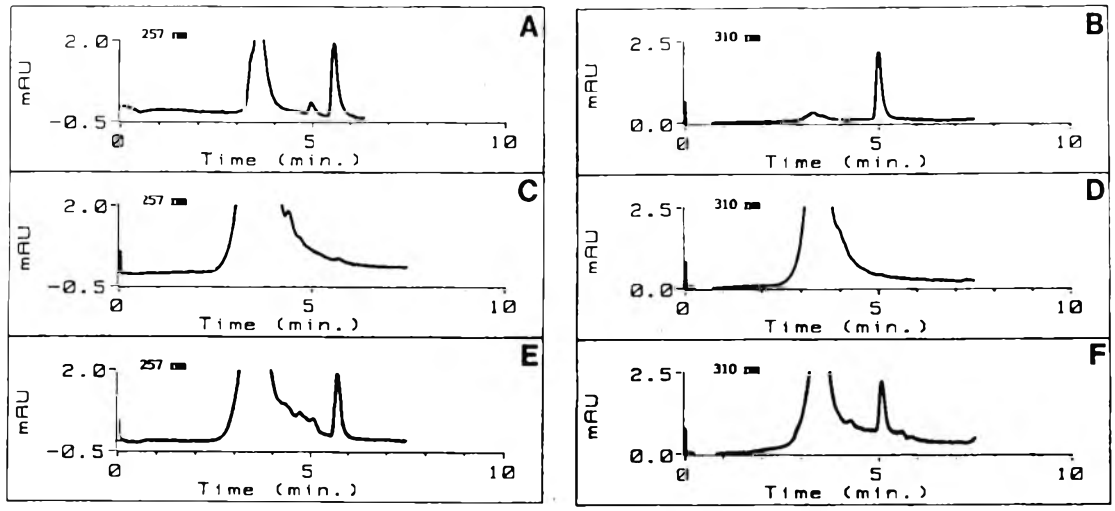

Figure 3. LC-diode array UV chromatograms displayed at 257 nm (PQ determination) and 310 nm (DQ determination) for analysis of potato fortified at screening level. A and B, standard mixture (10 ng each of PQ and DQ); C and D, potato control; E and F, fortified potato (0.01 ppm each of PQ and DQ).

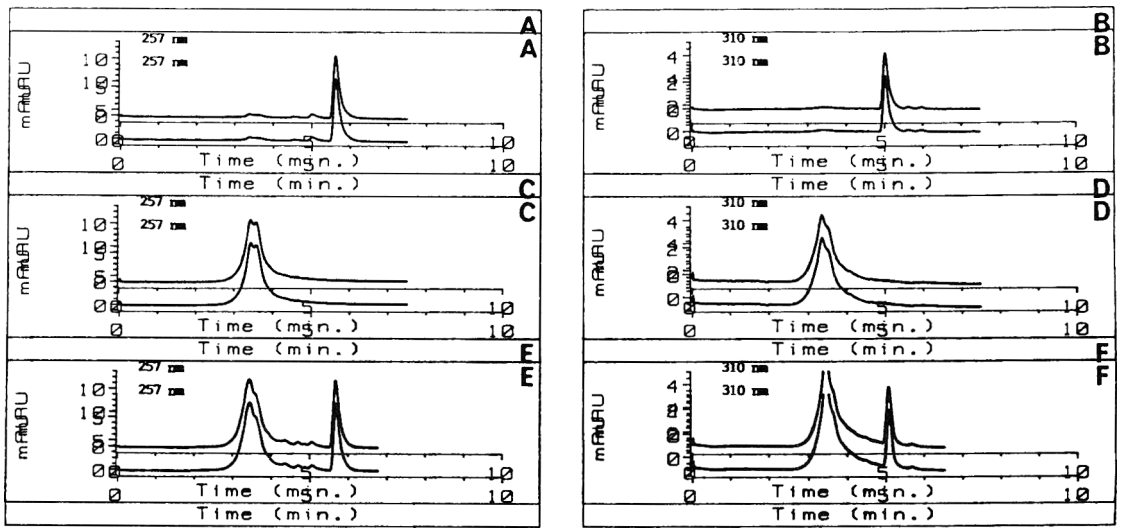

Figure 4. LC-diode array UV chromatograms displayed at 257 nm (PQ determination) and 310 nm (DQ determination) for analysis of corn fortified at tolerance levels. A and B, standard mixture (50 ng PQ and 20 ng DQ); C and D, corn control; E and F, fortified corn (0.05 ppm PQ and 0.02 ppm DQ).

crop matrix peaks, adequate resolution of PQ and DQ, and symmetrical peak shapes. At concentrations of <30 and >50%, however, increased retention was accompanied by band broadening and tailing.

Before incorporating the 0.1M methanolic HCl wash, which removed the majority of salt from the sorbent in the SPE cleanup, NaCl in the sample residues caused irreproducible analyte responses because the final concentration of salt in the dissolved residue was greater than in the mobile phase. This effect was controlled by increasing the concentration of NaCl in the mobile phase (e.g., 280mM). However, this was not desirable because of the potentially corrosive effects of excessive NaCl on LC hardware. The alternative was to eliminate or reduce the salt concentration in the sample residues. Because a small amount of NaCl is likely to be present in the final sample eluate, the salt concentration specified in the method (85.5mM) was selected to compensate for any possible effects of the residues on chromatographic consistency and to achieve optimum efficiency. Corrosion from NaCl in the mobile phase at this lower concentration was not encountered with routine maintenance of LC hardware and thorough rinsing of the system.

The pH of the mobile phase also influenced the retention and peak shape of PQ and DQ in the proposed LC system. In general, alterations in pH affect the protonation of basic analytes and the ionization of silanols, both of which affect retention (15). Because PQ and DQ maintain constant positive charges, however, they are not directly affected by pH. Hence, the extent to which ionization of silanols occurs is, in part, responsible for the retention and peak shape of these analytes. With increasing pH, retention increased and peak shapes deteriorated. Without pH adjustment (apparent pH of mobile phase approximately 4.5), retention times were not reproducible. Efficient and reproducible chromatograms

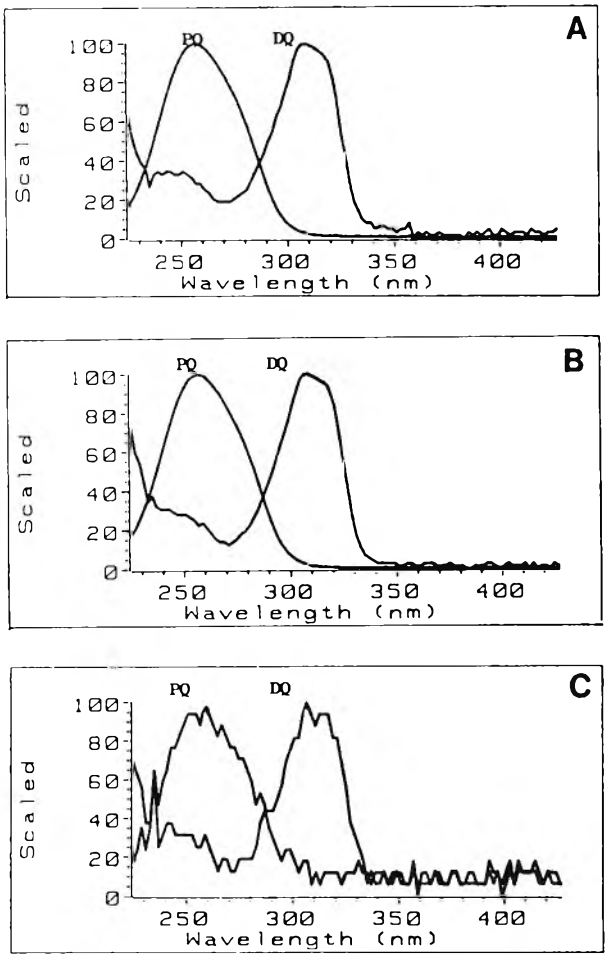

Figure 5. Diode array UV detector spectra obtained at chromatographic maxima for PQ and DQ. A, standard mixture (250 ng PQ and 50 ng DQ); B, potato fortified with 0.5 ppm PQ and 0.1 ppm DQ; C, asparagus fortified with 0.01 ppm each of PQ and DQ.

were achieved by adjusting the water used in the mobile phase to pH 2.2.

Several silica analytical columns from different manufacturers were evaluated for this method. The Du Pont Zorbax silica column produced the most symmetrical peaks of those columns tested. Columns packed with different batches of this silica produced identical chromatograms under the conditions of the method. The columns were stable with repetitive usage; a single column was subjected to approximately 1000 injections of sample and standard solutions before showing a notable loss of efficiency. New Zorbax columns with new guard cartridges typically produced >5000 theoretical plates per column using the formula:

$$N = 5.54 \times (\text{retention time/peak width at half high height})^2$$

for both PQ and DQ. Efficiency lost after repetitive injections was restored by replacement of the guard cartridge until significant analytical column deterioration occurred.

The LC-diode array detector system produced linear peak area responses for both PQ and DQ over the range of 2–500 ng using a constant injection volume of 100 µL. The correlation coefficient for both analytes was 0.999.

Typical UV diode array chromatograms of standard and sample solutions are shown in Figures 3 and 4. The diode array detector allows simultaneous detection of PQ and DQ at 257 and 310 nm, respectively. PQ and DQ are adequately resolved and separated from early-eluting sample coextractants in a single chromatographic run of less than 6 min.

Diode array UV spectra provided additional qualitative data for PQ and DQ in sample injections as shown in Figure 5. Maximum absorbance at the correct wavelengths for PQ and DQ are discernible even at the low screening level of 0.01 ppm in crop extract.

Table 1. Recovery of standards

Standard	Added, µg	Av. rec., %	CV, %	N
PQ	0.1	96.8	4.8	5
PQ	5.0	93.7	2.9	9
DQ	0.1	90.7	6.6	5
DQ	1.0	83.5	5.0	9

Recovery of Standards

Recoveries of standards in the absence of crop matrix are given in Table 1. Fortifications of 0.1–5 µg were equivalent to levels of 0.01–0.5 ppm in 10 g of crop. Determinations were made on sets of either 2 or 3 fortified reagent blanks that were analyzed on different days over a period of 2 weeks. Average recoveries ranged from 90.7% for DQ at 0.01 ppm to 96.8% for PQ at 0.01 ppm. DQ recoveries were typically somewhat lower for both fortified reagent blanks and crop samples.

Table 2. Recoveries of paraquat and diquat from fortified crops

	Paraquat			Diquat		
Crop	Added, ppm	Av. rec., %	CV, %	Added, ppm	Av. rec., %	CV, %
Potato	0.01	88.7 (12)[a]	13.7	0.01	82.6 (12)	11.8
	0.50[b]	89.0 (9)	1.6	0.10[b]	86.8 (9)	4.3
Corn	0.01	104.8 (6)	11.4	0.01	86.2 (6)	7.8
	0.05[b]	95.4 (6)	3.2	0.02[b]	85.3 (6)	5.9
Turnip	0.01	98.6 (6)	12.6	0.01	79.3 (3)	3.4
	0.05[b]	91.5 (3)	3.7	0.02[b]	80.4 (3)	3.8
Asparagus	0.01	85.3 (3)	4.8	0.01	84.8 (3)	5.0
	0.50[b]	89.5 (9)	2.7	0.50	84.2 (9)	2.2

[a] Number of samples is in parentheses.
[b] EPA tolerance level.

Recovery from Fortified Crops

Average recoveries for PQ and DQ in 4 crops are given in Table 2. Crop samples (10 g) were fortified with 1 mL aliquots of appropriately diluted standards in methanol and allowed to stand overnight under refrigeration. Samples were fortified at levels ranging from 0.01–0.5 ppm and, in most cases, were analyzed in groups of 3. The relatively high coefficients of variation (e.g., for 12 determinations of PQ and DQ at 0.01 ppm in potatoes) reflect day-to-day variability for runs made on several different days over a period of several months. The variability for determinations made concurrently (i.e., sets of 3 samples) are ≤5%. Individual recoveries ranged from 71.7% (DQ in potatoes at 0.01 ppm) to 120.1% (PQ in corn at 0.01 ppm). Average recoveries ranged from 79.3% (DQ in turnips at 0.01 ppm) to 104.8% (PQ in corn at 0.01 ppm).

Conclusion

The method described provides a rapid, specific, multiresidue assay for the determination of PQ and DQ in several high moisture crops. Reproducible recoveries were obtained for both analytes using a pH-controlled silica SPE procedure. Ion-pairing LC using a silica column and sodium chloride as an ion-pairing reagent in a reversed-phase eluent provided sensitivity at a screening level of 0.01 ppm and good selectivity for the analytes in crop extracts. UV diode array detection provided simultaneous determination of PQ and DQ with qualitative analysis. The application of this method to other high moisture crops and to dry products such as wheat and dry beans is currently under investigation.

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Improved Method for Determination of Polynuclear Aromatic Hydrocarbons in Pharmacopoeial Paraffin and Mineral Oils

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An improved method has been developed for quantitative determination of polynuclear aromatic hydrocarbons (PAHs) in pharmacopoeial paraffin and medicinal white oil samples. This new method combines 2 liquid-liquid partition and adsorption chromatography procedures with a 2-step purification on Sephadex LH 20 and liquid chromatography with fluorometric determination. Selective elution of PAHs results in absence of background fluorescence. The minimum detectable level ranges from 0.2 ppt for benzofluoranthene isomers to 200 ppt for acenaphthene. Recoveries of PAHs added at 7 ppm varied from 92.1 to 111.4%. When a variety of medicinal white oil samples were analyzed by this improved method, 27 PAHs were identified, including 11 suspected carcinogens. Their identities were confirmed by capillary gas chromatography.

Polynuclear aromatic hydrocarbons (PAHs) of great structural variety occur in the environment from many different sources. Because of their carcinogenic activity, PAHs are among the most intensively studied compounds in environmental samples. A number of these compounds, as well as their derivatives, are potent carcinogens (1). They can occur in fresh and used lubricating oils, cutting oils, waxy distillates, raffinates, and medicinal oils (2). A number of fully-refined petroleum products, with a potential trace level content of PAHs, constitute ingredients of many cosmetics, such as cold creams, cleansing creams, lipsticks, massaging oils, baby lotions or creams, and suntan oils (3).

Several analytical methods for PAHs in environmental samples and petroleum oils are reported in the literature (4–9). Few methods are described for PAH determination in medicinal white oils and paraffin (3, 10–13). Monarca et al. (3) extracted PAHs by frontal elution chromatography, purified the PAHs present in the aromatic concentrate on aluminum oxide, and concentrated the front fraction and chromatographed it on glass plates precoated with acetylated cellulose. After the spots were visualized by UV and scraped off, PAHs were determined by fluorescence spectrophotometry. Colin et al. (11) and Colin and Vion (14) used frontal elution chromatography with a 2-step purification including adsorption chromatography and partition chromatography. Concentrated extracts were analyzed by liquid chromatography (LC), and fractions collected from the LC column were analyzed by high resolution Shpolskii fluorometry (15). Grimmer and Bohnke (16) determined PAHs by gas chromatography, using different concentrated fractions chromatographed on Sephadex.

In this paper, a modification of the described methods (4–6) for determining PAHs in mineral oils is reported. The extraction (see Figure 1) was slightly modified by changing solvent ratios in the extracting mixtures. The degree of purification was greatly increased by combining adsorption chromatography with a 2-step liquid partition on Sephadex. The 20–150 mL fraction collected was concentrated and directly analyzed by LC with fluorometric detection. LC allowed a good and selective separation of more than 20 PAHs, with no interference from endogenous compounds.

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Experimental

Reagents

(a) *Solvents.*—Reagent grade dimethylformamide, isopropanol, dimethyl sulfoxide (Carlo Erba, Paris La Defense, France); distill in glass apparatus before use. Pesticide grade cyclohexane, *n*-hexane, acetone, and toluene (SDS, Peypin, France). For LC elution solvent, use chromatographic grade acetonitrile (SDS) and quartz bidistilled water.

(b) *Sodium sulfate.*—Na_2SO_4, analytical grade (E. Merck, Darmstadt, Germany).

(c) *Silica gel.*—35–70 mesh (Merck).

(d) *Sephadex LH 20.*—(Pharmacia, Science Park, Cambridge, England). *Column 1*: Add 10 g Sephadex LH 20 to 50 mL acetone and outgas. Transfer to 150 × 20 mm glass chromatographic column and allow Sephadex to settle. Drain off excess acetone. Pour 200 mL stationary phase (water–dimethylformamide (DMF), 15 + 85) onto column to replace acetone. Let column equilibrate 24 h. Pour 50 mL *n*-hexane onto column. *Column 2*: Add 10 g Sephadex LH 20 to 50 mL acetone and outgas. Transfer to 150 × 20 mm glass chromatographic column and allow Sephadex to settle. Drain off excess acetone. Pour 200 mL isopropanol onto column to replace acetone. Let column equilibrate 24 h. Pour 50 mL isopropanol onto column.

(e) *Internal standard.*—Benzo(b)chrysene (Community Bureau of References, Commission of the European Communities). Prepare 1 mg/mL solution by dissolving 100 mg benzo(b)chrysene in 100 mL toluene.

(f) *Polynuclear aromatic hydrocarbons.*—Acenaphthene, acenaphthylene, and benzo(g,h,i)perylene (EgaChemie, Steinheim, Albuch, Germany); fluorene, fluoranthene, and coronene (Janssen Chimica, Beers, Belgium); benzo(e)pyrene, benzo(b)fluorene, and cyclopenta(d,e,f)phenanthrene (Aldrich Chemical Co., Milwaukee, WI 53201); anthracene and phenanthrene (Prolabo, Paris, France); dimethyl-2,3-naphthalene, methyl-3-pyrene, dimethyl-3,6-phenanthrene, and methyl-2-anthracene (Fluka, Buchs, Switzerland); pyrene and methyl-3-cholanthrene (Eastman Organic Chemicals, Rochester, NY, 14650); benzo(a)anthracene and perylene (Koch-Light Laboratories, Colnbrook, Buchs, England); chrysene (Schuchardt, Munchen, Germany); dimethyl-7,12-benzo(a)anthracene (Sigma Chemical Co., St. Louis, MO 63178); benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, anthanthrene, indeno(1,2,3 cd) pyrene, and cyclopenta(cd)pyrene (Community Bureau of References, Commission of the European Communities). Prepare all stock solutions in toluene at 21 mg/mL. Keep in dark, frozen at −20°C. Pipet aliquots from stock solutions into

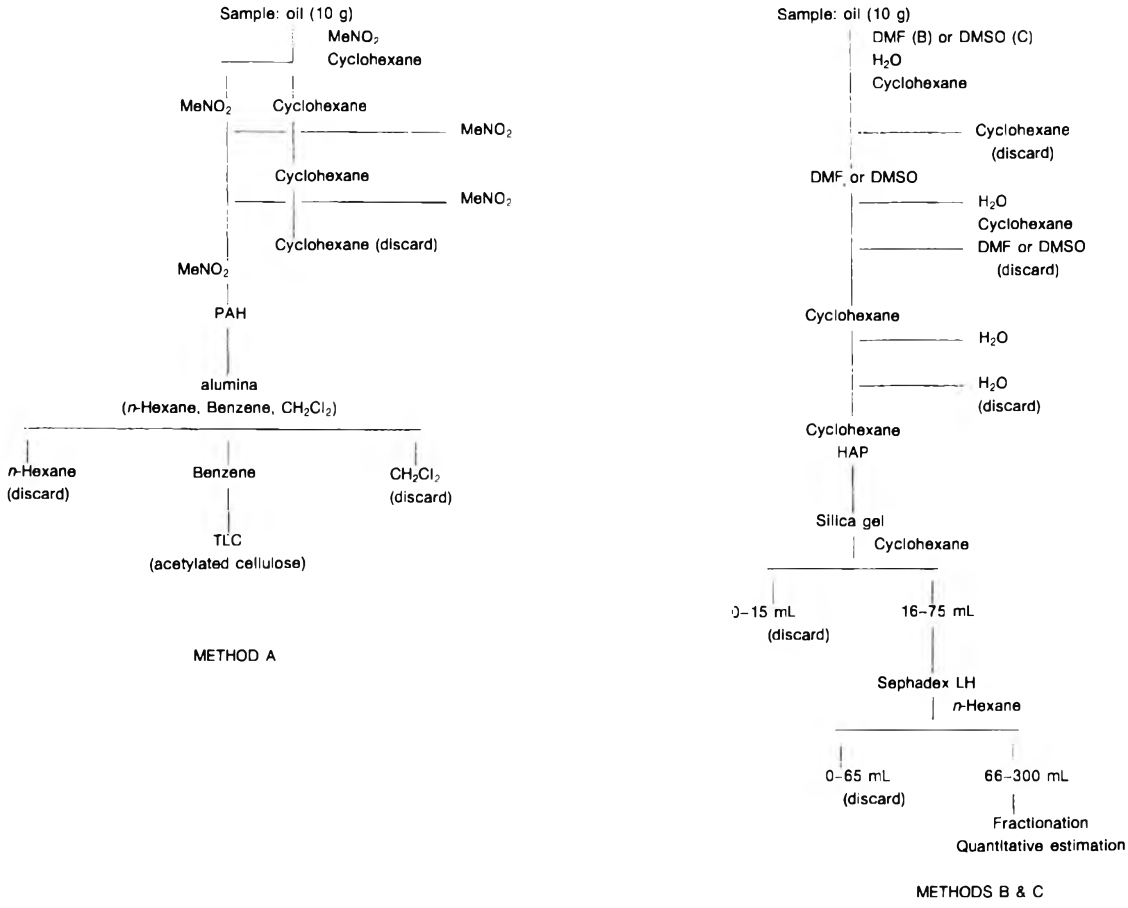

Figure 1. Different extraction and cleanup procedures described by Grimmer and Bohnke (4) and Grimmer et al. (5, 6).

100 mL volumetric flasks and dilute with toluene to make final working solutions (see Table 1; 93 mL toluene was used instead of 100 g PAHs-free mineral oil).

Warning: Some PAHs are carcinogens. All operations involving handling of PAHs or their solutions should be carried out with disposable rubber surgical gloves in ventilated enclosures. PAHs are degraded by UV light. Avoid exposure of samples, extracts, and standard solutions to sunlight.

Apparatus

(a) *Gas chromatograph.*—HRGC Fractovap Model 4160 gas chromatograph (Farmitalia-Carlo-Erba, Paris, La Defense, France) equipped with flame ionization detector and on-column capillary column injection port. GC column: fused silica, 25 m × 0.32 mm id, CP SIL 5 CB (Chrompack, Les Ulis, France), with 0.11 μm film thickness. Operating conditions: Temperature program as follows—initial, 80°C; programming rate, 3°C/min; final, 270°C. Carrier gas flow, hydrogen, 4 mL/min at 80°C. Capillary detector, 300°C: hydrogen flow 30 mL/min, air flow 400 mL/min. Detector make-up gas, hydrogen at 30 mL/min. Data collection, digital integrator with least squares capability.

(b) *Liquid chromatograph.*—Model 200 programmer, binary gradient with 2 Model 414 T pumps (Kontron Instruments, Everett, MA 02149) equipped with Model 7010 syringe-loading sample injector (Rheodyne Inc., Cotati, CA 94931) and coupled to Model SFM 3 spectrofluorometer (excitation 305 nm, emission cut-off filter >415 nm) (Kontron). Operating conditions: flow rate, 1 mL/min; column temperature, ambient; eluant system, acetonitrile (A) and water (B). Gradient program: hold at 60% B for 7.5 min; from 60 to 100% B in 60 min; from 100 to 60% B in 0.1 min; hold at 60% B for 5 min, then start injection. Column: 250 × 4.6 mm id, stainless steel packed by slurry technique with 5 μm Ultrasphere ODS (Beckman Instruments Inc., Fullerton, CA 92634).

(c) *Computing integrator.*—Hitachi-Merck 2000 and HP3390A (Hewlett-Packard Co., Palo Alto, CA 94303).

(d) *Glassware.*—Clean with 30% nitric acid, and wash twice with quartz bidistilled water.

Sample Extraction

See Figure 2. To 10 g mineral oil in 500 mL separatory funnel, add 50 μL internal standard solution, 80 mL cyclohexane, 80 mL DMF, and 100 mL water. Shake 3 min and allow layers to separate. Drain lower layer into second separatory funnel, add 80 mL water and 180 mL cyclohexane, and repeat extraction. Allow layers to separate and discard lower layer (water + DMF). Wash cyclohexane layer twice, using 30 mL water. Discard washings. Filter cyclohexane layer over anhydrous Na_2SO_4, and concentrate to ca 1 mL with rotary evaporator under reduced pressure in water bath at 40°C.

Table 1. Concentrations (μg/10 g) of various PAHs in standard mineral oil

Analyte	Concn, μg/10 g	Standard stock solution added to PAHs-free mineral oil, μL[a]
1. Phenanthrene	91.84	918.4
2. Anthracene	3.24	32.4
3. Fluoranthene	0.52	5.2
4. Pyrene	5.00	50.0
5. Methyl-2-anthracene	5.03	50.3
6. Methyl-9-anthracene	3.11	31.1
7. Dimethyl-3,6-phenanthrene	10.1	101.0
8. Benzo(k)fluoranthane	0.02	0.2
9. Perylene	1.02	10.2
10. Benzo(a)pyrene	0.58	5.8
11. Benzo(e)pyrene	4.32	43.2
12. Benzo(b)fluoranthene	0.02	0.2
13. Benzo(a)anthracene	0.41	4.1
14. Dibenzo(a,h)anthracene	0.59	5.9
15. Methyl-3-pyrene	7.8	78.0
16. Benzo(a)fluorene	6.27	62.7
17. Methyl-3-cholanthrene	0.11	1.1
18. Dimethyl-7,12-benzo(a)anthracene	0.22	2.2
19. Anthranthene	0.02	0.2
20. Indeno(1,2,3,cd)pyrene	0.29	2.9
21. Internal standard	(50)	—
22. Coronene	0.74	7.4
23. Benzo(g,h,i)perylene	0.44	4.4
24. Chrysene	8.2	82.0
25. Cyclopenta(c,d)pyrene	111.0	1110.0
26. Benzo(j)fluoranthene	1.0	10.0
27. Acenaphthene	220.8	2208.0
28. Acenaphthylene	111.0	1110.0
29. Benzo(b)fluorene	9.9	99.0
30. Cyclopenta(d,e,f)phenanthrene	43.6	436.0
Total	696.39	

[a] Toluene (93 mL) was used instead of 100 g PAHs-free mineral oil for the preparation of the final working standard solution.

Chromatography of Sample Extracts on silica Gel

Shake 6 g silica gel with ca 20 mL cyclohexane, transfer to 300 × 10 mm chromatographic column, and drain cyclohexane by gravity. Transfer concentrated extract to top of column. Rinse evaporation flask with two 1 mL portions of cyclohexane and add to same column. Add 100 mL cyclohexane to column. Discard first fraction (0–10 mL), and collect fraction from 10 to 100 mL. Concentrate eluate to 1 mL in rotary evaporator under reduced pressure at 40°C.

Chromatography of Sample Extracts on Sephadex LH 20

Place residue on top of Column 1. Rinse flask twice with 1 mL *n*-hexane saturated with water–DMF (15 + 85). Elute with 300 mL *n*-hexane equilibrated with same mixture. Discard first 20 mL and collect 20–300 mL fraction. Evaporate eluate almost to dryness in rotary evaporator under reduced pressure. Dissolve residue in 1 mL isopropanol. Transfer dissolved residue to top of Column 2. Rinse flask twice with 1 mL isopropanol and elute with 150 mL isopropanol. Discard first 20 mL and collect 20–150 mL fraction. Evaporate

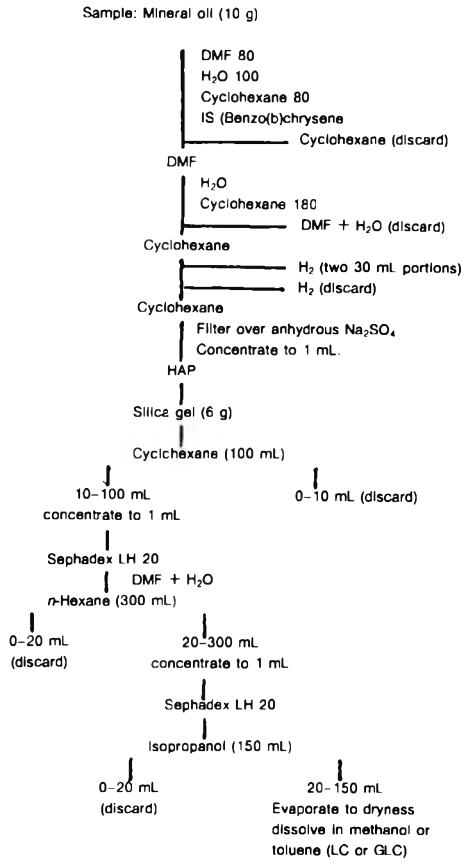

Figure 2. Extraction and cleanup steps as described in *Sample Extraction*.

to 1 mL in rotary evaporator under reduced pressure, and then to dryness under nitrogen stream at 37°C. Add 1 mL methanol and retain for LC.

LC and Capillary GC

Aliquots of 20 μL organic layer were subjected to LC with fluorometric detection. For capillary GC, 0.5 μL residue dissolved in toluene was injected.

Standard Preparation

To 10 g PAHs-free mineral oil (paraffin, French Codex, purified and pretested), add all 29 PAHs-mineral oil samples with respective PAH concentrations (see Table 1). Add 50 μg benzo(b)chrysene and continue as in *Sample Extraction*. For recovery study, prepare sample at 7 ppm level of total PAHs by adding 90 g PAHs-free mineral oil to 10 g spiked sample as described above.

Results and Discussion

Sample Extraction

Initial methods reported by Grimmer and Bohnke (4) and Grimmer et al. (5, 6) were reviewed (see Figure 1). Method A was found to be less selective than B and C, although recoveries for methyl-2-anthracene, indeno(1,2,3,cd)pyrene,

Table 2. Recoveries of 13 PAHs obtained by 3 different methods (A, B, and C)[a]

Analyte	Method A	Method B	Method C
Phenanthrene + anthracene	31	53	28
Fluoranthene	100	100	39
Methyl-9-anthracene	11.5	36	8
Methyl-2-anthracene	97	43	100
Benzo(a)anthracene	0	14	39
Benzo(f)fluoranthene	33	14	38
Indeno(1,2,3,cd)pyrene + benzo(g,h,i)perylene	28	16	15
Dimethyl-7,12-benzo(a) anthracene + benzo(k) fluoranthene	22	10	49
Benzo(a)pyrene	11.5	12	23
Methyl-3-cholanthrene	100	100	100

[a] Methods are described in Figure 2.

and benzo(ghi)perylene were higher (Table 2). Recoveries for all other PAHs were similar, and ranged from 12 to 100%. Errors arising from bad recoveries were avoided by adding a known amount of internal standard to unknown samples and to purified and pretested liquid paraffin spiked with different PAHs. Stable emulsions formed during the partition step in all methods. Good separation of aqueous and organic layers was obtained by increasing the water and DMF in the extraction mixture.

Extract Cleanup

Many oil components, mainly heterocyclic aromatic compounds, are extracted with PAHs and interfere with quantitative fluorometric determination. Interferences from such impurities were avoided by combining column chromatography on silica gel with 2-step column chromatography on Sephadex LH 20. Selective elution with *n*-hexane and isopropanol resulted in absence of background fluorescence, which allowed direct coupling of LC and fluorometry.

Resolution of PAHs on LC Column

Different column packings for LC were tested. Ultrasphere ODS gave the best results; high resolution of more than 20 PAHs was observed (Figures 3 and 4). Compounds with similar retention times were identified by capillary GC (Figures 5 and 6).

Precision, Detection Limit, and Recoveries

Precision of the modified method was verified with the results obtained from 10 determinations of a pretested mineral oil. Spiked samples were prepared at the 7 ppm level of added total PAHs. Phenanthrene, cyclopenta(c,d)pyrene, acenaphthene, and acenaphthylene were used at relatively high concentrations because of lack of sensitivity. Recoveries for 29 PAHs ranged from 39% for benzo(a)pyrene to 103% for methylcholanthrene. Relative standard deviations ranged from 1.8% for benzo(j)fluoranthene to 7.9% (n = 10) for phenanthrene and anthracene (Table 3). Total recoveries (estimated by the internal standard method) ranged from 92.1 to

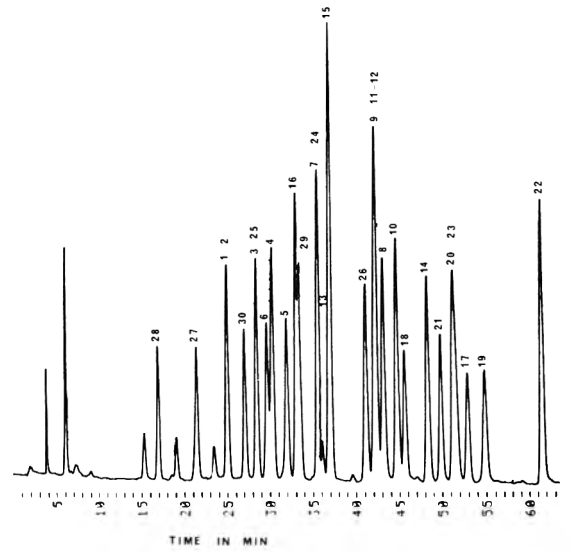

Figure 3. Liquid chromatogram of 30 PAHs on column packed with 5 μm Ultrasphere ODS (see details in Table 1).

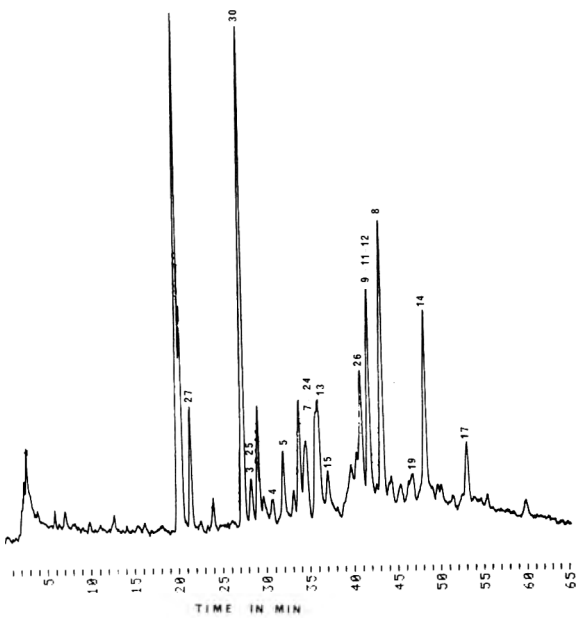

Figure 4. Liquid chromatogram of extracted white mineral oil sample (see details in Table 1).

111.4% (n = 10), with a mean of 7.02 ppm and a relative standard deviation of 5.29%.

Limit of detection ranged from 0.2 ppt for different benzofluoranthene isomers to 200 ppt for acenaphthene. The method was sensitive enough to detect trace amounts of PAHs in various samples subjected to analysis in our laboratory.

Application to Medicinal White Mineral Oils

Six white mineral oils were analyzed by the described modified method. Results are reported in Table 4. Twenty-seven PAHs were identified, and 11 suspected carcinogenic PAHs were quantified. No correlation between individual PAH concentrations was observed. Benzo(a)pyrene, in-

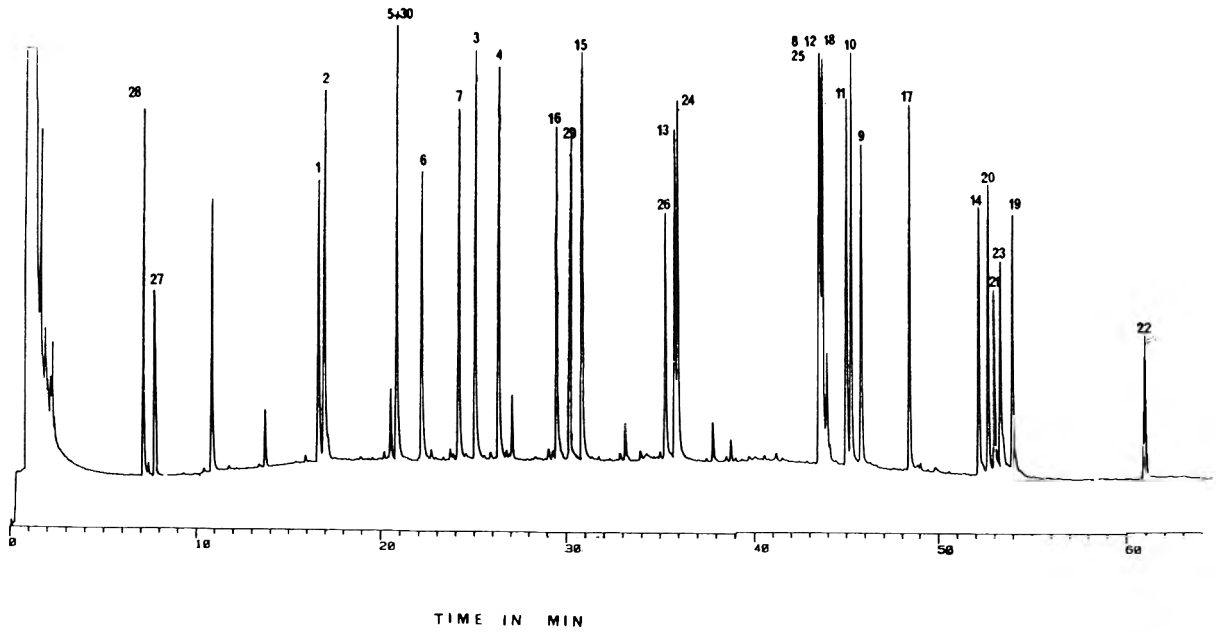

Figure 5. Gas chromatogram of 30 PAHs on CP SIL 5 CB capillary column (see details in Table 1).

