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Books in Brief

Capillary Electrophoresis. By Dale R. Baker. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158, USA, 1995. 256 pp. Price: $49.95. ISBN 0-471-11763-3.

Capillary electrophoresis is rapidly replacing liquid chromatography and slab gel electrophoresis as the analytical method of choice. Its advantages over traditional techniques are legion, including speed, high efficiency, small sample size requirements, and low reagent consumption. In addition, it provides high resolution separations for a wide variety of compounds ranging from polar and nonpolar ionic and nonpolar nonionic compounds to high molecular weight biomolecules and chiral compounds. Compared to LC columns, capillaries are relatively inexpensive, easy to use, and last a long time. Because the technique is relatively new—only recently being used for routine analysis of "real" samples, this book offers a practical overview and detailed explanation of this precise, versatile, and highly useful technique. With numerous figures to illustrate concepts, it presents clearly and in a straightforward manner: the theory and principles behind the methodology along with its application; a comparison of capillary electrophoresis to other separation techniques; a description of the instruments used for analysis; various types of capillary electrophoresis, including capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary gel electrophoresis, capillary isoelectric focusing, capillary isotachophoresis; and hands-on guidance in designing, developing, and optimizing a capillary electrophoretic method of analysis.

GMP/ISO 9000 Quality Audit Manual for Healthcare Manufacturers and Their Suppliers, Fourth Edition. By Leonard Steinborn. Published by Interpharm Press, 1358 Busch Parkway, Buffalo Grove, IL 60089, USA, 1995. 657 pp. Price: $289.00. Order Ref.: IPGMPQ4.

Significantly revised, expanded, and updated, GMP/ISO 9000 Quality Audit Manual for Health Manufacturers and Their Suppliers, features easy-to-use audit checklists to help users determine whether their documentation, manufacturing procedures, systems, suppliers, and quality controls meet GMP and ISO 9000 standards. Thousands of professionals already rely on previous editions to measure their level of compliance and documentation of pharmaceutical, medical device, and bulk chemical companies in over 50 countries. The fourth edition adds audit checklists for electronic components, software, and the new proposed medical device GMPs. For the first time, all the audit questions are included on computer disk for use on your PC or laptop. The computer version of the audit checklists is provided on two 3 1/2 in. IMB-PC 1.44 MB floppy disks in WordPerfect 6.0 and ASCII formats.

Methods to Assess Quality and Stability of Oils and Fat-Containing Foods. Edited by Kathleen Warner and N.A. Michael Eskin. Published by AOCS Press, PO Box 3489, Champaign, IL 61826-3489, USA, 1995. 240 pp. Price: $80.00. ISBN 0-935315-58-6.

There is an ever-present need for valid and reliable methods to assess the oxidation of fats and oils. Chapters 1 and 2 provide a broad perspective from which to consider the information in the methodology chapters. In subsequent chapters, authorities in measuring lipid oxidation describe the primary methods as well as their advantages and limitations. The editors have chosen to include only the most representative methods of oxidative deterioration in foods. Sensory analysis is the ultimate analytical test of oil quality for food-grade products. All aspects of sensory analysis are included, as well as those chemical and instrumental tests that relate most closely to the actual sensory quality and stability. Finally, the last 2 chapters present the critical procedures essential before and after any analysis.

ISO 9001, The Standard Interpretation, Second Edition. Published by ISO Easy, PO Box 21, Middletown, NJ 07748, USA, 1995. 128 pp. Price: $19.95. ISBN 0-9636003-7-0.

A complete step-by-step guide to interpreting and implementing the requirements of ISO 9001 has been revised to include the 1994 version of the standard. The 128-page book includes the full text of the latest version of the standard. Each section of the standard is introduced with an illustration showing its required elements. Each element is then clearly explained side-by-side with the exact text of the standard. This is followed by a discussion of common practice in carrying out the standard and an extensive checklist of audit questions. This book provides the quality manager and the compliance team with the complete information they need to direct the registration effort and conduct internal audits. A calendar of conformance activities provides the detailed outline for a compliance project plan. A suggested assignment of department responsibilities for compliance, advice on establishing the scope of registration, and selecting a registration agency, provides more guidance for planning your registration effort.

Quantitative Treatments of Solute/Solvent Interactions. Edited by P. Politzer and J.S. Murray. Published by Elsevier Science, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1994. 380 pp. Price: $228.50. ISBN 0-444-82054-X.

The primary objective of this volume, the first in a series entitled Theoretical and Computational Chemistry, is to survey some effective approaches to understanding, describing, and predicting ways in which solutes and solvents interact and the effects they have upon each other. The treatment of solute/solvent interactions presented emphasizes a synergism between theory and experiment. Experimentally obtained data are used as a basis for developing quantitative theoretical models that permit the correlation and interpretation of the data, and also provide a predictive capability. Linear solvation energy relationships have been quite successful in this respect and accordingly receive considerable attention. Other effective approaches, including computational ones, are also being pursued, and are discussed in several chapters. This is an area that is continually evolving, and it is hoped that the present volume will convey a sense of its dynamic nature.

Hydrocarbon Chemistry. By George Olah. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158, USA, 1995. 450 pp. Price: $69.95. ISBN 0-471-11359-X.

Hydrocarbons are the basis of natural gas and oil, essential to our everyday lives not only as fuels, but also as raw materials ranging from plastics to pharmaceuticals. Chemists today face tough new challenges involving the industrial applications of hydrocarbons, including diminishing oil reserves, an array of environmental hazards, and ever more stringent government regulations. *Hydrocarbon Chemistry*, by brining together all major aspects of contemporary hydrocarbon chemistry, will help readers understand both the fundamentals and applied aspects of the field. Each of *Hydrocarbon Chemistry's* 12 chapters treats a specific type of hydrocarbon transformation and reviews, in depth, all related basic chemistry, including reactivity, selectivity, stereochemistry, and numerous mechanistic aspects, as well as wide range of practical applications.

New Products

Moisture Monitor

The Strandberg 6711 microwave moisture sensor detects moisture in virtually any substance, including liquids that contain solids mixed with water and chemical solutions that contain water. The sensor sends out low-energy microwaves that are reflected back to it by water molecules in the substance. The reflected energy is then converted to a standard 4–20 mA dc signal that is directly proportional to the percentage of moisture in the substance. Contact or noncontact operation is permissible. Moisture can also be detected using waveguides or ducts that permit the sensor to be remotely located away from heat, inside ovens and furnaces. Strandberg Engineering Laboratories, Inc.
Circle No. 329 on reader service card.

Fisher Scientific Releases General Catalog

The 1995–1996 edition of the Fisher Catalog is the most comprehensive source of laboratory equipment, containing over 45 000 catalog numbers. It is packed with an outstanding selection of instrumentation, supplies, and reagents—virtually anything needed to equip a laboratory. New to the 1995–1996 edition are over 350 product pages and more than 15 300 catalog numbers. The Chemical Section has been expanded to include over 700 new chemicals and more than 1700 new catalog numbers from several new suppliers. Plus, all catalog numbers in the Chemical Section are now indexed in the Fisher Catalog Number Index. Fisher Scientific.
Circle No. 330 on reader service card.

Varian's LIMS Software Speeds Laboratory Operations

Varian introduces the latest version of its StarLIMS information management software, offering 3 major new features that enhance its capabilities in pharmaceutical laboratories and other industrial settings. StarLIMS version 6.1 features new stability-study software to greatly simplify the planning and execution of complex time/condition-based testing protocols. The new software also offers an innovative spreadsheet-style sample login feature to speed data management tasks. StarLIMS may be used in either a Windows- or DOS-based environment, enhancing ease of use and providing extensive hardware compatibility. Varian Associates, Inc.
Circle No. 331 on reader service card.

New LEICA Microscope

LEICA MZ12, the only stereo microscope with 12.5:1 zoom, opens new application possibilities. The continuous observation of 3 dimensional objects from lowest to highest magnification uncovers previously hidden information. For the first time, the magnification of an object can be increased without interruption from 80 to 1000 times in a single zooming movement. The LEICA MZ12 has a wider zoom range and a higher maximum magnification than any other stereo microscope on the market. A complete range of main objectives are available, allowing a maximum magnification of 640 times. Leica.
Circle No. 332 on reader service card.

Unique Enzyme Substrate Provides Rapid *E. coli* 0157:H7 Testing

Biosynth is currently manufacturing a unique enzyme substrate that quickly and easily recognizes the dangerous *E. coli* 0157:H7 strain in meat and poultry products. When added to MacConkey sorbitol agar plates and incubated with a meat or poultry sample at 35°C for 24 h, Biosynth's enzyme substrate will produce a deep blue color to indicate normal *E. coli* colonies. Conversely, the lack of any color change in the colonies indicates the presumptive presence of *E. coli* 01257:H7 in the test sample. This method is part of the official U.S. Food and Drug Administration testing procedure for *E. coli* 0157:H7. Biosynth International, Inc.
Circle No. 333 on reader service card.

Beckman Bulletin Explains Mathematics of Full Spectrum Quantitation

A new technical information bulletin, T-1795A, is available from Beckman Instruments, Inc., that presents the mathematics of Full Spectrum Quantitation (FSQ) as an advanced method for multicomponent analysis (MCA) on the DU Series 600 and 7000 spectrophotometer from Beckman. The 8-page bulletin describes a simple case of quantitating one component from the absorbance at one wavelength and the quantitation of multiple components at multiple discrete or continuous wavelengths. The brochure introduces principal component analysis (PCA) and explains this method for vector analysis of multiple spectra. Also, FSQ, which uses PCA, is described. Beckman Instruments, Inc.
Circle No. 334 on reader service card.

Finger Tight High Pressure Vessels

A new high pressure vessel with a unique self sealing design requires very little force to open and close. It is a true finger tight vessel. The "c" cup type seal is typically made of graphite reinforced Teflon with an energizing spring. The seal actually works better at higher pressures. The spring seal can be replaced with an O-ring for applications requiring little metal contact. The vessel is available in different materials, and 17-4 PH

stainless steel is standard material. This material is 60% stronger than regular stainless steel, making it smaller and lighter. Thar Designs, Inc.
Circle No. 335 on reader service card.

Varian Offers 1995–1996 Analytical Supplies Catalog

The Varian 1995–1996 analytical supplies catalog features hundreds of new parts and accessories designed to extend the life of analytical instruments and systems. Available free of charge, the catalog describes Varian's latest product offerings, from supercritical fluid extraction instruments and liquid chromatography columns to the new Liberty 150 AX Turbo ICP spectrometer. The catalog features an easy-to-use directory for placing orders and obtaining additional customer support. An international version of the catalog, which includes information specific to customers outside the United States, is available as well. Varian Analytical Instruments.
Circle No. 336 on reader service card.

Low-Cost Portable Balance

The SV series of top loading balances offers a rugged design, simple operation, and the ultimate in portability. In fact, the balances themselves weigh less than 1 pound. Due to their compact design, the balances are ideal for thousands of applications, including portion control and dietary management, sample preparation, environmental field testing, quality control, and education, to name a few. A&D Weighing.
Circle No. 337 on reader service card.

State-of-the-Art pH Tester

The Sentron 501 pH PocketFet, a low-cost, pocket-sized pH meter, uses the revolutionary Ion Sensitive Field Effect Transistor (ISFET) pH sensor. It is ideal for food, soil, wastewater, or spot-checking pH. The 501 calibrates easily with only one drop of buffer solution. It stores dry, requires no fill solutions, responds instantly, and resists breakage, eliminating the hassles as associated with glass pH electrodes. It can be calibrated at 1, 2, or 3 points. Features include auto power-off, calibration backup, 200 h battery life, and a replaceable sensor tip. Thomas Scientific.
Circle No. 338 on reader service card.

Rapid *E. coli* Contamination Testing Kit for Water

Celsis has announced the development of a revolutionary new system for the rapid identification and counting of the harmful bacteria *Escherichia coli* (*E. coli*) and related species known as Coliforms in water. The presence of *E. coli* in water can indicate human fecal contamination. Over 150 million Coliform tests are carried out every year in the developed world. Celsis has adapted its novel, rapid microbiological counting system, Celsis Digital System, to identify and count *E. coli* and Coliforms in a single procedure. These tests can be carried out in 24 h, compared to 2 to 4 days for traditional methods. Celsis, Inc.
Circle No. 339 on reader service card.

Cartridge System for LC Columns

New LC columns are packed with the popular NUCLEOSIL manufactured by Macherey-Nagel. They are available with bonded phases of C_{18}, C_8, cyano, phenyl, and amino, as well as bare silica. The packings are manufactured from base silica of either narrow pore (100Å) or wide pore (300 Å) of 3 and 5 µm particles. They are available packed in semi-microbore (2 and 3 mm id), as well as standard analytical (4.6 mm id) hardware. DyChrom.
Circle No. 340 on reader service card.

Pressure Manifolds Reduce Connection Components and Potential Leak Joints

A line of specialty pressure manifolds incorporates several needle valve, fitting, or adapter-type connection components into a single component. Specialty pressure manifolds minimize space requirements and reduce installation time necessary to plumb a pressure system. In addition, by reducing the number of components in a system, manifolds reduce the number of potential leak joints. Manifolds are capable of withstanding vacuum pressures up to 60 000 psi and are available in a variety of materials and sizes. Autoclave Engineers.
Circle No. 341 on reader service card.

Catalytic Process Removes TCE, Other Harmful Chemicals from Groundwater

An inexpensive new catalytic process rapidly and completely destroys chlorinated organics such as trichloroethylene in water, without forming harmful by-products that require costly disposal. These organics foul groundwater in at least half of the 1400 Superfund sites, and the National Research Council estimates that up to 40 000 sites have contaminated groundwater that may require remediation. The new catalytic process reductively dechlorinates compounds of 1-2 carbon atoms in aqueous systems at 20 ppm, a much higher level than one would expect in groundwater. This method destroys compounds such as TCE, PCE, TCA, dichloroethylene, chloroform, methyl chloride, and carbon tetrachloride, yielding environmentally benign methane, ethane, and chloride ions. The process also completely and rapidly dechlorinates pentachlorophenol (PCP) and polychlorinated biphenyls (PCBs). Research Corporation Technologies.
Circle No. 342 on reader service card.

New Reference Booklet on Drying Compressed Air

A comprehensive guide explaining how to optimize compressed air systems is now available from Whatman, Inc. The guide includes articles and detailed information on methods and capabilities for drying compressed air, filtration recommendations, techniques on condensation prevention, and types of drying systems available. For further information, contact Whatman, Inc., 260 Neck Rd, Box 8223, Haverhill, MA 01835-0723, USA, telephone +1-800-343-4048, fax +1-508-374-7070. Whatman, Inc.
Circle No. 343 on reader service card.

Run SFE System Economically with Liquid CO2 and No Helium

The Spe-ed Chiller, a new option for the Spe-ed SFE, allows the operator to use an entire cylinder of liquid CO_2 resulting in a $100 to $150 per cylinder savings. Most current systems require the use of helium head pressure in CO_2 cylinders. One-quarter to 1/3 of liquid CO_2 in a cylinder is left unavailable because of helium pressure dropping off as the cylinder is used. With the Spe-ed Chiller, no helium head pressure is required, resulting in an additional savings of $30 per cylinder cost for helium. By eliminating helium, the integrity of the CO_2 is maintained. Helium reduces the solubility of CO_2. Studies have shown that CO_2 density leads to higher solubility, resulting in shorter extraction times, less CO_2 consumption, and improved recovery of polar extractions. Greater variability in analytical recoveries has been observed in CO_2 diluted with helium, resulting in standard deviations increasing from 9 to 32%. Applied Separations.
Circle No. 344 on reader service card.

Qual and Quant LC Computer-Based Program

A training program on qualitative and quantitative LC techniques represents the newest addition to SAVANT Audiovisuals' computer-based learning series. The program, entitled "Identification and Quantitation Techniques in HPLC," presents identification techniques, retention times, spiking, and on-line versus off-line spectroscopic confirmation methods. The quantifitation section incorporates sample preparation, sources of error, peak measurements, data systems, and calculations methods. All topics are fully demonstrated with interactive examples. Savant Audiovisuals, Inc.
Circle No. 345 on reader service card.

Automated SPME II Speeds Sample Preparation

Varian's SPME II, a new version of the revolutionary solvent-free solid phase microextraction device for gas chromatography, eases method development, enhances sensitivity, and speeds the sample preparation process. The SPME technology dramatically changes the way laboratories perform sample analysis. It replaces time-consuming, manual methods that rely on large amounts of environment-damaging solvents with an automated, rapid, solvent-free sample preparation method. By eliminating the need for many manual steps, SPME reduces the labor costs required to produce samples. Because it is a solvent-free method, SPME minimizes the need for costly high-purity solvents. Automating the sample preparation process speeds results from the laboratory. SPME II allows the use of automated solid phase microextraction for a broader range of analytes with improved analytical performance.
Circle No. 346 on reader service card.

For Your Information

Meetings

May 18–20, 1995: Official Methods Board Meeting, Montreal, Quebec, Canada. Contact: Nancy Palmer (npalmer@aoac.org), AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA, +1-703-522-3032

June 13–15, 1995: Board of Directors Meeting, Arlington, VA, USA. Contact: Faye North (fnorth@aoac.org), AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA, +1-703-522-3032

June 15, 1995: AOAC MidCanada Regional Section Meeting, Winnipeg, Manitoba, Canada. Contact: Nelson Barchuk, Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main St, Winnipeg, MB, R3C 3G8, Canada, telephone +1-204-983-3036

June 22–23, 1995: AOAC América Latina y El Caribe Section Meeting, Sao Paulo, Brazil. Contact: Maria Ines R.M. Santoro, University de Sao Paulo, Ciudade University, CP 30786, Conj Quim B13, Sao Paulo 01000, Brazil, telephone +55-11-2118986

June 26–29, 1995: AOAC Midwest Regional Section Meeting, St. Paul, Minnesota, USA. Contact: Larry Felice, University of Minnesota, 1333 Gorner Ave, St. Paul, MN 55108, USA, telephone +1-612-625-9791

June 29–30, 1995: AOAC Pacific Northwest Regional Section Meeting, Olympia, Washington, USA. Contact: Kathleen Wickman, Oregon Department of Agriculture, Laboratory Services Division, 635 Capital St, NE, Salem, OR 97310-0110, USA, telephone +1-503-378-3793

September 17, 1995: Board of Directors Meeting, Nashville, TN, USA (in conjunction with AOAC annual meeting). Contact: Faye North (fnorth@aoac.org), AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA, +1-703-522-3032

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

CD-ROM Version of 16th Edition of OMA Soon to be Available

A CD-ROM version of AOAC INTERNATIONAL's newly released 16th Edition of the *Official Methods of Analysis* (OMA) will be available mid-1995. It will be a full-text searchable database of all AOAC Official Methods.

Using Adobe Acrobat™ Software, program users will be able to retrieve any method in OMA searching by method title, method number, chapter number, or any word or combination of words or phrases. The result of a search will list all methods that pertain to the search, or, if the search is conducted on only a single method title, that method title alone will appear. Then, you simply click on the method desired for full viewing.

A fully formatted method will appear—NO cumbersome ASCII text! Tables and figures will also be fully formatted and reside within the text just as they do in the printed product.

As for finding methods referenced within a method, the referenced method will be boxed, indicating a "hypertext" link. By clicking on the box, the referenced method will be brought up on the screen. Thus, users will have instantaneous access to all referenced methods and will be able to toggle back and forth between referenced methods and the original.

Other features will include: zooming in and out on a page or any portion of a page; creating "bookmarks" by opening up a column and creating an icon (bookmark) for future reference; creating "sticky notes" or messages to come back to; and printing out a method, or any number of methods.

Those who buy the CD-ROM of OMA will be able to subscribe to updates and will receive a new disk when a supplement is issued. This will mean that subscribers will always have a current version of OMA.

Windows and Macintosh platforms will be available. Minimum requirements for the Windows version are as follows: 486 IBM or compatible personal computer, with Microsoft® Windows 3.1, 4 M of RAM, a CD-ROM drive with 400 ms access time, and 6 M hard disk space. Minimum requirements for the Macintosh version are 68020 CPU, System 7.0 software, 2 M Application RAM, and 6 M hard disk space.

With either format, user instructions and help menu will be available for viewing or printing, as well as a quick reference guide card that will indicate installation and start-up, and application overview. Available user-support will include +1-800#, fax, and on-line numbers to a "Help Desk," which will answer users' questions regarding installation and Adobe Acrobat™ software features.

The initial CD-ROM single user fee will be $575 for those in North America and $595 for those outside North America. Local area network versions will also be made available at additional cost.

If there is enough demand and the initial product is successful, enhancements will soon follow. In 1996, all surplus methods will be included on the CD-ROM. And AOAC INTERNATIONAL plans to include the full collaborative studies of methods as published in the *Journal of AOAC INTERNATIONAL.*

Codex Committee on Food Import and Export Inspection and Certification Systems Meets in Canberra, Australia

The third session of the Codex Committee on Food Import and Export Inspection and Certification Systems (CCFICS) was held in Canberra, Australia, February 27–March 3, 1995. The meeting was attended by 197 delegates and advisors from 47 Codex member countries and 19 observers from 10 international organizations. AOAC INTERNATIONAL was represented by Ronald R. Christensen, Executive Director.

Opening statements at the meeting recognized the relevance of the work of the CCFICS in light of implications arising from the Final Act of the Uruguay round of multilateral trade negotiations, which went into effect in April 1994, commonly known as the General Agreement on Tariffs and Trade (GATT) The Final Act places obligations on member nations in their conduct of international trade.

Of specific relevance to the Codex Alimentarius Commission, food importing and exporting nations, and food importers and exporters, are two separate agreements that are part of the Final Act, namely the *Agreement on the Application of Sanitary and Phytosanitary Measures* (SPS) and the *Agreement on Technical Barriers to Trade* (TBT). These agreements are likely to become fully effective during 1995.

The SPS Agreement deals with measures necessary to protect human life or health while the TBT Agreement deals with other technical measures, such as those necessary to prevent deceptive practices. In some cases, especially with respect to food, provisions of the two agreements overlap. For example, a labelling issue could be seen as related to both consumer health and consumer deception.

The basic objective of the two agreements is to limit the use of protective measures that do, or may, restrict trade to only those measures that are justified to provide importing countries the level of protection that is necessary. At the same time, however, the fundamental right of member nations to protect themselves, at the level they determine necessary, is recognized.

Except under designated circumstances, GATT member countries are required to base their SPS and TBT measures relating to food safety on Codex standards, guidelines, and recommendations, where such Codex provisions exist. Hence, the importance of the work of the CCFICS and other Codex committees.

Within an ambitious agenda, the CCFICS addressed the following important subject matters:

- Draft Principles for Food Import and Export Inspection and Certification.
- Draft Guidelines for Information Exchange in Food Control Emergency Situations.
- Proposed Draft Glossary of Terms Based on Internationally Accepted Definitions.
- Proposed Draft Harmonized Guidelines on Import and Export Inspection and Certification Systems.
- Proposed Draft Guidelines for the Exchange of Information on [Import] Rejections.
- Proposed Draft Guidelines on the Principle Elements in an Electronic Documentation System.
- Proposed Draft Guidelines on the Application of the ISO 9000 Series to Food Inspection and Certification Systems.

Work on the "Draft Principles for Food Import and Export Inspection and Certification" and "Draft Guidelines for Information Exchange in Food Control Emergency Situations" was completed after extended discussion, debate, and revision. These two documents are to be submitted in July 1995 to the 21st Session of the Codex Alimentarius Commission for adoption at Step 8 as **Codex standards**.

With regard to the "Proposed Draft Glossary of Terms based on Internationally Accepted Definitions," it was decided to discontinue development of the Glossary for the present time. There was little current interest among the Committee members in developing it into an official Codex document. It was agreed that the Australian Secretariat would revise and maintain the Glossary for use by the Committee as a future reference source if and when required.

The "Proposed Draft Harmonized Guidelines on Import and Export Inspection and Certification Systems," prepared by the delegation from Canada and representatives of the European Communities, received comprehensive review. At the conclusion of this review, the Committee requested the document be redrafted in light of the comments and suggestions presented. It was agreed that the new draft would be circulated for further comment prior to being discussed again at the next session of CCFICS.

The delegation of Sweden presented the "Proposed Draft Guidelines for the Exchange of Information on Rejections." This document also received thorough review, and it was agreed to forward the proposed draft to the 21st Session of the Commission for adoption at Step 5 as a **draft standard**.

The "Proposed Draft Guidelines on the Principle Elements in an Electronic Documentation System" met with some concern being expressed over terminology and assumptions regarding centralization of electronic reception facilities within countries. It was agreed to hold the document at Step 3 of the Codex approval process, which calls for the document to be circulated to members and interested international organizations for comments on all aspects of the proposal.

The delegation from France presented the "Proposed Draft Guidelines on the Application of the ISO 9000 Se-

ries to Food Inspection and Certification Systems." Several delegations stressed the appropriateness and importance of the ISO 9000 series in general quality systems registration. However, a number of these same delegations questioned the usefulness, based on their experiences, of the ISO 9000 series in the food sector. These countries were of the opinion that the main quality system required for protection of consumer health was the HACCP-based system guidelines already adopted by Codex. Other delegations pointed out, on the other hand, that the HACCP system and the ISO 9000 standards were fully compatible; that HACCP could be incorporated into an ISO 9000 designed system to meet regulatory requirements. In the end, the Committee agreed it was premature to consider the document for consideration as a draft Codex standard. It was requested the document be further revised in light of the discussions and be considered as a proposal at the next session of the Committee. It was further agreed that information on the application of ISO 9000 within the foods sector would be sought and presented at the next session of the Committee.

The Committee concluded the session by discussing its future program of work. In addition to the ongoing projects, it was agreed that work on developing a model inspection certificate would have value. The Chairman of the Codex Committee on Fish and Fishery Products, who was in attendance, agreed to draft a paper for the next meeting of CCFICS identifying the requirements to be included in such a model certificate document. Also, the delegation from the United States proposed elaboration of draft guidelines for the development of bi- or multilateral agreements between exporting and importing countries. There was strong support for this proposal, and the Committee asked the U.S. delegation to prepare a draft for consideration at the next session.

Pending evaluation of alternative dates and approval of the Codex Alimentarius Commission, it is expected that the 4th Session of the CCFICS will be held in Canberra, Australia, in early 1996.

AOAC Connects to the Internet

AOAC INTERNATIONAL may now be reached directly through the Internet.

In April, e-mail has been activated. Members and others can now reach various AOAC departments or individual staff from anywhere in the world.

AOAC INTERNATIONAL will accept e-mail in a variety of formats, but WP5.1 and ASCII text files are preferred.

Direct Internet Addresses Now Available

Communicate with us via the Internet by sending your e-mail to one of the following AOAC INTERNATIONAL Internet addresses:

INFO@AOAC.ORG—AOAC e-mail address for obtaining general information.

PUBSALES@AOAC.ORG—To place orders and make inquiries regarding AOAC books or the *Journal of AOAC INTERNATIONAL.*

JAOAC@AOAC.ORG—To electronically submit papers for publication in the *Journal of AOAC INTERNATIONAL.*

RI@AOAC.ORG—To contact the AOAC Research Institute regarding the AOAC Test Kit Performance Testing Program.

Individual staff addresses—To correspond with individual AOAC staff, address the e-mail directly to that employee's mailbox by using his or her first initial and last name followed by @AOAC.ORG (for example, to send an e-mail to Nancy Palmer, AOAC Director of Technical Services, address it to: npalmer@aoac.org).

To be Available in May

Come May, look on the Internet for the AOAC annual meeting preliminary program, AOAC officer and committee lists, a meetings and deadlines calendar, publications catalog, short course listing, and information on AOAC INTERNATIONAL and its programs. All you will need is a Web viewer such as Mosaic or Netscape. The address will be: www.aoac.org. For example, you will be able to reach the AOAC "home" page by typing: **http://www.aoac.org**

Later in the Year

Other information, for example, *The Referee* and the AOAC Membership Directory will follow. Future plans include AOAC scientific discussion groups, bulletin boards, and AOAC member-generated information on the Internet, and, for a fee, the *Journal of AOAC INTERNATIONAL*, AOAC Official Methods, and AOAC Peer-Verified Methods are planned to be available, as well.

World-Wide Web

"All these services will be available from AOAC INTERNATIONAL via a new World-Wide Web Server," according to Chris Mass of Potomac Consulting Group, Inc., AOAC's computer consultant. He says, "If you have not yet had occasion to work on a Web Server, we think you will be really pleased with the amount, the quality, and the convenience of the information offered by this technology."

Currently, over 30,000 Web Server sites are up and running all over the world. They offer a huge range of information—from the latest scientific development to popular music reviews. Much of this information has long been available on the Internet. However, it was difficult to find and access, and was not 'user friendly' (users had to know many arcane, complex commands). The World-Wide Web technology has dra-

matically changed this picture for Internet users. "As a user, you only need Windows (or a Macintosh), an Internet Connection, and an inexpensive Web 'Viewer,' such as Mosaic (offered for free)," says Mass. "Once installed, Web viewers are ridiculously easy to use. Yet, they are extremely powerful tools for finding, reading, printing, and downloading information from the millions of pages of materials available on the Internet."

Links to the Internet have been made possible for AOAC INTERNATIONAL through system hardware and software upgrades, including Microsoft Windows and a variety of Windows-based applications. AOAC has changed to WordPerfect 6.1 for Windows, and its e-mail capabilities have been upgraded to Novell Groupwise 4.0 (formerly Wordperfect) for Windows.

New Sustaining Members

AOAC welcomes the following new Sustaining Members:

- ANRESCO, Inc., San Francisco, California, USA
- CETAC Technologies, Inc., Omaha, Nebraska, USA
- Chilean Nuclear Energy Commission, Santiago, Chile
- Fundación Chile, Santiago, Chile
- LECO Corporation, St. Joseph, Michigan, USA
- National Seafood Inspection Laboratory, Pascagoula, Mississippi, USA
- OMIC USA, Inc., Portland, Oregon, USA
- Promochem GmbH, Wesel, Germany

AOAC Thanks Wiley Fund Contributors

The Association thanks the members of AOAC INTERNATIONAL listed below for their contributions to the Harvey W. Wiley Award, which rewards scientists for outstanding contributions to analytical methodology, and the Harvey W. Wiley scholarship, which provides support to deserving students majoring in fields relevant to the mission of AOAC INTERNATIONAL:

Rafael Amon, Laboratorio Quimico Lambda, San Jose, Costa Rica

Karl Heil, Hoechst Veterinaer GmbH, Frankfurt, Germany

Anthony T. Pavel, London, Ontario, Canada

Willard Wesley Weeks, North Carolina State University, Raleigh, North Carolina, USA

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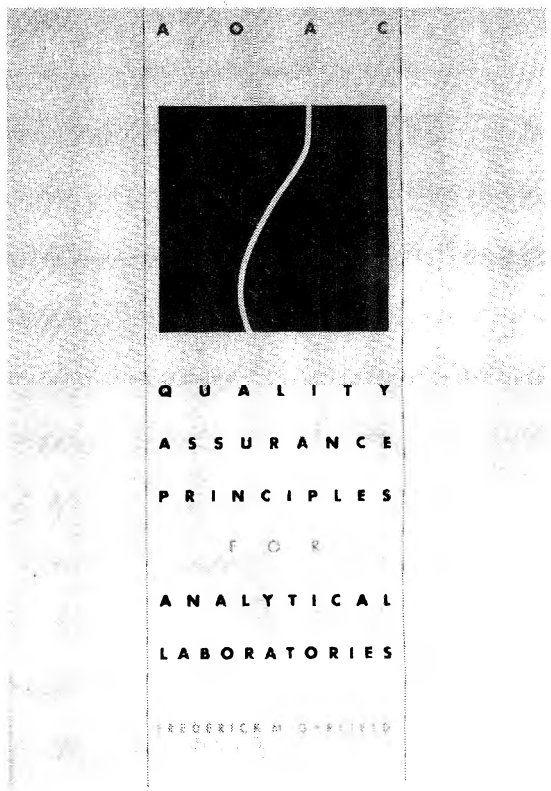

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SPECIAL REPORT

Integration of Immunochemical Methods with Other Analytical Techniques for Pesticide Residue Determination

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The growing volume of literature concerning immunoassay analysis for trace levels of agrochemicals and other low molecular weight contaminants in various matrixes is indicative of the tremendous interest in and utility of this analytical technique. Most immunoassay methods described in the literature analyze compounds directly, for example, a herbicide in water, or involve solvent exchange of an organic sample extract or dilution of an aqueous-based sample to minimize the matrix effect. As immunoassay for small molecules becomes widely accepted and applied, new challenges involving more complex chemicals in more difficult matrixes arise. The integration of "classical" analytical chemistry with immunochemistry can provide new techniques and approaches useful in discovering the movement, mode of action, and ultimate impact of certain chemicals on humans and the environment.

Immunoassay is beginning to achieve its enormous potential in the field of contaminant residue chemistry. Because immunochemical approaches are based on attraction between an analyte (or analyte derivative) and an antibody to that analyte or derivatized analyte, immunochemical techniques can be applied in virtually all stages of trace analysis. An immunochemical approach to trace analysis has even broader applicability than simply rapid quantitation in aqueous media. Such applications can include sample extraction, sample cleanup, and combining immunoassay with other techniques to take advantage of the strengths of individual methods. In this regard, immunochemistry is as fundamental as chromatography with a range of applicabilities rivaling those of chromatography. Distinct advantages will accrue as we learn to exploit the full range of capabilities of immunochemistry in the analytical process.

Low cost of analysis per sample, high sample turnover rate, and sensitivity have been extensively detailed as advantages of immunoassay. As with other analytical techniques, approximate concentration of sample is interpolated from a standard curve from a single signal response (e.g., absorbance). An erroneous result may be produced by matrix effects or the inability to differentiate between structurally similar compounds (i.e., cross-reactivity). To use the advantages of an immunoassay, understanding how an assay is developed and validated, and learning the correct use of commercially available kits are critical. These concepts have been the subject of numerous reviews (1–5).

The general approach for sample preparation and characterization of an immunoassay for specific matrixes has involved simple dilution of sample until no matrix effect is observed. However, as more complex systems are analyzed, careful sample preparation becomes critical for reliable results. Analytical chemists generally determine the best means of sample preparation (i.e., by exploiting size, volatility, functional groups, etc., in extracting the analyte), keeping in mind the final means of detection. When simple dilution of a sample prior to immunoassay does not eliminate matrix effects, sample preparation methods already used in classical analytical chemistry should be explored. This review will discuss sample extraction methods used prior to immunoassay analysis. In addition, use of antibodies as an active site for extraction or cleanup prior to analysis by immunoassay or other means is also examined. By exploring and exploiting the strengths and weaknesses of both classical and immunochemical techniques with regard to extraction, matrix effects, derivatization, and sensitivity, better methods of sample preparation can be developed.

Interfacing Immunoassays with Sample Preparation and Cleanup Techniques

When assessing sample preparation methods for immunoassay, approaches that emphasize speed and minimal steps are most desirable to keep cost down. Even when column or solid-phase extraction steps are needed, immunoassay is still relatively inexpensive compared with other analytical techniques. The ability of immunoassay to perform sample determinations in parallel (as opposed to sequential determination by traditional chromatographic methods) can tremendously reduce the time required for analysis. The coupling of a sample

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extraction and/or cleanup step to immunoassay, even in water where direct determination can be done, also improves the confidence in the analytical results over that of direct measurement.

In some cases, by minimizing or eliminating sample preparation and handling, immunoassays can appear to overestimate analyte concentration when compared with other methods. This may be due, for example, to compound loss in phase transfer, adsorption to various surfaces, or incomplete retention on a solid extraction phase, resulting in reduced recovery for the traditional method.

For samples in more complex matrixes such as foodstuff and soil, more preparation is generally needed prior to analysis. For immunoassay, matrix effects are manifested as a decrease in assay sensitivity or reduction in color development. These effects may be the result of nonspecific binding of the analyte to the matrix, nonspecific binding of the matrix to the antibody or enzyme, or to denaturation of the antibody and/or enzyme. When such signal changes occur, sample extraction or cleanup is indicated. The general approach is to begin with an established means of extraction for a particular analyte, such as homogenizing foodstuff or Soxhlet extraction of tissue with solvent. The analyst can begin from this step to apply immunoassay. An example of this approach is described for the analysis of tetrachlorodibenzodioxin (TCDD; 6). Depending on the analyte and the assay, further cleanup after extraction may or may not be needed. For example, after extraction of soil for bromacil with 1% NaOH and neutralization of the extract, an immunoassay was performed without subsequent cleanup steps that were required for liquid chromatographic (LC) and gas chromatographic (GC) analysis (7).

The advantages of immunoassay for analysis of drugs in blood and urine are well documented. Extension of this technology to environmental compounds in water became prominent when monitoring of groundwater became mandatory. Sample preparation techniques have gradually become more complex as immunoassay is applied to more lipophilic compounds and to traditionally more difficult matrixes. The strength of immunoassays is in analysis of aqueous media; the first immunoassays followed clinical practices of using simple dilution of aqueous samples and direct analysis in the immunoassay (Table 1). These assays had to be validated with classical GC or LC techniques. However, the solvents used for these techniques were often water immiscible and relatively nonpolar, making them unsuitable for use in immunoassay. In addition, to decrease variability due to application of different extraction methods, a common sample preparation technique was sought. Samples were often extracted with water-soluble solvents (such as methanol or acetonitrile), and these extracts were diluted for immunoassay (Table 2). With more lipophilic compounds, this approach is not feasible, and solvent exchange is used (Table 3). For example, an atrazine-treated soil was extracted with ethyl acetate, the ethyl acetate extract was evaporated, and the residue was taken up in buffer for immunoassay analysis or methanol for LC analysis (8). More recently, analysts have been using solid-phase or other column cleanup steps and subsequent analysis of the eluate by immunoassay.

Table 1. Assays with no sample preparation

Compound	Matrix	LLD[a]	Reference
Atrazine	Tap water	0.1 ng/mL	29
Atrazine	Water	0.05 ng/mL	30
Alachlor	Surface water	<0.2 ppb	31
Fenpropimorph	Soil leachates	13 ng/mL	32
2,4-D	River water, urine	<0.25 ng/mL	33
Diclofop-methyl	Urine	115 ng/mL	34
Paraquat	Serum	<0.08 ng/mL	35
Terbutryn	Pond water	4.8 ng per assay	36
Molinate	Field water	21 ng/mL	37
Paraquat	Plasma, urine, milk	1–7 ng/mL	21, 38
Carbaryl	Municipal and surface water	0.05 ng/mL	39
Metolachlor	River water, soil	2 ng/mL	40

[a] Lowest limit of detectability reported.

Liquid–Liquid Partitioning

A great strength of immunoassay is the ability to analyze an aqueous sample directly. Separation or extraction steps prior to analysis, however, provide further evidence of analyte identity based on physical and chemical behavior. Partitioning a sample between immiscible solvents is an example. For example, when a urine sample from a person with a known exposure to atrazine was analyzed directly, a large immunoreactive response was obtained. To identify the immunoreactive material, the urine was partitioned with chloroform, which separated atrazine from hydrophilic metabolites (9). Most of the immunoreactive material was in the aqueous layer. Previous research indicated that people could be monitored for exposure to atrazine by analyzing their urine(10). This new evidence based on partitioning prior to immunoassay uncovered a urinary metabolite that was more abundant than the parent. By using immunoassay in conjunction with a simple partitioning step, a key chemical characteristic (hydrophilicity) of the metabolite was evident, ruling out atrazine as the immunoreactive compound. In addition, immunoassay was a valuable technique for quickly screening urine for *s*-triazine exposure.

Solvent partitioning has also been used to remove interferences from sample extracts when analyzing for the appearance of hydroxyatrazine during atrazine biodegradation (11). The organic extract of the digestion material is dried and reconstituted in assay buffer. Partitioning this aqueous extract with chloroform reduces by a factor of 2 the amount of buffer needed to dilute a blank sample to obtain minimal matrix effects. This partitioning step also removes unmetabolized atrazine, so that cross-reactive response from the parent compound is eliminated.

Solid-Phase Extraction

Solid-phase extraction (SPE) columns as well as silica, alumina, and other cleanup columns are well suited to analysis of water samples by immunoassay. They provide rapid cleanup and concentration when used for sample or extract preparation prior to enzyme immunoassay analysis. Molinate, a thiocarbamate herbicide used in rice culture, is routinely monitored in

Table 2. Assays with dilution of water-soluble organic extracts for sample preparation

Compound	Matrix	Extraction Solvent	LLD[a]	Reference
Glycoalkaloids	Potato tubers	Methanol	<0.01 mM	41
Methoprene	Tobacco	Acetonitrile or methanol	0.64 ppm	42, 43
Histamine	Fish, cheese, wine, beer	Perchloric acid precipitate, collect supernatant	7 ng/mL	44
Imazethapyr	Soil	Water extraction, neutralization	0.3 ng/mL	45
Thiabendazole	Potatoes, apples	Blended in water, centrifuge	<0.2 ppm	46
Cyanazine	Soil/water	Methanol, water	3.5 ppb, 0.035 ppb	47
Thiabendazole	Liver	Dimethyl sulfoxide–water	20 ppb	46
Ergot Alkaloids	Seeds, flour	Neutral buffer	<10 ng/g	48
Fumagillin	Honey	Ethanol–water	20 ppb	49
Fenitrothion	Wheat	Methanol	<0.2 ppb	50, 51
Lupin alkaloids	Lupin seed	5% Trichloroacetic acid, neutralization	<10 μg/g	52

[a] Lowest limit of detectability reported.

paddy water and irrigation, canal, and river effluent to prevent contamination of drinking water supplies. An immunoassay has been reported that used C_{18} SPE extraction. The methanol eluate was diluted with buffer and assayed. Immunoassay and GC determinations showed comparable results. Immunoassay results were slightly lower in precision, partly because of variations in the binding of coating antigen and to antigen–antibody reactions. The same immunoassay, when used to analyze spiked soil samples, gave results that were 2–4 times higher than the spike level. The interference was attributed to a matrix effect, although the nature of the interference was not reported (12).

An assay for linuron describes an approach to evaluating sample preparation schemes for use with immunoassay (13). After methanol extraction of foodstuff, monolinuron or diuron could be analyzed by immunoassay, but linuron required further purification to achieve accurate quantitation at the maximum residue limit of 0.1 ppm. After CH_2Cl_2 extraction, passing the extract over a silica gel column, evaporating the eluate, and reconstituting with methanol, linuron could be analyzed by immunoassay. By examining established methods and applying immunoassay at each step, suitable sample preparation for immunoassay can be easily selected. Other examples where SPE was used are given in Table 4.

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a relatively new and powerful alternative to organic solvent extraction (14). Typically CO_2 or N_2O at ambient temperatures under increased pressures is used for extraction. This method is an ideal complement to immunoassay because the extraction medium is volatile. Two major advantages over traditional solvent extraction and GC determination were evident when examining soil for 4-nitrophenol and parathion (15). First, SFE was much easier for sample extraction and handling. Samples were extracted in a flow-through cell, and the extracts, trapped in a small volume of methanol, could be analyzed directly by GC or diluted and analyzed by immunoassay. This procedure circumvented the more arduous method of shaking the soil with ethyl acetate, filtering, and concentrating the extract prior to analysis. Second, immunoassay was more sensitive and faster than GC methods. Integration of SFE and immunoassay demonstrated significant improvement over other extraction and analytical methods for 4-nitrophenol and parathion in soil.

Immunoassays for Derivatives and Complexes

Techniques to derivatize a compound to change volatility or introduce a group to the analyte to increase end detector sensitivity has been applied to GC and LC for years. In immunoassay, if an analyte is too small, it may need to be derivatized to provide recognition sites for the antibody. Often, this derivatized analyte is better recognized by the antibody than the underivatized analyte. To take advantage of this characteristic, samples can be derivatized and the derivative analyzed sensitively by immunoassay. The herbicide bentazon is an example. Three sites were available for placement of a spacer arm for

Table 3. Assays with 1-step extraction and solvent exchange for sample preparation

Compound	Matrix	Extraction Solvent	LLD[a]	Reference
Metalochlor	Soil	Methanol	0.1 ppb	53, 54
Clenbuterol	Urine	*tert*-Butyl methyl ether	0.15 ppb	55
Methyl 2-benzimidazolecarbamate	Blueberries	Methanol	18 ppb	56
Molinate	Municipal water	Dichloromethane	3 ng/mL	57
Atrazine	Fresh and canned pineapple and corn	Acetonitrile–water	<2 ppb	58
Paraquat	Potatoes, beef	HCl	1–2 ppb	38

[a] Lowest limit of detectability reported.

Table 4. Assays with column cleanup and solid-phase extraction for sample preparation

Compound	Matrix	Column	LLD[a]	Reference
Atrazine	Water	SPE, C_{18}	0.1 ng/mL	8, 59
Linuron	Potatoes, carrots	Silica	11 ng/mL, 61 ng/mL	13
Molinate	Water	SPE, C_{18}	15 ppb	12
Picloram	Soil, plant	SPE, C_{18}	5 ng/mL	60
Atrazine	Urine	SPE, C_2	0.02 ng/mL	9
Bentazon	Water	SPE, C_8	2 ppb	16
Pyrethroids	Meat	Alumina	50 ppb	61

[a] Lowest limit of detectability reported.

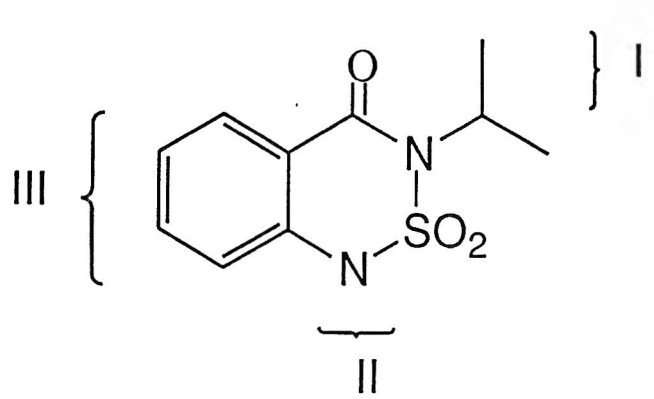

Figure 1. Structure of bentazon with 3 areas for placement of a spacer arm for protein attachment indicated.

subsequent protein attachment (Figure 1). Position I was not used because it was a unique and relatively unstable portion of the molecule. The second site was a convenient position, but changing the secondary amine to a tertiary amine eliminated the presentation of this physiologically and environmentally ionized group to the immune system for antibody production. For development of antibodies selective for *N*-derivatized analogues, this location is ideal. Site III was a rational location for spacer arm attachment, because it is distal from the electronically distinct heterocycle of bentazon. However, in the synthesis of a spacer arm on site III, protection and deprotection of the nitrogen at site II was necessary to prevent reactions at this location. Immunogens prepared from haptens with spacer arms at locations II and III were used to raise antibodies. No usable antibodies were generated with the hapten possessing a spacer arm at site III, possibly because of dimerization of the immunizing conjugate. The antibodies raised against the hapten with the spacer arm at site II provided a sensitive immunoassay for *N*-methyl and *N*-ethyl derivatized bentazon (16).

Immunoassays for metals have been developed by using a similar approach. EDTA chelates of indium are used as immunogens to obtain antibodies that demonstrate a remarkable preference for indium–EDTA chelates over EDTA chelated with other metals (17). Similarly an immunoassay selective for $HgCl_2$ was obtained by using mercury coordinately bound to a sulfhydryl group of a glutathione-KLH (keyhole limpet hemocyanin) molecule as the immunizing agent (18). These reports demonstrate the ability of the antibody to discriminate between the free chelate and the chelate bound to the target metal.

Using Antibodies for Sample Preparation

The preceding techniques all describe methods to prepare a sample prior to analysis by immunoassay. A common application in clinical research analysis, and now just beginning to be used in residue immunoassay, is the use of antibodies to prepare a sample prior to analysis with other detectors. Affinity chromatography has been used successfully for years to isolate and characterize proteins, hormones, viral and bacterial agents, and certain toxins such as aflatoxin. The variety of commercially available affinity solid supports and the number of books on affinity chromatography speak of its value as a separation technique.

Affinity chromatography using monoclonal antibodies has been used to isolate aflatoxin metabolites and adducts (19). Highly specific antibodies were bound to a Sepharose packing. Urine from people and animals exposed to aflatoxin was passed through the column. DNA–aflatoxin adducts as well as the oxidative metabolites M_1 and P_1 were trapped on the column. Subsequent elution from the column provided an extract suitable for quantitative determination by immunoassay or LC, thus circumventing lengthy and complex procedures used to purify aflatoxins before analysis. A similar technique has been used for detection of 17-β-estradiol (20).

Antibodies in solution have been used to extract samples (21). Glass fiber filters were used in air samplers to estimate worker exposure to paraquat. The filters were macerated, and the anti-paraquat antibodies in a phosphate buffer "extracted" paraquat from the filters. Subsequent immunoassay yielded recoveries superior to traditional acid extraction followed by GC determination. A drawback of this technique is the relatively large amount of antibody needed for extraction. Use of monoclonal, or eventually recombinant antibodies, can circumvent this problem. Other potential limitations, such as the amount of time to efficiently extract an analyte (hours to days) and matrix effects, depend on individual antibody and sample characterization.

By using immunoassay microtiter plate wells coated with an antibody, analyte in a sample can bind to the immobilized antibody. The wells can then be washed to remove interfering material and then "eluted" with a solvent or solution that would disrupt the antibody–analyte binding. The resulting affinity-extracted material may be removed and either analyzed directly or purified further. This method was used to extract and identify immunoreactive material found in urine of humans exposed to atrazine. LC of solvent-extracted urine indicated that the *N*-acetyl-L-cysteine conjugate of atrazine was possibly the predominant metabolite. The acidity of the LC mobile phase (pH 2.5) due to the trifluoroacetic acid used as an ion-pairing reagent severely restricted the sensitivity of the immunoassay analysis of fractions. Matrix effects from the urine prevented mass spec-

trometric (MS) analysis of the LC fractions or of the SPE extracts. Affinity extraction provided an extract suitable for fast atom bombardment MS (FAB MS) confirmation. The simplicity of this form of affinity extraction is remarkable. Urine diluted with buffer was placed in antibody-coated wells, and after the analyte had bound to the immobilized antibody, an acidified water solution was then placed in the wells to extract the analyte from the antibody (9).

Hyphenated Techniques

Exploiting the strengths of individual techniques has begun to yield exciting new information. For example, LC detectors are relatively nonspecific and immunoassay generates only a single signal (22). When the chromatographic power of LC is coupled to the analyte selectivity of immunoassay, these problems are solved. This hybridization of methods has been a trend in analytical chemistry for years. Examples of this integration include GC/MS, LC/MS, and MS/MS.

Thin-Layer Chromatography

Silica gel thin-layer chromatographic (TLC) plates have been used to separate analytes prior to immunoassay (23, 24). After a plate is developed with organic solvents to separate sample components, the area corresponding to the R_f of the compound of interest is scraped off and eluted with immunoassay buffer. Although the effects of developing solvent, silica gel, and elution conditions will vary from assay to assay, these 2 simple methods can generate high-quality results. The ability of TLC to separate a series of compounds coupled with immunoassay determination of samples in parallel can provide an efficient, inexpensive, and rapid means of quantifying samples in more complex matrixes.

Liquid Chromatography

Another logical step for sample preparation is to use the resolving power of LC. This topic was discussed in detail in a recent review (25), and only the major concepts are presented here. There are 3 basic approaches to the coupling of LC and immunoassay. One method called affinity subtraction, compares liquid chromatograms before and after the sample has passed through an immunosorbent cartridge. Peaks lost from the chromatogram are associated with the known affinity of the extracting antibody. Another technique analyzes compounds eluted from immunosorbent cartridges. This method can provide extremely clean chromatograms. The third method uses immunoassay as a postcolumn detector. This method is of particular utility when the peak of interest is obscured by coextracted material or the sensitivity of the LC detector is not sufficient.

Immunotechnology developed for the clinical field is being slowly adapted for immunoassay of residues. A good example is the use of affinity techniques by analytical chemists, which can result in automated, highly precise, and accurate methods. Placing an affinity column before a reversed-phase column in an LC system, Janis and Regnier (26) developed a means to purify IgG and the analyte (human transferrin) to which the IgGs were raised. Subsequent reversed-phase chromatography isolated IgG and transferrin. The development of high-resolution immunoaffinity chromatography (HRIAC) has yielded limits of detection in the femtomole to attomole range (27). Several variations using immunodiscrimination preceding traditional chromatography are detailed by de Frutos and Regnier (25). For routine analysis of a significant number of samples, the cost of setting up and characterizing HRIAC can be justified. However, affinity chromatography is not without drawbacks. The affinity packings can be expensive and operating limitations regarding pH and solvent concentrations requires a significant amount of time for development and characterization for each affinity chromatography method. In addition, the life of a column can range from one to hundreds of samples, depending on the antibody and the matrix. When a limited number of samples are to be analyzed or when the analyst needs to identify the proper antibodies and operating conditions for analyte isolation and identification, recently developed simplified affinity extraction techniques are appropriate (9).

Immunoassay can also be a selective and sensitive detector for quantitation of LC fractions. As an example, microbial media was fractionated by LC, and the fractions were analyzed for *Bacillus thuringiensis* β-exotoxin by immunoassay. A single response, such as UV absorbance, cannot provide unequivocal identification of the analyte. By performing an immunoassay on the collected peak of interest, the presence of the β-exotoxin was confirmed (28).

Conclusions

The speed, simplicity, and low cost of immunoassay make it an ideal tool for sample screening. Immunoassay is also attractive as a confirmation technique for nondestructive classical analyses such as LC or TLC. Once antibodies are raised and characterized, they can be used for quantitative analysis and as an active site for specific analyte extraction. Difficult analytes, not amenable to routine chromatographic methods can be isolated with specific antibodies and confirmed by appropriate MS or GC analysis. These methods than can be used both for initial screening and as a confirmation method.

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AGRICULTURAL MATERIALS

Practical Assay of Feed Premixes for Selenite Adsorbed on Reduced Iron

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This method assays feed premixes for added selenite in 5–10 min. It requires that selenite be added in a form easily isolated from the premix. Sodium selenite adsorbed on a reduced iron carrier serves this purpose since it can be retrieved magnetically from samples. The assay is done either indirectly by weighing the iron and calculating the amount of Se or directly by extracting the selenite from the iron carrier and determining it by titration. The indirect assay may be done any time after production of premix. The direct method requires retrieval of the additive from the premix soon after production. The actual assay may be done later. The indirect method gives high results with some matrixes unless adjusted for background ferromagnetic material. The direct method gives results with an accuracy and precision equal to those of the U.S. Food and Drug Administration's Center for Veterinary Medicine regulatory enforcement method with all matrixes studied.

The Food Drug Administration allows addition of sodium selenite to animal feeds at levels between 0.1 and 0.3 ppm. The selenite must be added as a premix, the premix must be added to the feed at no less than 1 lb/ton, and every batch of premix must be assayed for Se. For 20 years, many premix manufacturers have satisfied this requirement by using an indirect assay.

Sodium selenite adsorbed on reduced Fe (RFSe, available as Microtracer MTRFSE-2%, or 2% RFSE, and MTRFSE-4% or 4% RFSE) is a prototype product with microingredients that are magnetically retrievable and available for assay free from matrix interference. RFSe is free flowing and relatively dust-free. The Se contents should be controlled closely to conform to specifications. Premixes produced with RFSe are easily assayed for selenite.

The proposed assay uses no reagents for the indirect method and only a few common reagents for the direct method. A technician can complete 6–12 determinations per hour. The assays can be conducted in a premix-manufacturing environment. The practical procedures give timely and accurate analyses for every batch of premix.

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By contrast, the U.S. Food and Drug Administration's Center for Veterinary Medicine (FDA–CVM) regulatory enforcement method developed by FDA's Denver District (1) relies on classical but potentially explosive perchloric acid digestion (2), development of a colored complex with diaminobenzidine (a suspected carcinogen), extraction of the resulting complex with toluene, and spectrophotometric determination (2–4). An experienced analyst can assay 10 samples per day, but the method requires a perchloric acid hood and glassware.

METHOD

Apparatus

(a) *Rotary detector kit.*—Available from Microtracers, Inc., (San Francisco, CA), or equivalent. The commercial kit includes a rotary detector for retrieving at least 95% of 100–300 mesh ferromagnetic particulates from premixes. The device consists of a 2-part housing and an inner assembly of a 7 cm diameter ceramic speaker magnet mounted on the vertical shaft of a $^1/_{83}$ horsepower, 1550 revolution-per-minute unit bearing motor. The motor is rigidly supported by the lower member of the housing. The active face of the magnet is horizontal. Its vertical concentric poles are roughly 25 mm in diameter with a 1.25 mm gap. The entire face is covered with a 22 gauge aluminum sheet to protect the poles from contamination. A central spindle, $^3/_{16}$ in. (4.76 mm) in diameter, holds a circular sheet of filter paper much as a turntable holds a phonograph record. The upper part of the housing contains a funnel, the tip of which is concentric with the magnet's spindle and 5 mm above its face. Samples are introduced through the funnel, scanned by the rotating magnet, and captured in a plastic bin held in the lower housing. Iron is retained on the face of the filter paper just above the magnetic gap.

Also included in the commercial kit are a 25 mm fantail brush, an aluminum weighing scoop, a circular magnet as above but without spindle, and qualitative filter paper (7.5 cm diameter with a 4 mm hole in center).

(b) *Balance.*—Able to weigh to nearest milligram.

(c) *Sieve.*—U.S. sieve No. 6.

For the direct method, the following also are needed.

(d) *Glassware.*—Standard laboratory glassware.

(e) *Fluted filter paper.*—18.5 cm, Whatman No. 2V, or equivalent.

Reagents for Direct Method Only

(a) *Sodium thiosulfate.*—0.008N, made and standardized daily from a 0.1N stock.

(b) *Iodine.*—0.005N iodine in 2.5% KI.

(c) *HCl.*—20 mL diluted to 100 mL to make 2.5N.

(d) *Starch indicator.*—1%.

Sample Preparation

Four commercial premixes were used as matrixes. RFSe, weighed to ± 0.2 mg, was added to 25 g subsamples in pint mason jars to produce mixes of specified Se content. Each jar was capped and shaken for 2 min while being rotated and inverted. Because the entire mixture was analyzed, this procedure was a simple and accurate way to produce samples containing a known amount of analyte. For stability studies, samples with Se at 200 ppm were incubated at 35°C for various times before analysis. For calibration curves, samples with Se ranging from 0 to 400 ppm were used without incubation.

Commercial Samples

The resistance of RFSe to attrition was evaluated by examining 2 commercial premixes: a vitamin premix formulated with 8 lb of 2% RFSe per ton (80 ppm Se) and a mineral premix formulated with 80 lb of 4% RFSe per 3 tons (533 ppm Se).

Procedure

(a) *Indirect method.*—Weigh premix (ca 25 g for 200 ppm) to the nearest 0.1 g. Break up lumps and sift through a 6 mesh sieve, if necessary, before passing it through the rotary detector. Carefully remove the paper from the magnet and brush the retrieved iron into the counterpoised scoop. Make a second pass and add this retrieved iron to the scoop. Try a third pass to be sure there is no significant residual iron. If there is, add it to the scoop. If not, omit the third pass when assaying that matrix. Place the scoop on the face of the separate magnet to retain the iron. Remove extraneous matter by gentle blowing while moving the scoop about. If w is the net weight of retrieved iron (g), W is the weight of premix (g), and F is the percentage of Se in RFSe, then the amount of Se in sample (ppm) is calculated as follows:

$$\text{Se, ppm} = 10\,000 \times F \times \frac{w}{W} \qquad (1)$$

(b) *Direct method.*—Transfer the weighed iron to a 25 mL volumetric flask (or a 25 mL graduated glass-stoppered cylinder), make to volume with deionized water, and shake for 10 s to extract selenite. Filter. Prepare the solutions listed in Table 1 and titrate each with the iodine reagent.

Calculate the amount of Se in sample as follows:

Table 1. Sequence of reagent addition for the Norris-Fay titration

Reagent	Reagent blank	Sample
Sample filtrate, mL	0.00	10.00
H_2O, mL	40.00	30.00
Thiosulfate, mL	20.00	20.00
Starch indicator, drops[a]	6	6
HCl, mL[a]	2.5	2.5
Iodine titrant, mL	V_b	V_s[b]

[a] Add just before titrating.
[b] If less than 5 mL, add 5.00 mL more of thiosulfate and continue titration; adjust ($V_b - V_s$) by multiplying V_b by 25/20.

$$\text{Se, \%} = 100 \times \frac{20}{V_b} \times N \times 0.01974 \times \frac{R(V_b - V_s)}{W} \qquad (2)$$

$$= \frac{0.790}{V_b} \times \frac{V_b - V_s}{W} \qquad (3)$$

$$\text{Se, ppm} = 10\,000 \times (\text{Se,\%}) \qquad (4)$$

In equation 2, $20/V_b$ is the thiosulfate/iodine ratio determined during blank titration. When multiplied by N, the thiosulfate normality, it gives the normality of the iodine reagent. The dilution ratio, R, is the total volume of extract divided by the aliquot taken for titration, 25/10 in the procedure described. The milliequivalent weight of Se is 0.01974.

Results and Discussion

Table 2 illustrates the repeatability of the Norris-Fay titration (5) in assays of sodium selenite and RFSe.

Method precision, as measured by within-laboratory relative standard deviation, is ± 0.3%.

For all matrixes and all conditions, the reduced iron in RFSe was stable during the 15 days incubation and probably remained so during the life of the premix. Indirect assays should, therefore, be equally time-independent.

Table 2. Selenium in sodium selenite and RFSe determined by Norris-Fay titration

Sample	Selenium declared, %	n	Moisture, %	Selenium found, %	SD[a]
Sodium selenite[b]	>44.7	10	0.20	45.01	0.126
RFSe, 2%[c]	1.90–2.20	10	0.12	2.021	0.052
RFSe, 4%[d]	3.80–4.40	7	0.14	4.183	0.068

[a] Standard deviation.
[b] Spectrum lot CF 228.
[c] Microtracer lot C 2370.
[d] Microtracer lot C 2386.

In some premixes, sodium selenite appeared to migrate from the RFSe to the matrix. This movement imposed a time limit on sampling and retrieval of RFSe for the direct method. For other matrixes, possibly because of the presence of moisture or fat, retrieval of iron was less efficient. More than 2 passes through the rotary detector may be required to achieve the specified >95% recovery.

The selenite did not migrate in a limestone matrix with essentially zero moisture (Tables 3A and 3B). Recoveries were 103–121% by the indirect method and 91–102% by the direct method at incubations of up to 360 h for 2% RFSe and up to 28 h for 4% RFSe.

The vitamin premix contained 14 supplements plus rice hulls and mineral oil, each a potential interference. Recoveries were 92–102% (Tables 4A and 4B) by the indirect method. With the direct method, recoveries of 2% RFSe were 79–91% and recoveries of 4% RFSe were 74–82% during the first 4 h of incubation. These low recoveries may be due to migration of selenite from RFSE to the matrix. The possibility of fat interfering with retrieval of RFSe was minimized by passing the sample through the rotary detector 3 times.

A medicated premix with rice hulls as carrier (Tables 5A and 5B) gave recoveries of 101–112% by the indirect method over a 240 h incubation for 2% RFSe and a 28 h incubation for 4% RFSe and 94–101% by the direct method for the same incubations.

A mineral premix with 10 components and with added mineral oil (Tables 6A and 6B) yielded recoveries of 105–165% with the indirect method throughout the 24 h incubation and over a concentration range of 50–400 ppm. This premix con-

Table 3A. Stability of 2% RFSe in limestone premix incubated at 35°C

Time, h	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect[a]	Direct[b]	Indirect	Direct
0	206	243	202	118	98
	202	237	206	117	102
2	214	233	207	109	97
	200	235	198	117	99
5	203	230	198	113	98
	204	232	187	114	92
19	205	237	201	116	98
	204	237	201	116	98
24	205	210	201	103	98
	202	211	196	105	97
48	203	233	200	115	99
	204	246	197	121	97
216	200	218	200	105	98
	201	208	184	103	91
360	200	208	199	104	99
	198	214	196	108	99
Average ± standard deviation				112 ± 6.1	98 ± 2.6

[a] Here and in following tables, selenium proportion of retrieved iron.
[b] Here and in following tables, Norris-Fay titration of extract from retrieved iron.