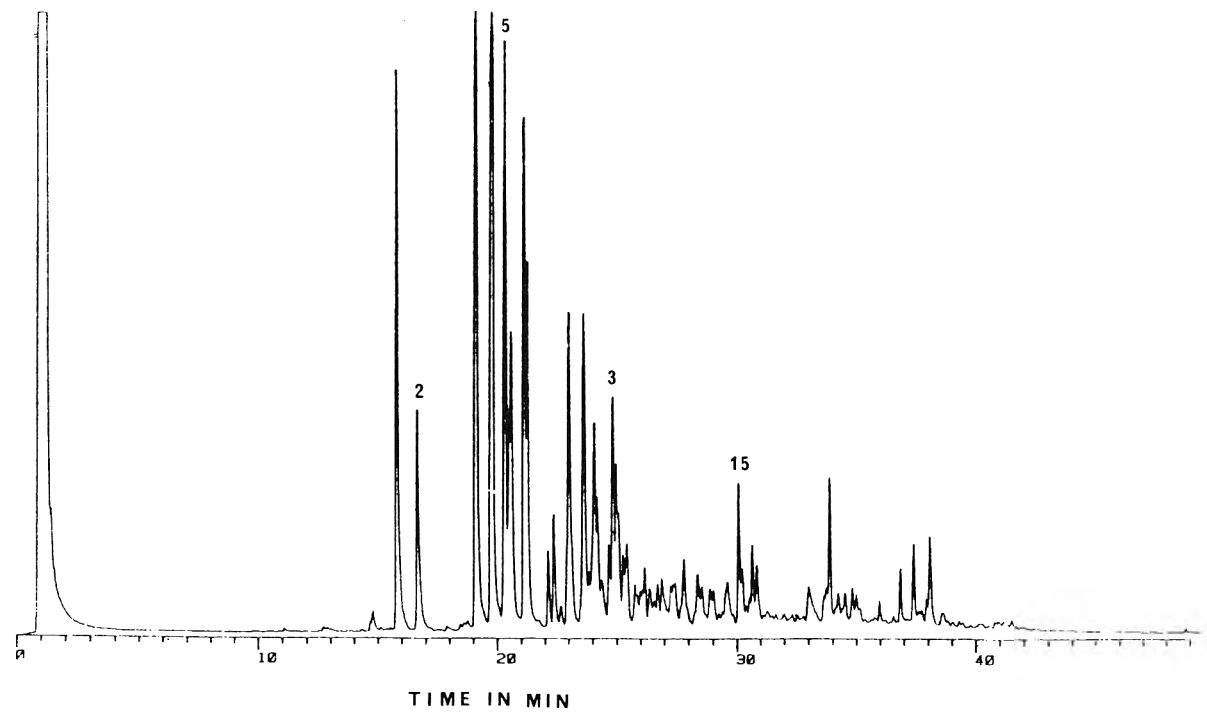

Figure 6. Gas chromatogram of extracted white mineral oil sample (see details in Table 1).

Table 3. Recoveries of 29 PAHs from 10 replicate analyses[a]

Analyte	Rec., %	RSD, %
Phenanthrene + anthracene	72.8	7.9
Fluoranthene + cyclopenta(d)pyrene	102.0	6.8
Pyrene	93.1	6.7
Methyl-2-anthracene	101.0	5.9
Methyl-9-anthracene	100.5	6.1
Dimethyl-3,6-phenanthrene + chrysene	90.8	5.9
Benzo(k)fluoranthene	69.0	4.3
Perylene + benzo(e)pyrene + benzo(b)fluoranthene	78.0	5.1
Benzo(a)pyrene	49.1	4.0
Benzo(a)anthracene	39.7	3.9
Dibenzo(a,h)anthracene	68.0	3.4
Methyl-3-pyrene	87.2	4.4
Benzo(a)fluorene	93.1	2.8
Methyl-3-cholanthrene	103.0	4.9
Dimethyl-7,12-benzo(a)anthracene	96.2	3.9
Anthranthene	98.4	3.7
Indeno(1,2,3,cd)pyrene + benzo(g,h,i)perylene	54.0	4.4
Coronene	99.1	2.1
Benzo(j)fluoranthene	99.7	1.8
Acenaphthene	91.6	2.6
Acenapthylene	93.1	2.8

[a] Mineral oil sample (10 g) was spiked at 7 ppm: 650 μg total PAHs + 50 μg internal standard benzo(b)chrysene.

deno(1,2,3,cd)pyrene, benzo(g,h,i)perylene, perylene, benzo(e)pyrene, benzo(k)fluoranthene, and pyrene were present in all samples.

Acknowledgment

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Table 4. PAH residues in 6 white mineral oil samples

Analyte	Analyte concn in mineral oil sample, μg/kg					
	1	2	3	4	5	6
Phenanthrene + anthracene	4400	38.4	—	—	1064	128
Fluoranthene + cyclopenta(cd)pyrene[a]	240	22500	—	—	87100	166610
Methyl-9-anthracene	—	29.6	56	233	602	80
Pyrene	726	45.5	267	333	255	80
Methyl-2-anthracene	—	17.3	—	161	141	38
Benzo(a)fluorene	854	40.8	—	161	135	31
Dimethyl-3,6-phenanthrene + chrysene + benzo(a)anthracene[a]	—	30.2	280	912	3143	716
Methyl-3-pyrene	—	49	659	275	129	59
Benzo(j)fluoranthene[a]	—	14.5	9	55	65	38
Perylene + benzo(e)pyrene[a] + benzo(b)fluoranthene[a]	1120	54.7	33	155	398	438
Benzo(k)fluoranthene[a]	4.5	0.08	—	—	1.8	2.9
Dimethyl-7,12-benzo(a)anthracene[a]	—	2.04	13	10.2	5.1	—
Benzo(a)pyrene[a]	142	4.3	58	22.5	5.5	9.6
Dibenzo(a,h)anthracene[a]	90	2.04	—	—	—	18
Indeno(1,2,3,cd)pyrene[a] + benzo(g,h,i)perylene	14	0.82	22	6.1	4.9	18
Methyl-3-cholanthrene[a]	40	0.41	—	—	1.2	3.5
Anthranthene	3	0.08	—	—	0.2	2.5
Coronene	76	0.82	—	—	0.4	9.6
Acenaphthene	—	—	—	—	—	151
Acenaphthylene	—	—	287	—	—	—

[a] Suspected carcinogen.

Analysis of Organochlorine Pesticide Residues Using Simultaneous Injection of Two Capillary Columns with Electron Capture and Electrolytic Conductivity Detectors

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A system has been developed that will allow low level screening of 31 organochlorine pesticide residues using simultaneous injection on 2 dissimilar capillary columns. An electron capture detector was attached to a DB-1701 column, and an electrolytic conductivity detector in the halogen mode was attached to a DB-5 column. Chlorinated pesticide amounts ranging from 0.05 ng for γ-BHC to 1.5 ng for decamethrin can easily be quantitated and confirmed. The system can be used in either the column programmed mode or the isothermal column mode. Good reproducibility was obtained for injections in both modes. This system can easily be retrofitted to any gas chromatograph using on column or split/splitless injectors.

The U.S. Food and Drug Administration (FDA) initiated the Total Diet Study (TDS) in May 1961 (1). TDS continually monitors levels of various selected nutrients, toxic elements, industrial chemicals, and pesticide residues in table ready foods. This information is used to reconstruct the daily intake of these substances for specific age/sex groups. The study provides baseline information that is used to identify trends in the food supply and in diets over time. This information assists FDA in identifying potential public health problems.

The food items of TDS are most often examined by using one or more multiresidue methods. These methods were developed for this purpose over the years and are described in the FDA Pesticide Analytical Manual, Volume I (PAM I) (2), which is compiled and issued by FDA. All fat samples (>2% fat) are extracted by PAM I, sec. 211, which involves mixed ether extractions and cleanup with gel permeation chromatography (3). The nonfat samples (<2% fat) are extracted with acetonitrile according to PAM I, sec. **212**. A final Florisil cleanup (PAM I, sec. **252**) is used with all fat and nonfat samples before the confirmation and quantitation steps.

TDS screens 234 food items at low ppb levels for organochlorine industrial chemicals and pesticide residues. This screening level can easily be achieved with ^{63}Ni-electron capture detection (ECD) by injecting 100 mg sample equivalents into columns with different polar phases. Confirmation and quantitation of chlorinated residues at these low levels are also possible with electrolytic conductivity detection (ELCD) in the halogen mode. ELCD is specific to compounds containing halogen elements and does not respond to most nonhalogenated compounds. ECD responds to chlorinated compounds and many nonhalogenated compounds present in complex sample matrixes. Thus, each detection method responds to chlorinated compounds, but each offers slightly different specificity. Confirmation and quantitation can then be obtained using different columns and detectors.

Dual-channel capillary gas chromatography (GC) systems that use different column phases and detectors have been developed (4–6). These systems inject 2 capillary columns simultaneously, using 1 injection port. The 2 columns are connected to the split/splitless injection port through a 2-hole ferrule.

This work describes a dual-column capillary GC system that can be used with conventional packed column injection ports. Low level quantitation and confirmation of organochlorine industrial chemicals and pesticide residues are achieved on this system by using a column programmed run after making a simultaneous flash on-column injection (7) into a retention gap connected to a glass "Y" splitter. The splitter is connected to 2 dissimilar phase capillary columns, with an electron capture detector attached to one column and an electrolytic conductivity detector with a special cell attached to the other.

The work includes statistical results on injection reproducibility using a column programmed run and an isothermal run. Incurred residue results obtained for samples analyzed with the dual-column capillary programmed run and the current individual column isothermal method will be presented for comparison.

Experimental

Principle

Quantitation and confirmation of organochlorine industrial chemicals and pesticide residues in samples are achieved using the PAM I extraction and cleanup procedures plus injection into a dual-channel GC system as previously described. The gas chromatograph initiates the 2-step linear program, the autosampler, the electrolytic conductivity detector vent, and the integrators attached to each detector. Quantitation and confirmation of each peak are determined after comparison against the retention times and areas of external standards injected on the system.

Apparatus and Reagents

(a) *Dual-column capillary system.*—Shown in Figure 1. 5880A gas chromatograph equipped with ^{63}Ni-electron capture detector (Hewlett-Packard Co., Palo Alto, CA 94303); Model 4420 electrolytic conductivity detector in the halogen mode with special cell containing a 0.127 mm (0.005 in.) solvent orifice (O.I. Analytical, College Station, TX 77841); Model 7672A autosampler (Hewlett-Packard); Model SP 4270 integrator (Spectra-Physics, Inc., San Jose, CA 93412); Model HP 5880A gas chromatograph Terminal 1 and a packed column injector (Hewlett-Packard). Integrator and terminal were operated at 1 cm/min chart speed with an attenuation of 2 for the integrator and 7 for the terminal.

The injection port is modified by installing a precut on-column liner (6.25 mm or 1/4 in. port, 210–1066, new version, 210–1070) with an installation Kit C (J&W Scientific, Inc., Folsom, CA 95630). A plug of presilanized glass wool (2–0409) is inserted in the on-column liner just above

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Mention of trade names and suppliers is for information only and does not imply endorsement by the Food and Drug Administration.

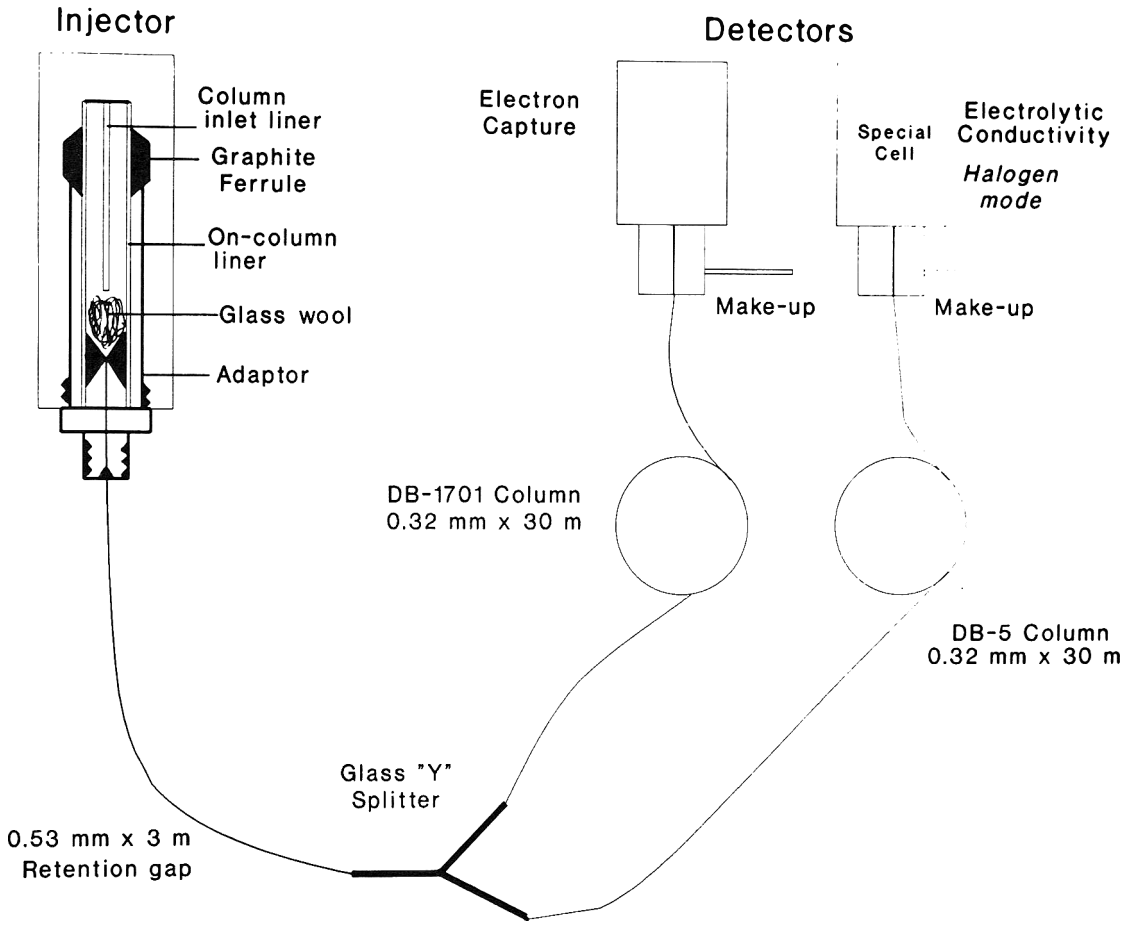

Figure 1. Components used in the dual-column capillary system.

the restricted end and a precut column inlet liner (2–0540, Supelco, Inc., Bellefonte, PA 16823) is inserted at the top. On-column liner is installed in the injector with a special adaptor (220–1120, J&W Scientific, Inc.) and a double beveled graphite ferrule. The restricted end of the on-column liner is installed so it will be at the bottom of the injection port. All components are contained within the injector, which is operated at 230°C.

One end of the retention gap (3 m × 0.53 mm id, presilanized, uncoated fused silica, 1602535, J&W Scientific, Inc.) is inserted into the on-column liner restriction through the bottom of the special adaptor and held in place with a graphite ferrule and nut. The other end of the retention gap is connected to the common leg of a quick-seal glass "Y" splitter (4797, Chrompack B.V., Delft, The Netherlands). One leg of the splitter is connected to a 30 m × 0.32 mm id, DB-1701 bonded phase fused silica capillary column (123-0732, J&W Scientific, Inc.) with 0.25 μm film thickness. The other leg of the splitter is connected to a 30 m × 0.32 mm id, DB-5 bonded phase fused silica capillary column (123-5032, J&W Scientific, Inc.) with 0.25 μm film thickness.

The DB-1701 column, with a flow rate of 1.6 mL/min hydrogen (ca 39 cm/s) is connected to the electron capture detector with a make-up gas fitting (103462, Scientific Glass Engineering, Austin, TX). The electron capture detector uses 5% methane in argon as the make-up gas, with a flow rate of 72 mL/min at 350°C. The DB-5 column, with a flow rate of 1.5 mL/min hydrogen (ca 41 cm/s), is connected to the electrolytic conductivity detector using the above make-up gas fitting and 27 mL/min of hydrogen make-up gas. The electrolytic conductivity detector is set with a base temperature of 270°C, a furnace reaction temperature of 950°C, and a hydrogen reaction gas flow rate of 48 mL/min. All hydrogen supplied to the HP 5880A gas chromatograph and electrolytic conductivity detector is first conditioned through a hydrogen purifier (Model 560, AADCO, Clearwater, FL 34625). Cell flow rate of the electrolytic conductivity detector is 6 μL/min *n*-propyl alcohol. This flow rate is achieved by using a 390 × 0.8 mm id Teflon bypass solvent tube with the pumping system. Attenuation of the electrolytic conductivity detector is set at "H" × 1, with the signal output cable connected to the 1 V terminal, which is in turn connected to the SP 4270 integrator signal input. The vent is set full clockwise, which provides ca 2 min of venting.

Keyboard terminals of the "Inj A" button on the SP 4270 were interconnected to the external events valve 7 connectors on the HP 5880A gas chromatograph with wires a and b. Wires were soldered to the keyboard terminals. The Model 4420 electrolytic conductivity detector vent was interconnected to the external events valve 8 of the HP 5880A gas chromatograph with wires c and d. The connection involved unsoldering one end of resistor 33 of the vent circuitry and removing that end from the printed circuit board. Wire c was soldered to that end of the resistor. Then, wire d was soldered

to the printed circuit board where the resistor was unsoldered. Valves 7 and 8 on the HP 5880A gas chromatograph signify connections for computer controlled switch closures.

A run table is programmed to operate the integrator (valve 7), the vent (valve 8), and run stop. The integrator is turned on at 0.00 min, when valve 7 is turned on, thus closing the switch contacts. The integrator is turned off at 0.05 min, when the contacts are opened. The vent is turned on at 0.07 min, when valve 8 is turned off, thus opening the switch contacts, and turned on at 0.10 min, when the valve 8 switch contacts are closed. An integrator report for the electrolytic conductivity detector is generated by turning valve 7 on at 44.00 min and off at 44.10 min. The stop-run command, at 50.00 min, generates an ECD report, stops the current programmed run, and initiates the starting of the next programmed run.

The HP 7672A autosampler parameters were set for a 4.8 μL injection from a 10 μL syringe, 5 prewashes, 5 filling pumps, and 5 postwashes. The first and last sample vial and injections per vial were set for each series of samples or standards.

(b) *Fatty sample extractants.*—Extractants were cleaned-up on an Auto-Prep 1002B gel permeation chromatograph. The gel permeation chromatograph is equipped with a 60 × 2.5 cm id column (Analytical Bio-Chemistry Laboratories, Columbia, MO 65205), slurry packed with 33 g Bio-Beads SX-3 resin (200–400 mesh, Bio-Rad Laboratories, Richmond, CA), and compressed to a bed length of ca 20 cm. The eluting solvent was methylene chloride–hexane (1 + 1, v/v) pumped at a flow rate of 5.0 mL/min with an operating pressure range of 8 to 11 psig. The gel permeation chromatograph was set up with a 12 min dump, 16 min collect, and 0 min wash cycle.

(c) *Pesticide standards.*—Prepared from 1 mg/mL stock solutions of 10% acetone in isooctane. All standards were obtained from the Pesticide and Industrial Chemicals Repository, Environmental Protection Agency, Research Triangle Park, NC 27711.

(d) *Solvents.*—USP 95% ethanol and pesticide grade methylene chloride, *n*-hexane, diethyl ether, petroleum ether, acetonitrile, acetone, and isooctane were used in the procedures.

(e) *Sample preparation.*—Fatty and nonfatty food items used in this study were obtained from TDS. Each item was initially prepared according to package instructions and common recipes found in American households (8). A few of the recipe items prepared for TDS include homemade beef and vegetable stew, lasagna, and meat loaf. Large quantities of each prepared item were ground or blended into a composite. Each composite represents items purchased from 3 different locations within a specific region of the United States. The United States is divided into 7 regions by FDA, which are systematically sampled.

Programmed Run

A programmed run was developed that best resolved 31 chlorinated pesticides routinely encountered in TDS. A 2-step linear temperature program was found to be the most efficient for separating chlorinated pesticides on both columns. Each GC run is cycled through a 2-step linear program beginning at 110°C for 1 min, ramped to 190°C at 15°C/min, held for 0 min, then ramped to 270°C at 3°C/min, and held at this temperature until the 50 min stop-run command is initiated. The programmed run gave optimum separation on the DB-1701 column without coelution of pesticides. Less efficient separation was achieved on the DB-5 column, where 10 analytes coeluted. The DB-5 column did not separate dicloran and quintozene, DCPA and *p,p*-dicofol, octachlor epoxide and heptachlor epoxide, endosulfan I and *cis*-chlordane, and endosulfan sulfate and *p,p'*-DDT. Confirmation may not be established in instances where these analyte pairs are present. The Florisil cleanup procedure (PAM I, sec. 252) used in the sample analysis helps separate pesticides into different eluates eliminating several coelution possibilities. All coeluting pesticides above, except for DCPA and p,p-dicofol, can be separated with the Florisil cleanup procedure into fractions A and B. The Florisil procedure splits the pesticides into fractions A, B, and C. The concentration of each chlorinated pesticide standard, retention times of each pesticide standard for each column, and numerical elution order of standards on the DB-1701 column are presented in Table 1.

A study was conducted to determine the reproducibility of peak areas and retention times for the 31 organochlorine pesticides using the 2-column system with a 2-step linear program. The autosampler was used to make five 4.8 μL sequential injections of 2 mixed standards. The mixed standards gave complete separation for each pesticide and eliminated any coelution of pesticides. Reproducibility of the standards is presented in Tables 2 and 3.

Isothermal Run

A study was also conducted to determine reproducibility of the peak areas and retention times for 8 organochlorine pesticides using the 2-column system in the isothermal mode, where combined column flow rates are ≥10 mL/min and the column oven temperature is held at 200°C. All other GC conditions were held constant while the autosampler was used to make five 4.8 μL sequential injections of a mixed standard at 3 different concentrations. Compounds were selected because their elution profile is a good indicator of how other organochlorine compounds would behave on this system. Reproducibility of the standard injection is presented in Table 4.

Analysis Comparison for Incurred Residues

Eluates from samples analyzed in TDS containing incurred organochlorine pesticide residues were reanalyzed on the programmed 2-column simultaneous injection system. Quantitation and confirmation were determined using external standards injected on the system. Results for incurred residues found in 17 TDS samples are presented in Tables 5–8.

Discussion

This dual-capillary system is installed on an HP 5880A gas chromatograph but can easily be retrofitted to almost any other gas chromatograph. The HP 5880A packed column injector requires Kit C, while the majority of the other packed column injectors on the market require Kit A. The adaptor that holds the precut on-column liner is the only difference in the 2 kits. Routine maintenance of this injection system is simplified by the ease with which the on-column liner can be

Table 1. Elution order of pesticides

Compound	Peak No.	Concn, µg/mL	Retention time, min/col DB–1701	DB–5
Pentachlorobenzene	1	0.002	6.93	10.42
Tecnazene	2	0.003	7.91	11.73
HCB	3	0.003	8.47	13.38
Pentachloroanisole	4	0.002	8.72	13.52
α-BHC	5	0.006	9.21	12.74
Quintozene (PCNB)	6	0.004	9.63	14.29
γ-BHC	7	0.010	10.21	13.72
Heptachlor	8	0.005	10.70	16.16
Dicloran	9	0.010	11.21	14.14
Pentachloroaniline	10	0.005	11.48	15.28
Pentachlorothioanisole	11	0.004	11.65	17.09
Chlorpyrifos	12	0.030	12.62	17.49
DCPA (dacthal)	13	0.005	13.02	17.84
Octachlor epoxide	14	0.050	13.17	19.18
p,p–Dicofol	15	0.010	13.41	17.74
Heptachlor epoxide	16	0.020	13.67	19.06
Endosulfan I	17	0.010	14.66	21.12
trans-Chlordane	18	0.010	14.80	20.05
cis-Chlordane	19	0.020	15.10	20.70
trans-Nonachlor	20	0.010	15.20	20.90
p,p'–DDE	21	0.015	15.52	21.76
Dieldrin	22	0.030	16.02	22.00
Endrin	23	0.030	16.82	22.76
p,p'–TDE	24	0.020	18.80	23.96
Endosulfan II	25	0.020	18.95	23.67
p,p'–DDT	26	0.030	19.53	25.20
Endosulfan sulfate	27	0.020	22.29	25.64
p,p'–Methoxychlor	28	0.100	22.67	28.05
cis-Permethrin	29	0.200	26.64	32.68
Cypermethrin	30	0.200	30.98	35.92
Deltamethrin	31	0.300	36.73	43.06

Table 2. Relative standard deviation (%) of area and retention times for 5 injections of mixed standard using 2-step linear program

Pesticide	Simultaneous injection[a] ECD DB–1701 col. Area	Retention time	ELCD (halogen mode) DB–5 col. Area	Retention time
HCB	3.5	0.08	8.9	0.20
α-BHC	1.0	0.08	5.4	0.19
γ-BHC	0.9	0.10	4.9	0.21
Chlorpyrifos	0.6	0.09	7.4	0.16
Heptachlor	0.4	0.11	5.6	0.21
p,p–Dicofol	7.2	0.07	27	0.16
Heptachlor epoxide	0.7	0.08	6.2	0.18
trans-Chlordane	0.7	0.08	7.6	0.17
cis-Chlordane	0.7	0.10	3.0	0.16
trans-Nonachlor	0.8	0.07	6.1	0.17
Dieldrin	1.0	0.10	6.1	0.17
Endrin	2.2	0.08	9.4	0.18
p,p'–DDT	2.0	0.08	10	0.15
p,p'–Methoxychlor	2.6	0.06	3.1	0.14
cis–Permethrin	3.1	0.06	6.0	0.10
Cypermethrin	5.4	0.04	3.6	0.09
Decamethrin	6.7	0.07	7.1	0.11

[a] 4.8 µL injectec with autosampler.

Table 3. Relative standard deviation (%) of area and retention times for 5 injections of mixed standard 2 using 2-step linear program

Pesticide	Simultaneous injection[a] ECD DB–1701 col. Area	Retention time	ELCD (halogen mode) DB–5 col. Area	Retention time
Pentachlorobenzene	9.4	0.06	1.5	0.12
Tecnazene	2.1	0.00	2.2	0.16
Pentachloroanisole	1.8	0.07	7.0	0.14
Quintozene (PCNB)	1.1	0.05	2.7	0.12
Dicloran[b]	—	—	—	—
Pentachloroaniline	2.0	0.04	3.0	0.14
Pentachlorothioanisole	2.0	0.04	3.0	0.12
DCPA (dacthal)	1.6	0.06	1.9	0.14
Octachlor epoxide	4.1	0.06	5.3	0.12
Endosulfan I	1.8	0.07	11	0.14
p,p'–DDE	2.1	0.07	6.3	0.15
p,p'–TDE	3.4	0.08	6.2	0.10
Endosulfan II	1.1	0.08	6.6	0.11
Endosulfan sulfate	4.0	0.06	14	0.12

[a] 4.8 µL injected with autosampler.
[b] Not determined because of low response at 0.01 µg/mL

disassembled and cleaned. While disassembled, the precut column inlet liner and glasswool contained in the on-column liner can be replaced, thus removing septum material and sample residues.

Extended use of the injection system requires replacement of the retention gap, which is accomplished by grasping the common leg of the glass "Y" splitter with one hand and the retention gap with the other hand. Exert force in the opposite direction while holding the polyimide seal (dark ring) of the compression fit over a small flame and separation will occur upon heating. The glass "Y" splitter can then be reused with a new piece of retention gap. Sometimes, small pieces of the polyimide are left in the constriction. These can be removed with a fine wire or small needle. The same technique can be applied to changing columns.

With the flash, on-column, simultaneous injection system, large injections can be used in conjunction with a programmed mode or an isothermal mode without changing any injector components. Large injections can be used with this injection system because of the combined internal volume of the on-column liner and the 0.53 mm id retention gap. This internal volume accommodates solvent expansion, which allows simultaneous injections to be made into the 0.32 mm id fused silica capillary columns. Maximum resolution and sensitivity is obtained in the programmed mode because the capillary columns generate the most theoretical plates in this mode. Also, a wide range of compounds can be analyzed at one time in the programmed mode.

The simultaneous injection system (Figure 1) is reproducible for programmed runs of 4.8 µL injections of

Table 4. Relative standard deviation (%) of area and retention times for 5 injections of mixed standard using isothermal run[a]

		Simultaneous injection[b]			
		ECD		ELCD (halogen mode)	
		DB–1701 col.[c]		DB–5 col.[c]	
Pesticide	Standard concn, µg/mL	Area	Retention time	Area	Retention time
	1 × dilution				
α-BHC	0.060	1.0	0.28	2.6	0.40
γ-BHC	0.100	0.8	0.14	2.8	0.20
Heptachlor	0.100	1.0	0.13	2.8	0.00
Chlorpyrifos	0.300	0.8	0.05	3.2	0.00
Heptachlor epoxide	0.200	1.1	0.14	3.9	0.08
cis-Chlordane	0.200	0.8	0.06	4.2	0.08
Dieldrin	0.300	2.3	0.08	4.1	0.07
Endrin	0.300	0.2	0.08	5.4	0.06
	1/5 × dilution				
α-BHC	0.012	3.4	—	11	—
γ-BHC	0.020	1.0	—	5.4	—
Heptachlor	0.020	1.6	—	2.3	—
Chlorpyrifos	0.060	0.8	—	3.0	—
Heptachlor epoxide	0.040	2.7	—	3.3	—
cis-Chlordane	0.040	0.7	—	2.2	—
Dieldrin	0.060	2.3	—	2.5	—
Endrin	0.060	0.4	—	2.2	—
	1/10 × dilution				
α-BHC	0.006	7.6	—	1.7	—
γ-BHC	0.010	0.9	—	2.9	—
Heptachlor	0.010	1.6	—	3.2	—
Chlorpyrifos	0.030	1.0	—	2.7	—
Heptachlor epoxide	0.020	2.6	—	3.1	—
cis-Chlordane	0.020	0.8	—	3.3	—
Dieldrin	0.030	6.4	—	3.8	—
Endrin	0.030	1.0	—	3.7	—

[a] Column oven temperature = 200°C.
[b] 4.8 µL injected with autosampler.
[c] Combined individual flow rates from both columns = 10 mL/min.

organochlorine pesticides. Relative standard deviation (RSD) of the peak areas for ECD in Tables 2 and 3 are very good for low level amounts of pesticides injected per column. RSD for peak areas of ELCD in the halogen mode is not as good as ECD, but is still very acceptable for low level confirmation. The large variation of RSD of the area for *p,p*-dicofol can be attributed to poor peak shape. Chromatograms obtained for a programmed run of mixed standards from ECD and ELCD with the special cell are shown in Figures 2 and 3. Peak numbers in Table 1 correspond to peak numbers in Figures 2 and 3.

The simultaneous injection system can be used in the more conventional isothermal mode, which will give good resolution and sensitivity for a less complex analysis. The isothermal mode is achieved in the injection system by increasing the combined column flow rates to 10 mL/min or greater, and adjusting the oven temperature to 200°C. Results in Table 4 for five 4.8 µL sequential injections of a mixed standard demonstrated that the isothermal mode can be used for the quantitation and confirmation of organochlorine pesticides. RSD for retention times and peak areas for 3 different concentrations of 8 pesticides are good. The high RSD for dieldrin was caused by an interfering peak. Results also show that ELCD with the special cell performed well at the 3 standard concentrations that cover the range needed for ppm and sub-ppm pesticide analysis. The ELCD special cell was found to be approximately 1 to 1 1/2 times more sensitive than the regular production cell. The only difference between the ELCD special cell and the regular production cell is the size of the solvent orifice. The ELCD special cell has a 0.127 mm (0.005 in.) solvent orifice, as compared to a 0.254 mm (0.010 in.) solvent orifice for the production cell.

TDS routinely analyzes food items for organochlorine pesticides in the ppm and sub-ppm range, according to the

Table 5. Analysis comparison for incurred residues (ppm)[a] using 2 different columns

		Isothermal individual injections		Programmed simultaneous injection	
			Confirmation		Confirmation
Item	Pesticide residue[b]	EC	EC or ELCD	EC	ELCD
Milk	*p,p'*–DDE	0.0020	0.0020	0.0014	0.0014
Low fat milk	p,p'–DDE	0.0009	0.0009	0.0008	0.0009
Chocolate shake	p,p'–DDE	0.0040	0.0050	0.0030	0.0030
Evaporated milk	p,p'–DDE	0.0020	0.0020	0.0020	0.0020
Cheddar cheese	p,p'–DDE	0.0310	Conf.[c]	0.0180	0.0220
	HCB	0.0020	0.001	0.0012	0.0007
Lamb chop	HCB	0.0010	Conf.	0.0008	0.0013
	Octachlor epoxide	0.0004	Conf.	0.0004	0.0004
	p,p'–DDE	0.0180	Conf.	0.0190	0.0260
	Dieldrin	0.0005	Conf.	0.0010	0.0008
Breaded, fried shrimp	Pentachloroanisole	0.0001	0.0002	0.0001	0.0001
	α-BHC	0.0003	Conf.	0.0003	0.0005
	Chlorpyrifos methyl	0.0010	Conf.	0.0008	0.0010
	p,p'–DDE	0.0010	Conf.	0.0006	0.0010
Cooked fish sticks	Chlorpyrifos methyl	0.0040	Conf.	0.0030	0.0040

[a] Single determination.
[b] All residues were quantitated against external standards.
[c] Confirmed on a different column but not quantitated.

procedures contained in PAM I. Preanalyzed sample eluates from food items in TDS were reanalyzed with the simultaneous injection system. Results in Tables 5–8 for 17 food items show, in most cases, good agreement between the original TDS individual column analysis and the reanalysis using the simultaneous injection system. Results of the simultaneous injection system with ECD and ELCD with the special cell also showed good agreement in most cases. Results for the simultaneous injection system were determined by comparing area counts of sample peaks to area counts of external standards injected in the system. Some inconsistencies in sample results can be attributed to the level of quantitation and to sample matrix effects that are also observed in packed column chromatography. Variabilities from approximately

Table 6. Analysis comparison for incurred residues (ppm)[a] using 2 different columns

		Isothermal individual injections		Programmed simultaneous injection	
			Confirmation		Confirmation
Item	Pesticide residue[b]	EC	EC or ELCD	EC	ELCD
Peanut butter	Pentachlorobenzene	0.0007	Conf.[c]	0.0007	0.0006
	HCB	0.0007	Conf.	0.0005	0.0005
	Pentachloroanisole	0.0009	Conf.	0.0006	0.0004
	Quintozene (PCNB)	0.0004	Conf.	0.0003	0.0003
	Pentachloroaniline	0.0010	Conf.	0.0017	0.0020
	Pentachlorothioanisole	0.0004	Conf.	0.0006	0.0005
	p,p'–DDE	0.0010	Conf.	0.0039	0.0060
	Dieldrin	0.0010	Conf.	0.0030	0.0030
	Chlorpyrifos	0.0060	Conf.	0.0060	0.0040
	Heptachlor epoxide	0.0003	Conf.	0.0006	0.0006
Peanuts	Pentachlorobenzene	0.0030	Conf.	0.0020	0.0020
	HCB	0.0007	Conf.	0.0008	0.0020
	Pentachloroanisole	0.0020	Conf.	0.0010	0.0003
	Quintozene (PCNB)	0.0006	Conf.	0.0003	0.0002
	Pentachloroaniline	0.0050	Conf.	0.0030	0.0040
	Pentachlorothioanisole	0.0010	Conf.	0.0010	0.0010
	p,p'–DDE	0.0010	0.0020	0.0030	0.0020
	Chlorpyrifos	0.0040	Conf.	0.0030	0.0020

[a] Single determination.
[b] All residues were quantitated against external standards.
[c] Confirmed on a different column but not quantitated.

Table 7. Analysis comparison for incurred residues (ppm)[a] using 2 different columns

		Isothermal individual injections		Programmed simultaneous injection	
			Confirmation		Confirmation
Item	Pesticide residue[b]	EC	EC or ELCD	EC	ELCD
Boiled spinach	*p,p'*–DDE	0.0150	Conf.[c]	0.0130	0.0200
	Dieldrin	0.0030	Conf.	0.0086	0.0060
	cis-Permethrin	0.0110	Conf.	0.0210	0.0220
	trans-Permethrin	0.0120	Conf.	0.0093	0.0096
Collards	DCPA (dacthal)	0.0540	Conf.	0.0310	0.0850
	Pentachloroaniline	0.0003	Conf.	0.0002	0.0006
	p,p'–DDE	0.0100	Conf.	0.0140	0.0360
	Dieldrin	0.0009	Conf.	0.0009	0.0010
	cis-Permethrin	0.0110	Conf.	0.0110	0.0140
Raw celery	Dicloran	0.0280	Conf.	0.0257	0.0400
	p,p'–DDE	0.0060	Conf.	0.0014	0.0020
	Endosulfan I	0.0060	Conf.	0.0020	0.0026
	Endosulfan II	0.0040	Conf.	0.0075	0.0040
	Endosulfan sulfate	0.0060	Conf.	0.0070	0.0080
Cauliflower	DCPA (dacthal)	0.0003	Conf.	0.0004	0.0004
	Dieldrin	0.0002	Conf.	0.0004	0.0003

[a] Single determination.
[b] All residues were quantitated against external standards.
[c] Confirmed on a different column but not quantitated.

30–45% are not uncommon for sample analyses in the low ppb level (9). The higher level of dicloran found in raw celery is easily quantitated and confirmed.

Conclusion

The simultaneous injection system with programmed run has shown that it is a viable system for quantitating and confirming ppm and sub-ppm pesticide residues in samples prepared using PAM I procedures. Simultaneous injection into different column phases and detectors reduces the possibility of false identifications caused by matrix interferences. Each column, in most cases, gives a different elution time for the same compound. ELCD is essential in a dual-column, dual-detector system because it is much less sensitive to matrix interferences than ECD; therefore, it further reduces the chance of false identifications. This system allows the laboratory to quantitate and confirm a wide range of organochlorine pesticide residues with one injection without significantly increasing the time and cost of analysis. Further time savings can be achieved by automating the simultaneous injection system.

Table 8. Analysis comparison for incurred residues (ppm)[a] using 2 different columns

		Isothermal individual injections		Programmed simultaneous injection	
			Confirmation		Confirmation
Item	Pesticide residue[b]	EC	EC or ELCD	EC	ELCD
Raw tomato	Endosulfan I	0.0020	Conf.[c]	0.0027	0.0029
	Endosulfan II	0.0070	Conf.	0.0090	0.0110
	Endosulfan sulfate	0.0080	Conf.	0.0098	0.0130
Tomato sauce	Endosulfan I	0.0020	Conf.	0.0009	0.0011
Canned spinach	*p,p'*–DDE	0.0120	Conf.	0.0100	0.0130
	cis-Permethrin	0.8670	Conf.	0.6000	0.9150

[a] Single determination.
[b] All residues were quantitated against external standards.
[c] Confirmed on a different column but not quantitated.

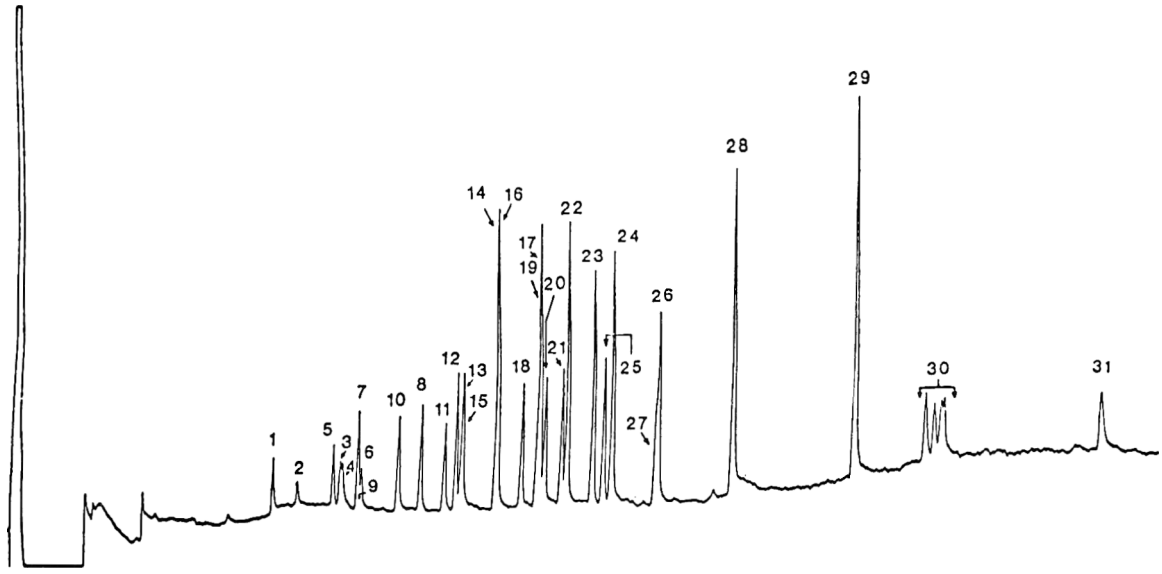

Figure 2. ELCD (halogen mode) chromatogram of 31 chlorinated pesticides on DB-5 column.

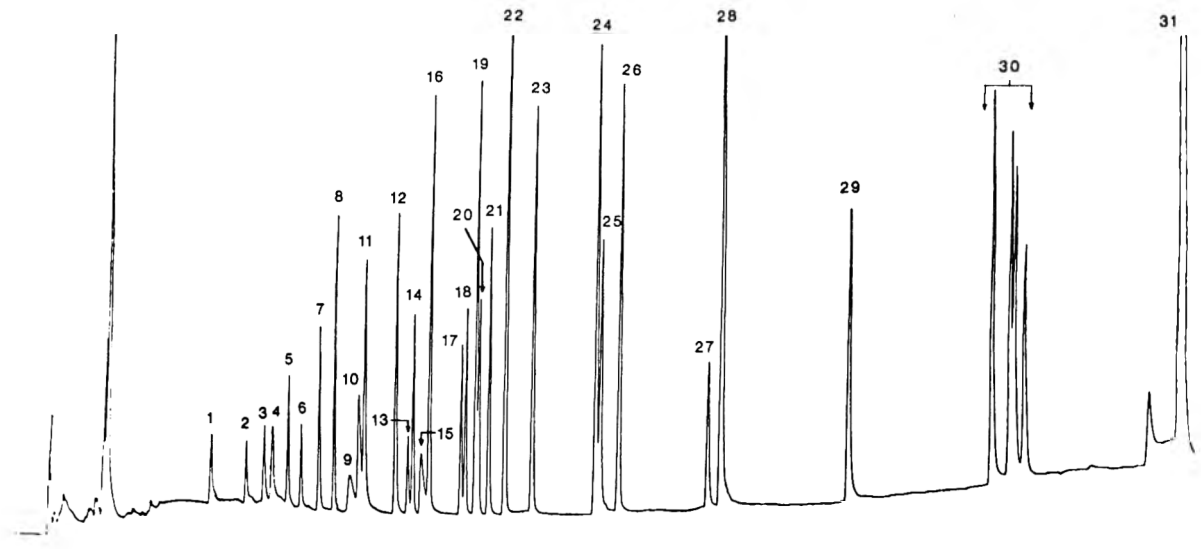

Figure 3. ECD chromatogram of 31 chlorinated pesticides on DB-1701 column.

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Multipesticide Determination in Surface Water by Gas Chromatography/Chemical Ionization/Mass Spectrometry/Ion Trap Detection

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An improved method for the determination of trace levels of pesticides in surface water has been developed and was used to analyze 20 target pesticides in New Jersey. Pesticides were extracted from 2 L water samples, using a mixture of XAD-2 and XAD-7 resins, and were determined by gas chromatography/chemical ionization mass spectrometry with ion trap detection. Average recoveries (performed in triplicate at the 1 ppb level, except for captan and chlorothalonil at 5 ppb) were between 75 and 113%, with an average coefficient of variation of 9%. Most of the pesticides (alachlor, atrazine, butylate, carbofuran, chlorpyrifos, diazinon, fonofos, isofenphos, metolachlor, metribuzin, parathion, and simazine) had limits of detection (LODs) of 0.005 ppb or lower, while some (carbaryl, cyanazine, fenamiphos, linuron, pendimethalin, and terbufos) had LODs between 0.005 and 0.05 ppb. Captan and chlorothalonil had LODs of 1 ppb. Of 31 samples analyzed, 29 contained one or more of the following pesticides: alachlor, atrazine, carbaryl, chlorpyrifos, cyanazine, diazinon, isofenphos, linuron, metolachlor, and simazine in concentrations between trace (<0.025 ppb) and 5.48 ppb.

Most methods for the analysis of pesticides in water, such as EPA method 508 for organochlorine pesticides (1), use liquid/liquid extraction. Samples are extracted with methylene chloride, concentrated, and then analyzed by gas chromatography (GC) with electron capture detection. Limits of detection (LODs) range from 0.0015 ppb for chlordane to 5 ppb for chlorobenzilate. Another method for organonitrogen and organophosphorus pesticides uses a nitrogen–phosphorus detector and has an estimated LOD between 0.075 ppb for simazine and 5.0 ppb for mevinphos (2). Several pesticides, including linuron, cyanazine, carbofuran, and carbaryl, require liquid chromatography (LC) methods for their determination.

Recently, solid-phase extraction (SPE) was used to extract numerous pesticides from water. These methods are more rapid and economical than liquid–liquid extraction methods (3–7). However, the SPE cartridges allow only a limited amount of sample to be used, and this decreases the sensitivity of the analysis. XAD resins have also been widely used for multiresidue pesticide extraction from water (8–10). These resins can extract a wide variety of pesticides, and, depending on the amount of resin used for the extraction, large sample volumes can be analyzed. To avoid using a variety of element selective detectors for detection and quantitation, relatively inexpensive mass spectrometers, such as the mass selective detector, have been used for multiresidue pesticide analysis (11, 12). Selected ion monitoring (SIM) is required to obtain the desired sensitivity. The disadvantage of this technique is that only relatively few ions can be monitored, thus limiting the number of pesticides that can be determined at one time. Ion trap detection (ITD), on the other hand, has outstanding inherent sensitivity when operated in the full scan mode, and it does not require SIM techniques. Pereira et al. (13) used ion trap detection/mass spectrometry to determine several herbicides in surface and ground waters. Full scan electron ionization spectra were obtained for less than 1 ng of the compounds. Detection limits were in the sub-ppb range. Tandem mass spectrometry (MS/MS) was also used (14), and LODs as low as 0.01 ppb were obtained. Even better sensitivity for screening purposes can be obtained by operating the ITD in the chemical ionization (CI) mode, as we have demonstrated for multiresidue pesticide determination in fruits and vegetables (15, 16).

In this study, pesticides were extracted from surface water using XAD-2 and XAD-7 resins and were analyzed by GC/CIMS with an ion trap detector.

Experimental

Reagents

(a) *Pesticide reference standards.*—(U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.)

(b) *Sodium sulfate, methylene chloride, methanol, and water.*—High purity anhydrous sodium sulfate and LC grade methylene chloride, methanol, and water (Fisher Scientific Co., Pittsburgh, PA 15219).

(c) *Fluorene.*—(Aldrich Chemical Co., Milwaukee, WI 53201.)

(d) *XAD-2 and XAD-7 resins.*—Supelco, Inc., Bellefonte, PA 16823.

Samples

Thirty-one samples were collected from rivers at various points in New Jersey. Samples include 3 from the South Branch Raritan River, 5 from the Main Branch Raritan River, 7 from the Manasquan River, 4 from the Millstone River, 3 from the Matchaponix Brook, and 9 from the Lower Mine Hill River. In addition, "transportation blanks" of double distilled water were transported from the laboratory to 3 of the collection sites and back. They were then analyzed as reagent blanks. No pesticides were found in these blanks.

Instrumentation

(a) *Gas chromatograph.*—Model 3400 (Varian Instrument Group, Sunnyvale, CA 94034) interfaced to an ion trap detector in CI mode (Finnigan MAT, San Jose, CA) and controlled by an IBM PC/AT. The analyses and quantitations were performed with Finnigan ion trap software Version 3.15. A splitless on-column injector held at 50°C was used, and a 2 m × 0.53 mm id deactivated fused silica precolumn

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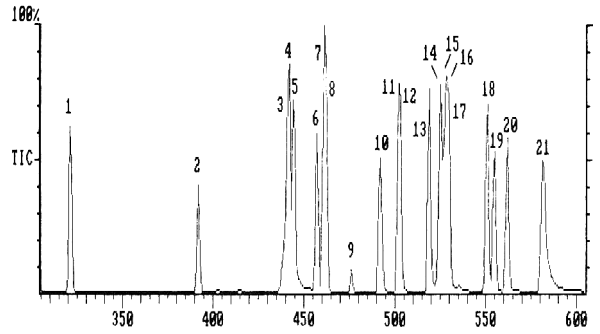

Figure 1. Total ion chromatogram of standard solution containing 10 ng/μL of pesticides determined in surface water, and 20 ng/μL internal standard: butylate (1), fluorene internal standard (2), simazine (3), carbofuran (4), atrazine (5), terbufos (6), diazinon (7), fonofos (8), chlorothalonil (9), metribuzin (10), carbaryl (11), alachlor (12), linuron (13), cyanazine (14), metolachlor (15), chlorpyrifos (16), parathion (17), pendimethalin (18), isofenphos (19) captan (20), and fenamiphos (21). X-axis, scan number; Y-axis, total ion intensity.

was fitted between the injector and capillary column. A 15 m × 0.25 mm id DB-5 fused silica capillary column (1 μm film thickness) (J & W Scientific, Folsom, CA 95630) was temperature programmed from 80 to 260°C at 20°C/min. Carrier gas (He) velocity was 25 cm/s, and the injection volume was 1 μL. A 15 cm syringe needle was used for the on-column injections.

(b) *Mass spectrometer.*—Operated in the CI mode using isobutane reagent gas at a source pressure that gave a 2:1 ratio for m/z 43 to m/z 57. The filament voltage and current were 70 eV and 80 μA, respectively. Electron multiplier gain was 1×10^5. Scan range was 100–400 amu at 1 s/scan. Transfer line and manifold temperatures were 250 and 220°C, respectively.

Table 1. Average recovery (%) of pesticides from surface waters

Pesticide	Rec., %[a]	CV, %[b]	LOD, ppb[c]
Alachlor	105.0	12.4	0.005
Atrazine	83.0	8.6	0.005
Butylate	101.1	11.4	0.005
Captan	74.8	2.8	1.0
Carbaryl	97.9	8.5	0.05
Carbofuran	80.4	4.4	0.005
Chlorothalonil	90.9	10.2	1.0
Chlorpyrifos	86.7	17.4	0.005
Cyanazine	85.0	10.9	0.05
Diazinon	103.8	14.0	0.0005
Fenamiphos	113.0	1.3	0.05
Fonofos	108.6	4.0	0.005
Isofenphos	81.5	8.8	0.005
Linuron	98.7	14.9	0.05
Metolachlor	102.5	14.6	0.005
Metribuzin	91.3	13.9	0.005
Parathion	109.3	3.4	0.005
Pendimethalin	88.9	12.1	0.05
Simazine	81.0	3.7	0.005
Terbufos	84.3	8.6	0.05

[a] At the 1 ppb level, except 5 ppb for captan and chlorothalonil.
[b] Coefficient of variation: each analysis performed in triplicate.
[c] Limit of detection: as detectable in 1.0, 0.05, 0.005, or 0.0005 ppb spikes.

Preparation of Calibration Curves

A 50 μg/mL stock solution was prepared by dissolving 25 mg of each reference standard in 500 mL dichloromethane. Stock solution was serially diluted, and the appropriate amount of internal standard stock solution was added. Standard solutions then contained 0.1, 0.5, 1.0, 5.0, 10.0, and 20.0 ng/μL each pesticide, and 20 ng/μL of the fluorene internal standard. All pesticides could be detected at 0.1 ng on-column except for captan and chlorthalonil (0.5 ng). The standard solutions were analyzed in duplicate at each concentration level. Peak areas were obtained from mass chromatograms of each analyte's quantitation ion. Response factors (area of pesticide/area of internal standard) were calculated by the computer. Linear calibration curves were generated with linear correlation coefficients between 0.95 (atrazine) and 0.99 (metolachlor and parathion).

Ions used for detection and quantitation were as follows: alachlor, m/z 238; atrazine, m/z 216; butylate, m/z 218; captan, m/z 264; carbaryl, m/z 202; carbofuran, m/z 222; chlorothalonil, m/z 267; chlorpyrifos, m/z 352; cyanazine, m/z 241; diazinon, m/z 305; fenamiphos, m/z 304; fluorene, m/z 167; fonofos, m/z 247; isofenphos, m/z 245; linuron, m/z 249; metolachlor, m/z 284; metribuzin, m/z 215; parathion, m/z 292; pendimethalin, m/z 282; simazine, m/z 202; and terbufos, m/z 103.

Extraction Procedures

Pesticides were extracted from water using XAD-2 and XAD-7 resins. Before each sample analysis, a 30 × 1 cm id glass Chromaflex column was filled with 5 g of both XAD-2 and XAD- 7 resins, packed tightly, and plugged at both ends with glass wool. Solvents were pumped through the column using a stainless steel tank pressurized with nitrogen. The column was first conditioned with 300 mL each of methylene chloride, methanol, and water. Sample water (2 L) was then pumped through the column at a rate of 75–100 mL/min. Tank pressure required for this flow rate was ca 25 psig. Pesticides were eluted from column with 300 mL methylene chloride. The eluate was collected, and 25 μL of 0.4 mg/mL fluorene internal standard stock solution was added. The solution was dried over anhydrous sodium sulfate and concentrated to 0.5 mL in a Kuderna-Danish concentrator on a steam bath. These extracts were analyzed by GC/CIMS.

Recovery Studies

Recovery studies were performed for each pesticide at the 1 ppb level, except for captan and chlorothalonil (5 ppb). The appropriate volume of pesticide stock solution was added to 2 L sampled water, which was then extracted as described above. Pesticide concentrations present in the final extracts were obtained by the instrument software, and relative responses of each pesticide were applied to the calibration curves. Corresponding concentrations (ppb) of pesticides in water samples were calculated by dividing the concentration in ng/μL by the overall concentration factor resulting from the extraction. This factor is 4000, because 2 L water was concentrated to 0.5 mL final extract.

Sample Analyses

Samples were analyzed by GC/CIMS, with a solvent run between each sample to assure the absence of any residual pesticide. After GC/CIMS analysis, the computer searched for each pesticide at the correct retention time (±15 s) by

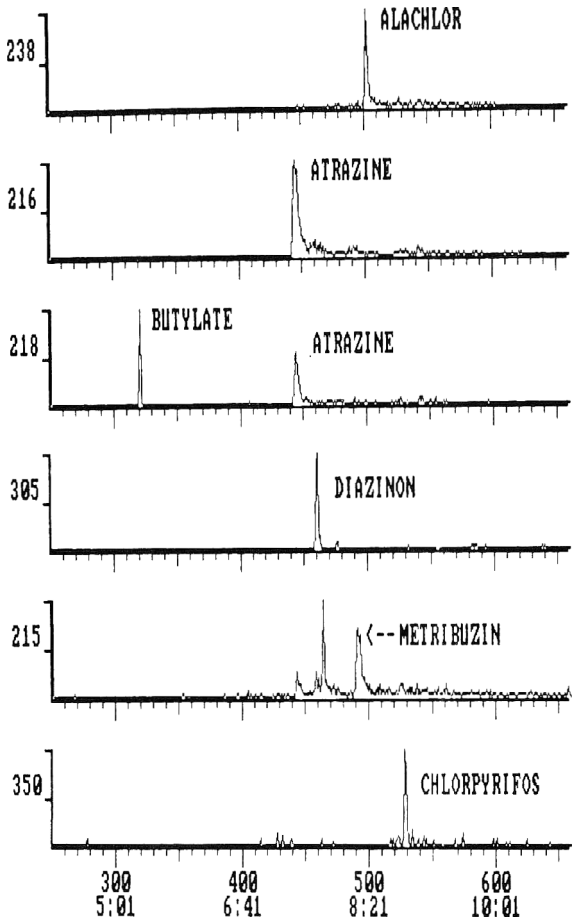

Figure 2. Mass chromatograms of selected pesticides at the 0.05 ppb level in surface water. X-axis, (upper) scan number and (lower) retention time; Y-axis, intensities of quantitation ions.

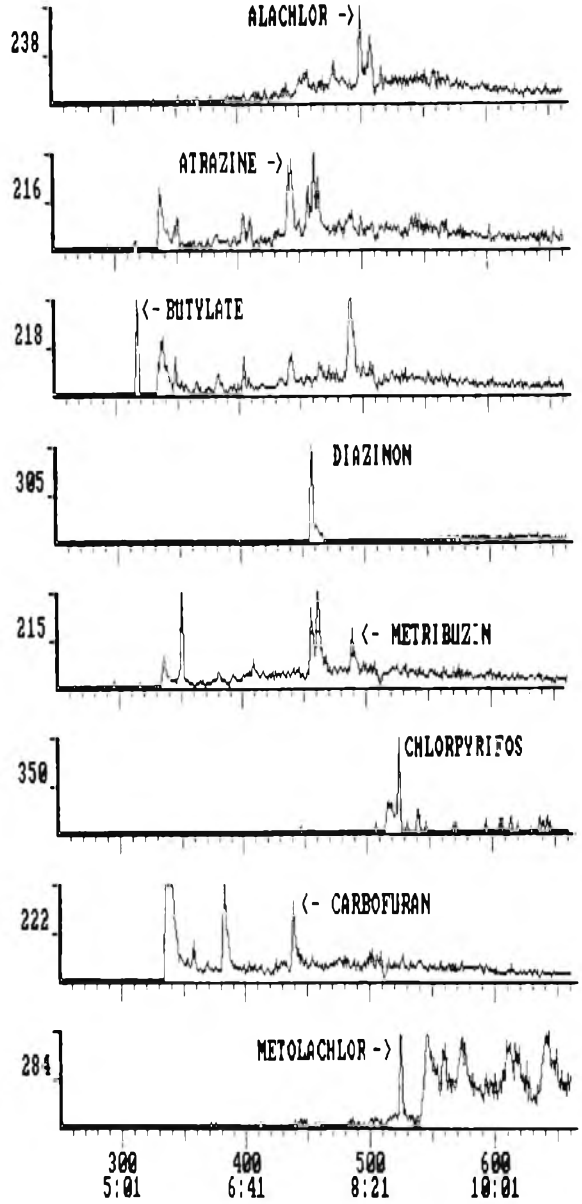

Figure 3. Mass chromatograms of selected pesticides at the 0.005 ppb level in surface water. X- and Y-axes as in Fig. 2.

mass chromatography of the pesticide's quantitation ion. The mass spectrum of each peak (if present) was compared to the standard CI spectrum for that pesticide in the search program. Pesticides found were quantitated by the computer. Confirmation of some pesticides could be obtained by comparing the mass chromatograms of the confirmation ions to the mass chromatogram of the quantitation ion at the exact retention time of the pesticide. These pesticides could be confirmed if the intensity ratios of the analyte's major ions were within 20% of known intensity ratios. A full-scan CI mass spectrum could also be observed. Confirmation ions used were as follows: alachlor, m/z 240 and 270; atrazine, m/z 218; butylate, m/z 156; captan, m/z 266, 152, and 236; carbaryl, m/z 145; carbofuran, m/z 165; chlorothalonil, m/z 265 and 269; chlorpyrifos, m/z 350 and 354; cyanazine, m/z 214; isofenphos, m/z 287; linuron, m/z 251 and 253; metolachlor, m/z 286 and 252; metribuzin, m/z 198; pendimethalin, m/z 264 and 212; simazine, m/z 204; terbufos, m/z 103, 233, 245, and 199. Diazinon, fenamiphos, fonofos, and parathion gave no significant fragmentation; therefore, they had no confirmation ions.

Sensitivity Determinations

Pesticide free water samples were spiked at the 1.0, 0.5, 0.05, 0.005, and 0.0005 ppb levels to determine LODs. For the 0.005 and 0.0005 ppb spiked samples, final extracts were concentrated to 100 μL. For a pesticide to be considered detectable, its mass chromatogram had to be distinguishable from background, and the chromatogram had to have a signal to noise ratio of ≥5.

Results and Discussion

Pesticides targeted for determination in surface waters were chosen because of their previously demonstrated runoff ability, widespread use in New Jersey, and their potential hazard to human health. Figure 1 shows the total ion chromatogram obtained from GC/CIMS analysis of a standard solution containing 10 ng/μL of the 20 pesticides and 20 ng/μL of the internal standard, fluorene. Results of the recovery studies at the 1 ppb level (5 ppb for captan and

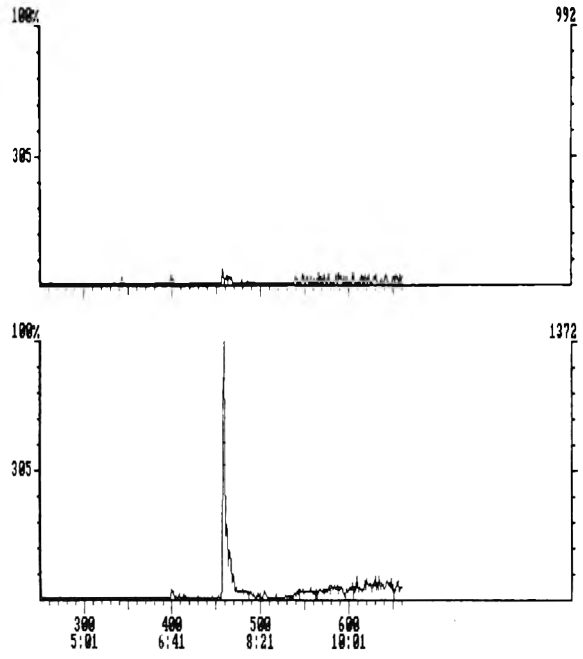

Figure 4. Mass chromatograms at m/z 305 of an unspiked surface water sample (upper trace), and the same sample spiked with 0.0005 ppb diazinon (lower trace). X- and Y-axes as in Fig. 2.

chlorothalonil) are given in Table 1. Recoveries were between 75 and 113%, with an average coefficient of variation of 9%. LODs in surface water were less than 0.05 ppb, except for captan and chlorothalonil, which had an LOD of 1 ppb. Alachlor, atrazine, butylate, carbofuran, chlorpyrifos, diazinon, fonofos, isofenphos, metolachlor, metribuzin, parathion, and simazine were all detectable at the 0.005 ppb level. The LOD of diazinon was less than 0.0005 ppb. Figure 2 displays mass chromatograms obtained from analysis of a water sample spiked at the 0.05 ppb level. Mass chromatograms of pesticides in a spiked water sample at the 0.005 ppb level are shown in Figure 3. The upper trace of Figure 4 shows a mass chromatogram for an unspiked surface water sample at m/z 305. The lower trace of Figure 4 shows a chromatogram of the same sample spiked with 0.0005 ppb diazinon.

Results of the pesticide analysis of New Jersey surface waters are summarized in Table 2. A total of 29 out of 31 samples analyzed contained one or more pesticides. Levels found ranged from trace (<0.025 ppb) to 5.48 ppb, as follows: alachlor, trace (9), 0.03, 0.06, and 0.13 ppb; atrazine, trace (6), 0.03 (2), 0.04, 0.05, 0.06 (4), 0.07 (2), 0.08 (2), 0.14 (2), 0.15, 0.18, 0.26, 0.35, and 0.61 ppb; carbaryl, trace (1), 0.23, 0.26, 0.33, 0.50, and 5.48 ppb; chlorpyrifos, trace (1); cyanazine, trace (1), 0.06 (2), and 0.07 ppb; diazinon, trace (1), 0.04, 0.05, 0.11, 0.21, 0.22, and 1.11 ppb; isofenphos, 0.10 and 0.61 ppb; linuron, 0.07 and 0.25 ppb; metolachlor, trace (3), 0.03 (2), 0.04 (3), 0.05 (2), 0.08, 0.10, 0.11, 0.13, 0.21, 0.36, 0.45, 0.80, 1.74, and 2.70 ppb; and simazine, trace (8), 0.04, 0.05 (2), 0.25, and 0.32 ppb..

The XAD extraction method can extract a wide variety of pesticides, including the triazine herbicides, atrazine, cyanazine, metribuzin, and simazine; the acetamide and aniline herbicides, alachlor, metolachlor, and pendimethalin; the

Table 2. Pesticides found during analysis of 31 surface water samples

Pesticide	No. positive findings	Found, ppb	Av., ppb[a]
Alachlor	12	trace – 0.13	0.014
Atrazine	25	trace – 0.61	0.087
Carbaryl	6	trace – 5.48	0.220
Chlorpyrifos	1	trace	0.001
Cyanazine	4	trace – 0.07	0.007
Diazinon	7	trace – 1.11	0.057
Isofenphos	2	0.10 – 0.61	0.023
Linuron	2	0.07 – 0.25	0.010
Metolachlor	20	trace – 2.70	0.227
Simazine	13	trace – 0.32	0.029

[a] Assuming trace = 0.025 ppb.

carbamates, butylate, carbaryl, and carbofuran; the phenylurea herbicide, linuron; the organophosphates, chlorpyrifos, diazinon, fenamiphos, fonofos, isofenphos, parathion, and terbufos; and the miscellaneous pesticides, captan and chlorothalonil. Preliminary studies using only XAD-2 resin resulted in low recoveries for the more polar pesticides, such as the organophosphates and chlorothalonil. XAD-7, which has a higher affinity for polar organic compounds, gave significantly higher recoveries of the polar pesticides.

The ion trap detector is a single, selective detector that can detect pg quantities of chemicals in complex matrixes. Because of excellent sensitivity in the full scan mode, SIM is unnecessary. The method is not limited in the number of pesticides that can be determined, as long as each pesticide can be extracted by XAD-resin, chromatographed by cold, on-column injection GC, and ionized by CI. Analysis of extracts with the ion trap detector gave excellent sensitivity for most of the pesticides studied. Except for captan and chlorothalonil, all pesticides were detectable at 0.05 ppb or below. Most pesticides were detectable between 0.005 and 0.0005 ppb. In comparison, published EPA methods result in the following higher LODs: alachlor (0.38 ppb), atrazine (0.13 ppb), butylate (0.15 ppb), diazinon (0.25 ppb), fenamiphos (1.0 ppb), metolachlor (0.75 ppb), metribuzin (0.15 ppb), and simazine (0.075 ppb). In addition, EPA requires LC methods for linuron (LOD, 0.25 ppb), cyanazine (LOD, 0.58 ppb), carbaryl (LOD, 2.0 ppb), and carbofuran (LOD, 1.5 ppb).

In conclusion, we have demonstrated that low-pressure XAD extraction coupled with GC/CIMS with ITD provides a rapid, sensitive, and accurate method for the determination of pesticides in surface water.

Acknowledgments

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Separation and Determination of Diesel Contaminants in Various Fish Products by Capillary Gas Chromatography

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A semiquantitative capillary column gas chromatographic method is described for the determination of diesel fuel contamination in various canned seafood products. The diesel contaminants are separated from the fish sample by steam distillation, with little carry-over of interfering intrinsic materials such as fish oils. The diesel fuel is extracted from the condensate with *n*-hexane, and the extract is analyzed on an SPB-1 fused silica capillary column. The efficiency of recovery of diesel fuel added to canned seafood at levels of 40–400 ppt ranged from 72 to 102%. With the additional step of concentrating the hexane extract, the sensitivity of this procedure may be increased at least 10-fold. This procedure can detect the differences among diesel fuel grades No. 1, 2, and 5, and variations within diesel grade No. 2, and thus may be useful in determining the type of petroleum contaminants present in various canned fish products.

The recent oil spill in Alaskan waters and the problems associated with storing fish next to diesel fuel on boats have led to the possibility of contamination of fish with petroleum products. Indeed, organoleptic analyses (smelling of samples by a technician trained to detect and identify odors), usually aimed at detecting food decomposition, have detected odors that resemble those of petroleum distillates in some canned fish samples. However, chemical testing of petroleum contaminants in food samples has been impossible with currently available protocols.

Currently, no official analytical methods for detecting and/or confirming petroleum contamination in fish are available, and a literature review did not reveal any rapid or simple methods for conducting such analyses. Most of the analytical procedures found were those used by the petroleum industry (1–8), the U.S. Environmental Protection Agency (9), the U.S. Coast Guard (10, 11), and others (12) to identify and match oil spill samples or classify unknown crude oils (13). There were, however, a few published articles dealing with the evaluation of petroleum contaminants in various foods (14–16).

To extract petroleum contaminates from food samples, Farrington and Meyer (14) mixed the samples with organic solvents such as diethyl ether, then steam distilled the extract to obtain a volatile hydrocarbon fraction. Other methods include saponification of samples with alcoholic KOH (14) or alcoholic NaOH (15) to eliminate interferences from lipids, followed by partitioning of hydrocarbons and unsaponifiable lipids into a nonpolar solvent such as hexane. Other techniques used to separate the petroleum hydrocarbons from coextracted lipids include column and thin-layer chromatography (TLC), individually or in combination (1, 14).

The application and relative merits of various analytical methods used to characterize petroleum hydrocarbons to identify oil spills or crude oils were published recently in reviews by Bentz (1) and Clark and Jurs (13). Hites and Bieman (17) and Karger et al. (18) successfully used liquid chromatography to separate and detect aromatic hydrocarbons. Blumer et al. (19) used gel permeation chromatography for the analysis of high molecular weight components of petroleum in marine samples. IR, UV, and fluorescence spectroscopy, TLC, and gas chromatography (GC), by itself or in combination with mass spectrometry (GC/MS), are also among the analytical methods most commonly suggested. On the basis of the advantages and limitations of each technique, GC was chosen as the most suitable method for a rapid examination of petroleum contaminants.

Oil-derived petroleum fuels are highly complex mixtures of aliphatic and aromatic hydrocarbons, alkenes, napthenes, and alkylated aromatic compounds (20). A gas chromatograph equipped with capillary column can separate the saturated hydrocarbons approximately in the order of their boiling points (1, 11). Because diesel fuels may contain over a hundred different hydrocarbon compounds, the use of a temperature program enhances GC resolution, and can produce a "finger print" unique to the particular petroleum product being analyzed. The typical petroleum chromato-

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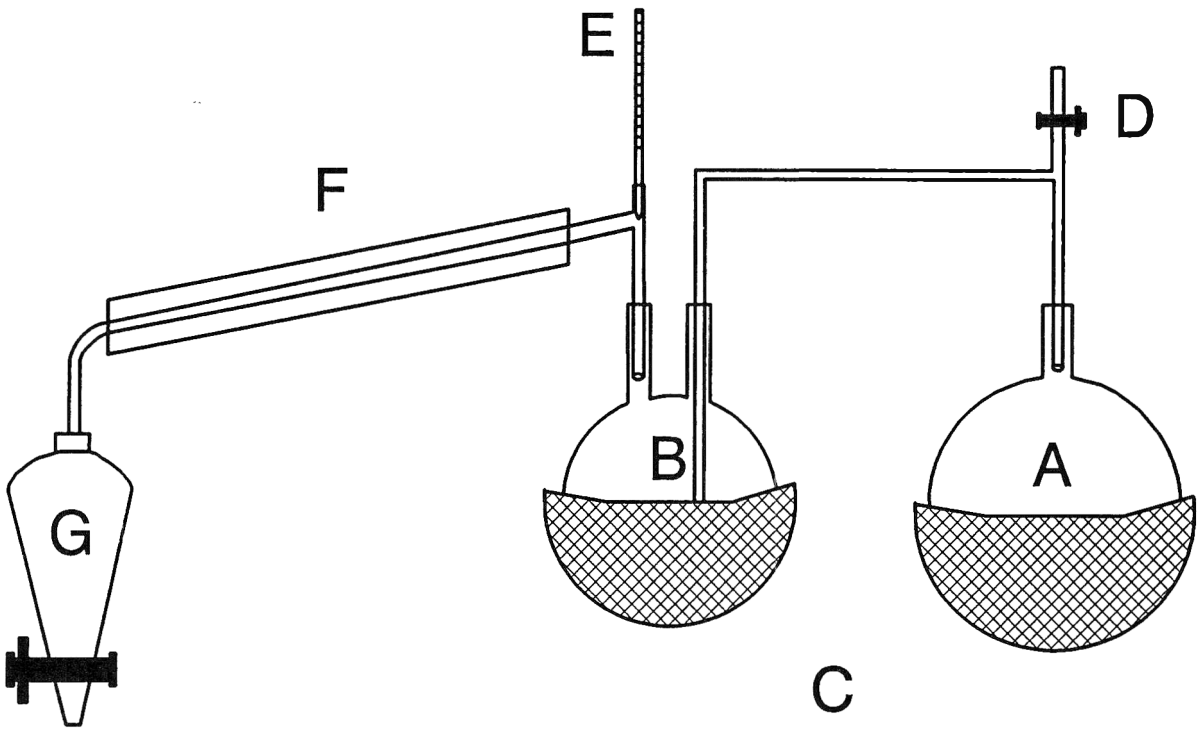

Figure 1. Schematic diagram of steam distillation apparatus: A, 3 L boiling flask for generating steam; B, 500 mL 2-neck distillation flask holds 50 g fish sample and 100 mL water; C, heating mantles; D, pressure relief valve; E, thermometer; F, condenser; and G, separatory funnel.

gram contains a number of dominant peaks corresponding to a homologous series of straight chain (*n*-) alkanes, interspersed with a large number of peaks corresponding to unsaturated paraffins, isoprenoid hydrocarbons, and aromatic hydrocarbons. The size and relative abundance of the *n*-alkane peaks depends on the type of fuel analyzed. The entire set of peaks arise from a broad baseline peak (envelope), caused presumably by the incomplete resolution of the petroleum peaks.

Experimental

Apparatus

(a) *GC apparatus.*—Perkin-Elmer Sigma Model 2000 equipped with flame ionization detection (FID) system and adapted for SPB-1 fused silica capillary column, 30 m × 0.32 mm id, with film thickness of 0.25 μm (Supelco, Inc. Bellefonte, PA 16823). Operating conditions: Initial column temperature, 60°C. Hold 5 min, program to increase at 5°C/min to 220°C. Hold 5 min. Maintain injector at 230°C, and detector at 270°C. Adjust sample splitter to 1:50, with helium carrier entering splitter at 20 mL/min. Set FID attenuation to 256, and chart speed to 0.5 cm/min. Set hydrogen flow to FID at 150 mL/min and air at 100 mL/min.

(b) *Integrator.*—Model 427 (Beckman Instruments, Inc., Fullerton, CA 92634).

(c) *Blender.*—Osterizer Model 548-51A, 1/2 hp motor, set at "Liquefy," with 16 oz glass blending container.

(d) *Steam distillation apparatus.*—See Figure 1. Assembly consists of 3 L boiler flask heated with mantle, connected to 1 neck of 500 mL 2-neck distillation flask heated with mantle. The boiler flask provides steam at constant rate to produce constant rate of distillation. Each heating mantle is connected to its own variable power supply. Second neck of distillation flask is connected to condenser, and 500 mL separation funnel receives distillate.

Reagents

(a) *Hexane.*—Glass distilled grade (EM Science, Gibbstown, NJ 08027). A 5 μL sample concentrated 1:1 or 10:1 shows only one peak when analyzed by GC.

(b) *Sodium sulfate.*—Reagent grade, anhydrous, granular (Mallinkrodt, Inc., St. Louis, MO 63134).

(c) *Sodium chloride.*—Saturated solution.

(d) *Diesel standards.*—Diesel No. 1 and No. 5 grade were obtained from Sheldon Oil Corp., Fairfield, CA. Five diesel No. 2 samples were obtained from Chevron Oil Corp., Richmond, CA. Dissolve 2 g petroleum standard in 100 mL *n*-hexane. Dilute 10 mL into 100 mL *n*-hexane to yield 2 mg/mL standard.