Table 3B. Stability of 4% RFSe in limestone premix incubated at 35°C

Time, h	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	204	235	199	115	97
	205	242	202	118	98
2	202	226	194	112	96
	197	215	191	109	97
4	199	218	191	109	96
	210	222	198	111	98
19	206	231	196	112	95
	213	235	203	110	95
24	205	216	196	105	95
	200	204	192	102	96
28	202	223	192	110	95
	214	231	200	108	93
Average ± standard deviation				110 ± 4.2	96 ± 1.4

Table 3C. Norris-Fay titrations of aqueous extracts of retrieved 2% RFSe from limestone premix, 2% RFSe variable

Se added, ppm	Selenium, ppm		Selenium recovery, %	
	Indirect	Direct	Indirect	Direct
0.0	0.0	—	Blank	—
0.0	0.0	—	Blank	—
52.2	48.9	43.6	93.7	88.7
50.7	51.6	47.3	101.7	93.3
99.3	96.4	95.1	97.1	95.8
96.8	98.7	96.2	102.0	99.4
204.8	193.0	181.8	94.2	88.8
207.2	194.1	182.3	93.7	88.1
300.4	282.6	249.9	94.1	83.2
301.0	294.9	270.2	98.0	89.8
401.0	390.7	358.6	97.4	89.5
408.9	410.3	361.4	100.3	88.2
Average ± standard deviation			97 ± 3.2	90 ± 4.6

Table 4A. Stability of 2% RFSe in vitamin premix incubated at 35°C

Time, h	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	199	194	176	98	88
	199	193	173	97	87
2	206	210	188	102	91
	202	205	179	102	89
4	206	199	164	96	79
	200	201	164	101	82
24	205	195	138	95	67[a]
	208	211	153	101	73[a]
Average ± standard deviation				99 ± 2.8	86 ± 4.6

[a] Low recoveries resulting from selenite migration, more pronounced with increasing time, were omitted from average.

tained background ferromagnetic material and gave false high values with the indirect method. A background correction can be made by deducting a blank, the amount of ferromagnetic material in the premix prior to addition of RFSe. The blank can also be estimated as the difference between the indirect and direct assays, particularly at low addition levels. In Table 6B, for Se added at 50 ppm, by subtracting data in the third column from those in the second column, we find blanks of 83.0 – 47.3 = 35.7 and 86.8 – 48.3 = 38.5 ppm, averaging 37.1 ppm. With this value, recoveries for the entire range, 50–400 ppm, become 90–108%. The direct method gave recoveries of 83–98%.

Calibration data are reported in Tables 3C, 4C, 5C, and 6B. Data are summarized in Tables 7 and 8. Table 9 demonstrates the linearity of both indirect and direct methods. The regression

Table 4B. Stability of 4% RFSe in a vitamin premix incubated at 35°C

Time, h	Se, ppm added	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	211	194	164	92	78
	209	195	169	93	81
2	201	193	158	97	79
	213	206	175	97	82
4	198	193	156	97	79
	206	196	153	95	74
24	213	204	123	96	58[a]
	201	187	105	93	52[a]
Average ± standard deviation				95 ± 2.1	79 ± 2.8

[a] Low recoveries result from selenite migration, more pronounced with increasing time. They are omitted from average.

Table 4C. Norris-Fay titrations of aqueous extracts of retrieved 2% RFSe from vitamin premix, 2% RFSe variable

Se added, ppm	Selenium, ppm		Selenium recovery, %		
	Indirect	Direct	Indirect	Indirect, net	Direct
0.0	0.6	—	Blank	—	—
0.0	0.9	—	Blank	—	—
53.2	53.2	45.7	100.0	98.6	85.9
52.1	55.1	47.6	105.7	104.3	91.4
101.4	102.8	86.6	101.5	100.6	85.5
98.9	98.8	84.7	99.9	99.1	85.6
199.4	194.3	175.9	97.5	97.1	88.3
198.5	192.7	173.2	97.1	96.7	87.3
302.4	301.8	274.4	99.8	99.6	90.7
302.5	299.8	247.6	99.1	98.9	81.9
405.6	412.5	340.2	101.7	101.5	83.9
395.1	398.7	327.8	100.9	100.7	83.0
Average			100.3	99.7	86.4
Standard deviation			± 2.4	± 2.2	± 3.1

intercept differed significantly from zero only for the mineral premix analyzed by the indirect method, reflecting the presence of extraneous ferromagnetic material. The slopes measure recoveries ranging from 84 to and 110% for both indirect and direct methods. Standard errors of the estimate for Se vary between ± 2.6 and ± 7.2 ppm for the 4 matrixes.

Recoveries and coefficients of variation (CVs) for data from indirect assay are summarized in Table 7. Recoveries vary from

Table 5A. Stability of 2% RFSe in medicated premix incubated at 35°C

Time, h	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	206	208	206	101	100
	200	204	201	102	101
2	201	202	201	101	100
	197	202	196	103	100
5	195	202	191	104	98
	202	205	201	102	100
24	206	213	201	103	97
	200	204	199	102	100
240	201	211	203	105	101
	203	211	199	104	98
Average ± standard deviation				103 ± 1.3	100 ± 1.4

Table 5B. Stability of 4% RFSe in a medicated premix incubated at 35°C

Time, hrs	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	206	221	199	107	96
	206	217	203	105	99
2	202	211	201	105	100
	212	227	207	107	98
4	212	237	201	112	95
	209	226	201	108	96
19	201	215	193	107	96
	210	218	203	109	97
24	215	231	202	107	94
	206	228	200	111	97
28	207	226	202	109	98
	206	225	200	109	97
Average ± standard deviation				108 ± 2.1	96 ± 2.6

92 to 121% (only background-adjusted data for the mineral premix were considered). CVs range between ± 0.8 and ± 2.9%. Two commercial premixes made with RFSe were assayed for Se to evaluate resistance of RFSe to mechanical abuse. The vitamin premix made with 2% RFSe was 2 days old when assayed. The mineral premix made with 4% RFSe was 12 days old. Se in the vitamin premix was 103% of the specified Se content as determined by the indirect method and 83% by the direct method. This low recovery by the direct method was probably due to the age of the sample. The mineral premix contained much extraneous ferromagnetic material as expected, and the direct method was used. Fifteen samples taken from one batch were each assayed to evaluate the uniformity of the mix. The Se found was 87% of the value specified, and the CV was ± 3.3%.

Table 6A. Stability of 4% RFSe in mineral premix incubated at 35°C

Time, h	Se added, ppm	Selenium, ppm		Selenium recovery, %	
		Indirect	Direct	Indirect	Direct
0	211	259	192	123	91
	200	210	165	105	83
2	209	251	186	120	89
	213	235	186	111	87
4	207	255	199	123	96
	204	247	189	121	93
24	210	283	205	135	98
	211	268	206	127	98
Average ± standard deviation				121 ± 9.2	92 ± 5.4

Table 5C. Norris-Fay titrations of aqueous extracts of retrieved 2% RFSe from medicated premix, 2% RFSe variable

Se added, ppm	Selenium, ppm		Selenium recovery, %		
	Indirect	Direct	Indirect	Indirect, net	Direct
0.0	1.4	—	Blank	—	—
0.0	1.7	—	Blank	—	—
50.4	52.9	50.0	104.9	101.2	99.2
49.9	51.4	49.1	103.1	101.7	98.4
100.1	103.5	96.9	103.4	101.9	96.7
99.9	103.1	99.6	103.2	101.6	99.8
207.9	209.9	194.3	100.9	100.2	93.5
202.5	206.0	196.3	101.7	101.0	97.0
299.9	296.4	272.0	98.8	98.3	90.7
295.1	297.1	272.9	100.7	100.2	92.5
410.3	409.5	396.1	99.8	99.4	96.6
399.4	406.4	391.9	101.8	101.4	98.2
Average			101.8	100.7	96.3
Standard deviation			± 1.9	± 1.2	± 3.0

Table 6B. Norris-Fay titrations of aqueous extracts of retrieved 2% RFSe from mineral premix, 2% RFSe variable

Se added, ppm	Selenium, ppm		Selenium recovery, %		
	Indirect	Direct	Indirect	Indirect, net	Direct
0.0	23.6	0.0	Blank	—	—
0.0	26.5	0.0	Blank	—	—
50.9	83.0	47.3	163.6	113.8	92.9
52.6	86.8	48.3	165.2	117.3	91.8
101.1	132.7	94.1	132.3	106.4	93.1
96.5	132.0	94.5	136.7	110.8	97.9
199.2	238.2	192.8	119.6	107.0	96.8
201.2	234.3	192.2	116.5	104.0	95.5
303.4	355.2	292.3	117.0	108.8	96.3
298.1	356.2	274.9	119.5	102.8	92.2
405.4	466.9	374.1	115.2	109.0	92.3
405.4	476.5	376.1	111.4	111.3	92.8
Average			129.7	109.1	94.2
Standard deviation			± 19.9	± 4.4	± 2.2

Table 7. Summary of recoveries by indirect method, Se added at 200 ppm unless otherwise indicated

Table	*n*	Incubation, h	Recovery, %	Pooled CV, %
		Limestone		
3A	16	0–360	103–121	1.9
3B	12	0–28	102–118	1.6
3C[a]	10	0	94–102	2.9
		Vitamin premix		
4A	8	0–24	93–102	2.2
4B	8	0–24	92–97	1.1
4C[a]	10	0	97–106	2.7
		Medicated premix		
5A	10	0–240	101–105	1.0
5B	12	0–28	105–112	1.5
5C[a]	10	0	99–105	0.8
		Mineral premix		
6A	8	0–24	105–135	5.5
6B[a]	10	0	111–165	1.8
6B[a] less background	10	0	104–117	2.5

[a] Selenium varied from 0 to 400 ppm.

Table 8. Summary of recoveries by direct method, Se added at 200 ppm unless otherwise indicated

Table	*n*	Incubation, h	Recovery, %	Pooled CV, %
		Limestone		
3A	16	0–360	91–102	3.0
3B	12	0–28	93–98	0.9
3C[a]	10	0	83–99	2.7
		Vitamin premix		
4A	6	0–4	79–91	1.7
4B	6	0–4	74–82	3.3
4C[a]	10	0	83–91	2.7
		Medicated premix		
5A	10	0–240	97–101	1.3
5B	12	0–28	94–100	1.3
5C[a]	10	0	91–99	1.6
		Mineral premix		
6A	8	0–24	83–98	2.6
6B[a]	10	0	92–98	1.8

[a] Selenium varied from 0 to 400 ppm.

Table 9. Linear regressions derived from calibration data[a] (Se, ppm)$_{calc}$ = b_0 + b_1(Se, ppm)$_{present}$, *n* = 12

Matrix/table	b_0	$b_1 \pm SE^b$	r^{2c}	SE of est.
Limestone, 3C				
Indirect	−1.235	0.987 ± 0.012	0.998	± 6.1
Direct	2.266	0.876 ± 0.013	0.998	± 6.6
Vitamin, 4C				
Indirect	−0.545	1.004 ± 0.008	0.999	± 3.7
Direct	2.477	0.841 ± 0.015	0.997	± 7.2
Medicated, 5C				
Indirect	2.125	0.999 ± 0.005	1.000	± 2.6
Direct	0.177	0.954 ± 0.014	0.998	± 6.8
Mineral, 6B				
Indirect	24.40	1.097 ± 0.011	0.999	± 5.2
Direct	1.642	0.931 ± 0.009	0.999	± 4.3

[a] Derived from data in Tables 3C, 4C, 5C, and 6B.
[b] SE, the standard error of the estimate.
[c] r^2, the correlation coefficient squared (coefficient of determination).

Recoveries with the direct method for all matrixes and conditions ranged from 74 to 102%, and CVs ranged from 0.9 to 3.3% (Table 8). These results compare favorably with those obtained by 2 more arduous procedures being proposed for regulatory monitoring of Se premixes (FDA–CVM method): using Procedure 1 applicable only to mineral premixes, recoveries in a 4-laboratory trial ranged from 82 to 101%, with CVs ranging from 0.8 to 9.9%. Using Procedure 2 applicable to feed-based premixes, recoveries in a single laboratory study ranged from 76.5 to 104%, with CVs ranging from 0.2 to 9.4%.

Conclusions

The direct method permits practical, accurate, and timely in-plant Se assays of selenite-containing premixes produced with sodium selenite adsorbed on reduced iron. It compares in accuracy with methods proposed by FDA–CVM. The indirect method may give false high recoveries in matrixes containing extraneous ferromagnetic material unless appropriate blanks are deducted.

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AGRICULTURAL MATERIALS

Determination of Boron in Fertilizers by Inductively Coupled Plasma-Atomic Emission Spectrometry: Studies of Some Spectral Interferences at Different Wavelengths

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The most sensitive analytical wavelengths of boron cannot be used in the determination of boron in fertilizers by inductively coupled plasma-atomic emission spectrometry because of spectral interference by potassium, phosphorus, and iron. For the 4 wavelengths of boron investigated, it was noticed that, at the same wavelength, the pattern of spectral interference changes according to fertilizer composition. The spectral interference patterns at the 4 analytical boron wavelengths were studied by adding matrix elements typically found in fertilizers to real fertilizer samples. When levels of added matrix elements correlate less than 0.2 to each other, the effect of added matrix elements on boron determination can be calculated by multiple linear regression. The best analytical wavelength for determination of boron in fertilizer is 208.959 nm. If wavelength is selected only according to calibration data, without doing interference studies, the best wavelength should be 249.773 nm. Multiple linear regression in conjunction with experimental design may be used to determine the best analytical wavelength for a sample matrix under analysis, examine the interference elements, and verify the concentration detected.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) is widely used for routine environmental analysis (1). However, the use of this method for analysis of boron (B) in fertilizers, or to fertilizer analysis at all, has not been widely documented (2). Because B is an essential plant nutrient, its measurement at low concentrations by any technique is of importance in soil and plant analysis (1).

Determination of B by methyl borate distillation or by azomethine H spectrometry is lengthy (3). The argon plasma techniques, including ICP-AES, are supposedly effective procedures for the rapid and precise determination of the B content of fertilizers (3–6). Nevertheless, ICP-AES has not been standardized for fertilizer analysis.

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The argon plasma technique was first proposed for determination of B in fertilizer by Melton et al. (6). They claimed that, of the elements normally found in fertilizers, only alkali metals significantly enhanced B emission. They found that adding excess Li to the sample during preparation masked the enhancement caused by alkali metals. Woodis et al. (3) also determined the effects of some ions commonly found in fertilizers on B determination: only K caused significant enhancement. According to Woodis et al., Li is not effective in masking the B enhancement caused by alkali metals; masking is effective only when B and K are present.

Jones (4, 5) was the first to report the determination of boron and other elements in fertilizers by ICP-AES. He reported results that compared well with the known Magruder value for the Fertilizer Standards. According to Jones, K and P may affect emissions of other elements in fertilizer.

Four analytical wavelengths are available for routine B determination by ICP-AES: 249.773, 249.678, 208.959, and 208.893 nm. But the 2 most sensitive lines (249.773 and 249.678 nm) have severe Fe interferences, which are either total overlap or spectral wing interferences (7).

The original aim of this study was to develop a standard method for determination of B in fertilizers by ICP-AES. The method was tested in 4 ICP-AES laboratories. Two commercial fertilizers, NPK 20-4-8 and NPK 25-4-4, were used in both collaborative studies and matrix effects studies. Because of variation between and within laboratories, more information about calibration procedure and matrix constituents interferences was needed. Ions thought to interfere were added to fertilizer samples, and data obtained at 4 B wavelengths were analyzed by multiple linear regression (8).

Experimental

(a) *Fertilizer matrix solution.*—Prepared by dissolving 44 g KH_2PO_4, 12.6 g KCl, and 66 g NH_4NO_3 in ca 600 mL water and diluted to 1 L with water.

(b) *Boron standard solutions.*—Stock solution was made according to the AOAC official final action method (9). Working calibration solutions were prepared in 200 mL volumetric flasks, resulting in final concentrations of 0, 5, 10, 15, 20, and 25 μg B/mL when diluted to volume. To each working calibration solution 6.5 mL fertilizer matrix solution was added and

acid concentration was adjusted to that of the samples. Working calibration solutions contained P at 326 mg/L, K at 625 mg/L, and N at 750 mg/L.

(c) *Interference solutions.*—The following 5 solutions were prepared by dissolving in 1 L of water 263.5948 g KH_2PO_4, 110.8007 g $CaCl_2$, 244.1042 g KCl, 10.1701 g NaCl, and 24.4057 g LiCl. The solutions contained P at 60 000 mg/L, Ca at 40 000 mg/L, K at 128 000 mg/L, Na at 4000 mg/L, and Li at 4000 mg/L, respectively. Also, a Mg solution was prepared by dissolving 20.0054 g Mg powder in 5M HCl and diluting to 1 L with water, and an Fe solution by dissolving 1.9999 g Fe powder in a mixture of HCl (38%) and HNO_3 (68%) and diluting to 1 L with water. The solutions contained Mg at 20 000 mg/L and Fe at 2000 mg/L, respectively.

(d) *Apparatus.*—A Perkin-Elmer ICP 1000/2000 apparatus was used in our laboratory. Instrument parameters were optimized.

(f) *Sample preparation.*—Samples for water-soluble boron (WS-B) and acid-soluble boron (AS-B) were prepared according to the AOAC official final action method (9).

The procedure does not involve any special safety demands.

Results and Discussion

The procedure (sample preparation, calibration, and measurements with ICP-AES) was thought to be ready for standardization. To evaluate the procedure, collaborative measurements were done. Each participating laboratory used its own optimized ICP-AES instrument for B determination. Various B wavelengths were used (e.g., 249.773, 249.683, and 249.678 nm). A uniform wavelength was not specified, because the effects of wavelength and of fertilizer matrix at different wavelengths were not fully understood. The major fertilizer components N, P, and K, which were thought to eliminate enhancements in the B spectrum caused by other fertilizer components, were added to the calibration solutions. Table 1 shows matrix element concentrations in fertilizer sample solutions.

Boron determination was carried out in 4 independent ICP-AES laboratories. The WS-B and AS-B were determined in 3 groups, each group including 6 samples. The statistical techniques proposed by Youden and Steiner (10) and Miller and Miller (11) were used to investigate measurement differences within and between laboratories. Statistical calculations were made with SPSS' one-way ANOVA (analysis of variance) and multiple linear regression procedures (8).

Analytical results from the 4 laboratories and calculated statistical test values are shown in Tables 2–5. The numerals I, II, and III in Tables 2–5 represent the determination group. The repeatability of the method is excellent, but reproducibility is poor. In each laboratory, the method precision is good, but differences in analytical results between laboratories are not ac-

Table 1. Matrix elements in fertilizer sample solutions

Fertilizer	Concentration, ppm					
	P	Ca	K	Na	Mg	Fe
NPK 20-4-8	620[a] 878[b]	400	1660	40	200	20
NPK 25-4-4	561[a] 800[b]	380	800	40	100	20

[a] Water soluble.
[b] Acid soluble.

Table 2. Results and statistical analyses for WS-B in fertilizer NPK 20-4-8

A. Boron concentrations found

Laboratory	Determination group	Mean[a] B conc, mg/kg	RSD[b]
1	I	325	1.1
	II	336	0.9
	III	331	1.5
2	I	333	3.1
	II	310	12.6
	III	305	13.4
3	I	327	0.9
	II	318	1.3
	III	337	1.5
4	I	338	2.6
	II	413	7.8
	III	319	0.8

[a] Means of 6 determinations.
[b] Relative standard deviation.

B. *t*- and *F*-test values

Laboratory	Groups being compared	*t* value[a]	*F* value[b]
1	I, II	6.87	1.51
	I, III	1.84	1.91
	II, III	2.19	2.89
2	I, II	1.53	14.5
	I, III	1.76	15.8
	II, III	1.90	1.09
3	I, II	3.53	1.00
	I, III	3.57	2.70
	II, III	5.44	1.60
4	I, II	4.96	13.0
	I, III	4.84	12.4
	II, III	6.68	16.1

[a] Paired *t*-test, $t_{0.05}$ (5) = 2.57.
[b] Two-tailed *F*-test, $F_{0.05}$ (5, 5) = 7.146.

C. ANOVA table[a]

Source	Degrees of freedom	Sum of squares	Mean squares	*F* ratio	*F* probability
Between lab.	3	16111.75	5370.58	6.472	0.0006
Within lab.	68	56424.06	829.77		
Total	71	72535.81			

[a] For 18 determinations.

Table 3. Results and statistical analyses for AS-B in fertilizer NPK 20-4-8

A. Boron concentrations found

Laboratory	Determination group	Mean[a] B conc, mg/kg	RSD[b]
1	I	447	3.0
	II	392	2.4
	III	396	3.1
2	I	406	1.4
	II	414	3.6
	III	404	3.6
3	I	381	2.4
	II	342	2.9
	III	382	3.1
4	I	394	4.9
	II	373	1.7
	III	384	2.6

[a] Means of 6 determinations.
[b] Relative standard deviation.

B. *t*- and *F*-test values

Laboratory	Groups being compared	*t* value[a]	*F* value[b]
1	I, II	6.96	2.03
	I, III	7.07	1.19
	II, III	1.11	1.71
2	I, II	1.27	7.26
	I, III	0.48	6.85
	II, III	5.35	1.06
3	I, II	7.33	1.24
	I, III	0.12	1.57
	II, III	6.32	1.41
4	I, II	2.18	8.62
	I, III	0.90	3.76
	II, III	2.02	2.35

[a] Paired *t*-test, $t_{0.05}$ (5) = 2.57.
[b] Two-tailed *F*-test, $F_{0.05}$ (5, 5) = 7.146.

C. ANOVA table[a]

Source	Degrees of freedom	Sum of squares	Mean squares	*F* ratio	*F* probability
Between lab.	3	22975.12	7658.37	18.616	0.0000
Within lab.	68	27974.42	411.39		
Total	71	50949.54			

[a] For 18 determinations.

Table 4. Results and statistical analyses for WS-B in fertilizer NPK 25-4-4

A. Boron concentrations found

Laboratory	Determination group	Mean[a] B conc, mg/kg	RSD[b]
1	I	247	0.7
	II	242	1.7
	III	250	0.9
2	I	258	3.0
	II	259	1.2
	III	248	1.1
3	I	245	0.9
	II	240	0.0
	III	248	1.6
4	I	242	2.3
	II	315	2.0
	III	240	1.2

[a] Means of 6 determinations.
[b] Relative standard deviation.

B. *t*- and *F*-test values

Laboratory	Groups being compared	*t* value[a]	*F* value[b]
1	I, II	2.46	6.49
	I, III	2.16	1.82
	II, III	3.29	3.51
2	I, II	0.22	6.05
	I, III	3.25	8.35
	II, III	10.4	1.38
3	I, II	1.44	1.00
	I, III	2.42	3.00
	II, III	4.56	4.30
4	I, II	16.9	1.32
	I, III	0.90	4.56
	II, III	22.9	6.01

[a] Paired *t*-test, $t_{0.05}$ (5) = 2.57.
[b] Two-tailed *F*-test, $F_{0.05}$ (5, 5) = 7.146.

C. ANOVA table[a]

Source	Degrees of freedom	Sum of squares	Mean squares	*F* ratio	*F* probability
Between lab.	3	4930.58	1643.53	4.705	0.0048
Within lab.	68	23753.96	149.32		
Total	71	28684.53			

[a] For 18 determination.

ceptable for a standard procedure. Bonferroni tests obtained with one-way ANOVA gives a homogeneous subset to identify subsets of means that are not different from each other at the 0.05 significance level (8). From Bonferroni tests of results, we found that results of 3 of 4 laboratories for WS-B are not significantly different, while for AS-B, results of only 2 of 4 laboratories are not significantly different. So the requirements of method standardization are not fulfilled.

Collaborative studies show that the amount of B found in fertilizers is a function of the wavelength and the ICP-AES instrument used. Boron was detected in the homogeneous fertilizer solution samples sent to each laboratory and also in self-made fertilizer samples; the correlation between measurements was 0.817. Variations in data, especially between laboratories, may have been due to use of different wavelengths, differences in fertilizer matrix composition, or calibration problems.

Table 5. Results and statistical analyses for AS-B in fertilizer NPK 25-4-4

A. Boron concentrations found

Laboratory	Determination group	Mean[a] B conc, mg/kg	RSD[b]
1	I	314	4.4
	II	283	6.5
	III	288	8.6
2	I	280	1.6
	II	292	1.2
	III	285	1.8
3	I	271	1.8
	II	248	1.6
	III	272	1.5
4	I	253	2.1
	II	270	2.8
	III	259	3.2

[a] Means of 6 determinations.
[b] Relative standard deviation.

B. *t*- and *F*-test values

Laboratory	Groups being compared	*t* value[a]	*F* value[b]
1	I, II	4.54	1.79
	I, III	3.30	3.20
	II, III	0.33	1.79
2	I, II	3.86	1.55
	I, III	1.30	1.34
	II, III	3.96	2.08
3	I, II	6.44	2.40
	I, III	0.28	1.53
	II, III	10.1	1.00
4	I, II	10.2	1.95
	I, III	2.23	2.41
	II, III	3.74	1.25

[a] Paired *t*-test, $t_{0.05}$ (5) = 2.57.
[b] Two-tailed *F*-test, $F_{0.05}$ (5, 5) = 7.146.

C. ANOVA table[a]

Source	Degrees of freedom	Sum of squares	Mean squares	*F* ratio	*F* probability
Between lab.	3	15319.75	5106.58	25.060	0.000
Within lab.	68	13856.67	203.77		
Total	71	29176.42			

[a] For 18 determinations.

The effects of calibration in spectrometric determination are well known. Two-point calibration is widely used, but in our opinion, it is not the best for every measurement. We used 5-point calibration in our measurements. It is more time-consuming than 2-point calibration, but the data obtained can be treated with more confidence. The calibration data are assumed to take the algebraic form $y = bx + a$, where b is the slope of the line and a is the intercept. Let s_b and s_a be the standard deviations of the slope and the intercept, respectively. The limit of determination, LOD, is obtained by replacing the y with the term $a + 3s_a$; the limit of quantitation, LOQ, is obtained by replacing y with the term $a + 10s_a$ (10). Difficulties in calibration are seen from the calculated LOD and LOQ, not from the correlation coefficient. The lower LOD and LOQ are, the more successful is the application. LOD helps to decide the best analytical concentration range where measurements should be done. Table 6 shows the best and worst calibration data at 4 wavelengths. An acceptable correlation coefficient is >0.9998. If the correlation coefficient is <0.9998, LOD and LOQ are too high. One may note here the differences that appeared between calibrations of the same kind: Variation in analytical data is explicable if measurements are made with many calibrations. Ideally, replicate measurements should be made with one acceptable calibration, but this is not always possible because of difficulties in calibration. The best calibration data are obtained at 249.773 nm (Table 6), but B calibration solutions contain only the fertilizer major elements N, P, and K, not other possible interference ions.

The effects of P, Ca, K, Na, Li, Fe, and Mg at 5 concentration levels on B determination were investigated. These matrix elements were selected because they are thought to affect B determination (3–6). Nitrate was not included because it was not thought to affect B determination appreciably (6). The effect of sulfate on boron determination was not examined.

The lowest added concentration level of each matrix element represents the average range that would be present in the fertilizer samples used, and the highest added level represents 5 times the level that would be present in the fertilizer samples used. Matrix element levels were arranged in random order, so that they do not correlate with each other. If 7 matrix ions are selected as variables, 78 125 combinations are possible when ions are studied at 5 levels; with correlation models, a subset of 21 combinations can be chosen to give a sample matrix, where levels of added matrix elements correlate less than 0.2 to each other.

Matrix elements were added to fertilizer sample solutions during preparation, before diluting to volume. WS-B and AS-B in both fertilizers were determined by ICP-AES at 4 wavelengths. Boron calibration solutions were similar to those used in collaborative studies: They contain the major fertilizer elements N, P, and K. Determinations were made both from interference solutions ($2 \times 2 \times 21$ solutions) and fertilizer sample solutions to which matrix elements had not been added ($2 \times 2 \times 13$ solutions). The matrix effects of combinations of added elements were examined by multiple linear regression with the added elements as independent variables (8). The matrix element combinations used in samples are shown in Table 7.

Multiple regression lines at 4 wavelengths for each sample type are given in Table 8. F_{Prob} in the last column shows the goodness of linearity: The smaller the F_{Prob}, the better the linearity. The correlation coefficient also shows the goodness of linearity. Because the number of samples is 21, almost all the correlation coefficients are good enough. Table 8 shows that fertilizer NPK 20-4-8 is more complicated than fertilizer NPK 25-4-4. For fertilizer NPK 20-4-8, no regression was found at 249.678 nm for any sample, and no regression was

Table 6. Best and worst calibration data for determination of WS-B and AS-B by ICP-AES at 4 wavelengths

Wavelength, nm	Sample	$y = (b \pm s_b)x + (a \pm s_a)$	r	LOD[a]	LOQ[b]
208.893	WS-B	$(466.38 \pm 2.85)x + (207.12 \pm 47.34)$	0.9999	0.304	1.015
		$(533.59 \pm 9.65)x + (-102.52 \pm 160.06)$	0.9995	0.900	3.000
	AS-B	$(484.32 \pm 2.60)x + (231.94 \pm 43.11)$	1.0000	0.267	0.890
		$(708.38 \pm 58.17)x + (749.61 \pm 964.71)$	0.9900	4.086	13.619
208.959	WS-B	$(802.50 \pm 7.12)x + (444.21 \pm 118.08)$	0.9999	0.441	1.471
		$(763.74 \pm 29.40)x + (820.92 \pm 487.57)$	0.9978	1.915	6.384
	AS-B	$(901.54 \pm 10.48)x + (1283.58 \pm 173.85)$	0.9998	0.578	1.928
		$(873.77 \pm 34.71)x + (106.22 \pm 575.54)$	0.9976	1.976	6.587
249.678	WS-B	$(985.98 \pm 17.20)x + (537.87 \pm 285.28)$	0.9995	0.868	2.893
		$(1093.20 \pm 10.78)x + (574.26 \pm 178.70)$	0.9999	0.490	1.635
	AS-B	$(1042.72 \pm 8.35)x + (393.28 \pm 138.52)$	0.9999	0.399	1.328
		$(1519.35 \pm 49.96)x + (2660.04 \pm 828.56)$	0.9984	1.636	5.453
249.773	WS-B	$(1866.81 \pm 10.45)x + (830.89 \pm 173.28)$	1.0000	0.278	0.928
		$(1399.96 \pm 17.42)x + (749.55 \pm 288.88)$	0.9998	0.619	2.063
	AS-B	$(1537.19 \pm 11.70)x + (780.80 \pm 194.12)$	0.9999	0.379	1.263
		$(4702.17 \pm 63.49)x + (7199.44 \pm 1052.93)$	0.9997	0.672	2.239

[a] Limit of detection, mg/L.
[b] Limit of quantitation, mg/L.

found at 249.773 nm for the AS-B sample. At the 2 remaining wavelengths, regression lines were functions of 2 or 3 variables. For fertilizer NPK 25-4-4, multiple regression lines typically are functions of P and K; for fertilizer NPK 20-4-8, they are functions of Ca, Fe, P, and K.

According to Xu and Rao (7), Fe disturbs the B lines at 249.678 and 249.773 nm, but our studies show that Fe disturbs notably at 249.773 nm. When Fe causes a disturbance, the coefficient of Fe concentration is, at its maximum, 100 times stronger than those of other elements. Because the Fe concentration of fertilizer samples is about 20 ppm, the effect of Fe on the B concentration determined is similar to those of other interference elements.

Generally, the effects of interference elements on B concentration are at the 4th decimal position. The best analytical wavelength for determination of B in fertilizer samples by ICP-

Table 7. Matrix elements used in determination of spectral interferences

	Concentration, ppm						
Sample	P	Ca	K	Na	Mg	Fe	Li
1	1800	1200	8033	160	200	40	160
2	600	1600	4598	120	600	20	80
3	600	800	4598	80	200	100	120
4	1800	400	8033	120	600	40	200
5	600	1600	3318	160	200	60	80
6	3000	400	7627	120	200	20	40
7	2400	2000	5590	80	1000	20	160
8	600	800	6518	40	800	80	80
9	1200	1200	7916	120	400	100	120
10	1200	1600	7276	200	400	20	40
11	2400	1200	8150	160	800	100	80
12	1200	1200	7276	40	1000	80	120
13	3000	800	6347	160	400	100	120
14	3000	400	8907	40	600	40	40
15	2400	1600	9430	80	200	60	160
16	1800	400	7393	200	800	80	200
17	1800	2000	4832	40	400	60	200
18	2400	2000	8150	200	1000	100	40
19	3000	1600	10188	200	800	60	160
20	3000	2000	9548	80	600	80	160
21	1200	800	5355	200	1000	40	200

Table 8. Multiple regression data calculated from 21 matrix element solutions

Wavelength, nm	Sample[a]	Multiple regression line	R^b	$F_{Prob}{}^c$
208.893	WS-B(1)	$0.0338 - (1.070 \times 10^{-5}) \times [Fe] - (5.493 \times 10^{-7}) \times [Ca] - (6.591 \times 10^{-7}) \times [P]$	0.7899	0.0004
	AS-B(1)	$0.0422 - (1.522 \times 10^{-6}) \times [Ca] - (4.306 \times 10^{-7}) \times [K] + (2.680 \times 10^{-6}) \times [Mg]$	0.7612	0.0011
	WS-B(2)	$0.0247 - (7.551 \times 10^{-7}) \times [P]$	0.6285	0.0017
	AS-B(2)	$0.0281 - (5.576 \times 10^{-7}) \times [P]$	0.4683	0.0279
208.959	WS-B(1)	$0.0348 - (8.531 \times 10^{-7}) \times [Ca] - (1.067 \times 10^{-6}) \times [P] - (1.063 \times 10^{-5}) \times [Fe]$	0.8877	0.0000
	AS-B(1)	$0.0434 - (5.041 \times 10^{-7}) \times [K] - (1.246 \times 10^{-6}) \times [Ca]$	0.8099	0.0000
	WS-B(2)	$0.0241 - (6.004 \times 10^{-7}) \times [P]$	0.5977	0.0033
	AS-B(2)	$0.0273 - (1.108 \times 10^{-6}) \times [P] + (2.361 \times 10^{-7}) \times [K]$	0.6328	0.0078
249.678	WS-B(1)	No regression		
	AS-B(1)	No regression		
	WS-B(2)	$0.0268 + (7.826 \times 10^{-7}) \times [K] - (1.261 \times 10^{-6}) \times [P]$	0.7082	0.0013
	AS-B(2)	$0.0309 + (1.151 \times 10^{-6}) \times [K] - (1.691 \times 10^{-6}) \times [P]$	0.7880	0.0001
249.773	WS-B(1)	$0.0418 + (4.917 \times 10^{-5}) \times [Fe]$	0.5391	0.0096
	AS-B(1)	No regression		
	WS-B(2)	$0.0286 + (2.668 \times 10^{-5}) \times [Fe] + (4.208 \times 10^{-7}) \times [K]$	0.6355	0.0073
	AS-B(2)	$0.0335 - (1.385 \times 10^{-6}) \times [P] + (1.209 \times 10^{-6}) \times [K]$	0.6940	0.0019

[a] (1), fertilizer NPK 20-4-8, and (2), fertilizer NPK 25-4-4.
[b] Multiple correlation coefficient.
[c] Probability associated with the *F* statistic.

AES is 208.959 nm. The effects of matrix elements on B determination can be modeled by multiple linear regression, and the disturbance at this wavelength is very weak when calculated with the real fertilizer sample matrix concentrations (Table 1).

Table 9 shows B concentrations of fertilizers determined at 4 wavelengths by ICP-AES and B concentrations calculated by multiple linear regression. Calculated values are obtained by relating real fertilizer matrix concentrations of samples to multiple linear regression lines. From Table 9, the differences between determined and calculated B concentrations are insignificant at 208.959 nm. At 208.959 nm, the determined B concentration corresponds to the real B concentration, so interference of matrix elements is negligible.

Multiple linear regression may be used in selecting the best analytical wavelength, examining interference elements, and verifying the concentration detected. In this work, 208.959 nm was found to be the best wavelength for B determination in fertilizer by ICP-AES. The most sensitive lines have matrix effects caused by K, P, and Fe, depending on fertilizer composition.

Table 9. Determined and calculated boron levels of 2 fertilizers at 4 wavelengths

Wavelength, nm	Sample[a]	Determined,[b] %	Calculated,[c] %	Difference, %
208.893	WS-B(1)	0.0337	0.0329	0.0008
	AS-B(1)	0.0412	0.0414	–0.0002
	WS-B(2)	0.0233	0.0243	–0.0010
	AS-S(2)	0.0264	0.0276	–0.0012
208.959	WS-B(1)	0.0330	0.0334	–0.0004
	AS-B(1)	0.0389	0.0420	–0.0031
	WS-B(2)	0.0233	0.0237	–0.0004
	AS-B(2)	0.0270	0.0266	0.0004
249.678	WS-B(1)	0.0372	—	—
	AS-B(1)	0.0457	—	—
	WS-B(2)	0.0235	0.0267	–0.0032
	AS-B(2)	0.0276	0.0304	–0.0022
249.773	WS-B(1)	0.0380	0.0427	–0.0047
	AS-B(1)	0.0461	—	—
	WS-B(2)	0.0242	0.0295	–0.0053
	AS-B(2)	0.0278	0.0334	–0.0056

[a] (1), fertilizer NPK 20-4-8, (2), fertilizer NPK 25-4-4.
[b] Mean of 13 determinations.
[c] From linear multiple regression.

We believe that the sample preparation and the calibration solutions used in this work are suitable for determination of B in fertilizers.

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AGRICULTURAL MATERIALS

Simultaneous Determination of Chlorothalonil and Hexachlorobenzene in Technical and Formulated Materials by Capillary Gas Chromatography

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A capillary gas chromatographic method using flame ionization detection was developed for simultaneous assay of chlorothalonil and hexachlorobenzene in technical material and formulated products. Method precision is excellent, and the method allows simultaneous determination of one chemical impurity. The method uses an internal normalization reagent (*n*-butyl phthalate). Modification and extension of the method to formulated products, including wettable powders, water-based flowables, and dispersible (or dry flowable) granular formulations, are discussed. Preliminary statistical analysis of intra- and interlaboratory comparisons was performed to evaluate method ruggedness. Average relative standard deviations for technical materials were 0.34 and 0.17% from 2 laboratories. Additional method validation studies were conducted to examine the influence of pH, solvent systems, gas chromatographic systems, and temperature conditions on different formulations.

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Chlorothalonil (tetrachloroisophthalonitrile; CTL) is a commercially important contact fungicide registered for use on a wide variety of fruit crops, vegetables, ornamentals, turf, and specialty applications in the United States and around the world. It is also being used to control mildew in paints and sap stain on wood. Early gas chromatographic (GC) analysis of CTL used packed metal columns and flame ionization detectors (FIDs).

This approach was used extensively for active-ingredient analysis and modified to include electron capture detection for trace-level analysis after spray deposition, in formulation tenacity experiments and in residue studies. Hexachlorobenzene

(HCB) is a chemical impurity present in CTL at relatively low levels. The early packed column methods do not have the sensitivity necessary to simultaneously quantitate HCB at levels deemed significant by the Environmental Protection Agency and other regulatory organizations. Worldwide regulations and economics require extremely accurate and precise analyses of CTL and HCB. Earlier methods required 2 separate preparation schemes for these analyses. More modern technologies, such as capillary GC, allow simultaneous analysis of the 2 compounds and lower detection limits for HCB. A recent literature search identified many articles about analysis of CTL on plant tissues (3–7) and methods for surveying residues in plants and soils (8, 9), monitoring air (10), and determining occupational exposure (11).

The purpose of this research was to develop a reliable and rugged AOAC method for confirming the concentration of CTL active ingredient in formulations and technical materials. The method requirements include excellent precision and accuracy, while maintaining sufficient resolution and sensitivity to detect the trace impurity, HCB. Four categories of samples are commonly analyzed for CTL: technical materials, wettable powders, dispersible (dry flowable) granular formulations, and water-based flowables. CTL is minimally soluble in water and has low solubility in most organic solvents. The solvent systems chosen must dissolve CTL completely and must be miscible with water in water-based formulations. Experiments were conducted to evaluate the effect of pH, solvents, and solvent mixtures on CTL stability. This paper describes a specific procedure for analysis of CTL and HCB in technical materials, with minor modifications for use with formulated products. The method was tested by 2 laboratories and found to be free from interference by any related compounds that may be present as impurities.

METHOD

Safety

Normal laboratory procedures should be observed when handling volatile solvents, compressed gases, and GC equipment. Normal laboratory procedures can be used when handling purified technical materials or formulations containing chlorothalonil. The acute oral median lethal dose (LD_{50}) for chlorothalonil is >10 000 mg/kg in albino rats (12).

Reagents

Reagents include toluene, methanol, *n*-butyl phthalate (DBP; for use as an internal normalization reagent [INR]), and phosphoric acid, all ACS reagent grade or better. CTL and HCB of known purity are the reference standards, and are available through ISK Biosciences Corp., the primary manufacturer of CTL. The current approved CTL standard is SDS-2787-1501, as designated by ISK. The purity of this standard is 99.6%, as characterized under Good Laboratory Practice (GLP) guideline 160.105. The concentration of HCB in this reference CTL was determined by capillary GC by preparing SDS-2787-1501 at ca 20 mg/mL in toluene for analysis. The mean result was 0.009% by area normalization, subsequently corroborated by external-standard capillary GC. The current HCB standard is SDS-1497-0301, as designated by ISK, and was determined by GC to be 100% pure. This compound also was characterized under GLP guideline 160.105.

Apparatus

(a) *Gas chromatograph.*—Equipped with FID and split–splitless injector. Temperatures: column, 205°C; injection port, 330°C; detector, 300°C. Gas flows: hydrogen carrier at 25 psi column head pressure; split flow, 140 mL/min. Linear gas velocity, 74 cm/s. Sample size, 1 μL. Adjust hydrogen and air for flame gases per manufacturer's specifications. Makeup gas, nitrogen at 30 mL/min; split liner, single taper with glass wool.

To simultaneously quantitate HCB and CTL, the sample must be prepared at the relatively high concentration of 20 mg/mL with respect to CTL. Multiple analyses of high levels of dissolved solids can lead to plugging of the split line in some GCs. In Laboratory B, a 1/4 in. od × 1 in. long span of nickel tubing was attached to the split line coming off the injector. This acts as a trap to prevent plugging of this line.

(b) *Column.*—30 m, DB-17, 0.25 mm id, 0.5 μm film thickness (J&W Scientific, Folsom, CA). Approximate retention times are HCB, 3.4 min; CTL, 6.0 min; DBP (INR), 7.0 min.

(c) *Integrator.*—Automatic digital or chromatographic data system or personal computer and software package.

Preparation of INR

Prepare INR solution by weighing 2.0 g (± 0.01) DBP and quantitatively transferring to a 1000 mL volumetric flask. Dissolve in toluene.

Preparation of Calibration Standards

Prepare 2 HCB stock solutions. Weigh and record 30 mg (± 3 mg) directly into two 100 mL volumetric flasks. Dilute these stock preparations to volume with INR. Mix well until all HCB dissolves. Dilute the stock solutions with INR (2 + 98) in each 100 mL volumetric flask. Label these solutions Diluent 1 and Diluent 2.

Weigh in duplicate and record to 4 decimal places 1.0 g (± 0.01 g) analytical grade CTL of known purity into each of 2 bottles (4 oz. Qorpak bottles were used in Laboratory B). Correct each weight for label purity and identify these bottles as STD 1 and STD 2, respectively. Pipet 50.0 mL Diluent 1 into STD 1 and 50.0 mL Diluent 2 into STD 2.

CTL and HCB concentrations in STD 1 (and STD 2) are expressed as weight (mg/50 mL) after correction for purity. CTL standard SDS-2787-1501 contains 0.009% HCB. Therefore, the final concentration of HCB in STD 1 (and STD 2) is the sum of HCB from the CTL source and the amount of HCB weighed and contained in the diluent solutions.

The total HCB concentration in STD 1 (and STD 2) is determined as follows:

$$(A \times 0.009\%) + B$$

where A is the amount of SDS-2787-1501 in STD 1 (or STD 2), mg/mL, and B is the amount of HCB in Diluent 1 (or Diluent 2), mg/mL.

Example:

$$A \times 0.009\% = \frac{1.0\ g}{50\ mL}\ CTL \times 0.009\%$$

$$= 1.8 \times 10^{-6}\ g$$

$$= 1.8 \times 10^{-3}\ mg\ HCB$$

B = mg HCB/100 mL followed by a (2 + 98) dilution
$= 6.0 \times 10^{-3}$ mg/mL HCB

Total HCB = 0.0018 ($A \times 0.009\%$) + 0.0060 (B)
= 0.0078 mg/mL in STD × 50 mL,
or 0.390 mg/50 mL

Chromatograph all STD solutions to ensure that they are properly prepared. Each is considered properly prepared if the relative response factors (RRFs) for each solution are within 1% of the other for CTL and 10% for HCB:

$$RRF = \frac{\text{analyte weight per 50 mL solvent}}{\text{area of analyte}} \times \text{area of INR}$$

If the RRFs meet these criteria, then combine STD 1 and STD 2 (1 + 1) for use as a calibration solution. This solution is stable for 7 days under normal, ambient laboratory conditions. Laboratory B combined the standards by pipetting 40 mL of each into a clean 4 oz. bottle. The weights used for this calibration solution are the average corrected weights for the 2 compounds in each of the 2 standards per 50 mL solvent.

Preparation of Technical Samples

Grind sample with a mortar and pestle prior to weighing. Weigh samples directly into a vessel. Qorpak bottles (2 or 4 oz. size) were used in Laboratory B.

Weigh in duplicate and record to 4 decimal places 1.0 g sample and then dissolve with 50.0 mL INR solution. Mix samples until CTL dissolves. Some solids in samples may not dissolve in the INR solution. An ultrasonic bath may be used to dissolve solids. Be sure to allow enough mixing time to ensure that all CTL is in solution.

In this study, Laboratory B placed sample solutions in an ultrasonic bath for ca 30 min and then allowed them to sit on a laboratory bench for another 30 min to equilibrate to room temperature. Laboratory A used a magnetic stirring motor. Technical CTL samples may contain small amounts of carbon black, and the preparation may take on a dark gray hue when agitated. After returning to room temperature, sample aliquots were filtered when necessary (in Laboratory B, 0.45 µm Acrodisc CR filters were used to filter the standard and sample preparations, if filtering was required) or transferred to appropriate autosampler vials for analysis.

Modifications for Dry Formulations

Wettable powders and dispersible granular formulations must be ground with a mortar and pestle prior to dissolution. Modifications in sample weights were made for each formulation to allow for CTL in solution to remain at 20 mg/mL (Table 1). Preparations are allowed to settle prior to analysis, or samples may be filtered as noted earlier.

Table 1. Formulations and proposed sample weights

Formulation	Chlorothalonil, wt %	Sample weight, g
Technical	>97	1.0
90-DG	90	1.1
Bravo W-75	75	1.3
Bravo 720	54	1.9
Bravo 500	40.4	2.4

Modifications for Water-Based Flowable Formulations

Samples must be shaken well prior to subsampling to ensure homogeneity. Water-based formulations may contain over 40% water. The solvent or solvent system used must be miscible with water and must effectively solubilize CTL. The INR solution is prepared as described earlier with regard to the amount of DBP per volume, but for these aqueous formulations, methanol–toluene (1 + 1, v/v) is used. Add 4 to 6 drops phosphoric acid for each liter of INR. Sample weights are adjusted upward to allow for CTL in solution to remain at 20 mg/mL. *See* Table 1 for a description of formulation concentrations and sample weights needed to achieve this concentration. Prepared samples must be allowed to settle prior to analysis, or samples may be filtered.

Analysis

Injections (1 µL) were made with automatic sampling equipment. The analysis sequence was CS, CS, S1, S2, S3, CS, S4, S5, S6, CS, etc., where CS is calibration standard and S1, S2, S3, etc., are sample solutions 1, 2, 3, etc. Sample injections may commence when the calibration standard RRF agrees within 5% for CTL.

Calculations

The integration devices used are programmed to calculate results on the basis of INR methodology. RRF is calculated with the following formula:

$$RRF = \frac{\text{weight 1}}{\text{area 1}} \times \text{area 2}$$

where weight 1 is weight of SDS-2787-1501 in CS (mg/50 mL), area 1 is chromatographic peak area of SDS-2787-1501 in CS, and area 2 is chromatographic peak area of DBP (INR) in CS.

Weight percentage results for sample preparations are then calculated with the following formula:

$$CTL,\ wt\ \% = \frac{\text{area 3} \times RRF}{\text{weight 3} \times \text{area 4}} \times 100$$

where area 3 is chromatographic peak area of CTL in sample, weight 3 is sample weight (mg/50 mL), and area 4 is chromatographic peak area of DBP (INR) in sample.

Calculations are performed identically for quantitation of HCB.

Initially, the RRF is determined with the following formula:

$$\text{RRF} = \frac{\text{weight 1}}{\text{area 1}} \times \text{area 2}$$

where weight 1 is weight of HCB in CS (mg/50 mL), area 1 is chromatographic peak area of HCB in CS, and area 2 is chromatographic peak area of DBP (INR) in CS.

Weight percentage results for sample preparations are then calculated with the following formula:

$$\text{HCB, wt \%} = \frac{\text{area 3} \times \text{RRF}}{\text{weight 3} \times \text{area 4}} \times 100$$

where area 3 is chromatographic peak area of HCB in sample, weight 3 is sample weight (mg/50 mL), and area 4 is chromatographic peak area of DBP (INR) in sample.

Results and Discussion

Laboratory A (ISK Biosciences Corp., Greens Bayou Plant, Houston, TX) analyzed each sample in duplicate. Laboratory B (Ricerca, Inc., Painesville, OH) analyzed 8 preparations of each sample. Laboratory B initially analyzed all sample types (except 2 aqueous formulations, Bravo 500 and Bravo 720 which were initially unavailable) and then reanalyzed them 4–5 months later. Results of the initial and follow up analyses (4 to 5 months apart) done by Laboratory B are shown in Table 2. The mean value of the 2 injections made for each preparation was used in calculations, therefore, $n = 8$.

Table 3 presents CTL data from the 2 laboratories using the described method. These results represent the overall mean values generated by each laboratory. The results and relative standard deviations (RSDs) listed for laboratory B are the simple averages of analyses conducted 5 months apart, as presented in Table 2. Results shown in Table 3 for laboratory A are based on 2 preparations of each sample and 2 injections of each preparation. Because n therefore equals 2, the reported RSD may not be meaningful. Overall, the 2 laboratories produced similar results. The largest absolute difference (0.4%) between the 2 laboratories was for a 90-DG formulation. Results for all other sample types were within 0.2% or better.

Table 4 presents the HCB data generated by laboratory B. These results were simultaneously generated during chromatographic analyses previously described and for which results are listed in Table 2. The largest absolute difference between the 2 analyses generated by laboratory B over the 4–5 month period was 0.006% (technical sample 1).

Table 5 presents HCB data from the 2 laboratories using the described method. As with CTL data, these HCB data are, for laboratory B, the average of results from 2 analysis dates, and for laboratory A, the result of the double preparation and dual injections. The largest absolute difference between laboratories (0.004%) again was for technical sample 1 and is approximately 10% relative to the reported HCB concentration, which is within the expected method precision for that compound.

Table 2. Chlorothalonil results from Laboratory B

Formulation	Chlorothalonil,[a] wt %	
	Initial	5 Months later
Technical sample 1	98.6 (0.24)	98.2 (0.16)
Technical sample 2	98.4 (0.17)	97.7 (0.11)
90-DG sample 1	90.7 (0.11)	90.3 (0.24)
90-DG sample 2	90.3 (0.18)	90.6 (0.10)
Bravo W-75 sample 1	75.7 (0.17)	76.0 (0.18)
Bravo W-75 sample 2	75.4 (0.18)	75.5 (0.09)
Bravo 720	—[b]	53.9 (0.83)
Bravo 500	—[b]	40.6 (0.49)

[a] Values are means (RSDs); $n = 8$.
[b] Samples were not available at the time of analysis.

Table 3. Interlaboratory comparison of chlorothalonil results

Formulation	Chlorothalonil,[a] wt %	
	Laboratory A[b]	Laboratory B[c]
Technical sample 1	98.4 (0.14)	98.4 (0.20)
Technical sample 2	98.0 (0.53)	98.1 (0.14)
90-DG sample 1	90.9 (0.03)	90.5 (0.18)
90-DG sample 2	90.4 (0.21)	90.5 (0.14)
Bravo W-75 sample 1	75.8 (0.23)	75.9 (0.018)
Bravo W-75 sample 2	75.3 (0.02)	75.5 (0.14)
Bravo 720	53.8 (0.18)	53.9 (0.83)[d]
Bravo 500	40.3 (0.04)	40.6 (0.49)[d]

[a] Values are means (RSDs).
[b] $n = 2$.
[c] $n = 16$.
[d] One analysis only; $n = 8$.

Table 4. Hexachlorobenzene results from Laboratory B

Formulation	Hexachlorobenzene,[a] wt %	
	Initial	5 Months later
Technical sample 1	0.038	0.032
Technical sample 2	0.034	0.032
90-DG sample 1	0.029	0.026
90-DG sample 2	0.035	0.030
Bravo W-75 sample 1	0.022	0.021
Bravo W-75 sample 2	0.026	0.022
Bravo 720	—[b]	0.019
Bravo 500	—[b]	0.013

[a] Values are means; $n = 8$.
[b] Samples were not available at the time of analysis.

Table 5. Interlaboratory comparison of hexachlorobenzene results

Formulation	Hexachlorobenzene,[a] wt %	
	Laboratory A[b]	Laboratory B[c]
Technical sample 1	0.032	0.036
Technical sample 2	0.030	0.033
90-DG sample 1	0.024	0.027
90-DG sample 2	0.029	0.033
Bravo W-75 sample 1	0.018	0.022
Bravo W-75 sample 2	0.021	0.024
Bravo 720	0.016	0.019[d]
Bravo 500	0.012	0.013[d]

[a] Values are means.
[b] $n = 2$.
[c] $n = 16$.
[d] One analysis only; $n = 8$.

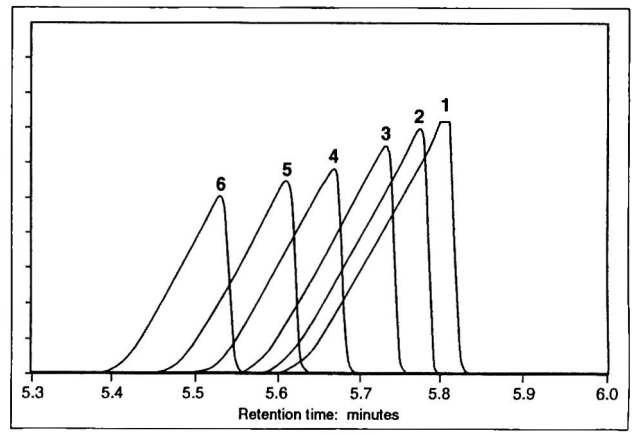


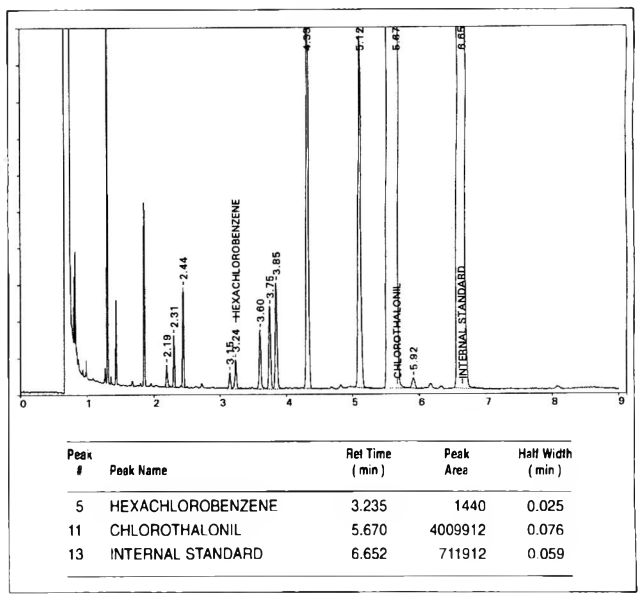
Figure 1. Split flow experiment conducted to determine the split ratio necessary to prevent integrator analog to digital (A-D) converter overload. Split flow (mL/min): 1, 100; 2, 120; 3, 140; 4, 160; 5, 180; 6, 200.

Both laboratories evaluated GC injection port temperatures. Reasonable results were obtained throughout the temperature range studied, –250° to 330°C. Because of the boiling points of the compounds of interest (HCB, 323°–326°C; DBP, 340°C; and CTL, 350°C), an injection port temperature of 330°C was used.

Experiments were conducted with regard to split flow. Initially, Laboratory B used a very low split flow, which lowered the overall RSD for HCB analysis. However, CTL precision suffered. Laboratory B discovered that the CTL response was overloading the integrator, which resulted in a "clipped" peak for that compound. An example of this phenomenon can be seen in Figure 1, which was produced by Chrom Perfect software, manufactured by Justice Innovations. This figure presents a computer-generated overlay of 6 chromatograms at 6 split flows. However, only a very small section of the chromatogram (5.3 to 6.0 min) is presented and at a relatively insensitive attenuation to bring the peak of interest on scale. Consequently, the split flow was raised from the initial 50 mL/min to 140 mL/min. The CTL peak apex became acceptable at around 120 mL/min. A split flow of 140 mL/min was selected to ensure that integrator overloading would not be a concern. Some minor column overload for CTL can be seen in this highly attenuated Figure 1, but this is of little concern, and a high concentration of CTL must be prepared to see HCB. Split flow must be optimized by each laboratory to ensure that similar overloading problems do not occur. Figure 2 shows a representative chromatogram of Bravo 720, generated by laboratory B, at the desired detector range and attenuation.

Laboratory B determined the linearity of the method for both CTL and HCB. Five solutions were prepared containing both analytes from approximately 80 to 120% of concentrations expected when preparing standards and samples as described in the method. CTL concentrations ranged from ~16 to 25 mg/mL. The data were plotted by using a first-order equation. The correlation coefficient for the range was 1.00. HCB concentrations ranged from ~0.006 to 0.009 mg/mL. The correlation coefficient for the range was 0.990. Figures 3 and 4 show the curves generated from the data.

Laboratory A conducted experiments and generated data showing improved precision when detector makeup gas is turned off. These method validation studies were conducted to determine the GC response of CTL in single (toluene) and double (methanol–toluene) solvents without makeup gas flow to the detector and at a split flow of 50 mL/min. With the technical and dry formulations, where use of a single solvent is appropriate, linear regression analysis of calculated standard concentration versus the CTL/DBP response ratio gave a correlation coefficient of 1.00 for the 8.0 to 40 mg/mL range. The slope of the least-squares curve was 0.135879, with a standard error coefficient of 0.000526 and a y intercept of 0.0068. For aqueous formulations requiring double solvent, linear regression analysis of calculated standard concentration versus the CTL/DBP re-



Peak #	Peak Name	Ret Time (min)	Peak Area	Half Width (min)
5	HEXACHLOROBENZENE	3.235	1440	0.025
11	CHLOROTHALONIL	5.670	4009912	0.076
13	INTERNAL STANDARD	6.652	711912	0.059

Figure 2. Typical gas chromatogram from analysis of Bravo 720.

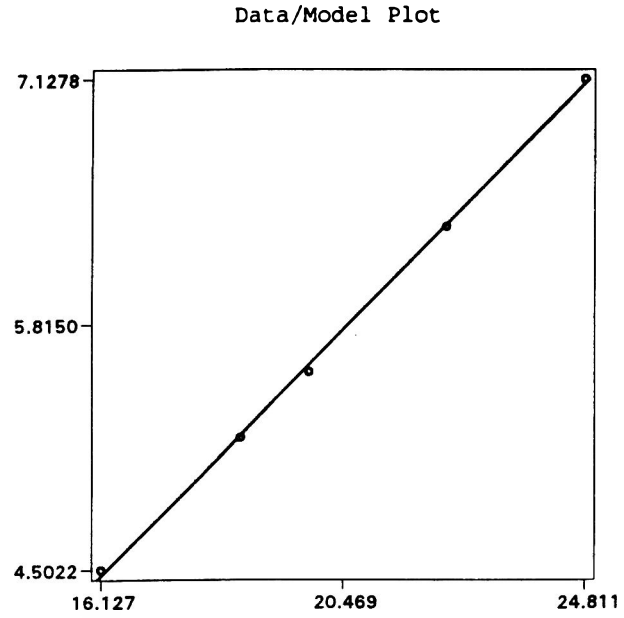


Figure 3. Linear regression curve from analysis of chlorothalonil.

sponse ratio gave a correlation coefficient of 1.00 for the range 0.51 to 10 mg/mL. Excellent correlation coefficients (better than 0.9999) from both experiments indicate that GC responses were linear without overloading the system. However, one disadvantage of this technique (makeup gas off) is the significant loss of detector sensitivity. Again, after operating conditions have been finalized, careful attention must be given to the CTL peak to ensure that no severe column, electrometer, or integrator overload occurs. The use of FID makeup gas is specified by GC manufacturers and was done for all analyses.

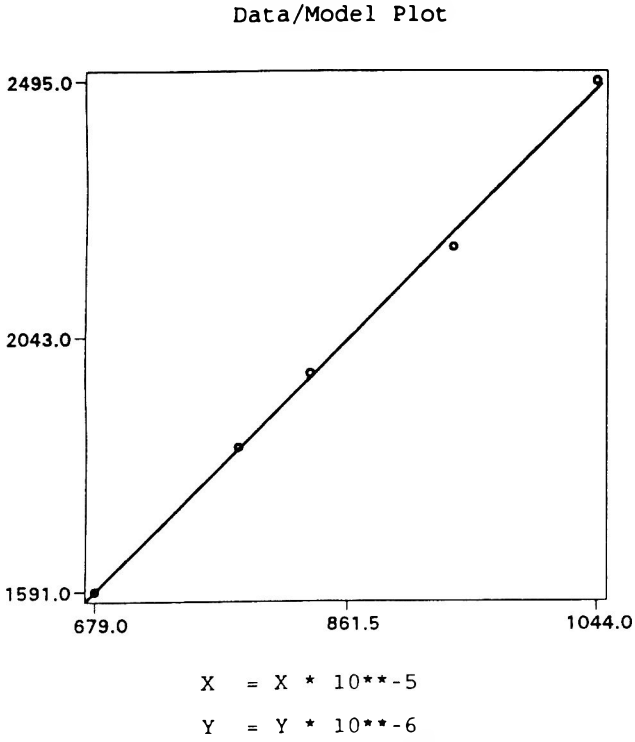


Figure 4. Linear regression curve from analysis of hexachlorobenzene.

Solutions containing CTL were stable for at least 24 h under normal, ambient laboratory conditions. When prepared standards and samples were allowed to sit on a laboratory bench overnight and reanalyzed, results before and after overnight standing were identical. Laboratory B showed that, in organic solvent, standard preparations are stable for at least 7 days. Experiments have shown that CTL does not hydrolyze in either neutral or acidic aqueous media (1). Hydrolysis occurs slowly in moderate alkali (pH 9) solutions (13).

Direct exposure of the prepared solutions to ultraviolet light will adversely affect CTL concentration, via CTL photodegradation in the presence of solvents (14).

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We thank Myron Bliss, ISK Biosciences Corporation, for his continued efforts and encouragement to complete this project.

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CHEMICAL CONTAMINANTS MONITORING

U.S. Food and Drug Administration Monitoring of Lead in Domestic and Imported Ceramic Dinnerware

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The U.S. Food and Drug Administration (FDA) conducted a survey of domestic and imported ceramic dinnerware from January to February 1992 to determine the status of lead leaching from this ware. Ceramicware was screened at the collection point by using the Quick Color Test (QCT); if the QCT was positive, the ware was analyzed in the laboratory by using atomic absorption spectroscopy (AAS). For imports, 5222 lots were examined using the QCT. Of these lots, 46 exceeded FDA's 1991 guidelines as determined by using AAS. For domestic ware, 676 lots were examined using the QCT, and 17 lots exceeded the 1991 guidelines. The violation rates, 0.9% for imports and 2.5% for domestic ware, were about twice as high as they would have been under the 1980 guidelines.

Lead glazes are used to produce a smooth, lustrous, and sometimes decorative coating on ceramicware such as bone china, earthenware, porcelain, and pottery (1). The nonporous, glazed surfaces permit easy cleaning and contribute to good sanitation. If a glaze is improperly formulated or applied, however, or if the object is improperly fired during the manufacturing process, excessive amounts of lead may leach from the glaze into food contained in the vessel (2). Even with properly glazed ware, some lead may migrate into food; however, the amounts will be much lower than with improperly glazed items.

Since the 1930s, the U.S. Food and Drug Administration (FDA) has acted to protect the public from the hazards associated with excessive exposure to lead. These efforts, which have focused on lead from sources such as agricultural chemicals, lead-soldered cans, and glazes for ceramicware, have resulted in a substantial decrease in dietary lead exposure (3).

In 1971, FDA set informal guidelines for levels of lead leaching from ceramicware products. These levels were further restricted in 1980 (4). Since that time, research has revealed that lead could adversely affect the fetus, young children, and adults in amounts well below those previously believed harmful (5). In 1991, FDA reduced the guideline levels that had been issued in 1980 as follows (6): from 7 to 3 ppm for plates, saucers, and other flatware; from 5 to 2 ppm for small hollowware such as cereal bowls (but not cups and mugs); from 5.0 to 0.5 ppm for cups and mugs; from 2.5 to 1.0 ppm for large (>1.1 L) hollowware such as bowls (but not pitchers); and from 2.5 to 0.5 ppm for pitchers.