(e) *GC standards.*—Homologous series of *n*-alkane standards from *n*-undecane (*n*-C_{11}) to *n*-eicosane (*n*-C_{20}) and *n*-tricosane (*n*-C_{23}) (Sigma Chemical Co., St. Louis, MO 63178). Add 5–10 mg each standard to 100 mL *n*-hexane so that no 2 peaks have more than 3:1 ratio of heights, and inject 5 μL as chromatographic standard (not shown).

(f) *Canned fish samples.*—Samples were obtained from commercial sources in the San Francisco, CA, and Seattle, WA, areas from January 1984 to December 1989.

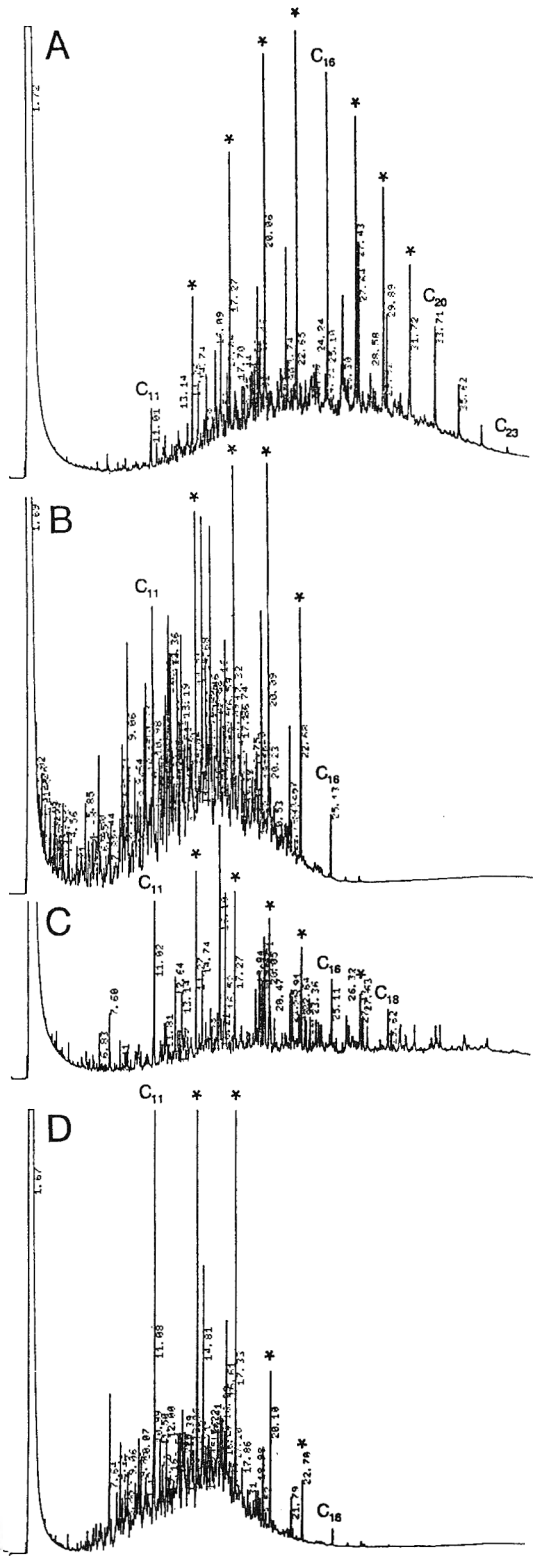

Figure 2. Chromatograms of diesel fuel standards: A, diesel fuel No. 2 (0689-5C); B, diesel fuel No. 1; C, diesel fuel No. 5; and D, kerosene. In this figure and Figure 4, elution time in min is printed to the right of the corresponding peak, and identified *n*-alkane peaks are labeled by name or an asterisk.

Procedure

Add 50 g fish sample, 50 mL water, and, when spiking, 20–220 mg diesel fuel to blending container. Blend 2 min at room temperature (21–24°C). Using a nickel spatula, transfer homogenate to 2-neck distillation flask (Figure 1B). Rinse blending container with 25 mL water, add rinsed material to distillation flask with aid of spatula, and add 100 mL water to distillation flask. Steam distill until ca 150 mL liquid collects in separatory funnel. Extract distillate 3 times each with 25 mL *n*-hexane. Pass combined extracts through glass column (9.5 × 1 in. id) packed with 2 in. anhydrous sodium sulfate to remove any remaining water. Collect extract in 100 mL volumetric flask and add fresh *n*-hexane to volume. Column is packed as follows: insert glass wool plug above column stem, and pack dry sodium sulfate on top of glass wool by tapping column on a padded surface; sodium sulfate does not need to be rinsed with hexane before or after packing.

Inject 5.0 μL fish sample extract into GC apparatus and run temperature program. After column has re-equilibrated to 60°C, inject 5.0 μL diesel No. 2 standard, and compare the 2 chromatograms. This linking of sample and standard will allow proper identification of *n*-alkane peaks in the event that chromatographic conditions (e.g., capillary column length) have changed slightly between runs carried out on different days.

If the sample chromatogram has peaks corresponding to diesel fuel, but peak heights are < 5% full-scale deflection, concentrate sample by evaporation to 10 mL using a Rotavapor at 35°C under vacuum. Inject 5.0 μL sample and compare chromatogram with that of petroleum standard. Quantify amount of petroleum contamination in fish samples by comparing heights (or areas) of a dominant *n*-alkane peak in sample and standard. For example, if the contaminant is diesel No. 2, use *n*-C_{14}, *n*-C_{15}, or *n*-C_{16} peaks. In cases where relative abundance of *n*-alkanes is markedly different between sample and standard, the total of identified *n*-alkane peak heights rather than the height of a single peak should be the basis of quantitation.

Table 1. Relative abundance of *n*-alkanes in 5 grade No. 2 diesel standards[a]

n-Alkane	7413–6	7383–15	7384–27	7415–33	0689–5C
n-C_{11}	9	8	4	9	10
n-C_{12}	7	7	2	7	7
n-C_{13}	5	5	1	5	5
n-C_{14}	3	2	3	2	2
n-C_{15}	1	1	5	1	1
n-C_{16}	2	3	6	3	3
n-C_{17}	4	4	7	4	4
n-C_{18}	6	6	8	6	6
n-C_{19}	8	9	9	8	8
n-C_{20}	10	10	10	10	9

[a] 1 is the most abundant; 10 is the least abundant.

Calculation

Calculate percent contamination as follows:

$$\text{Percent contamination (w/w)} = (100P_{SP}C_{ST})/(P_{ST}C_{SP}K)$$

where P_{SP} and P_{ST} are the bases of quantitation (reference *n*-alkane peak heights or total *n*-alkane peak heights) in the fish sample and standard solution, respectively; C_{ST} is the amount of diesel standard per 100 mL hexane (0.2 g); C_{SP} is the number of grams of fish sample added to the distillation

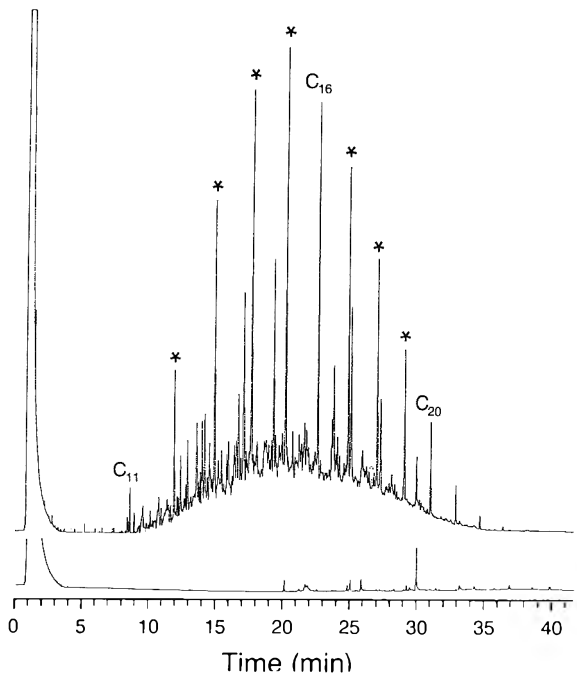

Figure 3. Top: Chromatogram of an extract of canned albacore spiked with 200 mg diesel No. 2 fuel. Bottom: Chromatogram of unspiked canned albacore extract from same source as used in top. Elution time is indicated on the X-axis.

flask (50 g); and K is the amount by which the fish sample extract has been concentrated (1- or 10-fold).

Results and Discussion

A number of extraction techniques were investigated before steam distillation was chosen. Headspace chromatography was attempted, but did not yield material suitable for GC analysis. This technique is limited by its inability to recover the less volatile components of diesel fuel, and because the yield of the more volatile components is too low for routine GC analysis. A second technique, blending fish with hexane followed by saponification, also was not useful. It did not adequately resolve fish oils from the diesel standard, and recoveries of diesel fuels were very low.

To attempt to identify the type of petroleum contaminant found in fish samples, chromatograms of several different petroleum fuels (Figure 2) were compared to that of a series of homologous n-alkane standards ranging in size from n-C_{11}–n-C_{23} (not shown). In all cases, n-alkane peaks elute in order of increasing size with increasing time. Differences in retention times of corresponding n-alkanes in this figure and all subsequent figures are due to the slight shortening of the capillary column when it is refit onto the GC system. This type of analysis can detect differences among diesel oils No. 1, 2, 5, and kerosene, and variations within diesel No. 2. With regard to the latter point, diesel No. 2 shows a series of dominant peaks corresponding to n-C_{11}–n-C_{20} and n-C_{23}, and varying amounts of unsaturated paraffins, isoprenoids, and aromatics (Figure 2A). On the basis of the relative abundance of n-C_{11}–n-C_{20} (Table 1), all 5 diesel No. 2 standards can be distinguished. The chromatogram of diesel No. 1 (Figure 2B) is distinguished from that of diesel No. 2 by dominant peaks

Table 2. Recoveries of diesel No. 2 in various canned fish samples

Species of canned fish	50 g sample, added, mg	Rec., mg	Rec., %
Chunk yellow fin tuna in oil	207.7[a]	209.0	100.6
	103.9[a]	106.1	102.1
	20.8[a]	17.3	83.2
Grated tuna in oil	203.3[a]	200.6	98.7
	101.7[a]	101.1	99.4
Sardines in tomato sauce	207.7[a]	191.6	92.2
	103.9[a]	104.2	100.3
Sardines in oil	207.7[a]	189.9	91.4
	103.9[a]	92.4	88.9
Mackerel in oil	203.3[a]	197.8	97.3
	101.7[a]	98.0	96.4
Milk fish in oil	200.2[a]	154.2	77.0
	100.1[a]	72.0	71.9
Chunk blue fin tuna in oil	219.7[b]	219.7	100.0
	109.8[b]	93.2	84.9
Albacore in water	221.4[b]	212.7	96.1
	110.7[b]	107.7	97.3

[a] Diesel No. 2 (0689-5C).
[b] Diesel No. 2 (7383-15).

corresponding to the series n-C_{11}–n-C_{16}, the absence of n-alkane peaks larger than n-C_{16}, and the existence of many additional peaks probably corresponding to unsaturated paraffins, isoprenoids, and aromatics. Diesel No. 5 (Figure 2C) differs from diesel No. 1 by having relatively higher levels of n-alkanes, having n-C_{12} rather than n-C_{13} and n-C_{14} as its dominant n-alkane, and having additional peaks corresponding to n-C_{17} and n-C_{18}. The chromatogram of kerosene (Figure 2D) is similar to that of diesel No. 1, but has a relatively lower abundance of peaks corresponding to unsaturated paraffins, isoprenoids, and aromatics.

The steam distillation/GC technique was first tested with fish samples considered uncontaminated by organoleptic analysis that were spiked with diesel No. 2. Diesel No. 2 was chosen because it is the fuel of choice for most commercial fishing boats. A chromatogram obtained from a 50 g canned albacore sample spiked with 200 mg diesel No. 2 is shown in Figure 3 (top). The pattern of peaks obtained from the spiked sample corresponds closely to that of the diesel No. 2 used for the spiking (Figure 2A). A chromatogram of an unspiked sample of the same fish (Figure 3, bottom) shows a flat base line and only a few small peaks that do not correspond to the diesel No. 2 standard. This indicates that GC analysis of diesel contamination is not complicated by interference from intrinsic substances such as fish oils.

A summary of the analysis of 17 fish samples spiked with 0.04–0.4% (w/w, 40–400 ppt) diesel fuel is presented in Table 2. Recoveries of diesel fuel ranged from 72 to 102%. For each sample, all identified n-alkane components were recovered with similar efficiencies (86–104%). By concentrating the sample extract 10-fold, a corresponding increase in the sensitivity of contaminant detection is found. A further check on the reliability of the GC analysis is performed by injecting varying sized samples of standard diesel solutions. A plot of n-C16 peak height vs amount of diesel standard injected was linear within the 0.1–2.0 mg/mL range (not shown). These findings indicate that the steam distilla-

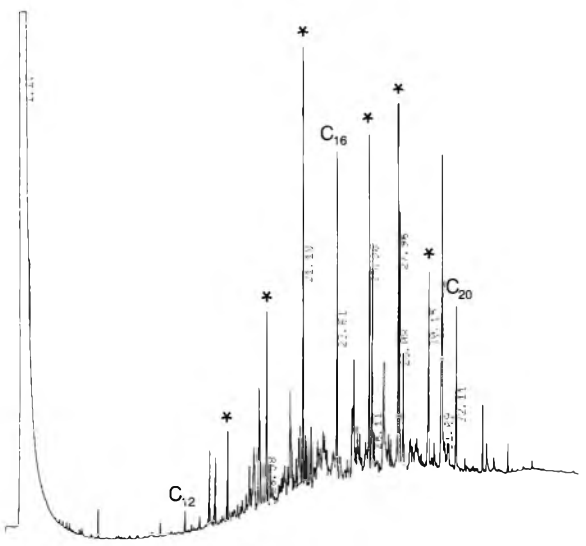

Figure 4. Chromatogram of canned albacore extract suspected by organoleptic analysis to be contaminated by diesel fuel.

tion/GC procedure yields results that are semiquantitative in nature.

An example of a chromatogram of an unspiked albacore sample that tested positive for diesel contamination by organoleptic analysis is presented in Figure 4. Because of the low yield of material on initial GC analysis (not shown), the *n*-hexane extract of this sample was concentrated 10-fold. The pattern of major peaks corresponds most closely to that of diesel No. 2 (Figure 2A). However, we cannot be certain of the identification of this contaminant, and we cannot rule out additional sources of contamination. When individual peak heights are normalized to the totals of n-C_{12}–n-C_{20} peak heights in each chromatogram, n-C_{12}–n-C_{14} are relatively underrepresented and n-C_{18}–n-C_{20} are relatively overrepresented in the fish sample. Likely causes of these differences are: (*1*) differences in the original compositions of the standard and contaminating diesel fuels, (*2*) weathering of the contaminating fuel, and (*3*) degradation of the contaminating fuel in the canning process. Comparison of the n-C_{16} peaks of the spiked and contaminated samples indicates that the level of diesel contamination is approximately 0.03% (w/w, 30 ppt). The same result is obtained when the totals of n-C_{12}–n-C_{20} peak heights are compared for the 2 chromatograms. Although we did not determine the limit of detection of our procedure, increased sensitivity may be gained by a combination of further concentrating the sample, injecting a larger sample volume, and increasing the sensitivity of the FID.

In summary, this paper presents a simple capillary GC procedure for detecting and approximating the quantity of diesel contamination of various canned fish products. It may be used to complement organoleptic analysis of these products, and may reveal the type of contaminating petroleum fuel.

Acknowledgments

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GC/MIP/AED Method for Pesticide Residue Determination in Fruits and Vegetables

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This research describes the results of a gas chromatography/microwave induced plasma/atomic emission detection (GC/MIP/AED) method performed on a Hewlett-Packard 5921A system for pesticide residue analysis in fruits and vegetables. A total of 6 experiments were conducted: (*1*) sensitivity and linearity studies for elements S, P, Cl, and N by analyzing dursban; (*2*) a study of instrument response to Cl concentration in pesticide molecules; (*3*) organochlorinated pesticide recoveries; (*4*) organophosphate pesticide recoveries; (*5*) carbamate pesticide recoveries; and (*6*) investigation of metallic pesticides with plictran and vendex as standards. The rank according to sensitivity and linearity was found to be as follows: S-181>P-178>Cl-479>N-174. Instrument response to the concentration of chlorine atoms in the pesticide molecule was linear, with a correlation coefficient of 0.89. Recoveries of organochlorinated pesticides were 91.7–109.3%, with the exception of citrus, whose recovery was affected by coeluting interferences. Organophosphate recoveries were 73.2% or higher, except for the cygon oxygen analog, which degraded in the GC system under all circumstances. Carbamate recoveries were inconsistent quantitatively; however, the information generated from elements N and S were useful for qualitative confirmation of other methods, such as LC postcolumn derivatization analysis. Overall, the GC/MIP/AED method is powerful for qualitative confirmation in pesticide residue analysis. The instrument's capability of acquiring multi-elements (Cl and P) selectively and accurately is an alternative method for organochlorinated and organophosphate pesticide residue analyses. In addition, the GC/MIP/AED system is easy to use, simple to maintain, and its chromatograms can be interpreted by any chromatography analyst without much prior training.

Spectrochemical analysis is not new; it was first reported by Talbot in 1820 (1). Gas chromatography (GC) with atomic emission detection (AED) is one type of spectrochemical method; however, the incorporation of a microwave induced plasma (MIP) generator and a photodiode array is a creative approach for analyzing many volatile compounds. With multielement analysis capability and 3 dimensional spectrum display, the GC/MIP/AED system is the first instrument that possesses the flexibility to be both general and selective. As a result, the primary test and the confirmation can, in principle, be accomplished together.

Since the 1985 California watermelon crises (2) and the 1988 CBS *60 Minutes* report on alar tainted apples, the pesticide residue monitoring program has attracted enormous publicity. As a result, food safety has become a focal issue on the national agenda. The consensus of the U.S. Congress Technology Assessment Team (3) is that development of a greater number of methods for examining pesticide residue in food crops is essential; the methods must analyze perishable commodities faster and/or have lower detection limits to better assess the trace contaminates.

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In November 1989, a joint project was teamed by the Hewlett-Packard Co., Neely Sales Region in Fullerton, CA, and the California Department of Food and Agriculture (CDFA), Anaheim Chemistry Laboratory. Hewlett-Packard supplied the instruments, including the HP 5921A atomic emission detector, HP 5890A gas chromatograph, work related accessories, and maintenance services. The Anaheim Chemistry Laboratory staff provided the plan, location, and work force for research experiments. The objectives were to gain a fundamental understanding of GC/MIP/AED operation and to explore the feasibility of adapting this technique to our existing pesticide residue analysis program.

Experimental

Materials and Reagents

(a) *Solvents.*—Pesticide grade (Fisher Scientific, Pittsburgh, PA 15219).

(b) *Standards.*—(U.S. Environmental Protection Agency, Pesticide and Industrial Chemical Repository.)

(c) *Hydrobromic acid.*—48%, ACS grade (Spectrum Chemical Mfg. Corp., Redondo Beach, CA 90278).

(d) *Filter paper.*—Sharkskin (Schleicher & Schuell, Inc., Keene, NH 03431).

Apparatus

(a) *Solid phase extraction.*—Bond Elut C18 (Analytichem International, Harbor City, CA 90710).

(b) *Food chopper.*—Model 8181-D (Hobart Mfg. Co., Troy, OH 45374).

(c) *Homogenizer.*—Omni-Mixer (Du Pont Co., Bio Medical Division, Newton, CT 06410).

(d) *Centrifuge.*—Model CS (International Equipment Co., Needham Heights, MA 02194).

(e) *Water bath.*—Model WBT-100, equipped with thermostat (Bamstead Still & Sterilizer Co., Boston, MA 02132).

(f) *Atomic emission detector.*—Model 5921A (Hewlett-Packard Co., Avondale, PA 19311-0900). Parameters: window purge flow, 30 mL/min; ferrule purge flow, 40 mL/min; liquid nitrogen flow, 2 L/min; transfer line temperature, 230°C; cavity temperature, 283°C; cavity water temperature, 63°C; and solvent dump on and off, 0–2 min.

(g) *Gas chromatograph.*—Model 5890A (Hewlett-Packard Co.). Parameters (Experiments 1–3): Column, Ultra 2 (Crosslinked 5% Ph Me Silicone), 25 m × 0.32 mm × 0.52 m film thickness (Hewlett-Packard Co.). Temperature program: initial temperature, 200°C; hold 2 min; ramp rate, 10°C/min; final temperature, 280°C; hold 2 min. Column flow, 1.95 mL/min; septum purge, 2.29 mL/min; split vent, 45 mL/min; injection mode, splitless; injection volume, 2 μL. Parameters (Experiments 4–6): Column, HP-1 (Methyl Silicone Gum), 10 m × 0.53 mm × 2.65 m film thickness

Table 1. Four element GC/MIP/AED responses to dursban at 9 levels by peak area

Net wt., ng	Cl-479	S-181	N-174	P-178
0.20	—[a]	—	—	—
0.20	—	28.45	—	13.85
0.20	—	—	—	—
0.20	—	—	—	—
0.62	—	80.82	—	47.39
0.62	38.48	61.38	—	46.40
0.62	—	71.98	—	51.62
0.62	—	62.03	—	46.08
0.98	—	74.14	—	113.68
0.98	—	75.56	—	117.48
0.98	44.06	96.60	—	121.57
0.98	—	109.35	—	94.89
2.06	18.58	207.82	—	265.50
2.06	62.64	196.10	—	280.91
2.06	20.87	177.35	—	254.70
2.06	53.60	242.57	—	296.07
6.58	112.50	1104.15	—	1389.30
6.58	124.75	1073.37	—	1199.70
6.58	110.71	1137.09	—	1235.75
6.58	115.24	1187.10	18.29	1234.78
10.30	169.51	1733.38	33.20	2162.00
10.30	146.36	1786.97	64.31	2286.46
10.30	173.14	1694.24	32.90	2240.46
10.30	140.66	1667.62	—	2262.06
24.70	359.08	4526.77	189.69	6828.48
24.70	343.63	4465.99	106.03	7153.63
24.70	359.86	4558.13	186.29	6760.64
24.70	357.14	4529.97	97.35	7214.91
32.96	520.15	6158.63	139.14	10362.00
32.96	483.60	6244.26	143.42	9697.59
32.96	496.13	6123.55	142.28	9456.53
32.96	498.34	6370.24	197.11	9674.06
41.20	644.67	—	—	—
41.20	537.81	7929.60	96.28	—
41.20	533.21	10765.00	131.89	—
41.20	556.96	8088.60	91.17	—
Constant	20.41	-138.29	-5.51	-422.7
Std err. Y	26.12	84.58	35.97	320.82
R^2	0.985	0.998	0.714	0.993
No. Obs.	26	28	12	28
Degree of freedom	24	26	10	26
X coeff.	13.73	190.82	5.28	302.85
Std err. X	0.35	1.35	1.06	5.13

[a] No response in low levels or erroneous value in high levels.

(Hewlett-Packard Co.). Temperature program: initial temperature, 100°C (66°C for Experiment 6); hold 0 min; ramp rate, 17°C/min; final temperature, 270°C; hold 5 min (3 min for Experiment 6). Column flow, 12 mL/min; septum purge, 2.29 mL/min; split vent, 45 mL/min; injection mode, splitless; injection volume, 4 μL.

(h) *Auto sample.*—Model 7673A (Hewlett-Packard Co.).
(i) *ChemStation.*—Model 5895 (Hewlett-Packard Co.).
(j) *Printer.*—Think Jet (Hewlett-Packard Co.).

Preparation of Sample

Experiment 3–Organochlorinated pesticide residue analysis. Chop and mix vegetable or fruit in food chopper. Weigh 50 g sample into 1 pt Mason jar. Add 100 mL acetonitrile to jar and blend 2 min with Omni-Mixer. Let solids settle for a few minutes. Pour top aqueous phase through sharkskin filter paper and collect filtrate in 4 oz glass bottle. Add ca 15 g NaCl to bottle. Cork and shake bottle vigorously for 1 min. Place bottle in centrifuge for 2 min at ca 1500 rpm. Pipet 40 mL of top acetonitrile layer into 100 mL beaker and evaporate to near dryness in 100°C water bath with gentle air stream. Let final evaporation to dryness occur at ambient temperature. Transfer sample completely with isooctane into 2 mL volumetric flask and add isooctane to volume.

Experiments 4 and 5–Organophosphate and carbamate pesticide residue analyses. Incorporate Bond Elut C18 (ca 0.3 g) solid phase cleanup step into the above sample preparation procedure to minimize interferences. Add cleanup step after separating aqueous phase from solid with sharkskin filter paper. Pass aliquot through C18 cartridge prewetted with water–methanol (1 + 1) under slight vacuum (ca 50 mL/min). Continue succeeding procedures as described above.

Results and Discussion

The HP 5921A GC/MIP/AED system has only recently been introduced for commercial use. Every operator must first understand the capabilities of the instrument to be able to develop a legitimate method. Therefore, Experiments 1 and 2 were designed to be basic instrument studies that included the sensitivity, linearity, and elemental response as criteria. Experiments 3–5 were pesticide residue recovery studies carried out by fortifying pesticides and metabolites into 5 groups of produce. Experiment 6 was an investigation of metallic pesticide (e.g., plictran and vendex) analyses by GC/MIP/AED.

Experiment 1–Sensitivity and Linearity Study

In general, the elemental composition of pesticides is C, H, O, Cl, P, S, N, and/or others. The first 3 (C, H, and O) are also building blocks of every living organism, including the tissues of fruits and vegetables. Therefore, C, H, and O can be called common elements among both human-made pesticides and naturally existing plant tissues. These elements are of little advantage or interest in pesticide analyses by a selective detector because coeluting natural components of the sample make identification and quantitation impossible. The remaining elements (Cl, P, S, N, and others) may be called functional elements because the potency or toxicity of pesticides is often generated by functional groups containing these elements. Usually, plant tissue contains little of these elements in conjugated form, or macromolecules; therefore, they can be separated easily from pesticides of small molecular size by a chromatographic column before detection and quantitation. For this reason, pesticide analysts use selective detection exclusively, such as: electron capture detection

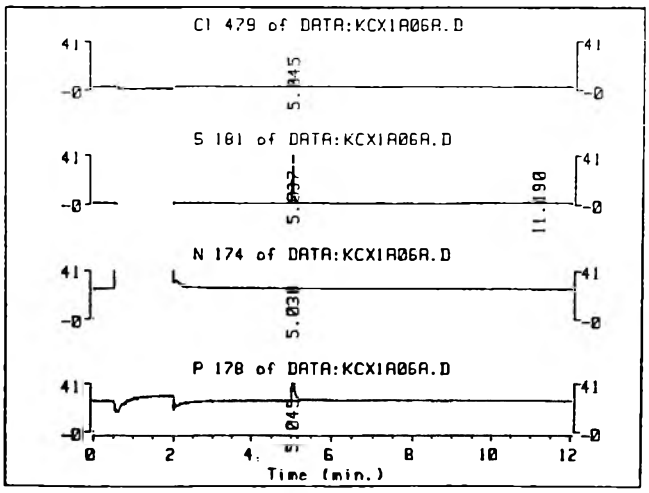

Figure 1. Multielement chromatogram of dursban by GC/MIP/AED displaying retention times (min): Cl-479, 5.045; S-181, 5.037; N-174, 5.038; and P-178, 5.045.

(ECD), electrolytic conductivity detection (ELCD), flame photometric detection, thermionic nitrogen/phosphorus detection, and fluorescent detection with liquid chromatography (LC) for determining Cl, P, S, and N in pesticide residue on food crops. One typical pesticide residue that contains 4 functional elements (Cl, P, S, and N) is dursban (commonly referred to as chlorpyrifos). For this reason, most pesticide analysts like to use dursban as the reference compound for the development of various methods. Dursban was also chosen as the reference compound in this experiment.

A total of 9 levels of standards (0.1, 0.31, 0.49, 1.03, 3.29, 5.15, 12.35, 16.48, and 20.60 μg/mL) were made from 0.93 mg/mL dursban stock. For AED element and wavelength selections, Cl-479, S-181, N-174, and P-178 were activated. Each level of standards was acquired 4 times consecutively to obtain statistical information. The results are presented in Table 1. Rather than concentration, the values in the table are expressed in net weight (ng), which was calculated by multiplying the standard concentration (ng/μL) by the injection volume (2 μL). The values of the elements Cl-479, S-181, N-174, and P-178 in Table 1 are expressed as the area printout generated by the instrument in response to each level of standard. The dashes in the table indicated no response in lower level standards due to lack of sensitivity. For higher level standards, the dashes indicate erroneous values because of overload.

In terms of sensitivity and linearity, the 4 elements ranked as follows: S-181 > P-178 > Cl-479 > N-174 as shown in Table 1. S-181 was not only the most sensitive, it also had a wider linear range (0.62–41.20 ng). P-178 ranked second; its area values were nearly 1/3 less than S-181 in the lower ng levels (0.20 and 0.62 ng). The linear range of P-178 was also smaller (0.62–32.96 ng). Cl-479 ranked third; the linear range could not be determined in this experiment because the higher level did not reach the maximum. N-174 was least sensitive; in addition, its linear dynamic range was narrower (10.30–32.96 ng) in comparison to the others. In Table 1, the correlation coefficients derived from linear regressions between areas and concentrations reveal the following ranking by atomic species: S-181 (R = 0.998), P-178 (R = 0.993), Cl-479 (R = 0.985), and N-174 (R = 0.714). Figure 1 shows that the 4 elements are determined together. Retention times are printed on top of the peaks and are almost identical. The effect of multielement identification is displayed.

Experiment 2–The Response of Cl-479 to Organochlorinated Pesticides

This experiment was conducted with one question in mind: Did AED respond to element concentration of chemical compounds in a linear function? The demonstration of the response from Cl in this trial can be assumed to be similar to the response of other elements.

In this experiment, organochlorinated pesticides with 1–7 chlorine atoms per molecule were chosen. The pesticides chosen were vegadex (1-Cl), botran (2-Cl), dyrene (3-Cl), bravo (4-Cl), PCNB (5-Cl), endrin (6-Cl), and heptachlor (7-Cl). The total amount of pesticide injected into the GC/MIP/AED system varied from 8.20 to 11.58 ng. The area values of 10 replicates are presented in Table 2.

To demonstrate the relationship between Cl concentration and area response, the data were processed with the following equations:

Table 2. GC/MIP/AED Cl-479 responses (area) to 7 organochlorinated pesticides

Pesticides	Cl, No.	Concn, ng	Area A	B	C	D	E	F	G	H	I	J	Mean	SD	RSD
Vegadex	1	9.82	96.24	229.57	98.34	83.36	90.08	95.85	79.94	115.55	123.77	90.51	110.32	41.75	37.85
Botran	2	11.18	238.48	367.47	230.69	206.46	213.34	227.96	185.27	232.48	200.49	211.03	231.37	47.98	20.74
Dyrene	3	11.32	174.11	257.36	192.31	199.42	201.14	190.79	170.90	233.75	189.51	187.83	199.71	25.20	12.62
Bravo	4	11.58	356.38	487.25	368.15	335.29	323.74	323.53	348.93	340.60	274.40	317.87	347.61	52.53	15.11
PCNB	5	8.2	315.72	413.69	280.11	249.61	251.64	272.84	230.28	246.94	263.73	272.76	279.73	49.81	17.81
Endrin	6	11.64	233.09	241.82	251.6	231.75	222.79	218.35	189.88	242.63	248.94	236.07			
Endrin breakdowr		—[a]	86.15	75.36	68.51	91.20	60.29	76.10	44.21	66.23	78.54	82.43			
Endrin breakdowr		—	103.40	79.47	68.00	66.89	70.30	66.21	71.04	76.25	78.56	102.24			
Added areas		—	422.64	396.65	388.11	389.84	353.38	360.66	305.13	385.11	406.04	420.74	382.83	33.51	8.75
Heptachlor	7	9.38	469.81	551.14	444.54	425.98	415.20	432.58	383.21	448.20	411.60	441.12	442.34	42.63	9.64

[a] Not applicable.

Table 3. Liner regression of area response (area/concn) to Cl concentration

Pesticides	Cl, No.	Cl, %	Response, area/concn	Best fit	Regression output	
					Constant	-2.44
Vegadex	1	16	11.23	7.97	Std err. of Y est.	4.37
Botran	2	34	20.69	19.68	R^2	0.89
Dyrene	3	39	17.64	22.94	No. of obs.	7
Bravo	4	53	30.02	32.05	Degrees of freedom	5
PCNB	5	56	32.89	34.00	X coefficient(s)	0.65
Endrin	6	60	34.11	36.60	Std err. of coefficient	0.10
Heptachlor	7	66	47.16	40.51	Y = -2.44 + 0.65X	

Cl% = number of Cl atoms in the pesticide × atomic wt (35.451) × 100%/pesticide molecular wt (*1*)

Area response = average of 10 replicate area values/amount of pesticide injected (ng) (*2*)

The linear regression was calculated between the percentage of Cl and the area response in Table 3. The correlation coefficient was 0.89, which is a high value for this relationship. The response of the GC/MIP/AED as a function of percent of Cl atoms in the pesticides is clearly linear. Note that the sum of 3 areas was used for endrin because this pesticide decomposes on a hot injector. Figure 2 shows the 7 organochlorinated pesticides and the endrin-breakdown products.

Experiment 3—Organochlorinated Pesticide Recoveries

Note that the previous experiments did not involve real samples; in other words, they were not subjected to coelution interferences from sample matrixes. Experiment 3 was designed to evaluate the selectivity, sensitivity, and overall applicability of Cl-479 in pesticide residue analyses with complicated matrixes like those found in fruits and vegetables.

This experiment was of 2-way factorial design because pesticide residue recovery studies in food crops are affected by 2 factors. The first factor is the difference between various pesticides; the second is the variance among crops. A total of 14 pesticides and metabolites were fortified into 10 commodities that were grouped into 5 classes: leafy vegetables, roots, fruits, citrus, and spices. The percentage recovery data are presented in Table 4 and the chromatogram is shown in Figure 3. The statistics on the right side of the table evaluate the accuracy and precision of each pesticide among fruits and vegetables. The statistics below the table measure pesticide recovery difficulties within each commodity. The dashes represent the recovery data below 69%; these data were excluded from statistical calculation.

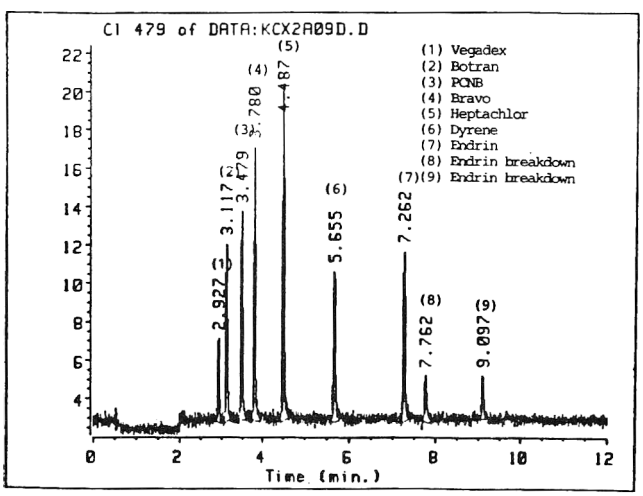

Figure 2. Chromatogram of 7 organochlorinated pesticide standards by element-wavelength Cl-479 of GC/MIP/AED.

The results and statistical data on the right of Table 4 show that the percentage recovery among all commodities range from 91.7 to 109.3%. Considering that the procedure was carried out without any kind of cleanup, the recovery data are good. Poor standard deviation in dursban and thiodan sulfate (15.38 and 30.63, respectively) indicated that the spiked levels (0.07 and 0.16 ppm, respectively) were low. Therefore, the AED could not avoid the noisy background effect of matrix interference. The data suggest that cleanup may be needed for low level pesticide residue determination. The statistical data found in Table 4 show that there are a lot of inconsistencies in pesticide residue analyses among different fruits and vegetables. Lemon was a difficult sample for GC/MIP/AED analyses, as was obvious by the number of dashes. Not surprisingly, tangerine also had problems. The GC/MIP/AED system was apparently subject to more interferences from citrus than any other produce.

Experiment 4—Organophosphate Pesticide Recoveries

Results from Experiment 1 suggest that P-178 in AED is more sensitive than Cl-479. In addition, the results of Experiment 3 suggest that cleanup may be needed during testing of low level pesticide residues in food crops. On the basis of this information, Experiment 4 was designed for the testing of lower level pesticides than Experiment 3. With the cleanup step, a noticeable amount of plant pigment was trapped in the C18 solid phase; consequently, the filtrate became a lighter color. Because organophosphates are more polar than organochlorinated pesticides, acetone, instead of isooctane, was used to transfer each sample into a 2 mL volumetric flask in the final sample preparation step.

The recovery results are presented in Table 5 and the chromatogram is shown in Figure 4. Percent recoveries for both pesticides and commodities are 73.2% or higher, except in the case of the cygon oxygen analog. This metabolite of cygon is unstable and degrades very rapidly in the presence of sample residue deposits located in the injection port. Better recovery results were achieved by placing a clean glass insert in the injection port. Therefore, the low recovery of this compound was not a result of a loss of sensitivity on the part of the AED apparatus.