From January to February 1992, FDA conducted a survey of imported and domestic dinnerware to determine the status of leachable lead in these types of ware. The objectives of the survey were to examine, if possible, 100% of imported lots of food-use ceramicware offered for entry into the United States during an approximately 4-week period. An attempt was also made to examine multiple lots of domestic food-use ceramicware at manufacturing establishments throughout the United States within the designated time frame. All the ware was to be screened by using the Quick Color Test (QCT) (7), and positive QCT findings were to be confirmed by using atomic absorption spectroscopy (AAS) (8). Another objective of the survey was to identify import manufacturers, shippers, and importers and domestic manufacturers producing violative food-use ceramicware and take appropriate regulatory action. Also, a handbook of color photographs of violative items and patterns was to be produced for use as an inspection aid.

Collection of Ceramicware

Up to 10 domestic manufacturers' lots of food-use ceramicware were examined by using the QCT (7). For imports, all lots of food-use ceramicware were targeted, and if the QCT was positive, 6 identical units (same color, design, shape, and size) were collected from that lot. For domestic ware, 12 identical units were collected, and emphasis was placed on highly decorated ware. In both instances, 6 units were analyzed in the laboratory by using the official AOAC AAS method (8). (The extra set of 6 domestic units was retained as required by FDA in case regulatory action was taken on the basis of the lead

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analyses. No extra units are required for regulatory action imposed on imports.)

The samples were only those types that are used for foods (but not those used for dry foods, e.g., salt and pepper shakers, sugar bowls, or plates manufactured for decorative display). When complete dinnerware sets were encountered, the QCT was applied to the item with the largest decorated food-contact surface. If the dinnerware did not include a decorated food-contact surface (i.e., whiteware), the QCT was used on cups or mugs in the set.

Imported and domestic ceramicwares were collected by personnel in 19 and 11 FDA districts, respectively. The ware was analyzed by 11 FDA laboratories.

Analysis of Ceramicware

Ware was quantitatively screened for leachable lead at the collection point by using the QCT (7). The QCT use of filter paper slips that are placed on the food-contact surface of the ware to be tested. The slip is wet with citric acid solution, and after ≤30 min the slip is removed and spotted with a chromogen solution. The immediate appearance of a rose to rose–red stain on the paper slip indicates the presence of leachable lead. (An initial yellow color that fades within 1–2 min is a negative test.) The sensitivity of the QCT was arbitrarily defined as the lowest lead concentration at or above which the QCT is at least 95% reliable. The sensitivity of the QCT is 1.7 ppm lead (9).

Ware that tested positive by the QCT was further tested in the laboratory by using the official AOAC AAS method (8). The unit to be tested was filled with 4% (v/v) acetic acid and allowed to stand at room temperature for 24 h. The lead in the acetic acid solution was measured by using AAS. In the analyzing laboratories, contamination control as outlined by Boyer and Horwitz (10) was practiced.

As prescribed by the revised guidelines (6), in each category of ware except flatware, the maximum value of the 6 analyzed units was reported. In the case of flatware, the average of the 6 units was reported.

Levels of lead exceeding the guidelines were reported to FDA headquarters for possible regulatory action. In addition, color photographs of such ware were taken to develop a handbook as an inspection aid.

Results

Imported Ware

During the survey, shipments from 29 countries were examined. Shipments from Canada, Denmark, Greece, India, Luxembourg, Mexico, Morocco, and Democratic People's Republic of Korea had no ware that tested positive by the QCT. The other 21 countries, Brazil, Czechoslovakia, France, Germany, Hong Kong (colony), Hungary, Indonesia, Ireland, Italy, Japan, Malaysia, The Philippines, Poland, Portugal, People's Republic of China, Republic of Korea, Spain, Sri Lanka, Taiwan, Thailand, and United Kingdom, had various numbers of shipments with wares that tested positive with the QCT. Twelve countries had >100 lots examined during the survey. Table 1 lists the 12 countries and, for each country, the number of lots examined, the number of lots that tested positive as determined by the QCT, the number and percentage of lots that exceeded the 1991 guidelines, and the product categories in which lead levels that exceeded the 1991 guidelines occurred.

A total of 5222 lots were examined using the QCT. Of those, 4936 (94.5%) yielded negative QCTs, whereas 286 (5.5%) were positive using the field test and, thus, were subject to confirmatory analysis by the AAS method (8).

Of these 286 lots analyzed, 46 lots exceeded the 1991 guidelines (6). Table 2 lists the product category, country of origin, and results of AAS lead analysis (given as either the maximum or average amount found) and which samples exceeded the 1980 and 1991 guidelines.

Table 1. Imported dinnerware from 12 countries with >100 lots examined

Country	No. lots examined	No. lots with positive QCT test	No. lots (and %) exceeding 1991 guidelines	Product category[a]
Japan	1897	102	6 (0.3)	Flatware, cups/mugs, small hollowware, pitchers
People's Republic of China	613	31	11 (2)	Flatware, small hollowware
Taiwan	413	14	2 (0.5)	Flatware, large hollowware
Italy	367	9	5 (1)	Flatware
Republic of Korea	367	11	1 (0.3)	Flatware
Portugal	157	10	2 (1)	Flatware
Thailand	157	2	0	—
United Kingdom	130	28	4 (3)	Flatware
France	111	12	2 (2)	Flatware, cups/mugs
The Philippines	110	4	0	—
Hong Kong	108	2	2 (2)	Flatware, large hollowware
Germany	102	45	6 (6)	Cups/mugs

[a] If multiple categories of ware had lead levels that exceeded the 1991 guidelines, the product categories are listed in descending order.

Table 2. Lead leached[a] from imported dinnerware as determined by atomic absorption spectroscopy

Product	Country of origin	Pb max., ppm	Pb av., ppm
Cups/mugs	France	0.74[b]	
	France	0.10	
	Germany	0.90[b]	
	Germany	0.83[b]	
	Germany	1.3[b]	
	Germany	0.59[b]	
	Germany	0.19	
	Germany	0.21	
	Germany	0.26	
	Germany	0.43	
	Germany	0.15	
	Germany	0.18	
	Germany	0.20	
	Germany	0.14	
	Germany	0.18	
	Germany	0.45	
	Germany	0.44	
	Germany	1.2[b]	
	Germany	0.22	
	Germany	0.47	
	Germany	0.12	
	Germany	0.10	
	Germany	0.10	
	Germany	0.80[b]	
	Germany	0.14	
	Germany	0.12	
	Germany	0.11	
	Hungary	16[b,c]	
	Japan	0.10	
	Japan	0.20	
	Japan	0.18	
	Japan	1.0[b]	
	Republic of Korea	0.20	
	Spain	6.2[b,c]	
	United Kingdom	0.10	
	United Kingdom	0.10	
Flatware	Brazil		8.8[b,c]
	Brazil		3.4[b]
	Brazil		4.4[b]
	Brazil		1.9
	Brazil		3.0
	Czechoslovakia		7.7[b,c]
	France		4.7[b]
	France		1.2
	France		2.3
	France		2.7
	France		1.8
	France		2.4
	Hong Kong		9.6[b,c]
	Italy		45[b,c]
	Italy		1.2
	Italy		12[b,c]
	Italy		4.4[b]
	Italy		11[b,c]
	Italy		5.6[b]
	Japan		1.0
	Japan		1.0
	Japan		1.9
	Japan		2.7
	Japan		15[b,c]
	Japan		27[b,c]
	Japan		8.0[b,c]
	Japan		2.4
	Japan		1.6
	Japan		1.3
	Japan		1.6
	Japan		1.4
	Japan		1.8
	Japan		1.3
	Japan		1.7
	Poland		1.1
	Portugal		1.7
	Portugal		4.0[b]
	Portugal		11[b,c]
	People's Republic of China		25[b,c]
	People's Republic of China		15[b,c]
	People's Republic of China		1.2
	People's Republic of China		2.2
	People's Republic of China		6.6[b]
	People's Republic of China		9.9[b,c]
	People's Republic of China		1.4
	People's Republic of China		7.5[b,c]
	People's Republic of China		12[b,c]
	People's Republic of China		4.1[b]
	People's Republic of China		1.6
	People's Republic of China		2.4
	People's Republic of China		1.3
	People's Republic of China		2.8
	Republic of Korea		4.9[b]
	Republic of Korea		2.3
	Sri Lanka		1.7
	Taiwan		12[b,c]
	United Kingdom		1.0
	United Kingdom		3.5[b]
	United Kingdom		2.8
	United Kingdom		80[b,c]
	United Kingdom		33[b,c]
	United Kingdom		1.1
	United Kingdom		3.3[b]
Large hollowware	Hong Kong	1.6[b]	
	Taiwan	7.4[b,c]	
Pitchers	Hungary	11[b,c]	
	Japan	0.9[b]	
Small hollowware	Brazil	2.2[b]	
	Hungary	17[b,c]	
	Hungary	1.0	
	Hungary	172[b,c]	
	Hungary	50[b,c]	
	Japan	5.5[b,c]	
	People's Republic of China	4.2[b]	

Table 2. *(continued)*

Product	Country of origin	Pb max., ppm	Pb av., ppm
	People's Republic of China	3.1[b]	
	People's Republic of China	5.1[b,c]	
	People's Republic of China	1.0	
	People's Republic of China	1.4	
	People's Republic of China	1.2	
	People's Republic of China	19[b,c]	
	People's Republic of China	7.2[b,c]	
	People's Republic of China	9.0[b,c]	
	People's Republic of China	4.5[b]	
	People's Republic of China	8.9[b,c]	
	Taiwan	1.8	

[a] For cups/mugs and pitchers, values <0.10 μg/mL (limit of quantitation [LOQ] of AAS method) are not listed. For flatware and small and large hollowware, values <1.0 μg/mL (LOQ of AAS method) are not listed.
[b] Lead level exceeds 1991 guidelines (6).
[c] Lead level exceeds 1980 guidelines (4).

Domestic Ware

Examinations of food-use ceramicware were conducted at 88 domestic manufacturers' establishments, during which 676 lots were tested using the QCT. Of these lots, 70 (10.4%) had a positive QCT. When ceramicware from these lots was analyzed in the laboratory by using AAS, 17 lots exceeded the 1991 guidelines (6).

Table 3 lists the domestic product category and results of AAS lead analysis (given as either the maximum or average amount found), and indicates which samples exceeded the 1980 and 1991 guidelines.

Discussion

The results of this survey show that, for imported ware, most countries were shipping ceramic dinnerware to the United States that did not leach lead in amounts that exceeded the 1991 guidelines.

For both imported and domestic ware, pitchers as a product category exhibited the greatest percentage of lots exceeding the 1991 guidelines. However, this category was represented by such a small number of lots (a total of 4) that no significance can be attached to that finding without testing additional lots. Domestic cups/mugs and imported and domestic small hollowware were the product categories that had the next highest percentages of lots exceeding the 1991 guidelines.

When violation rates are based on the 1991 guidelines (6), they are about twice as high as when they are based on the 1980 guidelines (4): 0.9 versus 0.4% for imported ware and 2.5 versus 1% for domestic ware, respectively. The higher rates for domestic ware compared with imported ware may result from the increased examination of highly decorated domestic ware. For both domestic ware and imports, the violation rates calculated by using the 1991 guidelines might have been higher if

Table 3. Lead leached[a] from domestic dinnerware as determined by atomic absorption spectroscopy

Product	Pb max., ppm	Pb av., ppm
Cups/mugs	1.0[b]	
	4.0[b]	
	0.78[b]	
	0.23	
	0.11	
	0.20	
	0.11	
	34[b,c]	
	75[b,c]	
	5.0[b]	
	2.2[b]	
	124[b,c]	
	1.7[b]	
	7.0[b,c]	
	0.40	
Flatware		1.3
		2.5
		1.0
		1.4
		2.1
		5.5[b]
		18[b,c]
		2.7
Large hollowware	0.37	
Pitchers	4.3[b,c]	
Small hollowware	1.2	
	2.1[b]	
	5.8[b,c]	
	3.4[b]	
	5.7[b,c]	

[a] For cups/mugs and pitchers, values <0.10 μg/mL (limit of quantitation [LOQ] of AAS method) are not listed. For flatware and small and large hollowware, values <1.0 μg/mL (LOQ of AAS method) are not listed.
[b] Lead level exceeds 1991 guidelines (6).
[c] Lead level exceeds 1980 guidelines (4).

the ware in this survey had not been selected on the basis of QCT screening. Also, most if not all of the ware sampled in this survey probably was manufactured before the issuance of the 1991 guidelines and would have been "in the pipeline" at the time of sampling.

Adoption of the lower lead guidelines should enable FDA to identify and remove from commerce those items that could be a potential health hazard. A notebook containing colored photographs of the violative ware, which was compiled as part of this survey, can be used as an aid in identifying potentially violative items.

This survey represents a "snapshot" view of the status of leachable lead in imported and domestic ceramicware in commercial channels during a relatively short period of time, and the findings cannot be extrapolated to conclude that similar results would be found over a longer time period. FDA will continue to monitor imported and domestic ceramicware for leach-

able lead as well as other potential food-related contributors to dietary lead.

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CHEMICAL CONTAMINANTS MONITORING

Accumulated Pesticide and Industrial Chemical Findings from a Ten-Year Study of Ready-to-Eat Foods

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This report lists the pesticide and industrial chemicals found in the ready-to-eat foods tested repetitively for 10 years through the U.S. Food and Drug Administrations's Revised Market Basket Study. The study operated from 1982 to 1991. During that time 37 market baskets, each containing 234 food items that represented about 5000 food types in American diets covering all age groups, including infants and children, were collected. Each food item was individually prepared for eating; that is, it was opened, unwrapped, washed, peeled, sliced, formulated by recipe, or cooked. Each item was then composited and analytically screened for about 300 different chemicals, including chlorphenoxy acids, ethylenethiourea, methyl carbamates, organochlorines, organophosphates, organosulfurs, phenylureas, and pyrethroids. Overall, less than 1% of the potential of 2.5 million findings occurred for the 10-year study period. In total, 138 different chemical residues accounted for 17 050 accumulated findings. Most findings were less than 1 μg/g, which is considered a low-level finding. Each food item averaged about 2 low-level findings per analysis.

The Total Diet Study (TDS) began in 1961. The study, part of the Pesticides and Chemical Contaminants Program, monitors the human intake of selected nutrients, essential and toxic elements, and radioactive and pesticide contaminants in the United States. The domestic and imported foods selected for the study are analyzed by multielemental and multiresidue methods at screening levels about 5 times below normal regulatory requirements.

The study underwent minor revision in 1970. Most of the analytical work was assigned to the Kansas City District Laboratory. Ready-to-eat foods (120) were divided into 12 food-group diets (1), e.g., dairy products, fruits, meats, and vegetables. Each group of samples was composited into one food group sample. Each sample was then screened for about 15 essential and toxic elements and 300 pesticides by using established analytical methods (2). Infant and toddler food groups were added to the study in 1975 (1).

The study underwent major revisions in 1982 (3, 4). Diets were changed to represent more age/sex groups in the U.S. population. The number of food items was increased from 120 to 234. Each item was screened individually instead of screen-

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Table 1. Ten-year accumulated frequency of 138 pesticide and industrial chemicals found in 234 ready-to-eat foods tested 37 times each

Residue code	Times found[a]	No. foods[b]	Average found,[c] µg/g	Chemical name
1	17	5	0.0049	1,2,4,5-Tetrachloro-3-(methylthio)benzene
2	37	10	0.0069	2,3,5,6-Tetrachloroaniline
3	23	6	0.0037	2,3,5,6-Tetrachloroanisidine
4	2	2	0.0002	2,3,5,6-Tetrachloroanisole
5	5	2	0.0038	2,4,5-T
6	1	1	0.0060	2,4-D
7	115	14	0.0031	2,4-Dichloro-6-nitrobenzenamine
8	8	2	0.0080	2-Chloroethyl caprate
9	24	7	0.0066	2-Chloroethyl laurate
10	291	33	0.1152	2-Chloroethyl linoleate
11	89	11	0.0145	2-Chloroethyl myristate
12	215	25	0.0275	2-Chloroethyl palmitate
13	3	3	0.0263	3-Hydroxycarbofuran
14	222	33	0.0170	Acephate
15	1	1	0.0110	Aldicarb
16	1	1	0.0250	Aldoxycarb
17	5	5	0.0016	Aldrin
18	1	1	0.0040	Atrazine
19	37	6	0.0469	Azinphos-methyl
20	584	88	0.0010	BHC, alpha
21	14	9	0.0027	BHC, beta
22	1	1	0.0030	BHC, delta
23	3	1	0.0020	Bromophos-ethyl
24	66	13	0.0835	Captan
25	298	30	0.0604	Carbaryl
26	6	3	0.0251	Carbofuran
27	1	1	0.0050	Carbophenothion
28	27	11	0.0130	Chlordane
29	32	11	0.0017	Chlordane, *cis*-
30	40	13	0.0011	Chlordane, *trans*-
31	1	1	0.0007	Chlordene
32	73	7	0.0045	Chlorobenzilate
33	4	1	0.0105	Chlorothalonil
34	1	1	0.1300	Chlorowax 500C
35	333	54	0.1261	Chlorpropham
36	718	121	0.0036	Chlorpyrifos
37	439	46	0.0042	Chlorpyrifos methyl
38	147	32	0.0033	DCPA
39	8	4	0.0025	DDE, *o,p'*-
40	1700	142	0.0026	DDE, *p,p'*-
41	5	4	0.0053	DDT, *o,p'*-
42	98	31	0.0045	DDT, *p,p'*-
43	10	2	0.0035	DEF
44	2	2	0.0155	Demeton-S
45	11	7	0.0216	Demeton-S sulfone
46	894	144	0.0019	Diazinon
47	1	1	2.5000	Dibutyl phthalate
48	82	22	0.2353	Dichlorooctadecanoic acid
49	113	25	3.6475	Dichlorooctadecenoic acids
50	1	1	0.0100	Dichlorvos
51	450	74	0.0340	Dicloran
52	40	13	0.0072	Dicofol, *o,p'*-
53	202	41	0.0238	Dicofol, *p,p'*-
54	925	117	0.0015	Dieldrin

Table 1. ***(continued)***

Residue code	Times found[a]	No. foods[b]	Average found,[c] µg/g	Chemical name
55	236	35	0.0094	Dimethoate
56	146	41	0.5120	Diphenyl 2-ethylhexyl phosphate
57	1	1	0.6700	Diphenylamine
58	2	2	0.0089	Disulfoton
59	4	4	0.0080	Disulfoton sulfone
60	388	52	0.0038	Endosulfan I
61	437	50	0.0060	Endosulfan II
62	429	51	0.0083	Endosulfan sulfate
63	26	9	0.0027	Endrin
64	3	2	0.0050	EPN
65	199	31	0.0053	Ethion
66	8	5	0.0029	Ethion oxygen analogue
67	5	4	0.0256	Ethylenethiourea[d]
68	35	8	0.0037	Fenitrothion
69	3	3	0.0650	Fenuron[d]
70	6	3	0.2068	Fenvalerate
71	6	3	0.0831	Folpet
72	31	4	0.0010	Fonofos
73	1	1	0.0050	Gardona
74	2	2	0.0028	Heptachlor
75	450	71	0.0010	Heptachlor epoxide
76	618	81	0.0006	Hexachlorobenzene
77	22	4	0.0700	Iprodione
78	9	4	0.0487	Iprodione metabolite isomer
79	40	7	0.0098	Chlorpropham metabolite
80	1	1	0.2200	Isopropylphenyl phenyl phosphates, mixed
81	369	81	0.0012	Lindane
82	25	4	0.0095	Linuron[d]
83	1794	110	0.0111	Malathion
84	427	49	0.0111	Methamidophos
85	8	4	0.0040	Methidathion
86	3	3	0.0573	Methiocarb
87	43	11	0.0451	Methomyl
88	50	24	0.0152	Methoxychlor, *p,p'*-
89	1	1	0.0400	Metobromuron
90	19	4	0.0501	Mevinphos,(*E*)-
91	31	8	0.0251	Mevinphos,(*Z*)-
92	2	2	0.0050	Monocrotophos
93	1	1	0.0030	Neburon[d]
94	4	3	0.0013	Nonachlor, *cis*-
95	34	18	0.0030	Nonachlor, *trans*-
96	303	54	0.0007	Octachlor epoxide
97	126	26	0.0260	Omethoate
98	2	2	0.0210	Oxamyl
99	173	53	0.0043	Parathion
100	2	1	0.0100	Parathion oxygen analogue
101	12	8	0.0035	Parathion-methyl
102	127	27	0.0024	Pentachloroaniline
103	83	15	0.0012	Pentachlorobenzene
104	6	5	0.0014	Pentachlorobenzonitrile
105	485	128	0.0073	Pentachlorophenol
106	150	33	0.0006	Pentachlorophenyl methyl ether
107	73	9	0.0016	Pentachlorophenyl methyl sulfide
108	98	15	0.1365	Permethrin, *cis*-[d]
109	96	14	0.1415	Permethrin, *trans*-[d]
110	5	1	0.1736	Perthane

Table 1. ***(continued)***

Residue code	Times found[a]	No. foods[b]	Average found,[c] μg/g	Chemical name
111	2	1	0.0015	Phorate sulfone
112	2	1	0.0080	Phorate sulfoxide
113	107	18	0.0252	Phosalone
114	36	7	0.0276	Phosmet
115	3	2	0.0190	Phosphamidon
116	49	24	0.0103	Pirimiphos-methyl
117	27	24	0.0179	Polychlorinated biphenyls
118	1	1	0.0160	Procymidone
119	1	1	0.0320	Profenofos
120	71	15	0.1131	Propargite[d]
121	74	14	0.0016	Quintozene
122	2	2	0.0035	Ronnel
123	20	8	0.2259	Sulfur
124	101	35	0.0020	TDE, *p,p'*-
125	76	22	0.0057	Tecnazene
126	2	2	0.0025	Tetrachlorobenzenes
127	2	1	0.0140	Tetradifon
128	65	15	0.1871	Thiabendazole[d]
129	138	18	0.0410	Toxaphene
130	5	5	0.2804	Tri(2-butoxyethyl)phosphate
131	1	1	0.0040	Tri-allate
132	73	25	0.0137	Tributylphosphate
133	2	2	0.0015	Trichlorobenzenes
134	19	8	0.2803	Triphenylphosphate
135	23	22	0.1910	Tris(2-ethylhexyl)phosphate
136	3	2	0.0600	Tris(beta-chloroethyl)phosphate
137	3	3	0.0093	Tris(chloropropyl)phosphate
138	55	6	0.0443	Vinclozolin

[a] Total findings, 17 050.
[b] Number of foods the residue was found in, of the 230 food items having findings.
[c] Per residue finding.
[d] Analysis added after 1982.

ing a food-group composite. All findings (in μg/g) went into a database for the determination of the daily intake of nutrients and contaminants by diet or food-group type (5). The revised study was completed in 1991, when it underwent further revision and improvement. This report summarizes the findings for the 1982–1991 study period.

METHOD

Because the validated analytical methods used in the study (2) are updated continuously, they are available upon request. These methods can be classified as 2 main types: those used for analyzing fat-containing foods, and those used for analyzing nonfat foods. Generally, all food items are analyzed by one of these method types. Certain items may be cross-analyzed. That is, certain low-fat-containing foods might be analyzed by a nonfat method if that procedure effectively screens that item. Similarly, certain nonfat foods might be put through a fat-method cleanup step for best results. All food items are screened by gas or liquid chromatography, using ion-selective detectors. All findings are confirmed by a second or third instrument; unusual findings are confirmed by gas chromatography/mass spectroscopy.

Results

Long listings of accumulated chemical findings in ready-to-eat foods can give the impression of highly contaminated food. Such lists must be viewed carefully, because they represent information gradually accumulated over time. Table 1, for example, shows that 2 chemical residues accounted for about 20% of the 10-year findings: malathion (1794) and *p,p'*-DDE (1700). Similarly, Table 2 shows that 16 food items having more than 190 findings each, accounted for about 22% of the total findings. Moreover, most of these and the other 10-year findings are <1 μg/g (ppm) and are well below established tolerances. On average, the screening of each food item during the study period yielded about 2 low-level findings per item (17 050 total findings per 37 baskets per 230 items having findings).

Table 2. Ten-year accumulated frequency of chemical findings listed by the 234 ready-to-eat foods tested 37 times each

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
194	28	Nonfat	0.0611	**Apple**, red, raw: **14**, 1, 0.0008; **19**, 14, 0.0333; **24**, 3, 0.0190; **25**, 10, 0.0268; **36**, 16, 0.0083; **37**, 1, 0.0020; **38**, 1, 0.0004; **40**, 3, 0.0007; **46**, 6, 0.0019; **51**, 4, 0.0043; **52**, 5, 0.0088; **53**, 7, 0.0469; **55**, 1, 0.0110; **57**, 1, 0.6700; **60**, 27, 0.0039; **61**, 30, 0.0078; **62**, 25, 0.0034; **65**, 1, 0.0060; **76**, 1, 0.0004; **84**, 1, 0.0009; **97**, 3, 0.0033; **99**, 4, 0.0022; **106**, 4, 0.0003; **113**, 6, 0.0758; **114**, 2, 0.0295; **115**, 2, 0.0320; **120**, 8, 0.1939; **128**, 7, 0.5171
35	10	Nonfat	0.0136	**Applesauce**, canned: **25**, 12, 0.0127; **46**, 1, 0.0010; **51**, 2, 0.0022; **53**, 1, 0.0060; **55**, 10, 0.0030; **56**, 1, 0.0470; **99**, 2, 0.0025; **113**, 3, 0.0033; **120**, 1, 0.0100; **128**, 2, 0.0480
26	13	Nonfat	0.0237	**Asparagus**, boiled: **35**, 1, 0.0020; **36**, 4, 0.1753; **38**, 2, 0.0007; **40**, 7, 0.0017; **46**, 3, 0.0033; **51**, 2, 0.0044; **54**, 1, 0.0006; **56**, 1, 0.0600; **60**, 1, 0.0006; **82**, 1, 0.0160; **93**, 1, 0.0080; **108**, 1, 0.0150; **109**, 1, 0.0210
4	3	Fat	0.0021	**Avocado**, raw: **38**, 1, 0.0008; **81**, 1, 0.0005; **105**, 2, 0.0050
48	6	Nonfat	0.0161	**Baby food**, apple/apple–cherry/apple–grape juice: **14**, 2, 0.0020; **25**, 18, 0.0129; **55**, 19, 0.0041; **83**, 1, 0.0040; **97**, 7, 0.0043; **128**, 1, 0.0690
100	17	Nonfat	0.0125	**Baby food**, applesauce: **14**, 1, 0.0020; **36**, 24, 0.0025; **40**, 4, 0.0020; **52**, 1, 0.0020; **53**, 4, 0.0065; **55**, 8, 0.0064; **60**, 3, 0.0005; **61**, 5, 0.0011; **62**, 3, 0.0015; **65**, 3, 0.0013; **97**, 3, 0.0077; **99**, 16, 0.0025; **113**, 15, 0.0096; **114**, 1, 0.0030; **120**, 7, 0.0631; **124**, 1, 0.0020; **128**, 1, 0.0980
1	1	Nonfat	0.0050	**Baby food**, banana and pineapple or tapioca: **83**, 1, 0.0050
48	6	Fat	0.0018	**Baby food**, beef: **40**, 36, 0.0034; **54**, 2, 0.0007; **76**, 3, 0.0002; **81**, 1, 0.0004; **96**, 1, 0.0005; **124**, 5, 0.0057
9	4	Nonfat	0.0010	**Baby food**, carrots: **40**, 5, 0.0010; **76**, 1, 0.0002; **99**, 2, 0.0009; **104**, 1, 0.0020
19	7	Nonfat	0.0504	**Baby food**, chicken and noodles: **10**, 2, 0.2755; **12**, 2, 0.0175; **35**, 5, 0.0042; **36**, 7, 0.0010; **46**, 1, 0.0003; **99**, 1, 0.0004; **130**, 1, 0.0540
52	7	Fat	0.0007	**Baby food**, chicken or turkey: **40**, 27, 0.0014; **51**, 1, 0.0010; **54**, 9, 0.0006; **75**, 2, 0.0004; **76**, 9, 0.0002; **96**, 1, 0.0003; **124**, 3, 0.0009
7	3	Nonfat	0.0021	**Baby food**, corn, creamed: **36**, 5, 0.0024; **83**, 1, 0.0030; **84**, 1, 0.0010
46	11	Nonfat	0.0032	**Baby food**, Dutch apple or apple Betty: **20**, 1, 0.0004; **36**, 13, 0.0016; **40**, 2, 0.0005; **51**, 1, 0.0010; **53**, 9, 0.0058; **61**, 1, 0.0004; **62**, 1, 0.0008; **65**, 2, 0.0010; **99**, 11, 0.0021; **101**, 1, 0.0007; **113**, 4, 0.0208
15	9	Nonfat	0.0058	**Baby food**, fruit dessert w/ tapioca: **36**, 3, 0.0017; **40**, 1, 0.0008; **51**, 2, 0.0015; **52**, 1, 0.0050; **53**, 1, 0.0330; **55**, 2, 0.0045; **61**, 1, 0.0010; **99**, 2, 0.0019; **113**, 2, 0.0030
59	12	Nonfat	0.0020	**Baby food**, green beans: **14**, 13, 0.0045; **20**, 1, 0.0007; **40**, 6, 0.0014; **51**, 1, 0.0050; **54**, 4, 0.0007; **60**, 2, 0.0015; **61**, 1, 0.0007; **62**, 1, 0.0010; **84**, 23, 0.0054; **99**, 3, 0.0020; **102**, 3, 0.0005; **124**, 1, 0.0010
49	13	Fat	0.0044	**Baby food**, high meat, beef and vegetables: **10**, 2, 0.0265; **12**, 1, 0.0050; **24**, 1, 0.0090; **35**, 10, 0.0037; **36**, 8, 0.0013; **40**, 15, 0.0016; **46**, 1, 0.0004; **54**, 3, 0.0007; **65**, 2, 0.0009; **75**, 1, 0.0003; **76**, 1, 0.0001; **83**, 1, 0.0020; **105**, 3, 0.0060
33	9	Fat	0.0310	**Baby food**, high meat, chicken, turkey, or vegetables: **35**, 5, 0.0030; **36**, 7, 0.0013; **40**, 12, 0.0008; **46**, 1, 0.0003; **54**, 2, 0.0007; **75**, 1, 0.0010; **76**, 3, 0.0002; **83**, 1, 0.0010; **134**, 1, 0.2710
33	7	Fat	0.0108	**Baby food**, high meat, ham and vegetables: **35**, 2, 0.0050; **36**, 5, 0.0013; **40**, 16, 0.0010; **54**, 4, 0.0006; **105**, 1, 0.0050; **124**, 4, 0.0007; **134**, 1, 0.0620
2	2	Fat	0.0090	**Baby food**, infant formula w/ iron: **46**, 1, 0.0020; **105**, 1, 0.0160
2	2	Fat	0.0065	**Baby food**, infant formula w/o iron: **35**, 1, 0.0090; **105**, 1, 0.0040
47	6	Nonfat	0.0021	**Baby food**, infant mixed cereal: **36**, 4, 0.0007; **37**, 1, 0.0004; **40**, 4, 0.0009; **46**, 3, 0.0005; **83**, 32, 0.0037; **105**, 3, 0.0063
21	8	Nonfat	0.0030	**Baby food**, mixed vegetables: **10**, 2, 0.0075; **35**, 9, 0.0051; **40**, 2, 0.0006; **51**, 1, 0.0050; **54**, 1, 0.0004; **83**, 2, 0.0040; **84**, 3, 0.0008; **99**, 1, 0.0010

Table 2. *(continued)*

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
36	11	Nonfat	0.0110	**Baby food**, oatmeal, applesauce, or banana: **36**, 4, 0.0013; **37**, 1, 0.0009; **60**, 1, 0.0020; **61**, 1, 0.0030; **62**, 1, 0.0020; **83**, 20, 0.0031; **84**, 1, 0.0010; **99**, 2, 0.0025; **113**, 2, 0.0035; **120**, 2, 0.0235; **128**, 1, 0.0780
50	6	Nonfat	0.0038	**Baby food**, orange or orange–pineapple juice: **32**, 19, 0.0063; **61**, 1, 0.0007; **65**, 27, 0.0032; **66**, 1, 0.0030; **86**, 1, 0.0090; **99**, 1, 0.0007
66	11	Nonfat	0.0269	**Baby food**, peaches: **7**, 1, 0.0008; **36**, 5, 0.0020; **40**, 6, 0.0012; **51**, 12, 0.0418; **53**, 1, 0.0070; **60**, **5**, 0.0019; **61**, 5, 0.0053; **62**, 4, 0.0078; **99**, 13, 0.0022; **113**, 10, 0.0505; **120**, 4, 0.1760
121	17	Nonfat	0.0089	**Baby food**, pears or pears w/ pineapple: **36**, 2, 0.0010; **38**, 1, 0.0020; **39**, 1, 0.0008; **40**, 7, 0.0009; **52**, 1, 0.0020; **53**, 4, 0.0060; **60**, 16, 0.0019; **61**, 25, 0.0054; **62**, 25, 0.0063; **65**, 4, 0.0018; **83**, 1, 0.0020; **99**, 6, 0.0017; **101**, 1, 0.0020; **108**, 1, 0.0240; **109**, 1, 0.0170; **113**, 21, 0.0326; **128**, 4, 0.0443
2	2	Nonfat	0.0083	**Baby food**, peas: **38**, 1, 0.0006; **132**, 1, 0.0160
76	10	Fat	0.0013	**Baby food**, pork: **40**, 23, 0.0022; **54**, 4, 0.0011; **75**, 3, 0.0005; **76**, 9, 0.0004; **95**, 1, 0.0006; **96**, 5, 0.0004; **103**, 1, 0.0001; **105**, 10, 0.0065; **106**, 2, 0.0002; **124**, 18, 0.0016
54	8	Nonfat	0.0150	**Baby food**, prunes or plums w/ tapioca: **40**, 6, 0.0017; **51**, 3, 0.0133; **52**, 3, 0.0030; **53**, 17, 0.0161; **62**, 2, 0.0035; **99**, 6, 0.0024; **113**, 9, 0.0069; **120**, 8, 0.0728
4	4	Nonfat	0.0044	**Baby food**, pudding or custard: **46**, 1, 0.0003; **53**, 1, 0.0090; **81**, 1, 0.0004; **113**, 1, 0.0080
46	9	Nonfat	0.0881	**Baby food**, spinach, creamed: **36**, 2, 0.0010; **40**, 21, 0.0017; **64**, 2, 0.0070; **67**, 1, 0.0640; **84**, 2, 0.0070; **102**, 1, 0.0007; **105**, 1, 0.0050; **108**, 8, 0.3531; **109**, 8, 0.3533
37	4	Nonfat	0.0046	**Baby food**, sweet potato: **14**, 1, 0.0010; **51**, 29, 0.0101; **54**, 6, 0.0051; **102**, 1, 0.0020
15	8	Nonfat	0.0031	**Baby food**, tomato, beef & macaroni: **36**, 6, 0.0010; **40**, 2, 0.0010; **46**, 1, 0.0010; **51**, 1, 0.0100; **83**, 1, 0.0010; **84**, 2, 0.0010; **124**, 1, 0.0010; **132**, 1, 0.0090
18	5	Nonfat	0.0079	**Baby food**, turkey and rice: **10**, 3, 0.0263; **12**, 1, 0.0030; **35**, 3, 0.0060; **36**, 9, 0.0028; **40**, 2, 0.0012
21	6	Nonfat	0.0018	**Baby food**, vegetables w/ bacon or ham: **35**, 5, 0.0046; **36**, 6, 0.0021; **46**, 2, 0.0004; **81**, 1, 0.0004; **83**, 6, 0.0029; **99**, 1, 0.0007
21	6	Nonfat	0.0146	**Baby food**, vegetables w/ turkey or chicken: **10**, 3, 0.0610; **12**, 1, 0.0100; **35**, 7, 0.0050; **36**, 8, 0.0028; **125**, 1, 0.0009; **132**, 1, 0.0080
33	11	Nonfat	0.0028	**Baby food**, vegetables with beef: **10**, 1, 0.0130; **20**, 1, 0.0010; **28**, 1, 0.0030; **35**, 9, 0.0051; **36**, 5, 0.0007; **40**, 10, 0.0008; **46**, 1, 0.0009; **54**, 1, 0.0003; **83**, 2, 0.0035; **84**, 1, 0.0020; **124**, 1, 0.0007
8	1	Nonfat	0.0879	**Banana**, raw: **128**, 8, 0.0879
99	22	Nonfat	0.0249	**Beans**, green, boiled: **14**, 12, 0.0547; **20**, 1, 0.0009; **25**, 3, 0.0283; **36**, 1, 0.0003; **40**, 6, 0.0019; **45**, 1, 0.0030; **46**, 6, 0.0021; **51**, 6, 0.0818; **53**, 1, 0.0260; **55**, 2, 0.0015; **56**, 1, 0.2760; **60**, 10, 0.0038; **61**, 9, 0.0021; **62**, 12, 0.0195; **84**, 15, 0.0271; **97**, 1, 0.0020; **99**, 3, 0.0063; **101**, 1, 0.0070; **102**, 2, 0.0007; **107**, 1, 0.0002; **121**, 1, 0.0003; **138**, 4, 0.0023
58	14	Nonfat	0.0140	**Beans**, green, canned: **14**, 18, 0.0127; **45**, 1, 0.0010; **51**, 1, 0.0007; **54**, 2, 0.0015; **55**, 3, 0.0070; **56**, 1, 0.1060; **60**, 1, 0.0010; **62**, 1, 0.0030; **70**, 1, 0.0410; **75**, 1, 0.0007; **81**, 1, 0.0005; **84**, 23, 0.0084; **99**, 1, 0.0050; **138**, 3, 0.0073
102	10	Nonfat	0.0057	**Beans**, lima, immature, boiled: **14**, 28, 0.0170; **38**, 5, 0.0013; **40**, 2, 0.0015; **46**, 1, 0.0020; **53**, 14, 0.0071; **55**, 8, 0.0104; **81**, 7, 0.0004; **83**, 1, 0.0040;**84**, 33, 0.0071; **97**, 3, 0.0060
56	11	Nonfat	0.0021	**Beans**, lima, mature, boiled: **36**, 4, 0.0075; **38**, 9, 0.0033; **46**, 3, 0.0013; **55**, 1, 0.0020; **81**, 20, 0.0006; **83**, 1, 0.0010; **84**, 14, 0.0012; **102**, **1**, 0.0004; **105**, 1, 0.0040; **121**, 1, 0.0007; **125**, 1, 0.0009
13	5	Nonfat	0.0008	**Beans**, navy, boiled: **20**, 1, 0.0005; **36**, 2, 0.0008; **46**, 6, 0.0018; **54**, 2, 0.0009; **81**, 2, 0.0003
5	2	Nonfat	0.0008	**Beans**, pinto, boiled: **20**, 1, 0.0008; **46**, 4, 0.0008

Table 2. ***(continued)***

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
12	8	Nonfat	0.0024	**Beans**, red, boiled: **14**, 1, 0.0007; **20**, 1, 0.0002; **46**, 3, 0.0025; **54**, 1, 0.0004; **62**, 2, 0.0015; **81**, 2, 0.0003; **102**, 1, 0.0130; **107**, 1, 0.0006
99	14	Fat	0.0304	**Beef**, chuck roast, roasted: **20**, 8, 0.0004; **36**, 1, 0.0006; **40**, 25, 0.0042; **46**, 3, 0.0015; **54**, 18, 0.0013; **56**, 2, 0.1605; 75, 9, 0.0006; **76**, 19, 0.0007; **96**, 8, 0.0005; **105**, 1, 0.0050; **106**, 2, 0.0004; **117**, 1, 0.0100; **124**, 1, 0.0030; **135**, 1, 0.2370
149	14	Fat	0.0114	**Beef**, ground, fried: **20**, 17, 0.0006; **36**, 2, 0.0007; **40**, 31, 0.0056; **46**, 5, 0.0006; **51**, 1, 0.0050; **54**, 22, 0.0011; **56**, 5, 0.1334; **75**, 18, 0.0007; **76**, 25, 0.0006; **81**, 3, 0.0010; **96**, 16, 0.0007; **105**, 2, 0.0045; **106**, 1, 0.0002; **124**, 1, 0.0050
84	13	Fat	0.0101	**Beef**, loin or sirloin, cooked: **20**, 7, 0.0015; **21**, 1, 0.0050; **36**, 1, 0.0002; **40**, 18, 0.0059; **46**, 4, 0.0010; **54**, 14, 0.0009; **75**, 11, 0.0006; **76**, 15, 0.0007; **83**, 1, 0.0030; **96**, 8, 0.0004; **105**, 1, 0.0080; **106**, 2, 0.0005; **135**, 1, 0.1030
73	13	Fat	0.0021	**Beef**, round steak, stewed: **20**, 6, 0.0005; **36**, 1, 0.0010; **40**, 19, 0.0033; **46**, 6, 0.0007; **54**, 6, 0.0008; **75**, 6, 0.0005; **76**, 12, 0.0004; **81**, 1, 0.0030; **96**, 9, 0.0005; **105**, 3, 0.0043; **106**, 1, 0.0001; **117**, 2, 0.0110; **124**, 1, 0.0009
1	1	Nonfat	0.0005	**Beer**, canned: **51**, 1, 0.0005
7	6	Nonfat	0.0386	**Beets**, canned: **14**, 1, 0.0010; **40**, 2, 0.0013; **54**, 1, 0.0004; **80**, 1, 0.2200; **102**, 1, 0.0007; **132**, 1, 0.0080
102	12	Fat	0.2361	**Biscuits**, baking powder: **36**, 10, 0.0009; **37**, 19, 0.0052; **40**, 3, 0.0017; **46**, 17, 0.0016; **48**, 4, 0.0950; **49**, 6, 2.6850; **54**, 1, 0.0003; **81**, 2, 0.0012; **83**, 35, 0.0100; **105**, 3, 0.0070; **116**, 1, 0.0010; **117**, 1, 0.0240
164	14	Fat	0.0084	**Bologna**: **10**, 9, 0.0666; **12**, 4, 0.0135; **20**, 14, 0.0007; **40**, 30, 0.0036; **42**, 2, 0.0060; **46**, 1, 0.0050; **54**, 23, 0.0013; **75**, 23, 0.0012; **76**, 18, 0.0010; **81**, 18, 0.0015; **96**, 16, 0.0008; **105**, 4, 0.0083; **117**, 1, 0.0080; **124**, 1, 0.0007
2	1	Nonfat	0.0060	**Bouillon**, beef: **105**, 2, 0.0060
116	19	Fat	0.0364	**Bread**, rye: **10**, 7, 0.0776; **12**, 2, 0.0060; **20**, 1, 0.0005; **23**, 3, 0.0020; **36**, 15, 0.0009; **37**, 18, 0.0175; **46**, 13, 0.0019; **48**, 1, 0.0300; **49**, 1, 0.4200; **50**, 1, 0.0100; **51**, 1, 0.0080; **68**, 9, 0.0037; **81**, 1, 0.0010; **83**, 36, 0.0573; **99**, 1, 0.0030; **105**, 1, 0.0060; **116**, 3, 0.0013; **132**, 1, 0.0090; **135**, 1, 0.0360
105	15	Fat	0.0184	**Bread**, white, enriched: **17**, 1, 0.0020; **34**, 1, 0.1300; **35**, 3, 0.0063; **36**, 13, 0.0019; **37**, 18, 0.0074; **46**, 21, 0.0027; **51**, 3, 0.0023; **56**, 2, 0.0580; **83**, 36, 0.0319; **88**, 1, 0.0060; **99**, 1, 0.0008; **105**, 1, 0.0040; **116**, 1, 0.0030; **132**, 2, 0.0070; **134**, 1, 0.0120
115	16	Fat	0.0129	**Bread**, whole wheat: **30**, 1, 0.0008; **36**, 13, 0.0021; **37**, 19, 0.0154; **46**, 16, 0.0030; **51**, 1, 0.0020; **56**, 1, 0.0150; **68**, 13, 0.0122; **81**, 1, 0.0003; **83**, 37, 0.1052; **88**, 1, 0.0020; **99**, 2, 0.0025; **105**, 3, 0.0113; **116**, 1, 0.0040; **131**, 1, 0.0040; **132**, 4, 0.0095; **135**, 1, 0.0170
90	20	Nonfat	0.0070	**Broccoli**, boiled: **13**, 1, 0.0150; **16**, 1, 0.0250; **20**, 1, 0.0010; **26**, 3, 0.0153; **36**, 4, 0.0014; **38**, 28, 0.0078; **40**, 19, 0.0017; **46**, 7, 0.0027; **53**, 2, 0.0065; **54**, 6, 0.0015; **55**, 1, 0.0310; **60**, 4, 0.0010; **61**, 2, 0.0006; **62**, 3, 0.0012; **63**, 3, 0.0011; **81**, 1, 0.0006; **97**, 1, 0.0100; **102**, 1, 0.0003; **108**, 1, 0.0090; **109**, 1, 0.0070
220	19	Fat	0.0462	**Butter**, stick type: **20**, 32, 0.0051; **40**, 34, 0.0185; **42**, 1, 0.0060; **54**, 33, 0.0061; **56**, 11, 0.7568; **62**, 1, 0.0050; **75**, 26, 0.0034; **76**, 34, 0.0021; **81**, 6, 0.0006; **83**, 1, 0.0040; **88**, 1, 0.0050; **88**, 1, 0.0080; **95**, 1, 0.0010; **96**, 25, 0.0022; **105**, 9, 0.0097; **106**, 1, 0.0003; **116**, 1, 0.0170; **117**, 1, 0.0240; **124**, 1, 0.0020
6	4	Fat	0.0034	**Buttermilk**: **40**, 3, 0.0008; **54**, 1, 0.0004; **75**, 1, 0.0002; **105**, 1, 0.0120
16	6	Nonfat	0.0041	**Cabbage**, boiled from raw: **45**, 1, 0.0160; **46**, 5, 0.0028; **51**, 1, 0.0006; **54**, 2, 0.0007; **81**, 1, 0.0009; **84**, 6, 0.0035
106	12	Fat	1.220	**Cake**, chocolate w/ chocolate icing: **20**, 13, 0.0006; **36**, 12, 0.0015; **37**, 6, 0.0018; **40**, 3, 0.0006; **46**, 14, 0.0016; **48**, 4, 0.8675; **49**, 5, 13.76; **81**, 11, 0.0006; **83**, 32, 0.0030; **105**, 4, 0.0048; **106**, 1, 0.0004; **116**, 1, 0.0010
78	10	Fat	1.328	**Cake**, yellow: **36**, 5, 0.0005; **37**, 7, 0.0014; **40**, 6, 0.0016; **46**, 8, 0.0012; **48**, 6, 0.8633; **49**, 7, 11.83; **54**, 1, 0.0008; **56**, 1, 0.5700; **83**, 34, 0.0066; **105**, 3, 0.0067

Table 2. *(continued)*

o. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
77	17	Fat	0.2488	**Candy**, caramels: **20**, 3, 0.0015; **36**, 2, 0.0007; **40**, 1, 0.0004; **46**, 1, 0.0005; **54**, 2, 0.0007; **56**, 35, 3.876; **76**, 1, 0.0010; **81**, 3, 0.0007; **83**, 2, 0.0065; **102**, 1, 0.0020; **103**, 1, 0.0010; **105**, 10, 0.0076; **106**, 1, 0.0008; **107**, 1, 0.0010; **121**, 1, 0.0007; **134**, 11, 0.2540; **135**, 1, 0.0740
31	18	Fat	0.0224	**Candy**, milk chocolate: **17**, 1, 0.0009; **20**, 37, 0.0112; **21**, 1, 0.0030; **22**, 1, 0.0030; **36**, 6, 0.0015; **40**, 22, 0.0024; **42**, 8, 0.0027; **46**, 8, 0.0008; **54**, 9, 0.0010; **75**, 9, 0.0009; **76**, 2, 0.0005; **81**, 37, 0.0098; **83**, 6, 0.0032; **96**, 2, 0.0006; **105**, 11, 0.0082; **106**, 7, 0.0011; **124**, 13, 0.0029; **135**, 1, 0.3500
10	21	Nonfat	0.0135	**Cantaloupe**, raw: **14**, 1, 0.0040; **20**, 2, 0.0007; **25**, 6, 0.0182; **28**, 1, 0.0040; **36**, 2, 0.0015; **38**, 7, 0.0026; **51**, 1, 0.0030; **53**, 5, 0.0098; **54**, 14, 0.0040; **55**, 3, 0.0023; **60**, 4, 0.0009; **61**, 3, 0.0009; **62**, 15, 0.0188; **63**, 4, 0.0018; **65**, 1, 0.0010; **81**, 1, 0.0008; **84**, 15, 0.0565; **87**, 3, 0.0247; **97**, 3, 0.0747; **102**, 1, 0.0007; **129**, 18, 0.0521
64	12	Nonfat	0.0108	**Carrots**, raw: **7**, 2, 0.0055; **35**, 1, 0.0300; **40**, 17, 0.0034; **46**, 5, 0.0065; **51**, 7, 0.0311; **54**, 2, 0.0007; **62**, 2, 0.0010; **82**, 21, 0.0179; **84**, 1, 0.0010; **99**, 2, 0.0007; **102**, 1, 0.0007; **129**, 3, 0.0313
01	19	Nonfat	0.0102	**Catsup: 10**, 4, 0.0210; **12**, 4, 0.0045; **14**, 5, 0.0042; **36**, 1, 0.0010; **38**, 1, 0.0008; **40**, 10, 0.0013; **51**, 1, 0.0010; **53**, 2, 0.0023; **60**, 19, 0.0020; **61**, 20, 0.0041; **62**, 8, 0.0011; **65**, 1, 0.0020; **84**, 17, 0.0087; **99**, 1, 0.0007; **105**, 3, 0.0047; **121**, 1, 0.0030; **124**, 1, 0.0010; **130**, 1, 0.1200; **137**, 1, 0.0100
14	8	Nonfat	0.0040	**Cauliflower**, boiled: **14**, 1, 0.0140; **38**, 4, 0.0006; **40**, 1, 0.0005; **46**, 2, 0.0020; **54**, 1, 0.0002; **84**, 3, 0.0029; **87**, 1, 0.0060; **115**, 1, 0.0060
94	22	Nonfat	0.0114	**Celery**, raw: **7**, 12, 0.0022; **14**, 20, 0.0462; **33**, 4, 0.0105; **35**, 1, 0.0020; **38**, 4, 0.0055; **40**, 26, 0.0025; **42**, 9, 0.0019; **46**, 13, 0.0044; **51**, 27, 0.0469; **54**, 1, 0.0009; **59**, 1, 0.0080; **60**, 10, 0.0062; **61**, 9, 0.0052; **62**, 8, 0.0084; **69**, 1, 0.0280; **84**, 21, 0.0045; **87**, 2, 0.0260; **99**, 3, 0.0077; **101**, 3, 0.0083; **104**, 1, 0.0020; **108**, 9, 0.0127; **109**, 9, 0.0114
11	5	Nonfat	0.0229	**Cereal**, cornflakes: **46**, 2, 0.0017; **56**, 1, 0.0820; **83**, 1, 0.0020; **105**, 1, 0.0090; **132**, 6, 0.0198
59	11	Nonfat	0.0164	**Cereal**, fruit-flavored: **32**, 2, 0.0090; **36**, 2, 0.0030; **46**, 1, 0.0004; **53**, 2, 0.0040; **56**, 2, 0.1125; **65**, 9, 0.0033; **83**, 30, 0.0065; **85**, 1, 0.0030; **88**, 1, 0.0050; **88**, 6, 0.0123; **132**, 3, 0.0210
7	5	Nonfat	0.0044	**Cereal**, crisped rice: **5**, 2, 0.0040; **35**, 1, 0.0070; **46**, 1, 0.0004; **83**, 1, 0.0010; **132**, 2, 0.0095
17	5	Nonfat	0.0088	**Cereal**, oat ring: **46**, 2, 0.0015; **51**, 1, 0.0080; **83**, 11, 0.0067; **105**, 1, 0.0070; **132**, 2, 0.0210
93	21	Nonfat	0.0309	**Cereal**, raisin bran: **24**, 1, 0.0200; **37**, 7, 0.0060; **40**, 8, 0.0011; **46**, 2, 0.0004; **51**, 3, 0.0033; **52**, 2, 0.0060; **53**, 11, 0.0248; **55**, 6, 0.0042; **56**, 1, 0.2430; **60**, 1, 0.0009; **61**, 6, 0.0023; **62**, 2, 0.0025; **65**, 1, 0.0150; **83**, 26, 0.0112; **88**, 1, 0.0190; **105**, 2, 0.0040; **113**, 3, 0.0283; **116**, 1, 0.0030; **120**, 6, 0.1237; **123**, 2, 0.1225; **132**, 1, 0.0070
64	11	Nonfat	0.0200	**Cereal**, shredded wheat: **36**, 4, 0.0017; **37**, 6, 0.0137; **46**, 4, 0.0011; **83**, 35, 0.0471; **88**, 3, 0.0110; **88**, 3, 0.0097; **99**, 1, 0.0004; **105**, 1, 0.0050; **116**, 1, 0.0520; **117**, 1, 0.0640; **132**, 5, 0.0142
83	11	Fat	0.0309	**Cheese**, American: **20**, 28, 0.0020; **40**, 32, 0.0025; **54**, 32, 0.0021; **75**, 28, 0.0013; **76**, 30, 0.0008; **81**, 3, 0.0005; **88**, 1, 0.0040; **96**, 17, 0.0009; **103**, 1, 0.0009; **105**, 10, 0.0086; **135**, 1, 0.3160
80	14	Fat	0.0580	**Cheese**, cheddar, sharp or mild: **20**, 29, 0.0028; **40**, 27, 0.0045; **54**, 32, 0.0022; **56**, 2, 0.4905; **75**, 29, 0.0013; **76**, 27, 0.0010; **81**, 2, 0.0050; **88**, 1, 0.0030; **88**, 1, 0.0070; **96**, 17, 0.0010; **105**, 10, 0.0129; **106**, 1, 0.0001; **117**, 1, 0.0200; **135**, 1, 0.2600
44	7	Fat	0.0016	**Cheese**, cottage, 4%: **20**, 9, 0.0003; **40**, 12, 0.0023; **54**, 4, 0.0010; **75**, 2, 0.0003; **76**, 8, 0.0003; **83**, 1, 0.0006; **105**, 8, 0.0061
69	32	Nonfat	0.2983	**Cherries**, sweet, raw: **7**, 19, 0.0041; **13**, 1, 0.0470; **14**, 2, 0.0215; **19**, 1, 0.0150; **20**, 1, 0.0010; **24**, 12, 0.0873; **25**, 3, 0.0457; **36**, 1, 0.0020; **40**, 2, 0.0007; **46**, 6, 0.0048; **51**, 28, 0.2772; **53**, 1, 0.1000; **55**, 1, 0.0580; **56**, 1, 7.290; **60**, 10, 0.0072; **61**, 14, 0.0130; **62**, 12, 0.0117; **65**, 1, 0.0010; **77**, 13, 0.1946; **78**, 5, 0.0156; **83**, 8, 0.0034; **84**, 1, 0.0005; **86**, 1, 0.0160; **97**, 1, 0.3680; **99**, 10, 0.0219; **100**, 2, 0.0100; **110**, 5, 0.1736; **113**, 2, 0.0125; **114**, 1, 0.0040; **123**, 2, 0.0250; **134**, 1, 0.2080; **135**, 1, 0.5050

Table 2. *(continued)*

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
130	19	Fat	0.0114	**Chicken noodle casserole**: **20**, 7, 0.0004; **35**, 1, 0.0030; **36**, 10, 0.0016; **37**, 7, 0.0016; **38**, 1, 0.0010; **40**, 14, 0.0012; **46**, 17, 0.0019; **49**, 1, 0.0400; **51**, 18, 0.0078; **54**, 6, 0.0009; **56**, 1, 0.1440; **60**, 2, 0.0008; **75**, 2, 0.0004; **76**, 4, 0.0002; **83**, 30, 0.0037; **96**, 1, 0.0002; **99**, 2, 0.0009; **101**, 1, 0.0009; **105**, 5, 0.0060
81	17	Fat	0.0364	**Chicken**, drum and breast, fried: **20**, 2, 0.0006; **36**, 3, 0.0004; **37**, 1, 0.0003; **40**, 15, 0.0020; **46**, 8, 0.0006; **48**, 3, 0.0233; **49**, 4, 0.5800; **51**, 1, 0.0030; **54**, 10, 0.0010; **75**, 5, 0.0003; **76**, 9, 0.0002; **81**, 1, 0.0010; **83**, 11, 0.0011; **96**, 1, 0.0003; **103**, 1, 0.0001; **105**, 5, 0.0050; **106**, 1, 0.0002
68	12	Fat	0.0020	**Chicken**, whole, roasted: **20**, 1, 0.0003; **40**, 16, 0.0028; **46**, 4, 0.0027; **51**, 2, 0.0014; **54**, 12, 0.0013; **75**, 8, 0.0005; **76**, 7, 0.0003; **81**, 2, 0.0017; **96**, 3, 0.0004; **103**, 1, 0.0001; **105**, 10, 0.0126; **106**, 2, 0.0003
176	19	Fat	0.0683	**Chill con carne**, beef: **8**, 4, 0.0033; **9**, 9, 0.0048; **10**, 33, 1.042; **11**, 23, 0.0161; **12**, 32, 0.2038; **20**, 9, 0.0012; **35**, 1, 0.0040; **36**, 3, 0.0006; **38**, 2, 0.0030; **40**, 29, 0.0022; **46**, 3, 0.0003; **54**, 3, 0.0009; **75**, 4, 0.0006; **76**, 6, 0.0003; **81**, 1, 0.0003; **83**, 6, 0.0038; **96**, 3, 0.0004; **105**, 2, 0.0100; **124**, 3, 0.0013
59	12	Fat	0.0094	**Chocolate powder**, sweetened: **20**, 19, 0.0007; **35**, 1, 0.0200; **36**, 4, 0.0028; **46**, 4, 0.0004; **54**, 1, 0.0020; **56**, 1, 0.0660; **81**, 19,0.0008; **83**, 1, 0.0020; **96**, 1, 0.0004; **105**, 1, 0.0160; **106**, 5, 0.0002; **116**, 2, 0.0020
70	17	Fat	0.0030	**Chow mein**, pork: 7, 2, 0.0007; **35**, 1, 0.0020; **36**, 3, 0.0013; **40**, 7, 0.0010; **42**, 1, 0.0020; **46**, 14, 0.0012; **51**, 24, 0.0158; **60**, 4, 0.0022; **61**, 1, 0.0020; **62**, 1, 0.0040; **81**, 2, 0.0009; **83**, 2, 0.0035; **99**, 1, 0.0030; **101**, 1, 0.0020; **105**, 2, 0.0065; **106**, 3, 0.0018; **124**, 1, 0.0007
1	1	Nonfat	0.0550	**Coffee**: **135**, 1, 0.0550
0	0	Nonfat	0.0000	**Coffee**, decaffeinated: No findings
143	14	Fat	0.5713	**Coffeecake**, ready to serve or frozen: **20**, 2, 0.0010; **36**, 23, 0.0028; **37**, 19, 0.0035; **40**, 4, 0.0008; **46**, 29, 0.0021; **48**, 6, 0.3933; **49**, 6, 7.567; **54**, 5, 0.0016;**75**, 4, 0.0007; **76**, 2, 0.0004; **81**, 1, 0.0005; **83**, 37, 0.0174; **96**, 2, 0.0006; **105**, 3, 0.0063
67	14	Fat	0.0065	**Coleslaw**, with dressing: **10**, 16, 0.0451; **12**, 12, 0.0115; **20**, 11, 0.0006; **35**, 1, 0.0050; **36**, 2, 0.0008; **38**, 1, 0.0020; **42**, 3, 0.0017; **46**, 6, 0.0024; **51**, 2, 0.0070;54, 2, 0.0007; **81**, 3, 0.0005; **83**, 2, 0.0004; **105**, 5, 0.0056; **123**, 1, 0.0070
211	39	Nonfat	0.0452	**Collards**, boiled: **14**, 3, 0.0803; **20**, 2, 0.0006; **25**, 1, 0.0170; **28**, 2, 0.0140; **29**, 2, 0.0006; **30**, 3, 0.0007; **36**, 7, 0.0184; **38**, 27, 0.0361; **39**, 3, 0.0033; **40**, 32, 0.0115; **42**, 3, 0.0025; **45**, 2, 0.1030; **46**, 12, 0.0124; **51**, 5, 0.0029; **54**, 12, 0.0028; **55**, 4, 0.0348; **60**, 6, 0.0395; **61**, 7, 0.0404; **62**, 9, 0.0678; **63**, 1, 0.0020;**70**, 4, 0.5225; **76**, 2, 0.0006; **81**, 1, 0.0002;**82**, 1, 0.0020; **83**, 1, 0.0470; **84**, 4, 0.0813;**87**, 1, 0.0700; **90**, 4, 0.0305; **91**, 6, 0.0315; **95**, 1, 0.0004; **97**, 4, 0.0318; **99**, 2, 0.0105; **102**, 5, 0.0018; **104**, 1, 0.0020; **108**, 13, 0.2150; **109**, 13, 0.2003; **121**, 1, 0.0006; **124**, 1, 0.0010; **129**, **3**, 0.0237
54	18	Fat	0.2240	**Cookies**, chocolate chip: **20**, 24, 0.0013; **36**, 11, 0.0036; **37**, 12, 0.0054; **40**, 5, 0.0012; **42**, 1, 0.0010; **46**, 8, 0.0045; **48**, 5, 0.2436; **49**, 7, 3.726; **54**, 2, 0.0010; **75**, 2, 0.0007; **76**, 2, 0.0004; **81**, 24, 0.0010; **83**, 37, 0.0297; **88**, 1, 0.0020; **105**, 5, 0.0076; **106**, 4, 0.0004; **116**, 2, 0.0020; **124**, 2, 0.0015
95	15	Fat	0.1770	**Cookies**, sandwich type: **20**, 2, 0.0003; **36**, 4, 0.0010; **37**, 12, 0.0094; **40**, 9, 0.0006; **46**, 12, 0.0012; **48**, 1, 0.2300; **49**, 2, 2.350; **54**, 3, 0.0009; **75**, 1, 0.0008; **76**, 1, 0.0002; **81**, 5, 0.0006; **83**, 37, 0.0301; **88**, 1, 0.0230; **105**, 4, 0.0060; **124**, 1, 0.0009
32	8	Fat	0.0032	**Cooking oil**, vegetable, corn: **20**, 1, 0.0007; **46**, 1, 0.0010; **54**, 3, 0.0050; **75**, 1, 0.0030; **76**, 1, 0.0004; **83**, 16, 0.0033; **103**, 1, 0.0020; **105**, 8, 0.0099
45	13	Fat	0.0432	**Corn chips**: **9**, 1, 0.0110; **10**, 2, 0.2330; **11**, **1**, 0.0290; **12**, 2, 0.0720; **28**, 1, 0.0070; **46**, 5, 0.0008; **56**, 1, 0.1000; **81**, 1, 0.0010; **83**, 21, 0.0234; **88**, 1, 0.0110; **105**, 4, 0.0080; **116**, 4, 0.0123; **129**, 1, 0.0530
33	6	Nonfat	0.0067	**Corn grits**, cooked: **36**, 1, 0.0020; **46**, 6, 0.0031; **81**, 1, 0.0004; **83**, 18, 0.0075; **116**, 2, 0.0025; **132**, 5, 0.0246
8	3	Nonfat	0.0016	**Corn**, boiled: **36**, 1, 0.0030; **46**, 6, 0.0016; **81**, 1, 0.0003

Table 2. *(continued)*

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
1	1	Nonfat	0.0020	**Corn**, canned: **46**, 1, 0.0020
1	1	Nonfat	0.0050	**Corn**, cream style: **105**, 1, 0.0050
90	10	Fat	0.6361	**Cornbread**, southern style: **36**, 3, 0.0005; **37**, 11, 0.0026; **40**, 4, 0.0027; **42**, 1, 0.0070; **46**, 13, 0.0008; **48**, 5, 0.1170; **49**, 8, 6.202; **83**, 37, 0.0133; **105**, 3, 0.0057; **116**, 5, 0.0088
81	14	Nonfat	0.0021	**Cowpeas**, boiled: **14**, 17, 0.0047; **40**, 1, 0.0007; **46**, 4, 0.0018; **54**, 1, 0.0010; **55**, 3, 0.0027; **60**, 1, 0.0008; **61**, 2, 0.0020; **62**, 4, 0.0048; **81**, 17, 0.0009; **83**, 2, 0.0020; **84**, 17, 0.0022; **99**, 4, 0.0038; **102**, 7, 0.0006; **121**, 1, 0.0010
86	10	Fat	0.1750	**Crackers**, saltine: **35**, 1, 0.0050; **36**, 7, 0.0011; **37**, 19, 0.0113; **46**, 10, 0.0018; **48**, 2, 0.0500; **49**, 4, 1.588; **83**, 37, 0.0771; **88**, 2, 0.0045; **105**, 2, 0.0090; **116**, 2, 0.0025
8	4	Fat	0.0028	**Cream substitute**: **36**, 1, 0.0002; **46**, 1, 0.0001; **83**, 1, 0.0007; **105**, 5, 0.0102
114	8	Fat	0.0015	**Cream**, half and half: **20**, 19, 0.0006; **40**, 26, 0.0041; **54**, 23, 0.0009; **75**, 13, 0.0007; **76**, 22, 0.0004; **81**, 2, 0.0005; **96**, 8, 0.0006; **105**, 1, 0.0040
191	33	Nonfat	0.0097	**Cucumber**, raw: **6**, 1, 0.0060; **14**, 7, 0.0110; **20**, 3, 0.0006; **21**, 2, 0.0019; **25**, 1, 0.0100; **28**, 6, 0.0052; **29**, 5, 0.0013; **30**, 6, 0.0012; **36**, 6, 0.0102; **38**, 2, 0.0050; **40**, 3, 0.0013; **46**, 1, 0.0060; **51**, 2, 0.0040; **54**, 26, 0.0047; **55**, 2, 0.0115; **60**, 24, 0.0056; **61**, 20, 0.0040; **62**, 21, 0.0137; **63**, 2, 0.0040; **65**, 2, 0.0115; **74**, 1, 0.0006; **75**, 8, 0.0032; **81**, 2, 0.0091; **84**, 20, 0.0608; **87**, 2, 0.0390; **91**, 1, 0.0020; **94**, 1, 0.0007; **95**, 3, 0.0030; **96**, 1, 0.0010; **97**, 2, 0.0070; **98**, 1, 0.0310; **125**, 1, 0.0010; **129**, 6, 0.0442
137	15	Fat	0.0574	**Donuts**, cake type, plain: **9**, 2, 0.0095; **10**, 7, 0.0643; **11**, 11, 0.0347; **12**, 7, 0.0249; **36**, 21, 0.0012; **37**, 16, 0.0030; **40**, 9, 0.0037; **46**, 16, 0.0024; **48**, 1, 0.1100; **49**, 2, 0.5500; **65**, 1, 0.0009; **83**, 36, 0.0267; **101**, 1, 0.0040; **105**, 6, 0.0085; **138**, 1, 0.0170
33	8	Fat	0.0016	**Eggs**, fried: **40**, 13, 0.0021; **46**, 2, 0.0007; **54**, 3, 0.0008; **75**, 1, 0.0003; **76**, 6, 0.0004; **81**, 1, 0.0002; **95**, 1, 0.0010; **105**, 6, 0.0073
29	11	Fat	0.0243	**Eggs**, scrambled: **40**, 11, 0.0027; **46**, 2, 0.0007; **54**, 1, 0.0007; **56**, 1, 0.2500; **75**, 1, 0.0005; **76**, 5, 0.0002; **81**, 1, 0.0002; **83**, 2, 0.0015; **95**, 1, 0.0010; **105**, 3, 0.0077; **133**, 1, 0.0020
22	9	Nonfat	0.0057	**Eggs**, soft boiled: **28**, 1, 0.0390; **36**, 1, 0.0010; **40**, 8, 0.0031; **46**, 1, 0.0004; **54**, 6, 0.0007; **75**, 1, 0.0010; **76**, 1, 0.0003; **95**, 1, 0.0010; **105**, 2, 0.0045
34	3	Nonfat	0.0074	**Farina**, cooked: **46**, 6, 0.0015; **83**, 14, 0.0024; **132**, 14, 0.0184
100	16	Fat	0.3269	**Fish sticks**, cooked: **20**, 4, 0.0002; **35**, 3, 0.0063; **36**, 5, 0.0016; **37**, 12, 0.0026; **40**, 1, 0.0030; **46**, 15, 0.0018; **48**, 4, 0.3825; **49**, 7, 4.771; **51**, 1, 0.0250; **68**, 1, 0.0030; **76**, 6, 0.0003; **81**, 1, 0.0004; **83**, 36, 0.0085; **105**, 2, 0.0140; **107**, 1, 0.0006; **117**, 1, 0.0090
53	11	Nonfat	0.3308	**Fish**, cod or haddock, cooked: **20**, 15, 0.0003; **35**, 1, 3.610; **40**, 5, 0.0070; **46**, 4, 0.0012; **76**, 19, 0.0004; **83**, 2, 0.0013; **95**, 1, 0.0010; **96**, 1, 0.0004; **105**, 2, 0.0055; **106**, 1, 0.0003; **117**, 2, 0.0115
42	9	Fat	0.0019	**Fish**, tuna, canned in oil: **20**, 1, 0.0002; **38**, 1, 0.0020; **40**, 20, 0.0025; **46**, 2, 0.0018; **54**, 2, 0.0006; **76**, 3, 0.0002; **95**, 2, 0.0020; **105**, 5, 0.0068; **124**, 6, 0.0015
206	20	Fat	0.0359	**Frankfurters**, cooked: **10**, 18, 0.5659; **11**, 4, 0.0083; **12**, 14, 0.0922; **20**, 18, 0.0011; **36**, 1, 0.0007; **40**, 31, 0.0054; **42**, 3, 0.0107; **46**, 1, 0.0004; **54**, 28, 0.0018; **65**, 1, 0.0020; **75**, 23, 0.0012; **76**, 23, 0.0011; **81**, 13, 0.0011; **83**, 1, 0.0010; **96**, 18, 0.0008; **105**, 3, 0.0060; **106**, 1, 0.0005; **124**, 2, 0.0008; **125**, 2, 0.0012; **129**, 1, 0.0160
102	14	Fat	0.0174	**Frozen dinner**, fried chicken: **10**, 11, 0.0449; **11**, 2, 0.0035; **12**, 5, 0.0108; **35**, 16, 0.0122; **36**, 5, 0.0006; **37**, 5, 0.0007; **40**, 5, 0.0006; **46**, 11, 0.0007; **51**, 2, 0.0015; **54**, 2, 0.0006; **83**, 34, 0.0029; **96**, 1, 0.0002; **105**, 2, 0.0050; **135**, 1, 0.1590
34	9	Nonfat	0.0045	**Fruit cocktail**: **25**, 23, 0.0157; **40**, 1, 0.0004; **53**, 1, 0.0030; **55**, 2, 0.0015; **60**, 1, 0.0010; **61**, 3, 0.0021; **65**, 1, 0.0020; **97**, 1, 0.0010; **120**, 1, 0.0140

Table 2. *(continued)*

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
7	3	Nonfat	0.0065	**Gelatin**, strawberry: **36**, 1, 0.0007; **105**, 4, 0.0063; **132**, 2, 0.0125
71	12	Fat	0.0189	**Granola**, plain: **30**, 1, 0.0020; **31**, 1, 0.0007; **36**, 9, 0.0059; **37**, 11, 0.0078; **46**, 7, 0.0012; **56**, 1, 0.1340; **68**, 1, 0.0020; **74**, 1, 0.0050; **83**, 35, 0.0526; **99**, 2, 0.0095; **116**, 1, 0.0040; **125**, 1, 0.0020
32	6	Nonfat	0.0287	**Grapefruit**, raw: **32**, 1, 0.0010; **51**, 1, 0.0006, **53**, 2, 0.0095; **65**, 17, 0.0032; **83**, 1, 0.0020; **128**, 10, 0.1558
52	32	Nonfat	0.0585	**Grapes**, purple or green: **7**, 2, 0.0020; **19**, 1, 0.0290; **20**, 2, 0.0006; **24**, 12, 0.1413; **25**, 2, 0.0525; **36**, 3, 0.0020; **40**, 12, 0.0015; **41**, 1, 0.0010; **42**, 2, 0.0060; **43**, 1, 0.0030; **46**, 2, 0.0025; **51**, 8, 0.0699; **52**, 5, 0.0052; **53**, 8, 0.0591; **55**, 16, 0.0097; **60**, 5, 0.0028; **61**, 7, 0.0069; **62**, 7, 0.0083; **71**, 2, 0.0105; **77**, 7, 0.0483; **78**, 2, 0.1050; **84**, 3, 0.0023; **87**, 9, 0.1008; **91**, 1, 0.0020; **97**, 16, 0.0255; **99**, 2, 0.0023; **113**, 1, 0.0920; **118**, 1, 0.0160; **120**, 3, 0.0990; **123**, 2, 0.9215; **124**, 1, 0.0007; **138**, 6, 0.0432
38	7	Nonfat	0.0035	**Gravy**, brown: **10**, 2, 0.0105; **37**, 3, 0.0013; **46**, 5, 0.0010; **60**, 1, 0.0040; **83**, 21, 0.0025; **105**, 5, 0.0044; **125**, 1, 0.0008
16	22	Fat	0.0021	**Hamburger**, ¼-lb: **20**, 15, 0.0007; **29**, 1, 0.0009; **30**, 1, 0.0006; **35**, 1, 0.0060; **36**, 19, 0.0009; **37**, 16, 0.0024; **38**, 1, 0.0010; **40**, 31, 0.0033; **46**, 20, 0.0009; **51**, 1, 0.0050; **54**, 20, 0.0014; **60**, 4, 0.0007; **61**, 4, 0.0012; **62**, 2, 0.0020; **68**, 3, 0.0016; **75**, 9, 0.0010; **76**, 14, 0.0004; **81**, 5, 0.0010; **83**, 37,0.0097; **96**, 10, 0.0007; **99**, 1, 0.0006; **105**, 1, 0.0040
9	4	Nonfat	0.0225	**Honey**: **20**, 1, 0.0003; **46**, 1, 0.0030; **105**, 6, 0.0067; **135**, 1, 0.0800
42	18	Fat	0.3088	**Ice cream sandwich**: **20**, 13, 0.0005; **36**, 1, 0.0006; **37**, 9, 0.0040; **40**, 25, 0.0020; **46**, 2, 0.0006; **48**, 7, 0.2657; **49**, **7**, 5.234; **54**, 11, 0.0011; **60**, 1, 0.0020; **68**, 2, 0.0020; **75**, 5, 0.0005; **76**, 8, 0.0003; **81**, 3, 0.0003; **83**, 36, 0.0238; **88**, 1, 0.0050; **96**, 5, 0.0005; **99**, 3, 0.0053; **105**, 3, 0.0097
11	14	Fat	0.0013	**Ice cream**, chocolate: **20**, 16, 0.0009; **36**, 3, 0.0004; **40**, 27, 0.0040; **42**, 1, 0.0007; **46**, 1, 0.0006; **54**, 15, 0.0008; **75**, 10, 0.0004; **76**, 15, 0.0004; **81**, 10, 0.0006; **83**, 2, 0.0028; **96**, 6, 0.0004; **99**, 1, 0.0005; **105**, 3, 0.0053; **116**, 1, 0.0010
50	9	Fat	0.0525	**Ice milk**, vanilla: **20**, 9, 0.0003; **40**, 16, 0.0021; **54**, 8, 0.0005; **75**, 4, 0.0004; **76**, 6, 0.0002; **83**, 2, 0.0035; **96**, 2, 0.0005; **105**, 2, 0.0065; **135**, 1, 0.4590
35	6	Nonfat	0.0047	**Jelly**, grape: **25**, 17, 0 0168; **51**, 3, 0.0011; **55**, 11, 0.0026; **65**, 1, 0.0005; **83**, 1, 0.0010; **105**, 2, 0.0060
43	6	Nonfat	0.0106	**Juice**, apple: **14**, 3, 0.0012; **25**, 18, 0.0155; **55**, 14, 0.0026; **84**, 2, 0.0008; **97**, 3, 0.0023; **128**, 3, 0.0410
62	4	Nonfat	0.0147	**Juice**, grape: **25**, 31, 0.0489; **55**, 21, 0.0033; **83**, 2, 0.0030; **97**, 8, 0.0036
57	5	Nonfat	0.0399	**Juice**, grapefruit: **32**, 15, 0.0043; **53**, 6, 0.0038; **65**, 33, 0.0026; **66**, 2, 0.0025; **130**, 1, 0.1860
77	7	Nonfat	0.0038	**Juice**, orange: **25**, 1, 0.0100; **32**, 21, 0.0054; **52**, 1, 0.0010; **53**, 18, 0.0042; **65**, 32, 0.0027; **66**, 3, 0.0020; **85**, 1, 0.0010
1	1	Nonfat	0.0050	**Juice**, pineapple: **105**, 1, 0.0050
6	4	Nonfat	0.0063	**Juice**, prune: **51**, 2, 0.0022; **83**, 1, 0.0050; **113**, 2, 0.0040; **120**, 1, 0.0140
72	13	Nonfat	0.0034	**Juice**, tomato: **14**, 3, 0.0013; **20**, 1, 0.0008; **25**, 13, 0.0132; **40**, 2, 0.0010; **53**, 1, 0.0130; **60**, 13, 0.0020; **61**, 15, 0.0022; **62**, 2, 0.0009; **65**, 2, 0.0040; **72**, 1, 0.0008; **84**, 16, 0.0023; **99**, 1, 0.0020; **104**, 2, 0.0005
03	14	Fat	0.0041	**Lamb chop**, cooked: **20**, 6, 0.0010; **36**, 1, 0.0006; **40**, 31, 0.0117; **42**, 1, 0.0020; **46**, 3, 0.0016; **54**, 5, 0.0007; **75**, 2, 0.0012; **76**, 27, 0.0008; **81**, 1, 0.0003; **96**, 19, 0.0009; **103**, 2, 0.0005; **105**, 3, 0.0243; **117**, 1, 0.0100; **124**, 1, 0.0020
70	15	Fat	0.0028	**Lasagna**: **10**, 3, 0.0143; **12**, 1, 0.0040; **20**, 7, 0.0004; **36**, 3, 0.0012; **40**, 21, 0.0020; **46**, 10, 0.0018; **51**, 1, 0.0030; **54**, 2, 0.0005; **60**, 2, 0.0012; **61**, 3, 0.0015; **76**, 4, 0.0003; **81**, 1, 0.0004; **83**, 8, 0.0017; **96**, 3, 0.0003; **105**, 1, 0.0090
17	8	Nonfat	0.0848	**Lemonade**: **25**, 2, 0.6100; **32**, 4, 0.0019; **53**, 3, 0.0016; **55**, 1, 0.0040; **65**, 3, 0.0013; **85**, 2, 0.0018; **97**, 1, 0.0030; **135**, 1, 0.0550
97	20	Nonfat	0.0515	**Lettuce**, raw: **14**, 15, 0.0050; **38**, 3, 0.0025; **40**, 2, 0.0010; **42**, 1, 0.0010; **45**, 2, 0.0170; **46**, 1, 0.0010; **54**, 1, 0.0006; **55**, 5, 0.0088; **59**, 1, 0.0040; **60**, 11, 0.0034; **61**, 12, 0.0024; **62**, 13, 0.0095; **84**, 13, 0.0018; **87**, 5, 0.0130; **91**, 5, 0.0063; **97**, 3, 0.0093; **102**, 1, 0.0010; **108**, 1, 0.4760; **109**, 1, 0.3720; **135**, 1, 0.0940

Table 2. *(continued)*

. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
6	12	Fat	0.0033	**Liver**, beef or calf, cooked: **20**, 3, 0.0003; **36**, 1, 0.0006; **40**, 12, 0.0020; **46**, 6, 0.0009; **54**, 11, 0.0011; **75**, 7, 0.0008; **76**, 8, 0.0003; **96**, 8, 0.0014; **105**, 15, 0.0265; **106**, 2, 0.0004; **122**, 1, 0.0050; **124**, 2, 0.0006
4	13	Fat	0.0464	**Macaroni and cheese**: **20**, 1, 0.0007; **24**, 1, 0.0090; **36**, 5, 0.0007; **37**, 12, 0.0013; **40**, 5, 0.0020; **46**, 8, 0.0013; **54**, 5, 0.0016; **56**, 7, 0.5749; **75**, 1, 0.0005; **76**, 1, 0.0003; **83**, 33, 0.0046; **105**, 4, 0.0053; **116**, 1, 0.0010
9	7	Nonfat	0.0041	**Macaroni**, cooked: **36**, 4, 0.0018; **37**, 3, 0.0033; **46**, 8, 0.0033; **81**, 4, 0.0010; **83**, 28, 0.0045; **105**, 1, 0.0060; **132**, 1, 0.0090
3	6	Fat	0.4227	**Margarine**, stick type: **56**, 21, 2.096; **76**, 1, 0.0005; **103**, 1, 0.0020; **105**, 8, 0.0074; **117**, 1, 0.0200; **134**, 1, 0.4100
8	6	Fat	0.0103	**Mayonnaise**, bottled: **10**, 4, 0.0423; **12**, 3, 0.0087; **54**, 3, 0.0016; **76**, 1, 0.0004; **83**, 1, 0.0020; **105**, 6, 0.0072
2	19	Fat	0.0120	**Meatloaf**, beef: **10**, 1, 0.0060; **20**, 8, 0.0005; **36**, 4, 0.0009; **37**, 1, 0.0030; **40**, 30, 0.0047; **46**, 7, 0.0005; **51**, 2, 0.0025; **54**, 19, 0.0011; **56**, 7, 0.1871; **60**, 1, 0.0020; **61**, 1, 0.0020; **75**, 12, 0.0007; **76**, 14, 0.0005; **81**, 1, 0.0010; **83**, 20, 0.0039; **96**, 10, 0.0007; **105**, 2, 0.0040; **106**, 1, 0.0004; **117**, 1, 0.0070
5	6	Fat	0.0009	**Milk**, chocolate: **20**, 2, 0.0005; **36**, 1, 0.0003; **40**, 6, 0.0020; **54**, 3, 0.0014; **75**, 2, 0.0008; **96**, 1, 0.0002
8	9	Fat	0.0012	**Milk**, evaporated: **20**, 17, 0.0004; **40**, 21, 0.0017; **54**, 17, 0.0008; **75**, 9, 0.0004; **76**, 12, 0.0002; **83**, 1, 0.0009; **96**, 5, 0.0004; **105**, 5, 0.0052; **133**, 1, 0.0010
1	8	Fat	0.0004	**Milk**, lowfat, 2% fat: **20**, 1, 0.0001; **36**, 1, 0.0004; **40**, 11, 0.0013; **54**, 8, 0.0003; **75**, 5, 0.0002; **76**, 3, 0.0001; **83**, 1, 0.0005; **96**, 1, 0.0001
4	3	Fat	0.0023	**Milk**, skim: **40**, 1, 0.0004; **96**, 1, 0.0004; **105**, 2, 0.0060
4	6	Fat	0.0005	**Milk**, whole: **20**, 9, 0.0002; **40**, 20, 0.0021; **54**, 10, 0.0003; **75**, 6, 0.0002; **76**, 7, 0.0001; **96**, 2, 0.0002
4	9	Fat	0.0042	**Milkshake**, chocolate: **20**, 5, 0.0004; **36**, 2, 0.0008; **40**, 12, 0.0022; **46**, 2, 0.0005; **54**, 5, 0.0004; **75**, 3, 0.0003; **76**, 3, 0.0003; **83**, 1, 0.0010; **105**, 1, 0.0320
7	15	Fat	0.5961	**Muffins**, blueberry or plain: **36**, 15, 0.0035; **37**, 19, 0.0029; **40**, 5, 0.0034; **46**, 17, 0.0023; **48**, 6, 0.3750; **49**, 7, 8.1286; **53**, 1, 0.0110; **54**, 1, 0.0020; **68**, 1, 0.0020; **83**, 36, 0.0183; **88**, 1, 0.1180; **105**, 4, 0.0048; **116**, 2, 0.0085; **125**, 1, 0.0030; **135**, 1, 0.2580
2	19	Nonfat	0.0344	**Mushrooms**, canned, sliced: **2**, 1, 0.0007; **14**, 1, 0.0050; **20**, 7, 0.0049; **21**, 1, 0.0030; **36**, 8, 0.0080; **40**, 1, 0.0007; **46**, 3, 0.0012; **60**, 1, 0.0050; **61**, 1, 0.0020; **67**, 1, 0.0090; **81**, 9, 0.0058; **84**, 1, 0.0010; **105**, 4, 0.0085; **106**, 25, 0.0026; **108**, 1, 0.0130; **109**, 1, 0.0100; **124**, 1, 0.0009; **128**, 4, 0.5683; **132**, 1, 0.0040
0	9	Nonfat	0.0035	**Noodles**, egg, cooked: **20**, 1, 0.0005; **35**, 2, 0.0060; **36**, 11, 0.0024; **37**, 8, 0.0061; **40**, 2, 0.0009; **46**, 18, 0.0037; **81**, 1, 0.0005; **83**, 36, 0.0082; **116**, 1, 0.0030
9	23	Fat	0.0135	**Nuts**, peanuts, dry-roasted: **10**, 12, 0.0793; **11**, 1, 0.0040; **12**, 7, 0.0251; **20**, 1, 0.0040; **35**, 1, 0.0070; **36**, 31, 0.0035; **40**, 10, 0.0027; **46**, 12, 0.0027; **51**, 13, 0.0055; **54**, 21, 0.0027; **72**, 14, 0.0014; **75**, 10, 0.0009; **76**, 33, 0.0025; **83**, 31, 0.0137; **102**, 35, 0.0083; **103**, 35, 0.0036; **105**, 9, 0.0100; **106**, 36, 0.0028; **107**, 34, 0.0030; **121**, 30, 0.0025; **125**, 9, 0.0010; **126**, 1, 0.0030; **129**, 33, 0.1212
0	7	Fat	0.0279	**Nuts**, pecans: **10**, 1, 0.1410; **46**, 8, 0.0021; **83**, 3, 0.0133; **88**, 1, 0.0270; **105**, 5, 0.0068; **121**, 1, 0.0030; **125**, 1, 0.0020
5	5	Nonfat	0.0119	**Oats**, rolled, cooked: **36**, 1, 0.0010; **46**, 2, 0.0070; **51**, 1, 0.0070; **83**, 33, 0.0048; **132**, 8, 0.0395
1	12	Fat	0.4880	**Onions rings**: **10**, 1, 0.0940; **36**, 4, 0.0026; **37**, 8, 0.0025; **40**, 4, 0.0015; **46**, 2, 0.0009; **48**, 5, 0.3660; **49**, 8, 5.350; **63**, 1, 0.0040; **76**, 1, 0.0004; **83**, 37, 0.0205; **88**, 1, 0.0060; **105**, 9, 0.0079
2	2	Nonfat	0.0040	**Onions**, raw: **21**, 1, 0.0050; **35**, 1, 0.0030
9	5	Nonfat	0.0025	**Orange drink**: **25**, 1, 0.0050; **32**, 11, 0.0034; **53**, 1, 0.0010; **65**, 25, 0.0024; **66**, 1, 0.0009
2	10	Nonfat	0.0296	**Orange**, navel or valencia: **25**, 12, 0.0249; **27**, 1, 0.0050; **36**, 6, 0.0014; **53**, 7, 0.0034; **55**, 4, 0.0020; **65**, 2, 0.0100; **85**, 4, 0.0100; **97**, 3, 0.0037; **99**, 3, 0.0012; **128**, 10, 0.2341

Table 2. ***(continued)***

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
87	11	Fat	0.2282	**Pancakes**: **35**, 1, 0.0020; **36**, 8, 0.0013; **37**, 9, 0.0019; **40**, 7, 0.0025; **46**, 14, 0.0013; **48**, 2, 0.1550; **49**, 3, 2.317; **54**, 1, 0.0010; **83**,37, 0.0208; **105**, 2, 0.0065; **116**, **3**, 0.0016
124	15	Fat	0.1269	**Pastry**, danish or sweet roll: **20**, 3, 0.0005; **36**, 23, 0.0019; **37**, 19, 0.0029; **40**, 2, 0.0007; **46**, 24, 0.0022; **48**, 3, 0.0633; **49**, 4, 1.762; **51**, 1, 0.0020; **53**, 1, 0.0330; **54**, 1, 0.0010; **81**, 1, 0.0002; **83**, 37, 0.0210; **96**, 1, 0.0020; **105**, 3, 0.0077; **121**, 1, 0.0020
32	3	Nonfat	0.0143	**Peach**, canned: **25**, 30, 0.0400; **54**, 1, 0.0009; **55**, 1, 0.0020
226	39	Nonfat	0.0591	**Peach**, raw: 7, 31, 0.0093; **19**, 7, 0.0940; **20**, 1, 0.0010; **24**, 4, 0.0450; **25**, 13, 0.1560; **36**, 7, 0.0058; **40**, 5, 0.0007; **46**, 10, 0.0020; **51**, 35, 0.9346; **52**, 1, 0.0030; **53**, 1, 0.0390; **55**, 1, 0.0030; **56**, 2, 0.0975; **60**, 15, 0.0012; **61**, 17, 0.0551; **62**, 9, 0.0056; **65**, 1, 0.0030; **73**, 1, 0.0050; **77**, 1, 0.0040; **78**, 1, 0.0360; **84**, 1, 0.0120; **88**, 1, 0.0340; **90**, 2, 0.0335; **91**, 2, 0.0135; **97**, 2, 0.0095; **99**, 19, 0.0206; **101**, 3, 0.0030; **102**, 1, 0.0010; **105**, 1, 0.0050; **106**, 1, 0.0006; **108**, 1, 0.0340; **109**, 1, 0.0300; **113**, 2, 0.0410; **114**, 5, 0.0583; **120**, 4, 0.1703; **123**, 3, 0.1963; **128**, 1, 0.0190; **136**, 2, 0.1130; **137**, 1, 0.0090
384	19	Fat	0.0110	**Peanut butter**, creamy: **20**, 1, 0.0002; **36**, 31, 0.0047; **40**, 11, 0.0035; **46**, 10, 0.0028; **51**, 4, 0.0148; **54**, 24, 0.0021; **72**, 15, 0.0013; **75**, 6, 0.0006; **76**, 32, 0.0021; **81**, 1, 0.0008; **83**, 35, 0.0620; **102**, 34, 0.0070; **103**, 34, 0.0029; **105**, 8, 0.0110; **106**, 34, 0.0022; **107**, 32, 0.0032; **121**, 32, 0.0021; **125**, 6,0.0009; **129**, 34, 0.0841
4	3	Nonfat	0.0013	**Pear**, canned: **51**, 1, 0.0010; **62**, 2, 0.0025; **84**, 1, 0.0003
167	28	Nonfat	0.0308	**Pear**, raw: **7**, 2, 0.0004; **19**, 12, 0.0612; **20**, 1, 0.0009; **24**, 3, 0.0093; **36**, 3, 0.0020; **40**, 4, 0.0010; **42**, 2, 0.0048; **46**, 8, 0.0017; **51**, 10, 0.0116; **52**, 5, 0.0106; **53**, 6, 0.0735; **54**, 1, 0.0005; **60**, 17, 0.0023; **61**, 25, 0.0053; **62**, 26, 0.0076; **65**, 10, 0.0336; **66**, 1, 0.0060; **76**, 1, 0.0010; **84**, 1, 0.0007; **87**, 3, 0.0273; **99**, 3, 0.0300; **105**, 1, 0.0070; **106**, 2, 0.0004; **108**, 1, 0.0100; **109**, 1, 0.0120; **114**, 8, 0.0411; **128**, 9, 0.4911; **137**, 1, 0.0090
20	7	Nonfat	0.0046	**Peas**, green, boiled: **14**, 1, 0.0020; **25**, 3, 0.0093; **46**, 5, 0.0015; **55**, 5, 0.0040; **84**, 1, 0.0010; **88**, 1, 0.0120; **99**, 4, 0.0022
1	1	Nonfat	0.0040	**Peas**, green, canned: **46**, 1, 0.0040
202	28	Nonfat	0.0246	**Pepper**, sweet, green, raw: **7**, 1, 0.0010; **14**, 30, 0.1831; **20**, 2, 0.0005; **25**, 5, 0.0228; **35**, 9, 0.0046; **36**, 12, 0.0212; **38**, 1, 0.0010; **40**, 9, 0.0013; **46**, 6, 0.0040; **51**, 9, 0.0043; **55**, 10, 0.0083; **56**, 1, 0.0670; **60**, 12, 0.0080; **61**, 10, 0.0119; **62**, 9, 0.0048; **65**, 4, 0.0085; **81**, 1, 0.0001; **84**, 34, 0.1298; **87**, 4, 0.0400; **97**, 13, 0.0319; **98**, 1, 0.0110; **99**, 2, 0.0130; **102**, 3, 0.0006; **105**, 1, 0.0040; **108**, 5, 0.0416; **109**, 5, 0.0476; **125**, 2, 0.0035; **132**, 1, 0.0140
146	20	Nonfat	0.0051	**Pickles**, dill: **14**, 1, 0.0080; **20**, 2, 0.0007; **28**, 1, 0.0020; **29**, 2, 0.0013; **30**, 1, 0.0010; **38**, 1, 0.0020; **40**, 3, 0.0030;**54**, 36, 0.0069; **60**, 21, 0.0047; **61**, 21, 0.0033; **62**, 21, 0.0108; **63**, 4, 0.0020; **75**, 14, 0.0014; **81**, 4, 0.0047; **84**, 1, 0.0010; **95**, 1, 0.0020; **96**, 2, 0.0012; **105**, 1, 0.0040; **106**, 1, 0.0004; **129**, 8, 0.0414
72	14	Fat	0.0034	**Pie**, apple, frozen: **20**, 1, 0.0003; **36**, 1, 0.0007; **37**, 13, 0.0068; **40**, 1, 0.0009; **46**, 7, 0.0006; **51**, 1, 0.0005; **53**, 1, 0.0020; **54**, 1, 0.0020; **75**, 1, 0.0020; **76**, 1, 0.0006; **81**, 1, 0.0005; **83**, 37, 0.0222; **99**, 1, 0.0010; **105**, 5, 0.0080
187	21	Fat	0.0067	**Pie**, pumpkin, frozen: **9**, 7, 0.0030; **10**, 16, 0.0355; **11**, 25, 0.0265; **12**, 15, 0.0231; **20**, 8, 0.0013; **28**, 1, 0.0100; **29**, 3, 0.0007; **30**, 1, 0.0007; **37**, 9, 0.0059; **40**, 3, 0.0006; **42**, 1, 0.0010; **46**, 5, 0.0011; **54**, 31, 0.0048; **63**, 1, 0.0030; **75**, 12, 0.0013; **81**, 4, 0.0005; **83**, 37, 0.0136; **95**, 3, 0.0008; **96**, 1, 0.0005; **105**, 3, 0.0053; **116**, 1, 0.0010
1	1	Nonfat	0.0400	**Pineapple**, canned: **135**, 1, 0.0400
194	22	Fat	0.0091	**Pizza**, cheese, cooked: **8**, 4, 0.0128; **9**, 3, 0.0110; **10**, 28, 0.0983; **11**, 7, 0.0087; **12**, 24, 0.0220; **20**, 12, 0.0006; **35**, 1, 0.0030; **36**, 6, 0.0008; **37**, 15, 0.0040; **40**, 7, 0.0011; **46**, 17, 0.0013; **54**, 7, 0.0010; **60**, 4, 0.0015; **61**, 4, 0.0019; **75**, 6, 0.0005; **76**, 2, 0.0003; **81**, 2, 0.0018; **83**, 37, 0.0125; **96**, 1, 0.0004; **99**, 1, 0.0005; **105**, 5, 0.0066; **117**, 1, 0.0100
135	28	Nonfat	0.0240	**Plums**, purple, raw: **7**, 15, 0.0058; **19**, 2, 0.0490; **20**, 1, 0.0003; **24**, 1, 0.0310; **36**, 10, 0.0021; **40**, 4, 0.0006; **42**, 1, 0.0020; **46**, 2, 0.0065; **51**, 36, 0.2395; **53**, 2, 0.0060; **55**, 4, 0.0018; **56**, 2, 0.0590; **60**, 8, 0.0017; **61**, 8, 0.0015; **62**, 13, 0.0105; **65**, 2, 0.0135; **71**, 1, 0.0140; **77**, 1, 0.0330; **78**, 1, 0.0380; **88**, 3, 0.0107; **97**, 1, 0.0020; **99**, 2, 0.0090; **105**, 1, 0.0050; **106**, 1, 0.0003; **113**, 1, 0.0080; **114**, 7, 0.0087; **120**, 4, 0.1048; **136**, 1, 0.0070

Table 2. ***(continued)***

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
72	11	Fat	0.0201	**Popcorn**, popped: **26**, 1, 0.0220; **36**, 3, 0.0039; **46**, 11, 0.0017; **54**, 2, 0.0014; **81**, 1, 0.0009; **83**, 32, 0.0337; **88**, 7, 0.0194; **88**, 3, 0.0113; **105**, 3, 0.0063; **116**, 8, 0.1113; **117**, 1, 0.0090
23	6	Nonfat	0.0110	**Pork and beans**, canned: **10**, 10, 0.0553; **11**, 1, 0.0010; **12**, 9, 0.0072; **38**, 1, 0.0005; **54**, 1, 0.0007; **105**, 1, 0.0010
69	15	Fat	0.0202	**Pork bacon**, cooked: **17**, 1, 0.0020; **20**, 1, 0.0004; **36**, 1, 0.0040; **40**, 16, 0.0044; **42**, 7, 0.0094; **54**, 7, 0.0019; **75**, 2, 0.0065; **76**, 4, 0.0007; **81**, 8, 0.0057; **88**, 1, 0.0040; **95**, 1, 0.0300; **96**, 8, 0.0039; **105**, 9, 0.0074; **124**, 2, 0.0020; **134**, 1, 0.2210
43	14	Fat	0.0039	**Pork chop**, cooked: **20**, 1, 0.0004; **40**, 4, 0.0065; **42**, 1, 0.0050; **46**, 6, 0.0008; **54**, 3, 0.0012; **75**, 2, 0.0006; **76**, 1, 0.0008; **81**, 6, 0.0021; **83**, 1, 0.0080; **96**, 4, 0.0006; **105**, 11, 0.0096; **117**, 1, 0.0160; **122**, 1, 0.0020; **124**, 1, 0.0010
32	11	Fat	0.0289	**Pork roast**, loin, cooked: **35**, 1, 0.0090; **36**, 1, 0.0002; **40**, 6, 0.0015; **42**, 1, 0.0210; **46**, 3, 0.0009; **56**, 1, 0.2350; **76**, 2, 0.0014; **83**, 1, 0.0040; **105**, 13, 0.0084; **117**, 1, 0.0340; **124**, 2, 0.0024
161	17	Fat	0.0204	**Pork sausage**, cooked: **9**, 1, 0.0030; **10**, 21, 0.2041; **11**, 4, 0.0065; **12**, 19, 0.0438; **20**, 7, 0.0018; **35**, 1, 0.0520; **40**, 26, 0.0091; **46**, 1, 0.0020; **54**, 6, 0.0020; **75**, 8, 0.0036; **76**, 8, 0.0008; **81**, 26, 0.0015; **95**, 1, 0.0008; **96**, 12, 0.0020; **103**, 1, 0.0007; **105**, 9, 0.0094; **124**, 10, 0.0037
33	9	Fat	0.1186	**Pork**, ham, cured, cooked: **40**, 5, 0.0008; **42**, 2, 0.0025; **46**, 3, 0.0004; **56**, 8, 0.2376; **76**, 1, 0.0007; **81**, 4, 0.0007; **105**, 7, 0.0070; **129**, 1, 0.0140; **134**, 2, 0.8040
112	24	Fat	0.0700	**Potato chips**: **1**, 1, 0.0020; **2**, 2, 0.0023; **3**, 1, 0.0002; **4**, 1, 0.0003; **29**, 1, 0.0007; **30**, 1, 0.0004; **35**, 22, 1.5804; **40**, 24, 0.0067; **43**, 9, 0.0040; **46**, 3, 0.0011; **54**, 14, 0.0026; **62**, 2, 0.0055; **75**, 2, 0.0006; **76**, 2, 0.0007; **79**, 8, 0.0154; **83**, 1, 0.0010; **95**, 1, 0.0006; **102**, 1, 0.0020; **103**, 1, 0.0010; **105**, 6, 0.0062; **107**, 1, 0.0050; **121**, 1, 0.0030; **125**, 6, 0.0081; **129**, 1, 0.0300
175	33	Nonfat	0.0384	**Potatoes**, baked: **1**, 6, 0.0144; **2**, 7, 0.0333; **3**, 5, 0.0118; **28**, 3, 0.0133; **35**, 32, 0.6793; **40**, 24, 0.0028; **41**, 2, 0.0030; **42**, 13, 0.0037; **44**, 1, 0.0020; **45**, 2, 0.0090; **46**, 2, 0.0008; **51**, 5, 0.0318; **54**, 10, 0.0018; **58**, 1, 0.0007; **59**, 1, 0.0110; **60**, 1, 0.0008; **61**, 3, 0.0083; **62**, 12, 0.0075; **67**, 1, 0.0090; **75**, 4, 0.0018; **76**, 1, 0.0010; **79**, 11, 0.0088; **84**, 1, 0.0010; **96**, 1, 0.0010; **102**, 3, 0.0055; **103**, 1, 0.0002; **106**, 2, 0.0005; **111**, 2, 0.0015; **112**, 2, 0.0080; **121**, 1, 0.0030; **124**, 2, 0.0010; **125**, 10, 0.0594; **128**, 3, 0.3313
99	25	Nonfat	0.0113	**Potatoes**, boiled: **1**, 4, 0.0043; **2**, 8, 0.0121; **3**, 7, 0.0042; **4**, 1, 0.0001; **15**, 1, 0.0110; **35**, 26, 0.0932; **40**, 7, 0.0014; **42**, 1, 0.0050; **44**, 1, 0.0290; **45**, 2, 0.0025; **46**, 1, 0.0010; **51**, 1, 0.0360; **54**, 4, 0.0008; **58**, 1, 0.0170; **59**, 1, 0.0090; **60**, 1, 0.0002; **61**, 1, 0.0030; **62**, 8, 0.0038; **75**, 3, 0.0006; **79**, 7, 0.0067; **96**, 1, 0.0005; **102**, 2, 0.0007; **105**, 1, 0.0040; **125**, 8, 0.0114; **128**, 1, 0.0240
130	21	Fat	0.0134	**Potatoes**, French fries: **1**, 4, 0.0015; **2**, 6, 0.0015; **3**, 5, 0.0026; **7**, 1, 0.0005; **17**, 1, 0.0020; **24**, 1, 0.0320; **35**, 33, 0.1922; **36**, 3, 0.0009; **38**, 1, 0.0007; **40**, 12, 0.0009; **46**, 9, 0.0008; **51**, 5, 0.0132; **54**, 13, 0.0010; **62**, 12, 0.0041; **75**, 3, 0.0005; **76**, 2, 0.0005; **79**, 4, 0.0132; **83**, 1, 0.0008; **103**, 1, 0.0020; **105**, 5, 0.0044; **125**, 8, 0.0056
75	11	Fat	0.0197	**Potatoes**, mashed: **35**, 32, 0.0602; **36**, 8, 0.0011; **40**, 10, 0.0021; **46**, 9, 0.0010; **51**, 1, 0.0020; **54**, 1, 0.0003; **56**, 5, 0.1382; **79**, 3, 0.0030; **83**, 1, 0.0006; **102**, 1, 0.0030; **105**, 4, 0.0058
127	23	Fat	0.0656	**Potatoes**, scalloped: **1**, 2, 0.0026; **2**, 6, 0.0077; **3**, 4, 0.0034; **20**, 4, 0.0003; **35**, 28, 0.0679; **36**, 1, 0.0020; **37**, 1, 0.0008; **40**, 16, 0.0022; **46**, 7, 0.0011; **48**, 3, 0.0600; **49**, 4, 1.300; **51**, 2, 0.0220; **54**, 10, 0.0007; **62**, 5, 0.0022; **75**, 2, 0.0010; **76**, 2, 0.0001; **79**, 5, 0.0188; **81**, 1, 0.0005; **83**, 12, 0.0012; **96**, 1, 0.0005; **99**, 1, 0.0008; **105**, 2, 0.0065; **125**, 8, 0.0078
104	15	Fat	0.0604	**Potpie**, chicken: **10**, 8, 0.0194; **12**, 3, 0.0033; **20**, 1, 0.0004; **35**, 10, 0.0105; **36**, 4, 0.0008; **37**, 12, 0.0045; **40**, 8, 0.0009; **46**, 10, 0.0017; **49**, 1, 0.8000; **51**, 1, 0.0010; **54**, 2, 0.0020; **81**, 5, 0.0013; **83**, 36, 0.0258; **105**, 2, 0.0100; **117**, 1, 0.0250

Table 2. ***(continued)***

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], μg/g	Food item name: residue code, times found, average μg/g
109	18	Nonfat	0.0178	**Prunes**, dried: **36**, 3, 0.0012; **40**, 4, 0.0009; **51**, 2, 0.0009; **52**, 1, 0.0030; **53**, 12, 0.0109; **56**, 4, 0.0713; **60**, 4, 0.0010; **61**, 16, 0.0014; **62**, 15, 0.0031; **65**, 2, 0.0020; **83**, 5, 0.0040; **105**, 6, 0.0082; **113**, 19, 0.0111; **117**, 1, 0.0120; **120**, 10, 0.0774; **123**, 1, 0.0020; **132**, 2, 0.0120; **135**, 2, 0.0990
25	10	Fat	0.0009	**Pudding**, instant, chocolate: **20**, 3, 0.0003; **40**, 12, 0.0020; **46**, 1, 0.0002; **54**, 2, 0.0008; **75**, 1, 0.0004; **76**, 1, 0.0001; **81**, 1, 0.0003; **96**, 2, 0.0003; **105**, 1, 0.0040; **125**, 1, 0.0009
76	21	Nonfat	0.0214	**Radish**, raw: **13**, 1, 0.0170; **25**, 1, 0.0110; **26**, 2, 0.0380; **35**, 1, 0.0240; **36**, 8, 0.0025; **38**, 10, 0.0083; **40**, 16, 0.0059; **42**, 4, 0.0060; **46**, 1, 0.0004; **54**, 11, 0.0016; **55**, 1, 0.0090; **60**, 2, 0.0093; **61**, 1, 0.0230; **62**, 3, 0.0503; **69**, 1, 0.0800; **75**, 3, 0.0015; **89**, 1, 0.0400; **91**, 1, 0.0560; **97**, 1, 0.0110; **99**, 1, 0.0150; **129**, 6, 0.0395
226	29	Nonfat	0.0417	**Raisins**, dried: **24**, 4, 0.0550; **25**, 23, 0.0968; **29**, 2, 0.0008; **30**, 2, 0.0008; **36**, 2, 0.0006; **40**, 31, 0.0040; **42**, 3, 0.0018; **46**, 2, 0.0006; **51**, 4, 0.0108; **52**, 7, 0.0141; **53**, 28, 0.0596; **55**, 25, 0.0107; **60**, 10, 0.0008; **61**, 23, 0.0046; **62**, 12, 0.0027; **65**, 5, 0.0132; **69**, 1, 0.0870; **83**, 2, 0.0015; **95**, 2, 0.0009; **97**, 5, 0.0076; **105**, 5, 0.0076; **113**, 4, 0.0420; **117**, 1, 0.0210; **119**, 1, 0.0320; **120**, 10, 0.3323; **123**, 7, 0.3656; **125**, 1, 0.0003; **127**, 2, 0.0140; **132**, 2, 0.0220
29	8	Nonfat	0.0383	**Rice**, white, cooked: **5**, 3, 0.0037; **35**, 1, 0.0020; **46**, 6, 0.0022; **56**, 2, 0.2730; **83**, 14, 0.0082; **88**, 1, 0.0020; **105**, 1, 0.0040; **132**, 1, 0.0110
115	12	Fat	0.1076	**Rolls**, white, soft: **35**, 1, 0.0350; **36**, 20, 0.0019; **37**, 20, 0.0084; **40**, 1, 0.0005; **46**, 22, 0.0044; **49**, 1, 1.200; **51**, 2, 0.0009; **68**, 5, 0.0031; **83**, 36, 0.0294; **99**, 2, 0.0010; **105**, 2, 0.0045; **116**, 3, 0.0016
44	5	Fat	0.1008	**Salad dressing**, Italian: **10**, 18, 0.0873; **12**, 14, 0.0251; **54**, 3, 0.0033; **105**, 8, 0.0085; **135**, 1, 0.3800
181	15	Fat	0.0162	**Salami**: **9**, 1, 0.0040; **10**, 21, 0.1653; **11**, 10, 0.0214; **12**, 12, 0.0225; **20**, 16, 0.0007; **40**, 30, 0.0028; **42**, 2, 0.0070; **54**, 18, 0.0015; **75**, 17, 0.0010; **76**, 14, 0.0008; **81**, 18, 0.0014; **96**, 14, 0.0008; **105**, 6, 0.0122; **106**, 1, 0.0002; **124**, 1, 0.0010
10	5	Nonfat	0.0136	**Sauerkraut**, canned: **20**, 2, 0.0007; **54**, 5, 0.0011; **56**, 1, 0.0640; **84**, 1, 0.0020; **107**, 1, 0.0004
80	19	Fat	0.1727	**Shrimp**, breaded, fried: **20**, 3, 0.0009; **21**, 1, 0.0010; **35**, 1, 0.0090; **36**, 2, 0.0006; **37**, 4, 0.0015; **38**, 1, 0.0040; **40**, 10, 0.0017; **46**, 10, 0.0009; **48**, 4, 0.1900; **49**, 6, 2.845; **54**, 2, 0.0007; **56**, 1, 0.1960; **76**, 1, 0.0004; **81**, 1, 0.0004; **83**, 26, 0.0037; **105**, 4, 0.0078; **106**, 1, 0.0001; **117**, 1, 0.0130; **124**, 1, 0.0050
1	1	Nonfat	0.0130	**Soda**, lemon–lime: **25**, 1, 0.0130
1	1	Nonfat	0.0080	**Soda**, low-calorie, cola: **25**, 1, 0.0080
0	0	Nonfat	0.0080	**Soft drink**, cola soda: No findings
0	0	Nonfat	0.0080	**Soft drink**, cherry: No findings
12	7	Nonfat	0.0032	**Soup**, chicken noodle: **35**, 1, 0.0110; **36**, 1, 0.0010; **40**, 1, 0.0009; **46**, 3, 0.0030; **81**, 1, 0.0005; **83**, 3, 0.0010; **105**, 2, 0.0050
61	17	Nonfat	0.0164	**Soup**, cream of tomato: **14**, 1, 0.0009; **20**, 1, 0.0004; **36**, 1, 0.0010; **37**, 1, 0.0020; **40**, 7, 0.0014; **46**, 4, 0.0019; **48**, 2, 0.0150; **49**, 2, 0.2450; **54**, 1, 0.0006; **60**, 1, 0.0003; **61**, 2, 0.0007; **62**, 1, 0.0005; **75**, 1, 0.0005; **81**, 3, 0.0004; **83**, 23, 0.0045; **84**, 9, 0.0012; **99**, 1, 0.0020
12	5	Nonfat	0.0023	**Soup**, vegetable beef: **35**, 4, 0.0058; 36, 2, 0.0035; **46**, 4, 0.0017; **54**, 1, 0.0004; **84**, 1, 0.0004
37	9	Nonfat	0.0044	**Spaghetti**, w/ tomato sauce, canned: **10**, 2, 0.0255; **36**, 2, 0.0018; **37**, 1, 0.0010; **46**, 1, 0.0003; **55**, 1, 0.0030; **60**, 2, 0.0006; **61**, 3, 0.0010; **83**, 15, 0.0053; **84**, 10, 0.0015
80	18	Fat	0.0069	**Spaghetti**, with meat sauce: **10**, 2, 0.0680; **12**, 2, 0.0195; **20**, 1, 0.0001;**35**, 1, 0.0130; 36, 11, 0.0019; **40**, 20, 0.0013; **46**, 9, 0.0021; **51**, 3, 0.0017; **54**, 1, 0.0007; **60**, 1, 0.0030; **61**, 1, 0.0010; **75**, 1, 0.0006; **76**, 2, 0.0002; **81**, 2, 0.0007; **83**, 19, 0.0030; **96**, 1, 0.0004; **105**, 2, 0.0060; **116**, 1, 0.0020

Table 2. *(continued)*

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
245	36	Nonfat	0.0258	**Spinach**, boiled: **14**, 1, 0.0370; **20**, 2, 0.0011; **21**, 1, 0.0020; **25**, 1, 0.0110; **29**, 4, 0.0025; **30**, 7, 0.0010; **35**, 1, 0.0770; **36**, 10, 0.0062; **38**, 13, 0.0020; **39**, 2, 0.0040; **40**, 37, 0.0193; **41**, 1, 0.0150; **42**, 18, 0.0074; **46**, 9, 0.0043; **51**, 4, 0.0035; **53**, 1, 0.0830; **54**, 15, 0.0035; **55**, 2, 0.0285; **60**, 8, 0.0222; **61**, 9, 0.0127; **62**, 19, 0.0476; **75**, 2, 0.0019; **76**, 1, 0.0007; **81**, 3, 0.0011; **84**, 4, 0.0096; **90**, 2, 0.0115; **91**, 2, 0.0085; **95**, 3, 0.0008; **97**, 3, 0.0197; **99**, 4, 0.0055; **102**, 5, 0.0008; **106**, 1, 0.0004; **108**, 20, 0.2011; **109**, 20, 0.2414; **124**, 7, 0.0057; **129**, 3, 0.0290
108	17	Nonfat	0.0835	**Spinach**, canned: **21**, 5, 0.0030; **29**, 1, 0.0010; **30**, 1, 0.0007; **35**, 1, 0.0380; **38**, 5, 0.0011; **39**, 2, 0.0020; **40**, 34, 0.0139; **51**, 1, 0.0010; **67**, 2, 0.0205; **70**, 1, 0.0570; **75**, 1, 0.0004; **76**, 2, 0.0013; **84**, 2, 0.0305; **99**, 3, 0.0050; **102**, 3, 0.0013; **108**, 22, 0.6116; **109**, 22, 0.6313
213	32	Nonfat	0.0072	**Squash**, summer, boiled: **14**, 3, 0.0016; **28**, 6, 0.0355; **29**, 6, 0.0021; **30**, 9, 0.0015; **36**, 2, 0.0015; **38**, 3, 0.0014; **40**, 5, 0.0032; **42**, 1, 0.0070; **46**, 5, 0.0018; **51**, 2, 0.0330; **53**, 1, 0.0060; **54**, 32, 0.0087; **60**, 27, 0.0073; **61**, 15, 0.0022;**62**, 26, 0.0212; **63**, 8, 0.0041; **65**, 1, 0.0010; **75**, 10, 0.0019; **76**, 8, 0.0010; **81**, 2, 0.0001; **84**, 6, 0.0020; **92**, 1, 0.0020; **94**, 1, 0.0008; **95**, 5, 0.0028; **96**, 1, 0.0010; **99**, 6, 0.0037; **102**, 5, 0.0060; **107**, 1, 0.0008; **108**, 1, 0.0070; **121**, 1, 0.0003; **124**, 1, 0.0080; **129**, 12, 0.0525
116	32	Nonfat	0.0050	**Squash**, winter, boiled: **2**, 1, 0.0005; **14**, 6, 0.0118; **20**, 1, 0.0030; **28**, 4, 0.0098; **29**, 5, 0.0066; **30**, 6, 0.0032; **35**, 1, 0.0020; **36**, 2, 0.0009; **38**, 2, 0.0010; **40**, 4, 0.0024; **41**, 1, 0.0020; **42**, 1, 0.0030; **46**, 5, 0.0017; **51**, 3, 0.0008; **53**, 1, 0.0030; **54**, 23, 0.0108; **60**, 2, 0.0008; **61**, 1, 0.0020; **62**, 8, 0.0041; **63**, 2, 0.0024; **75**, 5, 0.0019; **76**, 2, 0.0010; **84**, 8, 0.0053; **94**, 2, 0.0025; **95**, 5, 0.0034; **99**, 1, 0.0010; **102**, 4, 0.0015; **103**, 1, 0.0010; **117**, 1, 0.0260; **121**, 1, 0.0007; **125**, 2, 0.0019; **129**, 5, 0.0408
102	24	Fat	0.0526	**Stew**, beef and vegetable: **2**, 2, 0.0030; **7**, 1, 0.0040; **10**, 1, 0.0380; **17**, 1, 0.0010; **35**, 22, 0.0346; **36**, 6, 0.0020; **37**, 1, 0.0010; **40**, 19, 0.0018; **46**, 9, 0.0018; **48**, 2, 0.0300; **49**, 3, 0.6700; **51**, 4, 0.0193; **54**, 7, 0.0008; **62**, 2, 0.0015; **72**, 1, 0.0007; **75**, 1, 0.0008; **76**, 1, 0.0050; **79**, 2, 0.0030; **81**, 2, 0.0004; **82**, 2, 0.0020; **83**, 8, 0.0009; **106**, 1, 0.0003; **125**, 3, 0.0067; **135**, 1, 0.4330
206	29	Nonfat	0.1380	**Strawberries**, raw: **20**, 1, 0.0020; **21**, 1, 0.0005: **24**, 22, 0.6230; **25**, 8, 0.4158; **36**, 1, 0.0010; **38**, 1, 0.0007; **40**, 10, 0.0016; **46**, 2, 0.0105; **51**, 1, 0.0360; **52**, 7, 0.0301; **53**, 13, 0.2172; **54**, 4, 0.0023; **56**, 1, 0.7240; **60**, 16, 0.0181; **61**, 18, 0.0317; **62**, 19, 0.0103; **71**, 3, 0.2247; **83**, 11, 0.0071; **84**, 1, 0.0090; **87**, 11, 0.1381; **88**, 1, 0.0330; **90**, 11, 0.1251; **91**, 13, 0.0893; **99**, 3, 0.0016; **120**, 2, 0.2220; **123**, 2, 0.1675; **129**, 1, 0.0050; **130**, 1, 0.6620; **138**, 21, 0.1918
12	5	Nonfat	0.0372	**Sugar**, white: **46**, 1, 0.0050; **56**, 2, 0.0630; **105**, 3, 0.0050; **132**, 5, 0.0108; **135**, 1, 0.1020
81	12	Nonfat	0.0289	**Sweet potatoes**, baked: **2**, 1, 0.0004; **7**, 21, 0.0058; **35**, 2, 0.0060; **36**, 2, 0.0009; **40**, 5, 0.0012; **42**, 2, 0.0010; **46**, 2, 0.0012; **51**, 35, 0.2779; **54**, 7, 0.0015; **62**, 1, 0.0009; **114**, 2, 0.0485; **125**, 1, 0.0020
85	15	Fat	0.0134	**Sweet potatoes**, candied: 7, 5, 0.0012; **20**, 5, 0.0003; **35**, 3, 0.0013; **36**, 1, 0.0005; **40**, 14, 0.0015; **42**, 1, 0.0020; **46**, 5, 0.0005; **51**, 35, 0.0562; **54**, 8, 0.0024; **56**, 1, 0.1120; **75**, 1, 0.0003; **76**, 2, 0.0003; **83**, 1, 0.0170; **96**, 2, 0.0005; **105**, 1, 0.0050
4	2	Nonfat	0.0473	**Syrup**, cane: **105**, 3, 0.0047; **135**, 1, 0.0900
2	2	Nonfat	0.0024	**Tea**: **84**, 1, 0.0007; **105**, 1, 0.0040
133	14	Nonfat	0.0080	**Tomato sauce**: **10**, 20, 0.0463; **12**, 19, 0.0094; **14**, 2, 0.0050; **38**, 6, 0.0033; **40**, 18, 0.0014; **53**, 1, 0.0070; **54**, 1, 0.0004; **60**, 20, 0.0016; **61**, 19, 0.0026; **62**, 4, 0.0015; **83**, 1, 0.0010; **84**, 20, 0.0023; **104**, 1, 0.0007; **129**, 1, 0.0300
125	21	Nonfat	0.0269	**Tomatoes**, raw: **14**, 8, 0.0104; **20**, 1, 0.0050; **36**, 7, 0.0069; **40**, 1, 0.0009; **46**, 3, 0.0079; **51**, 6, 0.0160; **53**, 2, 0.0055; **60**, 9, 0.0018; **61**, 14, 0.0033; **62**, 9, 0.0044; **64**, 1, 0.0030; **65**, 2, 0.0090; **81**, 2, 0.0009; **84**, 29, 0.0281; **97**, 2, 0.0090; **99**, 1, 0.0020; **106**, 1, 0.0002; **108**, 13, 0.0245; **109**, 12, 0.0262; **117**, 1, 0.0200; **130**, 1, 0.3800
23	5	Nonfat	0.0047	**Tomatoes**, canned: **14**, 2, 0.0019; **25**, 8, 0.0124; **60**, 2, 0.0013; **61**, 3, 0.0011; **84**, 8, 0.0067
81	12	Fat	3.5579	**Tortilla**, flour: **36**, 5, 0.0010; **37**, 18, 0.0055; **40**, 2, 0.0007; **46**, 12, 0.0040; **47**, 1, 42.50; **54**, 1, 0.0020; **56**, 1, 0.1050; **81**, 1, 0.0020; **83**, 37, 0.0360; **88**, 1, 0.0070;**88**, 1, 0.0240; **105**, 1, 0.0080

Table 2. ***(continued)***

No. findings[a]	Different findings[b]	Analysis type[c]	Av. found[d], µg/g	Food item name: residue code, times found, average µg/g
23	9	Fat	0.0311	**Turkey**, breasts, roasted: **40**, 7, 0.0015; **46**, 4, 0.0008; **54**, 3, 0.0005; **56**, 1, 0.2520; **75**, 1, 0.0003; **76**, 2, 0.0002; **96**, 1, 0.0002; **105**, 2, 0.0080; **117**, 2, 0.0165
75	15	Fat	0.0089	**Veal cutlet**, cooked: **20**, 3, 0.0008; **36**, 4, 0.0006; **37**, 4, 0.0013; **40**, **6**, 0.0080; **46**, 10, 0.0008; **54**, 3, 0.0006; **56**, 2, 0.0665; **75**, 2, 0.0007; **76**, 4, 0.0007; **81**, 3, 0.0023; **83**, 24, 0.0024; **96**, 3, 0.0008; **105**, 5, 0.0078; **117**, 1, 0.0090; **129**, 1, 0.0310
36	9	Nonfat	0.0034	**Vegetables**, mixed, canned: **2**, 3, 0.0073; **3**, 1, 0.0002; **14**, 11, 0.0030; **24**, 1, 0.0050; **35**, 3, 0.0063; **81**, 1, 0.0020; **84**, 13, 0.0013; **125**, 2, 0.0033; **126**, 1, 0.0020
1	1	Nonfat	0.0040	**Water**: **18**, 1, 0.0040
31	9	Nonfat	0.0041	**Watermelon**, raw: **51**, 1, 0.0050; **54**, 1, 0.0009; **55**, 1, 0.0040; **76**, 5, 0.0004; **84**, 15, 0.0051; **87**, 2, 0.0110; **97**, 2, 0.0045; **102**, 3, 0.0014; **132**, 1, 0.0050
0	0	Nonfat	0.0000	**Whiskey**: No findings
168	15	Fat	0.3964	**White sauce**: **20**, 19, 0.0009; **36**, 3, 0.0032; **37**, 5, 0.0014; **40**, 25, 0.0045; **46**, 8, 0.0026; **48**, 6, 0.2500; **49**, 7, 5.267; **54**, 16, 0.0014; **56**, 3, 0.4017; **75**, 10, 0.0006; **76**, 17, 0.0004; **81**, 3, 0.0010; **83**, 32, 0.0048; **96**, 9, 0.0005; **105**, 5, 0.0064
108	7	Nonfat	0.0332	**Wine**, table, 12.2% alcohol: **25**, 30, 0.0331; **51**, 4, 0.0015; **55**, 37, 0.0251; **86**, 1, 0.1470; **92**, 1, 0.0080; **97**, 34, 0.0171; **105**, 1, 0.0008
10	4	Fat	0.0035	**Yogurt**, plain, lowfat: **40**, 7, 0.0026; **54**, 1, 0.0003; **76**, 1, 0.0001; **105**, 1, 0.0110
62	7	Fat	0.0017	**Yogurt**, sweet, strawberry: **40**, 8, 0.0011; **53**, 1, 0.0020; **60**, 14, 0.0010; **61**, 14, 0.0015; **62**, 4, 0.0017; **76**, 1, 0.0001; **138**, 20, 0.0043

[a] Total findings, 17 050.
[b] Different residue findings, 138.
[c] Findings (44%) in 127 nonfat foods, 7486; findings (56%) in 103 fatty foods, 9564.
[d] Per food item (all findings).

This report lists the 138 different chemical residues found in 230 of the 234 ready-to-eat foods that were each tested 37 times over a 10-year period. The average amounts found are also listed. Essentially, except for the accumulation of low-level findings as analytical results, the American food supply is free of detectable pesticide and industrial chemical contamination.

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CHEMICAL CONTAMINANTS MONITORING

Survey of Deoxynivalenol in U.S. 1993 Wheat and Barley Crops by Enzyme-Linked Immunosorbent Assay

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Wheat and barley from the 1993 crop year were analyzed for deoxynivalenol (DON). A total of 630 samples were collected by the Federal Grain Inspection Service in 25 states and analyzed using a commercially available, direct competitive, enzyme-linked immunosorbent assay. The limit of determination was about 0.5 μg/g. DON contamination in the 483 wheat samples averaged 2.0 μg/g and ranged from <0.5 to 18 μg/g. DON contamination in the 147 barley samples averaged 4.2 μg/g and ranged from <0.5 to 26 μg/g. About 40% of the wheat samples and 57% of the barley samples contained DON levels that were greater than the U.S. Food and Drug Administration 1982 advisory level of 2 μg/g for DON in wheat designated for milling (human consumption).

Deoxynivalenol (DON or vomitoxin) is one of the toxic 12,13-epoxytrichothecenes produced by various species of *Fusarium*, especially *F. graminearum*. DON is often found in wheat, corn, barley, oats, and rye. *Fusarium* infections are characterized by shriveled, discolored kernels called scab, tombstone, or head blight. DON-contaminated feed causes emesis, feed refusal, and growth depression in swine (1, 2). Pure DON causes emesis in swine at 0.05–0.1 ng/g body weight (2, 3). Other toxic effects observed in laboratory animals include immunotoxicity (4) and embryotoxicity (5). Reports from China (6) and India (7) associated DON with outbreaks of acute gastrointestinal illness in humans.

The unusual wet weather in many areas of the midwestern United States in 1993 provided favorable conditions for proliferation of *F. graminearum*. By September 1993 numerous reports indicated occurrence of scab damage or head blight in wheat and barley and the presence of high levels of DON in many samples, particularly in those from Minnesota, North Dakota, and South Dakota. Although scab damage is often associ-

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ated with mycotoxin contamination, sometimes grains with no visible scab damage contain high levels of mycotoxins. To determine the extent of the outbreak, the U.S. Food and Drug Administration (FDA) requested the U.S. Department of Agriculture (USDA), Federal Grain Inspection Service (FGIS), Field Management Division, to collect wheat and barley samples from these and other states where winter wheat, spring wheat, and barley crops are grown. This sampling plan was not designed to be representative of the entire U.S. wheat and barley crops, nor was it targeted for known problem areas. No specific instructions were given for sampling of the different lots involved.

Several methods for the determination of DON in grains have been reported, including those based on the use of thin-layer chromatography (TLC) (8–10), capillary gas chromatography (GC) (11–13), GC/mass spectrometry (GC/MS) (14), liquid chromatography (LC) (15), and immunochemical methods (16, 17). The TLC, GC, GC/MS, and LC methods require extensive cleanup procedures and, with the exception of the LC method, include a derivatization step before separation and quantitation. Because of the time requirements, these methods are not practical when analysis of large numbers of test samples is needed in a relatively short time. Immunochemical methods are simple, quick, and suitable for simultaneous, multiple analyses. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (18) was evaluated and found satisfactory for use in this survey. The use of quick test kits for DON was approved by FGIS for use under the Grain Standards Act (19).

The ELISA method is a direct, competitive, microtiter well assay. DON present in the test extract competes with enzyme-labeled DON for the binding sites on the antibodies. The amount of enzyme-labeled DON is determined by adding a chromogen substrate to generate a colored product. The color development is proportional to the concentration of the enzyme-labeled DON and inversely proportional to the concentration of DON in the test sample. The color is measured with a microwell strip reader. A log/logit data transformation program and linear regression calibration data are used to derive analyte concentrations.

Experimental

Samples

Samples were collected by USDA, FGIS, from 25 states and consisted of 194 hard red winter wheats. 59 soft red winter wheats, 201 hard red spring wheats, 28 soft white wheats, 1 mixed wheat, 118 barleys, and 29 malting barleys. Each sample consisted of multiple subsamples selected from different sampling sites. The subsamples were pooled and mixed by the collector. Approximately 100 g of each composite was sent to FDA for analysis. The entire composite was ground to pass a No. 20 sieve by using a Retsch mill with a 2 mm screen. A 50 g test portion was taken for analysis.

Apparatus

(**a**) *Blender.*—Waring blender with 1 L jar and cover.

(**b**) *Filter paper.*—18 cm, prefolded (Whatman 2V performed satisfactorily).

(**c**) *ELISA reader.*—Microtiter strip reader (Model EL301, Neogen Corp., Lansing, MI).