Table 4. Recoveries (%) of organochlorinated pesticides in produce

		Leafy vegetables		Roots		Fruits		Citrus		Spices		Statistics		
Pesticides	Spiked amt, ppm	Cabbage	Red leaf lettuce	Carrot	Potato	Apple	Nectarine	Lemon	Tangerine	Green onion	Chill pepper	Mean	SD	RSD
Vegadex	0.49	104.9	111.9	96.5	98.4	96.7	99.3	92.1	99.8	117.2	100.1	101.7	7.2	7.1
PCNB	0.24	96.3	107.3	91.9	98.1	96.5	102.9	105.6	108.5	94.1	94.9	99.6	5.7	5.7
Ronilan	0.51	116.1	112.6	107.5	108.9	106.2	116.4	96.5	116.7	127.9	114.5	112.3	7.9	7.0
Heptachlor	0.19	108.2	111.1	109.2	106.8	95.2	103.6	87.8	93.5	112.3	106.7	103.4	7.9	7.7
Dursban	0.07	113.4	122.9	86.9	109.3	85.5	103.9	79.7	92.1	119.9	120.6	103.4	15.4	14.9
Dacthal	0.51	107.3	121.5	98.9	111.2	91.5	109.9	98.9	102.1	111.9	112.2	106.5	8.3	7.7
Thiodan I	0.24	95.9	114.4	101.1	108.3	94.7	98.6	—[a]	108.2	103.9	123.8	105.4	9.4	8.9
Endrin	0.27	101.5	121.7	107.7	110.6	94.8	112.6	—[a]	104.6	121.6	108.8	109.3	8.8	8.1
Thiodan II	0.26	103.7	113.8	103.6	93.8	92.9	102.9	—[a]	77.6	92.3	102.8	98.2	10.3	10.5
Thiodan SO_4	0.16	142.7	146.8	103.2	98.6	120.8	75.7	—[a]	—[a]	71.3	71.5	103.8	30.6	29.5
Permethrin C	0.52	93.2	105.7	92.3	91.1	83.3	97.5	82.1	96.3	98.1	107.3	94.7	7.8	8.3
Permethrin T	0.55	87.6	106.5	92.3	87.2	80.8	95.5	84.9	88.1	93.9	100.2	91.7	7.3	7.9
Cypermethrin C	0.27	108.8	122.9	111.6	97.5	91.4	96.8	—	78.1	109.2	95.7	101.3	13.2	13.0
Cypermethrin T	0.27	120.4	117.3	104.9	100.4	90.1	114.4	69.3	90.1	108.2	86.2	100.1	15.3	15.3
Mean		107.1	116.9	100.5	101.4	94.3	102.1			105.8	103.2			
SD		13.2	10.1	7.3	7.5	9.5	9.8			14.3	13.2			
RSD		12.3	8.6	7.3	7.4	10.1	9.6			13.5	12.8			

[a] Recoveries below 69%. Excluded from statistical calculation.

The statistic results in Table 5 unveil some difficulty in analyzing citrus by GC/MIP/AED. Grapefruit had the lowest average recovery (75.0%) with the highest standard deviation (39.7).

Another problem is the tailing of the P-178 chromatogram peaks. This is an inherent characteristic of the AED (4). P-178 peak shape can be improved by increasing the helium makeup gas flow; however, tailing peaks cannot be totally eliminated. Therefore, unlike the last experiment, peak heights rather than areas were used for quantitation. Thus, the results noticeably improved.

Because most organophosphate pesticides are also thio compounds, S-181 was activated for confirmation (see Figure 4). As was described previously in Experiment 1, S-181 was the most sensitive wavelength, but S-181 peaks might also be subject to interference from other sulfur containing compounds, such as onions and the brassica (cole) leafy vegetables. The S-181 mode should, therefore, only be used for confirmation purposes in these cases.

Experiment 5—Carbamate Pesticide Recoveries

In general, carbamates are thermolabile and incompatible with GC analyses. Therefore, LC with postcolumn derivatization (5) is always the first choice for this group of pesticide residues. However, any good method may occasionally fail; consequently, a second (or confirmation) method should be established to guard against uncertainty. Carbamates are esters of carbamic acid, and many of them also contain sulfur atoms in their structure; therefore, both N-174 and S-181 were activated during the testing process. Because nitrogenous compounds are polar in nature, methanol was used to transfer the carbamate sample into a 2 mL volumetric flask in this experiment. As expected, the quantitative results in Tables 6 and 7 are unacceptable with or without the cleanup step. The poor showing of the quantitative data may be due to several reasons. (*1*) The N-174 mode lacks sensitivity, as was learned in Experiment 1. The spiked pesticide levels are too low for consistent AED analyses. (*2*) The N-174 mode lacks selectivity. It is very susceptible or vulnerable to the presence of carbon in plant tissue. (*3*) Carbamates are thermolabile. They partially degrade in the GC oven before ever reaching the AED. (*4*) Most fruits and vegetables contain nitrogenous moieties, the coelution of which interferes with carbamate analyses.

Although N-174 had a quantitatively poor showing in carbamate recovery data, the GC/MIP/AED system is still a fast and powerful tool for confirmation. The system is especially suited for compounds with 2 or more distinguishable elements, such as the nitrogen and sulfur atoms in aldicarb sulfone and methiocarb (Figure 5). The information generated by this method is complementary to LC postcolumn derivatization analyses.

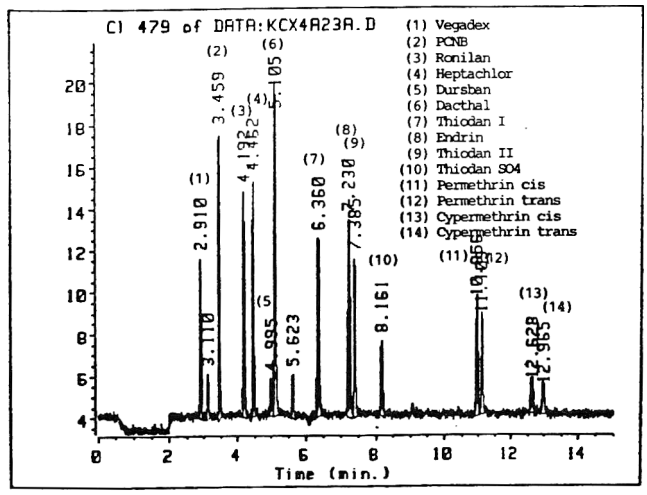

Figure 3. Chromatogram of green onion with spiked organochlorinated pesticides and metabolites by CI-479 of GC/MIP/AED.

Table 5. Recoveries (%) of organophosphate pesticides in produce

		Leafy vegetables		Roots		Fruits		Citrus		Spices		Statistics		
Pesticides	Spiked amt, ppm	Cabbage	Lettuce	Carrot	Potato	Apple	Grape	Orange	Grapefruit	Green onion	Chili pepper	Mean	SD	RSD
Cygon oxygen	0.30	46.9	55.8	47.9	59.7	40.0	38.0	38.6	51.2	69.8	45.6	49.4	9.6	19.5
Cygon	0.12	94.1	92.8	106.3	79.7	106.2	63.5	91.8	37.6	45.3	86.0	80.3	22.8	28.4
Diazinon	0.02	136.5	87.7	136.9	82.4	62.0	109.7	156.6	101.2	175.2	156.3	120.5	35.4	29.4
Parathion	0.05	93.6	82.1	98.2	70.6	89.5	86.3	106.7	78.1	89.2	93.4	88.8	9.7	11.0
Malathion	0.03	104.9	106.1	127.5	124.9	81.7	96.4	140.3	116.5	84.5	121.8	110.5	18.2	16.5
Dursban	0.04	110.9	96.4	117.7	102.9	100.9	104.2	144.4	159.2	106.9	137.8	118.1	20.3	17.2
Supracide	0.02	105.7	118.4	101.5	105.6	70.6	112.2	113.7	78.3	99.2	103.9	100.9	14.4	14.3
Ethion	0.05	92.6	89.2	93.7	85.3	100.6	101.8	100.5	70.2	81.5	104.7	92.0	10.2	11.1
EPN	0.20	107.0	95.3	74.5	69.6	73.9	67.6	52.5	30.4	70.3	90.8	73.2	20.6	28.2
Phosalone	0.20	102.9	100.5	88.8	77.9	43.3	85.9	56.1	27.4	62.5	103.0	74.8	25.1	33.6
Mean		99.5	92.4	99.3	85.9	76.9	86.6	100.1	75.0	88.4	104.3			
SD		21.2	15.6	24.5	18.7	22.2	22.5	38.8	39.7	33.6	28.7			
RSD		21.3	16.9	24.7	21.8	28.9	26.0	38.8	52.9	37.9	27.5			

Experiment 6—Metallic Pesticide Investigation

Because chemists have been able to synthesize new compounds around active metals, the use of metallic pesticides has been on the rise. Most metallic pesticides are nonvolatile and are impossible for an ordinary GC system to detect. There is no simple method that can be used to analyze this group of compounds in produce. For example, plictran (tricyclohexyl tin hydroxide) and vendex (di[tri-2, 2-dimethyl-2-phenylethyl tin] oxide) must react with HBr to prepare a brominated derivative (6). The derivatives can then be analyzed by GC with halogen sensitive detectors, such as the ECD or ELCD. A resulting signal is detected from bromide, but no information is received about metallic Sn. Regardless, this is still the best method for today's primary test; however, a problem appears when confirmation is required. The available method for confirmation relies upon atomic absorption spectroscopy (AAS) in testing for Sn. However, the presence of vast amounts of Sn in the environment may interfere with a nonspecific AAS method. Experiment 6, therefore, demonstrated the superiority of using GC/MIP/AED for the analyses of these compounds.

The chromatograms of plictran and vendex are presented in Figures 6 and 7, respectively. Each figure consists of 2 chromatograms; the top is Sn-303 and the bottom is Br-478. The retention times are printed on top of the peaks. The peaks from Sn-303 and Br-478 elute at almost the same time. On the basis of the information from 2 distinguished elements, the chance of misidentification is very slim.

The operation of the photodiode array in the AED hardware must be thoroughly understood. The array is not a fixed unit; it moves up or down vertically depending on the wavelengths of the elements to be acquired. If the distances of the 2 wavelengths are within 24 nm, the 2 elements are detected simultaneously. Otherwise, the elements are scanned separately with 2 or more consecutive GC injections. The entire operation is controlled by the AED computer in the ChemStation.

In the case of plictran analyses, the distance in wavelengths for Sn-303 and Br-478 is 175 nm. Hence, the data are acquired by 2 consecutive GC injections. The peak retention times, which for Sn-303 was 3.849 min and for Br-478 was 3.712 min, were nearly the same. Again, the peak retention times for vendex, which for Sn-303 was 12.289 min and Br-478 was 12.160 min, are close, but not identical. Small discrepancies in retention time were a normal aspect of GC/MIP/AED analysis throughout the entire study.

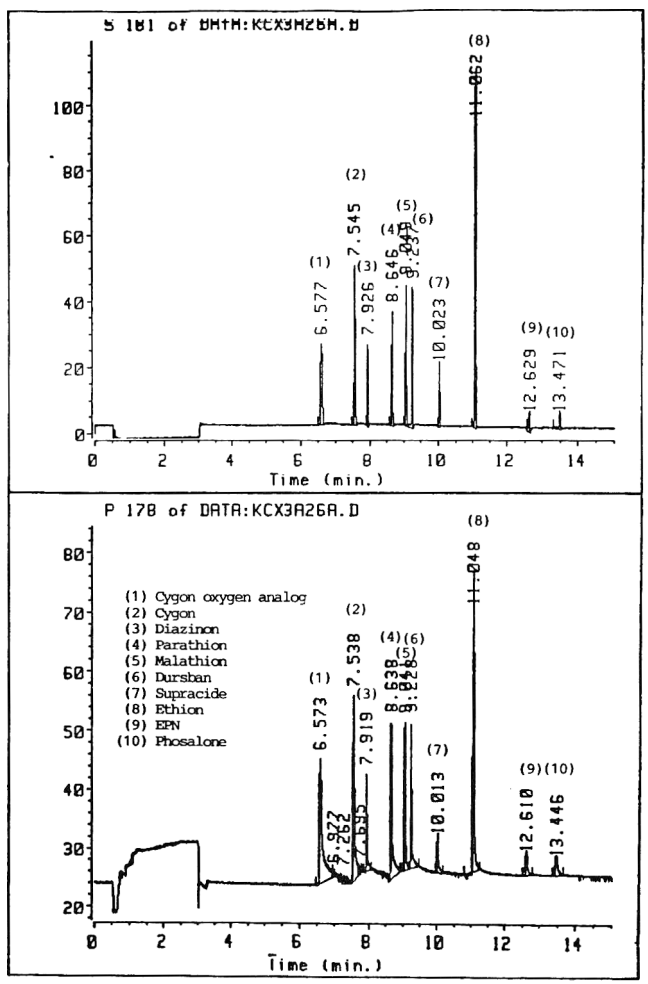

Figure 4. Chromatograms of grapes with spiked organophosphate pesticides and metabolite by S-181 (top) and P-178 (bottom) of GC/MIP/AED. Notice tailing peaks in P-178 chromatogram.

Table 6. Recoveries (%) of carbamate pesticides in produce (without cleanup)

		Leafy vegetables		Roots		Fruits		Citrus		Spices		Statistics		
Pesticides	Spiked amt, ppm	Spinach	Lettuce	Carrot	Yam	Apple	Cantaloup	Orange	Lemon	Green onion	Parsley	Mean	SD	RSD
Aldic sulfone	1.71	95.7	225.4	17.1	64.8	107.9	184.6	85.3	—[a]	135.6	30.3	105.2	68.2	64.8
Bendiocarb	2.88	58.8	82.9	28.7	420.4	69.0	88.0	95.7	163.8	106.0	1082.1	219.5	322.6	146.9
Carbaryl	2.84	—	57.2	28.4	—	25.2	333.6	66.0	289.8	—	—	133.4	127.6	95.6
Methiocarb	3.18	—	32.8	31.8	—	35.3	41.0	—	137.3	24.1	596.6	128.4	210.1	163.6
Propoxur	2.85	150.3	448.9	28.5	117.5	320.6	394.1	144.7	167.5	124.2	73.5	197.0	140.9	71.5
Pirimicarb	0.64	117.3	193.6	6.4	95.8	112.0	168.0	97.5	—	92.3	44.0	103.0	53.5	51.9
Mean		105.5	173.5	23.5	174.6	111.7	201.6	97.8	189.6	96.4	365.3			
SD		38.4	141.7	9.8	165.3	108.4	137.6	29.0	68.1	43.7	465.8			
RSD		36.4	81.7	41.7	94.6	97.1	68.2	29.7	35.9	45.3	127.5			

[a] Not determined.

Conclusion

The GC/MIP/AED system is a powerful multielement analyzer. Because of its 3-dimensional spectrum display, GC/MIP/AED possesses the flexibility to be both general and selective. In many cases, the primary test and confirmation can be accomplished together; consequently, half of the operation time for an entire analysis may be saved. In comparison to gas chromatography/mass spectrometry, the instrument is simpler to operate, easier to maintain, and its chromatograms can be interpreted by an analyst without much prior training.

On the basis of pesticide recovery data, this method has demonstrated its accuracy, reproducibility, and simplicity; therefore, it is considered to be an alternative method for organochlorinated and organophosphate pesticide residue analyses. Although the method is not recommended for quantitative carbamate pesticide residue analysis, it is a simple and fast confirmation method for LC postcolumn derivatization analysis. One superb feature of the instrument is its capability to detect metallic pesticides, such as plictran and vendex. More research studies should be focused in this area.

Overall, GC/MIP/AED is a powerful instrument to have for identification because of its unique ability to provide molecular elemental composition information (7). During the course of this study, a reference regarding LC/AED (8) has come to my attention. Therefore, many new developments associated with AED can be expected in the future.

Acknowledgments

The author wishes to thank Dennis Dingle (District Sales Manager, Hewlett-Packard) for lending the HP 5921A system to this laboratory. Larry Casey (Service Engineer, Hewlett-Packard) gave a lecture on the operation and maintenance of GC/MIP/AED system that was invaluable. Appreciation is extended to Jim Crabtree (Application Engineer, Hewlett-Packard) for his technological advice and consultation. The author also wishes to thank Diane Sanders (Sales Representative, Hewlett-Packard) for her patience and coordination skills that made this study possible. Finally, the author wishes to thank Bill Cusick (Chief of Chemistry, CDFA) for his permission and encouragement of this research work.

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Table 7. Recoveries (%) of carbamate pesticides in produce (with cleanup)

		Leafy vegetables		Roots		Fruits		Citrus		Spices		Statistics		
Pesticides	Spiked amt, ppm	Bok choy	Lettuce	Carrot	Radish	Tomariilo	Cantaloup	Orange	Lemon	Green onion	Chili pepper	Mean	SD	RSD
Aldic sulfone	1.71	103.9	84.4	66.4	98.6	105.9	68.5	47.2	67.1	221.4	103.3	96.7	48.2	49.9
Bendiocarb	2.88	—[a]	44.7	12.6	—	51.5	73.9	12.0	—	—	166.7	60.2	57.3	95.2
Carbaryl	2.84	46.4	36.9	16.5	28.8	54.3	—	32.7	165.3	26.3	—	50.9	47.7	93.7
Methiocarb	3.18	35.1	18.2	422.2	20.2	43.2	22.4	17.9	26.3	39.5	64.3	70.9	124.3	175.2
Propoxur	2.85	111.5	127.0	100.8	128.8	232.6	91.7	67.3	110.7	374.9	207.2	155.3	92.5	59.6
Pirimicarb	0.64	101.3	102.4	81.6	100.1	8.3	79.8	57.5	—	45.0	53.3	69.9	31.7	45.3
Mean		79.6	68.9	116.7	75.3	82.7	67.3	39.1	92.4	141.4	119.0			
SD		35.9	42.2	153.8	48.0	79.8	26.5	22.0	59.6	153.2	66.4			
RSD		45.1	61.3	131.8	63.8	96.6	39.4	56.3	64.5	108.3	55.8			

[a] Not determined.

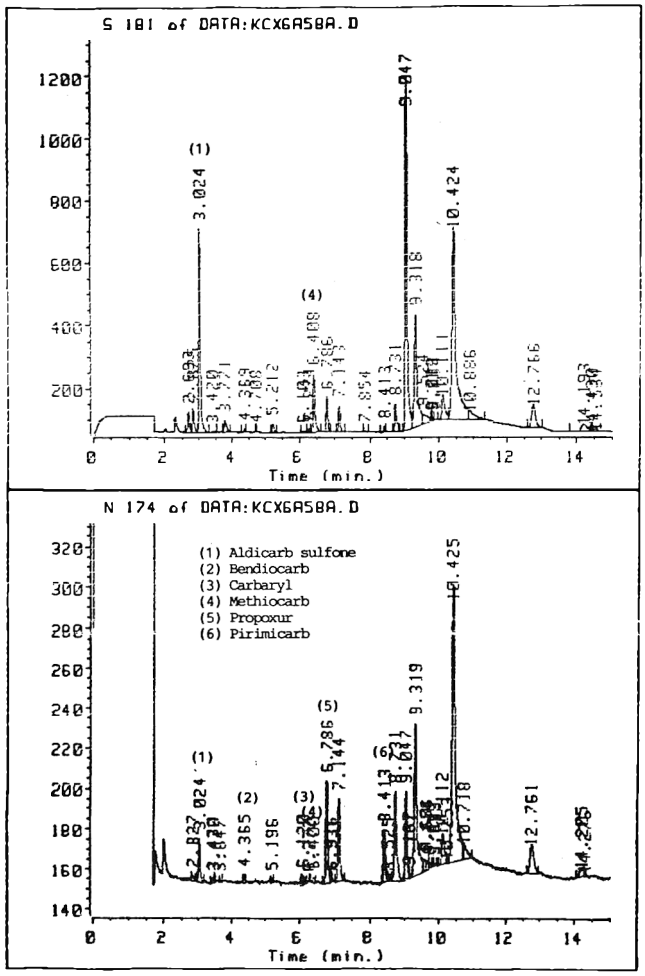

Figure 5. Chromatograms of head lettuce with spiked carbamate pesticides by S-181 (top) and N-174 (bottom) of GC/MIP/AED.

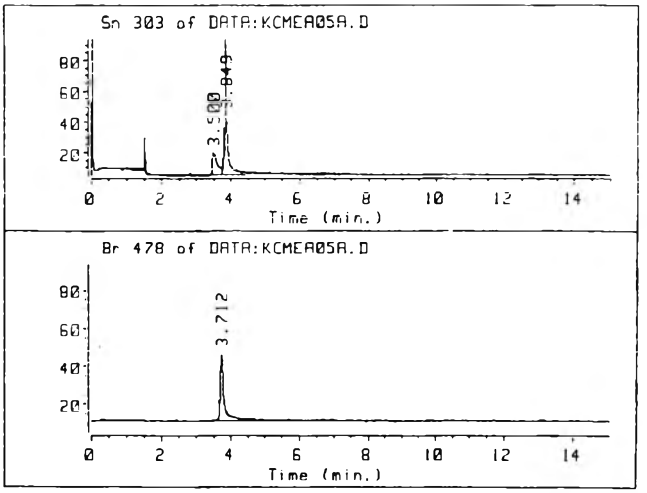

Figure 6. Chromatograms of plictran by element-wavelength Sn-303 (top) and Br-478 (bottom) of GC/MIP/AED.

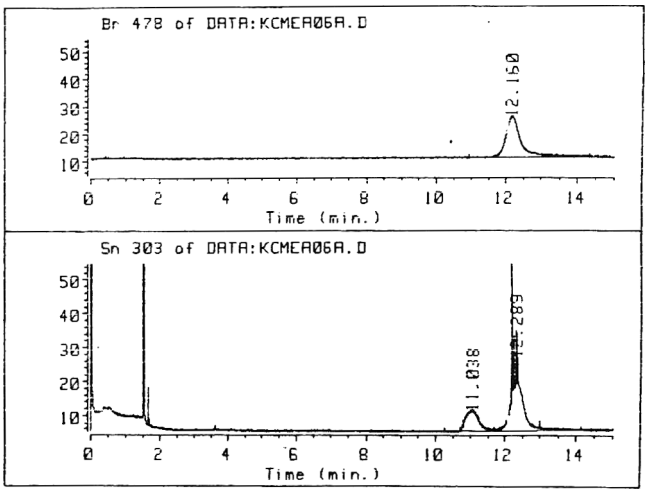

Figure 7. Chromatograms of vendex by element-wavelength Br-478 (top) and Sn-303 (bottom) of GC/MIP/AED.

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PESTICIDE FORMULATIONS

Separation of Clethodim Herbicide from Acid and Photodegradation Products by Liquid Chromatography

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Clethodim, (*E,E*)-(±)-2-[1-[[(3-chloro-2-propenyl)oxy]imino] propyl]-5[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one, is stable in acetonitrile, but the inclusion of water induced degradation in the dark. Addition of acetic acid to the mobile phase did not increase degradation; however, it did improve peak symmetry and liquid chromatographic (LC) separation, with the optimum resolution at 0.75% acetic acid. The extinction coefficient for clethodim at 254 nm did not change in acetonitrile with addition of water or acetic acid. The LC detector responded linearly to clethodim in the 0.7–400 ppm range. Separate solvent gradient programs (40 min each) were developed for separation of acid degradation products (ADPs) and the photodegradation products (PDPs). The inclusion of cobalt or silver nitrate to the mobile phase did not improve separations. A minimum of 31 and 19 products formed during photolytic and hydrolytic degradation of clethodim, respectively. PDPs were more polar than ADPs. Reproducibility of this method was considered to be acceptable for separation of ADPs and PDPs of clethodim.

Cyclohexanediones are bioactive compounds originally discovered in insects. Many homologs of this class have recently been synthesized, and some are effective herbicides. Clethodim selectively kills grasses in broadleaf crops (1). Field trials with clethodim and other cyclohexanedione herbicides resulted in inconsistent efficacy, which is possibly related to instability of the active ingredient. Sethoxydim, 2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio) propyl] -3-hydroxy-2-cyclohexen-1-one, another cyclohexane-1,3-dione herbicide, undergoes degradation (2, 3), including photodegradation. Clethodim is also degraded in aqueous solution by acid and light. Clethodim degradation increased as solution pH decreased, and photolysis was more rapid and more complete than hydrolysis. The previously used liquid chromatographic (LC) method did not adequately separate all degradation products (4). Therefore, we developed an LC method for the separation of both acid degradation products (ADPs) and photodegradation products (PDPs) of clethodim.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters (Millipore Corp., Bedford, MA 01730) system composed of 2 Model 501 solvent delivery systems, Model 700 Satellite WISP autosampler equipped with 200 µL sample loop injector, system interface module, and Model 484 tunable absorbance detector set at 254 nm (0.5 AUFS). Analytical column: 25 cm × 4.6 mm id, containing 5 µm Hypersil C18 (Applied Science Labs, State College, PA 16804).

(b) *pH meter.*—Model 701A/digital ionalyzer (Orion Research Inc., Boston, MA 02129), with research grade combination pH electrode (Fisher Scientific, Pittsburgh, PA 15219).

(c) *Solvent filtration apparatus.*—Millipore glass filter holder with Type HV, 0.45 µm membrane (Millipore). Pyrex 1 L suction flask. Aspirator pump, Model 7050-00 (Cole-Parmer Instrument Co., Chicago, IL 60648).

(d) *Plastic film.*—No. 9076 (Nugget Distributor, Stockton, CA).

Reagents

(a) *LC solvents.*—Acetonitrile, LC grade; glacial acetic acid, analytical grade (J.T. Baker, Phillipsburg, NJ 08865). Filtered through 0.45 µm membrane and degassed with sonication 15 min.

(b) *LC water.*—Deionized, distilled, filtered, and degassed as above solvents.

(c) *Cobalt nitrate (2.0%), silver nitrate (0.1 and 2.0%).*—Analytical grade (Baker), aqueous solutions filtered and degassed as for LC solvents.

(d) *Clethodim stock solution.*—10 000 ppm in acetonitrile, in actinic volumetric flask.

Solvent Effects

Stock clethodim aliquots were diluted to 100 ppm with appropriate solvent (Table 1) and pH was measured. Clethodim was diluted directly in LC vial and injection occurred within 1 min after mixing. The vial, wrapped in aluminum foil, remained in LC autosampler and was assayed repeatedly over the time course. Injection volume was 20 µL, with mobile phase of acetonitrile–water (1 + 1) or dilute acetic acid delivered at flow rate of 1.0 mL/min. Many solvent programs were investigated. Addition of 2% cobalt nitrate or 0.1 or 2.0% silver nitrate to mobile phase did not improve peak resolution. The following linear gradient program was selected: 30–65% acetonitrile in 20 min, held at 65% for 12 min, returned to 30% in 1 min, and re-equilibrated with ca 2.33 column volumes of solvent.

Methods Development

Stock clethodim aliquots were diluted to various concentrations (100–1000 ppm) with acetonitrile–0.75% acetic acid (1 + 1, v/v). To determine the influence of acid on clethodim degradation, clethodim was diluted with solvent in the LC vial. The foil wrapped vial remained in the autosampler for the reaction time periods (Table 1). To determine the influence of light on clethodim degradation, 10–20 mL clethodim

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Table 1. Effect of solvent composition on detector response (×10³) to clethodim (t_R = 30.9)[a] and clethodim recovery following degradation in the dark

Solvent[b]	pH	Reaction time, min	t_R (min) 13.3	18.2	30.9	Clethodim rec., %
			Detector response			
ACN: H20	5.5	0	12.8 ± 0.4	19.6 ± 0.6	216 ± 6	100
		45	13.0 ± 0.5	61.6 ± 0.9	180 ± 6	83
		90	13.0 ± 0.6	88.9 ± 1.3	158 ± 6	73
		135	13.0 ± 0.5	104 ± 3	146 ± 5	68
		180	13.1 ± 0.5	111 ± 4	141 ± 5	65
ACN: 2.6% HAc	3.18	0	13.1 ± 0.2	20.0 ± 0.6	223 ± 2	100
		45	13.1 ± 0.2	62.8 ± 1.5	185 ± 3	83
ACN: 1.4% HAc	3.25	0	12.8 ± 0.2	20.4 ± 0.5	220 ± 5	100
		45	12.6 ± 0.6	62.0 ± 2.3	185 ± 2	83
ACN	5.0	0	11.0 ± 0.1	17.0 ± 0.2	221 ± 2	100
		45	11.1 ± 0.1	17.6 ± 1.3	220 ± 2	100

[a] t_R = retention time.
[b] ACN = acetonitrile. HAc = glacial acetic acid. Ratio of ACN–water aqueous was 1:1.

was diluted with solvent to a concentration of 1000 ppm, placed in a 100 mL Pyrex beaker, covered with plastic film, and placed in sunlight (ca 1000 W/m² s) at Griffin, GA (latitude 33.4° N) for various time periods during July (Table 2). Following exposure to light, a portion was pipetted into an LC vial and assayed using an LC injection volume of 20 or 200 μL.

Linearity, Sensitivity, and Reproducibility

Instrument-response linearity and sensitivity were determined by methods used to determine the influence of acid on clethodim degradation, except the clethodim concentration ranged up to 400 ppm. Methods for determining method reproducibility were the same, except a 20 mL test solution was incubated in Pyrex petri dishes.

Data were collected and processed with Maxima 820 software (Dynamic Solutions Corp., Ventura, CA 93003) via a system interface module (Millipore) between the detector and an NEC APC IV/Powermate 1 computer.

Results and Discussion

Solvent Effects

Clethodim eluted at 30.9 min and was stable in acetonitrile with no degradation during 45 min in the dark (Table 1). Ap-

Table 2. Detector response (×10³) to clethodim (t_R = 31.2)[a] and ADPs separated by LC (acid method) following several treatment periods in 2 experiments

Exp.	Time	t_R (min) 11.3	14.7	16.0	26.6	27.0	27.6	31.2
1	0 h	18 ± 0.6	1.4 ± 0.4	31 ± 1.5	7.3 ± 0.5	7.2 ± 0.3	13 ± 0	470 ± 15
	1	18 ± 0.6	2.0 ± 0.4	97 ± 6	7.2 ± 0.5	6.8 ± 0.6	11 ± 0.6	377 ± 12
	2	19 ± 0.6	2.5 ± 0.2	128 ± 8	7.4 ± 0.2	6.7 ± 0.4	9.8 ± 0.2	337 ± 8
	4	19 ± 0.6	3.9 ± 0.1	143 ± 6	7.4 ± 0.2	6.4 ± 0.2	9.4 ± 0.1	314 ± 8
	8	19 ± 0.0	6.8 ± 0.1	141 ± 7	7.1 ± 0.2	6.1 ± 0.2	9.1 ± 0.2	312 ± 6
	1 day	20 ± 1	18 ± 1	156 ± 7	6.4 ± 0.4	5.4 ± 0.6	8.4 ± 0.3	308 ± 13
	2	22 ± 1	36 ± 3	161 ± 13	5.2 ± 0.5	3.9 ± 0.4	7.7 ± 0.1	306 ± 17
	4	26 ± 3	91 ± 20	178 ± 19	0	0	5.1 ± 0.4	269 ± 5
	8	28 ± 1	99 ± 4	203 ± 8	0	0	4.6 ± 0.1	245 ± 8
2	0 h	21 ± 1	1.6 ± 0.1	42 ± 3	7.6 ± 0.5	6.7 ± 0.2	13 ± 0.5	552 ± 17
	1	21 ± 1	2.1 ± 0.1	119 ± 8	7.7 ± 0.3	6.4 ± 1	10 ± 0.5	449 ± 9
	2	21 ± 0	2.8 ± 0.1	153 ± 2	7.4 ± 0.3	6.2 ± 0.2	8.9 ± 0.1	396 ± 8
	4	21 ± 0	3.9 ± 0.1	170 ± 9	7.1 ± 0.3	5.9 ± 0.2	8.1 ± 0.4	364 ± 3
	8	21 ± 0	6.5 ± 0.3	162 ± 1	5.8 ± 0.2	3.7 ± 0.1	8.7 ± 0.3	356 ± 9
	1 day	22 ± 0	16 ± 1	191 ± 2	7.0 ± 0.1	5.3 ± 0.2	7.7 ± 0.4	353 ± 5
	2	23 ± 1	32 ± 2	199 ± 10	5.6 ± 0.3	3.9 ± 0.2	7.3 ± 0.4	343 ± 2
	4	24 ± 0	66 ± 5	170 ± 8	4.8 ± 0.6	3.0 ± 0.6	6.1 ± 0.5	306 ± 2
	8	25 ± 1	123 ± 12	149 ± 6	3.4 ± 1	0	4.8 ± 0.3	244 ± 3

[a] t_R = retention time.

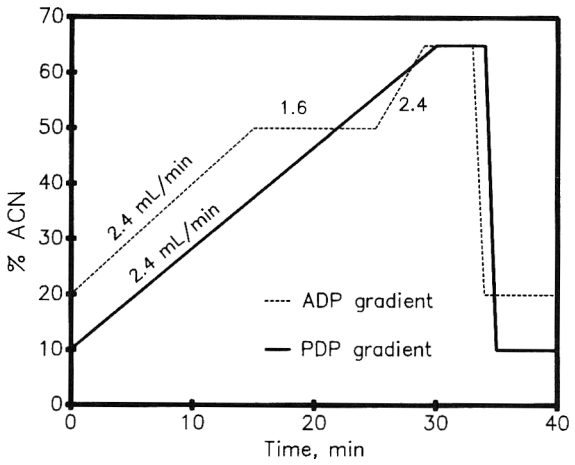

Figure 1. LC solvent gradients for separation of ADPs and PDPs.

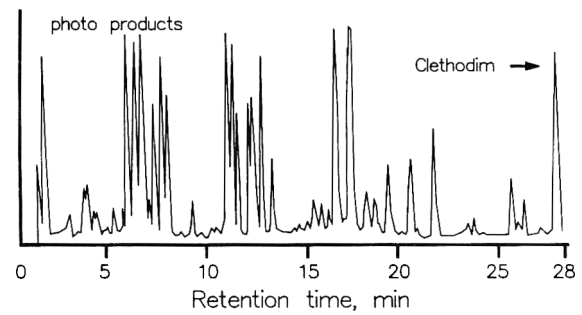

Figure 2. Chromatogram of PDPs of clethodim showing separation by LC with UV detection at 254 nm.

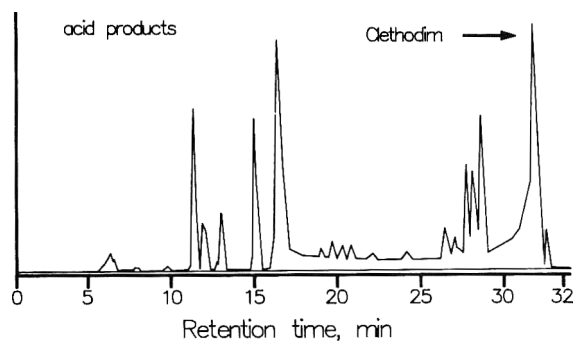

Figure 3. Chromatogram of ADPs of clethodim showing separation by LC with UV detection at 254 nm.

proximately 17% clethodim degradation occurred over 45 min when clethodim was placed in aqueous acetonitrile. Addition of low concentrations of acetic acid did not increase the degradation rate compared to the degradation rate in water alone over a 45 min period (an LC run was 40 min). Over a much longer time period (20 h), increased acidity resulted in increased clethodim degradation (4). Although low acetic acid concentrations (1.4 and 2.6%) did not increase the degradation rate over the short term, they did improve peak symmetry and resolution. The optimum acetic acid concentration was 0.75%; lower concentrations resulted in poor peak resolution, and higher concentrations did not affect resolution. Higher acetic acid concentrations increased clethodim degradation during long-term, multiple-injection LC separations (data not included). Therefore, additional studies were conducted using 0.75% acetic acid.

Clethodim absorbance was not affected by the 4 solvent systems at 0 h (Table 1). Significant acid degradation of clethodim did not seem to occur during LC processing because absorbance in acetonitrile was equal to the other 3 solvent systems at t = 0. Clethodim degradation resulted in an increase of the product at t_R = 18.2 min. The extinction coefficient of the product exceeded that of clethodim at 254 nm.

Acid and Photoproducts Method Development

During development of the method for determining ADPs, a water–acetonitrile solvent system resulted in improved resolution of peaks at t_R = 26–28 min, while a dilute acetonitrile–acetic acid solvent system gave the best resolution of the peaks at t_R = 13–20 min. Therefore, a solvent system of acetonitrile–0.75% acetic acid was used to separate both ADPs and PDPs. The inclusion of cobalt nitrate (2%) or silver nitrate (0.1 or 2.0%) to the dilute acetic acid solvent did not affect peak resolution. A 6-step gradient with a variable flow rate method and a 4-step gradient with a static flow rate method, using acetonitrile–0.75% acetic acid, were developed for separation of ADPs and PDPs, respectively (Figure 1).

The early eluting polar compounds of PDPs did not separate well using the acid method. Solution volumes in excess of 10 mL in a 100 mL beaker resulted in poor LC separations, indicating that increasing reaction volume resulted in decreased photodegradation. However, 20 mL clethodim readily degraded in shallow petri dishes (8 cm diameter). This difference was probably due to the differential accessibility of UV light to the clethodim molecules. Flow rate was 2.4 mL/min. Clethodim eluted at t_R = 31.2 min in both programs.

Using the solvent programs (Figure 1), 31 peaks were separated following photocatalysis (Figure 2), and 19 peaks were separated following acid catalysis (Figure 3). More peaks eluted sooner (5–10 min) in PDPs than in ADPs, possibly indicating increased polarity for PDPs. This corroborates earlier evidence in which addition of pure acetonitrile to aqueous solutions of PDPs caused some precipitation, but subsequent addition of water solubilized the precipitate (4).

Linearity, Sensitivity and Reproducibility

The detector responded linearly to clethodim in the 1–400 ppm range. Detector response at 0.25 ppm generated only random background noise. A clethodim concentration of 0.7 ppm yielded a detector response approximately 2 times background. Therefore, lower and upper detection limits of 0.7 and 400 ppm, respectively, were established (data not included).

Table 3. Detector response (×10^3) to clethodim (t_R = 31.2)[a] and PDPs separated by LC (photomethod) following several treatment periods in 2 experiments

		t_R (min)							
Exp.	Time, h	5.8	6.3	6.7	11.0	11.3	16.4	21.4	31.2
1	0	0	0	0	0	0	18 ± 0	31 ± 3	426 ± 8
	1	34 ± 6	3.3 ± 0.6	2.9 ± 1	18 ± 1	19 ± 1	112 ± 6	129 ± 20	156 ± 11
	2	112 ± 13	20 ± 2	19 ± 2	37 ± 2	36 ± 1	178 ± 5	125 ± 8	121 ± 8
	4	499 ± 30	155 ± 17	157 ± 16	75 ± 3	72 ± 2	299 ± 6	68 ± 2	66 ± 1
	6	822 ± 118	335 ± 69	348 ± 73	77 ± 5	76 ± 5	300 ± 17	32 ± 5	31 ± 2
2	0	0	0	0	0	0	18 ± 5	33 ± 3	439 ± 4
	1	26 ± 1	26 ± 0.1	2.3 ± 0.1	16 ± 0.5	16 ± 5	97 ± 2	161 ± 14	147 ± 15
	2	84 ± 10	15 ± 1	14 ± 1	34 ± 0.5	35 ± 1	162 ± 3	148 ± 14	113 ± 7
	4	409 ± 30	120 ± 6	121 ± 7	62 ± 10	60 ± 9	272 ± 20	69 ± 13	73 ± 3
	6	690 ± 23	263 ± 13	268 ± 14	65 ± 2	64 ± 2	286 ± 7	29 ± 2	35 ± 5

[a] t_R = retention time.

Reproducibility was determined by comparing the standard deviation of the mean as a percent of the mean. The reproducibility of analyzing clethodim and ADPs (Table 2) was excellent, with averages of less than 5% in the 2 experiments using the ADPs gradient program (Figure 1). Clethodim retention time (t_R = 31.2) generally varied less than 3%. Assuming that the same compounds were present in the replicates of treated solutions, the following observations were made: (*1*) Retention time of only one ADP (t_R = 14.7) showed significant variability, 11 and 6% in the 2 experiments, respectively; (*2*) retention time of PDPs varied slightly more, with overall variation averaging 8 and 6% in the 2 experiments using the PDPs program; and (*3*) one PDP (t_R = 21.4) gave a moderate level of variation (10%) (Table 3). The slightly higher variation with PDPs was probably caused by slight variations in environmental conditions during reaction. The method reproducibility was considered to be acceptable for separation of clethodim and ADPs and PDPs.

Acknowledgments

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SEAFOOD PRODUCTS

Liquid Chromatographic Determination of the Processing Aid 4-Hexylresorcinol in Shrimp

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A rapid, sensitive, liquid chromatographic (LC) method has been developed for determination of residuals of the processing aid, 4-hexylresorcinol, on shrimp meat. An aqueous homogenate of shrimp meat is extracted with ethyl acetate followed by precolumn preparation on a silica Sep-Pak cartridge. LC determination is preformed with a Nova-Pak C18 column, with UV detection at 214 nm. Sensitivity was 0.006 μg, and recovery from shrimp meat samples of known 4-hexylresorcinol addition was 94%. Shrimp treated with 4-hexylresorcinol under the recommended dip protocol had mean residuals of 1.18 ppm, with a standard deviation of 0.13 ppm.

4-Hexylresorcinol is an effective processing aid for the inhibition of shrimp melanosis (blackspot) in both laboratory and field trials in all species tested (1, 2). The compound is a potent specific inhibitor of the shrimp enzyme polyphenol oxidase, which catalyzes the formation of precursors of blackspot formation. The inhibitor has the potential for application in any food system in which enzymatic browning is problematic, such as apples, potatoes, avocados, and certain juices. A 1 min dip into 50 ppm 4-hexylresorcinol in sea water with subsequent storage on crushed ice inhibits blackspot formation on shrimp for up to 14 days, a typical storage time for fresh shrimp on boats. Experimental protocols using ^{14}C-labelled 4-hexylresorcinol showed that shrimp treated under recommended dip protocols had mean residues of ≤1 ppm (3).

4-Hexylresorcinol use in the food industry requires analytical methodology for routine testing of treated samples. An AOAC official method for titration of 4-hexylresorcinol in drugs has been published (4); however, sensitivity of the method is inadequate for determination of low 4-hexylresorcinol residuals on the edible portion of shrimp. This necessitated development of a method with greater sensitivity for detection and quantitation of 4-hexylresorcinol on shrimp meat.

Experimental

Apparatus

(a) *Liquid chromatography system.*—Series 400 (Perkin Elmer Corp., Norwalk, CT 06859) fitted with Model 7125 200 μL injection loop (Rheodyne, Inc., Cotati, CA 94931), ISS-100 autosampler with 200 μL injection loop, and LC 90 biospectrophotometric UV detector set at 214 nm and interfaced to a Kaypro 286i computer with Omega 2 software, version V1.50 (Perkin Elmer).

(b) *Column.*—Radial-Pak Nova-Pak C18 cartridge column, 8 × 100 mm cartridge, 4 μm particle size (Millipore Corp., Milford, MA 01757), connected to precolumn filter (Upchurch Scientific, Inc., Oak Harbor, WA 98277) equipped with 0.5 μm stainless steel frit.

(c) *Homogenizer.*—Polytron Model PT 10/35 (Kinematica AG, Littau-Luzern, Switzerland) equipped with 20 mm probe.

Reagents

(a) *Acetonitrile and methanol.*—LC grade (J. T. Baker Inc., Phillipsburg, NJ 08865).

(b) *Trifluoroacetic acid (TFA).*—Sigma Chemical Co., St. Louis, MO 63178.

(c) *4-Hexylresorcinol.*—98.4% pure (Riedel-de Haen, Seelze, Germany).

(d) *Water.*—Obtained from Milli-Q reagent water system (Millipore).

(e) *Mobile phases.*—0.1% (v/v) TFA in water, 0.1% (v/v) TFA in acetonitrile, and methanol. All solvents were continuously sparged with helium during operation. Flow rate, 1 mL/min.

Procurement of Shrimp

Pink shrimp (*Penaeus duorarum*) were caught and frozen in Key West, FL, by an individually quick frozen method (IQF) using liquid nitrogen, then shipped frozen to Opta Food Ingredients, Inc. (Cambridge, MA). Shrimp were stored frozen at −20°C and thawed before use.

4-Hexylresorcinol Standard Solutions

4-Hexylresorcinol stock solution was prepared by dissolving 1 mg/mL in water–methanol (9 + 1, v/v). An aliquot of stock solution was diluted 100-fold in water to give a working standard solution. Stock solution was prepared fresh before use.

LC Operation Conditions

Equilibrate column with initial mobile phase of 0.1% TFA, 5% acetonitrile. After injection of sample, develop column with linear gradient to 0.1% TFA, 20% acetonitrile over 15 min, followed by linear gradient to 0.1% TFA, 95% acetonitrile over the next 20 min. Following 2 min linear gradient to 100% methanol, wash column 2 min with methanol before re-equilibration, and wash with initial mobile phase 9 min before next injection.

A typical set of LC runs consisted of the following: a blank of the unknown sample matrix without added 4-hexylresorcinol, an unknown sample run in triplicate, and a calibration

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Table 1. Recovery of 4-hexylresorcinol from shrimp meat

Weight of shrimp meat, g	4-Hexylresorcinol detected, μg[a]
Unspiked	
12.64	0.278 ± 0.07
12.38	0.240 ± 0.04
12.40	0.116 ± 0.02
Cumulative mean	0.211 ± 0.08
Spiked[b]	
12.72	14.20 ± 0.08
12.21	14.67 ± 0.18
12.88	14.39 ± 0.05
Cumulative mean	14.42 ± 0.23

[a] Each value is the mean of 3 LC analyses as described in *Experimental.*
[b] Samples spiked with 15.12 μg 4-hexylresorcinol.

curve consisting of at least 5 concentrations of 4-hexylresorcinol run in duplicate. Standard samples were either run before and after unknowns or interspersed among unknown samples.

Calculations

A calibration curve consisting of at least 5 concentrations of 4-hexylresorcinol, from 0.25 to 2.0 μg, was run in duplicate. The standard curve was obtained by least squares regression of the peak integration area as a function of micrograms injected (Cricket Graph, Malvern, PA; version 1.3.1i). Concentration of an unknown sample was calculated from least squares regression of the standard curve.

Treatment of Shrimp with 4-Hexylresorcinol

Shrimp were treated according to the previously published method (1), which describes the recommended protocol for use in the shrimp industry. Approximately 1 lb shrimp (heads-on) was placed in a nylon mesh bag. Shrimp were dipped into 1 L 50 ppm 4-hexylresorcinol prepared in sea water (pH 7.5–8) for 1 min. Shrimp were then drained 15 s and stored on bed of crushed ice at 4°C. Random samples were removed after 4 days for analysis of 4-hexylresorcinol residuals.

Table 2. Residual analysis of 4-hexylresorcinol on treated shrimp meat

Weight of shrimp meat, g	4-Hexylresorcinol total, μg[a]	Residuals, ppm
10.36	13.00	1.25
10.04	10.62	1.06
11.66	12.92	1.11
8.25	11.24	1.36
9.73	10.66	1.10
Cumulative mean		1.18 ± 0.13

[a] Each value is the mean of 3 LC analyses as described in *Experimental.*

Extraction of 4-Hexylresorcinol from Shrimp Meat

Shrimp were peeled and deheaded manually. Water (25 mL) was added to shrimp meat (ca 10 g meat from 3 shrimp). Meat was then homogenized 5 min. To avoid contamination, probe was rinsed with fresh ethyl acetate between samples. The resulting white paste was transferred to a 50 mL centrifuge tube, vortexed vigorously with 15–20 mL ethyl acetate, and centrifuged at 3000 × *g* for 10 min. The ethyl acetate phase was removed and extraction was repeated twice more with fresh ethyl acetate. Combined ethyl acetate extracts were dried over anhydrous sodium sulfate before rotary evaporation to dryness. Resulting residue was dissolved in 2 mL 10% ethyl acetate in hexane. Solution was loaded onto silica Sep-Pak cartridge (Millipore). The cartridge was first washed with an additional 5 mL 10% ethyl acetate in hexane (eluant was discarded), then eluted with 7 mL 50% ethyl acetate in hexane. Latter eluant was rotary evaporated to dryness. Resulting residue was dissolved in 0.5 mL methanol, then 1 mL water was added. Solution was centrifuged in Eppendorf tubes for 10 min in microcentrifuge. An aliquot of supernatant (diluted with an equal volume of water) was analyzed for 4-hexylresorcinol by LC as described in *Experimental.*

Results and Discussion

Chromatography

4-Hexylresorcinol had a retention time of 32.1 ±0.2 min. The limit of detection was 0.006 μg (peak area of 2.062 × 10^5). Precision of the system was determined by making 6 replicate injections of 0.82 μg; the relative standard deviation of the peak area response was 1.2%. The chromatographic response was linear up to about 2.5–3.0 μg 4-hexylresorcinol injected. The square of the correlation coefficient for 4-hexylresorcinol from 0.25 to 2.0 μg was 0.995.

Detection of 4-Hexylresorcinol Residuals on Shrimp Meat

Control experiments were performed to determine the recovery of 4-hexylresorcinol from shrimp meat. Untreated samples were spiked with 15.12 μg 4-hexylresorcinol. Table 1 summarizes results, and Figure 1 shows typical chromatograms for unspiked (A) and spiked (B) samples. Overall recovery of 4-hexylresorcinol was 94%. Extraction and LC analysis of shrimp meat with no 4-hexylresorcinol added resulted in a peak with the same retention time as 4-hexylresorcinol (probably not endogenous 4-hexylresorcinol, as there are no reports of its presence in nature), and with an area corresponding to an average value of 0.013 ±0.005 μg injected (Figure 1A). This value corresponded to a total of 0.211 μg 4-hexylresorcinol (1.5% of the amount added), and was subtracted from spiked sample values.

Results of residual analyses performed on previously frozen shrimp that were dipped into 4-hexylresorcinol are summarized in Table 2. Heads-on shrimp that were treated according to the recommended method (1 min dip into 50 ppm 4-hexylresorcinol) had 4-hexylresorcinol residuals of 1.18 ±0.13 ppm. Samples of shrimp that were not treated with 4-hexylresorcinol were also analyzed by ethyl acetate extraction and LC analysis according to the method described in *Experimental.* In this case, no peak with the same retention time as 4-hexylresorcinol was detected.

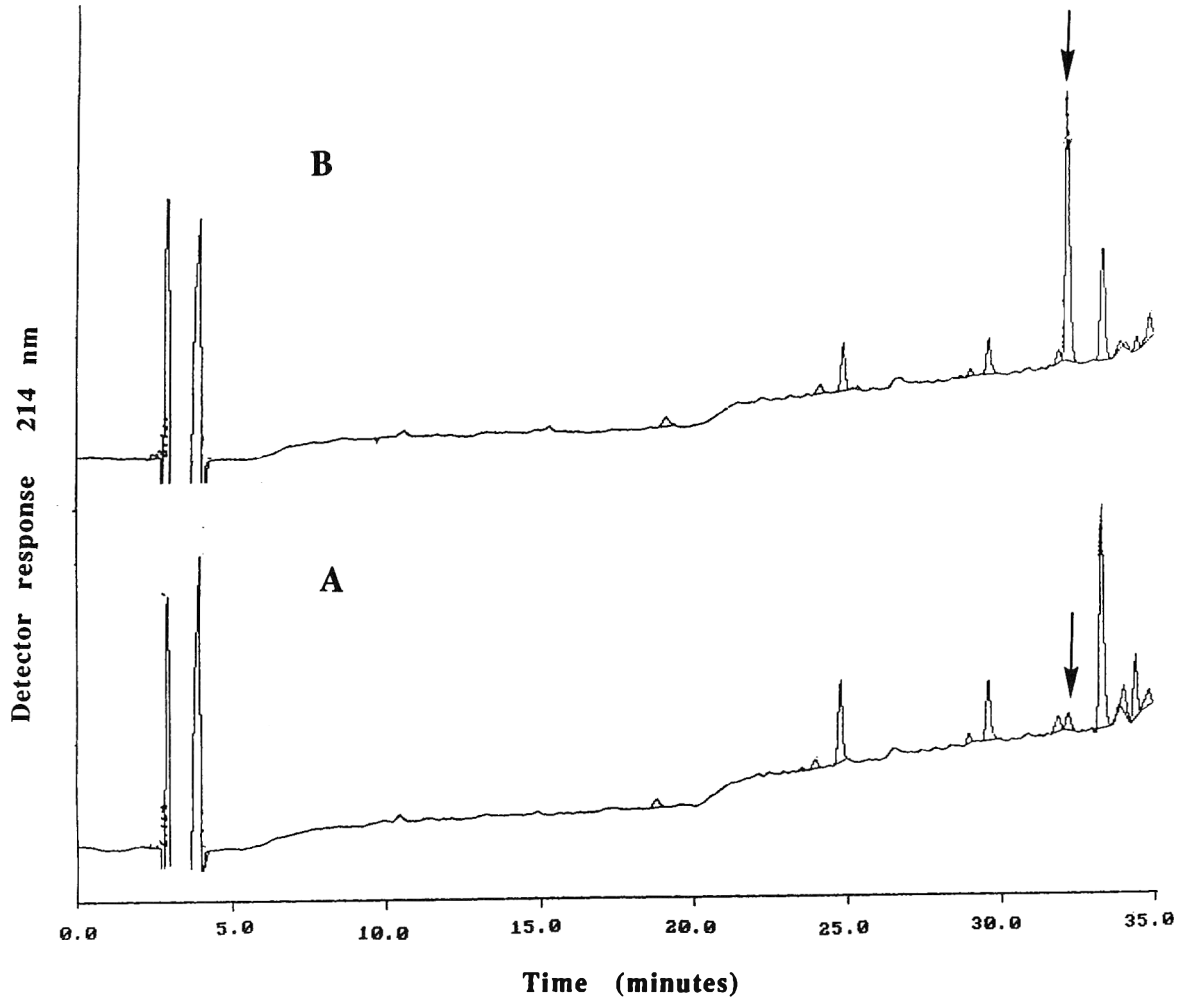

Figure 1. Representative liquid chromatogram of (A) unspiked shrimp meat and (B) shrimp meat spiked with 15.12 μg 4-hexylresorcinol. Arrows show peaks corresponding to 4-hexylresorcinol.

When used as a processing aid for the inhibition of shrimp melanosis, 4-hexylresorcinol results in extremely low residuals on shrimp meat. Mean residuals of 4-hexylresorcinol for previously frozen shrimp presented above are slightly higher than those reported elsewhere (3). In the latter study, which used 4-hexyl-[UL-^{14}C]-resorcinol, average residuals on frozen heads-on and headless shrimp following a 1 min dip were 0.6 ±0.17 ppm and 1.0 ±0.30 ppm, respectively. Higher residuals of 4-hexylresorcinol reported here could be due to the method of freezing the shrimp. The IQF technique could result in more tissue damage and, therefore, slightly higher residuals. Because of the low residuals, no adverse effects on product quality were determined by sensory panel evaluations. Taste, texture, visual appearance, and normal development of pink color upon cooking are unaffected by dipping in 4-hexylresorcinol. The method reported here for 4-hexylresorcinol determination on shrimp meat is simple, sensitive, and reproducible. It can be used for the 4-hexylresorcinol quantitation in other species of shrimp (data not shown). The method can also be adapted easily to 4-hexylresorcinol analysis in other food systems.

4-Hexylresorcinol is a functional alternative for sulfite in the shrimp industry. Present regulations prescribe a dip treatment of 1 min in 12 500 ppm (1.25%) sodium bisulfite solution with an allowable sulfite residual of 100 ppm (5, 6). Problems associated with adverse reactions in a selected proportion of the population that is hypersensitive to sulfites are well documented (7). Consumer awareness of dangers associated with sulfite use has created a need for a substitute, and the safe use of 4-hexylresorcinol in the shrimp industry is the subject of a recent review (8). In light of the toxicological data and very low residuals on shrimp meat, 4-hexylresorcinol is a safe, effective alternative to sulfites.

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SEAFOOD TOXINS

Liquid Chromatographic Determination of Paralytic Shellfish Poisons in Shellfish After Prechromatographic Oxidation

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A liquid chromatographic method for quantitating paralytic shellfish poison toxins in shellfish has been developed in which the toxins are converted to fluorescent purines by prechromatographic oxidation under mildly basic conditions with hydrogen peroxide or periodate. The addition of ammonium formate to the periodate oxidation reaction greatly improved the yield of fluorescent derivatives for neosaxitoxin, gonyautoxin-1, B-2, and C-3 compared to the same reaction without ammonium formate. As little as 3–6 ng of each of the nonhydroxylated toxins and 7–12 ng of the hydroxylated compounds per gram of shellfish could be detected. Reversed-phase chromatography using ammonium formate in the mobile phase improved the chromatography of neosaxitoxin and B-2 compared to results obtained earlier. Because the oxidation products of neosaxitoxin and B-2 could not be separated, parent compounds were separated before oxidation by using an SPE-COOH ion exchange cartridge. The repeatability coefficient of variation for the oxidation reactions ranged from 3 to 8% for the peroxide reaction, and from 4 to 11% for the periodate reaction, depending upon the individual toxin determined and its concentration in the extract (0.04–0.55 μg/g). The method was compared to the mouse bioassay and the postcolumn oxidation method. In most cases, results were comparable.

A number of methods for determination of paralytic shellfish poisons (PSP) have been reported in the literature. The most commonly used chemical method thus far involves liquid chromatography (LC) with gradient elution, followed by postcolumn oxidation and fluorescence detection (1). This method was modified to include isocratic separations on silica-based reversed-phase columns (2) and changes in the postcolumn reaction system (3). The method of Oshima et al. (2) is easier to use than the original procedure (1), it yields better sensitivities for individual toxins, and it provides excellent separation of all toxins, including several decarbamoyl toxins. To determine all toxins, 3 different mobile phases are required. For research purposes, this method is probably the best currently available; however, it can be somewhat time-consuming to setup and perform PSP determinations on an occasional basis. In addition, it has been reported that the postcolumn LC method (1) failed in a collaborative trial among 9 laboratories (4). As a result, we have been studying alternative means to determine PSP toxins that would require less equipment assembly and maintenance, but would be as sensitive as existing methods for determinations that are carried out only on an occasional basis.

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Recently, we reported results of an investigation of prechromatographic oxidation and LC of PSP toxins (5). That work dealt mainly with studying the oxidation products in terms of structure, fluorescence properties, reactions involved, and chromatographic separation of the individual products. Some attempts were made to apply the method to the quantitative determination of PSP toxins in shellfish. However, there were indications of sample matrix effects on recoveries of certain toxins, and there was some problem with the chromatography of neosaxitoxin (NEO) and B-2. In addition, the relative sensitivities of the *N*-1-hydroxylated toxins were far less than those of their nonhydroxylated analogues. The work presented here describes improvements to the prechromatographic oxidation technique for the quantitative determination of PSP toxins in shellfish.

Experimental

Apparatus

(a) *LC system.*—Consisted of 2 pumps (Model 110B) with gradient controller (Model 421A) and injection port (Altex Model 210A) with 20 μL loop (Beckman Instruments, Inc., San Ramon, CA 94583).

(b) *LC column.*—Supelcosil LC-18, 15 cm × 4.6 mm id, 5 μm (Supelco, Bellefonte, PA 16823).

(c) *Fluorometric detector.*—Model 820-FP; excitation, 330 nm; emission, 400 nm; and gain, 10 (Jasco, Inc., Easton, MD 21601). Used for monitoring LC effluent.

(d) *Integrating recorder.*—Model 4270; attenuation, 4 (Varian Instrument Group, Sunnyvale, CA 94034). Used for monitoring LC effluent.

Note: PSP oxidation products were eluted by using a linear gradient of 0–5% (v/v) acetonitrile in 0.1M ammonium formate (adjusted with formic or acetic acid to pH 6.0 with pH paper) over 15 min at 1.0 mL/min. Mobile phases were prepared fresh every 2 days.

(e) *Postcolumn LC system.*—Assembled and operated as described in ref. 5.

Reagents

All solvents and reagents were analytical or LC grade materials.

(a) *Water.*—Twice deionized (Milli-Q, Millipore, Bedford, MA 01730).

(b) *Purified standards.*—Saxitoxin, NEO, B-1, B-2, C-1, and C-3 (Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC 20204).

(c) *GTX-1, GTX-2, and GTX-3.*—Prepared by hydrolysis of "C" toxins (0.1N HCl, 10 min, 90°C), followed by adjust-

ment to ca pH 5. All standards yielded single peaks with postcolumn LC method (1). Table 1 shows structures of most common PSP toxins.

Sample Cleanup

Pass 0.5 mL shellfish extract, prepared according to mouse bioassay (6), through 3 mL SPE-C18 cartridge (Supelco) conditioned with 6 mL methanol followed by 6 mL water then 3 mL 0.1N HCl. Collect effluent (containing toxins) and rinse cartridge with 2 mL water to elute remaining toxins. Collect eluate and combine with first portion. Adjust volume to exactly 4 mL with water and remove 1 mL solution. Adjust to pH 8.0 (pH paper) with 1N NaOH (25–30 μL) before oxidation reactions.

Ion-Exchange Chromatography

Condition 3 mL SPE-COOH cartridge (J.T. Baker Inc., Phillipsburg, NJ 08865) with 3 mL hexane and dry with vacuum, followed by 3 mL methanol, 3 mL water, 3 mL 0.1M ammonium acetate (pH 7.5, pH paper), and 3 mL water. Pass 0.5 mL sample extract from SPE-C18 cleanup through column. Collect effluent. Pass 2 mL water through cartridge and collect with first effluent. This fraction contains C group toxins. Elute column with 3.5 mL 0.1M ammonium acetate and collect eluate. This fraction contains B-2. Elute remaining toxins with 2 mL 1.0M acetic acid. Adjust aliquots of first and third fractions to ca pH 8.0 (pH paper). Carry each fraction through periodate oxidation to determine C-3, GTX-1, NEO, and B-2.

Peroxide Oxidation

Measure all reagents and solutions (except for peroxide solution, which is measured with a syringe) with Eppendorf autopipets with disposable plastic tips. Prepare individual oxidation reactant solutions fresh weekly for both oxidation reactions. Perform peroxide reaction exactly as described in ref. 5.

Add 25 μL 10% (w/v) aqueous H_2O_2 to 250 μL 1N NaOH in 0.5 mL plastic microcentrifuge tube. Add 100 μL aqueous sample extract. (Actual volume can vary, depending upon anticipated PSP concentration.) Mix solution and allow to react at room temperature (20°C) for 2 min, then add 20 μL concentrated acetic acid and mix well. Inject 20 μL into LC system.

Periodate Oxidation

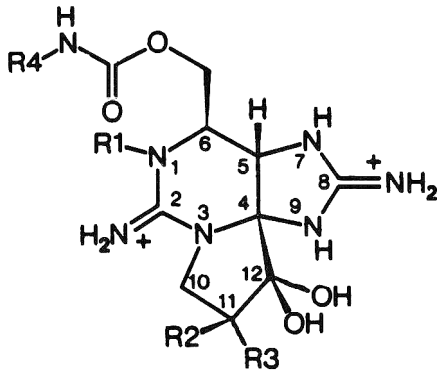
Adjust 500 μL 0.03M periodic acid–0.3M Na_2HPO_4–0.3 M ammonium formate (1 + 1 + 1, prepared daily) to pH 8 (pH paper) with 1N NaOH. Add this solution to 100 μL aqueous sample (actual volume can vary depending upon anticipated PSP concentration) in 1.5 mL plastic microcentrifuge tube, and mix well. Allow mixture to react at room temperature for 3 min, then add 10 μL concentrated acetic acid. Mix and inject 20 μL into LC system.

Results and Discussion

In the original chromatographic study using reversed-phase chromatography (5), the oxidation products of NEO and its sulfamate analogue, B-2, occasionally chromatographed poorly when mobile phases comprised of mixtures of acetonitrile and aqueous phosphate solutions were used. However, the addition of ion-pairing agents, such as tetrabutylammonium or heptane sulfonate, significantly improved peak shape and reproducibility (5). After examining a number of other mobile phase combinations, we found that by replacing the phosphate salts with ammonium acetate or ammonium formate, chromatography and reproducibility of both NEO and B-2 improved significantly and became comparable to those of the other PSP oxidation products. Combinations of aqueous ammonium formate and water were chosen for the mobile phases for the remainder of the study.

An attempt was made to improve the sensitivity of the periodate oxidation reaction for *N*-1-hydroxy toxins, because they were 10–30 times less sensitive than their nonhydroxy analogues. The addition of ammonium formate and formamide to the oxidation mixture was examined because of reports that they significantly improved *N*-1-hydroxy PSP sensitivity in the postcolumn oxidation method (3). Formamide did not have a significant effect. However, a substantial improvement was discovered with the addition of ammonium formate to the reaction mixture. Table 2 lists the sensitivities obtained with the new periodate reaction compared to the peroxide reaction and the old periodate reaction (5). Sensitivities of *N*-1-hydroxy toxins improved about 10-fold, although there

Table 1. Structures of 12 PSP toxins



Substituents				
R1	R2	R3	R4	Toxin
H	H	H	H	Saxitoxin (SAX)
H	H	H	SO_3	B1
H	H	OSO_3	H	Gonyautoxin 2 (GTX 2)
H	H	OSO_3	SO_3	C1
H	OSO_3	H	H	Gonyautoxin 3 (GTX 3)
H	OSO_3	H	SO_3	C2
OH	H	H	H	Neosaxitoxin (NEO)
OH	H	H	SO_3	B2
OH	H	OSO_3	H	Gonyautoxin 1 (GTX 1)
OH	H	OSO_3	SO_3	C3
OH	OSO_3	H	H	Gonyautoxin 4 (GTX 4)
OH	OSO_3	H	SO_3	C4

was some reduction in responses of non-*N*-1-hydroxylated toxins, particularly B-1, C-1, and C-2. The reduction in sensitivity of the nonhydroxylated toxins is not a problem because they are best quantitated by the peroxide oxidation. Sensitivities of all the most toxic analogues (saxitoxin, NEO, and the GTX's) are much more than adequate for monitoring at 80 μg PSP/100 g.

Figure 1 shows chromatograms of 2 standard mixtures carried through the periodate and peroxide reactions. Under the optimum conditions, each non-*N*-1-hydroxy toxin produced a single major peak with 1 or 2 minor peaks, the total of which amounted to less than 10% of the major peak in area for both the peroxide and periodate reactions, except B-1. B-1 gave a very low response with the periodate reaction. It also showed a second earlier eluting peak that represented about 30% (peak height) of the major peak and eluted at the same time as the major peak of B-2. However, the peroxide reaction is used to quantitate B-1 because of the vastly greater sensitivity for B-1 with that reaction. One of the minor peaks of saxitoxin eluted at the same time as the major peak for NEO. This was taken into account when NEO (or B-2) was quantitated.

The origin of the minor peaks is unknown at present. They appear to be secondary reaction products from the oxidations. Some of them may also arise from impurities in the standards.

As previously reported, *N*-1-hydroxy toxins, except GTX-1, did not produce fluorescent derivatives with the peroxide reaction. GTX-1 produced a derivative with a relatively weak response (see Table 2). All *N*-1-hydroxy toxins yielded products with good sensitivity in the optimized periodate reaction. NEO and B-2 produced the same products (see Figure 1), 1 major peak and 2 minor peaks. One of the minor peaks was a broad peak that eluted before the major peak, with a peak height equivalent to about 20% of the major peak. The other minor peak eluted with the same retention time as saxitoxin. The peak represented about 10% of the major peak of B-2, and 40% of the major peak of NEO. The last peak was not a result of saxitoxin traces because, with the peroxide reaction, it decreased in peak height. Saxitoxin, however, produces a taller peak with peroxide oxidation (see Table 2). The early eluting broad peak (see Figure 1) is useful in confirming the presence of NEO or B-2 in sample extracts.

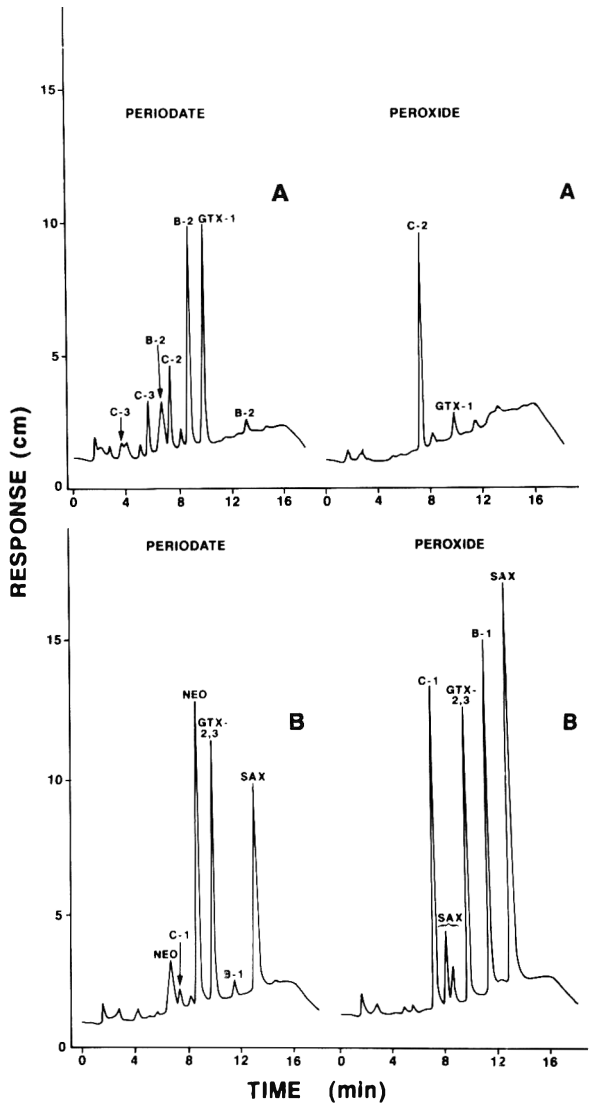


Figure 1. Chromatograms of standard mixtures of PSP toxins after periodate and peroxide oxidation. (A) Quantities injected (periodate): C-3, 32 pg; C-2, 90 pg; B-2, 179 pg; and GTX-1, 109 pg. Quantities injected (peroxide): C-3, 49 pg; C-2, 138 pg; B-2, 277 pg; and GTX-1, 168 pg. (B) Quantities injected (periodate): C-1, 72 pg; NEO, 186 pg; GTX-2/3, 25 pg (each); B-1, 63 pg; and SAX, 53 pg. Quantities injected (peroxide): C-1, 111 pg; NEO, 288 pg; GTX-2/3, 38 pg (each); B-1, 98 pg; and SAX, 81 pg. Conditions as described in the text.

Table 2. Approximate sensitivities of PSP toxins by prechromatographic oxidation

Toxin	Sensitivity (peak height), cm/ng[a]		
		Periodate	
	Peroxide[b]	Old reaction[b,c]	New reaction[d]
Saxitoxin	154	90	60
Neosaxitoxin	< 0.1	2.5	62
B–2	< 0.1	5	41
B–1	141	89	9
GTX–3	105	118	188
GTX–2	111	157	100
GTX–1	1.4	7	71
C–3	< 0.1	7	68
C–2	52	82	27
C–1	74	31	6

[a] Baseline noise approximately 1 mm (peak to peak).
[b] Data from ref. 5.
[c] Reaction using 0.02M phosphate.
[d] Reaction using 0.1M ammonium formate.

Both *N*-1-hydroxy toxins, C-3 and GTX-1, gave the same 3 peaks with the optimized periodate reaction. However, the peak at 10 min (same retention time as the oxidation products of GTX-2 and GTX-3, see Figure 1) was predominant for GTX-1, and was used for its quantitation. The peak eluting at about 6 min was predominant for C-3; therefore, it was used for C-3 quantitation. The earliest eluting (broad) peak (at about 4 min) served as a confirmation of the presence of either C-3 or GTX-1.

In a rapid screening program for regulatory purposes, the peaks eluting at the retention times of NEO/B-2 or C-3/GTX-

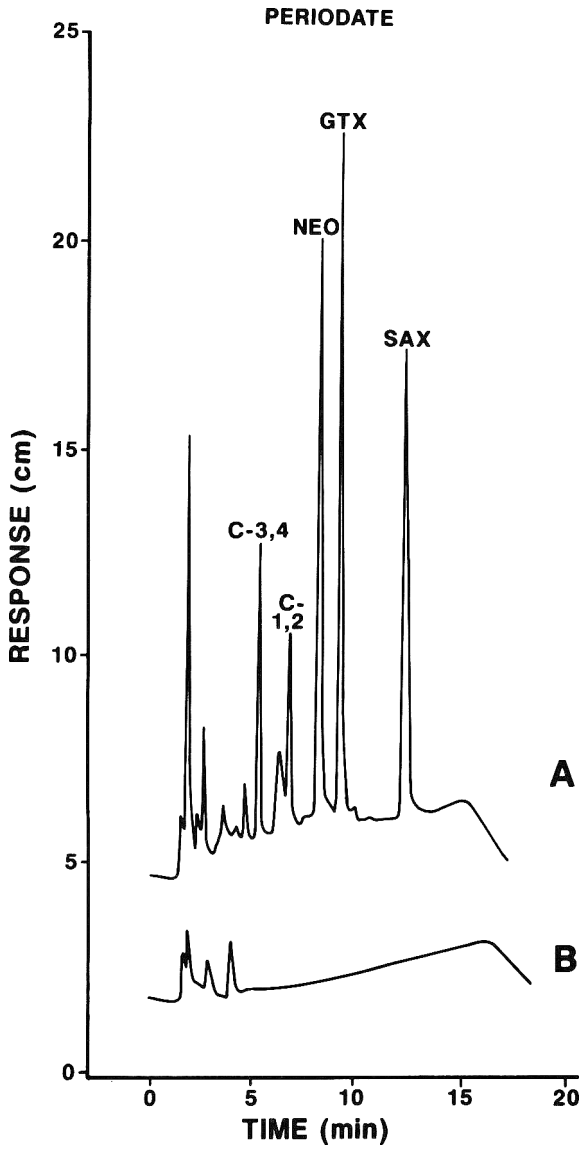


Figure 2. Chromatograms of extracts of (A) contaminated (total calculated PSP equivalent to 1 μg saxitoxin/g) and (B) uncontaminated mussels with periodate oxidation (0.41 mg equivalent sample injected). Conditions as described in text.

1 should be assumed to be the more toxic analogues, NEO or GTX-1. Any samples producing total yields greater than the regulatory limit of 80 μg/100 g (0.8 μg/g), which includes values derived from NEO or GTX-1, can be confirmed by using the SPE-COOH cartridge for separation. The SPE-COOH treatment is essentially a modification of the ion-exchange method used to isolate individual toxins on a preparative scale (7). Cleanup removes the C toxins and B-2 from their respective carbamate analogues, allowing GTX-1 and NEO to be identified.

GTX-2 and GTX-3 yield the same oxidation product. Therefore, the prechromatographic oxidation method cannot distinguish between them, and they are not resolved using the SPE-COOH cartridge. This might lead to some error in true toxicity, because GTX-2 is only about 55% as toxic as GTX-3 (8).