(**d**) *Personal computer.*—IBM 486 with cable for coupling to ELISA reader.

(**e**) *Computer software.*—Log/logit transformation and linear regression calibration program (Neogen Corp.).

(**f**) *Multichannel pipet.*—12 channels (Finnpipette, Labsystem, Finland).

(**g**) *Pipet.*—200 µL.

(**h**) *Pipet tips.*—200 µL.

(**i**) *Eppendorf tubes.*—1.5 mL.

(**j**) *Microcentrifuge.*—Maximum speed, 14 000 rpm.

(**k**) *Wash bottle.*—Polypropylene, 500 mL.

Reagents

(**a**) *ELISA kit.*—Veratox for DON (Neogen Corp.). Kit includes 0, 1, 2, and 4 ppm DON standards, mixing microtiter wells, antibody wells, DON–enzyme conjugate (horseradish peroxidase) solution, substrate (tetramethylbenzidine) solution, and acid stopping solution.

(**b**) *Milli-Q water.*

(**c**) *DON standard.*—From FDA, Division of Natural Products, Washington, DC.

Extraction

Weigh 50 g ground wheat or barley in blender jar. Add 250 mL water and blend 2 min at high speed. Filter through prefolded paper. Pour ca 1 mL filtrate into Eppendorf tube and centrifuge at 14 000 rpm for 6 min. Proceed with ELISA.

ELISA

Follow the 3-step procedure provided by the manufacturer: (*1*) Use 1 strip of wells (12 wells) at a time. Pipet 100 µL DON standard solution provided in kit, or test filtrate, and 100 µL DON–enzyme conjugate solution into a mixing well and mix. Use 12 channel pipet to transfer 100 µL from mixing wells to antibody wells. Mix and let stand 10 min. DON in the test solutions competes with the DON–enzyme conjugate for binding sites on the antibodies. (*2*) Empty antibody wells and wash 5 times with Milli-Q water. Wrap strips of wells in paper towel and tap on table to shake off water. Wipe back of strips with clean towel. (*3*) Add 100 µL substrate to antibody wells, mix, and let stand 10 min. Add 100 µL acid stopping solution and mix. Set ELISA reader at 650 nm and read absorbance of test solutions immediately.

Results and Discussion

Recoveries of DON from ground wheat spiked at 1, 2, and 4 µg/g were 65, 70, and 78% (average of 3 determinations), respectively, whereas recoveries of DON from barley spiked at the same levels were 40, 70, and 68%, respectively. The limit

of determination was about 0.5 μg/g. To evaluate this procedure for wheat with high levels (≥10 μg/g) of contamination, 5 samples were obtained from the FDA Minneapolis District Office. The extracts were analyzed by the ELISA procedure, and the test solution was diluted and reanalyzed. These data are shown in Table 1. The within-laboratory relative standard deviations for the analysis of these 5 test samples (average of 5 analyses of each) ranged from 4.5 to 16.4%. On the basis of the recovery data and the linearity between the amount of DON and the volume of extract analyzed, the ELISA method was considered suitable for analysis for DON in wheat and barley. No other chemical analyses were performed.

A single analysis was performed for all test samples in this survey. Apparent DON concentrations of extracts of some barleys were lower than that of the reagent blank. The reason for this phenomenon is unknown. Although preliminary calculations demonstrated that this procedure is satisfactory for screening for DON contamination of wheat and barley, until more thorough interlaboratory evaluations are reported, we recommend that the results be confirmed by validated procedures in cases involving regulatory or other legal actions.

A total of 630 samples of wheat and barley were analyzed. A summary of the results for 5 types of wheat and 2 categories of barley is shown in Table 2. About 75% of the 201 hard red spring wheats contained DON in excess of 2 μg/g, whereas only 13.4% of the hard red winter wheats showed comparable contamination. Both the soft winter wheats and the soft white wheats showed lower incidences and levels of DON. All of the 29 malting barleys showed severe scab damage, and DON levels ranged from 0.7 to 26 μg/g and averaged 9 μg/g. Of the 118 barleys, 22 were graded as "better barley" and were found to contain DON at <0.5–0.7 μg/g; the "other barley" samples were found to contain DON at levels of <0.5–14 μg/g.

Table 3 gives DON levels in wheat from 24 states and barley from 4 states. Most of the wheat samples were collected from Kansas, Minnesota, North Dakota, South Dakota, and Washington. The highest occurrence and incidence of DON were in wheat from Minnesota; 86% of the Minnesota wheat samples contained DON in excess of 2 μg/g, and all Minnesota wheat samples averaged 4.7 μg/g. About 66 and 78% of wheat samples from North Dakota and South Dakota, respectively, showed DON contamination >2 μg/g. Wheat samples from California, Colorado, Georgia, Michigan, Oklahoma, Texas, and Washington were almost free (<0.5 μg/g) from DON contamination at the limit of determination. Only a few samples of wheat were collected from each of the other states, because no unusual wet weather or occurrences of scab damage in wheat were reported and the DON contamination levels for these states were found to be low. About 40% of the wheat samples in this survey had DON levels >2 μg/g.

Although some correlation is found between average DON levels and wheat grade, some samples in all grades contained DON at levels greater than 2 μg/g (Table 4). These results indicate considerably greater DON contamination of wheat than that found in previous FDA surveys of the 1982 (20), 1984–85 (21), and 1991 (22) crops. Table 5 gives additional data for the 1991 wheat crop. The highest occurrence and incidence of DON were in wheat from Illinois and Missouri.

Table 3 includes DON levels of barley samples collected from 4 states. High levels of DON were found in barley sam-

Table 1. Results of replicate analyses for deoxynivalenol (DON) in wheat

Sample[a]	Dilution	DON, μg/g
1	1	10.9
	1	14.9
Average		12.9
	2	11.4
	4	11.6
	8	10.8
Average		11.3
RSD, %		14.3
2	1	12.5
	1	15.2
Average		13.9
	2	10.4
	4	13.6
	8	15.2
Average		13.1
RSD, %		16.4
3	1	13.3
	1	15.5
Average		14.4
	2	10.0
	4	11.6
	8	12.8
Average		11.5
RSD, %		16.9
4	1	10.1
	1	10.8
Average		10.5
	2	11.0
	4	11.6
	8	11.2
Average		11.3
RSD, %		5.1
5	1	8.7
	1	9.3
Average		9.0
	2	8.6
	4	9.2
	8	9.6
Average		9.1
RSD, %		4.5

[a] Averages are of duplicate analyses and serial dilution analyses. RSD is the relative standard deviation for the 5 analyses of each test sample.

Table 2. Deoxynivalenol (DON) found in 1993 grain sorted by type

Commodity	No. samples			DON, μg/g	
	Analyzed	DON, ≥0.5 μg/g	DON, >2 μg/g[a]	Av.[b]	Max.
Barley	118	79	57	3.0	14.0
Malting barley	29	29	26	9.0	25.8
Hard spring wheat	201	180	150	3.7	18.4
Hard winter wheat	194	94	26	0.8	7.6
Mixed wheat	1	1	1	2.3	2.3
Soft winter wheat	59	50	16	1.4	14.6
Soft white wheat	28	8	0	0.1	0.7
Total	630	441	276		

[a] FDA advisory level of 2 g/g set in 1982 for DON in wheat designated for milling (human consumption).
[b] Average DON level for all samples, including those with DON levels of <0.05 μg/g (0 μg/g used in calculations).

Table 3. Deoxynivalenol (DON) levels in 1993 U.S. wheat and barley

Commodity	State	No. samples			DON, μg/g	
		Analyzed	DON, ≥0.5 μg/g	DON, >2 μg/g[a]	Av.[b]	Max.
Barley	Idaho	12	0	0	ND[c]	ND
	Minnesota	54	54	46	5.5	14.0
	Montana	16	12	2	0.7	5.1
	North Dakota	65	42	35	4.8	25.8
Wheat	Arkansas	7	7	5	2.4	3.6
	California	7	0	0	ND	ND
	Colorado	16	1	0	0.1	0.1
	Georgia	4	3	0	0.4	1.2
	Indiana	6	5	1	3.3	14.6
	Kansas	73	47	19	1.3	7.3
	Kentucky	7	7	5	2.4	3.0
	Michigan	4	1	0	0.2	0.7
	Minnesota	94	92	81	4.7	18.4
	Missouri	12	11	1	1.0	2.8
	Mississippi	1	1	0	1.3	1.3
	Montana	4	2	2	1.1	2.8
	North Carolina	5	5	1	0.7	2.0
	North Dakota	62	55	41	2.7	9.1
	Nebraska	12	8	5	2.1	6.5
	Ohio	9	9	1	1.1	3.3
	Oklahoma	29	19	0	0.3	0.7
	Pennsylvania	2	0	0	ND	ND
	South Carolina	2	0	0	ND	ND
	South Dakota	37	37	29	3.7	7.6
	Texas	21	3	0	0.1	0.7
	Virginia	2	2	1	1.6	2.0
	Washington	66	18	1	0.1	3.8
	Wyoming	1	0	0	ND	ND
	Total	630	441	276		

[a] FDA advisory level of 2 μg/g set in 1982 for DON in wheat designated for milling (human consumption).
[b] Average DON level for all samples, including those with DON levels of <0.05 μg/g (0 μg/g used in calculations).
[c] ND, <0.5 μg/g.

Table 4. Deoxynivalenol (DON) found in 1993 wheat sorted by grade

Wheat grade	No. samples[a]	DON, μg/g	
		Range[b]	Av.
1	118	ND–3.4	0.4
2	120	ND–7.5	1.3
3	60	ND–14.6	3.0
4	46	ND–11.9	2.2
5	24	ND–13.8	3.8

[a] Some of the wheat samples collected were not graded.
[b] ND, <0.5 μg/g.

ples collected from Minnesota (85% of the samples contained DON levels >2 μg/g) and North Dakota (54% of the samples contained DON levels >2 μg/g). Most barley samples collected from Minnesota and North Dakota were malting barley. Most barley samples (better barley) collected from Idaho and Montana either were free from contamination or were contaminated at low levels.

Following the completion of risk analyses initiated in 1991, FDA issued a new advisory for DON on September 16, 1993 (personal communication, Letter of September 16, 1993, from R.G. Chesemore, Associate Commissioner for Regulatory Affairs, FDA, to State Agricultural Directors, State Food Central Officials, and Food, Feed, and Grain Trade Organizations). This advisory recommended a maximum DON level of 1 μg/g in finished wheat products (e.g., flour, bran, and germ) that po-

Table 5. Deoxynivalenol (DON) levels in 1991 U.S. wheat[a]

State	No. samples			DON, μg/g	
	Analyzed	DON, ≥0.5 μg/g	DON, >2 μg/g[b]	Av.[c]	Max.
			Winter wheat		
Alabama	4	4	2	1.7	4.1
Arkansas	13	13	7	2.6	4.5
Georgia	9	6	0	0.1	0.2
Illinois	29	29	14	4.9	40.3
Indiana	22	22	9	2.4	6.9
Kansas	4	4	2	2.4	4.9
Kentucky	7	7	3	2.3	3.9
Louisiana	2	2	0	0.6	0.8
Maryland	6	4	0	<0.1	0.1
Michigan	2	2	0	0.2	0.3
Minnesota	1	1	0	—	1.3
Missouri	36	35	26	4.5	11.5
Mississippi	2	2	1	1.7	2.0
Ohio	34	34	0	0.7	2.0
North Carolina	12	12	2	1.2	2.1
South Carloina	6	6	0	0.2	0.3
Tennessee	6	6	2	2.4	4.8
Virginia	7	7	0	0.5	1.6
Wisconsin	5	5	0	0.3	0.5
Total	207	201	68		
			Spring wheat		
Idaho	6	3	0	<0.1	0.1
Minnesota	16	15	0	0.4	1.0
Montana	34	1	0	<0.1	0.1
North Dakota	105	64	5	0.8	7.6
South Dakota	23	21	2	0.9	2.6
Washington	6	1	0	<0.1	0.2
Wisconsin	16	15	0	0.2	0.4
Total	206	120	7		

[a] Data compiled by G.E. Wood. Samples collected by FGIS. Analyses by FDA Kansas City, Minneapolis, New Orleans, New York, and Seattle District Laboratories.
[b] FDA advisory level of 2 g/g set in 1982 for DON in wheat designated for milling (human consumption).
[c] Average DON level for all samples, including those with DON levels of <0.05 μg/g (0 μg/g used in calculations).

tentially may be consumed by humans. No recommendation for unprocessed wheat was made even though high levels (>2 μg/g) of DON were found in many wheat samples. Reported studies demonstrate that DON levels can be reduced substantially (24–75%) during the milling process (23). Because the variability of industry practices could result in considerable differences in the reduction of DON levels in the finished product, no advisory was issued for the wheat itself. With regard to animal feeds, the advisory levels were 10 μg/g in grains and grain by-products fed to cattle and chickens and a recommendation limit of 50% of the diet, and 5 μg/g in grains and grain by-products fed to swine and all other animals and a 20% limit in swine rations, and a 40% limit in other animal feeds.

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461	462	463	464	465	466	467	468	469	470
471	472	473	474	475	476	477	478	479	480
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DRUGS, COSMETICS, FORENSIC SCIENCES

Electrochemical Behavior and Polarographic Assay of Nitroxynil

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University of Chile, Faculty of Chemical and Pharmaceutical Sciences, Laboratory of Bioelectrochemistry and Pharmacology, PO Box 233, Santiago-1, Chile

Nitroxynil is an anthelmintic veterinary drug used in prophylaxis and treatment of hepatic distomatosis. A simple method for its identification and determination in pharmaceutical parenteral solutions is described. The method is based on electrochemical reduction of the nitro group at the dropping mercury electrode. The ac and dc polarographic and cyclic voltammetric responses of the drug are reported. Accuracy and precision of the method are good, and sample preparation is easy.

Nitroxynil, 4-hydroxy-3-iodo-5-nitrobenzonitrile (Figure 1), is an anthelmintic drug widely used in prophylaxis and treatment of hepatic distomatosis, caused by the trematode *Fasciola hepatica* (1–3).

The drug is metabolized in the liver to 3-iodo-5-hydroxy-5-aminobenzonitrile and 3-iodo-4-hydroxy-5-nitrobenzamide by the action of enzymes in the cytosol and endoplasmic reticulum and cytochrome P450 (4). Cytochrome P450 is also the main system in the hepatic cell for detoxication of the hydroxylamine derivative, produced during in vivo reduction of nitroxynil and, consequently, the more susceptible cellular site that can be attacked by this reactive species (4, 5).

Nitroxynil has been quantitatively assayed by different methods, including gas chromatography/mass spectrometry for detection in cow milk, with a detection limit of 1.46 ppm (6); heterogeneous-phase titrimetry of the pure drug and its pharmaceutical form (7); and ultraviolet spectrophotometry for determination in plasma, urine, and tissues (8). The last technique appears as *official methodology* in the *British Pharmacopoeia (Veterinary)* (9).

Several reviews about polarographic and voltammetric analysis of drugs (10–12) show the wide applicability of these techniques in pharmaceutical analysis. Specifically, organic molecules with an aromatic nitro group are good candidates for electrochemical analyses, because they can be reduced easily and produce a good signal. Most papers deal with the well-defined polarographic reduction of the nitro group, adapted for direct quantitative determinations (13–16) or for determinations after an accumulation step (17, 18). However, no electrochemical method for nitroxynil has been reported.

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Our study reports the cathodic behavior of nitroxynil and describes a quantitative polarographic assay for determination of the drug in pharmaceuticals.

METHOD

Reagents and Solutions

(a) *Nitroxynil.*—99.6% activity, 100% chromatographically pure (Rhodia Mérieux Laboratories, Santiago, Chile).

(b) *Stock solutions.*—Prepare solutions of nitroxynil at a constant concentration of 2.5 mM in dimethylformamide (DMF).

(c) *Routine solutions*—Take 1 mL nitroxynil stock solution and dilute to 25 mL with 0.1M phosphoric acid–0.1M acetic acid buffer at pH 6.0 to obtain a final concentration of 0.1 mM in 10% DMF.

(d) *Buffer solutions.—(1) 0.1M acetic acid–0.1M phosphoric acid.*—pH 2–8. *(2) 0.1M phosphoric acid–0.1M sodium carbonate.*—pH 8.5–12. *(3) Universal buffer for pH 2–12.*—Containing 21.01 g citric acid, 13.61 g potassium phosphate, 6.18 g boric acid, 12.1 g Tris, and 7.46 g potassium chloride per liter.

(e) *Synthetic samples.*—Add excipients to the drug for recovery studies according to manufacturer's batch formulas for a nitroxynil concentration of 25 mg/100 mL.

All reagents used were analytical grade.

Equipment

(a) *Spectrophotometer.*—Shimadzu UV 160 A spectrophotometer with a 1 cm quartz cell.

(b) *Polarograph.*—An ac/dc-operated Tacussel assembly consisting of an EPL-3 recorder equipped with a TI-PULS module, similar to one previously described (19). Operating conditions: pulse amplitude, 60 mV; potential scan rate, 5 mV/s; drop time, 1 s; voltage range, 0 to −2000 mV; current range, 1.25 to 5 μA; temperature, 25°C.

(c) *Polarographic cell.*—Tacussel CPRA measuring cell with dropping mercury electrode as a working electrode, a platinum wire counterelectrode, and a saturated calomel reference electrode (SCE).

(d) *Cyclic voltammetry.*—This technique was carried out with a totally automated Inelecsa assembly similar to one previously described (20).

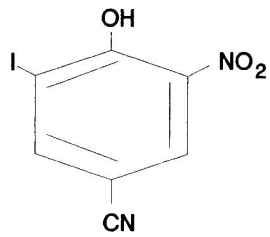


Figure 1. Structure of nitroxynil.

Quantitative Assay

(**a**) *Polarographic assay.*—Take 1 mL pharmaceutical solution (Dovenix, Rhodia Mèrieux Laboratories; injectable solution with a declared amount of 25 mg/100 mL nitroxynil) and dilute with distilled water to 100 mL. Take 2 mL of this solution and dilute to 100 mL with a mixture of DMF (10%) and 0.1M phosphoric acid–0.1M acetic acid buffer solution (90%); keep the ionic strength at 0.3M with KCl. Adjust final pH to 6.0, Then, add no less than 20 mL of the buffered solution to a dry polarographic cell and degas by bubbling nitrogen through the solution for 5 min. Scan the sample solution at least twice from –150 to –450 mV. Calculate the amount (mg) of nitroxynil in the sample solution from the standard calibration curve.

(**b**) *Spectrophotometric assay.*—Prepare the sample as described for *Polarographic assay.* Measure the sample solution absorptivity at 271 nm, with a mixture of DMF (10%) and 0.1M phosphoric acid–0.1M acetic acid buffer solution at pH 6.0 (90%) as a blank. Calculate the amount of nitroxynil in the sample solution (mg) from the standard calibration curve.

(**c**) *Calibration curve preparation for polarography and spectrophotometry.*—Prepare a 10-solution series containing nitroxynil concentrations ranging from 0.1 and 0.5 mM in a mixture of DMF 10% and 0.1M phosphoric acid–0.1M acetic acid buffer at pH 6.0 (90%).

(**d**) *Determination of apparent* pK_a.—For this study, the 271 nm UV band was selected and the pH range studied was from 2.0 to 12.0, varying each 0.5 pH unit. Near the pK_a zone, the pH was changed in increments of 0.25 pH unit per determination. The temperature was kept constant at 25°C. The nitroxynil concentration was 0.1 mM for the entire pH range; the nitroxyl was in a mixture of DMF (10%) and 0.1M acetic acid–0.1M phosphoric acid buffer (90%). The absorptivity values were used in the expression:

$$pK_a = pH - \log \frac{A - A_{min}}{A_{max} - A} \qquad (21)$$

Results and Discussion

Nitroxynil is reduced electrochemically at the dropping mercury electrode (DME) both in ac and dc mode (Figure 2). The polarographic response is strongly pH dependent. In acidic media (pH 2–5), 2 waves or peaks are observed (waves 1 and 2). However, at pH >6, the more cathodic wave disappears and

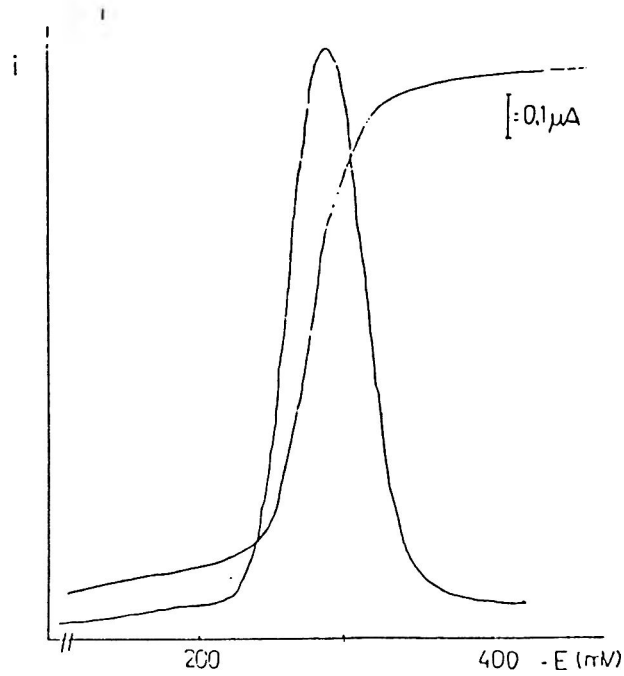


Figure 2. Typical dc wave and ac peak polarograms of 0.1 mM nitroxynil at pH 6.0.

only one peak (wave 1) remains. In dc mode, wave 1 is well resolved in the pH range studied (pH 2–12). The peak shifts to a more cathodic potential with increasing pH, showing a linear dependence of peak potential on pH in both ac and dc modes (Figure 3). The lines show a break at pH 4.5, with slopes of –79.0 and –50.8 mv/pH, before and after the break, respectively. This break implies a change in the mechanism of the cathodic reaction, probably due to a nitro group protonation, as observed with other nitro compounds such as nitrobenzene (22).

Figure 4 shows that the limiting current (i_{lim}) is not affected by the pH in both waves. This result disproves the concept of kinetic or catalytic control of the limiting current. Because the limiting current of the second wave is approximately twice that of the first wave, then the electron transfer that produces the second wave involves half the electrons involved in the first

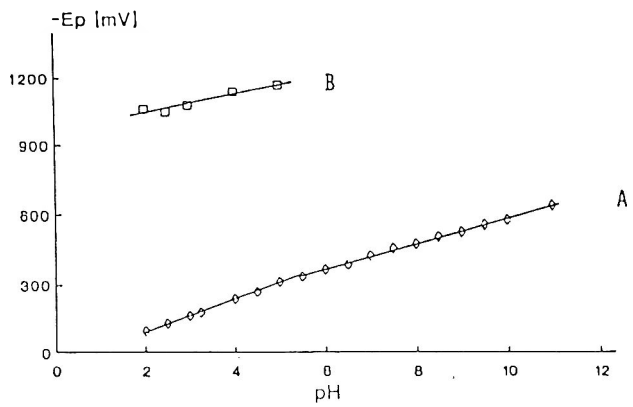


Figure 3. Dependence of peak potential on pH. (A) Peak 1; (B) peak 2.

wave. This result supports the hypothesis that the first wave is caused by 4-electron reduction to give the hydroxyl derivative, in agreement with nitro group reduction in protic media described for nitro compounds (23). The second wave in acidic medium is due to 2-electron reduction of the protonated hydroxylamine derivative to give the amine derivative as a final product.

With these results and the well-known nitroaromatic behavior (24), the electrode mechanism for nitroxynil reduction can be represented as follows:

First peak

pH <4.5

$$Ar\text{-}NO_2H^+ + 3H^+ + 4e^- \rightarrow Ar\text{-}NHOH + H_2O$$

pH >4.5

$$Ar\text{-}NO_2 + H^+ \rightleftharpoons Ar\text{-}NO_2H^+$$

$$Ar\text{-}NO_2H^+ + 3H^+ + 4e^- \rightarrow Ar\text{-}NHOH + H_2O$$

Second peak

pH <6.0

$$Ar\text{-}NH_2OH^+ + 2H^+ + 2e^- \rightarrow Ar\text{-}NH_3^+ + H_2O$$

The mechanism agrees with both the peak potential or the half-wave potential dependence on pH and the break observed at pH 4.5. The 2:1 current ratio found for the first and the second peaks is consistent with such a reduction.

On the basis of the current–pH behavior and the peak characteristics, peak 1 at pH 6.0 was selected for quantitative studies. Under this condition, the current is linearly dependent on the square root of the mercury column height ($r = 0.99$; slope = 0.242; intercept = –0.68). A similar dependence also is observed between current and temperature, with a temperature coefficient of <2%. Consistently, a plot of log i_p versus log t (drop time) is linear, with a slope of 0.68. These results support a diffusion-controlled electrode process (25).

To elucidate the contribution of adsorption of the electroactive species on the DME, a range of drug concentrations was tested (1.0–0.001 mM). The peak potential clearly depends on nitroxynil concentrations above 0.1 mM. Probably, the adsorption phenomenon operates at higher nitroxyl concentrations. However, the peak current is linearly dependent on drug concentration in this range.

Figure 5, a typical cyclic voltammogram of 1 mM nitroxynil at pH 6.0, shows a single irreversible peak with a cathodic potential of –370 mV. The peak shape suggests an adsorption phenomenon, which was indicated by the polarographic experiments. Also, the linear relationships between both potential peak (E_p) and peak current (i_p) and the logarithm of the sweep rate confirms a weak adsorption of the electroactive species according to Laviron's theory (26).

When the sweep rate was varied, the irreversible character of the cyclic voltammogram did not change, thus confirming that the reduction is totally irreversible, according to the following equation:

$$Ar\text{-}NO_2 + 4H^+ + 4e^- \rightarrow Ar\text{-}NHOH + H_2O$$

To elucidate the mechanism of action of this antiparasitic drug, we explored the feasibility of nitro radical anion formation. Several works (27, 28) report cyclic voltammetric evidence of nitro radical anion formation from nitroaromatic compounds in mixed media. However, despite our use of mixed media (DMF–aqueous buffer at pH 6.0, 20 + 80), no radical species was detected. This behavior could be explained by intramolecular protonation of the nitro radical anion by the proton of the neighboring hydroxyl group, blocking stabilization of the nitro radical anion, according to Scheme 1.

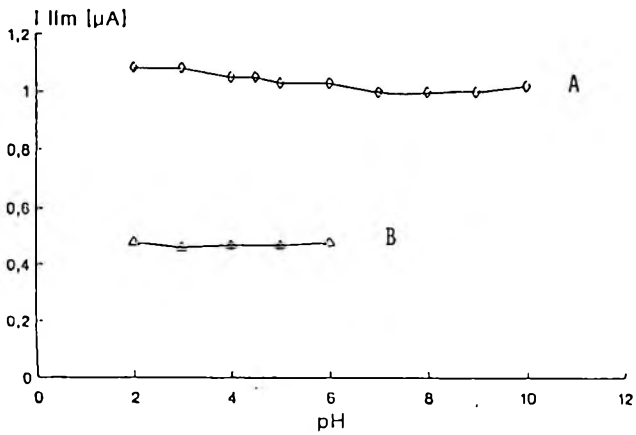


Figure 4. Dependence of limiting current on pH. (A) wave 1; (B) wave 2.

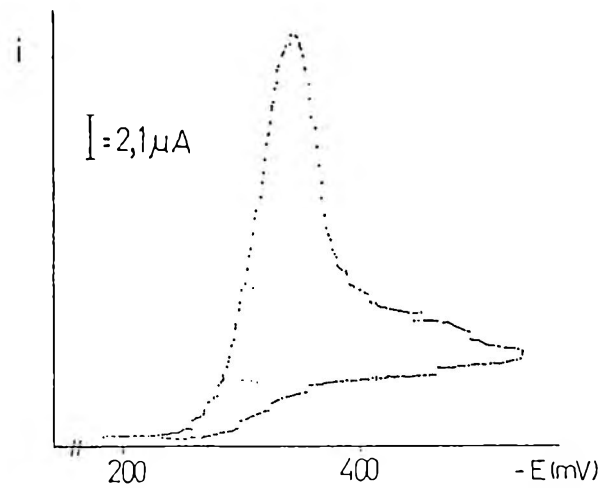
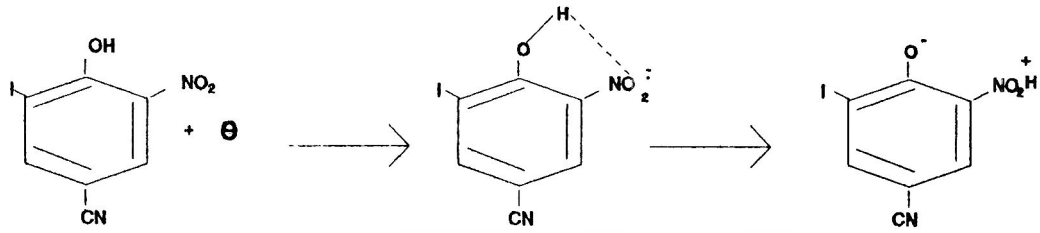


Figure 5. Cyclic voltammogram of 1 mM nitroxynil at pH 6.0 and a sweep rate of 1 V/s.



Scheme 1. Intramolecular protonation of the nitro radical anion in nitroaromatic compounds by the neighboring hydroxyl group.

To quantitate nitroxynil in dosage forms, the relation between current and concentration was determined. The calibration curve is described by the following regression curve:

$$i_p = 2153.13c + 0.097$$

where the correlation coefficient is 0.997 for 10 points between 0.1 and 0.5 mM, i_p is the peak current (in μA), and c is nitroxynil concentration (in mM).

Table 1 shows recoveries of synthetic samples containing nitroxynil at 25 mg/mL. The average recovery (100.6%) and standard deviation (1.6%) indicate good accuracy and precision. Reproducibility is adequate, with a coefficient of variation of 1.4%. Results of assays of individual parenteral solutions are shown in Table 2. Excipients do not interfere with nitroxynil determination. Method specificity is adequate because of the absence of interference from related compounds such as synthetic intermediates or by-products. The *British Pharmacopoeia (Veterinary)* procedure for nitroxynil suggests detection of both inorganic iodide and sulfated ash (9). In our polarographic conditions, any response due to these compounds is detected. Interference from other nitro drugs is not possible, because nitroxynil is the lone nitro drug in commercial formulations.

For comparison (Table 2), we used UV spectrophotometry method based on nitroxynil absorption at 271 nm (a procedure similar to that of the *British Pharmacopoeia [Veterinary]*). A 0.1M solution of nitroxynil in a mixture of aqueous acetic acid–phosphoric acid buffer (pH 6.0) (90%) DMF (10%) was used. For analytical determination, a calibration curve was used for nitroxynil concentrations between 0.4 and 1.2 mg/100 mL. The following regression line was obtained:

$$A_{271} = 0.60\ [c, \text{mg/100 mL}] - 0.0035$$

where the correlation coefficient is 0.9996, A_{271} is absorbance for 10 points, c is nitroxynil concentration, and 0.0035 is the intercept of the calibration curve.

From the pH-dependent absorption at 271 nm (Figure 6) it was possible to determine the pK_a of nitroxynil. The apparent pK_a was 2.7, similar to one previously obtained under different experimental conditions (100% ethanolic solution) (4).

Table 1. Recovery of synthetic nitroxynil[a]

Sample	Amount found, mg/mL	Recovery, %
1	26.00	104.0
2	25.10	100.4
3	25.25	101.0
4	24.90	99.6
5	25.63	101.2
6	25.05	100.2
7	25.15	100.6
8	24.80	99.2
9	25.13	100.5
10	24.55	98.2
Mean	25.16	100.62
Standard deviation	0.41	1.64
Coefficient of variation, %	1.63	1.63

[a] Each synthetic mix contained nitroxynil at 25 mg/mL, plus excipients.

Table 2. Assay for nitroxynil in individual parenteral solution[a]

	Amount found, mg/mL	
Sample	Polarography	UV spectrophotometry
1	25.5	25.5
2	26.0	25.2
3	25.4	25.7
4	25.7	26.0
5	26.7	25.0
6	25.3	25.5
7	25.7	25.9
8	25.5	26.2
9	25.8	26.2
10	25.6	25.1
Mean	25.7	25.6
Standard deviation	0.4	0.44
Coefficient of variation, %	1.6	1.73

[a] Dovenix, Rhodia Mèrieux, Santiago-Chile; declared amount, 25 mg/mL nitroxynil.

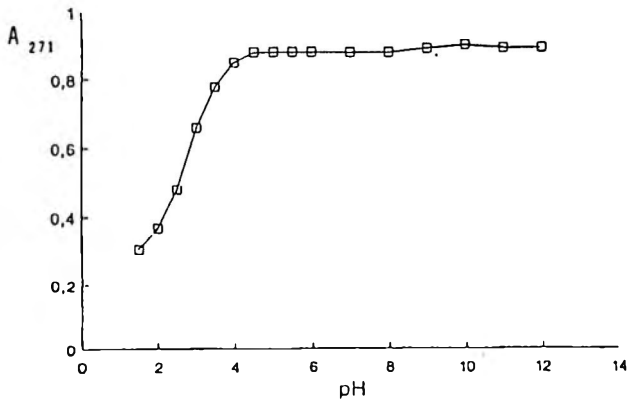


Figure 6. Dependence of nitroxynil (0.1 mM) absorptivity at 271 nm on pH.

Acknowledgments

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Liquid Chromatographic Fluorescence Method for Multiresidue Determination of Thiabendazole and 5-Hydroxythiabendazole in Milk

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A novel liquid chromatographic multiresidue method has been developed for quantitation of thiabendazole (TBZ), the metabolite 5-hydroxythiabendazole (5-OH-TBZ), and the sulfate conjugate of 5-hydroxythiabendazole (5-HSO_4-TBZ) in raw cow's milk. The 5-HSO_4-TBZ is hydrolyzed quantitatively under acidic conditions to 5-OH-TBZ. TBZ and 5-OH-TBZ are extracted from milk at pH 8.0 with ethyl acetate followed by cleanup of the extract on a cation-exchange solid-phase extraction column. Analytes are quantitated by liquid chromatography with a cation-exchange stationary phase and fluorescence detection. Recoveries from raw cow's milk samples fortified with TBZ and 5-OH-TBZ or TBZ and 5-HSO_4-TBZ at 0.05 to 2 ppm ranged from 87 to 103% for TBZ, 98–109% for 5-OH-TBZ, and 96–115% for 5-HSO_4-TBZ (quantitated as 5-OH-TBZ). The assay provides a simple, rapid, and sensitive multiresidue method for monitoring total residues of TBZ, 5-OH-TBZ, and 5-HSO_4-TBZ in milk.

Thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole; CAS registry number 148-79-8; TBZ] was discovered in 1961 by Merck scientists (1, 2). TBZ has been widely used as a broad-spectrum anthelmintic agent for domestic animals (1, 3–7) and as a pre- or postharvest fungicide for the control of a wide range of fungi affecting field crops and stored fruits and vegetables (8–11). TBZ use patterns provide an entry route for residues into the human food chain, either through direct exposure or through exposure of food animals to treated feed. Analytical procedures are needed to monitor TBZ residues and metabolites in agricultural commodities.

TBZ is metabolized in farm animals to 5-hydroxythiabendazole (5-OH-TBZ) and conjugated 5-OH-TBZ (glucuronide- and sulfate-conjugated forms) (Figure 1; 5, 12, 13). A recent study (14) of the metabolism of [^{14}C]-thiabendazole (^{14}C-TBZ) in lactating dairy goats, dosed orally with 120 mg ^{14}C-TBZ per day for 7 consecutive days, showed that only about 1% of the accountable administered dose is found in milk. Levels of ^{14}C-TBZ-related residues reached a plateau (about 1 ppm) within 3 days. The major TBZ metabolite found in milk was the sulfate conjugate of 5-OH-TBZ (5-HSO_4-TBZ). No significant residues of unconjugated 5-OH-TBZ was detected prior to hydrolytic treatment of 5-HSO_4-TBZ, and little, if any, parent TBZ or the glucuronide conjugate of 5-OH-TBZ was found. The finding that conjugated 5-OH-TBZ is the major TBZ metabolite in milk is consistent with earlier results of Tocco et al. (12) for a similar TBZ metabolism study conducted with lactating goats and dairy cows.

Numerous analytical methods using fluorescence spectrophotometry and liquid chromatography (LC) have been reported for quantitation of TBZ in plant and animal products (13, 15–23). At present, 2 methods (12. 24, 25) have been reported for determination of TBZ and 5-OH-TBZ in milk. The current method cited in the Food and Drug Administration (FDA) *Pesticide Analytical Manual* (24) for analysis of TBZ and 5-OH-TBZ residues in milk uses glusulase (an enzyme mixture of β-glucuronidase and sulfatase) to convert glucuronide and sulfate conjugates of 5-OH-TBZ to unconjugated 5-OH-TBZ, followed by liquid–liquid partitioning between ethyl acetate and 0.1N HCl. TBZ and 5-OH-TBZ are quantitated by spectrophotofluorometry. The FDA spectrofluorometric method for analysis of TBZ and 5-OH-TBZ (conjugated and unconjugated) residues is based on a method developed by Tocco et al. (12). The more recent (LC) method developed by Tai et al. (25) for determination of benzimidazoles in cow's milk quantitates TBZ and unconjugated 5-OH-TBZ in milk but does not quantitate conjugated 5-OH-TBZ (sulfate ester).

We describe a simple LC multiresidue method that uses the cationic nature of TBZ and 5-OH-TBZ for extract cleanup and inherent fluorescence for LC detection and quantitation of total residues of TBZ, 5-OH-TBZ, and 5-HSO_4-TBZ in milk. The method is based on a similar LC method for determination of TBZ residues in bananas (26).

METHOD

Apparatus

(a) *Homogenizer.*—Waring commercial blender, Model 31BL41 (Waring Products Division, New Hartford, CT).

(b) *Variable-speed touch mixer.*—Vortex-Genie, Model K-550-G (Scientific Industries, Bohemia, NY).

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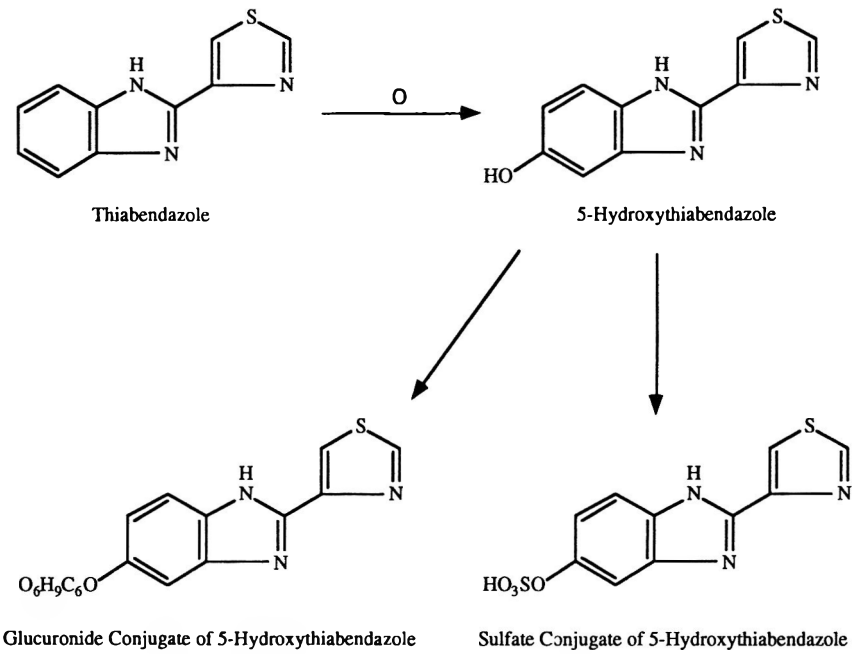

Figure 1. Metabolism of thiabendazole (13).

(c) *General purpose utility oven.*—Type 3-H, style 288 (Despatch Oven, Minneapolis, MN).

(d) *pH meter.*—Model 611 (Orion Research, Cambridge, MA).

(e) *Reciprocating shaker.*—Model 6000 (Eberbach, Ann Arbor, MI).

(f) *Centrifuge.*—IEC Model HN-SII (International Equipment, Needham Heights, MA).

(g) *Vacuum manifold.*—United Chemical Technologies, Horsham, PA.

(h) *Solid-phase extraction (SPE) column.*—Regular Bond Elut, PRS (propylsulfonic acid); 500 mg/2.8 mL (Varian Sample Preparation Products, Harbor City, CA). Regular Bond Elut accessories: reservoir (empty), 25 mL capacity; regular Bond Elut adaptor; Luer stopcock (Varian Sample Preparation Products).

(i) *LC column.*—PartiSphere SCX (benzenesulfonic acid); particle size, 5 μm; 12.5 cm × 4.6 mm (Whatman, Inc., Clifton, NJ).

(j) *LC system.*—Model 114M solvent delivery module–analytical (Beckman Instruments, Fullerton, CA); WISP Model 710B autosampler (Waters Associates, Milford, MA); golden foil CH-1530 basic model column heater (Systec, Minneapolis, MN); fluorescence detector, Model RF-551, equipped with a 12 μL flow cell (Shimadzu, Japan); ChromJet integrator/SP4400 integrator (Thermo Separation Products, San Jose, CA).

(k) *LC conditions.*—Flow rate, 1.0 mL/min; injection volume, 50 μL; column temperature, 25.0°C or ambient; TBZ detection: 305 nm excitation, 380 nm emission; 5-OH-TBZ detection: 318 nm excitation, 525 nm emission, 1.5 s response, high sensitivity.

Reagents

(a) *TBZ and 5-OH-TBZ.*—Analytical standard (Merck, Rahway, NJ).

(b) *5-HSO_4-TBZ, sodium salt.*—Prepared by a modification of the procedure of Tocco et al. (27). To 210 mg 5-OH-TBZ in 6 mL dry pyridine, add 1.0 g sulfur trioxide trimethylamine complex and shake vigorously for 24 h on a mechanical shaker. To the reaction mixture, add 7 mL water and adjust pH to 8.2 with 1N NaOH. Extract the clear yellow solution 3 times with 20 mL portions of ethyl acetate and discard the ethyl acetate extracts. Transfer the aqueous solution to a Petri dish and concentrate to dryness under a fume hood overnight. Wash the pale yellow precipitate twice with 5 mL portions of methanol and air-dry the solids. Transfer the white powder to a test tube and wash once with 2 mL water and twice with 5 mL portions of acetone, and dry in a vacuum oven for 2 h at 40°C. Approximately 155 mg of nearly white sodium salt of 5-HSO_4-TBZ is obtained. Purity is 65.8%, based on HCl hydrolysis to 5-OH-TBZ (a similar purity based on sulfatase hydrolysis was obtained).

(c) *Sulfur trioxide trimethylamine complex.*—98% (Aldrich Chemical, Milwaukee, WI).

(d) *Pyridine, anhydrous.*—99% (Aldrich).

(e) *Ethyl acetate, methanol (MeOH), acetonitrile (ACN), and water (H_2O).*—LC grade.

(f) *HCl.*—37%, ACS reagent.

(g) *H_3PO_4.*—85%, LC grade.

(h) *NaOH.*—Certified ACS.

(i) *KH_2PO_4.*—LC grade.

(j) *pH 8 buffer.*—Mix 250 mL 0.2M KH_2PO_4 and 234 mL 0.2M NaOH in a 1 L volumetric flask and dilute to the mark with water.

(k) *SPE column-conditioning solution.*—1% H_3PO_4 in MeOH–H_2O (80 + 20). To a 200 mL volumetric flask, add 40 mL water and 2 mL H_3PO_4 (85%). Dilute to 200 mL with MeOH.

(**l**) *SPE column elution solution.*—0.1M KH_2PO_4 in ACN–H_2O (30 + 70). Dissolve 2.8 g KH_2PO_4 in ca 100 mL water. Add 60 mL ACN and dilute to 200 mL with water.

(**m**) *LC mobile phase.*—Dissolve 6.8 g KH_2PO_4 in 800 mL water. Add 200 mL ACN, shake, and adjust the pH of the solution to about 3.8 with H_3PO_4..

Preparation of Standard Stock Solutions

(**a**) *TBZ and 5-OH-TBZ stock solutions (100 μg/mL).*—Accurately weigh ca 20 mg each TBZ and 5-OH-TBZ reference standards into separate 200 mL volumetric flasks. Dissolve the solids in ca 100 mL MeOH by sonication and dilute each flask to the 200 mL mark with MeOH.

(**b**) *5-HSO_4-TBZ stock solution (100 μg/mL).*—Accurately weigh ca 30 mg 5-HSO_4-TBZ (sodium salt) reagent into a 200 mL volumetric flask. Dissolve the solids in ca 100 mL MeOH by sonication and dilute the flask to the 200 mL mark with MeOH.

(**c**) *Intermediate stock solutions (5 μg/mL).*—Transfer 2.50 mL of the 100 μg/mL TBZ, 5-OH-TBZ, and 5-HSO_4-TBZ stock solutions to separate 50 mL volumetric flasks and dilute each flask to the 50 mL mark with MeOH.

Preparation of LC Working Standards

Transfer 250, 500, 750, 1000, and 1300 μL aliquots of the 5 μg/mL TBZ and 5-OH-TBZ intermediate stock solutions into separate 25 mL volumetric flasks. Dilute each flask to the 25 mL mark with LC mobile phase. Each flask contains a mixture of TBZ and 5-OH-TBZ at ca 50, 100, 150, 200, and 250 ng/mL.

Sample Preparation

The raw milk sample obtained from Holstein cows (Merck, Branchburg, NJ) is homogenized with a high-speed blender and stored at −10°C or lower if not used immediately.

Fortifications (Method Recovery Samples)

To 5 g aliquots of control raw cow's milk in separate 50 mL glass centrifuge tubes, add TBZ and 5-OH-TBZ or TBZ and 5-HSO_4-TBZ at the following fortification levels for each analyte: 0 (control), 0.05, 0.4, and 2 ppm. Recovery of analyte is determined by comparison of the amount of analyte added with the amount of analyte found.

Hydrolysis of 5-HSO_4-TBZ

To 5 g milk in a 50 mL glass centrifuge tube, add 2.5 mL concentrated HCl and heat for 4 h at 85°–90°C. Cool the solution to ambient temperature. Add 5 mL 6M NaOH, shake, and cool the mixture to room temperature. Adjust the pH of the hydrolyzed milk sample to pH 8.0 with appropriate volumes of 6M and 0.2M NaOH. Add 5 mL of pH 8 phosphate buffer.

Extraction and Cleanup

Add 20 mL ethyl acetate, cap, and shake on a reciprocating shaker for 15 min. Centrifuge at 3200 × *g* (4000 rpm) for 5 min. With a Pasteur pipet with an attached rubber bulb, quantitatively transfer the ethyl acetate layer to a PRS SPE column preconditioned with (*1*) 10 mL 1% H_3PO_4 in MeOH–H_2O (80 + 20), (*2*) 3 mL MeOH, and (*3*) 5 mL ethyl acetate. Allow the extract to drain from the SPE column. Repeat extraction of TBZ and 5-OH-TBZ from the hydrolyzed milk sample with another 20 mL portion of ethyl acetate and quantitatively transfer the ethyl acetate layer to the loaded PRS SPE column. Wash the SPE column with 5 mL ethyl acetate. Air dry the loaded PRS SPE column with vacuum. Elute the analytes from the SPE column with 9.5 mL 0.1M KH_2PO_4 in ACN–H_2O (30 + 70). Collect the eluate in a 10 mL volumetric flask and dilute to volume with the SPE column elution solution.

Determination

If required, dilute appropriate volumes of the final sample extract with LC mobile phase to give an analyte concentration of ca 150–200 ng/mL. Determine the linear regression coefficients for the standard calibration curve from the plot of the analyte chromatographic peak response (area or height) versus the corresponding concentration (ng/mL) of analyte in LC working standards (50–250 ng/mL). Curves should be linear with a coefficient of determination (r^2) greater than 0.98. Calculate the amount of analyte in the sample with the following equation:

$$\text{Analyte in sample, ppm} = C \times V \times 10/W \times 1000$$

where *C*, concentration of analyte in the LC sample solution (ng/mL); *V*, volume (mL) to which 1 mL of final extract is diluted or the dilution factor (*V* is equal to 1 if no further dilution of the sample extract is made); and *W*, weight of sample (g).

Results and Discussion

Results from the study of the metabolism of ^{14}C-TBZ in lactating dairy goats (14) indicate that ethyl acetate extraction of untreated milk (or raw milk) yields an organic fraction free from radioactivity, whereas ethyl acetate extraction of milk pretreated with either glusulase, sulfatase, or 6N HCl digestion gives an organic fraction containing radioactivity, with 5-OH-TBZ as the primary component of the organic-extractable fraction. Ethyl acetate extraction of milk treated with β-glucuronidase gives a nonradioactive organic fraction, indicating that little, if any, of the glucuronide conjugate of 5-OH-TBZ is present in milk. Because metabolism studies in lactating dairy goats (14) indicate that TBZ is metabolized chiefly to the 5-HSO_4-TBZ in milk, an analytical procedure was developed to quantitate not only parent TBZ but also the free and sulfate-conjugated 5-OH-TBZ in milk. The 5-HSO_4-TBZ can be hydrolyzed to 5-OH-TBZ either by overnight enzymatic digestion with glusulase or by acid hydrolysis (12, 27). We used acid hydrolysis, because it can be completed in 4 h compared with overnight reaction required by the enzymatic digestion. Hydrolysis of 5-HSO_4-TBZ with HCl at 85°–90°C for 4 h gave quantitative conversion of 5-HSO_4-TBZ to 5-OH-TBZ, as measured by LC (Figure 2).

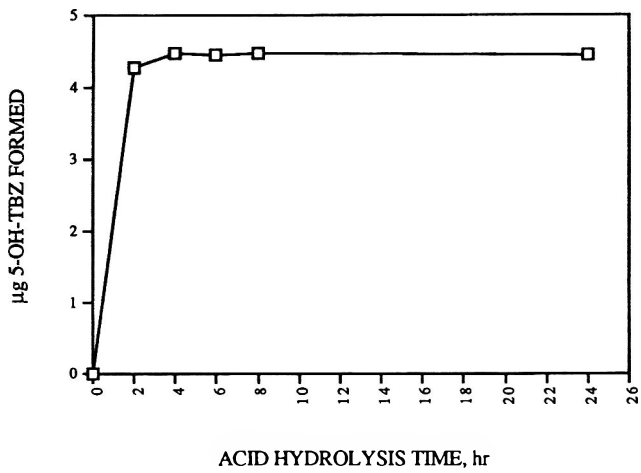

Figure 2. Time course for conversion of 5-hydroxythiabendazole sulfate (sodium salt) to 5-hydroxythiabendazole; hydrolysis conditions: 12M HCl, 85°–90°C.

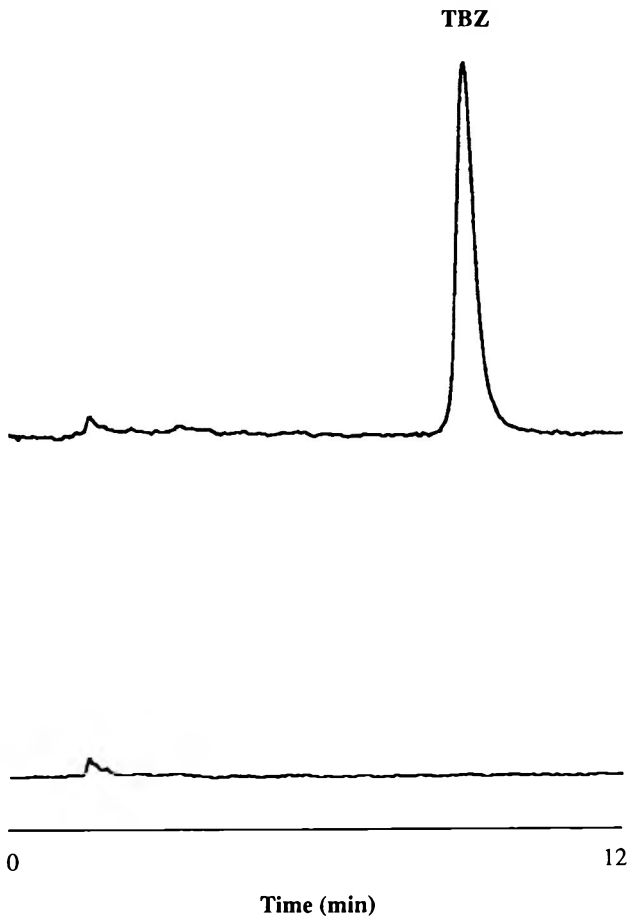

Figure 3. Typical chromatograms for milk fortified with TBZ at 0.4 ppm (top) and control milk (bottom). Approximate retention time for TBZ is 9.0 min. LC conditions: column, PartiSphere SCX (benzenesulfonic acid), 125 × 4.6 mm, particle size, 5 µm; mobile phase, 0.05M KH_2PO_4 in ACN–H_2O, 20 + 80 (pH = 3.8); flow rate, 1.0 mL/min; column temperature, 25.0°C or ambient; detector, fluorescence, TBZ excitation, λ = 305 nm, TBZ emission, λ = 380 nm; injected volume, 50 µL.

Chromatography and Analysis

LC with a cation-exchange stationary phase and fluorescence detection was used to analyze TBZ and 5-OH-TBZ in all milk samples. Milk samples fortified with TBZ, 5-OH-TBZ, or 5-HSO_4-TBZ (sodium salt) gave sharp TBZ and 5-OH-TBZ peaks, after acid hydrolysis of 5-HSO_4-TBZ, with elution times of about 9.0 and 5.6 min, respectively. Because TBZ and 5-OH-TBZ have significantly different fluorescence emission spectra ($TBZ_{emission\ max}$ = 380 nm, 5-OH-$TBZ_{emission\ max}$ = 525 nm), 2 separate LC runs were necessary to quantitate both TBZ and 5-OH-TBZ with a grating monochromator fluorescence detector. Alternatively, a dual-grating monochromator fluorescence detector with a time-programmable wavelength function or a filter fluorescence detector equipped with a 308 nm excitation filter and a 345 nm emission filter could be used to quantitate both TBZ and 5-OH-TBZ in the same chromatographic run. Figures 3 and 4 show typical chromatograms for raw cow's milk fortified with TBZ and 5-OH-TBZ at 0.4 ppm each and control milk. No chromatographic interferences (<0.005 ppm apparent TBZ or 5-OH-TBZ) from the control milk were observed in the region of TBZ and 5-OH-TBZ elution.

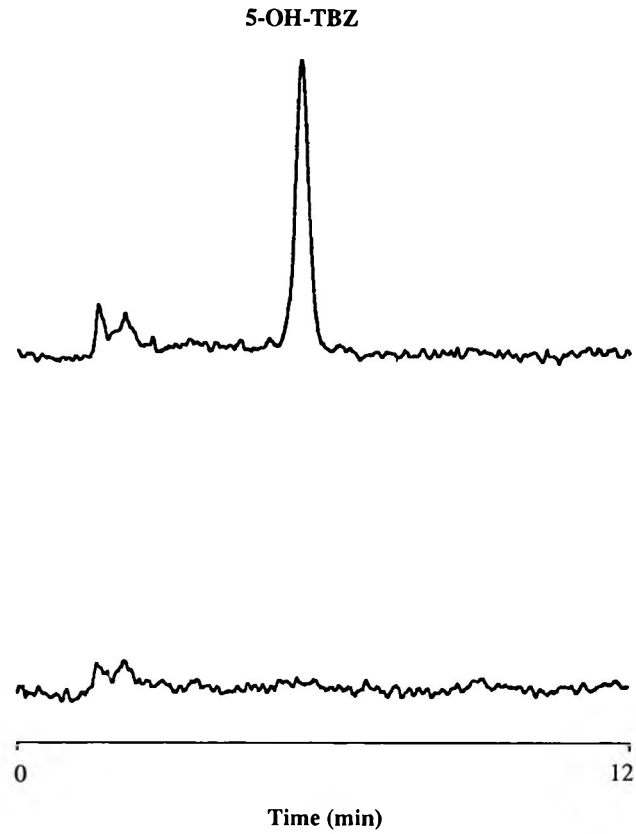

Figure 4. Typical chromatograms for milk fortified with 5-OH-TBZ at 0.4 ppm (top) and control milk (bottom). Approximate retention time for 5-OH-TBZ is 5.6 min. LC conditions: as described in the caption for Figure 3, except detector, fluorescence, 5-OH-TBZ excitation, λ = 318 nm, 5-OH-TBZ emission, λ = 525 nm.

Table 1. Recovery of thiabendazole residues from raw cow's milk fortified with TBZ, 5-OH-TBZ, and 5-HSO_4-TBZ (sodium salt)

Analyte	Fortification level, ppm	Recovery range, %	n^a	Mean, %
TBZ	0	ND[c]	4	—
	0.05[b]	87–92	4	90
	0.4	88–92	4	90
	2	94–103	4	98
5-OH-TBZ	0	ND	2	—
	0.05[b]	100–106	2	103
	0.4	105–109	2	107
	2	98–101	2	100
5-HSO_4-TBZ (Sodium salt)	0	ND	2	—
	0.05[b]	96–104	2	100
	0.4	102–115	2	108
	2	108–108	2	108

[a] Number of replicates.
[b] Final eluted sample volume from PRS SPE column, 2.0 mL.
[c] Not detectable, <0.005 ppm.

Standard calibration curves for each analyte were linear (r^2 >0.99) over a range of 2.5–12.5 ng TBZ and 5-OH-TBZ injected. The limit of quantitation (LOQ), defined as the lowest TBZ and 5-OH-TBZ fortification levels for which recovery data were deemed acceptable, was 0.05 ppm for both TBZ and 5-OH-TBZ in raw cow's milk. LOQ could be much lower than those stated; however, no recoveries of TBZ, 5-OH-TBZ, or 5-HSO_4-TBZ below the 0.05 ppm level in milk were determined. Because the method uses fluorescence detection, other pesticides or fungicides that are not inherently fluorescent are not expected to interfere.

Recoveries from Milk

Average recoveries of TBZ and 5-OH-TBZ or TBZ and 5-HSO_4-TBZ from untreated milk fortified in duplicate with 0.05, 0.4, and 2 ppm each of TBZ and 5-OH-TBZ or TBZ and 5-HSO_4-TBZ (sodium salt) are tabulated in Table 1. Recoveries of TBZ ranged from 87 to 103%, with an overall average recovery of 92% and a coefficient of variation (CV, 1σ) of 5.4%. Recoveries of 5-OH-TBZ ranged from 98 to 109%, with an overall average recovery of 103% and a CV (1σ) of 4.0%. Recoveries of 5-HSO_4-TBZ ranged from 96 to 115%, with an overall average recovery of 106% and a CV (1σ) of 6.1%.

Acknowledgments

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DRUGS, COSMETICS, FORENSIC SCIENCES

Liquid Chromatographic Determination of Tolrestat and Related Compounds in Raw Materials

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A liquid chromatographic method was developed for the determination of tolrestat and 7 related compounds. The lower limit of quantitation of the related compounds in the presence of the drug was <0.05%. The coefficient of variation on the assay of drug raw material analyzed on 5 different days was 0.75%. Total impurities found in 2 samples of tolrestat from different sources were 0.12 and 0.54%, respectively. The method was validated by a second laboratory.

Tolrestat is an aldose reductase inhibitor used in the treatment of complications due to diabetes. Chemical names and structures of the drug and related compounds available for method development at the time of this work are given in Figure 1. Tolrestat (compound VIII) can be synthesized from compound V by treatment of the acid chloride of comound V with methyl sarcosinate followed by further treatment with phosphorus pentoxide to give the methyl ester, which is hydrolyzed to tolrestat with potassium hydroxide (1). Of the related compounds available, V is a starting material, and I, II, and III are potential impurities in V. Compounds IV and VI are side-reaction products and VII is an intermediate. Tolrestat exists in *trans* form in the solid state. However, in solution, it rapidly equilibrates to a mixture of *cis* and *trans* rotamers of the thioamide functionality (2). Most of the available related compounds also have amide functionalities, and they too equilibrate in solution to a mixture of rotamers.

A liquid chromatographic (LC) method for the determination of degradation products in tolrestat was published (3), but this method does not provide for the quantitation of the related compounds available to us. We describe a validated method for the determination of the drug and of the related compounds in the presence of the drug. A major problem during method development was to control the rate of isomerization from the *trans* to the *cis* rotamer so that the related compounds could be determined. No monographs exist for tolrestat in the United States Pharmacopeia (4) or British Pharmacopoeia (5).

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METHOD

Apparatus

(a) *LC system.*—Model 5060 pump (Varian, Walnut Creek, CA) fitted with a 5 µL loop injector (Rheodyne, Model 7126, Cotati, CA), a UV detector set at 229 nm (Varian Model UV-100), an autosampler (Spectra Physics, Model SP8780 XR, San Jose, CA), and a data station (Varian Vista; Model 650).

(b) *LC column.*—Octadecyl silane bonded phase, 150 × 3.9 mm, 5 µm thickness (Waters Resolve, Milford, MA). used at ambient temperature.

(c) *Other equipment.*—UV/vis spectrophotometer (Varian; Model DMS 90) connected to an HP-85 computer and HP-7470A plotter (Hewlett-Packard, Avondale, PA); Sybron–Barnstead water purification system.

Reagents

(a) *Chemicals.*—LC grade acetonitrile (J.T. Baker Co., Phillipsburg, NJ), tetrahydrofuran and tetrabutylammonium hydroxide, 40% in water (Aldrich Chemical Co., Inc., Milwaukee, WI), and ammonium phosphate monobasic and phosphoric acid (85%) (Fisher Scientific, Nepean, ON, Canada). Ammonium hydroxide (30%) (J.T. Baker).

(b) *Tolrestat related compounds.*—Compounds I, II, III, and IV were from Ayerst (Montreal, PQ, Canada); compounds I, V, VI, and VII, from Finorga (Rhone, France). Infrared and mass spectra of these compounds were consistent with their respective structures. Tolrestat raw materials were obtained directly from the manufacturers.

(c) *Mobile phase buffer solution.*—Ammonium phosphate monobasic, 0.05M, adjusted to pH 3.5 with 0.05M phosphoric acid.

(d) *Mobile phase.*—Buffer–acetonitrile–tetrahydrofuran–tetrabutylammonium hydroxide (40% in water) (615 + 205 + 185 + 3). Filter solution through a 0.45 µm filter. Mobile phase flow rate was 1.0 mL/min.

(e) *Dissolution solution buffer.*—Phosphoric acid, 0.01M, adjusted to pH 7.0 with 0.01M ammonium hydroxide.

(f) *Dissolution solution.*—A 350 mL aliquot of acetonitrile made up to 1 L with dissolution solution buffer.

(g) *System suitability solution.*—Tolrestat, 0.1 mg/mL, and 0.1 mg/mL propylparaben in dissolution solution. Sonication with gentle shaking may be necessary for complete dissolution of the drug.

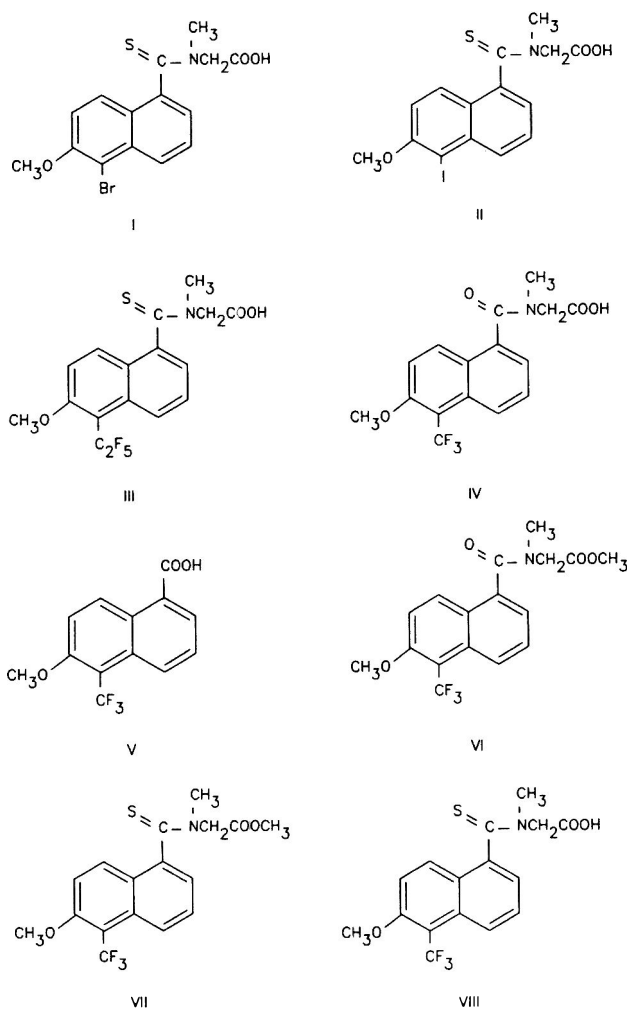

Figure 1. Structure of tolrestat, *N*-{[6-methoxy-5-(trifluoromethyl)-1-naphthalenyl]thioxomethyl}-N-methylglycine (VIII), and available related compounds: I, *N*-[(6-methoxy-5-bromo-1-naphthalenyl)thioxomethyl]-*N*-methylglycine; II, *N*-[(6-methoxy-5-iodo-1-naphthalenyl)-thioxomethyl]-*N*-methylglycine; III, *N*-{[6-methoxy-5-(pentafluoroethyl)-1-naphthalenyl]thioxomethyl}-*N*-methylglycine; IV, *N*-{[6-methoxy-5-(trifluoromethyl)-1-naphthalenyl]carbonyl}-*N*-methylglycine; V, 6-methoxy-5-trifluoromethyl-1-naphthoic acid; VI, *N*-{[6-methoxy-5-(trifluoromethyl)-1-naphthalenyl]carbonyl}-*N*-methylglycine methyl ester; and VII, *N*-{[6-methoxy-5-(trifluoromethyl)-1-naphthalenyl] thioxomethyl}-N-methylglycine methyl ester.