During the course of this work, it became clear that pH had a significant influence on oxidation yield. We found

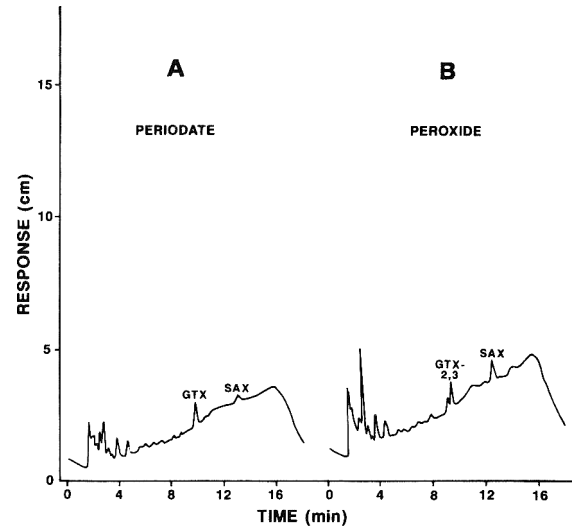


Figure 3. Chromatograms of scallop adductor muscle extract [0.013 μg total calculated PSP/g (saxitoxin equivalents)]. (A) Prechromatographic oxidation with periodate; 0.48 mg sample injected. Attenuation × 4. (B) With peroxide; 0.75 mg injected. Attenuation × 4.

that the sample matrix effects observed earlier (5) were, in fact, due to pH changes in the reaction mixture. These pH changes were caused by different buffering capacities in the acidic sample extracts. After some experimentation, overall reproducibility and yield of oxidation products of the toxins were greatly improved by adjusting the pH of the sample extracts to pH 8 before addition of the oxidation reagents. Under the optimized conditions (described in *Experimental*), the yields of periodate oxidation products for spiked extracts (0.2–2.0 μg/g individual toxin) of scallop roe and mussel tissue compared to yields of pure standards were, respectively:

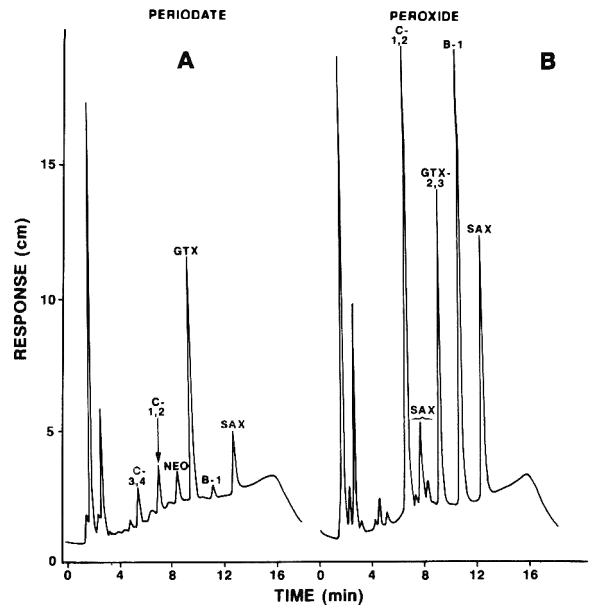


Figure 4. Chromatograms of contaminated oyster extract [0.29 μg total calculated PSP/g (saxitoxin equivalents)]. (A) Prechromatographic oxidation with periodate; 0.41 mg sample injected. Attenuation × 4. (B) With peroxide; 0.63 mg injected. Attenuation × 4.

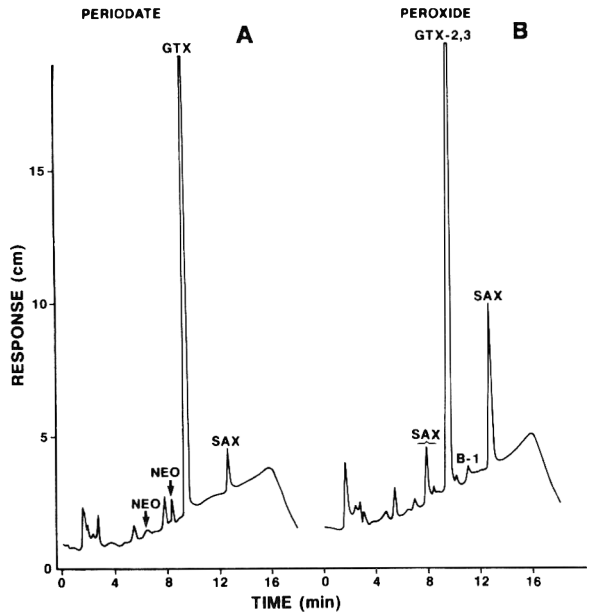


Figure 5. Chromatograms of contaminated scallop viscera extract [0.80 μg total calculated PSP/g (saxitoxin equivalents)]. (A) Prechromatographic oxidation with periodate; 0.21 mg sample injected. Attenuation × 4. (B) With peroxide; 0.32 mg injected. Attenuation × 4.

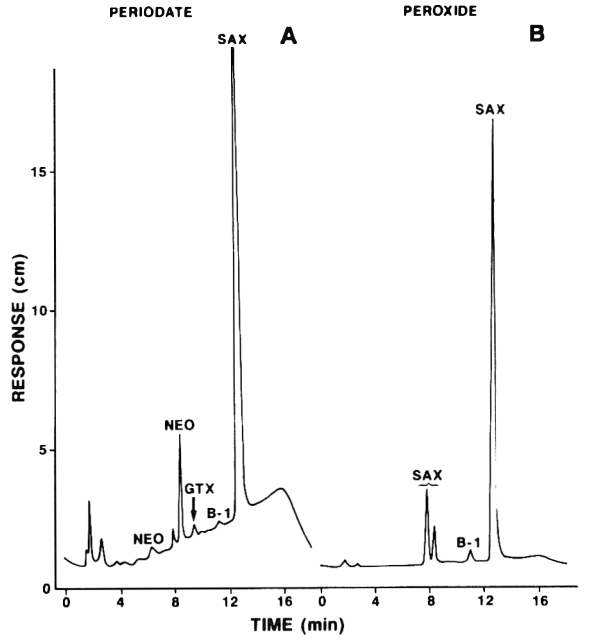


Figure 6. Chromatograms of contaminated butterclam chowder extract [3.57 μg total calculated PSP/g (saxitoxin equivalents)]. (A) Prechromatographic oxidation with periodate; 0.21 mg sample injected. Attenuation × 4. (B) With peroxide; 0.32 mg injected. Attenuation × 32.

C-1, 83 and 64%; C-2, 60 and 55%; C-3, 104 and 104%; GTX-1, 98 and 120%; GTX-2/3, 80 and 58%; B-1, 90 and 70%; B-2, 139 and 138%; NEO, 91 and 108%; and saxitoxin, 91 and 75%. Product yields for non-*N*-1-hydroxy toxins spiked into extracts of oyster tissue at the 0.2–2.0 μg/g per individual toxin level and carried through the peroxide oxidation were as follows: C-1, 104%; C-2, 122%; GTX-2, 106%; GTX-3, 98%; B-1, 98%; and saxitoxin, 106%.

Figure 2 shows periodate oxidation results for an uncontaminated and a contaminated mussel sample (total PSP equivalent to approximately 1 μg saxitoxin/g). As can be seen, the blank chromatogram is clean in the area where the toxin oxidation products elute. In some samples, a naturally fluorescent coextractive peak eluted just before NEO, causing some difficulty with its quantitation. The SPE C18 cleanup proved to be effective in removing the peak if 0.5 mL sample extract or less was passed through the cartridge. We found that with extract volumes of 1.0 mL or more, the cartridge became overloaded and was not effective in removing the peak if it was present in large quantities. The peak was confirmed not to be a PSP toxin by injecting an aliquot of the extracts without oxidation. It was found to be naturally fluorescent, unlike the PSP toxins.

The detection limit (3:1, signal:noise) in samples after SPE-C18 cleanup was estimated to be about 3–6 ng/g each for non-*N*-1 hydroxylated toxins, and 7–12 ng/g for *N*-1-hydroxy analogues. The SPE-C18 cleanup of the extracts before oxidation led to cleaner chromatograms and decreased overall contamination of the LC column, extending its lifetime over that of noncleaned up extracts. The C18 column operated well for over 1000 injections, including several hundred samples. The detection limits after ion-exchange treatment (for separation of B-2 and NEO) were 4–7 times higher because of dilution of the samples. However, they

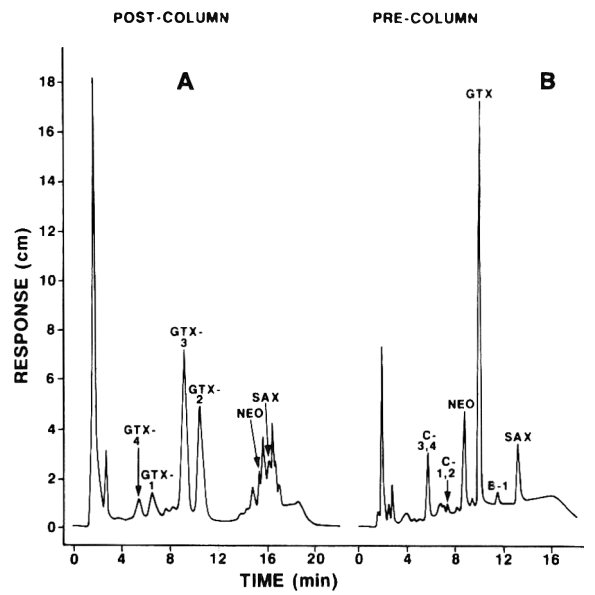


Figure 7. Chromatograms of contaminated mussel extract [2.0 μg total calculated PSP/g (saxitoxin equivalents)]. (A) Postcolumn oxidation; 10 mg sample injected. Attenuation × 32. (B) Prechromatographic oxidation (periodate); 0.21 mg injected. Attenuation × 4.

were still much more than adequate for detection of the toxins at the regulatory action level. We also found that the fractions could be concentrated, if necessary, without significant loss of toxins.

Table 3. Comparison of pre- and postcolumn LC methods with mouse bioassay

	Sample[a]	Total PSP found (saxitoxin equivalent, μg/g) Post-column	Pre-column	Mouse bioassay
1	Scallop adductor	0.10	0.048	< 0.40
2	Scallop adductor	0.32	0.190	< 0.40
3	Scallop adductor	0.09	0.006	< 0.40
4	Scallop adductor	0.06	0.013	< 0.40
5	Scallop adductor	0.19	0.170	< 0.40
6	Scallop roe	0.22	0.027	< 0.40
7	Scallop roe	0.47	0.31	0.45
8	Scallop roe	0.97	0.64	< 0.40
9	Scallop viscera	0.95	0.80	1.19
10	Scallop viscera	8.52	5.85	5.70
11	Scallop viscera	48.83	37.44	20.91
12	Mahogany clams	3.58	2.66	4.80
13	Mahogany clams	4.71	2.70	3.64
14	Mahogany clams	5.56	3.14	4.76
15	Ocean quahogs	1.14	1.12	< 0.40
16	Sea clams	4.54	4.98	6.01
17	Clams	3.48	2.26	2.30
18	Clams	8.06	8.03	6.20
19	Mussels	3.16	2.54	1.60
20	Mussels	4.41	3.42	0.98
21	Mussels	4.83	4.49	4.60
22	Mussels	6.55	4.04	3.00
23	Mussels	14.65	15.17	7.50
24	Mussels	16.35	10.07	10.00

[a] Samples 1–17 are from the North Atlantic coast of the United States; 18–24 are from the Gulf of St. Lawrence.

The repeatability coefficient of variation of replicate analyses (n = 4) of extracts taken through the SPE-C18 cleanup and peroxide oxidation reaction were as follows: saxitoxin, 3.0%; GTX-2, 7.5%; C-2, 8.0%; and B-1, 2.9%. The corresponding values after the cleanup and periodate oxidation reaction were as follows: saxitoxin, 3.6%; NEO, 8.6%; GTX-2, 5.3%; C-1, 10.9%; and C-3, 8.9%. The individual toxin concentrations ranged from 0.04 to 0.55 μg/g in the sample extracts. Both oxidation reactions produced linear results for all toxins over a range of 1 to 10 μg total toxin/g in a naturally contaminated sample of mussel that was serially diluted with an extract of uncontaminated mussels.

For quantitating unknown samples, the periodate reaction is best used as an initial screen to observe peaks appearing at the retention times of the most toxic PSPs, saxitoxin, NEO, and the gonyautoxins. If present, they should be quantitated as saxitoxin, NEO (not B-2), and GTX-1 (not GTX-2 or GTX-3, because GTX-1 has the weakest fluorescence response of the 3 and is the most toxic). If the total toxin concentration is greater than the action level of 80 μg/100 g, the peroxide oxidation is carried out to determine how much GTX-2 and GTX-3 is present in the gonyautoxin peak (and to determine the presence of C-1/C-2 and B-1, as well as quantitating saxitoxin). Once the total GTX-2/GTX-3 concentration is determined, the true concentration of GTX-1 (and/or GTX-4) is determined by subtracting the response of GTX-2 and GTX-3 in the periodate reaction. To confirm the presence of NEO and GTX-1 (and/or GTX-4), the SPE-COOH cleanup is used to remove B-2 and the C toxins from extracts before periodate oxidation. A particular advantage of using both the peroxidate and periodate reactions is that one reaction acts as a confirmation for the other.

The method was applied to the determination of PSP toxins in a number of different samples, including mussels, clams, scallops, oysters, whelks, quahogs, and a variety of chowders and pickled and smoked shellfish products. Figures 3–6 show typical chromatograms obtained with both periodate and peroxide reactions. The patterns are quite varied, as would be expected in view of species and geographic differences. Saxitoxin was present in all PSP-positive samples. The toxin peaks are easily quantifiable in the samples over a wide range of total PSP concentrations.

The method was compared to both the mouse bioassay and the postcolumn LC method (1). Extracts of shellfish obtained from the Gulf of St. Lawrence and from the North Atlantic coast of the United States that had been analyzed by the mouse bioassay (6) were analyzed in the authors' laboratory by the pre- and postcolumn LC methods. Table 3 lists the results obtained. In making the comparisons, the toxicity values used to convert individual PSP toxins to saxitoxin equivalents were those reported by Boyer et al. (8). Those values are generally intermediate to values reported by others (9, 10). However, the uncertainty as to the true (absolute) toxicities of the toxins will have a direct effect on the correlation of the LC methods with the mouse bioassay. For the precolumn oxidation method, GTX-4 was quantitated as GTX-1 because no separate standard was available for GTX-4. To quantitate GTX-2 and GTX-3, average fluorescence response factors (Table 2) and an average toxicity factor were used to convert to saxitoxin equivalents. Figure 7 shows chromatograms obtained for an extract of contaminated mussels using pre- and postchromatographic oxidation methods. NEO and saxitoxin are easily quantitated by the prechromatographic oxidation, whereas some difficulty was

encountered with the postcolumn method. The modified postcolumn method by Oshima et al. (2) may provide better results than the Sullivan and Wekell method (1), which was used in this work.

In general, the results in Table 3 show a reasonable correlation among the 3 methods. Postcolumn results were almost always higher than those obtained by the prechromatographic oxidation method, mainly because of the lack of sensitivity of the postcolumn method to the *N*-1-hydroxy toxins. In some cases, very small peaks in the chromatograms close to the detection limits were assumed to be PSP toxins if they appeared at the correct retention times. These would contribute significantly to the total toxin content. The most frequently found gonyautoxins were GTX-2 (predominant) and GTX-3. GTX-1 was found occasionally, and GTX-4 was only observed in the mahogany clams by the postcolumn method. Quantitation of GTX-2 and GTX-3 together with the prechromatographic oxidation method did not appear to be a significant problem. Usually, both were present in the samples, although GTX-2 was present in much greater quantity in some, as determined by the postcolumn LC method.

An additional 32 samples obtained from the Pacific Coast of Canada that were analyzed by the mouse bioassay were analyzed by prechromatographic oxidation LC. Although the overall correlation was similar to that shown in Table 3, the results were, on the average, 50% higher by LC, regardless of total PSP concentration (from less than 0.44 to 2.3 μg/g by the mouse bioassay) and type of sample (mussels, oysters, butterclams, and long neck clams). The reason for this difference is not clear at present. The chromatograms were uncomplicated. Several of the samples were analyzed by the postcolumn methods, and the results correlated well with the precolumn LC method. It is possible that the difference between the methods results in part from a low bias of the mouse bioassay resulting from the effect of salt on the toxicity of the extracts when low levels of PSP toxin are present. It has been clearly demonstrated that the mouse bioassay can underestimate PSP concentration by as much as 60% at levels of 400 μg/100 g (4 μg/g) or less (11). Also, some hydrolysis of the "B" and "C" toxins to their more toxic analogues may have occurred during storage of the extracts before LC analysis.

The method was applied to a survey of a number of fresh and frozen domestic and imported shellfish products, and to smoked and pickled shellfish and chowders in cans or glass jars. No PSPs were found above 80 μg/100 g in any of the 29 samples analyzed. No difficulty was experienced in analyzing any of the samples.

Overall, the prechromatographic LC method performed as well as the postcolumn method or the mouse bioassay. Although it requires more sample pretreatment before LC analysis, we have found that the oxidation reactions can be performed on-line automatically, with a programmable autoinjector. The most time-consuming part of the prechromatographic oxidation method is the ion exchange separation of B-2 and NEO. However, for regulatory purposes, this needs to be done only if the total PSP content is above the 80 μg/100 g action level, as mentioned earlier. This method is especially suited to PSP determination on an occasional basis. The postcolumn method is still superior for research purposes where the separation of individual toxins is required.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Supercritical Fluid Extraction/Enzyme Assay: A Novel Technique to Screen for Pesticide Residues in Meat Products

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The novel combination of supercritical fluid extraction (SFE) with an enzyme assay system has been used to screen meat products to detect the presence of pesticides. Analytes are collected in water by expanding supercritical carbon dioxide to atmospheric pressure through a restrictor and into an aqueous phase. The solution is then tested for the presence of pesticide residues by enzyme assay. Two experimental approaches have been used. Alachlor-fortified lard and bovine liver were monitored by static SFE coupled with an enzyme immunoassay. SFE of carbofuran-fortified frankfurters was coupled with an enzyme assay based on cholinesterase inhibition. A major benefit of the SFE/enzyme assay technique over conventional screening techniques is that the analyst is not exposed to organic solvents.

The increasing demand for monitoring pesticide residues in food products is accompanied by the need for novel analytical techniques that screen foods for the presence of pesticides. Such techniques can ultimately be developed into screening methods that determine whether a compound or class of compounds is present at some designated tolerance level in a meat matrix (1). In general, screening methods are useful because they allow a larger number of samples to be surveyed than could be accomplished with conventional analyses. Such screening methods are usually designed to detect the presence of an analyte but not necessarily the exact quantity of residue in the food matrix.

Enzyme assays, such as enzyme inhibition detection and enzyme-linked immunosorbent assays, are expected to play a prominent role in future screening methodologies (2). In fact, the number of immunoassays developed for use in trace pesticide analysis has continued to increase (3). However, one problem arises in attempting to integrate enzyme immunoassays with pesticide residue analyses. The classical methods for determining pesticide residues in fatty samples generally rely on organic solvent extractions to separate the compound(s) of interest from the sample matrix. Immunochemical assays, on the other hand, are conducted in aqueous media, although minor amounts of certain polar, water-miscible solvents can be tolerated (4). Organic solvents are known to disrupt the antigen-antibody reaction and can also denature the enzymes. Consequently, the integration of organic solvent-based extraction techniques with enzyme assays in a proposed screening method requires that additional steps be incorporated. For example, the organic solvent must be evaporated to dryness and the pesticide residues redissolved in an aqueous medium before the enzyme assay can be performed.

An alternative technique is to use supercritical fluid extraction (SFE) with carbon dioxide in place of organic solvents. Additional benefits can also be realized by using supercritical carbon dioxide (SC-CO_2) as the extraction solvent. Safety concerns associated with the use of organic solvents, such as waste disposal, flammability, and toxicity, are avoided.

SFE was recently investigated for use in pesticide residue analyses. For example, it was coupled with supercritical fluid chromatography to separate sulfonylurea herbicides and their metabolites from soil, cell culture media, and plant material (5). SFE was also used in conjunction with liquid chromatography, and it was shown to provide nearly quantitative recoveries for some pesticides (6). Organochlorine pesticides have been extracted from spiked lard (7) and fish tissue (8) by SFE.

In this communication, we describe 2 approaches to coupling SFE with enzyme assays. The purpose of this study was to demonstrate that SFE could be combined with enzyme assay methodology to yield a field-compatible technique for detecting the presence of pesticides in meat products. The described technique uses relatively nontoxic media (carbon dioxide, water) that allow it to be performed on-site in a food production facility. Because the extraction apparatus is very simple and inexpensive, it can be used by food inspectors at the plant level. No elaborate instrumentation or sample cleanup methods are required in this screening technique, and it can be used as a first step in determining whether violative levels of toxicants are present in a meat product.

Experimental

SC-CO_2 at moderate pressures (<151 atm) and temperatures (<69°C) is used to extract pesticides from fortified meat products. Analytes are solvated by SC-CO_2 and collected by expanding the pressurized CO_2 to atmospheric pressure through a restrictor directly into water in a collection vial. The aqueous solution is then tested for the presence of pesticides by an enzyme assay. Two experimental approaches were used. Alachlor-fortified lard and bovine liver were monitored by static SFE coupled with an enzyme immunoassay. In this approach, the sample is loaded into the extraction cell with dry ice. The sealed cell is then heated, which sublimes the dry ice, yielding SC-CO_2. In the second experimental approach, SFE of carbofuran-fortified frankfurters is coupled with an enzyme assay based on cholinesterase inhibition. In this instance, the extraction apparatus was equipped with a reservoir vessel filled with CO_2 from a storage cylinder with a dip tube.

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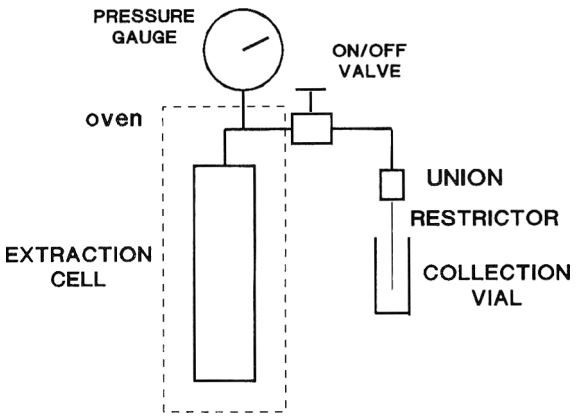

Figure 1. Schematic diagram of static SFE apparatus.

(a) *Pesticides.*—Alachlor and carbofuran (Pesticides and Industrial Chemicals Repository, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711).

(b) *Static SFE apparatus.*—Simple static extractions were performed with a 70 mL extraction vessel constructed of high pressure stainless steel tubing and fittings (Autoclave Engineers, Erie, PA 16512). One end of the extraction vessel was capped. The other end was connected to a pressure gauge and an exit line via a tee fitting, as schematically illustrated in Figure 1. Interconnecting lines consisted of 316 stainless steel tubing, 0.32 cm od, 0.159 cm id, having a more than adequate pressure rating. The extraction cell was positioned vertically in a Bendix gas chromatographic oven (Model 2600). The exit line was attached to a shut-off valve and connected to a length of PEEK tubing (Upchurch Scientific, Inc., Oak Harbor, WA 98277), 7 cm × 0.127 mm id, which served as a restrictor. The restrictor was vented into 5 mL deionized water held in 12 mL vial. The collection vial was placed in a beaker of water to moderate the cooling effect of the pressure drop. This step was required to prevent the water in the collection vial from solidifying into a mass of ice crystals. Dry ice (ca 40 g) was used to supply the CO_2 for SFE. Before the extraction cell was loaded, ca 40 g dry ice was used to initially cool the cell. This precaution limited CO_2 sublimation losses that occur before the cell is connected to the system.

(c) *Enzyme immunoassay (EIA).*—(Res-I-Quant, Alachlor Immunoassay Kit, Immunosystems, Scarborough, ME). Quantitative range of this assay is 0.5–20 ppb.

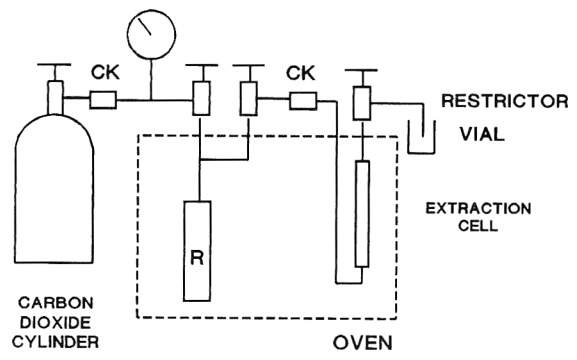

Figure 2. Schematic diagram of modified SFE apparatus (R = expansion reservoir, CK = check valve).

(d) *Microplate reader.*—Absorbances of the microtiter wells were measured with a microplate reader (Model EL-308, Bio-Tek Institutes, Winooski, VT).

(e) *Modified SFE apparatus.*—Extraction system was modified to enhance extraction efficiency to compensate for the decreased sensitivity exhibited by the enzyme inhibition assay. A schematic diagram of the modified SFE system is shown in Figure 2. The reservoir consisted of 316 stainless steel tubing (Part No. 15–009, Autoclave Engineers), 30.5 cm × 1.75 cm id. The extraction cell was 20.3 cm × 1.75 cm id. All other equipment is as cited in (a). To operate this system, the reservoir is cooled with an ice bath while being filled with CO_2 from a CO_2 cylinder equipped with a siphon tube. The head pressure (63 atm) of the cylinder is used to fill the reservoir. Concurrently, a sample is loaded into the extraction cell. Then, the appropriate valves are closed and the valve isolating the reservoir from the extraction cell is opened. The oven is heated to the extraction temperature and a supercritical fluid phase is produced. These extraction conditions are maintained for 15 min. CO_2 is then vented through the restrictor for ca 10 min until the pressure drops below the critical pressure (72.8 atm).

(f) *Enzyme inhibition assay.*—A commercial pesticide detection kit based on cholinesterase inhibition (Enzytec, Kansas City, KS) was also examined in conjunction with SFE. The assay can detect over 50 different pesticides, although it is not as sensitive as EIA. Carbofuran was detected in the collection water by a bioconcentration technique described by the manufacturer. In this approach, the detection limit for carbofuran in water is 0.003 ppm. Visual observation of the color of the test ticket indicated the presence or absence of the pesticide. The collection water for each sample was at room temperature for the enzyme assays.

(g) *Blender.*—Tissue samples were homogenized in a Model 1120 Waring blender (Waring Products Div., New Hartford, CT 06057) with dry ice (9).

(h) *Meat products.*—Lard samples were fortified with alachlor at levels up to 100 ppb, and they were supported on glass wool in the extraction cell. Frankfurter samples were fortified with carbofuran in amounts up to 0.3 ppm. Separate bovine liver samples were fortified with alachlor and carbofuran at 3.3 and 200 ppb, respectively.

Results and Discussion

Table 1 shows the screening results of the static SFE/EIA technique for some alachlor-fortified samples, using dry ice as the CO_2 source and the extraction apparatus as schematically illustrated in Figure 1. The technique confirmed the presence of alachlor in lard samples spiked at 10 ppb or greater. Blank extractions (no pesticide or sample) and negative control extractions (unspiked samples) yielded concentration values that in all cases were below those of the fortified samples. With the Student's paired *t*-test at a confidence limit of 95%, alachlor concentration in all of the spiked lard, except one sample spiked at 2.5 ppb, could be shown to be statistically greater than those in the negative control samples.

An estimate of the detection limit was determined for the alachlor EIA on the CO_2-bubbled water from the negative control and blank samples. The Shewhart chart (10) in Figure 3 graphically displays the results. The line at 1.1 ppb identifies the lower limit of detection. Samples yielding

Table 1. Screening results of static SFE/EIA on lard and bovine liver samples using SFE apparatus (Figure 1)

Sample	Alachlor detected[a]
Dry ice (× 3)	–
Lard (0.5–2.1 g), (× 3)	–
Alachlor (20 ng)	+
Lard (g), spiked with alachlor (ppb)	
1.9, 2.5	–
2.4, 10	+
2.5, 25	+
0.5, 50	+
1.1, 50	+
2.3, 50	+
0.5, 100	+
Bovine liver	
12 g, no spike	–
12 g, 3.3 ppb alachlor	+

[a] + = pesticide detected (concentraction of alachlor in collection water is above detection limit); – = pesticide not detected (concentration of alachlor in water is below detection limit).

pesticide concentrations in the collection water below this limit cannot be statistically differentiated from unspiked samples. This detection limit was used to classify the samples into 1 of 2 categories; alachlor is present (+) or absent (–). Of course, the absolute detection threshold will depend on a number of factors, such as the sample mass in the extraction cell, the volume of collection water, and the SFE methodology used.

The described static extraction technique eliminated the need for a pump or compressor, thereby keeping the SFE system as simple and inexpensive as possible. Static SFE involves filling the extraction cell with the extracting fluid, allowing the fluid to equilibrate with the sample, and then depressurizing the fluid from the cell into a suitable collection solvent. Static SFE was shown to produce recoveries of 80–100% for organochlorine pesticides in fish (8). In our studies, dry ice was used to fill the extraction cell with CO_2. For the bovine liver samples, dry ice was also used as an aid in homogenizing the sample (9). In this manner, dry ice served 2 purposes. It kept tissue brittle, so that the blender could produce a fine homogenate, and it was also the source of CO_2 for SFE. The relatively low extraction pressure was chosen to minimize coextraction of lipids. Under the cited extraction conditions (>86 atm, 55°C, and extraction time 1.0–3.5 h), solubility of pure alachlor in SC-CO_2 is greater than 0.01 wt% (11), sufficient for solubilizing trace amounts of alachlor.

Table 2. Screening results of SFE coupled with enzyme inhibition assay using modified SFE apparatus (Figure 2)

Spike level, ppm	Carbofuran detected[a]
Frankfurters (17 g)	
0.00	–
0.05	+
0.10	+
0.20	+
Bovine liver	
0.00	–
0.20	+

[a] + = enzyme ticket indicates presence of pesticide; – = enzyme ticket indicates absence of pesticide.

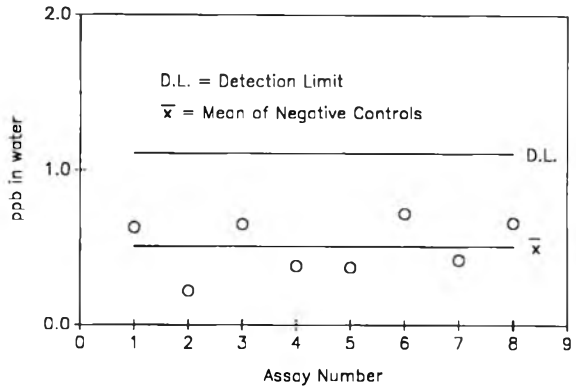

Figure 3. Shewhart chart of negative control collection water samples (enzyme immunoassay for alachlor, Res-I-Quant, Immunosystems).

A commercial pesticide residue detection system based on cholinesterase enzyme inhibition that is able to screen for a wide range of common pesticides (1) was also used in demonstrating the coupling of SFE and enzyme assay for pesticide residue screening. In general, the detection scheme used by this assay is not as sensitive as immunoassays. Hence, the static extraction system was modified to provide a more efficient sweeping of the extracting fluid from the extraction cell. Incorporation of a pump or compressor into the system was avoided to minimize equipment costs and to keep the SFE system simple. Similar approaches to fluid delivery have been reported (8, 12). The modified SFE system (Figure 2) supplied enough CO_2 to flush the volume of the extraction cell at least 3 times. Results from the SFE/enzyme inhibition assay on frankfurter and bovine liver samples spiked with carbofuran are given in Table 2 (extraction conditions: 150–72 atm and 68°C for 45 min.). For each spiked sample, a negative control sample was also extracted for comparison. The color of the ticket from the negative control sample was then compared to that of the spiked sample. Spiked samples were identified as having more of the enzyme inhibited than the negative controls.

In conclusion, several benefits of the SFE/enzyme assay technique are worth noting. The selective nature of enzyme assays allow inexpensive welding-grade CO_2 or dry ice to be used in screening for trace residues. The use of water and CO_2 avoids the need for any organic solvents in the extraction, concentration, or detection steps. The described technique also reduces the number of steps needed to perform enzyme assays for pesticide residue detection. The coupled technique was able to distinguish samples spiked at levels close to the tolerance limit for residues in meats (alachlor = 0.020 ppm; carbofuran = 0.050 ppm) (13). Further development of this screening technique for other food matrixes and toxicants will be forthcoming.

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Reducing Solvent Consumption in Automated Gel Permeation Chromatographic Cleanup for Pesticide Residue Analysis: A Modified GPC AutoPrep 1002

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Modifications to the gel permeation chromatographic apparatus recommended in AOAC method 984.21 for multiresidue analysis of organochlorine pesticides in animal fat are described. By using 1.0 mL sample loops and a 1.0 cm id column, organic solvent usage was reduced by 85%. Organochlorine pesticides were collected in a 14 mL fraction. Mean recoveries using the modified apparatus for aldrin, α-BHC, lindane, dieldrin, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT, heptachlor, heptachlor epoxide, methoxychlor, and mirex from beef fat fortified at 0.10–2.75 μg/g were in the 91.4–108% range, with CV of 3.3–10.0%.

Gel permeation chromatography (GPC) is one of the techniques used to separate the lipid fraction when preparing fats, oils, and plant and tissue extracts for multiresidue pesticide analysis. Because separation is based on molecular size, GPC has the advantage of being applicable to a wide range of structurally dissimilar compounds, unlike some adsorption techniques that are narrower in scope. GPC is also amenable to automation, and commercial multisample processors are available. A GPC method for determination of organochlorine residues in poultry, swine, and beef fat has been studied collaboratively (1, 2). The method was adopted in 1985 (3).

The official AOAC method specifies use of a 2.5 cm id chromatographic column on the AutoPrep Model 1002B, supplied by ABC Laboratories (3). With the specified bed length and mobile phase, each sample requires 250–300 mL solvent. A full run of 23 samples consumes up to 7 L organic solvent. For laboratories using this method on a routine basis, solvent costs and the volume of waste solvent produced are considerable.

Roos et al. (4) have shown that use of a narrower column (1.0 cm id) can substantially reduce solvent consumption without compromising the analysis. Such a column cannot be used on an unmodified AutoPrep system because the sample volume (5 mL loop) is too large to allow resolution of the lipid fraction from the pesticides. This paper describes modifications that were made to an AutoPrep system to accommodate a 1.0 cm id column.

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Experimental

Apparatus and Reagents

(a) *Automated GPC system.*—GPC AutoPrep Model 1002 (ABC Laboratories, Columbia, MO 65205).

(b) GPC Column.—60 × 1.0 cm jacketed glass column (Kontes, Vineland, NJ 08360). Column was slurry-packed with 9.6 g Bio-Beads SX-3, 200–400 mesh in CH_2Cl_2–C_6H_{12} (1 + 1) and compressed to 48 cm.

(c) *Gas chromatograph.*—Model 5890 (Hewlett-Packard Co., Mississauga, ON, Canada) with electron capture detector, autosampler, and 30 m × 0.53 mm id DB608 column (J&W Scientific, Folsom, CA 95630).

(d) *Evaporator.*—24-place nitrogen evaporator (Organomation Associates, Berlin, MA 01503).

(e) *Solvents.*—Methylene chloride, cyclohexane, and isooctane. High purity grade (Burdick and Jackson, Muskegon, MI 49442).

(f) *Valves.*—Hamilton HVP with 4-port housing and #5 plug (Chromatographic Specialties, Brockville, ON, Canada).

(g) *Flanging tool.*—(Milton Roy, LDC Division, Riviera Beach, FL 33419).

(h) *Fittings.*—Cheminert for 3 mm tubing (Supelco Inc., Oakville, ON, Canada).

Modifications—GPC AutoPrep 1002

The 1.58 mm id tubing in the sample introduction and flow systems was replaced with 0.79 mm id Teflon tubing. Connections were made with flanged fittings. Two Hamilton HVP valves were installed in the lines running from the load/run valve to the column inlet, and from the column outlet to the dump/collect solenoid, respectively. The 2 HVP valves were also connected directly by a bypass line so that the mobile phase could be directed through the column or

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through the bypass line, isolating the column from the flow system. Existing sample loops were shortened from ca 400 cm (5.0 mL) to 80 cm (1.0 mL), and calibrated. Loops were then shortened incrementally, if necessary, until each was 1.00 ±0.02 mL. Loop volume was determined as follows: All loops were filled with a standard solution of lindane; the valves were switched to direct the flow through the column bypass; each loop was flushed with hexane directly into 5 mL volumetric flasks and diluted to 5 mL with hexane; and the electron capture response of these solutions was compared to the response of 1.00 mL of the same standard solution made up to 5 mL in a volumetric flask.

GPC Calibration

To determine pesticide elution profiles, the flow rate was set to 1.0 mL/min, and a mixed pesticide standard was autofractionated into twenty-three 1 mL fractions over the 22–44 mL elution range. To collect the portion of each fraction remaining in the collection lines after the system had cycled through all 23 loops, the valves were switched to bypass the column, and the system was again cycled through all loops to flush each line into the collection vial. Fractions were evaporated to ca 0.5 mL, diluted to 1.0 mL with isooctane, and analyzed by gas chromatography.

To determine the elution profile for beef fat, a 1.5 g sample was prepared and an aliquot was loaded into a 1.0 mL loop, as described below. The aliquot was fractionated into 2 mL fractions over the 10–35 mL elution range, and fractions were collected in tared vials. Fractions were evaporated to dryness and weighed.

The minimum injection volume needed to fill the sample loops was determined by injecting varying volumes of a standard lindane solution, flushing the loop contents into collection vials, and comparing the electron capture response (as described earlier for loop volume determination).

Sample Preparation

A 1.5 g sample of liquid rendered fat was weighed into a 10 mL volumetric flask. Fortified samples were prepared at this point by adding standard solutions in CH_2Cl_2–C_6H_{12} (1 + 1). Samples were dissolved and diluted to 10 mL with CH_2Cl_2–C_6H_{12} (1 + 1).

Determination

Sample loops were loaded with 3 mL filtered, diluted samples (0.15 g sample in a 1 mL loop). Flow rate was adjusted to 1.0 mL/min, and dump and collect times were set to 26 and 14 min, respectively. Eluted fractions were collected in 15 mL graduated glass centrifuge tubes, evaporated to ca 0.5 mL at 35°C under a gentle stream of nitrogen, and adjusted to 1.0 mL (precalibrated) with isooctane.

Results and Discussion

Results of the minimum sample volume determination are shown in Figure 1. Data points represent a single injection at each volume, and they show that 2.2 mL was adequate. In practice, a 3 mL aliquot was used (2 mL overflow from a 1 mL loop). This is similar to the standard method for which a 7 mL injection into a 5 mL loop is recommended.