Related Compounds in Tolrestat Raw Material

(a) *Related compounds standard solution.*—Accurately weigh to obtain 0.005 mg/mL tolrestat in dissolution solution.

(b) *Related compounds test solution.*—Accurately weigh to obtain 2.0 mg/mL tolrestat in dissolution solution.

(c) *System suitability test.*—Inject a 5 µL aliquot of the system suitability solution into the LC system. The resolution between tolrestat and propylparaben is not less than 7. The efficiency of the column is not less than 12 000 plates per meter.

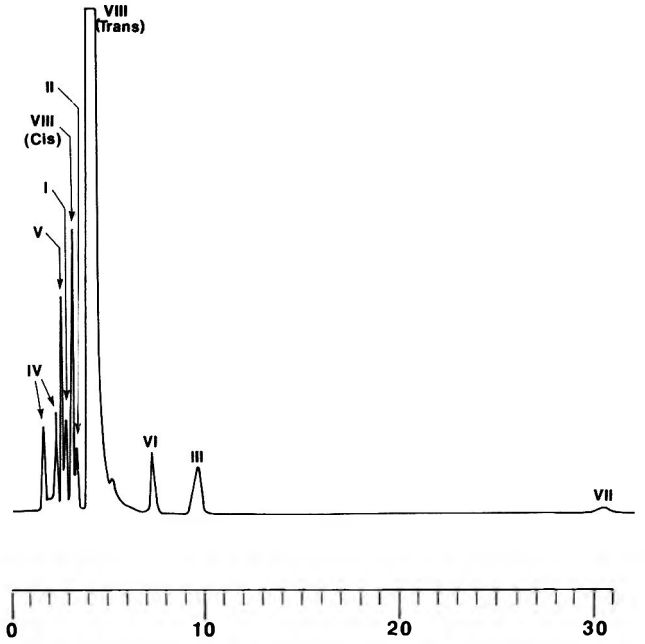

Figure 2. Chromatogram showing resolution of the related compounds and *cis*-tolrestat from the drug (VIII). Related compounds were at the 0.1% level relative to 20 µg tolrestat on-column.

The tailing factor is less than 2, calculated from the tolrestat peak. The retention time of tolrestat is between 4.0 and 5.3 min, and the relative retention time of propylparaben is 1.8. Inject 6 aliquots of the standard solution; the relative standard deviation is not more than 5.0%.

(d) *Procedure.*—Separately inject 5 µL of the standard and test solutions into the chromatograph and let run for 30 min. Calculate the amount of each impurity in the test solution as a percentage of the total amount of drug:

$$\text{Impurity},\% = 100\,\frac{A_i}{A_s} \times \frac{C_s}{C_u}$$

where A_i is the peak area response due to each individual impurity, A_s is the peak area response due to tolrestat in the standard solution, and C_s and C_u are the concentrations of tolrestat in the standard and test solutions, respectively.

Assay of Tolrestat Raw Material

(a) *Assay standard solution.*—Accurately weigh to obtain 0.1 mg/mL tolrestat reference standard in dissolution solution.

(b) *Assay test solution.*—Accurately weigh to obtain 0.1 mg/mL tolrestat in dissolution solution.

(c) *System suitability.*—The requirements for resolution, column efficiency, and tailing, stated previously for related compounds, are met. Inject 5 aliquots of the assay standard solution. The relative standard deviation is not more than 1.0%.

(d) *Procedure.*—Separately inject 5 µL of the standard and test solutions into the chromatograph and let run for 10 min.

Table 1. Impurities (%) in tolrestat raw materials

	Retention time relative to tolrestat at 4.3 min								
Sample	0.41	0.58	0.69	0.78	1.6	1.8	5.2	7.4	Total
A	0.11	0.14	0.02	NR[a]	0.01	0.19	0.04	0.03	0.54
B	0.05	0.07	—	NR[a]	—	—	—	—	0.12

[a] Not reported; this peak corresponds to the *cis* rotamer of tolrestat.

Calculate the percentage of tolrestat from the following equation:

$$\text{Tolrestat},\% = 100\frac{A_U}{A_S}\times\frac{C_S}{C_U}$$

where A_U and A_S are the peak area responses of the tolrestat peaks in the test and standard solutions, respectively; and C_S and C_U are the concentrations of tolrestat in the standard and test solutions, respectively.

Results and Discussion

A chromatogram showing the resolution of tolrestat from the available related compounds is shown in Figure 2. Two samples of tolrestat raw material were available at the time this work was done. Impurities found in these samples are listed in Table 1 and their UV characteristics are given in Table 2. Sample A was assayed using sample B as the standard because no other standards were available. The mean result of assays on 5 different days was 99.4%, and the coefficient of variation was 0.75%.

Relative retention times and responses on the LC system are given in Table 3. All compounds were resolved from the drug and each other, although compounds I and V were quite close to each other. Relative responses ranged from 0.63 to 1.59. Related compounds quantitated against tolrestat may be under or overestimated by approximately 40% (for compound II) to 60% (for compound V), but this variance is acceptable for low levels of nontoxic impurities. Response to all related compounds was linear from about 0.05 to 2.0% of the drug raw material. Minimum quantitatable amounts were less than 0.05%.

Table 2. UV characteristics of tolrestat and related compounds[a]

Compound	Maxima, nm	Absorbance[b]	Relative absorptivity	Conc, µg/mL
I	236, 279	0.966	0.94	8.8
II	205, 240, 277	0.427	0.62	5.9
III	228, 248, 270	0.886	0.95	8.0
IV	227, 284, 295	1.127	1.27	7.6
V	228, 287, 298	1.345	1.60	7.2
VI	227, 284, 295	1.185	1.27	8.0
VII	227, 245, 276	0.786	1.02	6.6
Tolrestat	228, 248, 272	1.208	1.00	10.3

[a] In a solution of methanol–acetonitrile–water (2 + 8 + 15).
[b] Absorbance measured at 229 nm.

Solution Stability

Tolrestat, which is *trans* in the solid state (2), and many of the related compounds isomerize to a *cis–trans* equilibrium in solution. To avoid increasing the complexity of the chromatogram and making the number and amount of impurities virtually impossible to determine, conditions that minimized isomerization over the time required to do the analysis were necessary. The rate of isomerization of drug in the dissolution solution was determined as a function of buffer type, concentration, and pH. Under the analytical conditions described in this report, the rate of formation of the *cis*-isomer at room temperature was 0.28% per hour, and the correlation coefficient was 0.999 (Table 4). Solutions were prepared just before use and were stored at 4°C.

System Suitability Parameters

Propylparaben was chosen as a component for the system suitability solution rather than one of the related compounds because it is commercially available, stable in the dissolution solution, does not undergo isomerization, and elutes at an appropriate retention time. System suitability parameters determined on 2 Resolve C_{18} columns illustrate the adequacy of the system suitability requirements specified in the method (Table 5).

Table 3. Linearity of tolrestat and related compounds

Compound	RRT[a]	Response[b]	Range[c]	Slope[d]	Intercept
I	0.69	0.88	0.05–2.0	1050	280
II	0.83	0.63	0.05–2.0	760	290
III	2.41	0.84	0.05–2.0	1000	–280
IV	0.41, 0.57[e]	0.82	0.05–1.0	1000	1270
V	0.66	1.59	0.05–2.0	1900	–40
VI	1.83	1.33	0.05–2.0	1600	60
VII	6.70	0.98	0.1–2.0	1170	–900
Tolrestat	1.00	1.00	0.05–2.0	1200	–330

[a] Relative retention time based on tolrestat at 4.6 min.
[b] Peak area response relative to that of tolrestat.
[c] As a percentage of the amount of tolrestat called for by the method and corresponding to 10 µg tolrestat on-column.
[d] The units of slope and intercept are area counts per nanogram and area counts, respectively. Correlation coefficients were 0.9990 or better.
[e] The sample of this compound contained 2 rotamers.

Table 4. Isomerization rate of *trans*-tolrestat[a]

Time, min	*cis*-Tolrestat, %[b]
0	0.03
30	0.16
60	0.31
90	0.45
120	0.60
150	0.73
180	0.86
210	0.99
240	1.12

[a] In dissolution solution at room temperature.
[b] Determined by LC method described in this report.

Ruggedness

A change in buffer component pH of the mobile phase from 3.5 to 7 decreased the retention time of tolrestat from 4.3 to 3.1 min and caused compound IV to elute at the solvent front. Decrease in tetrahydrofuran to 180 parts from 185 forced compound VII to elute at >30 min. An increase in tetrahydrofuran to 200 parts and a decrease in acetonitrile to 200 parts from 205 brought the retention time of tolrestat to 3.2 min and drove one rotamer of compound IV into the solvent front. When tetrahydrofuran was replaced by acetonitrile, tolrestat eluted at 7 min and compound VII did not elute at all. These changes affected the resolution of other related compounds from each other, but the compounds were still resolved from the drug.

Method Evaluation

The method was evaluated in a second laboratory by using completely different apparatus and chemicals. System suitability parameters measured on different days, compared to the requirements of the method, are shown in Table 6. Two sets of triplicate assays of sample A that used sample B as the standard, gave recovery (and standard deviation) results of 99.3 (0.8) and 98.4% (0.6%). Impurities found by the evaluating laboratory are given in Table 7. The chromatograms from the second laboratory showed similar impurity profiles as those obtained by the first analyst; however, 2 early eluting peaks were inadvertently omitted from the integration and were not quantitated by the second analyst. The differences in levels for the remaining impurities, as reported in Tables 1 and 7, are acceptable for low levels of nontoxic impurities.

Table 5. System suitability parameters on 2 columns[a]

Day	RT (Tol)	RT (PPB)	RRT	Resolution	Efficiency[b]
Waters Resolve No. T83471					
1	4.05	6.98	1.72	9.34	16200
2	4.49	7.40	1.65	9.33	20400
3	4.69	7.62	1.62	9.19	20800
4	4.91	7.86	1.60	9.18	24000
5	5.22	8.14	1.56	8.98	26700
Waters Resolve No. T13261					
1	3.98	7.46	1.87	9.66	12200
2	4.40	7.89	1.79	9.35	13600
3	4.74	8.32	1.75	9.06	14000
4	4.84	8.38	1.73	9.15	15800
5	5.22	8.74	1.67	8.62	16600

[a] Retention times of tolrestat (Tol) and propylparaben (PPB), in minutes, and RRT, relative retention time (Tol = 1.0).
[b] Units are plates per meter.

Table 6. External system suitability results

Parameter	Requirement	Day 1	Day 2	Day 3
Resolution	>7	8.5	7.47	7.42
Plates per meter	>12000	38600	37600	37800
Tailing	<2	1.2	1.1	1.2
RT, min[a]	4.0–5.3	4.5	4.4	4.5
RRT[b]	1.8	1.7	1.6	1.7
RSD (assay)[c]	<1.0	0.99	—	—
RSD (impurities)	<5.0	—	1.8	2.2

[a] RT, tolrestat retention time.
[b] RRT, propylparaben relative retention time.
[c] RSD, relative standard deviation.

Table 7. External related compound determination (%)[a]

	Retention time relative to tolrestat				
Sample	0.72	0.81[b]	1.47	1.81	Total
A	0.01	0.15	0.01	0.19	0.36
B	—	0.13	—	—	0.13

[a] Levels of 2 impurities eluting at relative retention times of less than 0.7 and present in each sample were not determined by the externa laboratory.
[b] This peak corresponds to the *cis* rotamer of tolrestat.

Acknowledgments

Infrared and mass spectra were obtained by H. Beckstead and J.C. Ethier, respectively. The method was evaluated by D.K.J. Gorecki, Pharmaceutical Research Analytical Laboratory, University of Saskatchewan, SK, Canada.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Sulfadimethoxine and Sulfamethazine Residues in Animal Tissues by Liquid Chromatography and Thermospray Mass Spectrometry

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A simple, sensitive, and rapid method for simultaneous determination of sulfamethazine and sulfadimethoxine residues in animal tissues by liquid chromatography (LC) with on-line confirmation by thermospray mass spectrometry is reported. Tissue extracts, after cleanup on C_{18} cartridges, were injected into a reversed-phase C_{18} column, and the sulfa drugs were determined by ultraviolet (UV) detection at 265 nm. On-line confirmation of sulfamethazine and sulfadimethoxine in the extracts by mass spectrometry was obtained by feeding the sulfa drugs eluting from the chromatographic column after UV detection directly into the ion source of a mass spectrometer with a thermospray ionization LC interface. Analytical results obtained with the LC method, which has detection limits of 2 and 10 ng/g of tissue, for sulfamethazine and sulfadimethoxine, respectively, compared favorably with those obtained with the official thin-layer chromatographic–densitometric method.

Our laboratory has for several years used the thin-layer chromatographic–densitometric (TLCD) method developed by Thomas et al. (1), considered the official method for the determination of sulfa drug residues in animal tissues. To confirm the identities of sulfa drug residues detected in tissues at concentrations in excess of the maximum residue limit (MRL) of 100 µg/kg in the United States (2) and Canada (3) in support of regulatory action, the non-volatile sulfa drugs, after analyses by TLCD, are chemically derivatized to form volatile analogues and subsequently analyzed by gas chromatography/mass spectrometry (GC/MS) (4). With the recent acquisition of a mass spectrometer with a thermospray ionization liquid chromatographic (TSP/LC) interface at our laboratory, we have endeavored to reduce to one the 2 experiments usually conducted to determine and confirm sulfa drug residues in animal tissues. As a prelude to the development of this method, we recently reported an LC method with ultraviolet (UV) detection with a detection limit of 2 ng/g for determination of sulfamethazine (SMZ) residues in bovine and porcine tissues (5). The analytical results obtained for SMZ in both incurred and fortified tissue samples with the LC method compared favorably with those obtained by 7 participating laboratories in the United States and Canada using the Thomas method. Consequently, we are now reporting an adaptation of the SMZ method for the simultaneous analysis of SMZ and sulfadimethoxine (SDM), another frequently detected sulfa drug in animal tissues.

SDM, a sulfonamide antibacterial like SMZ, is approved for therapeutic, prophylactic, and/or growth promotion use in horses, cattle, calves, and young pigs. In aquaculture, SDM is used to control enteric septicemia caused by *Edwardsiella ictaluri*. It is usually administered in combination with ormetoprim or trimethoprim to increase its effectiveness against the causative bacteria (6). It is recommended that land-based animals administered the label dose must not be slaughtered for use in food for at least 10 days after the last treatment with SDM or SMZ (7). To protect the consumer from the risk of unwanted sulfonamide residues including SMZ and SDM in edible meat and meat products, inspection programs have been implemented in federally inspected abattoirs in Canada and the United States. These programs include testing urine samples from randomly selected or suspect animals at slaughter for sulfa drug residues by using the Sulfa On Site (SOS) test (8), which is a sulfa drug-specific screening test. Carcasses of animals testing positive to the SOS test are detained in the abattoir until a regulatory laboratory identifies and confirms the presence of sulfa drug residue(s) in muscle and liver samples of the detained carcasses. Carcasses confirmed to contain sulfa drug residues in the muscle exceeding the MRL after laboratory testing are condemned and not allowed to enter the food chain.

Several methods, including TLC, gel permeation chromatography (GPC), GC/MS (9–12), GC/tandem mass spectrometry (GC/MS/MS) (13), LC with UV–visible detection (LC–UV) (14–24), capillary zone electrophoresis/mass spectrometry (25, 26), and collision-activated dissociation/tandem mass spectrometry (27, 28), have been described for analysis of SMZ and SDM, usually in combination with other sulfonamides, in meat, fish, and eggs in support of regulatory programs for veterinary drug residues in food. Because very few methods have been

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described for confirmation of sulfonamide residues by LC/MS (29, 30), this paper describes a simple modification of our previously reported SMZ method, which permits us to analyze SMZ and SDM in animal tissues simultaneously by isocratic LC with on-line confirmation by thermospray LC/MS.

METHOD

Reagents

(a) *Sulfadimethoxine and sulfamethazine.*—Sigma, St. Louis, MO.

(b) *Sulfaethoxypyridazine (SEPD).*—American Cyanamid, Pearl River, LA.

(c) *Sodium dihydrogen phosphate and ammonium acetate.*—Fisher Scientific, Toronto, ON, Canada.

(d) *Sodium chloride and sodium hydroxide.*—BDH, Toronto, ON, Canada.

(e) *Mobile phase for LC–UV.*—0.05M Sodium dihydrogen phosphate–acetonitrile (72 + 28, v/v) was filtered through 0.45 μm nylon filters, degassed, and continuously sparged with helium during LC analysis.

(f) *Mobile phase for LC/MS.*—0.01M Ammonium acetate (pH 4.6)–acetonitrile (72 + 28, v/v) was filtered through 0.22 μm nylon filters, degassed, and sparged intermittently with helium during LC/MS analysis.

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

(h) *All other reagents and solvents.*—LC grade and used without further purification.

Apparatus

(a) *Solvent evaporator.*—Zymark, Hopkinton, MA.

(b) *Sep-Pak C_{18}.*—3 mL, 500 mg capacity with 14% carbon loading solid-phase extraction cartridges (Waters Chromatography Division, Mississauga, ON, Canada).

(c) *MSE Coolspin 2 centrifuge.*—With fixed-angle rotors (Fisons, Sussex, UK).

(d) *Polytron homogenizer.*—With 20 mm probe (Brinkmann Instruments, Rexdale, ON, Canada).

(e) *0.45 μm Acrodisc filters.*—Gelman Sciences, Montréal, PQ, Canada.

(f) *LC–UV.*—A Waters 712 autosampler, a 501 pump (Waters, Mississauga, ON, Canada), a Kratos 783 variable UV detector (Kratos Analytical, Ramsay, NJ), a Spherisorb C_{18} ODS (2) (250 × 4.6 mm id, 5μm) column (Phenomenex, Torrance, CA). The LC was operated in an isocratic mode with a mobile phase flow rate of 1.2 mL/min with UV detection at 265 nm at a sensitivity setting of 0.003 absorbance unit full scale.

(g) *LC/MS.*—A Waters 600 MS multisolvent delivery LC system (Waters, Mississauga, ON, Canada) provided with a U6K injector was coupled directly to a single quadrupole mass spectrometer (VG TRIO 2, Fisons, Altrincham, UK) through a thermospray LC interface. The effluent from the UV detector was fed directly into the mass spectrometer ion source (temperature, 189°C) through a capillary jet whose temperature was held at 320°C to provide optimum ion intensity for the $M + H^+$ ion for SDM. The mass spectrometer was scanned from m/z = 100 to 320 in full-scan mode, and the pseudomolecular ions at m/z = 279, 295, and 311 for SMZ, SEPD, and SDM, respectively, were selectively monitored for quantitative analysis.

Preparation of Standard Solutions

(a) *Stock standard solutions of SMZ, SDM, and SEPD (1000 μg/mL).*—Dissolve accurately weighed amounts of each pure standard in methanol and store at −20°C in 4 mL polypropylene tubes.

(b) *Standard working solutions.*—SMZ and SDM (10 μg/mL) and SEPD (20 μg/mL). Dilute respective stock solutions with water.

Preparation of Tissue Samples for Extraction

Accurately weigh 5 g homogenized blank tissues into each of four 50 mL polypropylene centrifuge tubes. Add 10, 25, 50, and 100 μL of the 10 μg/mL SDM standard and 5, 25, 50, and 125 μL of the 10 μg/mL SMZ standard solution to corresponding tubes to provide samples with SDM at 20, 50, 100, and 200 ng/g and SMZ at 10, 50, 100, and 250 ng/g tissue equivalencies, respectively. Also, add 40 μL of the 20 μg/mL SEPD standard solution to each sample to provide a constant internal standard concentration of 160 ng/g in each sample. For incurred or test tissues, add 40 μL of the 20 μg/mL SEPD standard to accurately weighed 5 g homogenized samples.

Tissue Extraction

The procedure for extracting SMZ, SDM, and SEPD from animal tissues and the cleanup of tissue extracts on C_{18} solid-phase extraction (SPE) cartridges are identical to procedures described in detail elsewhere for SMZ (4). After the C_{18} SPE cartridge that has been loaded with the tissue extract is rinsed with 20 mL water, the sulfa drugs are eluted immediately with 1 mL acetonitrile (instead of methanol used in the SMZ procedure) into a 6 mL glass tube. In addition, the eluted sample is held in a 50° ± 1°C thermostated water bath and evaporated to dryness with high-purity nitrogen, after which the residue is redissolved in 2 mL mobile phase solution by vortex mixing at high speed for 20 s and left to sit in the 50°C bath for 5 min. It is then removed from the bath, cooled, and filtered through an Acrodisc filter for LC–UV analysis and/or TSP/LC/MS analysis.

LC–UV Analysis

Inject 20–50 μL of the filtered sample extract into the LC system. Measure peak heights of the chromatographic responses for SMZ, SDM, and SEPD and calculate the response ratio (peak height of SMZ or SDM/peak height of SEPD) for each spiked tissue sample. Plot a calibration curve of response ratio versus concentration of SMZ and SDM using regression analysis. Calculate also the response ratio for every test or incurred sample. Using the regression parameters for the standard curve, calculate the concentrations X_{SDM} and X_{SMZ} of SDM and SMZ, respectively, in the test samples with the following equations:

$$X_{SMZ} = \frac{Y_{SMZ} - (b)}{(m)}$$

$$X_{SDM} = \frac{Y_{SDM} - (b)}{(m)}$$

where Y_{SDM} and Y_{SMZ} are the response ratios for the samples and b and m are the y intercept and slope, respectively, of the regression line.

LC/MS Analysis

Inject 20–50 µL filtered sample extract into the LC/MS system. Measure the peak areas under the selected ion chromatogram peaks for SMZ (m/z = 279), SDM (m/z = 311), and SEPD (m/z = 295), the internal standard. Calculate the ion ratios for SMZ/SEPD and SDM/SEPD and plot calibration curves for SDM and SMZ by regression analysis. Concentrations of SMZ and/or SDM in the test samples can then be interpolated from their measured ion count ratios from the calibration curves.

Determination of Recovery, Interassay and Intra-assay Precision, and Accuracy

The recovery of SMZ and SDM from fortified tissue with the newly developed method was determined by comparing the UV responses from SMZ- and SDM- (n = 4 at each concentration) and SEPD- (n = 16) fortified tissue samples that were taken through the extraction–cleanup and LC analysis with those obtained from equivalent SMZ, SDM, and SEPD standards. Accuracy was assessed by calculating the amounts of SMZ and SDM found in the fortified blank samples from the calibration curve. Intra-assay precision was determined by replicate analyses (n = 4) on the same day of blank tissues fortified with SMZ and SDM at concentrations ranging from 10 to 250 ng/g. In addition, tissue samples blindly fortified with SMZ and SDM at concentrations above or below the MRL (i.e., 40 and 150 ng/g) were analyzed on 4 consecutive days to determine the day-to-day (interassay precision) variation.

Correlation between LC–UV and TSP/LC/MS Methods for Determination of SMZ and SDM

To determine the suitability of the TSP/LC/MS method for simultaneous determination of SMZ and SDM in animal tissues, blank tissue samples that had been fortified blindly with SMZ and SDM at concentrations within our calibration range and processed as described, were analyzed by LC–UV and TSP/LC/MS.

Determination of SMZ and SDM in Interlaboratory Check Samples

To evaluate the suitability of the LC method for simultaneous determination of SMZ and SDM in animal tissues, we were given incurred tissues or tissues that had been fortified with sulfa drugs as part of a Canada–United States interlaboratory sulfonamide check sample exchange program. We analyzed the sulfonamide content of the check samples with our newly developed LC–UV method. Other participating laboratories used the TLCD method described by Thomas et al. (1) to analyze the same samples.

Results and Discussion

The changes made to the previous SMZ method to permit simultaneous determination of SDM and SMZ in animal tissue consisted of increasing the mobile phase flow rate from 0.8 to 1.2 mL/min and replacing the methanol for eluting the sulfa drugs from the C_{18} cartridge and in the mobile phase with acetonitrile. These changes were made to improve SDM recovery from the C_{18} cartridge, which was about 60% with methanol, and to reduce the retention of the sulfa drugs on the analytical column. The solvent change, while increasing SDM recovery from the C_{18} cartridge, did not affect SMZ recovery from the C_{18} cartridge which was ≥85% from porcine tissues and ≥90% from bovine tissues with methanol as the elution solution. Because these changes did not affect recovery nor the analytical parameters previously determined for analysis of SMZ alone in animal tissues, the experimental results for SMZ in this multiresidue method evaluation have been omitted to avoid duplication of previously published data.

Figure 1 shows typical liquid chromatograms of extracts obtained from control porcine muscle tissue (Figure 1A) and control porcine muscle tissue fortified with SMZ and SDM at 20 ng/g each and with SEPD at 160 ng/g (Figure 1B) after extraction and cleanup as described. The 3 sulfa drugs, SMZ, SEPD (internal standard), and SDM, with retention times of 6.0, 12.8, and 23.0 min, respectively, are completely resolved from one another and from other endogenous components. Other sulfonamides likely to be used in animal food production, such as sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfadoxine, and sulfamethoxazole, were efficiently recovered from animal tissues by this method (Boison and Keng, unpublished data), and with retention times of 4.7, 4.9, 5.3, 6.5, 9.0, 10.4, and 11.7 min, respectively, on this analytical column, they do not interfere with the chromatographic analysis of SMZ and SDM. In our opinion, this characteristic feature of this method makes it potentially useful for the determination of multiple sulfonamide drug residues used in animal food production without having to make any significant changes to the procedure described.

Figure 2 shows the full-scan mass spectra (mass range, 100–320 amu) and chemical structures of the 3 compounds under thermospray ionization conditions. All 3 compounds, and those sulfonamides mentioned earlier that were tested for chromatographic interference, show intense $M + H^+$ ions as base peaks in their mass spectra and thus provide very useful molecular weight information for compound identification (Table 1). These spectra are very similar to those previously reported by Horie et al. (32) for determination of sulfa drug residues in meat by thermospray LC/MS; they are also similar to those obtained by Perkins et al. (29) under electrospray ionization (ESI) conditions and by Doerge et al. (33) under atmospheric pressure chemical ionization (APcI) conditions at low skimmer or cone voltages. They are, however, different in the low-mass region

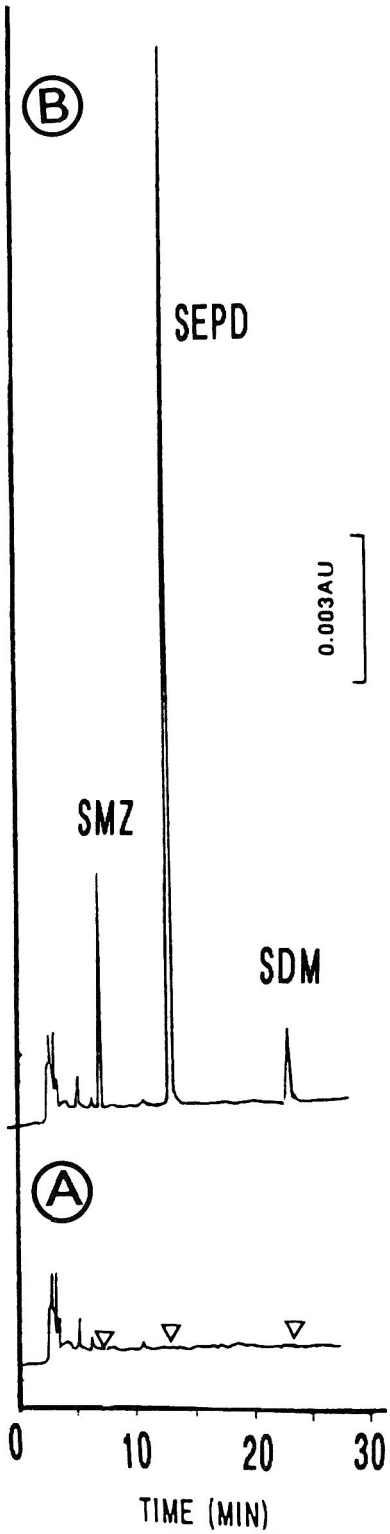

Figure 1. Typical chromatograms of a 50 μL injection of muscle tissue extract from a control (untreated) porcine muscle (A) and a control porcine muscle fortified with SMZ and SDM at 20 ng/g tissue and 160 ng/g of the internal standard, SEPD (B).

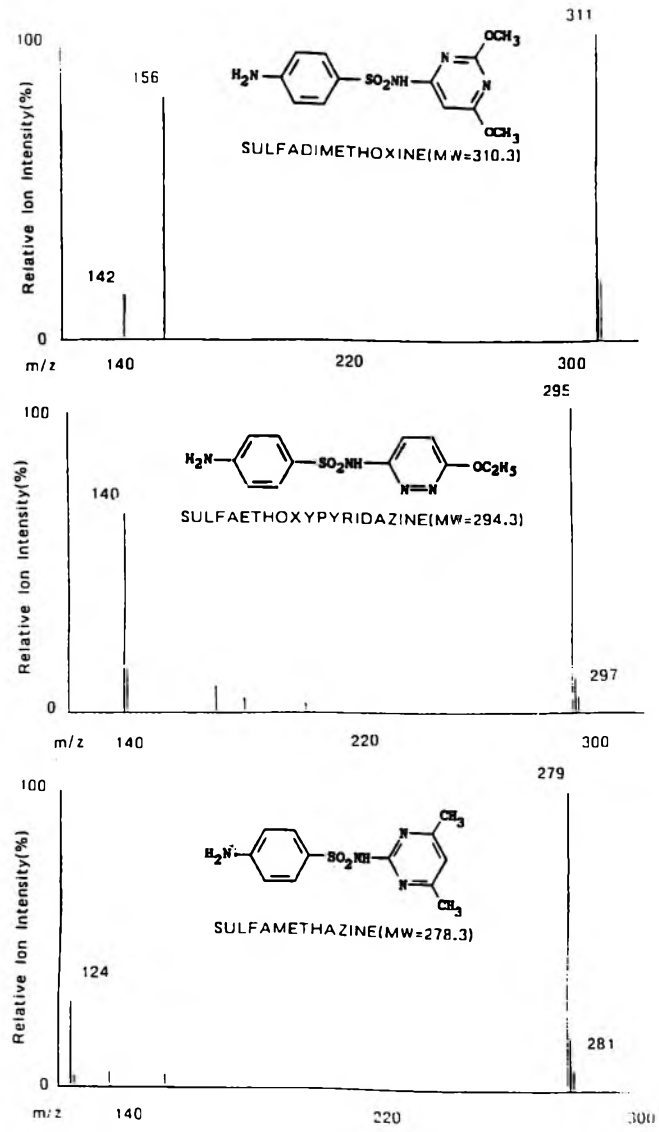

Figure 2. Mass spectra of SMZ, SDM, and SEPD (internal standard) under thermospray ionization conditions.

from the spectra obtained under these 2 latter ionization conditions at high skimmer or cone voltages in showing little or no fragmentation of the molecular ion. In ESI or APcI, unlike thermospray ionization, skimmer or cone voltages can be manipulated to induce more fragmentation to produce characteristic fragmentation patterns suitable for identification purposes. Ordinarily, for a mass spectrum of a compound to be suitable for confirmation and quantitative analysis in regulatory MS, the spectrum should contain no less than 3 characteristic ions with significant ion abundances. Nevertheless, even though TSP/LC/MS provides only one intense characteristic mass ion in the mass spectrum of each of the sulfa drugs, the specifity of the pseudomolecular ions coupled with the selectivity of the mass measurement technique makes the method acceptable for determination of the 2 sulfa drugs by TSP/LC/MS. Additionally, Figures 3 and 4 shown that there is a highly significant correlation between the LC–UV and the TSP/LC/MS methods. It can therefore be concluded that the TSP/LC/MS method of using ion ratios of the pseudomolecular ions to determine the concentrations of SMZ and SDM in animal tissues is a suitable method. Calibration curves obtained with the ion ratios of the

Table 1. Masses and relative abundances of ions used for thermospray analysis of SMZ and SDM

Compound	Chemical formula	Molecular weight	$M + H^+$ (abundance, %)	Others (abundance, %)
SMZ	$C_{12}H_{14}N_4O_2S$	278.3	279[a] (100)	124 (29), 280 (20)
SDM	$C_{12}H_{14}N_4O_4S$	310.3	311[a] (100)	156 (80), 142 (15)
SEPD	$C_{12}H_{14}N_4O_3S$	294.3	295[a] (100)	140 (65), 141 (15)

[a] Ions that were selectively monitored for quantitative analysis by TSP/LC/MS.

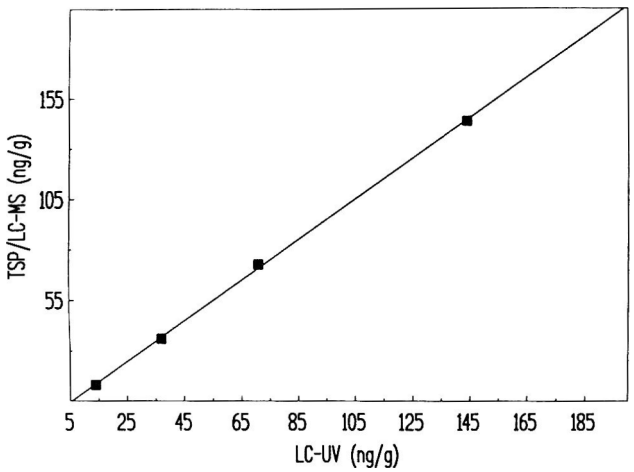

Figure 3. Correlation between LC–UV and TSP/LC/MS methods for determination of SMZ in animal tissues.

sulfa drugs to that of the internal standard were rectilinear at 10–250 and 20–200 ng/g tissues for SMZ and SDM, respectively.

calibration curves were also obtained for SMZ within the 10–250 ng/g calibration range and for SDM from 20 to 200 ng/g for the LC–UV method. For tissue samples with SMZ and SDM concentrations greater than the upper limit of our calibration curves, smaller sample sizes of 1–4 g should be used. This method accurately quantitates SMZ and SDM resi-

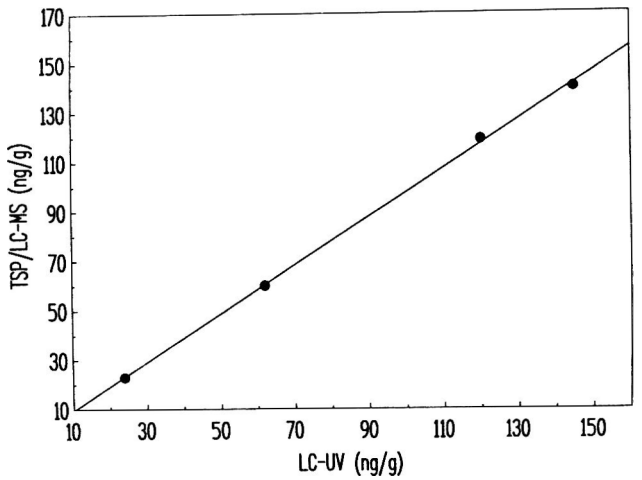

Figure 4. Correlation between LC–UV and TSP/LC/MS methods for determination of SDM in animal tissues.

dues at concentrations of ≥10 and 20 ng/g, respectively, in animal tissues; the detection limit (singal-to-noise ratio, 3) was 2 ng/g for SMZ and 10 ng/g for SDM added to animal tissues.

The average recovery of SDM added to animal tissues at concentrations ranging from 5 times below to 2 times above the MRL was ≥75% for porcine muscle and liver (Table 2). The internal standard, SEPD, was also consistently recovered from animal tissues with an efficiency of ≥80%. Tables 3 and 4 show the results of the intra- and interassay precision and accuracy estimations, respectively, of the method for determination of SDM in porcine muscle and liver. The tables show that concentrations of SDM added to animal tissues can be estimated to within ± 10% of the true values with a precision of ≤10% relative standard deviation.

Figures 5A and 5B show chromatograms of extracts obtained from check samples. Sample S-1142 contained SMZ at 103 ng/g but no detectable levels of SDM, and sample S-1143 contained SDM at 162 ng/g but no detectable levels of SMZ. The 8 participating laboratories determined that sample S-1142 contained SMZ at 106 ng/g and no detectable concentration of SDM, while sample S-1143 had no detectable concentrations

Table 2. Recovery of SDM and SEPD from fortified blank porcine tissues[a]

Tissue matrix	Drug added, ng/g tissue		UV responses from fortified tissue samples, mean ± SD		Mean recovery, %	
	SDM	SEPD	SDM	SEPD	SDM	SEPD
Muscle	20	160	5.0 ± 0.2 **[6.4 ± 0.2]**	143.0 ± 2.2 **[175 ± 5]**	78 ± 3	82 ± 1
	100	160	26.1 ± 0.8 **[33 ± 2]**	144.3 ± 4.8 **[175 ± 5]**	79 ± 2	82 ± 3
	200	160	52.6 ± 2.5 **[66 ± 3]**	140.3 ± 3.0 **[175 ± 5]**	80 ± 4	80 ± 2
Liver	20	160	4.8 ± 0.3 **[6.4 ± 0.2]**	144.8 ± 5.1 **[175 ± 5]**	75 ± 5	83 ± 3
	100	160	24.6 ± 0.6 **[33 ± 2]**	146.8 ± 5.0 **[175 ± 5]**	75 ± 2	84 ± 3
	200	160	50.3 ± 1.4 **[66 ± 3]**	140.8 ± 5.0 **[175 ± 5]**	76 ± 2	80 ± 3

[a] Four replicate analyses were conducted at each specified concentration level. The corresponding results for the mean UV response (peak height) for equivalent external standards ($n = 6$) are shown in square brackets after each entry. An external standard is one that has not been taken through the extraction–cleanup procedure described. Recovery is calculated as the experimentally determined UV detector response divided by the UV detector response of an equivalent external standard expressed as a percentage. Equivalent results for SMZ have been previously reported (5).

Table 3. Intra-assay precision and accuracy of the method for determination of SDM in porcine tissues

Tissue matrix[a]	SDM added, ng/g tissue	SDM found, ng/g of tissue (mean ± SD)	Coefficient of variation, %	Accuracy, %
Muscle	20	21 ± 2	10	+5
	50	49 ± 2	4	−2
	100	99 ± 2	2	−1
	200	201 ± 6	3	+1
QA(1)	40	39		−3
QA(2)	150	143		+5
Liver	20	19 ± 1	5	−5
	50	50 ± 3	6	0
	100	101 ± 2	2	+1
	200	200 ± 3	2	0
QA(3)	40	38		−5
QA(4)	150	137		−9

[a] QA(1) and QA(2) (muscle samples) and QA(3) and QA(4) (liver samples) were fortified blindly with SDM at 40 and 150 ng/g and analyzed with the rest of the samples. Quadruplicate analyses were conducted at each concentration level, except for the QA samples which were single analyses. Equivalent results for SMZ have been previously reported (5).

of SMZ but contained SDM at 159 ng/g. Chromatograms of extracts obtained from the test samples show that SMZ, SEPD, and SDM are all completely resolved from one another, making the method suitable for simultaneous determination of the 2 most commonly detected sulfonamide residues in animal tissues. Table 5 summarizes the analytical results obtained when sulfonamide residues in porcine muscles prepared for a Canada–United States interlaboratory sulfonamide check sample analysis program were analyzed with our LC method and the TLCD method used by the other 8 participating laboratories. The average results obtained by the 8 participating laboratories show that our LC results compared very favorably with those obtained with the official analytical method.

Our method has obvious advantages over the current official method. With detection sensitivities of ≤10 ng/g for SMZ and SDM, our LC method has higher detection sensitivitities than the TLCD method (detection limit, 20 ng/g) for these sulfa drugs; in addition, the method permits simultaneous determination and confirmation by thermospray LC/MS of the sulfa drugs in one experiment instead of the 2 experiments traditionally performed for analysis and confirmation of sulfa drug residues. Even though the TSP ion source does not permit generation of more than one intense, characteristic ion for multi-ion confirmation, as did the GC/MS method, it is realistic to mention that the multi-ion criterion required in regulatory MS confirmation can be achieved by simple replacement of the TSP source with an APcI or ESI source operated under high cone or skimmer voltages.

We have also included an internal standard, SEPD, which is efficiently extracted by the method described and has similar chromatographic and elution characteristics as the sulfa drugs of interest, to account for analyte losses in the procedure. Even more important, we have selected the concentration of the internal standard (160 ng/g) to be such that it shows similar absorbance as SMZ at 100 ng/g (i.e., the response ratio for SMZ to SEPD at these concentrations is 1; response ratios are 0.5 and 2 when the SMZ concentrations are 50 and 200 ng/g, respectively). This characteristic feature of the method enables an analyst to easily determine from casual observation of the detector responses of extracts from the calibration standards whether the method is working properly.

The disadvantage of the method as it stands now is the use of the chlorinated solvent chloroform to extract the sulfa drugs from animal tissues. In light of the recently signed international agreement, the Montreal Protocol on Substances That Deplete the Ozone Layer, to ban the production and use of ozone-depleting chemicals such as 1,1,1-trichloroethane and carbon tetrachloride in 1996 (31), we are trying to find alternative solvents for the efficient extraction of these sulfa drugs from animal tissues. Even though chloroform and dichloromethane (also used in the TLCD method) are not scheduled to be phased out yet, we believe it is our responsibility to try and reduce or eliminate the dependance of our analytical methods on these solvents.

Conclusions

A simple, sensitive, and rapid method for determination and confirmation of SMZ and SDM in animal tissues by thermospray LC/MS was developed. Results obtained with the LC method compared very favorably with those obtained with the current TLCD method for determination of sulfonamides in animal tissues.

Table 4. Interassay precision and accuracy of the method for determination of SDM in porcine tissues[a]

Tissue matrix	SDM added, ng/g tissue	SDM found, ng/g tissue on Day 1	Day 2	Day 3	Day 4	Mean recovery ± SD, ng/g	Coefficient of variation, %	Accuracy, %
Muscle	40	41	42	39	40	41 ± 1	3	+3
	150	148	153	143	151	149 ± 4	3	−1
Liver	40	42	43	38	39	41 ± 2	6	+3
	150	150	152	137	148	147 ± 7	5	−2

[a] Equivalent results for SMZ residues have been reported previously (5).

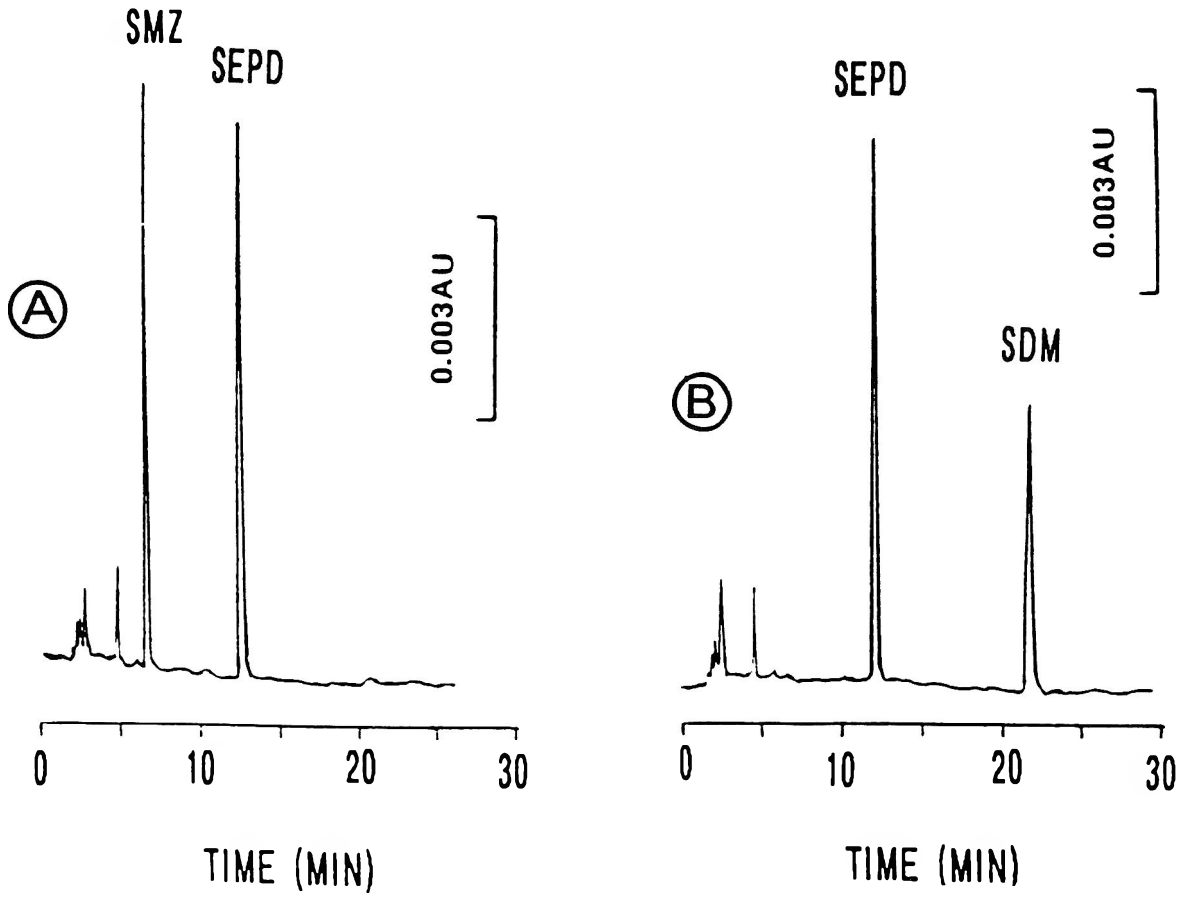

Figure 5. Chromatograms of a 25 μL injection of extracts obtained from check sample S-1142 (A) and check sample S-1143 (B) analyzed by our LC method as part of the interlaboratory check sample testing program. S-1142 was determined to contain SMZ at 103 ng/g and S-1143 was found to contain SDM at 162 ng/g.

Table 5. Comparison of results obtained for the determination of SMZ and SDM in porcine muscle by our LC method and the official TLCD method (1)

Sample identification	SMZ found, ng/g tissue		SDM found, ng/g tissue	
	LC[a]	TLCD[b]	LC[a]	TLCD[b]
UCS-221 (I[c])	153	145	152	159
UCS-222 (I[c])	177	164	ND[d]	ND
UCS-223 (I[c])	219	195	ND	ND
UCS-224 (I[c])	160	138	212[e]	195
S-1141 (I[c])	165	156	ND	ND
S-1142 (F[f])	103	106	ND	ND
S-1143 (F[f])	ND	ND	162	159
S-1144 (I[c])	379[e]	360	ND	ND
S-1145 (I[c])	154	155	ND	ND
S-1146 (I[c])	123	123	ND	ND
S-1147 (I[c])	169	174	ND	ND

[a] Our LC method; there was only enough sample for 1 analysis.
[b] The average result from the 8 independent participating laboratories using the official TLCD method.
[c] Sample is an incurred tissue.
[d] ND, none detected.
[e] This value was estimated because it fell outside the range of our calibration curves (10–200 ng/g).
[f] Tissue was fortified with the antibacterials.

Acknowledgments

We are sincerely grateful to Valerie Martz, Health of Animals Laboratory, for providing us with the check samples and the averaged results from the participating laboratories involved in the Canada–United States sulfa check sample exchange program. We also acknowledge the technical assistance of Ron Gedir for running the mass spectra of the sample extracts.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Microbiological Plate Assay for Determination of Tilmicosin in Bovine Serum

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A microbiological agar plate assay is described for determination of tilmicosin in bovine blood serum. The serum or serum dilution is added directly to wells cut in the agar plates. Tilmicosin activity is determined by measuring the zone of bacterial growth inhibition in agar medium inoculated with *Micrococcus luteus*, ATCC 9341. The assay was validated by evaluating the following parameters: accuracy, precision, linearity, parallelism, ruggedness, storage stability, and relative activity of isomers. Accuracy was evaluated with freshly collected bovine serum and with commercially available sera. Recoveries ranged from 93.4 to 97.5% across a fortification range of 0.08 to 1.28 μg/mL. Precision was estimated over a 6-day period with serum obtained from a tilmicosin-treated animal. Relative standard deviations were 0.63 to 3.13% within day and 5.23% across 6 days. Standard curves were linear with little variation in slope. No parallelism was observed between tilmicosin in serum and tilmicosin in buffered saline. The limit of detection was estimated to be 0.05 μg/mL, and the validated limit of quantitation was 0.08 μg/mL. Ruggedness was evaluated with different lots of antibiotic medium, different lots of sera, and different analysts. These variables did not affect method performance. Analyses of tilmicosin in frozen sera demonstrated that tilmicosin is stable for up to 16 days when stored at −20°C. A comparison of the relative microbiological activities of the purified *cis* and *trans* isomers of tilmicosin to that of the reference standard indicated no differences in microbiological activities, and showed a parallel response among the 3. The validation data demonstrate that this method is a rugged, reliable, and simple assay for tilmicosin in serum.

Tilmicosin phosphate (Micotil, Elanco) is a new semisynthetic macrolide antibiotic (Figure 1) that has been approved for treatment of bovine respiratory disease (BRD) complex. Its antibacterial activity and synthesis have been described previously by Kirst et al. (1–3) and Debono et al. (4). This antibiotic has high activity against *Pasteurella haemolytica*, which is the key pathogen in BRD complex (5–7).

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During development of a product like Micotil, studies are performed to evaluate product efficacy and safety. Methodology typically is needed to quantitate the analyte in animal tissues and body fluids such as urine and serum. A simple analytical method was needed to measure tilmicosin concentrations in serum. Method development includes resolution of issues including the type of detection system. Because of the structure similarity of tilmicosin and tylosin, initial method development was carried out with microbiological methods that are similar to or modifications of tylosin methods. The method for tilmicosin in serum is essentially a modification of the tylosin serum assay. It requires no sample preparation prior to analysis and is sensitive to tilmicosin at concentrations of 0.05 μg/mL. The method was validated by a more rigorous protocol than the tylosin methods of 20 years ago. It is reliable, reproducible, and easy to perform.

METHOD

Reagents

(a) *Solvent.*—Reagent grade methanol.

(b) *Bovine serum.*—Antibiotic-free normal bovine serum, commercial or other source.

(c) *Tilmicosin reference standard.*—Dry standard material for 3 h at 60°C in a vacuum oven. Accurately weigh dried standard to obtain a tilmicosin activity of 1024 μg/mL. Dissolve in methanol–water (10 + 90, v/v). This stock standard solution is stable for 2 weeks when refrigerated.

(d) *Standard solutions.*—On the day of assay, dilute stock standard with normal serum to obtain standard solutions with tilmicosin activity concentrations of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μg/mL.

(e) *Buffered saline.*—Phosphate buffered saline or equivalent.

(f) *Antibiotic media.*—Difco antibiotic medium No. 11, or equivalent; Difco Mueller Hinton Broth, or equivalent.

Apparatus

(a) *Antibiotic zone reader.*—Lilly-Fisher or equivalent, or an image analysis system such as an Omnicon 3000 image analysis system.

(b) *Analytical balance.*

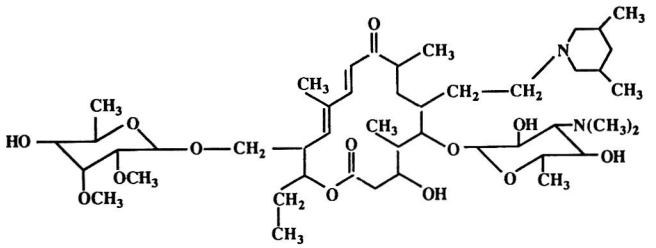

Figure 1. Structure of tilmicosin.

(**c**) *Petri dishes.*—100 mm × 20 mm.

(**d**) *Agar well cutter.*—9 mm wells.

(**e**) *Pipettes.*—Fixed or variable volume pipettes and disposable tips to deliver 100 µL.

(**f**) *Spectrophotometer.*—Spectronic 20, or equivalent.

(**g**) *Centrifuge.*

(**h**) *Agar-pouring device.*—Brewer semiautomatic plate pouring device or a manual syringe dispenser.

Microorganism and Media

Maintain *Micrococcus luteus* (ATCC 9341) on Difco antibiotic medium No. 11 slants. Prepare inoculum stock cultures by growing the organism on medium No. 11 in roux bottles at 37°C for 24 h. Remove cells by washing the agar surface with Mueller Hinton broth and concentrate by centrifugation. Dilute packed cells to 15% light transmission as measured by a Spectronic 20 spectrophotometer at 525 nm. Freeze diluted inoculum in 5 mL aliquots and store at −20°C. The frozen inoculum may be used for 2 months.

On the day of analyses, sterilize antibiotic medium No. 11 according to label directions and thaw inoculum at room temperature. Cool medium to 48°–50°C and inoculate with 10 mL of the *Micrococcus luteus* inoculum per liter of medium. The inoculum/medium ratio may be adjusted if necessary, to improve the size or contrast of the zones of inhibition. Maintain the agar temperature while dispensing 20 mL aliquots into each Petri dish. (A semiautomatic plate pouring device such as a Brewer or a manual syringe dispenser is recommended to improve volume uniformity.) Allow the medium to cool and solidify on a level surface. Cut 4 symmetrical 9 mm wells in each agar plate.

Sample Preparation

Mix serum samples well and dilute with control serum to within the standard curve range.

Assay Procedure

(**a**) Dose plates in clockwise order, starting with a well marked as the starting point.

(**b**) Prepare 20 plates for the standard curve. Dose each of 10 plates with 100 µL volumes of the 0.05, 0.1, 0.2, and 0.4 µg/mL standard solutions and each of the remaining 10 plates with 0.4, 0.8, 1.6, and 3.2 µg/mL solutions in clockwise order from the starting point.

(**c**) Prepare 5 plates for each serum sample. Dose 2 opposing wells of each of the 5 plates with 100 µL volumes of the reference concentration of the standard curve (0.4 µg/mL), starting at the well marked as the starting point. Dose the remaining 2 opposing wells with the serum sample.

(**d**) Run a maximum of 20 samples per test to minimize zone size variations caused by extended dosing times.

(**e**) Cover and incubate the plates at 37°C in a high-humidity (>50% relative humidity) incubator for 16 to 18 h.

(**f**) Measure the diameter of the resulting zones of inhibition to the nearest 0.1 mm and calculate the tilmicosin concentration for each sample.

Calculations

The calculations can be performed with a computer or manually, as follows: Calculate the average diameter of the zones of inhibition for each level of the standard curve. The average values are substituted into the following formulas to calculate the line of best fit representing the dose–response curve for the assay:

$$L \text{ (low concentration)} = \frac{13a + 10b + 7c + 4d + e - 2f - 5g}{28}$$

$$H \text{ (high concentration)} = \frac{-5a - 2b + c + 4d + 7e + 10f + 13g}{28}$$

where L is the calculated response value (in mm) of the low concentration (0.05 µg/g) and H is the calculated response value (in mm) of the high concentration (3.2 µg/g). Values a, b, c, d, e, f, and g are the observed average response values (in mm) for each respective concentration, ranging from the lowest to the highest dose in that order. The values obtained for L and H can be plotted on semilogarithmic graph paper with the abscissa representing the dose levels.

Calculate the average diameter of the zone of inhibition for both the unknown test solution and the 0.4 µg/g reference point from the sample plates. Correct the average size of the zone of inhibition for the unknown solution by adding or subtracting the difference between the average zone size of the sample plate reference standard and the theoretical value for this 0.4 µg/g standard. This latter value is indicated by the intercept of the ordinate for the 0.4 µg/g dose with the plotted response curve. Determine the potency of the unknown test solution by obtaining the curve reading of the adjusted average of the sample zone size. Calculate the potency of the original sample by multiplying this reading by the dilution factor for the particular sample assayed.

Results and Discussion

Accuracy

The accuracy of tilmicosin recovery from bovine serum was determined by fortifying control serum with tilmicosin reference standard. Tilmicosin concentrations of 0.08, 0.32, and 1.28 µg/mL serum were analyzed with 3 determinations per day. Analyses were carried out over 6 days with serum obtained from a commercial source and for 3 days with freshly obtained

Table 1. Accuracy of assay for tilmicosin in bovine serum

Serum source	Fortified level, µg/mL	*n*	Tilmicosin found, µg/mL	CV, %	Recovery, %
Commercial	0.08	18	0.075	6.1	93.8
Commercial	0.32	18	0.310	3.9	96.9
Commercial	1.28	18	1.196	4.0	93.4
Animal A	0.32	9	0.312	3.7	97.5
Animal B	0.32	9	0.305	2.6	95.3

serum. Results are listed in Table 1. Recoveries ranged from 93.4 to 97.5% across the concentrations tested in 2 sources of sera. Mean recovery for all concentrations and sera tested was 95.4%. Except for samples fortified at the limit of quantitation (0.08 µg/mL), the coefficients of variation (CVs) for fortified samples tested were ≤4%.

Precision

One animal was injected with tilmicosin at the recommended dose of 10 mg/kg. Blood was collected at 6 h postinjection, and a quantity of serum was prepared to provide a serum sample with incurred tilmicosin residue. The sample was divided into individual test tubes, frozen, and assayed in triplicate on 3 days over 2 time periods (Table 2). The within-day precision was very good, with CVs ranging from 0.7 to 3.1%. The mean of 18 determinations was 0.556 µg/mL, and the overall CV was 5.2%.

Parallelism

Tilmicosin standard diluted in serum and in buffered physiological saline were compared by preparing standard curves in each diluent. A standard curve in serum produced a slope of 3.69 and a correlation coefficient of 0.9986 (Table 3). The standards in buffered saline produced a slope of 5.18 with a correlation coefficient of 0.9935. Because of the nonparallelism between buffered saline and serum, the standard curve is prepared in control bovine serum to ensure parallel responses of samples and standards.

Table 2. Precision of assay for tilmicosin in bovine serum

Assay period	Assay day	*n*	Tilmicosin found, µg/mL	CV, %
1	1	3	0.548	1.0
1	2	3	0.582	2.0
1	3	3	0.590	3.1
2	1	3	0.569	0.6
2	2	3	0.521	3.0
2	3	3	0.526	0.7
Mean			0.556	5.2

Table 3. Parallelism of tilmicosin standard diluted in serum and saline

	Standard curve	
Diluent	Slope	Correlation coefficient
Bovine serum	3.69	0.9986
Buffered saline	5.18	0.9935

Linearity

Tilmicosin standard curves ($n = 9$) generated during validation of this method were analyzed to determine slopes and correlation coefficients (Table 4). The data show the standard curves to be consistently linear with very little variation in slope.

Sensitivity

The standard curve is linear from 0.05 to 3.2 µg tilmicosin/mL serum. Serum samples may be analyzed as is (without cleanup, dilution, or concentration) with a limit of detection of 0.05 µg tilmicosin/mL serum.

The validated limit of quantitation for this method is 0.08 µg tilmicosin/mL serum (Table 1). Mean recovery at this concentration was greater than 93% with a CV of 6.17%.

Ruggedness

Different lots of Difco antibiotic medium No. 11 were used for periods 1 and 2 of the precision, accuracy, and linearity evaluations. Serum from different animals was used to generate the accuracy data. No obvious differences were seen in the standard curves or assay results between periods or between animal tissues, indicating that different medium lots, animal sera, or time periods did not affect the assay results (Tables 1, 2, and 4)

Storage Stability

Aliquots of serum containing incurred residue and of commercial serum fortified with tilmicosin at 0.32 µg/mL were

Table 4. Standard curve linearity of tilmicosin serum assay

Test No.	Slope	Correlation coefficient
1	3.65	0.9964
2	3.66	0.9996
3	3.97	0.9993
4	3.65	0.9966
5	3.55	0.9950
6	3.67	0.9975
7	3.79	0.9991
8	3.59	0.9980
9	3.47	0.9990
Average	3.67	0.9978
Relative standard deviation, %	3.93	0.16

Table 5. Stability of tilmicosin in bovine serum during frozen storage

Sample	*n*	Age, days	Assay, µg/mL	Relative standard deviation, %	Percent of initial
Incurred residue	9	0	0.574	3.9	100.0
	9	7	0.539	4.5	93.9
	3	16	0.567	4.5	98.8
Fortified serum	9	0	0.316	2.1	100.0
(0.32 µg/mL)	9	7	0.304	4.5	96.2
	3	16	0.314	1.9	99.4

Table 6. Microbiological response of tilmicosin reference standard compared with *cis* and *trans* isomers of tilmicosin

Tilmicosin form	Day	Mean zone, mm 0.05 µg/ml	Mean zone, mm 3.20 µg/ml	Slope	Correlation coefficient
Standard	1	14.13	29.59	3.79	0.9991
trans	1	14.63	29.00	3.65	0.9991
cis	1	14.19	29.67	3.85	0.9988
Standard	2	12.97	27.54	3.59	0.9980
trans	2	13.02	27.80	3.66	0.9989
cis	2	13.15	28.05	3.64	0.9988

stored frozen at −20°C for up to 16 days (Table 5). The data indicate that tilmicosin is stable in frozen serum for up to 16 days.

Activity of cis and trans Isomers of Tilmicosin

Purified *cis* and *trans* isomers of tilmicosin were compared with tilmicosin reference standard by diluting each material with serum to each standard curve level. The tilmicosin reference standard is composed of 85% *cis* and 15% *trans* isomers. No obvious differences were observed with respect to microbiological activity against *M. luteus*. Average diameters of the zones of inhibition of the 0.05 and 3.2 µg/mL levels, the curve slope, and the correlation coefficients of each material are given in Table 6. The data demonstrate that the curves are linear, parallel, and nearly superimposable.

Conclusions

A simple, microbiological agar plate assay has been developed and validated for quantitation of tilmicosin in bovine serum. The method is sensitive with a validated tilmicosin limit of quantitation of 0.08 µg/mL.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Agarose Gel Electrophoretic Detection of Six β-Lactam Antibiotic Residues in Milk

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An electrophoretic method coupled with bioautography was developed for detection and identification of penicillin G, ampicillin, amoxicillin, cloxacillin, cephapirin, and ceftiofur residues in milk. The method uses a 2% agarose gel for electrophoresis, an overlay of PM indicator agar seeded with *Bacillus stearothermophilus* var. *calidolactis*, and incubation at 55°C for 16–18 h. The new method separated and detected residues in milk at the levels of concern for the Food and Drug Administration (FDA) for penicillin G (5 ppb), cephapirin (20 ppb), and ceftiofur (50 ppb). The method also detected ampicillin, amoxicillin, and cloxacillin at 20, 30, and 30 ppb, respectively, but these levels are above those of concern for FDA (10 ppb).

Existing methods adopted for antibiotic detection by the Food and Drug Administration (FDA) have remained unchanged for over 30 years (1–2). In general, these methods involve multiple extractions, various dilutions, agar diffusion plate assays, and microorganisms for antibiotic detection. These diffusion assay systems lack specificity and the proper antibiotic identification is based on special confirmation systems (3, 4). Newer methods capable of identifying specific antibiotics in tissues and other biological materials are needed and have been sought by various investigators.

Electrophoretic separation of antibiotics and detection by bioautography has been evaluated by several researchers since its development in the 1960s (3, 5–16). Electrophoresis separates charged molecules on the basis of their migration in a buffered gel when an electrical potential difference is applied (15). The migration distance depends on the size, shape and net charge of the molecules. The position of the antibiotic after migration is visualized by bioautography, which uses sensitive bacteria such as *Bacillus subtilis, B. cereus*, and *Micrococcus luteus*.

Grynne (8) used agar gel electrophoresis and bioautography to identify small amounts of 12 mixed antibiotics with limits of detection from 0.25 to 0.50 ppm. Smither and Vaughan (10) detected 50 antibacterial agents in animal tissues and feeds with high-voltage agar and agarose gel electrophoresis at pH 6.0 or 8.0. Kondo and Hayashi (16) evaluated agar gel electrophoresis and bioautography for identification of small amounts of 7 aminoglycoside antibiotics; limits of detection ranged from 0.078 to 0.31 ppm, and recoveries from extracts of bovine kidney tissue ranged from 59 to 90%. Another study (18) reported detection limits ranging from 3.2 to 83 ppb with *B. stearothermophilus* var. *calidolactis* (ATCC 10149) as indicator organism and with agarose gel at pH 6.0 or 8.0.