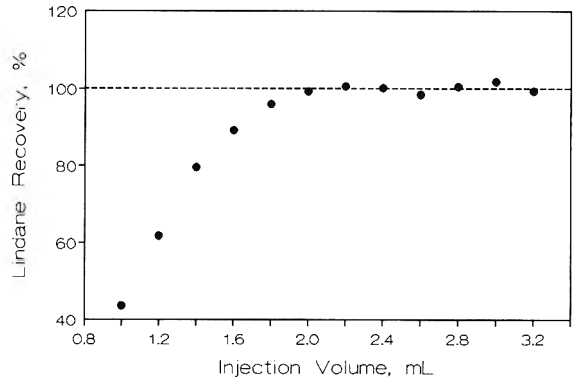

Figure 1. Minimum injection volume to fill 1 mL sample loop.

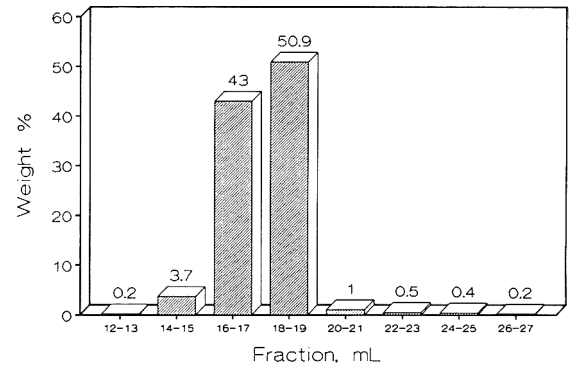

Figure 2. Beef fat elution profile from 9.6 g Bio-Beads SX–3 (48 cm bed × 1.0 cm id).

Table 1. Pesticide elution profiles[a] from 9.6 g Bio-Beads SX–3 (48 cm bed × 1.0 cm id)

Pesticide	Elution vol (mL) of CH_2Cl_2 – C_6H_{12} (1 + 1)
Aldrin	27–32
α-BHC	29–35
Lindane	30–36
Dieldrin	27–34
p,p'-DDD	28–35
p,p'-DDE	27–33
p,p'-DDT	27–33
Heptachlor	27–32
Heptachlor epoxide	27–33
Methoxychlor	27–33
Mirex	27–34

[a] In triplicate.

Elution profiles for beef fat and several organochlorine pesticides on the modified system are shown in Figure 2 and Table 1, respectively. Pesticide profiles were determined in triplicate, with the widest bands reported in Table 1. Data show that the pesticide fraction can be separated from the bulk of the lipids. Also, the combination of narrow column and reduced loop volume allows the pesticides to be eluted in a narrow band (about 10 mL), while consuming only 36 mL solvent. With the standard automated system, the same set of

Table 2. Recovery of organochlorine pesticides from fortified beef fat

Pesticide	Spike level, μg/g	Mean rec., %[a]	CV, %
Aldrin	0.16	98.9	5.6
	0.40	94.9	8.6
α-BHC	0.10	94.6	4.6
	0.25	96.1	4.9
Lindane	0.11	95.3	6.5
	0.28	99.2	4.1
Dieldrin	0.28	104	8.1
	0.70	101	8.0
p,p'-DDD	0.38	98.1	7.8
	0.95	108	9.7
p,p'-DDE	0.33	102	7.8
	0.83	107	7.7
p,p'-DDT	0.42	105	9.3
	1.05	107	6.3
Heptachlor	0.14	91.4	5.7
	0.35	97.2	6.0
Heptachlor epoxide	0.23	101	9.3
	0.58	95.0	10.0
Methoxychlor	1.10	106	5.7
	2.75	107	6.1
Mirex	0.56	96.4	3.3
	1.40	100	7.1

[a] n = 4.

pesticides is eluted in an 80 mL fraction, using 240 mL solvent (1). Also, the task of concentrating the extracts is simplified. They are small enough that simultaneous evaporation of a full run in a multiport nitrogen evaporator is feasible. The standard method requires rotary evaporation of 23 extracts of 80 mL each (more expensive automated options are available).

Recoveries for a variety of organochlorine pesticides spiked onto beef fat at concentrations in the 0.10–2.75 μg/g range are shown in Table 2. In all cases, mean recoveries were greater than 90% with good repeatability (CV ≤ 10%). Data from the collaborative study showed that for the same pesticides, recoveries from spiked beef fat were in the 96–107% range, and within-laboratory CV was 1.9–18.3% (2).

The intent of this work was to duplicate the standard method on a reduced scale, making only those changes needed to adapt the system to a narrow column while leaving the chemistry unmodified. The mobile phase, stationary phase, and column length were unchanged. Other specifications were adjusted to reflect the reduced column capacity. The weight of resin was in direct proportion to bed volume. Flow rate and the weight of lipid on-column were reduced in rough proportion to bed volume. This kept linear flow rate and system backpressure at levels similar to those for the standard system. This reduction also avoided overloading and compressing the gel. Sample loops were shortened to introduce the sample to the column in a more concentrated plug. Narrower tubing was used in the flow and sample introduction systems to minimize both system dead-volume and the injection volume needed to flush and refill a loop. The volume of the final extract was reduced from 5 to 1 mL to maintain similar sensitivity.

The miniaturized system can reduce solvent consumption by 85%, without sacrificing performance. It generates substantial cost savings in the purchase and disposal of organic solvents and lessens the environmental impact of pesticide residue analysis.

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Simple Colorimetric Determination of Carrageenan in Jellies and Salad Dressings

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A simple, rapid colorimetric method is described for the determination of carrageenan (CAR) in foods such as jellies and salad dressings. Alcian blue reagent is added to the sample solution prepared from these foods, then CAR is precipitated selectively as a CAR–alcian blue complex. The precipitate is dissolved in monoethanolamine and determined colorimetrically at 615 nm. Overall recovery for jelly was 93.3% with a coefficient of variation (CV) of 5.8%. Overall recovery for salad dressing was 91.9% with a CV of 3.0%. The detection limit was 0.05%. The results obtained by the proposed method were similar to those obtained by a capillary gas chromatography method.

Carrageenan (CAR) is a sulfated polysaccharide derived from certain types of seaweeds. In the food industry, plant and microbial polysaccharides including CAR are widely used as gelling effects in jellies, as emulsifiers in salad dressings, and as thickening agents in sauces (1).

In recent years, gas chromatography (GC) (2, 3), capillary GC (4, 5), liquid chromatography (6), and colorimetric (7) methods, and zonal electrophoresis (8, 9) have been reported for the determination and characterization of commercial polysaccharides.

Ramus (10), Gold (11), and Koburova et al. (12) developed colorimetric methods for the analysis of sulfated polysaccharides using an alcian blue reagent. Alcian blue, a cationic copper phthalocyanine dye, has been used as a histochemical stain. It complexes with sulfated polysaccharides in strongly acidic solution (pH 0.4) to form a precipitate (13). We found that the alcian blue reagent is also applicable to the determination of CAR in foods. This paper describes a simple, rapid colorimetric method for determination of CAR in jellies and salad dressings using the alcian blue reagent. In addition, results obtained by the proposed method were compared with those obtained by a capillary GC method (4).

METHOD

Apparatus

(a) *Spectrophotometer.*—Model UV-210 (Shimazu Instruments, Inc., Kyoto, Japan).

(b) *Membrane microfilters.*—0.45 μm pore size, resistant to attack by alcohol solutions.

(c) *Test tubes.*—10 × 100 mm glass, with Teflon-lined caps.

(d) *Gas chromatograph.*—Model 5890A gas chromatograph equipped with flame ionization detector and integrator; capillary column; Carbowax 20M phase, 25 × 0.2 mm id, 0.2 μm thickness (Hewlett-Packard Co., Palo Alto, CA 94303); and split injector. Operating conditions: nitrogen carrier gas at 100 mL/min; temperatures, injector 250°C, detector 270°C, oven 120°C (0.5 min) then 5°C/min to 180°C and hold for 1 min; injection volume 1 μL.

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Reagents

(a) *CAR standard.*—Dissolve 10 mg χ-CAR (Sigma Chemical Co., St. Louis, MO 63178) in 50 mL water in 50°C water bath. Cool to room temperature and dilute to 100 mL (100 μg/mL). Dilute aliquots of this solution to prepare 10–100 μg/mL solutions.

(b) *Alcian blue.*—Dissolve 1 g alcian blue 8GX (Sigma), 6.9 g disodium hydrogen phosphate, and 21 mL HCl in water and dilute to 100 mL. Use supernate. (After standing over night, alcian blue dye particles appear in the bottom of storage bottle; however, centrifugation or filtration step is not required.) Store at 5°C and prepare monthly.

(c) *Monoethanolamine.*—(Wako Chemicals, Inc., Tokyo, Japan.)

(d) *0.5N HCl–methanol.*—Bubble HCl gas slowly into anhydrous methanol until HCl normality is 0.5N. Check by titration with 0.5N methanolic NaOH. Store at 5°C (stable at least 3 months).

(e) *Trimethylsilyl (TMS).*—*N*-trimethylsilylimidazole (Gasukuro Kogyo Inc., Tokyo, Japan).

(f) *Internal standard.*—Dissolve 100 mg D-sorbitol (Kanto Chemical Co., Tokyo, Japan) in 2 mL water and dilute to 100 mL with methanol.

(g) *λ-Carrageenan, ι-carrageenan.*—Sigma.

Preparation of Sample Solutions

(a) *Jelly (fruit and wine jelly).*—Homogenize 2 g sample with 30 mL water. Adjust to pH 7 with 0.1N NaOH. Transfer to 100 mL volumetric flask and dilute to volume with water.

(b) *Salad dressing (separable and emulsion type).*—Weigh 2 g sample and add 30 mL water. Transfer to 100 mL separatory funnel and defat with 30 mL ether. Drain aqueous layer into 100 mL volumetric flask. Adjust to pH 7 with 0.1N NaOH and dilute to volume with water.

Colorimetric Determination

(a) *Standard curve.*—Pipet 1 mL CAR standard solution into 10 mL centrifuge tube. Add 0.2 mL alcian blue reagent and let stand for 10 min. Centrifuge at 3000 rpm for 5 min. Decant supernate and wash precipitate with 3 mL 80% ethanol. Centrifuge and decant supernate as before. Dissolve precipitate in 10 mL monoethanolamine. Measure absorbance at 615 nm against monoethanolamine. Plot absorbance vs CAR concentration (10–100 μg/mL) to obtain a standard curve.

(b) *Measurement of sample solution.*—Pipet 1 mL sample solution into 10 mL centrifuge tube and treat as standard curve. Determine sample concentration by comparison with the standard curve.

Sample Preparation for Capillary GC Method

(a) *Jelly.*—Homogenize 5–10 g sample with 30 mL water. Add 200 mL methanol to homogenate. Let stand overnight. Collect gelatinous precipitate on membrane filter and

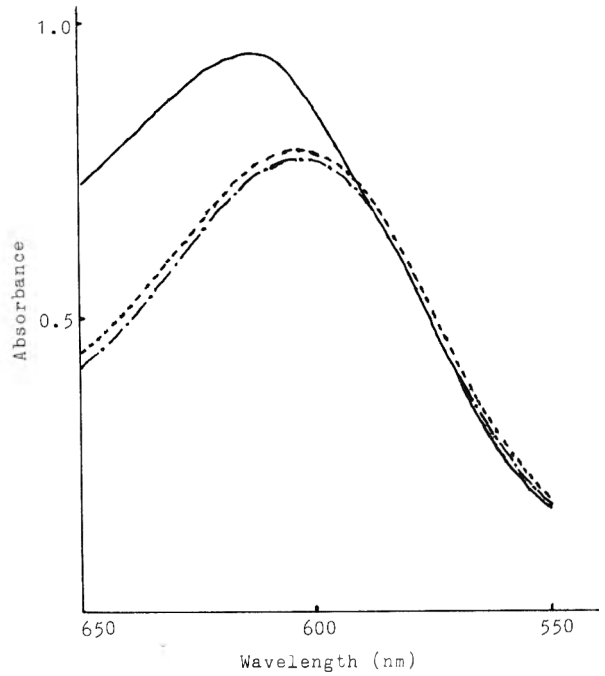

Figure 1. Absorption spectra of CAR (100 μg)–alcian blue complex. Solvent: — — monoethanolamine; ····1% cetylpyridinium chloride; ·—·— water.

transfer to test tube. Dry precipitate under reduced pressure at 40°C.

(b) *Salad dressing.*—Weigh 10 g sample and add 30 mL water. Transfer to 100 mL separatory funnel and defat with 30 mL ether. Drain aqueous layer into beaker. Add 200 mL methanol to aqueous layer and treat as jelly.

Table 1. Recovery of CAR from jelly and salad dressing

Sample	Added, mg/2 g	Found, mg/2 g	Rec., %
Jelly	0	2.44	—
	0	2.40	—
	0	2.36	—
	2	4.27	93.5
	2	4.22	91.0
	2	4.51	105.5
	4	5.95	88.8
	4	6.01	90.3
	4	6.08	92.0
	6	8.11	95.2
	6	8.15	95.8
	6	7.64	87.3
Overall rec., %			93.3
CV, %			5.8
Salad dressing	0	0	—
	0	0	—
	2	1.88	94.0
	2	1.82	91.0
	6	5.65	94.2
	6	5.31	88.5
Overall rec., %			91.9
CV, %			3.0

Table 2. Comparison of proposed and capillary GC methods for determination of CAR in jellies and salad dressings

No.	Sample	CAR, %			
		Proposed method		Capillary GC method	
1	Wine jelly	0.26	0.28	0.34	0.34
2	Wine jelly	0.28	0.30	0.34	0.33
3	Wine jelly	0.12	0.12	0.14	0.16
4	Orange jelly[a]	0.29	0.30	0.39	0.38
5	Apricot jelly[a]	0.36	0.38	0.38	0.36
6	Fruit jelly[a]	0.32	0.30	0.32	0.31
7	Orange jelly	0.23	0.23	0.26	0.23
1	Italian dressing	0.11	0.11	0.11	0.11

[a] Labeled to contain CAR.

Derivatization

TMS derivatization of polysaccharides, including CAR, was analyzed by Preuss and Thier (4) using capillary GC. The modified procedure for CAR is as follows.

Add 1 mL 0.5N HCL–methanol to dried sample precipitate and cap test tube. Place test tube on boiling water bath for 4 h. Cool to room temperature and transfer to 50 mL round bottomed flask. Add 50 μL pyridine and 1 mL internal standard. Evaporate to dryness. Dissolve residue in 0.5 mL pyridine and add 0.5 mL TMS. Let stand for 10 min at room temperature. Add 20 mL water, 1 mL saturated NaCl solution, and 2.0 mL *n*-hexane. Shake vigorously 30 s and let layers separate. Inject *n*-hexane layer into GC apparatus. To prepare standard used for GC quantitation, place 15 mg χ-CAR in test tube and convert to TMS derivative as dried sample precipitate. Calculate percentage CAR for each sample as follows:

$$SRF = 0.015 \text{ g}/(PHC/PHI)$$

$$CAR\ (\%) = (PHC'/PHI' \times SRF)/W \times 100$$

where *SRF* = standard response factor; *PHC* and *PHC'* = *CAR* peak height of standard and sample, respectively; *PHI* and *PHI'* = internal standard peak height of standard and sample, respectively; and *W* = sample weight (g).

Results and Discussion

First, we surveyed the formation of precipitate using 1% alcian blue solution as a histochemical dye (13) (add 4.2 mL HCl and 1.38 g disodium hydrogen phosphate/100 mL). With this alcian blue reagent, CAR, chondroitin sulfate (a mucopolysaccharide, containing sulfated groups), and sodium alginate (a carboxylated polysaccharide) formed a precipitate. Gum xanthan (a carboxylated polysaccharide) formed a slight precipitate. Gum locust bean, methyl cellulose, pectin, propylene glycol alginate, sodium carboxymethylcellulose, sodium carboxymethylstarch, and tamarind seed polysaccharide formed no precipitate. Subsequently, we increased by 5-fold the amount of HCl and disodium hydrogen phosphate. Under this strongly acidic condition, gum locust bean, gum xanthan, methyl cellulose, pectin, propylene glycol alginate, sodium alginate, sodium carboxymethylcellulose, sodium carboxymethylstarch, and tamarind seed polysaccharide formed no precipitate. CAR and chondroitin sulfate formed a precipitate with alcian blue.

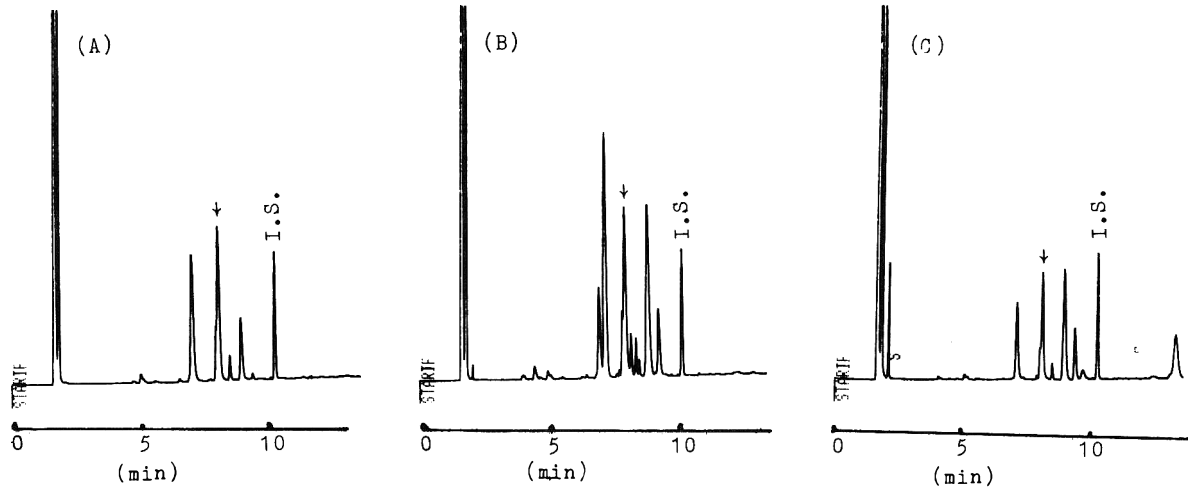

Figure 2. Capillary gas chromatograms of trimethylsilylated CAR (15 mg) (A), jelly extract No. 3 (B), and salad dressing extract No. 1 (C). ↓: Galactose peak; I.S.: Internal standard peak.

To select a solvent for dissolving the CAR–alcian blue precipitate, 9 different solvents were investigated. The CAR–alcian blue precipitate was soluble in water, 1% cetylpyridinium chloride, and monoethanolamine; but insoluble in 1N NaOH, 10% ammonia in water, *N, N*-dimethylformamide, tetrahydrofuran, acetone, and ethanol. Figure 1 shows the absorption spectra of the CAR–alcian blue complex dissolved in these solvents. We chose monoethanolamine as a solvent because it gave the highest absorption intensity at 615 nm.

In the proposed method, the calibration curve was linear from 10 to 100 μg CAR. The detection limit was 0.05%. CAR was spiked to jelly (wine jelly) and salad dressing (separate dressing) to demonstrate the recovery of the proposed method. Table 1 shows recoveries for jelly and salad dressing. Recoveries for jelly ranged from 87.3 to 105.5%. Recoveries for salad dressing ranged from 88.5 to 94.2%.

The proposed and capillary GC methods were applied to commercial jellies and salad dressings. All samples were purchased locally. Results were in close agreement, as shown in Table 2. Figure 2 shows capillary GC chromatograms of CAR, CAR from wine jelly (No. 3), and CAR from Italian dressing (No. 1) as TMS derivatives, respectively. CAR in samples was characterized by comparison of retention times and peak patterns. The indicated peak was the galactose peak. No significant interfering peaks were observed. Preuss and Thier (4) used 0.05% SE-30 (35 m × 0.2 mm id) as a capillary column to separate TMS derivatives. In the present study, we used Carbowax 20M phase to better characterize CAR.

In the proposed method, colorimetric response was slightly affected by the varying sulfate contents in different types of CAR. According to Ohe (1), the sulfate contents of χ-CAR, λ-CAR, and ι-CAR are 25, 35, and 32%, respectively. Figure 3 shows the comparison of the responses of each type of CAR. This different response was a critical point. Methods for more reliable values require further study. Absorption spectra and maxima of λ- and ι-CAR–alcian blue complex monoethanolamines were the same as χ-CAR, as shown in Figure 1. Individual CAR was not distinguished by absorption spectrum. Also, CAR was difficult to distinguish clearly by the capillary GC method. As a reference, Guevener et al. (14) proposed a metachromasy with thionine for CAR in toothpastes. They described how the absorption band of each type of CAR is changed.

The presence of proteins (e.g., gelatin) that bind CAR is considered to decrease CAR recovery from food stuff. However, the list of ingredients on the label of the commercial jellies and salad dressings that we analyzed in this study did not include any proteins. Therefore, we eliminated protein-removing steps such as enzymatic degradation (8) or trichloroacetic acid treatment (8, 9).

In the capillary GC method, the combination of other commercially used polysaccharides that yield galactose as their component, e.g., arabic, gum tragacanth, gum guar, and gum locust bean, is suspected to increase the galactose value. In such a case, the galactose peak is not reliable for quantitation of CAR.

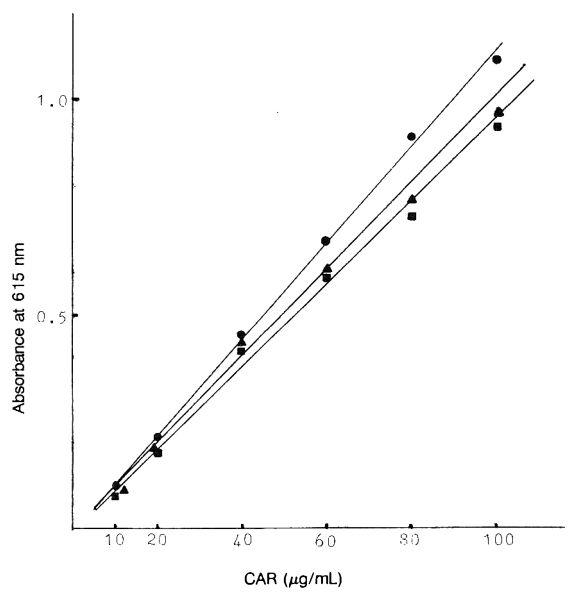

Figure 3. Comparison of the response of χ-CAR, λ-CAR, and ι-CAR to the proposed colorimetric method. —●— χ-CAR; —▲— λ-CAR; —■— ι-CAR.

In conclusion, the proposed method is based on the following steps; (a) formation of CAR–alcian blue precipitate; (b) dissolution of CAR–alcian blue precipitate in monoethanolamine; and (c) colorimetry of the monoethanolamine solution. The proposed method is convenient and rapid, although it gave slightly different values depending upon the type of CAR. The analytical time was shortened to about 2 h. The GC capillary method requires about 3 days for the entire analysis. Thus, the proposed method is suitable for routine analysis of CAR in jellies and salad dressings.

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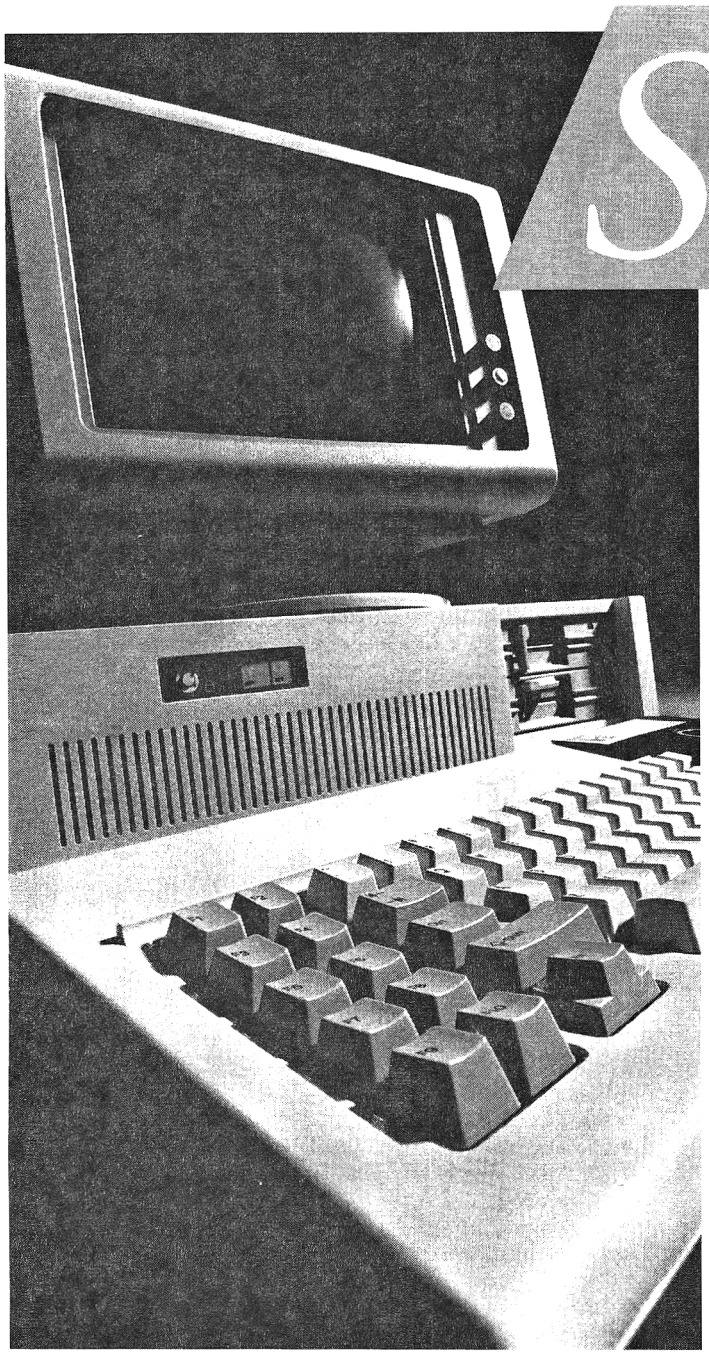

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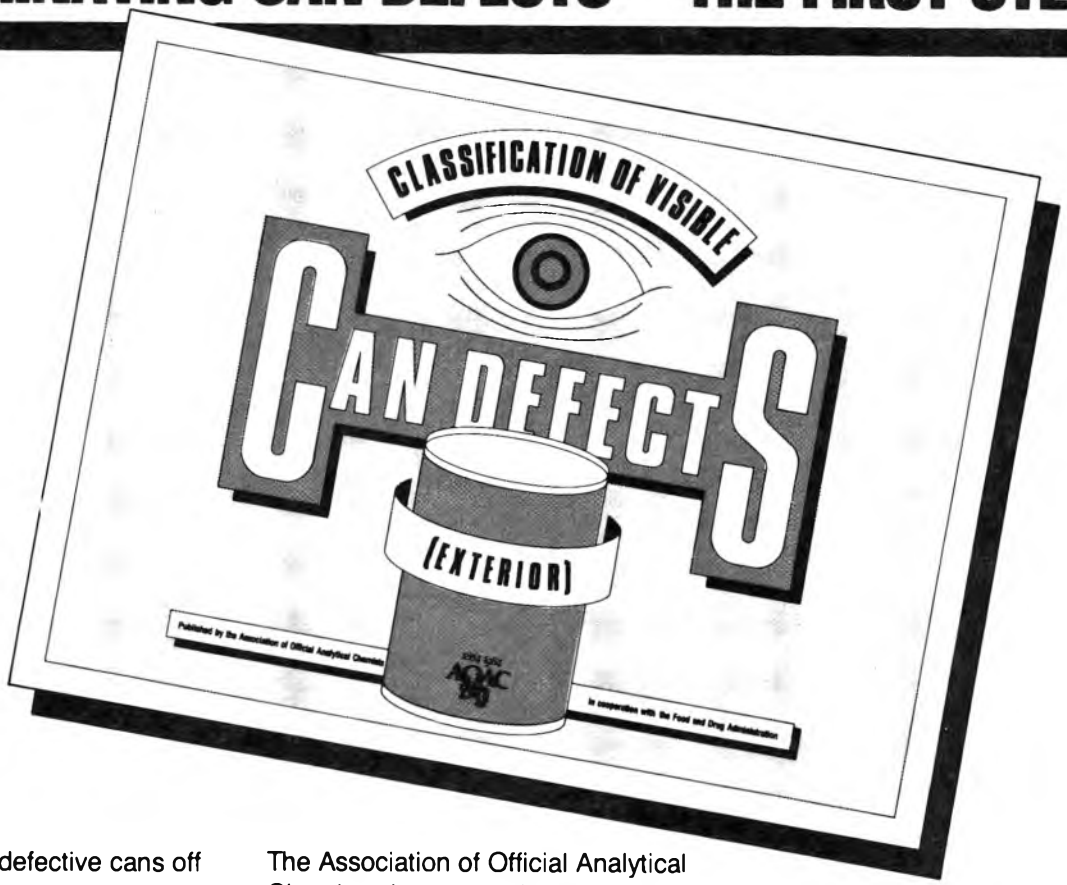

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Organization of AOAC regional sections was begun in 1981. The purpose was to provide a mechanism whereby AOAC members and other laboratory analysts could get together regularly and at low cost to share common interests and problems, find ways to solve these problems by providing practical education seminars and hands-on training workshops, and learn about and become involved in the AOAC methods validation process.

Who May Join

Membership in a regional section is open to any individual interested in the purpose of the regional section and residing or working within its boundaries. Officers of the sections must be members of AOAC.

BENEFITS

- ***Meet and talk with fellow scientists in a relaxed atmosphere***
- ***Learn and improve leadership skills***
- ***Gain exposure for your work and the work of your staff***
- ***Make valuable contacts***
- ***Share common problems and solutions with fellow analysts***
- ***Become better acquainted with the work of your peers***

Locations and Boundaries

There are currently eleven chartered AOAC regional sections in the U.S., Canada, Europe, and the Mediterranean area:

Central Regional Section

Indiana
Kentucky
Michigan
Ohio
West Virginia
Portions of Pennsylvania

Eastern Ontario-Quebec Regional Section

Quebec
Portions of Ontario

Europe Regional Section

Europe
Non-European countries on the Mediterranean Sea

Mid-Canada Regional Section

Manitoba

Midwest Regional Section

Illinois
Iowa
Kansas
Minnesota
Missouri
Nebraska
North Dakota
South Dakota
Wisconsin

New York-New Jersey Regional Section

New York City metropolitan area
New Jersey

Northeast Regional Section

Maine
Massachusetts
New Brunswick, Canada
New Hampshire
Nova Scotia, Canada
Prince Edward Island, Canada
Rhode Island
Vermont
Portions of Connecticut, New York, Pennsylvania and Ontario, Canada

Pacific Northwest Regional Section

Alaska
Alberta, Canada
British Columbia, Canada
Idaho
Oregon
Washington

Pacific Southwest Regional Section

Arizona
California
Hawaii
Nevada
Utah

Southeast-USA Regional Section

Alabama
Florida
Georgia
Mississippi
North Carolina
South Carolina
Tennessee
Virginia

Southwest-USA Regional Section

Arkansas
Louisiana
New Mexico
Oklahoma
Texas

Each regional section is organized and run by a local volunteer committee. Additional sections are in various stages of becoming organized.

For further information contact: **Coordinator, Membership and Regional Sections, AOAC, 2200 Wilson Blvd., Suite 400-J, Arlington, VA 22201-3301 USA, Phone (703) 522-3032.**

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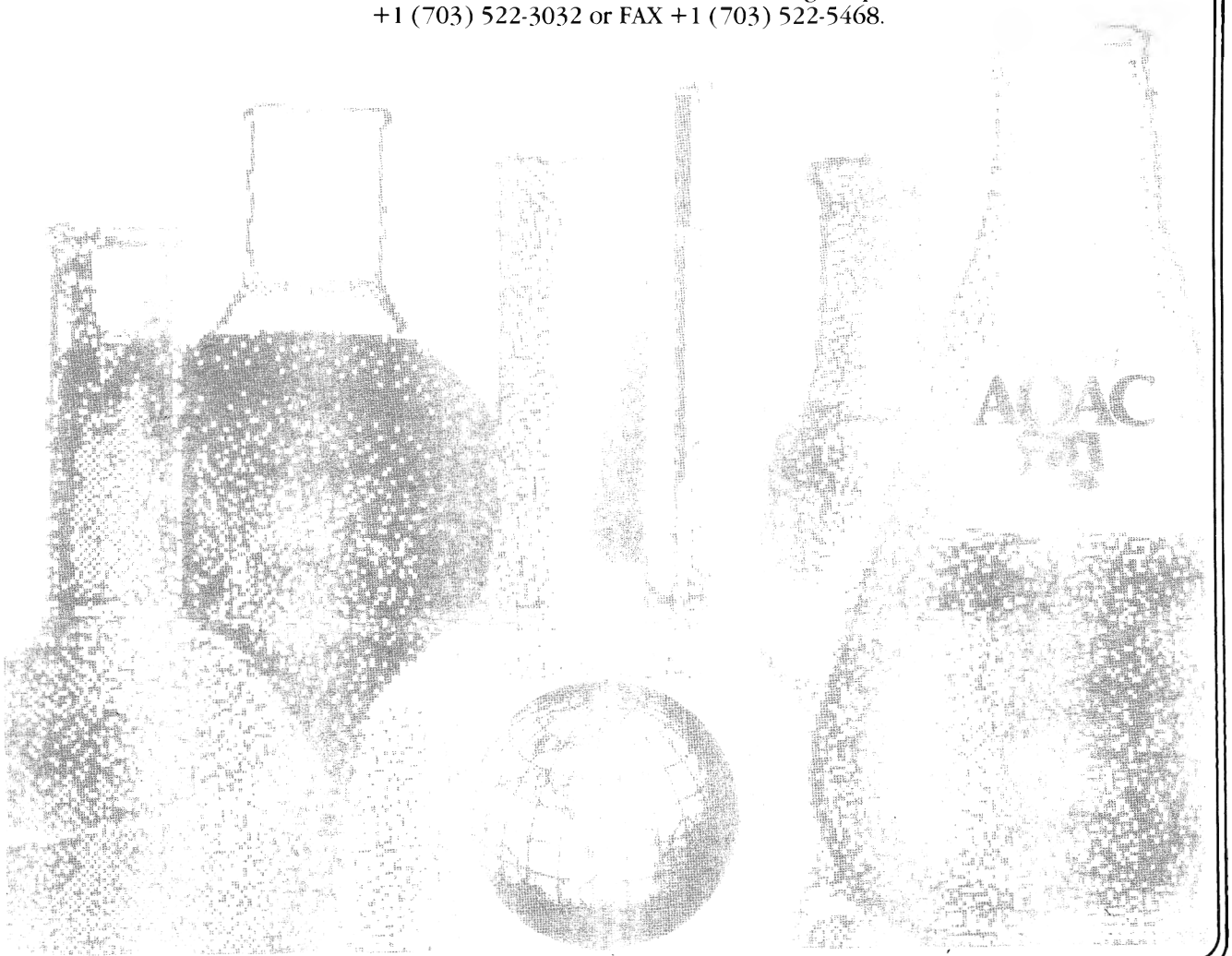
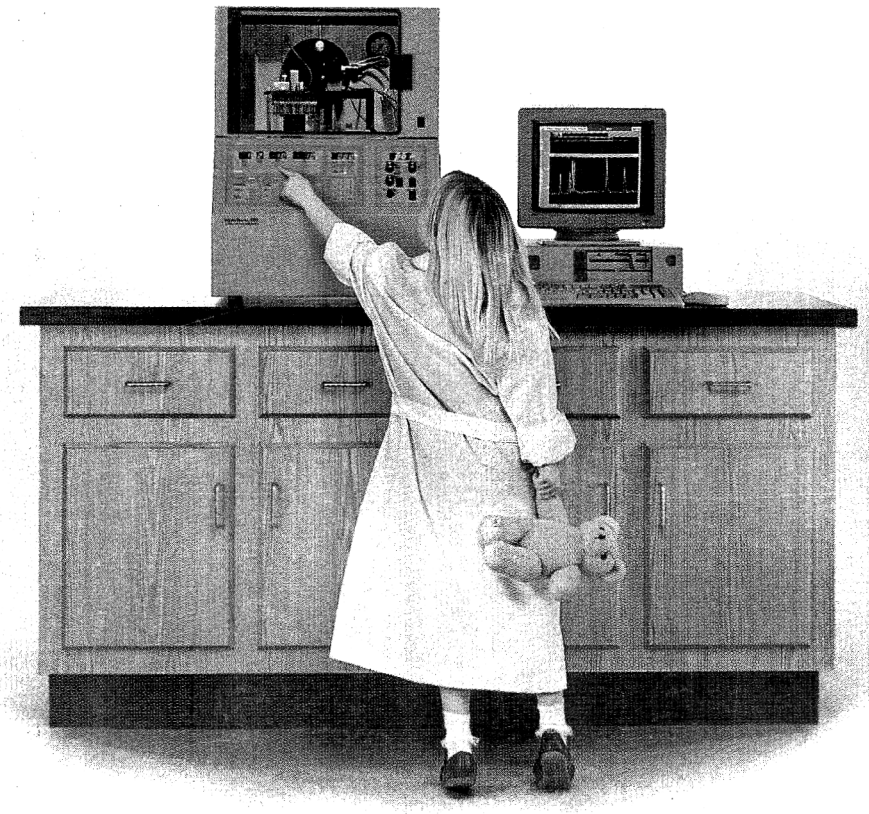


If our approach to organic acid analysis was any easier, you could call it child's play.



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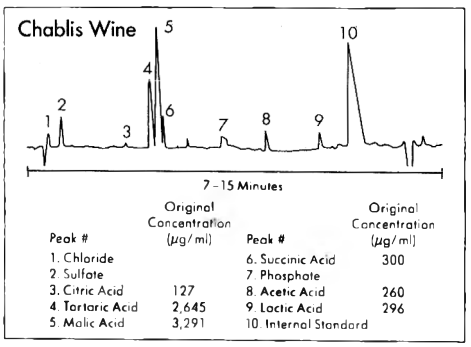
Measuring the organic acids in samples like wine or juice can be a complicated matter. So why bother with conventional methods when you can get good, quantitative results easily and quickly with capillary ion analysis (CIA).



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.

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