The lack of resolution between antibiotics with similar migration distances can be overcome using 2 supporting media of different electrophoretic activity and 2 pH levels (10). Salvatore and Katz (3) used agarose gel electrophoresis at pH 6.0 and 8.0 to distinguish 17 antibiotics accepted for use in animal feeds, including macrolides, aminoglycosides, tetracyclines, and one β-lactam. Microorganisms used for bioautographic detection were *Bacillus* spp., *Micrococcus* spp., and *Saccharomyces cerevisiae*. Salvatore and Katz (3) recommended that the simple, accurate, and precise electrophoretic system be used as a model to update existing methods for antibiotic detection.

Under the National Drug Residue Milk Monitoring Program, milk is routinely analyzed for β-lactam antibiotic residues by the FDA Grade A Pasteurized Milk Ordinance Microbiological Assay (17). This assay cannot distinguish among β-lactam antibiotics (e.g., amoxicillin, ampicillin, cloxacillin, cephapirin, penicillin, and ceftiofur) or other substances that may be present in milk and result in microbial inhibition. The method reported here can separate and give presumptive identification of the above-named 6 β-lactam antibiotics in milk at or slightly above tolerance levels by using an extraction and electrophoresis procedure coupled with bioautography.

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Experimental

Reagents

(a) *Electrolyte buffer, pH 6.0.*—1.82 g tris(hydroxymethyl)aminomethane (Tris) (Mallinckrodt, Paris, KY) and 0.98 g granular succinic acid (Mallinckrodt) in 1 L deionized water; if needed, the pH can be adjusted before use with 1N NaOH or 1N HCl.

(b) *Electrolyte buffer, pH 8.0.*—3.03 g Tris and 0.85 g succinic acid in 1 L deionized water; if needed, the pH can be adjusted before use with 1N NaOH or 1N HCl.

(c) *Phosphate buffer, 1%, pH 6.0.*—8.0 g anhydrous potassium phosphate monobasic and 2.0 g anhydrous potassium phosphate dibasic diluted to 1 L in deionized water; if needed, pH can be adjusted before use with 1N NaOH or 1N HCl.

(d) *PM indicator agar.*—Difco Laboratories (Detroit, MI), or equivalent.

(e) *Penase concentrate.*—Penicillinase, 10 000 000 international units/mL; Difco or equivalent.

(f) *Agarose electrophoresis reagent.*—Sigma Chemical Co. (St. Louis, MO; Catalog No. A-4679), or equivalent.

(g) *Standards.*—Ampicillin (anhydrous), amoxicillin, cephapirin Na, cloxacillin Na, and penicillin G-K from the United States Pharmacopeia (Rockville, MD); Ceftiofur HCl, from Upjohn Company (Kalamazoo, MI).

Materials

(a) *DNA Sub™ Cell electrophoresis system.*—Model 170-4304, Bio-Rad Laboratories (Richmond, CA), or equivalent.

(b) *Gel trays (15 × 20 × 2 cm).*—Cut from 0.25 in. glass and glued together with a thin bead of Elmer's clear silicone rubber sealer (aquarium safe or equivalent) along the outer edge of the 15 × 20 cm glass plate. The 2 cm glass edges are placed on the silicone and allowed to cure for 72 h before use.

(c) *EC 500, 0–2000 V DC power supply.*—E-C Apparatus Corp. (St. Petersburg, FL), or equivalent.

(d) *Rotary evaporator.*—Buchi/Brinkmann Rotavapor R110 (Westbury, NY), or equivalent.

(e) *Centrifuge bottles.*—250 mL maximum capacity, polypropylene (Nalgene, Co., Rochester, NY), or equivalent.

(f) *Pear-shaped flask.*—24/40, 150 mL, Kontes Scientific Glassware/Instruments (Vineland, NJ), or equivalent.

(g) *Automatic pipettor.*—Gilson P200 Pipetman, Rainin Instrument Company, Inc. (Emeryville, CA), or equivalent.

(h) *Bacterial spore suspension.*—Thermospore Suspension PM (*B. stearothermophilus* var. *calidolactis* [ATCC 10149]), Difco Laboratories, or equivalent.

(i) *Cotton cloth material from common wiping towels.*—Tightly woven cotton towel that was 35 cm long by 15 cm wide was folded over, giving a piece that was 10 cm long by 15 cm wide.

(j) *Glass microfiber filters.*—12.5 cm id, Whatman, Inc. (Clifton, NJ; Catalog No. 1827-125), or equivalent.

Preparation of Standards

Accurately weighed portions of ampicillin, amoxicillin, cephapirin Na, and cloxacillin were individually dissolved in 1% phosphate buffer, pH 6.0, giving a concentration of 1000 ppm activity. Penicillin G-K was first converted from units (U)/mg to ppm with 0.60 µg/U as conversion factor. An accurately weighed portion of the standard was then dissolved in 1% phosphate buffer, pH 6.0, as previously described, giving a concentration of 1000 ppm activity. An accurately weighed portion of ceftiofur HCl was dissolved in 95% methanol, giving a concentration of 1000 ppm activity. Each of the 6 standards was further diluted in 1% buffer, pH 6.0, to a working concentration that when spotted on the gel plate produced a visible clear zone of inhibition (Table 1). These solutions can be refrigerated at 4°C for 4 days. The volume (Table 1) of each standard applied was different, because the test microorganism shows different sensitivities against the 6 antibiotics. Adjustment of the volumes spotted prevented merging of the zones of inhibition.

Spiked Negative Milk Preparation

For spiked milk, 1.0 mL of a spike solution (Table 2) was added to 19 mL antibiotic-negative milk before extraction.

Identification of β-Lactam Antibiotics

Unknown milk samples and spiked milk samples (20 mL each) were either left untreated or treated with penase (1.0 mL), mixed, and incubated for 15 min at 35°C before extraction. When penicillinase is added to milk that is suspected to contain β-lactam antibiotic, the amide bond in the β-lactam is hydrolyzed and the antibiotic is inactivated. The penicillinase de-

Table 1. Amount of antibiotic spotted on agarose gel plates

		pH 6.0 plate		pH 8.0 plate		
Antibiotic standard	Concn, ppm	Amount spotted, µl	Concentration spotted, ng/well	Amount spotted, µL	Concentration spotted, ng/well	Zone diameters, mm
Amoxicillin	0.5	20	10	30	15	15
Ampicillin	0.5	20	10	30	15	15
Penicillin G-K	0.016	200	3.2	200	3.2	10
Cloxacillin Na	0.3	275	83	275	83	20
Ceftiofur HCl	0.3	250	75	250	75	15
Cephapirin Na	0.1	100	10	100	10	15

Table 2. Spike solutions and spiked milk levels

Antibiotic standard	Spike solution, ppm	Spiked milk level, ppb
Amoxicillin	0.6	30
Ampicillin	0.4	20
Penicillin G-K	0.1	5
Cloxacillin Na	0.6	30
Ceftiofur HCl	1.0	50
Cephapirin Na	0.4	20

grades the β-lactams studied, but it does not destroy many cephalosporins that may be present. Addition of penicillinase to the milk spike or the unknown sample identifies the β-lactam antibiotics studied and does not result in a spot on the electrophoresis plate.

Milk Extraction

Milk from healthy animals and free from antibiotics (20 mL) was placed into a 250 mL polypropylene centrifuge bottle, 40 mL acetonitrile was added, and the mixture was swirled gently for 2 min without any emulsion being formed. After the mixture was allowed to stand for 5 min, the clear supernatant was poured through a funnel with a glass microfiber filter into a 150 mL pear-shaped flask. The filtered supernatant was then evaporated under reduced pressure (Buchi/Brinkmann Rotavapor) in a 45°C water bath for ca 20–30 min to give an estimated final volume of 2–3 mL (19). The residue was then ready to be placed on an electrophoresis plate without any pH adjustment.

Preparation of Electrophoresis Gel Plate

The support consisted of 125 mL 2% agarose gel, prepared in either pH 6.0 or pH 8.0 electrolyte buffer, according to the method of Smither and Vaughan (10). After cooling to 60°C, the agarose gels were poured into a leveled glass gel plate, which had been masked with tape at both ends to give a uniform depth of 4 mm, and allowed to cool to room temperature for 2 h before use. The tape was then removed from the ends; the gel plate was ready to use.

Electrophoresis Procedure

Seven wells spaced 19 mm apart, each 13 mm id and 4 mm deep (all the way to the glass plate), were made in the agarose gel bed. The wells were made with a 13.0 ± 0.07 (standard deviation) mm diameter test tube (Catalog No. 73500, Kimble, Toledo, OH) in a row in the center of the 15 × 20 × 2 cm gel plate. The agarose gel cut by the tube was removed with needles. When standards were spotted on the gel plate, the working concentration was used. For each antibiotic, the amounts shown in Table 1 were spotted on the pH 6.0 and pH 8.0 plates with a Pipetman (the table also shows the concentration of each standard spotted in the wells).

Each well on each of the 2 (pH 6.0 and 8.0) gel plates was spotted in the following sequence with 300 µL of each of the following: spiked milk plus penicillinase, spiked milk, unknown milk plus penicillinase, unknown milk, negative milk, and negative milk plus penicillinase. The seventh well was spotted with spiked 1% buffer, pH 6.0, containing the corresponding amounts of each antibiotic shown in Table 1. Into each of the buffer chambers of the DNA Sub Cell electrophoresis system, 300 mL of the appropriate electrolyte buffer was added. The agarose gel was connected to the electrolyte buffer with the cotton fabric wick (6), and the lid on the system was closed. The electrophoresis procedure was then run normally for 3.5 h at a constant voltage of 150 V (16). After the electrophoresis procedure was completed, both ends of the glass gel plate were taped. PM indicator agar (125 mL) at 55°C was seeded with 0.2 mL thermospore suspension. This mixture was poured over the agarose gel and allowed to cool for 30 min at room temperature. Once the gel had cooled, the plate was incu-

Table 3. Migration of 6 β-lactam antibiotics from standards and corresponding spiked milk samples on pH 6.0 agarose gel plate

	Migration toward anode, mm								Migration toward cathode, mm			
	Penicillin G-K		Cloxacillin Na		Cephapirin Na		Ceftiofur HCl		Ampicillin		Amoxicillin	
Run	Std.[a]	Spiked milk (5 ppb)	Std.	Spiked milk (30 ppb)	Std.	Spiked milk (20 ppb)	Std.	Spiked milk (50 ppb)	Std.	Spiked milk (20 ppb)	Std.	Spiked milk (30 ppb)
1	74	75	68	70	55	55	52	52	7	7	10	10
2	75	75	65	65	51	50	49	50	5	4	10	10
3	77	75	70	69	53	53	55	57	11	11	10	10
4	68	66	55	55	45	45	34	35	10	10	10	10
5	65	65	55	55	45	45	45	45	7	7	7	7
6	70	70	60	60	47	47	41	45	10	10	10	10
7	75	75	55	55	50	50	35	35	10	10	10	10
Mean	72.0	71.6	61.1	61.3	49.4	49.3	44.4	45.6	8.6	8.4	9.6	9.6
SD[b]	4.4	4.5	6.5	6.7	3.9	3.9	8.2	8.3	2.2	2.5	1.1	1.1

[a] Std., standard.
[b] SD, standard deviation.

Table 4. Migration of 6 β-lactam antibiotics from standards and corresponding spiked milk samples on pH 8.0 agarose gel plate[a]

	Migration toward anode, mm											
	Penicillin G-K		Cloxacillin Na		Cephapirin Na		Ceftiofur HCl		Ampicillin		Amoxicillin	
Run	Std.[b]	Spikec milk (5 ppb)	Std.	Spiked milk (30 ppb)	Std.	Spiked milk (20 ppb)	Std.	Spiked milk (50 ppb)	Std.	Spiked milk (20 ppb)	Std.	Spiked milk (30 ppb)
1	77	76	55	55	65	65	52	54	45	45	25	25
2	72	71	65	65	66	65	54	53	45	45	28	25
3	79	79	65	65	65	65	57	58	45	45	25	25
4	65	65	55	55	55	55	30	30	50	44	40	30
5	70	70	60	60	65	65	41	45	50	45	45	35
6	70	70	50	50	60	60	33	35	50	35	45	30
7	70	70	56	56	60	60	30	32	55	50	45	30
Mean	71.8	71.6	58.0	58.0	62.3	62.1	42.4	43.8	48.6	44.1	36.1	28.6
SD[c]	4.7	4.6	5.6	6.0	4.1	3.9	11.8	11.8	3.8	4.5	9.7	3.8

[a] Penicillinase inactivates all 6 of the β-lactam antibiotics listed above.
[b] Std., standard.
[c] SD, standard deviation.

bated at 55°C overnight (16–18 h) in a moist incubator. The zones of inhibition were observed and the direction and distance of migration (from the center of the well to the center of the zone of inhibition) were recorded.

Results and Discussion

The procedure identifies the 6 β-lactam antibiotics tested. As Table 3 shows, penicillin and cloxacillin can be identified with the pH 6.0 agarose gel, while ceftiofur–cephapirin and amoxicillin–ampicillin are placed into 2 separate groups. By changing to a pH 8.0 agarose gel bed, one can further separate and presumptively identify the antibiotics in these groups (Table 4).

One problem with electrophoresis is keeping the agarose gel plate from overheating because of the high voltage. Overheating causes the agarose gel to warp and leads to uneven movement of antibiotics on the plate. By decreasing the voltage, increasing the run time to 3.5 h, and using a 2% agarose gel bed, these problems can be eliminated.

A problem with an electrophoretic run of 3.5 h is air contamination. Using *B. stearothermophilus* var. *calidolactis* incubated at 55°C overnight, this problem is eliminated by the high incubation temperature. Any surface growth can be washed gently with water.

The method allows presumptive identification of the 6 β-lactam antibiotics at the following levels (ppb): penicillin, 5; ceftiofur, 50; cloxacillin, 30; cephapirin, 20; amoxicillin, 30; and ampicillin, 20. With pH 6.0 agarose gel as a screen, one can first identify penicillin and cloxacillin, which move 71.6 ± 4.5 and 61.3 ± 6.7 mm, respectively, toward the anode. The pH 6.0 agarose gel also groups ceftiofur and cephapirin, which move about 45.6 ± 8.3 to 49.3 ± 3.9 mm toward the anode, and ampicillin and amoxicillin, which move about 8.4 ± 2.5 to 9.6 ± 1.1 mm toward the cathode (Table 3). Once the β-lactam antibiotics have been separated into these groups, ceftiofur and cephapirin can be separated further and identified by using a pH 8.0 agarose gel. Ceftiofur and cephapirin move 43.8 ± 11.5 and 62.1 ± 3.9 mm, respectively, toward the anode. Amoxicillin and ampicillin can also be separated further and identified with the pH 8.0 agarose gel. Amoxicillin moves 28.6 ± 3.8 mm

Table 5. Detection limits for β-lactam antibiotics in milk by various assays

Antibiotic standard	Tolerance level, ppb	Lowest detectable level by this method, ppb	Lowest detectable level by Idetek (20) B-L & Cef, ppb	Lowest detectable level by Charm sequential assay (21), ppb	Lowest detectable level by *B. stearothermophilus* disk assay (22), ppb
Amoxicillin	10	30	4	10	5–10[a]
Ampicillin	10	20	5	8	5–8
Penicillin G-K	5	5	4	4.8	2.4–4.8
Cloxacillin Na	10	30	8	50	50–80
Ceftiofur HCl	50	50	50	23	50[a]
Cephapirin Na	20	20	6	4.5	5–8

[a] Unpublished data from Denver District Laboratory.

toward the anode. and ampicillin moves 44.1 ± 4.5 mm toward the anode (Table 4). As shown in Table 4, for some amoxicillin and ampicillin replicates migration of the antibiotic extracted from standard differed from migration of the same antibiotic from the corresponding spiked milk sample when pH 8.0 plates were used. This variation was not observed at pH 6.0 (Table 3), and it may be due to variations in milk samples. However, even with this variation, amoxicillin and ampicillin could still be differentiated (Table 4).

Table 5 compares the method developed in this study with some commercially available test kits as well as the current method of detection. These tests detect some antibiotics at lower levels but do not identify individual antibiotics. By contrast, our method provides presumptive identification, which for 3 of the 6 antibiotics is at the tolerance level.

Method sensitivity can be improved when a large amount of sample is available by extracting double (40 mL) the amount of milk with double (80 mL) the amount of acetonitrile, filtering, and concentrating to 2–3 mL. The residue can then be placed on a different electrophoresis plate with 7 wells, 1.9 cm apart, each 7 mm id. For each sample, 100 µL can be spotted in the smaller wells in the pH 6.0 and pH 8.0 plates. Another approach to increase sensitivity is to concentrate the milk extract further by evaporation in a test tube. Evaporation, however, should be limited, because the volume needed for analysis is 200 or 600 µL (100 or 300 µL for each pH level).

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Determination of Monensin in Edible Bovine Tissues and Milk by Liquid Chromatography

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A method is described for detection and quantitation of monensin in bovine tissues by liquid chromatography (LC) with postcolumn derivatization (PCD) with vanillin. Monensin is extracted from the tissues by homogenization with methanol–water and is isolated and concentrated by liquid–liquid partition and sorbent extraction with silica gel. Monensin is mixed postcolumn with vanillin under acidic conditions and heated, and the resulting products are measured by a variable-wavelength detector at 520 nm. The method has a limit of quantitation of 5 ppb monensin in milk and 25 ppb monensin in bovine muscle, liver, kidney, and fat. Standard recovery over the levels and matrixes tested ranged from 80 to 88%. The method is an improvement in specificity, accuracy, and analysis time over existing monensin residue methods for bovine tissues.

Monensin (Figure 1) is a monocarboxylic polyether compound (1, 2). Sodium monensin is marketed as a feed additive for cattle (Rumensin) to improve feed efficiency. Monensin is typically incorporated into cattle feeds at concentrations ranging from 5 to 30 g/ton or placed into the rumen as a controlled-release capsule.

In 1973, Golab et al. (3) described a colorimetric method for the assay of monensin in feeds and premixes. This method, which is based on the chemical reaction of monensin with vanillin, was applicable at the higher feed concentrations. The standard procedure for the assay of monensin in animal tissues, developed by Donoho and Kline (4), is a thin-layer bioautographic method.

At the Eighth Annual Spring Workshop (1983) of AOAC, we described a method for determination of monensin by liquid chromatography (LC) with postcolumn derivatization (PCD). This method separates monensin from 2 structurally similar ionophores, narasin and salinomycin. In 1984, Goras and LaCourse (5) described a similar system for determination of salinomycin. At an AOAC workshop in 1985, we presented a paper on the confirmation of narasin tissue residues in poultry tissues by LC. Blanchflower et al. (6) reported a method for simultaneous LC determination of monensin, narasin, and salinomycin in feeds, using PCD. In 1992, Rodewald et al. (7) described a method for determination of monensin by LC–PCD with correlation to the official turbidimetric microbiological method (8).

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This LC method is an alternative to current microbiological methods for bovine tissue. It represents an improvement in specificity, analysis time, and precision over existing microbiological methods. This method also eliminates the need for carbon tetrachloride and chloroform in sample purification.

METHOD

Reagents

(a) *Solvents.*—Reagent and LC grade methanol and reagent grade dichloromethane.

(b) *Water.*—Distilled and deionized or LC grade.

(c) *Acids.*—Sulfuric acid and glacial acetic acid, reagent grade.

(d) *Vanillin.*—99% (e.g., Aldrich, Cat. No. V110-4).

(e) *Sodium chloride solution.*—100 g NaCl (reagent grade) dissolved in water to make 1000 mL.

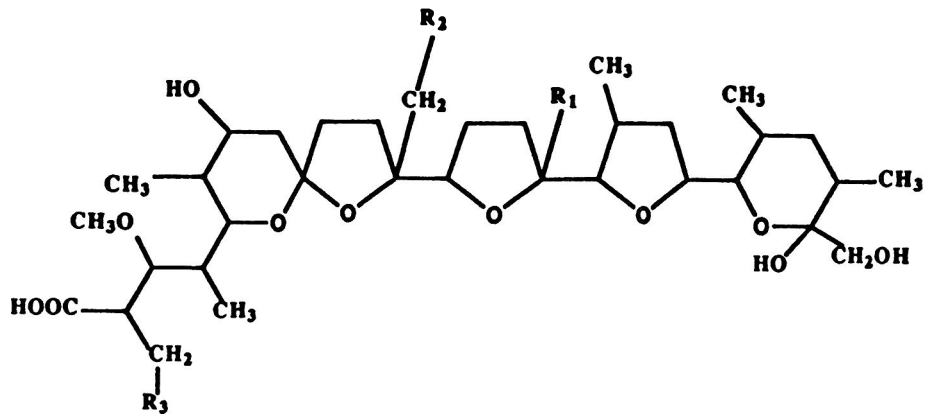
(f) *Mobile phase.*—Methanol–water–acetic acid (940 + 60 + 1). Filter under vacuum through 0.45 µm Nylon-66 filter (Cat. No. 38-114, Rainin Instrument, Woburn, MA). Degas by stirring for 5–10 min under vacuum or sparging with He for 5–10 min. Prepare fresh as required.

(g) *Vanillin reagent.*—While stirring gently, add slowly and carefully 20 mL concentrated sulfuric acid to 950 mL methanol. Allow the methanol–acid solution to cool to room temperature. Add 30.0 g vanillin while stirring. Degas by stirring for 5–10 min under vacuum or by sparging with He for 5–10 min.

(h) *Tissue extraction solution.*—Methanol–water (850 + 150, v/v).

(i) *Sample diluent.*—Methanol–water (900 + 100, v/v).

(j) *Monensin reference standard for LC.*—Accurately weigh an appropriate amount of monensin standard and quantitatively transfer to a volumetric flask. Dissolve and dilute to volume with methanol. Make quantitative dilutions in LC grade methanol–water (90 + 10, v/v) to obtain working solutions at 0.125, 0.25, 0.5, and 1.0 µg/mL. Use the 1.0 µg/mL standard solution to fortify tissues for standard recovery. These standard solutions may be stored for 1 month at room temperature when protected from direct sunlight or stored in a refrigerator.



Factor	R_1	R_2	R_3
A	C_2H_5	H	H
B	CH_3	H	H
C	C_2H_5	H	CH3

Figure 1. Structures of monensin factors.

Apparatus

(a) *Liquid chromatograph.*—With postcolumn reactor (Figure 2). A Beckman Model 110B pulse-dampened pump was used to deliver the mobile phase, and an LDC minipump was used to deliver the vanillin reagent. Both pumps were operated at 0.7 mL/min. A Varian Model 8055 autosampler equipped with a 100 µL injection loop was used. A mixing tee (SSI 01-0165) was placed into the system such that inlet flows directly opposed each another.

(b) *Chromatographic column.*—4.6 mm id × 25 cm Whatman Partisil 5 ODS-3 25 LC column.

(c) *Balances.*—Top loading and analytical.

(d) *Tissue grinder.*—Rival Grind O Matic, or equivalent.

(e) *High-speed blender.*—Hamilton Beach Model 909, or equivalent.

(f) *Centrifuge.*—IEC Model PR-2, or equivalent, capable of providing ca 2500 rpm with 250 mL bottles.

(g) *Rotary vacuum evaporators.*—Rinco, or equivalent, with temperature-controlled water bath.

(h) *Filters.*—Gelman Acrodisc CR and 0.45 µm Nylon-66.

(i) *Silica gel cartridges.*—Sep-Pak, Waters part No. 51900.

Extraction and Purification of Tissues

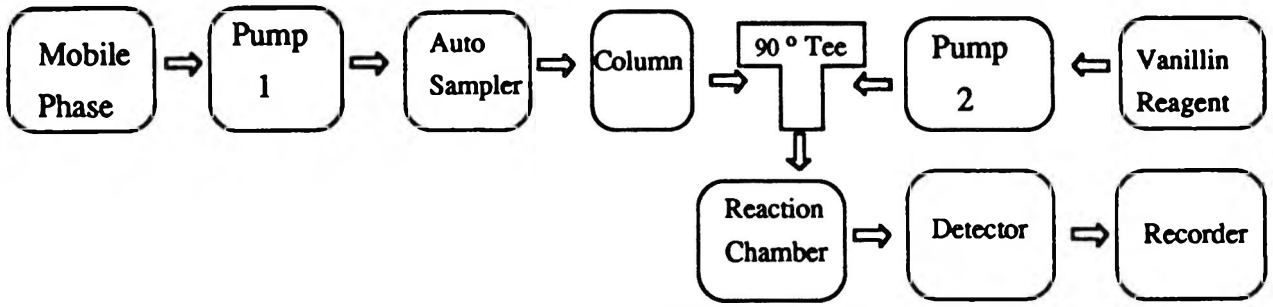
(a) *Tissue extraction.*—Weigh 10 g of a representative ground or minced tissue sample into a suitable container, such as a centrifuge bottle, with at least 100 mL capacity. Add 75 mL extraction solution to the sample and sonicate with an ultrasonic cell disrupter for ca 1 min. Alternatively, blend the tissue for 1 min with a suitable blender. Centrifuge the slurry at ca 2000 rpm for 10 min (exact speed and centrifugal force are not critical provided a good sediment pack and clear supernatant are obtained).

(b) *Liquid–liquid extraction.*—Decant the supernatant into a 250 mL separatory flask, add 50 mL NaCl solution for muscle and liver tissue, 40 mL for kidney, and 60 mL for fat tissue. Extract the aqueous methanol tissue supernatant with two 35 mL portions of dichloromethane (DCM). Collect the lower DCM phase in a 250 mL evaporating flask. Vacuum evaporate the DCM phase to dryness at a maximum water bath temperature of 45°C.

(c) *Solid-phase extraction (SPE).*—Prepare a silica gel SPE cartridge by adding 3 mL DCM and letting it drain to the surface of the cartridge. Dissolve the sample in ca 7 mL DCM and pass through a silica gel Sep-Pak cartridge at not more than 5 mL/min. Rinse the flask with an additional 3 mL DCM and add to the cartridge; discard the effluent. Wash the cartridge with 10 mL DCM; discard the effluent. Elute monensin from the cartridge with 5 mL DCM–methanol (95 + 5, v/v); collect the effluent in a 16 × 100 mm test tube. Evaporate the sample to dryness with an air or nitrogen stream at not more than 45°C. Dissolve the sample in 1 mL sample diluent and filter for LC analysis with a Gelman Acrodisc CR PTFE 0.45 µm filter.

Extraction and Purification of Milk

(a) *Milk extraction.*—Measure 40 mL of a representative, well-mixed milk sample into a suitable container, such as a centrifuge bottle, with at least 250 mL capacity. Add 160 mL



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

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

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

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

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

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

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

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

Reaction chamber = 2-mL stainless steel reaction chamber (0.02 in x 20 ft) enclosed in a 98°C oven/heater.

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

Recorder = Varian Model 9176, or equivalent

Figure 2. Diagram of LC–PCD system.

methanol to the sample and sonicate with an ultrasonic cell disrupter for ca 30 s. Alternatively, blend the sample for 30 s with a suitable blender. Let the sample stand at room temperature for 10–15 min. Centrifuge the slurry at ca 2000 rpm for 10 min (exact speed and centrifugal force are not critical provided a good sediment pack is obtained).

(**b**) *Liquid–liquid extraction.*—Decant the supernatant into a 500 mL separatory flask and add 60 mL NaCl solution. Extract the aqueous methanol supernatant with two 70 mL portions of DCM; collect the lower (DCM) phase in a 125 mL evaporating flask. Vacuum evaporate the DCM phase to dryness at a maximum water bath temperature of 45°C.

(**c**) *SPE extraction.*—Proceed as for tissue samples.

LC Determinative Step

Inject 100 µL LC standard solutions and analytical samples (Figure 2). Measure the peak area response (PR) at the retention volume of monensin for each sample. With the measured responses, construct a linear regression plot of the standard curve to determine the concentration of monensin in experimental samples.

Table 1. Recovery of monensin from bovine tissues[a]

Sample	Monensin present, ppb	Monensin found, ppb	Recovery, %	RSD,[b] %
Muscle	25	19.9	79.6	6.8
	50	42.4	84.8	6.6
	100	84.0	84.0	4.9
Liver	25	20.8	83.2	7.1
	50	41.0	82.0	4.1
	100	83.3	83.3	5.3
Kidney	25	21.6	86.4	7.3
	50	42.0	84.0	3.6
	100	82.8	82.8	3.6
Fat	25	21.7	86.8	9.1
	50	43.6	87.2	6.2
	100	81.5	81.5	4.6
Milk	25	4.4	88.0	5.9
	10	8.8	88.0	8.9
	20	17.2	86.0	5.6

[a] $n = 9$ for all samples at each level of monensin.
[b] Relative standard deviation.

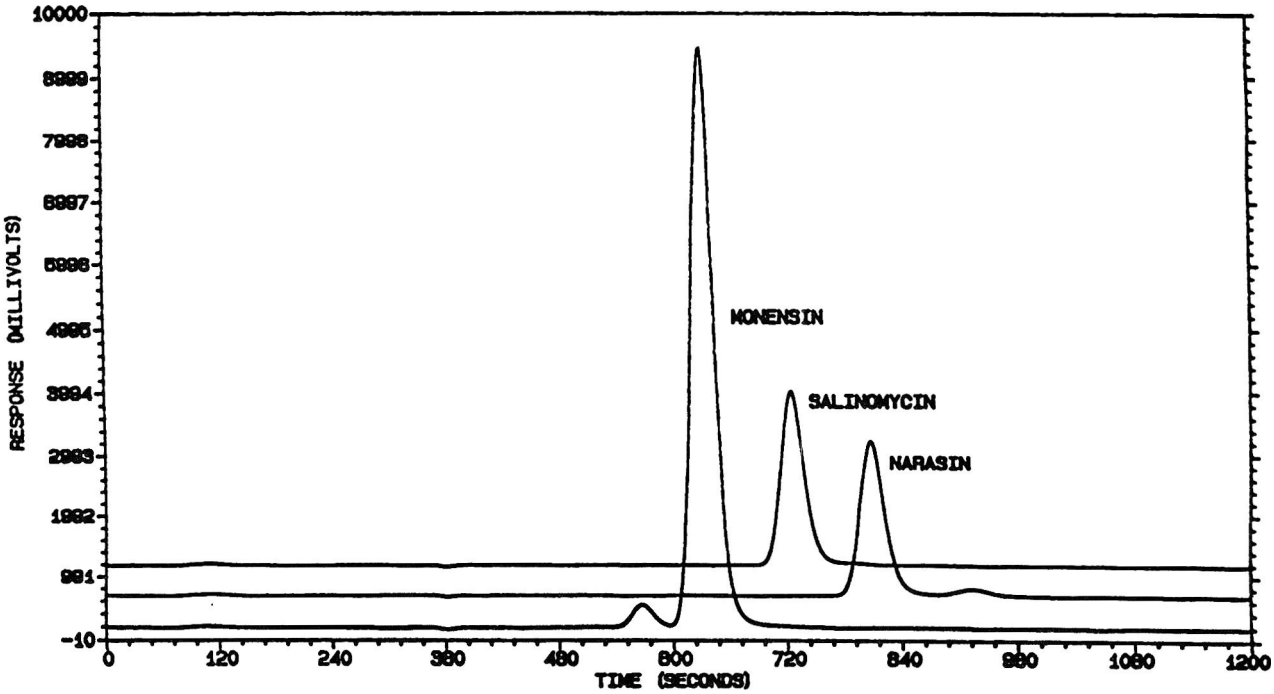


Figure 3. Chromatograms of monensin, narasin, and salinomycin.

Calculation of Monensin Concentration

$$\text{Monensin } (\mu g/g) = \frac{PR_{sa}}{PR_{std}} \times [\text{Std}] \times \frac{V}{wt}$$

where PR_{sa}, peak area response of sample; PR_{std}, peak area response of standard; [Std], concentration of monensin standard (μg/mL); V, sample volume (2 mL); and wt, sample weight (usually 20 g).

LC System Control Parameters

(a) *Retention time.*—The retention time for monensin should be between 400 and 600 s.

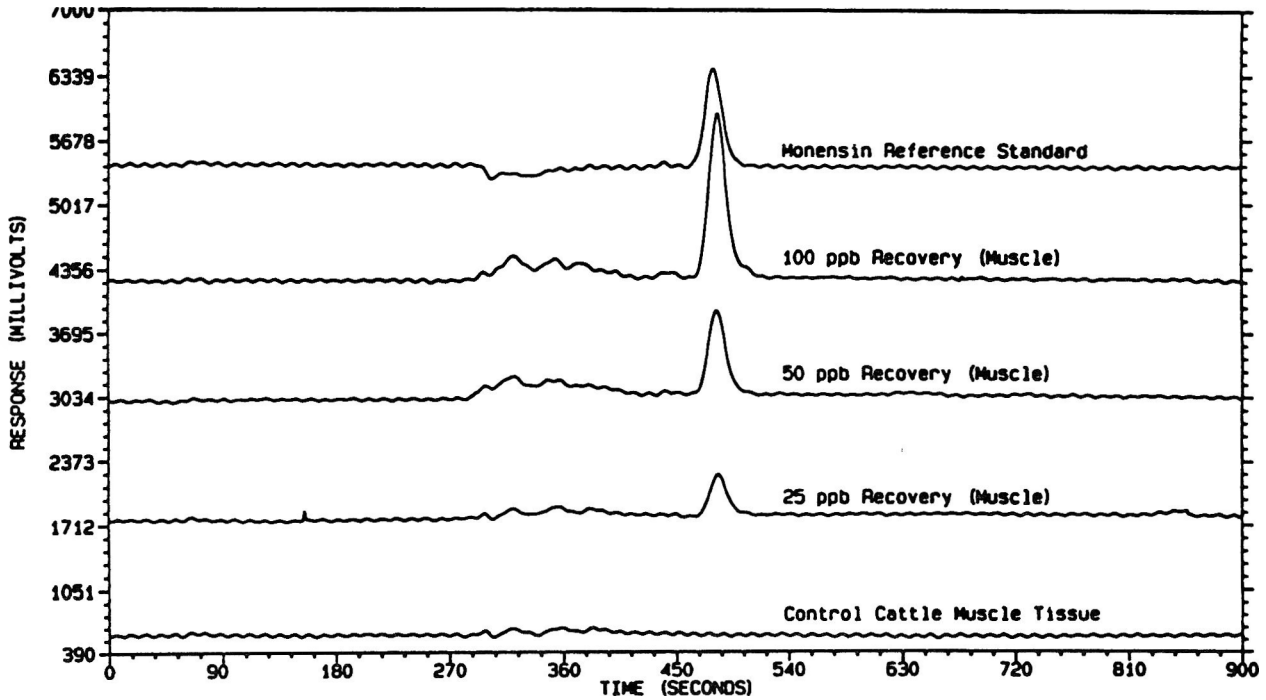


Figure 4. Typical chromatograms of bovine muscle samples.

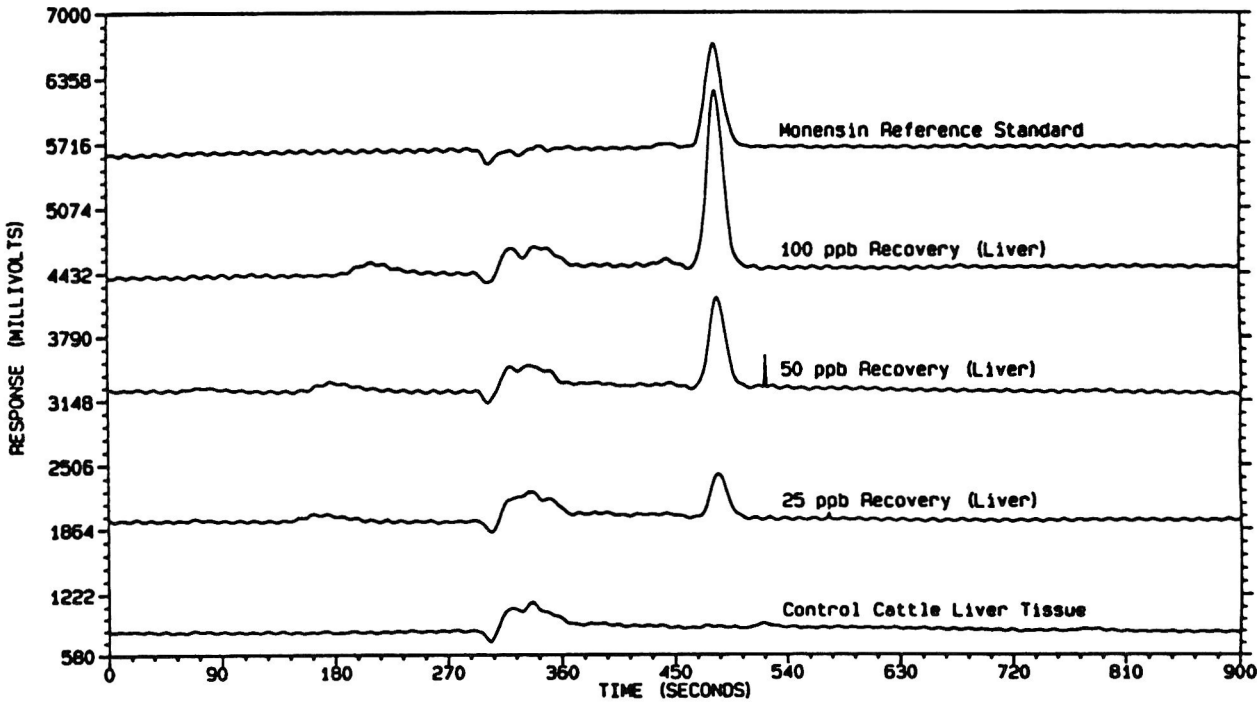


Figure 5. Typical chromatograms of bovine liver samples.

(**b**) *Tailing factor.*—The tailing factor for monensin reference standard should <1.5. Tailing is decreased by increasing the ratio of methanol to water in the mobile phase. Tailing is calculated with the following formula:

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

where retention time is time of the fitted Gaussian curve; 10% width is time of back 10% point – time of front 10% point; and

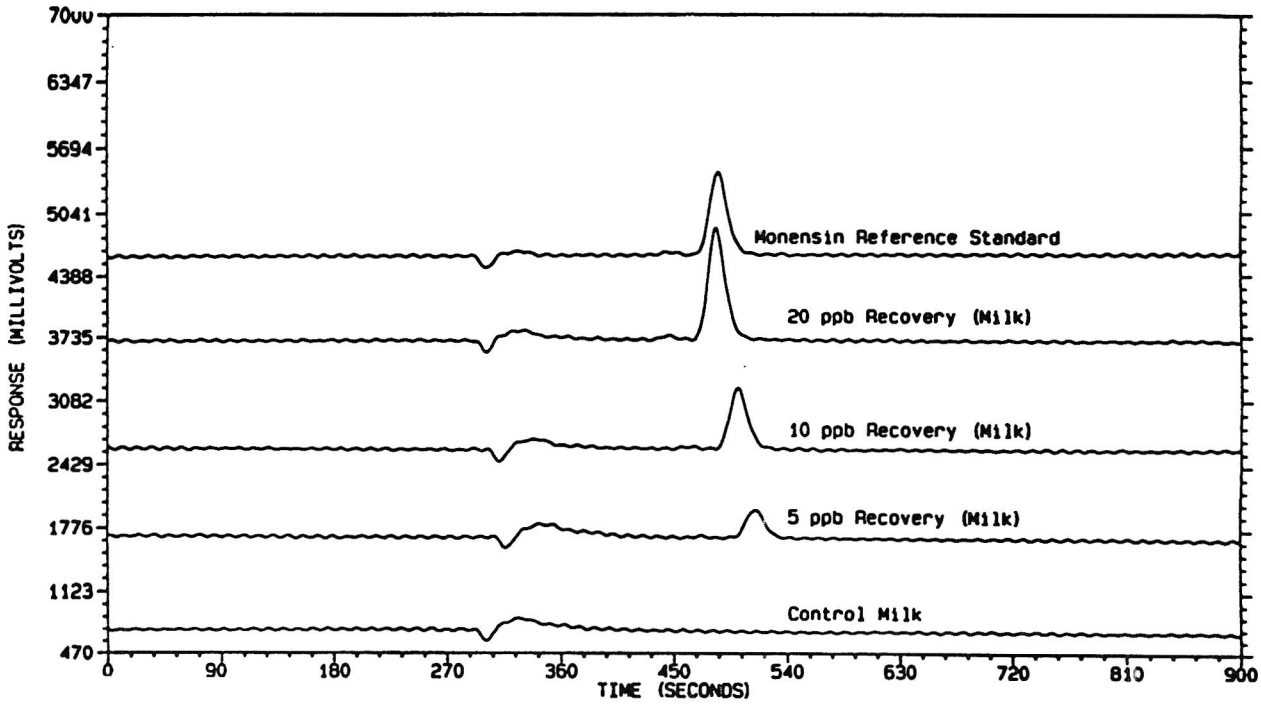


Figure 6. Typical chromatograms of bovine milk samples.

a 10% point is where the response of a side of the peak reaches a height equal to 10% of the peak maximum.

If either of these parameters is not met, the LC conditions must be adjusted. After adjustment, the specification for tailing and retention time must be met prior to analysis of samples. Failure to meet these criteria indicates unsatisfactory column performance.

Results and Discussion

Purpose and Scope

This method was developed for determination of monensin residue in bovine muscle, liver, kidney, and fat tissues and milk. The limit of quantitation (LOQ) in tissues is approximately 25 ppb and approximately 5 ppb in milk (response = 10 × baseline noise in this system). After sample preparation, these levels represent monensin at approximately 0.25 μg/mL in the injected sample.

Validation of LC–PCD Performance

(**a**) *Linearity.*—The linearity of standard response was determined with monensin reference standard concentrations of 0.125, 0.25, 0.5, and 1.0 μg/mL. These concentrations resulted in correlation coefficients ranging from 0.9981 to 0.9999 for the 17 standard curves analyzed.

(**b**) *Precision.*—The precision of standard response was determined by injecting 6 aliquots of the 0.5 μg/mL reference consecutively. The relative standard deviation of the 6 responses was 0.42%.

Validation of Method Performance

(**a**) *Accuracy and precision.*—The accuracy and precision data for this method when applied to fortified tissue samples were generated by analyses of 3 samples per day for 3 days (n = 9) at fortification levels of 25, 50, and 100 ppb in each tissue type. Mean monensin standard recovery from the 4 tissue types and milk ranged from 80 to 88% (Table 1).

(**b**) *Specificity.*—The method is specific for monensin in the presence of closely related ionophores narasin and salinomycin (Figure 3). A third ionophore, lasalocid, is not detected by the PCD system because it does not react with vanillin. Other antibiotics such as tylosin, nicarbazin, bacitracin, lincomycin, and bambermycin do not react in the system and therefore do not interfere. Matrix effect was determined by processing control tissue and dissolving final samples in 2 mL of each concentration used for the standard curve and analyzing against a standard curve in sample diluent only. The correlation coefficients and slopes of the curves show them to be linear and parallel with no significant response bias (Table 2). Chromatograms of control and recovery tissue samples indicate that sample matrixes produce no significant interfering substances at the retention volume of monensin (Figures 4–6).

Table 2. Determination of sample matrix effect on standard curve[a]

Sample matrix	Correlation coefficient	Slope
None	0.9998	31590
Muscle	0.9995	31690
Liver	0.9999	30670
Fat	1.0000	30090
Kidney	0.9998	31140
Milk	0.9997	30240
None	0.9999	31850

[a] Standard level, 0.125–1.0 μg/mL.

Summary

An LC–PCD method has been described for quantitation of monensin in bovine muscle, liver, kidney, and fat tissues and milk. The method is an improvement over existing methods in specificity, accuracy, precision, and analysis time. The validation data demonstrate that the method is selective and accurate for analysis of monensin residues in these tissues and an excellent alternative to existing methods.

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Liquid Chromatographic Determination of Fluorescent Derivatives of Six Sulfonamides in Bovine Serum and Milk

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A rapid and sensitive liquid chromatographic (LC) method with fluorometric detection was developed to detect sulfadiazine, sulfathiazole, sulfamethazine, sulfamonomethoxine, sulfamethoxazole, and sulfadimethoxine residues in bovine serum and milk. *p*-Aminobenzoic acid (PABA) was added as an internal standard. The sulfonamides were extracted from samples and derivatized with fluorescamine, and 50 μL was injected into a Nova-Pak C_{18} LC column and eluted with acetonitrile–10 mM potassium phosphate (30 + 70, v/v). The sulfonamides were detected fluorometricaliy (excitation, 390 nm; emission, 475 nm), and their retention times ranged from 6.2 to 16.5 min without interference from coextractives. The detection limit for standard sulfonamide solution was 0.1 ng/mL; the calibration curves were linear between 1 and 100 ng/mL in the presence of PABA as internal standard. Recovery rates of sulfonamides from spiked samples (1 and 10 ppb) were 95.4–107.2 and 81.4–89.6% for serum and 80.7–91.1 and 62.6–84.1% for milk, respectively.

Sulfonamides are used to prevent and treat infectious diseases in animals. Sulfamethazine (SMT), sulfamonomethoxine (SMM), and sulfadimethoxine (SDM) are used mainly as feed additives for animal production in Japan (1). The safety of sulfonamides to consumers has been questioned because of their apparent toxicity (2). In Belgium, a zero tolerance level for sulfonamides in edible animal tissues has been set (3), and in Japan, zero tolerance for antimicrobial agents in edible animal tissues has been established. Therefore, a sensitive and reliable method is needed to monitor edible animal tissues for sulfonamide residues.

Methods for determination of sulfonamides in animal tissue include bioassay (4), thin-layer chromatography (TLC) (3, 5–7), liquid chromatography (LC) (1, 2, 8, 9), enzyme immunoassay (10, 11), gas–liquid chromatography with mass spectrometry (12, 13), and postcolumn derivatization with dimethylaminobenzaldehyde (14, 15). However, none of these methods enables several sulfonamide residues at a concentration of 1 ppb in bovine serum and milk to be identified simultaneously. LC after fluorescamine derivatization does.

The use of fluorescamine was first reported in 1972 as a means of generating fluorescent derivatives of primary amino acids (16). Subsequently, Sigel et al. (6) detected sulfadiazine (SDZ) on a TLC plate by dipping it into a fluorescamine solution. Reimer et al. (5) detected sulfonamides after spraying TLC plates with a fluorescamine solution. In this study, we detected 6 sulfonamides that had been derivatized with fluorescamine by LC with a fluorescence detector. Bovine serum and milk samples were pretreated with acetonitrile to precipitate proteins and then centrifuged before LC analysis.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—A Model 6000E multisolvent delivery pump connected to a U6K injector (Waters Associates, Inc., Milord, MA); detector, Hitachi F-1050 fluorescence spectrophotometer (Hitachi Co., Tokyo) operated at excitation and emission wavelengths of 390 and 475 nm, respectively; chromatographic data system, Chromatopac C-R6A integrator (Shimadzu Seisaku Co., Kyoto, Japan); column, Nova-Pack C_{18}, prepacked 10 μm particle size, 300 mm × 3.9 mm id (Waters Associates); flow rate, 1.0 mL/min; and temperature, ambient.

(b) *Centrifuge.*—Model KS-5000P operated at 1000 × *g* (Kubota Co., Tokyo, Japan).

(c) *Milli-Q Labo.*—For production of LC grade water from distilled water (Millipore Corp., Bedford, MA).

Reagents

(a) *Water.*—Water was purified with a Milli-Q system from Millipore until a resistivity of 18 MΩ · cm was achieved.

(b) *Standard solutions.*—Sulfadiazine (SDZ), sulfamethazine (SMT), sulfamonomethoxine (SMM), sulfathiazole (SMX), and sulfadimethoxine (SDM) were from Sigma Chemicals Co., St. Louis, MO; sulfathiazole (STZ) was from Tokyo Kasei Kogyo Co., Tokyo, Japan. Samples (10 mg) of SMT, SMX, and STZ were dissolved in 1 mL, and 10 mg samples of SDZ, SMM, and SDM were dissolved in 2 mL, of *N,N'*-dimethylformamide; 9 mL dis-

tilled water was added to SMT, SMX, and STZ, and 8 mL was added to SDZ, SMM, and SDM. These stock solutions were stored in a refrigerator. Before use, each stock solution was diluted with distilled water to the required concentrations.

(c) *Solvents.*—*N,N'*-Dimethylformamide, acetonitrile, methanol (Wako Pure Chemical Industries, Tokyo, Japan), and trichloroacetic acid (TCA; Yoneyama Yakuhin Kogyo Co., Osaka, Japan).

(d) *Mobile phase.*—A mixture of 300 mL acetonitrile and 700 mL 10 mM potassium dihydrogen phosphate.

(e) *Derivative solution.*—This solution was prepared fresh each day by dissolving 10 mg fluorescamine (Sigma Chemicals) in 10 mL acetone.

(f) *Internal standard.*—Made by dissolving 10 mg *p*-aminobenzoic acid (PABA; Nakarai Tesque Co., Kyoto, Japan) in 1 mL *N,N'*-dimethylformamide and diluting with 9 mL distilled water.

Extraction from Sample

A 1.0 mL aliquot of serum or milk was transferred to a 10 mL test tube. Then, 4 mL acetonitrile was added for extraction and deproteinization, and the mixture was stirred on a Vortex mixer and centrifuged for 15 min at 1000 × *g*. The supernatant was evaporated to dryness under nitrogen at 40°C in a water bath, and the residue was dissolved in 0.05 mL distilled water and mixed vigorously. Acetonitrile (1 mL) was added, then the mixture was centrifuged at 1000 × *g* for 10 min. The upper layer was evaporated to dryness, and the residue was dissolved in 1 mL 0.01% TCA (w/v) containing PABA at 10 ng/mL (as internal standard) and centrifuged at 1000 × *g* for 10 min. A 0.5 mL sample of the clear layer was collected with a pipet and mixed with 0.1 mL fluorescamine solution for 1 min at room temperature to derivatize the sulfonamides, and then 50 μL was injected into the LC column.

Recovery Studies

A 0.1 mL aliquot of a solution of each sulfonamide (100 and 10 ng/mL) was added to 1.0 mL sulfonamide-free bovine serum and milk (spiked at 10 and 1 ppb, respectively) in a 10 mL test tube.

Calculation

Standard calibration curves (4 replicates each) for the 6 sulfonamides were plotted. The ratios of the peak height (*h*) of each sulfonamide to that of the internal standard (IS) were plotted against concentration (1, 2.5, 5, 10, 25, 50, and 100 ng/mL) according to the following equations:

$$Y = aX + b$$

and $$Y = h \text{ (of each sulfonamide)}/h \text{ (of IS)}$$

where X is the concentration, a is the slope, and b is the intercept. Recoveries of sulfonamides from spiked samples were calculated by comparing peak heights of extracts of spiked samples with those of standard controls under identical LC conditions.

Results and Discussion

To establish the optimal volume of sulfonamides for derivatization, we derivatized 9 volumes (0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL) of SMT (0.01 μg/mL) with 0.1 mL fluorescamine solution (1.0 mg/mL). Best results were achieved when the volume was 0.5–1.5 mL. We selected 0.5 mL as the volume of sulfonamide solution for derivatization. We ascertained the optimal fluorescamine concentration for derivatization by testing various levels: 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, and

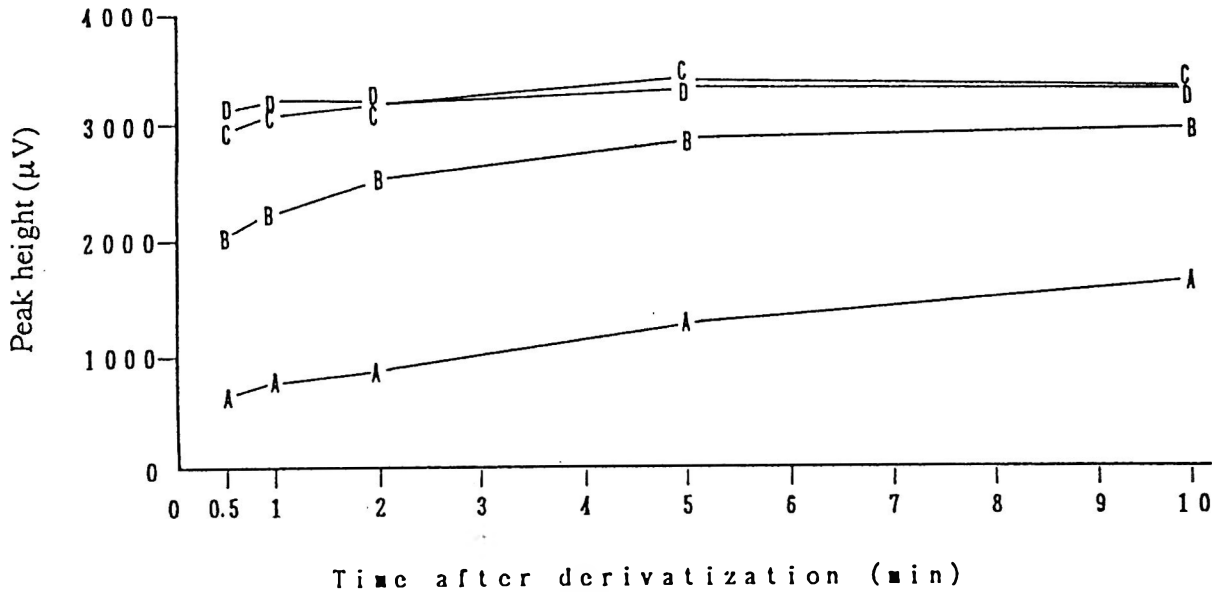

Figure 1. Stability of derivatives obtained by reacting 0.5 mL sulfamethazine (0.01 μg/mL) with 0.1 mL fluorescamine at different concentrations (A, 0.1; B, 0.5; C, 1.0; D, 1.5 mg/mL).

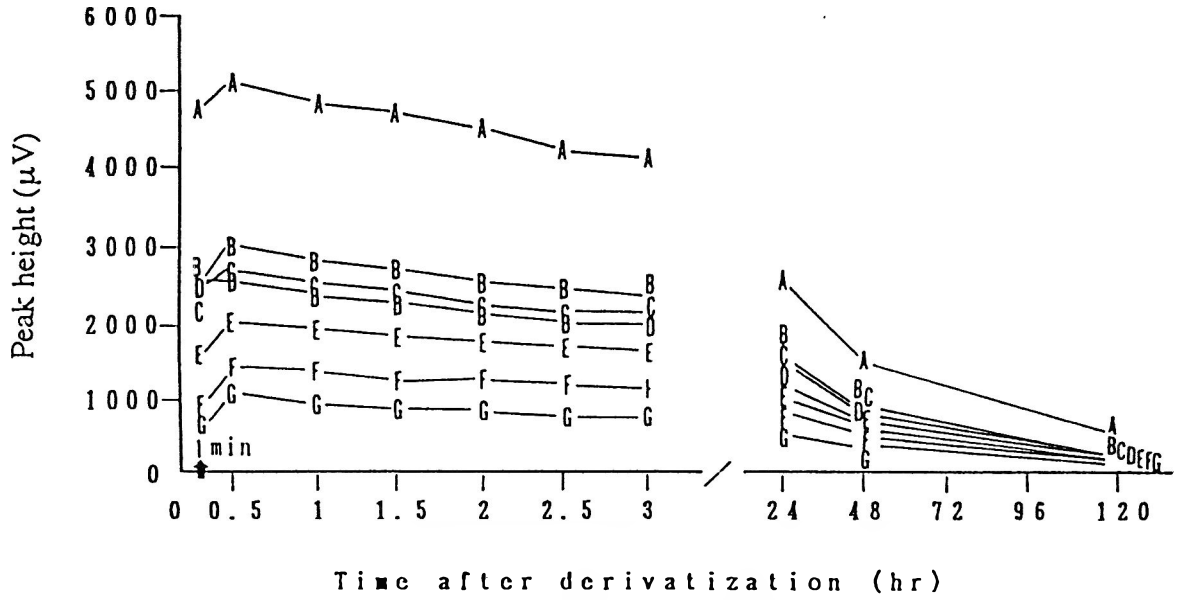

Figure 2. Stability of PABA (A) and derivatives of 6 sulfonamides (B, SDZ; C, STZ; D, SMT; E, SMM; F, SMX; and G, SDM) obtained by reacting 0.5 mL sulfonamide at a concentration of 0.01 μg/mL with 0.1 mL fluorescamine at 1 mg/mL.

4.0 mg/mL. In excess of 2.0 mg/mL, fluorescamine formed a precipitate with 0.5 mL SMT (0.01 μg/mL). Therefore, we used fluorescamine concentrations of 0.1, 0.5, 1.0, and 1.5 mg/mL (0.1 mL) and compared the results.

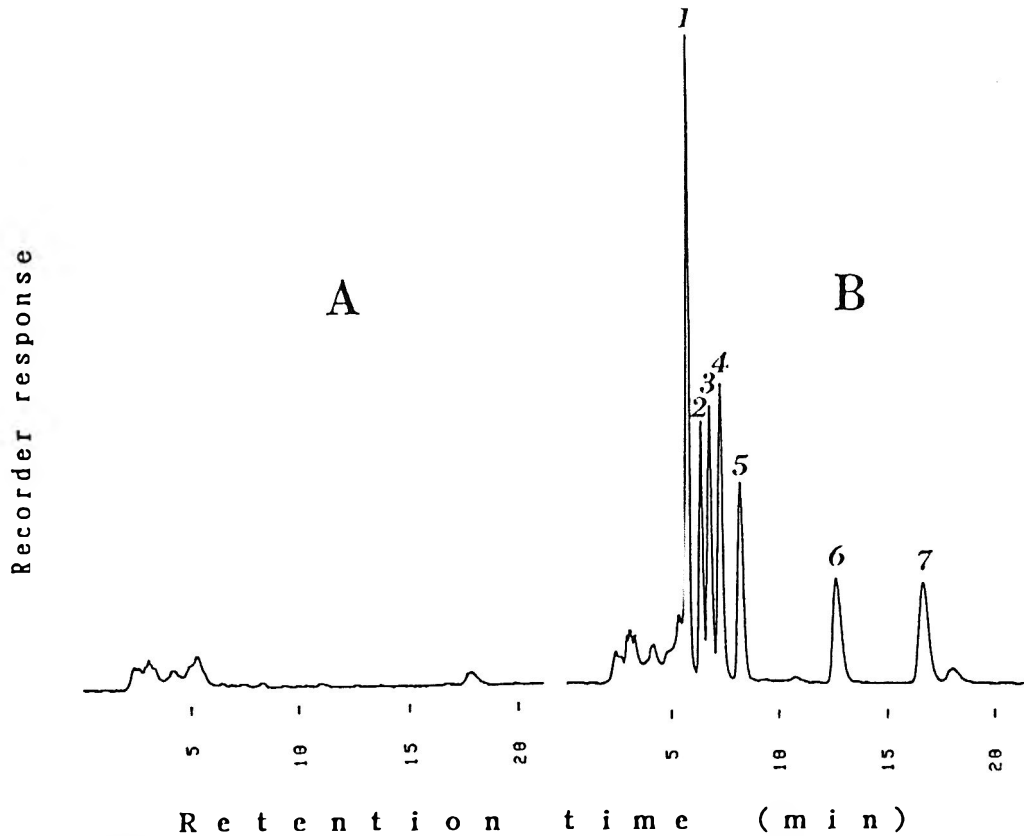

Figure 3. Chromatogram of blank (A) and spiked (10 ppb) bovine serum samples (B): (1) PABA (internal standard), (2) SDZ, (3) STZ, (4) SMT, (5) SMM, (6) SMX, and (7) SDM. Their retention times after acetonitrile extraction and fluorescamine derivatization were 5.6, 6.3, 6.7, 7.2, 8.1, 12.5, and 16.5 min, respectively.

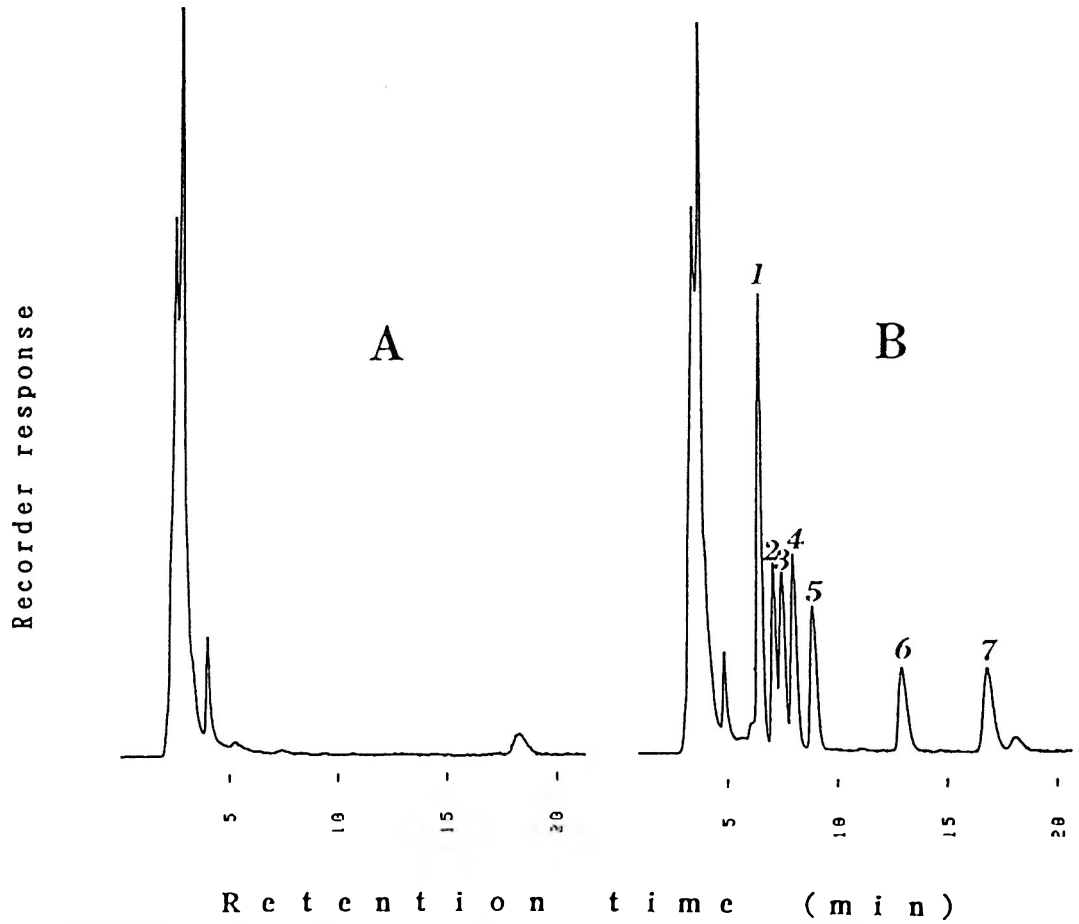

Figure 4. Chromatogram of blank (A) and spiked (10 ppb) milk samples (B): (1) PABA (internal standard), (2) SDZ, (3) STZ, (4) SMT, (5) SMM, (6) SMX, and (7) SDM. Their retention times after acetonitrile extraction and fluorescamine derivatization were 5.6, 6.3, 6.7, 7.2, 8.1, 12.5, and 16.5 min, respectively.

The fluorescamine concentration used for TLC was 0.1–0.25 mg/mL in acetone (3, 5–7). Figure 1 shows the stability (4 replicates) of the derivative from 0.5 mL SMT (0.01 μg/mL) reacted with 0.1 mL fluorescamine (0.1, 0.5, 1.0, and 1.5 mg/mL). The peak heights obtained with fluorescamine at 0.1 and 0.5 mg/mL were lower than those with 1.0 and 1.5 mg/mL after reacting for 30 s to 10 min. The peak heights with fluorescamine at 1.0 and 1.5 mg/mL were virtually identical (about 3000 μV) and were stable after reacting for 30 s to 10 min. Therefore, we selected the derivatization mixture obtained with 1.0 mg/mL fluorescamine and injected it into the LC column after a reaction time of 1 min, because the derivative obtained was stable and this time was convenient.

The stabilities of the PABA standard (10 ng/mL) and 6 sulfonamide (0.01 μg/mL each) derivatives with fluorescamine at 1.0 mg/mL are shown in Figure 2 (4 replicates). We used 0.1 mL fluorescamine (1.0 mg/mL) solution to derivatize 0.5 mL of a mixture of PABA and the 6 sulfonamides (0.01 μg/mL each) for 1 min, 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 1 day, 2 days, and 5 days to monitor the decline of the fluorophore. We found that the peak height, after derivatization for 1 day, was about half that after 1 min; it halved again from 1 to 2 days, and after 5 days, about 1 ppb was detected. Van Poucke et al. (3) reported that the fluorophore was stable for at least 30 min when TLC was used. In this study, it was stable for 3 h. Therefore, we used a derivatization time of 1 min before injection into the LC column, which resulted in a high peak. The PABA peak was the most intense and highest (about 5000 μV), and the peak heights of the 6 sulfonamides ranged from 1000 to 3000 μV.

The chromatograms of blank and sulfonamide-spiked (10 ppb) serum and milk samples are shown in Figures 3 and 4. Each peak was symmetrical. The retention times of the 6 sul-

Table 1. Recoveries[a] of 6 sulfonamides from spiked (1 and 10 ppb) bovine serum (n = 6)

Sulfonamides	Recovery (%) at indicated spiking level	
	1 ppb	10 ppb
SDZ	97.0 ± 4.2	81.4 ± 4.9
STZ	101.1 ± 6.3	89.6 ± 4.2
SMT	105.0 ± 4.2	89.3 ± 3.2
SMM	97.5 ± 3.5	83.9 ± 6.6
SMX	95.4 ± 4.9	87.0 ± 4.6
SDM	107.2 ± 11.2	86.3 ± 6.2

[a] Values are means ± standard deviations.

Table 2. Recoveries[a] of 6 sulfonamides from spiked (1 and 10 ppb) bovine milk (n = 6)

Sulfonamides	Recovery (%) at indicated spiking level	
	1 ppb	10 ppb
SDZ	82.3 ± 6.0	71.4 ± 1.7
STZ	82.9 ± 4.2	72.7 ± 1.5
SMT	80.7 ± 4.9	62.6 ± 2.6
SMM	85.2 ± 4.9	73.8 ± 2.6
SMX	87.5 ± 5.4	84.1 ± 3.1
SDM	91.1 ± 6.9	80.8 ± 3.2

[a] Values are means ± standard deviations.

fonamides ranged from 6.2 to 16.5 min, and their recovery rates from serum and milk are shown in Tables 1 and 2. The respective means ± standard deviations, and recoveries from 1 and 10 ppb-spiked samples ranged from 95.4 ± 4.9 to 107.2 ± 11.2% and 81.4 ± 4.9 to 89.6 ± 4.2%, respectively, for serum, and 80.7 ± 4.9 to 91.1 ± 6.9% and 62.6 ± 2.6 to 84.1 ± 3.1%, respectivelly, for milk. Recovery rates from serum were higher than from milk samples, and those from 1 ppb-spiked samples were a little higher than those from 10 ppb-spiked samples.

The standard calibration curves for the 6 sulfonamides were linear with correlation coefficients in excess of 0.99 as follows: SDZ, 0.9983 ± 0.0012; STZ, 0.9970 ± 0.0026; SMT, 0.9991 ± 0.0005; SMM, 0.9988 ± 0.0009; SMX, 0.9987 ± 0.0009 and SDM, 0.9987 ± 0.0006.

It was most important to dissolve the extracted residues that had been evaporated to dryness under nitrogen before derivatizing them with fluorescamine solution. We tested the following solutions: 0.01M HCl, 1% acetic acid, 0.01M KH_2PO_4, and 1% TCA. We found that 1% TCA solution produced the best result, but the LC peak was low and wide. So, we tried 5 concentrations of TCA (1, 0.5, 0.25, 0.1, and 0.01%, w/v) to dissolve the dried residues. The 0.01% solution was the best solvent for peak elution. The other concentrations affected fluorophore formation (reflected by low and wide peaks), perhaps because of their low pH values. When the residue solution made with 1% TCA was diluted to 0.01% TCA, the LC peaks were sharp and narrow, like the standards. This showed that a high concentration of TCA did not destroy the sulfonamides in the solution, but did prevent perfect fluorophore formation with fluorescamine. Therefore, we used 0.01% TCA to dissolve the residues after evaporation to dryness.

Gudding (4) demonstrated that PABA antagonized the bacterial inhibitory effect of sulfanilamide and was essential for dihydrofolic acid synthesis. He reported that adding PABA at 2 μg/mL to the medium reduced the detection sensitivity for sulfonamides. Therefore, we used PABA as internal standard in this experiment. Generally, the paper disk diffusion method is performed first to screen for sulfonamides in residues, and PABA and trimethoprim are often used for this screening. Next, LC analysis can be used to confirm whether residue-positive samples found with paper disk diffusion do contain sulfonamides and, if so, to identify them. Therefore, this LC method can be used with the paper disk diffusion method to detect sulfonamide residues.

Usually, sulfonamides are extracted from liquid samples by homogenizing the sample with solvent, such as acetone–chloroform (2, 8, 9). Munns et al. (17) reported that acetonitrile precipitates most of the casein and protein in animal tissue samples. In this study, we used acetonitrile for extraction and centrifugation and then evaporated the samples to dryness. The method enabled 6 sulfonamides to be extracted simultaneously from bovine serum and milk samples; a sample volume of only 1 mL and 5 mL of acetonitrile for extraction and centrifugation were required, followed by evaporation to dryness and an easy derivatization process. However, data from incurred samples are needed before the adequacy of the assay is documented. It is a simple, rapid, and sensitive method for simultaneously analyzing several sulfonamides in a sample and may be useful for routine laboratory testing for residual sulfonamides in food.

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FOOD BIOLOGICAL CONTAMINANTS

Relative Effectiveness of Selective Plating Agars for Recovery of *Salmonella* Species from Selected High-Moisture Foods

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The relative effectiveness of 6 selective plating media were compared for effectiveness in recovery of *Salmonella* spp. from selected high-moisture foods. Three new plating agars (EF-18, Rambach, and xylose lysine Tergitol-4) and 3 selective plating agars (bismuth sulfite, Hektoen enteric, and xylose lysine desoxycholate) recommended by AOAC INTERNATIONAL and the *Bacteriological Analytical Manual* (BAM) were compared. The agars were streaked from cultures selectively enriched in selenite cystine broth, tetrathionate broth, and Rappaport–Vassiliadis medium. The high-moisture foods studied were naturally contaminated pork sausage, chicken parts, turkey parts, and frog legs and artificially contaminated shrimp, oysters, egg yolks, and lettuce. The relative effectiveness of each selective plating agar was determined by recovery of *Salmonella* spp. and enumeration of false-positive and false-negative reactions. Although the new selective plating agars compared favorably with the AOAC/BAM-recommended agars, they offered no advantage. Incubation of selective enrichment broths at elevated temperatures decreased the numbers of false-positive and false-negative reactions for all 6 selective plating agars.

Several commercially available selective plating agars used to isolate and differentiate *Salmonella* spp. from other members of *Enterobacteriaceae* contain ingredients that restrict the growth of competing non-*Salmonella* spp. (1). The selective plating agars currently recommended by AOAC and the *Bacteriological Analytical Manual* (BAM) of the U.S. Food and Drug Administration (FDA) (bismuth sulfite [BS], Hektoen enteric [HE], and xylose lysine desoxycholate [XLD]) are used to isolate *Salmonella* spp. from foods (2, 3). The use of this combination of agars was based on results of an AOAC collaborative study (4); however, other agars have since been introduced. The present study compared the efficiency of 3 new agars (EF-18, Rambach, and xylose lysine Tergitol-4 [XLT-4]) with that of the AOAC/BAM-recommended agars.

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EF-18 medium is used as a selective plating agar in the AOAC-approved hydrophobic grid membrane filter method (5) for rapid identification of *Salmonella* spp. To our knowledge, EF-18 has not been evaluated as a streaking agar for the isolation of *Salmonella* spp.

Rambach medium uses a unique phenotypic characteristic, the formation of acid from propylene glycol (6), for identification of *Salmonella* spp. Most other selective plating agars rely on either lactose utilization or hydrogen sulfide production to distinguish *Salmonella* spp. from non-*Salmonella* spp. These characteristics, however, are frequently inadequate for differentiating *Salmonella* spp. from related enteric bacteria, particularly *Proteus* spp., which, like most *Salmonella* spp., are lactose-negative and hydrogen sulfide-positive.

XLT-4 medium was developed by Tate and Miller (7) to isolate *Salmonella* spp. from poultry in the presence of competitive organisms such as *Proteus*, *Pseudomonas*, and *Providencia* spp. Tergitol-4 added to XL agar base was reported to be superior for isolation of *Salmonella* spp. from poultry (8); however, its usefulness for a broader category of foods has not been investigated.

Experimental

Media and Reagents

Rappaport–Vassiliadis (RV) medium (9), Rambach agar (6), and XLT-4 agar (8) were prepared according to the developers' instructions. Remaining media and reagents were prepared according to methods recommended in *Official Methods of Analysis* (2).

Preparation of Inoculum

Brain heart infusion (BHI) broth (200 mL) was inoculated with one of 4 cultures of *Salmonella* spp. grown on BHI agar slants for 18–24 h at 35°C. Four *Salmonella* serovars (*S. enteritidis*, *S. kentucky*, *S. poona*, and *S. worthington*) were obtained from the FDA's regulatory stock culture collection of foodborne isolates.

After incubation of the BHI broth for 18–24 h at 35°C, two 10 mL aliquots of the broth culture were centrifuged 10 min at 5000 × *g*. Each was washed twice with 10 mL sterile Butterfield's phosphate buffer, pH 6.8–7.2. The contents of each tube were combined, and serial 10-fold dilutions were made for inoculation of certain food types.

Table 1. Components of selective plating agars[a]

Ingredient	Component	BS	HE	XLD	EF-18	Rambach	XLT-4
Carbohydrate	Glucose	X			X		
	Lactose		X	X			X
	Sucrose		X	X	X		X
	Xylose			X			X
	Propylene glycol					X	
	Salicin		X				
Protein	Peptone	X	X		X	X	
	Beef extract	X					
	Yeast extract		X	X	X	X	X
	Lysine			X	X		X
H_2S indicator system	Bismuth sulfite indicator	X					
	Ferric ammonium citrate		X	X			X
	Sodium thiosulfate		X	X			X
	Ferrous sulfate	X					
Selective agent	Bile salts				X		
	Bile salts No. 3		X				
	Sodium desoxycholate			X		X	
	Sulfapyridine				X		
Antibiotic	Novobiocin				X		
Surfactant	Tergitol-4						X
Indicator dye	Bromthymol blue		X		X		
	Acid fuchsin		X				
	Phenol red			X			X
	Brilliant green	X					
	Neutral red					X	
Inorganic salt	Disodium phosphate	X					
	Sodium chloride		X	X			X
	Magnesium sulfate				X		
Enzyme substrate	5-Bromo-4-chloro-3-indolyl β-galactopyranoside					X	

[a] BS, bismuth sulfite; HE, Hektoen enteric; XLD, xylose lysine desoxycholate; XLT-4, xylose lysine Tergitol-4.

Preparation of Foods

For this study, raw shrimp, raw oysters, egg yolks, and lettuce were artificially inoculated; other foods used were naturally contaminated. Foods were purchased at local retail stores in the Washington, DC, area. Contamination levels of the bulk amounts of artificially contaminated foods were determined by the 3-tube most-probable-number procedure on the day analysis was initiated (3).

(**a**) *Raw shrimp.*—A 5 mL aliquot of the appropriate serial 10-fold dilution of *Salmonella* spp. inoculum was added to a 1000 g bulk amount of thawed shrimp immersed in 3 L Butterfield's phosphate buffer. The mixture was gently stirred 15 min, drained thoroughly in colanders, and placed in a Stomacher 3500 (Tekmar Co., Cincinnati, OH) bag. The contents were frozen 2–3 days at −10° to −6°C.

(**b**) *Raw oysters.*—Oysters (200 g) and Butterfield's phosphate buffer (200 mL) were blended 2 min at ca 10 000–12 000 rpm. The mixture was poured into a sterile 6 L flask, and the process was repeated to a total volume of 2 L. A 5 mL aliquot of the appropriate serial 10-fold dilution of the washed *Salmonella* suspension was added, and the inoculated homogenate was mixed manually and refrigerated 2–3 days at 2°–5°C.

(**c**) *Egg yolk.*—Egg shells of intact whole eggs were surface-disinfected (10). Aseptic techniques were used to crack the eggs and separate the albumen from the yolk. Egg yolks were combined, and 1000 g portions were weighed into sterile 3 L flasks. A 5 mL aliquot of the appropriate serial 10-fold dilution of the washed *Salmonella* suspension was added, and the inoculated yolks were mixed manually and refrigerated 2–3 days at 2°–5°C.

(**d**) *Lettuce.*—Lettuce was shredded in a commercial food processor. A 1000 g bulk amount of shredded lettuce was placed in a 48 × 27 × 13 cm sterile plastic tray covered with foil. Butterfield's phosphate buffer (2 L) was inoculated with 5 mL of the appropriate serial 10-fold dilution of the washed *Salmonella* suspension; this suspension was added to the lettuce, and the material was mixed well for 15 min. The inoculated lettuce was drained thoroughly in colanders, placed in Stomacher 3500 bags, and refrigerated 2–3 days at 2°–5°C.

Comparative Efficiency of Selective Plating Agars

Raw pork sausage, raw chicken, raw turkey, raw shrimp, raw oysters, and lettuce (25 g each) were blended with 225 mL lactose broth (3) for 2 min at ca 10 000–12 000 rpm. Individual

Table 2. Colony characteristics of selected enteric bacteria on selective plating agars[a]

Culture[b]	BS	HE	XLD	EF-18	Rambach	XLT-4
Salmonella anatum	Black with metallic sheen	Dark green with black center	Pink with black center	Jade green with dark center	Red	Pink with black center
Salmonella arizonae (ATCC 12324) (Lac +)	Green with black center	Dark green with black center	Yellow with black center	Jade green with dark center	Pink	Yellow with black center
Salmonella arizonae (ATCC 13314)	Green with black center	Dark green with black center	Pink with black center	Jade green with dark center	Dark blue	Pink with black center
Salmonella cubana (H_2S –)	Black with metallic sheen	Dark green	Pink	Jade green with dark center	Red	Pink
Salmonella enteritidis	Black with metallic sheen	Dark green with black center	Pink with black center	Jade green with dark center	Red	Pink with black center
Salmonella tennessee (Lac +)	Green with black center	Yellow with black center	Yellow with black center	Jade green with dark center	Purple	Yellow with black center
Citrobacter freundii (ATCC 8090)	Green with black center	Yellow	Yellow	Yellow	Dark blue	Yellow
Edwardsiella tarda (ATCC 15947)	Green with black center	Black	Yellow with black center	Yellow	Dark blue	Yellow
Enterobacter aerogenes (ATCC 13048)	Green with black center	Yellow	Yellow	Yellow	Dark blue	Yellow
Enterobacter cloacae (ATCC 13047)	Green	Yellow with black center	Yellow	Yellow	Dark blue	Yellow
Escherichia coli (ATCC 8677)	Green with black center	Yellow	Yellow	Yellow	Dark blue	Yellow
Klebsiella pneumoniae (ATCC 9997)	Green with black center	Yellow	Yellow	Yellow	Dark blue	Yellow
Proteus mirabilis (ATCC 12453)	Green	Dark green	Pink with black center	Yellow	Pink	No growth
Proteus vulgaris	Green	Yellow	Yellow	Yellow	Pink	No growth
Providencia alcalifaciens (ATCC 9886)	Green with black center	Yellow with black center	Yellow	Yellow	Dark blue	Yellow

[a] BS, bismuth sulfite; HE, Hektoen enteric; XLD, xylose lysine desoxycholate; XLT-4, xylose lysine Tergitol-4.
[b] Unless specified otherwise, all *Salmonella* cultures were lactose-negative and hydrogen sulfide-positive.

frog legs were placed in lactose broth and shaken 15 min on a mechanical shaker at 100 rpm. The legs were removed, and additional lactose broth was added to a total volume of 250 mL. For egg yolks, 25 g was mixed manually with 225 mL trypticase soy broth (10). After 60 min, the pH of each food mixture was determined and adjusted, if necessary, to 6.8 ± 0.2. Test portions were incubated 24 ± 2 h at 35°C. After incubation, 1 mL aliquots of the preenriched test portions were subcultured to 10 mL selenite cystine (SC) broth and incubated at 35°C; 1 mL aliquots were also added to 10 mL tetrathionate (TT) broth and incubated at 35°, 41°, and 43°C; 0.1 mL aliquots of the incubated preenrichments were subcultured to 10 mL RV medium and incubated at 42° and 43°C. All selective enrichments were incubated for 24 ± 2 h. After incubation, cultures from the selective enrichments were streaked on BS, HE, XLD, EF-18, Rambach, and XLT-4 agars and incubated 24 ± 2 h at 35°C.

After incubation, the plating agars were examined for the presence of *Salmonella* colonies. BS agar was reincubated for an additional 24 h and reexamined. Suspect colonies on each of the 6 selective plating agars were transferred to triple sugar iron and lysine iron agars. Cultures giving reactions typical of *Salmonella* spp. were biochemically and serologically identified by the AOAC-recommended method.

Statistical Analysis

The data were analyzed by a Chi-square test for pairwise comparison of the media in terms of recovery and false-positive and false-negative rates. Significance was assessed at $p < 0.05$ (11).

Results and Discussion

Selective plating agars promote the growth of *Salmonella* spp. through formation of distinct colonies while repressing competing microflora. *Salmonella* spp. are differentiated by incorporating various dyes, bile salts, and other selective agents in the agars (12). Components of the 6 selective plating agars examined are shown

in Table 1. Each agar contains at least one source of carbohydrate and one source of protein, as well as an indicator dye; many also contain a hydrogen sulfide indicator and at least one inorganic salt. A few of these agars may be supplemented with a surfactant, a sulfa drug, or other selective agents. The mechanism of action of the BS, HE, and XLD agars is discussed in the Difco Manual (13); the actions of EF-18 (14), Rambach (6), and XLT-4 (8) agars are discussed elsewhere.

The appearance of *Salmonella* spp. and related enteric bacteria on the 6 selective plating agars is shown in Table 2. These descriptions, however, are subjective and appropriate only for the specific strains indicated. They may serve as a general guide, but they are not meant to replace familiarity gained by actual laboratory experience.

The criteria used to determine the relative effectiveness of the 6 selective plating agars were recovery of *Salmonella* spp. and enumeration of false-positive and false-negative reactions. The recovery of *Salmonella* spp. from 8 food types is shown in Table 3. Although the new selective plating agars compared favorably with the AOAC/BAM-recommended agars for certain

Table 3. Comparison of selective plating agars for recovery of *Salmonella* spp.

Food type	No. of test portions analyzed	Total No. of positive test portions	Selective enrichment/ temp. (°C)[a]	No. of positive test portions[b]					
				BS	HE	XLD	EF-18	Rambach	XLT-4
Pork sausage	60	37	SC/35	8	12	20[c,d]	16[c]	16[c]	26[c,d,e,f]
			TT/35	8	12	17[c]	21[c,d]	15	22[c,d]
			TT/41	ND[g]	ND	ND	ND	ND	22
			TT/43	8	14	18[c]	16[c]	18[c]	23[c,d,e]
			RV/42	ND[h]	ND	ND	ND	ND	ND
			RV/43	12	16	18	15	19[c]	18
Chicken parts	80	18	SC/35	1	4	4	9[c,d,i]	8[c]	7
			TT/35	2	4	6	10[c,d]	9[c,d]	7
			TT/41	ND	ND	ND	ND	ND	7
			TT/43	10	8	8	13	14[d,i]	10
			RV/42	8	10	7	13[c,f,i]	7	10
			RV/43	10	9	9	16[c,d,f,i]	9	12
Turkey parts	30	4	SC/35	1	0	0	0	2[d,e,i]	1
			TT/35	0	0	0	0	0	1
			TT/41	ND	ND	ND	ND	ND	2
			TT/43	0	0	0	2[c,d,i]	2[c,d,i]	3[c,d,i]
			RV/42	2[d,i]	0	0	2[d,i]	1	1
			RV/43	0	0	1	0	0	0
Frog legs	70	22	SC/35	0	9[c]	10[c]	8[c]	8[c]	8[c]
			TT/35	3	6	2	9[c,i]	7[i]	8[i]
			TT/41	ND	ND	ND	ND	ND	16
			TT/43	10	22[c]	22[c]	20[c]	18[c]	20[c]
			RV/42	21	21	21	20	19	21
			RV/43	20	20	22	21	21	21
Shrimp[j,k]	50	42	TT/35	21	26	30[c]	31[c,f]	24	31[c,f]
			TT/41	ND	ND	ND	ND	ND	32
			TT/43	23	31[c]	29	33[c]	28	29
			RV/42	32	34	34	35[f]	30	34
			RV/43	29	31	33	34[c]	32	34[c]
Oysters[j]	80	44	SC/35	24[f]	35[c,e,f,i,l]	19[f]	26[f]	9	24[f]
			TT/35	33	41[c]	40[c]	41[c]	37	36
			TT/41	ND	ND	ND	ND	ND	43
			TT/43	39	41[l]	39	41[l]	41[l]	37
			RV/42	40	40	39	40	40	38
			RV/43	37	38	37	38	38	37
Egg yolks[j]	40	39	SC/35	34[d,l]	24	34[d,l]	31[d]	33[d]	30[d]
			TT/35	33[d]	16	34[d]	33[d]	33[d]	30[d]
			TT/41	ND	ND	ND	ND	ND	30
			TT/43	32[d]	12	34[d,l]	33[d]	33[d,l]	29[d]
			RV/42	34[d]	4	34[d]	33[d]	33[d]	32[d]
			RV/43	33[d]	2	34[d]	33[d]	34[d]	30[d]

Table 3. *(continued)*

Food type	No. of test portions analyzed	Total No. of positive test portions	Selective enrichment/ temp. (°C)[a]	No. of positive test portions[b] BS	HE	XLD	EF-18	Rambach	XLT-4
Lettuce[j]	40	30	SC/35	4	13[c,e,f,i,l]	2	5	3	4
			TT/35	5	18[c,f,i]	11[f]	16[c,f]	7	17[c,f]
			TT/41	ND	ND	ND	ND	ND	29
			TT/43	5	26[c,f]	26[c,f]	24[c]	21[c]	23[c]
			RV/42	17	27[c]	29[c]	27[c]	29[c]	30[c]
			RV/43	17	27[c]	26[c]	26[c]	26[c]	26[c]

[a] SC, selenite cystine broth; TT, tetrathionate broth; RV, Rappaport–Vassiliadis medium.
[b] BS, bismuth sulfite agar; HE, Hektoen enteric agar; XLD, xylose lysine desoxycholate agar; XLT-4, xylose lysine Tergitol-4 agar.
[c] Significantly greater number of positives than BS.
[d] Significantly greater number of positives than HE.
[e] Significantly greater number of positives than EF-18.
[f] Significantly greater number of positives than Rambach.
[g] Not done. TT broth incubated at 41°C is specific for XLT-4 agar.
[h] Not done. RV medium at 42°C was not used initially but was added later according to commercial use.
[i] Significantly greater number of positives than XLD.
[j] Artificially contaminated.
[k] For analysis of shrimp, SC is not a recommended selective enrichment in BAM.
[l] Significantly greater number of positives than XLT-4.

foods, the results were not consistent. With raw chicken parts, raw frog legs, raw shrimp, raw oysters, and lettuce, incubation of the selective enrichment broths at elevated temperatures increased the recovery of *Salmonella* spp. on each of the 6 selective plating agars.

The enumeration of false-positive reactions is shown in Table 4. A false-positive reaction is defined as a typical colony that could not be confirmed as *Salmonella* spp. The occurrence of a large number of false-positive reactions is counterproductive and renders any method impractical. An increase in incubation temperature was effective in reducing the number of false-positive reactions for analysis of raw frog legs and raw oysters. Although enrichment at 35°C is widely used (1–3, 15), recovery of *Salmonella* spp. has been improved through incubation of enrichment broths at 41°–43°C (16–19); however, an incubation temperature of 43°C has been reported to be toxic to some *Salmonella* strains (1). RV medium incubated at 42° and 43°C has been reported to increase recovery of *Salmonella* spp. (20–22). This medium has been incubated at 43°C in some studies and at 42°C in others (9, 23). The efficiency of an incubated elevated temperature has been found to depend on the types of food being evaluated (1).

The enumeration of false-negative reactions is shown in Table 5. A false-negative reaction is defined as one in which a particular agar did not recover *Salmonella* spp., while the organism was recovered by one or more of the other selective plating agars. An elevated incubation temperature greatly reduced the number of false-negative reactions for raw frog legs, raw shrimp, raw oysters, and lettuce. This reduction may be explained by the absence of overgrowth by competitors on plates streaked from selective enrichments incubated at the elevated temperatures.

Any potential advantage of replacing the AOAC/BAM-recommended agars with one or more of the new agars is seen in Table 6. This table shows the number of *Salmonella*-positive test portions recovered by one or more of the 3 new plating agars but missed by all 3 of the AOAC/BAM-recommended agars. Conversely, Table 7 shows the number of *Salmonella*-positive test portions recovered by the AOAC/BAM-recommended agars but missed by each of the new plating agars. Although there were instances where one or more of the AOAC/BAM-recommended agars gave an additional number of positive test portions, this was not a general phenomenon.

Table 8 shows the frequency of isolation of various *Salmonella* serovars from the naturally contaminated foods. Particular serovars were not more readily recovered on one agar than on another. The fact that lactose-positive serovars are readily recovered on BS agar is the primary reason for this agar's retention in the AOAC/BAM-recommended combination of agars.

The results of this study show no apparent advantage of replacing the AOAC/BAM-recommended agars with any of the new plating agars. In addition to productivity and relative number of discrepant (false-positive and false-negative) reactions, other factors should be considered when deciding whether to replace one or more of the selective plating media: relative costs, actual time required for preparation, and amount of time required for the analyst to gain familiarity with the respective media. The relative costs of the agars in descending order were Rambach (most expensive), HE, BS, XLT-4, and EF-18 (least expensive). The times required to prepare each agar were comparable, except for XLT-4 agar, which required addition of several individual ingredients (ferric ammonium citrate, sodium thiosulfate, additional agar, and Tergitol 4). Because BS, HE,

and XLD agars have been in place for several years, most analysts are familiar with their performance. An analyst would need additional time to gain the familiarity required to use a new agar at its optimal level of effectiveness. Moreover, because one of the agars (XLT-4) is still undergoing modification, it may be prudent to wait until its formulation is finalized before making a final decision regarding its ultimate usefulness.

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Table 4. False-positive reactions on selective plating agars

Food type	No. of test portions analyzec	Selective enrichment/ temp. (°C)[a]	No. of false-positive reactions[b]					
			BS	HE	XLD	EF-18	Rambach	XLT-4
Pork sausage	60	SC/35	36	26	18[c]	28	24[c]	6[c,d,e,f,g]
		TT/35	38	39	16[c,d]	15[c,d,g]	24[c,d]	14[c,d,g]
		TT/41	ND[h]	ND	ND	ND	ND	13
		TT/43	19[d]	35	24[c]	31	16[d,e,f]	6[c,d,e,f,g]
		RV/42	ND[i]	ND	ND	ND	ND	ND
		RV/43	30	8[c,f]	8[c,f]	34	7[c,f]	3[c,f]
Chicken parts	80	SC/35	22	18	15	21	16	2[c,d,e,f,g]
		TT/35	26[d]	44	28[d]	22[d,e]	24[d]	11[c,d,e,f,g]
		TT/41	ND	ND	ND	ND	ND	14
		TT/43	20[d,e]	57	42[d]	20[d,e]	12[d,e]	12[d,e]
		RV/42	17	14	11	20	13	4[c,d,f,g]
		RV/43	15	22	14	15	12	1[c,d,e,f,g]
Turkey parts	30	SC/35	16	7[c]	9	8[c]	5[c]	1[c,d,e,f]
		TT/35	13	18	18	9[d,e]	10[d,e]	1[c,d,e,f]
		TT/41	ND	ND	ND	ND	ND	8
		TT/43	10[d,e]	23	23	8[d,e]	1[c,d,e,f]	0[c,d,e,f]
		RV/42	13	8	8	10	4[c]	1[c,d,e,f]
		RV/43	11	12	11	10	6	0[c,d,e,f,g]
Frog legs	70	SC/35	36	26	17	14[c,d]	13[c,d]	10[c,d,e]
		TT/35	25	20	19	13[c]	19	13[c]
		TT/41	ND	ND	ND	ND	ND	6
		TT/43	12	3[c]	4[c]	3[c]	5	2[c]
		RV/42	3	3	4	5	4	3
		RV/43	5	4	1	5	3	1
Shrimp[j,k]	50	TT/35	13	2[c]	3[c]	6	2[c]	2[c]
		TT/41	ND	ND	ND	ND	ND	3
		TT/43	11	2[c]	7	7	6	6
		RV/42	3	1	1	5	3	1
		RV/43	7	2	2	7	2	2
Oysters[j]	80	SC/35	15	3[c,e,f,l]	23	16	5[c,e,f,l]	13
		TT/35	13	11	6	3[c,d,g]	10	7
		TT/41	ND	ND	ND	ND	ND	0
		TT/43	2	1	3	1	0[l]	4
		RV/42	0	0	0	0	0	1
		RV/43	1	0	1	0	0	1
Egg yolks[j]	40	SC/35	0	1	0	0	0	0
		TT/35	0[d]	4	0[d]	0[d]	0[d]	1[d]
		TT/41	ND	ND	ND	ND	ND	0
		TT/43	0	3	0	0	0	0
		RV/42	0	1	0	0	0	0
		RV/43	0	0	0	0	0	0

Table 4. ***(continued)***

Food type	No. of test portions analyzed	Selective enrichment/ temp. (°C)[a]	No. of false-positive reactions[b]					
			BS	HE	XLD	EF-18	Rambach	XLT-4
Lettuce[j]	40	SC/35	8	1[c]	5	3	2[c]	2[c]
		TT/35	18[g]	1[c,g]	1[c,g]	1[c,g]	31	4[c,g]
		TT/41	ND	ND	ND	ND	ND	1
		TT/43	17	0[c,g]	0[c,g]	2[c]	4[c]	1[c]
		RV/42	11	2[c]	0[c]	0[c]	0[c]	0[c]
		RV/43	9	0[c]	0[c]	0[c]	2[c]	1[c]

[a] SC, selenite cystine broth; TT, tetrathionate broth; RV, Rappaport–Vassiliadis medium.
[b] BS, bismuth sulfite agar; HE, Hektoen enteric agar; XLD, xylose lysine desoxycholate agar; XLT-4, xylose lysine Tergitol-4 agar.
[c] Significantly lower number of false-positives than BS.
[d] Significantly lower number of false-positives than HE.
[e] Significantly lower number of false-positives than XLD.
[f] Significantly lower number of false-positives than EF-18.
[g] Significantly lower number of false-positives than Rambach.
[h] Not done. TT broth incubated at 41°C is specific for XLT-4 agar.
[i] Not done. RV medium at 42°C was not used initially but was added later according to commercial use.
[j] Artificially contaminated.
[k] For analysis of shrimp, SC is not a recommended selective enrichment in BAM.
[l] Significantly lower number of false-positives than XLT-4.

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Table 5. False-negative reactions on selective plating agars

Food type	No. of test portions analyzed	Selective enrichment/ temp. (°C)[a]	No. of false-negative reactions[b]					
			BS	HE	XLD	EF-18	Rambach	XLT-4
Pork sausage	60	SC/35	2	5	3	2	4	3
		TT/35	3	3	9	2[c]	4	2[c]
		TT/43	6	2	4	3	6	2
		RV/42	ND[d]	ND	ND	ND	ND	ND
		RV/43	0	3	1	1	1	3
Chicken parts	80	SC/35	4	6	7	1[c,e]	2[k]	5
		TT/35	2	4	5	1	1	3
		TT/43	1	0[c,f]	4	0[c,f]	1	5
		RV/42	2	5	6	0[c,e,h]	4	3
		RV/43	3	7	8	1[c,e]	4	5
Turkey parts	30	SC/35	0[c,f,g]	1	2	2	0[c,f,g]	2
		TT/35	0	0	0	0	0	0
		TT/43	3	2	2	1[k]	1[k]	0[c,e,k]
		RV/42	0	0	0	0	1	0
		RV/43	1	0	0	0	0	1
Frog legs	70	SC/35	2[h]	0[f,g,h]	2[h]	5	7	5
		TT/35	2[c,e]	7	7	0[c,e,h]	6	2[c,e]
		TT/43	3	2	1	2	2	3
		RV/42	0	0	0	0	0	0
		RV/43	0	0	0	0	0	1
Shrimp[i,j]	50	TT/35	4[h]	9	6	4[h]	12	4[h]
		TT/43	4	5	3	1	4	3
		RV/42	1	1	1	0	3	1
		RV/43	0	3	1	0	1	0
Oysters[i]	80	SC/35	3[h]	0[h]	2[h]	1[h]	24	2[h]
		TT/35	2	0[h]	0[h]	1	4	0[h]
		TT/43	0	0	0	0	0	0
		RV/42	0	0	1	0	0	1
		RV/43	0	0	0	0	0	0
Egg yolks[i]	40	SC/35	0[e,f,g]	9	0[e]	3	1[e]	4
		TT/35	2[e]	15	1[e]	2[e]	2[e]	4[e]
		TT/43	2[e]	19	0[e,f]	1[e]	0[e,f]	5[e]
		RV/42	0[e]	29	0[e]	1[e]	1[e]	2[e]
		RV/43	1[e]	32	0[e,f]	1[e]	0[e,f]	4[e]
Lettuce[i]	40	SC/35	5	1[c,f,g,h]	7	7	10	8
		TT/35	5[c]	5[c]	12	8	0[c,e,f,g,k]	3[c]
		TT/43	6	1[f,h]	1[f,h]	1[f,h]	3	3
		RV/42	2	1	1	3	1	0
		RV/43	1	0	1	1	0	0

[a] SC, selenite cystine broth; TT, tetrathionate broth; RV, Rappaport–Vassiliadis medium.
[b] BS, bismuth sulfite agar; HE, Hektoen enteric agar; XLD, xylose lysine desoxycholate agar; XLT-4, xylose lysine Tergitol-4 agar.
[c] Significantly lower number of false-negatives than XLD.
[d] Not done. RV medium at 42°C was not used initially but was added later according to commercial use.
[e] Significantly lower number of false-negatives than HE.
[f] Significantly lower number of false-negatives than XLT-4.
[g] Significantly lower number of false-negatives than EF-18.
[h] Significantly lower number of false-negatives than Rambach.
[i] Artificially contaminated.
[j] For analysis of shrimp, SC is not a recommended selective enrichment in BAM.
[k] Significantly lower number of false-negatives than BS.

Table 6. Number of test portions positive for *Salmonella* spp. on EF-18, Rambach, or XLT-4 agars but negative on all 3 agars recommended by BAM

Food type	No. of test portions analyzed	Total No. of positive test portions	Selective enrichment/temp. (°C)[a]	No. of positive test portions		
				EF-18	Rambach	XLT-4
Pork sausage	60	37	SC/35	4	4	7
			TT/35	6	2	7
			TT/43	1	1	3
			RV/42	ND[b]	ND	ND
			RV/43	0	1	1
Chicken parts	80	18	SC/35	4	4	1
			TT/35	4	3	1
			TT/43	2	2	1
			RV/42	1	1	1
			RV/43	5	1	3
Turkey parts	30	4	SC/35	1	2	0
			TT/35	0	0	1
			TT/43	0	0	0
			RV/42	3	2	2
			RV/43	0	0	0
Frog legs	70	22	SC/35	2	0	2
			TT/35	6	1	3
			TT/43	0	0	0
			RV/42	0	1	0
			RV/43	0	0	0
Shrimp[c,d]	50	42	TT/35	2	0	1
			TT/43	0	1	0
			RV/42	3	0	0
			RV/43	4	1	0
Oysters[c]	80	44	SC/35	0	0	0
			TT/35	0	0	0
			TT/43	0	0	0
			RV/42	0	0	0
			RV/43	0	0	0
Egg yolks[c]	40	39	SC/35	0	0	0
			TT/35	0	0	0
			TT/43	0	0	0
			RV/42	0	0	0
			RV/43	0	0	0
Lettuce[c]	40	30	SC/35	1	0	0
			TT/35	2	4	4
			TT/43	0	0	0
			RV/42	0	0	0
			RV/43	0	0	0

[a] SC, selenite cystine broth; TT, tetrathionate broth; RV, Rappaport–Vassiliadis medium.
[b] Not done. RV medium at 42°C was not used initially but was added later according to commercial use.
[c] Artificially contaminated.
[d] For analysis of shrimp by the BAM method, TT and RV media are the recommended selective enrichments.

Table 7. Number of test portions positive for *Salmonella* spp. on bismuth sulfite, Hektoen enteric, or xylose lysine desoxycholate agars but negative on each of the 3 new plating agars

Food type	No. of test portions analyzed	Total No. of positive test portions	New agar that was negative	Selective enrichment/ temp. (°C)[a]	No. of positive test portions		
					Bismuth sulfite	Hektoen enteric	Xylose lysine desoxycholate
Pork sausage	60	37	EF-18	SC/35	4	6	14
				TT/35	3	4	4
				TT/43	1	2	4
				RV/42	ND[b]	ND	ND
				RV/43	4	5	5
			Rambach	SC/35	3	6	12
				TT/35	4	4	4
				TT/43	0	0	2
				RV/42	ND	ND	ND
				RV/43	0	1	1
			XLT-4	SC/35	2	0	5
				TT/35	3	5	5
				TT/43	1	2	2
				RV/42	ND	ND	ND
				RV/43	0	1	2
Chicken parts	80	18	EF-18	SC/35	0	0	4
				TT/35	0	1	1
				TT/43	3	3	2
				RV/42	0	0	0
				RV/43	1	1	1
			Rambach	SC/35	0	0	0
				TT/35	0	2	1
				TT/43	2	3	2
				RV/42	1	3	2
				RV/43	3	1	1
			XLT-4	SC/35	1	0	0
				TT/35	0	0	0
				TT/43	3	2	2
				RV/42	2	1	1
				RV/43	2	0	0
Turkey parts	30	4	EF-18	SC/35	1	0	0
				TT/35	0	0	0
				TT/43	0	0	0
				RV/42	0	0	0
				RV/43	0	0	1
			Rambach	SC/35	1	0	0
				TT/35	0	0	0
				TT/43	0	0	0
				RV/42	1	0	0
				RV/43	0	0	1
			XLT-4	SC/35	1	0	0
				TT/35	0	0	0
				TT/43	0	0	0
				RV/42	1	0	0
				RV/43	0	0	0
Frog legs	70	22	EF-18	SC/35	0	2	2
				TT/35	1	3	1
				TT/43	1	2	2
				RV/42	1	1	1
				RV/43	1	1	1
			Rambach	SC/35	0	1	1
				TT/35	0	3	1
				TT/43	0	2	2

Table 7. ***(continued)***

Food type	No. of test portions analyzed	Total No. of positive test portions	New agar that was negative	Selective enrichment/ temp. (°C)[a]	No. of positive test portions		
					Bismuth sulfite	Hektoen enteric	Xylose lysine desoxycholate
				RV/42	1	1	1
				RV/43	1	1	1
			XLT-4	SC/35	0	0	0
				TT/35	0	0	0
				TT/43	0	0	0
				RV/42	0	0	0
				RV/43	0	0	0
Shrimp[c,d]	50	42	EF-18	TT/35	2	3	4
				TT/43	0	2	1
				RV/42	0	1	1
				RV/43	0	0	0
			Rambach	TT/35	7	4	6
				TT/43	2	4	4
				RV/42	3	3	3
				RV/43	0	1	0
			XLT-4	TT/35	1	1	2
				TT/43	0	5	2
				RV/42	0	0	0
				RV/43	0	0	0
Oysters[c]	80	44	EF-18	SC/35	5	8	5
				TT/35	1	1	2
				TT/43	0	0	0
				RV/42	0	0	0
				RV/43	0	0	0
			Rambach	SC/35	14	22	6
				TT/35	5	6	6
				TT/43	0	0	0
				RV/42	0	0	0
				RV/43	0	0	0
			XLT-4	SC/35	7	9	5
				TT/35	4	7	7
				TT/43	3	3	2
				RV/42	0	0	0
				RV/43	3	3	2
Egg yolks[c]	40	39	EF-18	SC/35	3	1	2
				TT/35	1	0	1
				TT/43	1	0	1
				RV/42	1	0	1
				RV/43	0	0	2
			Rambach	SC/35	1	0	1
				TT/35	1	0	1
				TT/43	0	0	0
				RV/42	1	0	1
				RV/43	0	0	0
			XLT-4	SC/35	4	4	4
				TT/35	4	4	4
				TT/43	4	4	4
				RV/42	2	2	2
				RV/43	4	4	4
Lettuce[c]	40	30	EF-18	SC/35	2	9	0
				TT/35	0	5	3
				TT/43	0	4	4
				RV/42	3	3	0
				RV/43	1	1	1

Table 7. *(continued)*

Food type	No. of test portions analyzed	Total No. of positive test portions	New agar that was negative	Selective enrichment/ temp. (°C)[a]	No. of positive test portions		
					Bismuth sulfite	Hektoen enteric	Xylose lysine desoxycholate
			Rambach	SC/35	3	10	1
				TT/35	3	13	8
				TT/43	2	5	5
				RV/42	1	1	0
				RV/43	0	1	1
			XLT-4	SC/35	3	9	1
				TT/35	1	5	3
				TT/43	1	3	3
				RV/42	0	0	0
				RV/43	0	1	1

[a] SC, selenite cystine broth; TT, tetrathionate broth; RV, Rappaport–Vassiliadis medium.
[b] Not done. RV medium at 42°C was not used initially but was added later according to commercial use.
[c] Artificially contaminated.
[d] For analysis of shrimp by the BAM method, TT and RV media are the recommended selective enrichments.

Table 8. Frequency of isolation of *Salmonella* serovars from naturally contaminated foods

Salmonella spp. isolated	Source(s)	No. of test portions containing serovar
S. typhimurium	Sausage	28
S. derby	Sausage, frog legs	28
S. enteritidis	Frog legs	24
S. heidelberg	Chicken, sausage, frog legs	21
S. anatum	Chicken, sausage	16
S. braenderup	Chicken, turkey, frog legs	11
S. infantis	Chicken	10
S. hadar	Turkey, chicken	9
S. paratyphi B	Frog legs	6
S. saint-paul	Turkey	6
S. montevideo	Chicken	5
S. bovis-morbificans	Frog legs	5
S. kentucky	Chicken	4
S. ohio	Sausage	4
S. reading	Sausage, turkey	4
S. brandenburg	Sausage	3
S. indiana	Chicken	3
S. thompson	Frog legs	3
S. senftenberg	Frog legs	2

FOOD CHEMICAL CONTAMINANTS

Identification of an Impurity in Commercial Sources of the Tomato Glycoalkaloid Tomatine

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Three commercial sources of tomatine showed 2 peaks of approximately equal size when determined by liquid chromatography (LC). Analysis by liquid chromatography/mass spectroscopy (LC/MS) showed one LC peak having an [M + H] ion at 1035, which corresponds to tomatine. The other peak had an [M + H] ion at 1033, which indicates a tomatine-like molecule that has tomatidenol for the aglycone instead of tomatidine or solasodine. Ultraviolet data support a delta C-5–C-6 double bond in the aglycone. LC of tomatine hydrolysate indicated the presence of tomatidine and tomatidenol and the absence of solasodine. Thus, the impurity was dehydrotomatine. Purity calculations based on mass spectral data showed that the tomatine standards were 80% pure.

Tomatine, a tetrasaccharide glycoalkaloid found in tomatoes, has been stirring interest again with the development of new bioengineered tomatoes (1). The U.S. Government requires that genetically engineered food containing natural toxins be monitored to ensure that toxin composition is not changed, either by increasing the level or creating different toxins.

During the development of a liquid chromatographic (LC) method for the analysis of tomatine in the FLAVR SAVR™ tomato for Calgene, Inc. (1), the tomatine standard yielded 2 peaks of almost equal size. After purchasing tomatine standards from 2 other commercial suppliers and observing the same effect, a study was begun to determine which peak was tomatine and the purity of these standards.

This paper describes the methods used to ascertain each chromatographic peak in the 3 commercial tomatine standards and the percentage of each.

Experimental

Reagents and standards

(a) *Reagents.*—All solvents were from EM Science (Gibbstown, NJ) and were LC grade except for glacial acetic acid and phosphoric acid (reagent grade). Tetrahydrofuran was nonstabilized UV grade.

(b) *Tomatine standards.*—Standards were from 3 commercial suppliers (Sigma Chemical Co., St. Louis, MO; Pfaltz and Bauer Co., New York, NY; and U.S. Biochemical, Inc.. Cleveland, OH). Label purities were 95%.

(c) *Tomatine standard stock solution.*—Accurately weigh 150 mg tomatine into a 50 mL volumetric flask, and dilute to volume with tetrahydrofuran–water–acetonitrile (50 + 30 + 20). Stock solution should be stable for 2 months at 4°C.

(d) *Tomatine working standard.*—Remove 1 mL stock standard and place into a 10 mL volumetric flask. Bring to volume with LC mobile phase (e).

(e) *Mobile phase.*—Water–acetonitrile–methanol–0.1M ammonium phosphate buffer (58 + 26 + 11 + 5) adjusted to pH 3.5 with phosphoric acid. Use extreme care in making this mobile phase because percentages are very critical.

Apparatus

(a) *Liquid chromatograph.*—A Hewlett-Packard (HP) (Avondale, PA) 1050 isocratic pump, an HP 1050 autosampler, and an HP 1040A photodiode array detector/integrator system with an updated quartz flow cell, computer, and software. Operating conditions were as follows: injection volume, 5 µL; flow rate, 1 mL/min; UV detection, 205 nm; and quantitation, peak area.

(b) *Chromatographic column.*—Ultremex C_6 5 µm (stainless steel, 15 cm × 4.6 mm id) (Phenomenex, Torrance, CA).

(c) *LC/mass spectroscopy (MS) system.*—A SCIEX API III Biomolecular Mass Analyzer, ion spray interface, Q1 positive MS (150–1500 amu) mode, parameters as follows: ISV = 4800, OR = 35, MU = 4200, and CGT = 0. LC conditions were as follows: column, Ultremex C_6; mobile phase, 550 mL water, 250 mL acetonitrile, 100 mL methanol, and 50 mL 0.1M ammonium acetate adjusted to pH 3.5 with acetic acid; flow rate, 0.85 mL/min; split ratio, 950:50 µL; injection volume, 20 µL; and run time, 16 min. Acetate buffer was used for LC/MS method instead of the phosphate buffer because phosphate buffer interferes with the MS system.

Results and Discussion

Purity of the standard is an extremely important quantity in any analytical calculation. When injecting our tomatine standard from Sigma Chemical Co., we notice that the tomatine

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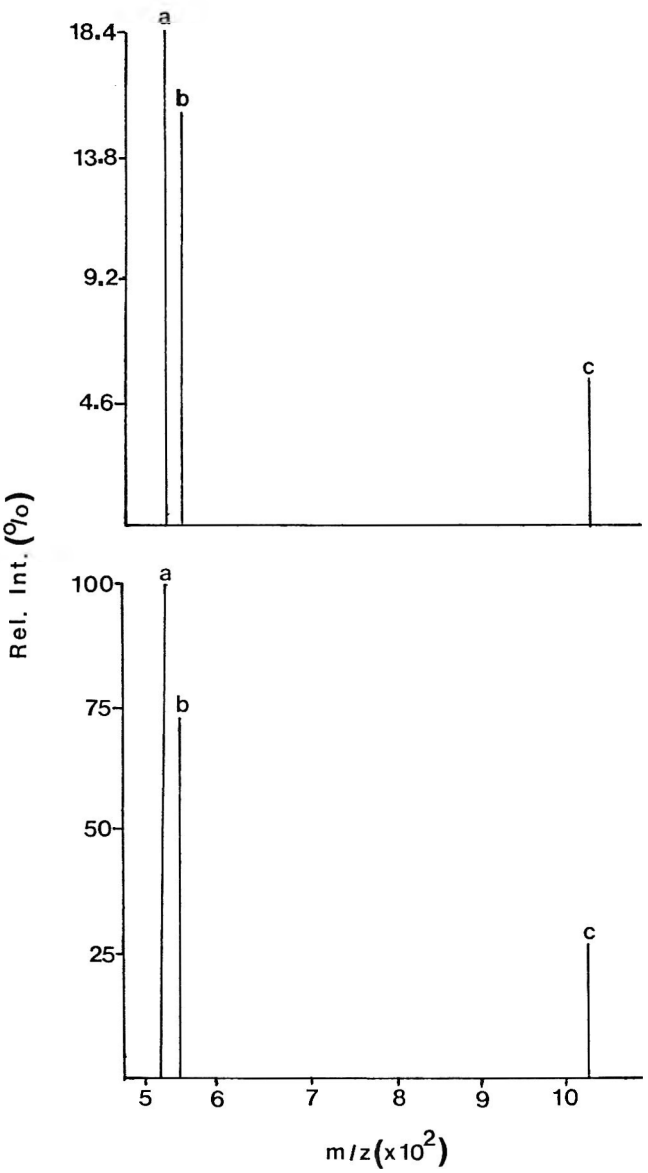

Figure 1. Positive ion LC/MS spectra [M + H] of tomatine standard. Spectra 1a (LC peak 1) is the tomatine-like compound with *m/z* ions at 526 (a), 546 (b), and 1033 (c). Spectra 1b (LC peak 2) is tomatine with *m/z* ions at 526 (a), 547 (b), and 1035 (c).

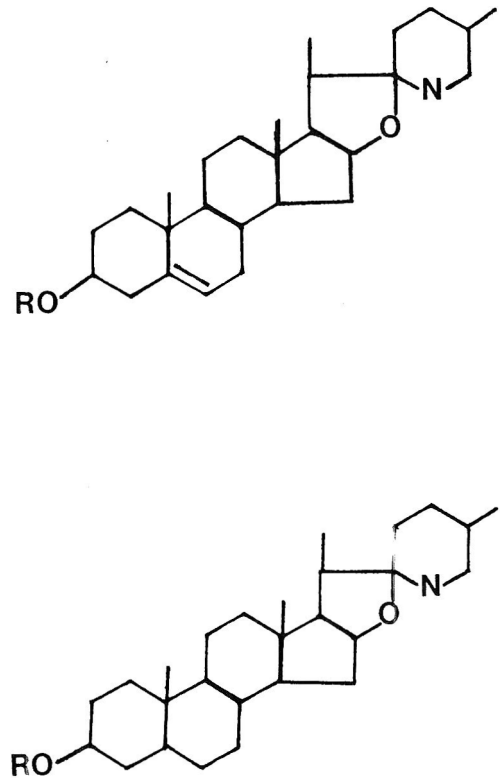

Figure 2. Structure 2a is the tomatine-like glycoalkaloid dehydrotomatine, in which tomatidine is replaced by tomatidenol. Structure 2b is tomatine. R is equal to galactose–glucose–glucose–xylose for both structures.

yielded 2 peaks. Therefore, we purchased another standard from Sigma (different lot), and the same observation was made. Thus, 2 more tomatine standards were obtained from other suppliers: one from Pfaltz and Bauer and the other from U.S. Biochemical. All standards yielded 2 peaks of equal area when analyzed by our LC procedure. By using the photodiode detector, we were able to take UV spectra from 190 to 350 nm of each peak. However, the spectra of both peaks were very similar. This finding was not surprising. UV spectra of glycoalkaloids are very simple and have no detail and very little absorption because of insufficient chromophores in their structures. The nitrogen, oxygen, and a double bond (present in the aglycone of only certain glycoalkaloids) are the only UV absorbing groups, and they absorb only weakly at wavelengths from 190 to 220 nm.

An LC/MS analysis was performed on the tomatine standards to identify each peak. Mass spectra of each peak are shown in Figure 1. The spectra of the first peak to elute from the LC system is presented in Figure 1a with ions at *m/z* 1033, 547, and 526. The spectra of LC peak 2 yielded ions at *m/z* 1035, 546, and 526. Mass spectral data indicated that the first LC peak with its [M + H] ion of 1033 is either a tomatine-like molecule having a tomatidenol (Figure 2a) aglycone instead of the normal tomatidine (Figure 2b) aglycone or a solasodine aglycone, which only differs from tomatidenol by the placement of functional groups in the last ring. LC peak 2 with the [M + H] ion at 1035 agrees with tomatine.

Further support for the idea that LC peak 1 is a tomatine-like glycoalkaloid and its aglycone is tomatidenol or solasodine in place of tomatidine comes from the LC/UV chromatogram (Figure 3). Both peaks with UV detection yielded areas that were about equal, whereas the mass spectral data indicated that LC peak 1 (impurity) comprised 20% of the total standard and peak 2 (tomatine) made up 80%. Glycoalkaloids containing a double bond in the delta C 5–C 6 position of the aglycone show a substantial increase in UV absorption at 205 nm, which is the wavelength used in our LC method. This increase in UV absorption would make peak 1 equal to peak 2 when analyzing with UV detection.

Finally a hydrolysis experiment was performed, whereby the tomatine standard was hydrolyzed according to the procedure of Gregory et al. (2). When injected into the LC system by

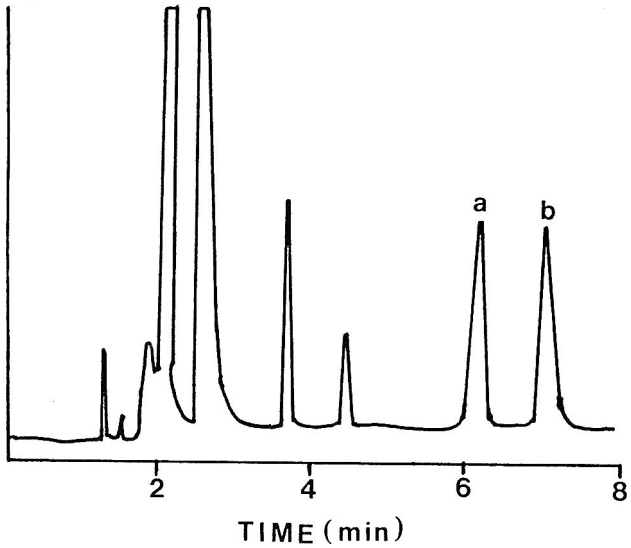

Figure 3. LC chromatogram of tomatine (conditions given in text). Peak a is the tomatine-like glycoalkaloid dehydrotomatine and peak b is the tomatine.

using the method of Osman and Sinden (3), the tomatine hydrolysate yielded 2 peaks that were identified as tomatidine and tomatidenol. No solasodine was present. Thus, the impurity was dehydrotomatine. Given the structure of tomatidine and tomatidenol, tomatidenol is probably not an artifact produced during sample preparation. Also, while this paper was being reviewed, Friedman et al. (4) observed a 10% level of dehydrotomatine in the Sigma standard. The difference between the levels of dehydrotomatine reported by us in this paper (20%) and the 10% reported by Friedman et al. could be due to different lot numbers or differences in analytical methods.

Because of increased interest in developing bioengineered tomatoes, this purity information should be very beneficial for quantitating tomatine in tomatoes.

Acknowledgments

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FOOD CHEMICAL CONTAMINANTS

Incidence of Aflatoxins in Animal Feedstuffs: A Decade's Scenario in India

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During the period 1983–1993, 4818 samples of agricultural commodities, comprising cereals, oilseed cakes, compound feeds, and other ingredients, were examined for aflatoxin contamination. High quantities of aflatoxins were found in groundnut cake, deoiled groundnut cake, maize, and mixed feeds. Highest incidence of aflatoxin contamination was observed in groundnut cake (96.35%) and deoiled groundnut cake (96.20%), and the highest level of aflatoxin B1, 8260 ppb, was observed in maize.

The effects of aflatoxins on animal production were recognized when it was shown that turkey "X" disease in the United Kingdom (1) was caused by a toxin produced by the mold *Aspergillus flavus*. Aflatoxins are secondary metabolites produced by strains of *A. flavus* and *A. parasiticus*. Aflatoxin contamination of agricultural commodities is one of the most serious problems associated with food products and storage. Fungi that form toxins occur worldwide. Levels of contamination vary from year to year. To reduce the risk, many

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Table 1. Aflatoxin in agricultural commodities in India, 1983–1993

Commodity	Total No. samples analyzed	No. positive samples	Positive samples, %	Range of aflatoxin concentration, ppb
Cereals and their products				
Maize	862	563	65.31	3–8260
Wheat	244	16	6.56	1–79
Rice polish	168	41	24.40	3–782
Deoiled rice bran	143	48	33.57	2–421
Sorgum	30	12	40.00	6–145
Wheat bran	108	20	18.52	5–67
Damaged rice	5	2	40.00	7–107
Cakes				
Groundnut cake	301	291	96.35	8–6280
Deoiled groundnut cake	79	76	96.20	9–1623
Sesame cake	107	52	48.60	2–527
Soybean meal	250	36	14.4	ND[a]–256
Deoiled mustard cake	4	None	—	—
Rapeseed cake	4	1	25.0	ND–22
Cottonseed extract	7	3	42.86	6–19
Cottonseed	4	None	—	—
Sunflower cake	4	3	75.0	6.33–45
Black sesame cake	1	None	—	—
Feeds				
Poultry feed	1368	1108	80.99	2–2410
Cattle feed	498	378	75.90	3–1754
Pig feed	163	146	89.57	3–1231
Prawn feed	14	None	—	—
Sheep feed	3	2	66.67	200.1–245.2
Rabbit feed	4	3	75.00	37–381
Husks				
Redgram husk	16	3	18.75	6–46
Blackgram husk	17	2	11.76	9–39
Bengalgram husk	8	2	25.0	ND–17
Maize husk	1	1	100	99.0
Birichunni	2	None	—	—
Forage				
Alfalfa	55	6	10.91	13–65
Groundnut leaves	1	None	—	—
Bagasse	2	1	50.0	ND–9.3
Animal products				
Fish meal	257	18	7.00	6–276
Liver samples	10	2	20.00	0.004–0.428
Liver meal	1	None	—	—
Meat meal	4	None	—	—
Protoliv	11	None	—	—
Miscellaneous				
Livol	1	1	100	18
Lito	1	None	—	—
Protopulp	1	None	—	—
Soji	2	None	—	—
Maida	1	None	—	—
Ragi	1	None	—	—
Green grass	1	None	—	—
Maize gluten	7	6	85.71	35.7–103
Molasses	7	None	—	—
Oil (groundnut)	1	1	100	113.0
Horse gram	35	16	45.71	ND–107
Maize cake	1	None	—	—

Table 1. (*continued*)

Commodity	Total No. samples analyzed	No. positive samples	Positive samples, %	Range of aflatoxin concentration, ppb
Tapioca	1	1	100	270.0
Corn flakes	2	2	100	51–145

[a] ND, not detected.

countries have regulated the maximum permissible levels of aflatoxins in foods and feeds.

Many researchers have reported analytical results of aflatoxin contamination, but most of them examined only a few sample types collected in a short period (2–6).

The aflatoxin data from 4818 samples reported here are for the period from the beginning of 1983 to the end of 1993 and cover a wide variety of products collected and/or received from different parts of India.

Table 2. Number of samples positive for aflatoxins B_1 and B_2 and average concentrations

Commodity	No. positive samples	No. samples positive for B_1	No. samples positive for B_2	Average concentration, ppb	
				B_1	B_2
Cereals and their products					
Maize	563	563	245	226.27	37.41
Wheat	16	16	4	17.36	4.90
Rice polish	41	41	9	58.86	44.51
Deoiled rice bran	48	48	13	31.10	10.58
Sorgum	12	12	3	33.81	15.40
Wheat bran	20	20	1	26.10	3.00
Damaged rice	2	2	—	57.0	—
Cakes					
Groundnut cake	291	291	142	449.95	80.40
Deoiled groundnut cake	76	76	39	239.57	49.26
Sesame cake	52	52	8	51.17	16.21
Soybean meal	36	36	11	26.74	9.21
Rapeseed cake	1	1	—	22.00	—
Cottonseed extract	3	3	1	8.67	5.0
Sunflower cake	3	3	—	31.49	—
Feeds					
Poultry feed	1108	1108	894	110.42	16.57
Cattle feed	378	378	234	103.02	19.34
Pig feed	146	146	115	91.34	18.91
Sheep feed	2	2	2	203.05	19.60
Rabbit feed	3	3	3	122.67	32.35
Husks					
Redgram husk	3	3	2	22.67	6.5
Blackgram husk	2	2	1	22.50	3.0
Bengalgram husk	2	2	—	17.0	—
Maize husk	1	1	1	76.0	23.0
Forage					
Alfalfa	6	6	—	34.67	—
Bagasse	1	1	—	9.3	—
Animal products					
Fishmeal	18	18	2	63.06	28.0
Liver samples	2	—	2	—	0.428
Miscellaneous					
Maize gluten	6	6	5	54.10	14.04
Horsegram	16	16	1	36.56	9.0
Oil (groundnut)	1	1	1	73.46	39.0
Corn flakes	2	2	2	86.57	11.87

Table 3. Level of aflatoxin B_1 in agricultural commodities

Ingredients (No. samples analyzed)	No. positives	No. samples with aflatoxin B_1 in the indicated range, ppb											
		Up to 25	26 to 50	51 to 100	101 to 200	201 to 500	501 to 1000	1001 to 1500	1501 to 2000	2001 to 3000	3001 to 4000	4001 to 5000	>5001
Cereals and their products													
Maize (862)	563	213	84	83	63	59	39	3	6	4	4	4	1
Wheat (244)	16	14	—	2	—	—	—	—	—	—	—	—	—
Rice polish (168)	41	25	7	3	3	2	1	—	—	—	—	—	—
Deoiled rice bran (143)	48	36	5	4	2	1	—	—	—	—	—	—	—
Sorgun (30)	12	7	3	1	1	—	—	—	—	—	—	—	—
Wheat bran (108)	20	10	7	3	—	—	—	—	—	—	—	—	—
Damaged rice (5)	2	1	—	—	1	—	—	—	—	—	—	—	—
Cakes													
Groundnut cake (301)	291	27	32	44	49	67	40	13	4	7	3	1	4
Deoiled groundnut cake (79)	76	12	10	17	15	10	7	5	—	—	—	—	—
Sesame cake (107)	52	24	16	5	5	1	1	—	—	—	—	—	—
Soybean meal (250)	36	28	3	3	1	1	—	—	—	—	—	—	—
Rapeseed cake (4)	1	1	—	—	—	—	—	—	—	—	—	—	—
Cottonseed extraction (7)	3	3	—	—	—	—	—	—	—	—	—	—	—
Sunflower cake (4)	3	1	2	—	—	—	—	—	—	—	—	—	—
Feeds													
Poultry feed (1368)	1108	371	212	224	134	112	45	7	1	2	—	—	—
Cattle feed (498)	378	131	87	61	60	25	9	4	1	—	—	—	—
Pig feed (163)	146	36	45	35	15	11	3	1	—	—	—	—	—
Sheep feed (3)	2	—	—	—	1	1	—	—	—	—	—	—	—
Rabbit feed (4)	3	—	2	—	—	1	—	—	—	—	—	—	—
Husks													
Redgram husk (16)	3	2	1	—	—	—	—	—	—	—	—	—	—
Blackgram husk (17)	2	1	1	—	—	—	—	—	—	—	—	—	—
Bengalgram husk (8)	2	2	—	—	—	—	—	—	—	—	—	—	—
Maize husk (1)	1	—	—	1	—	—	—	—	—	—	—	—	—
Forage													
Alfalfa (55)	6	2	3	1	—	—	—	—	—	—	—	—	—
Animal Producst													
Fishmeal (257)	18	6	4	4	3	1	—	—	—	—	—	—	—
Miscellaneous													
Maize gluten (7)	6	—	3	3	—	—	—	—	—	—	—	—	—
Horsegram (35)	16	8	2	5	1	—	—	—	—	—	—	—	—
Corn flakes (2)	2	—	—	1	1	—	—	—	—	—	—	—	—
Oil (1)	1	—	—	—	1	—	—	—	—	—	—	—	—
Tapioca (1)	1	—	—	—	—	1	—	—	—	—	—	—	—

Experimental

Samples were sent to Central Poultry Training Institute's laboratory from many sources such as millers, animal feed suppliers, farmers, and farm cooperatives. All materials were either currently in use or marketed. The quantity submitted for analysis was about 500 g. Samples were thoroughly mixed and finely ground in a sample grinding mill before analysis.

Of 4818 samples, 862 samples were maize, 301 were groundnut cake, 257 were fish meal, 168 were rice polish, 250 were soybean meal, 244 were wheat, 108 were wheat bran, 107 were sesame cake, 55 were alfalfa meal, 30 were sorgum, 79 were deoiled groundnut cake, 1368 were compound poultry feed, 498 were cattle feed, 163 were pig feed, and 328 were other feed ingredients or feed such as prawn feed, sunflower cake, liver samples, liver meal, molasses, meat meal, husks, and forages.

Toxin Extraction and Cleanup

Aflatoxin extraction and cleanup were carried out according to the method of Romer (7). The method involves extracting 50 g sample with 85% aqueous acetone followed by cleanup with cupric carbonate and ferric gel. Finally, the aflatoxins are taken into chloroform. A known volume of extract is evaporated to dryness, and the residue is dissolved in a known volume of chloroform and spotted on thin-layer chromatographic (TLC) plates.

Thin-Layer Chromatography

The aflatoxins were separated by TLC on silica gel G plates (20 × 20 cm; Merck, Cat. No. 5721) with chloroform–acetone (90 + 10) or alternative developing solvent systems (8). Detection was carried out by viewing under long-wave UV light.

The quantitation was by visual comparison with known concentration of standards (aflatoxins B_1, B_2, G_1, and G_2) run on the same chromatoplate. Mycotoxin standards were obtained from Sigma Chemical Co. (St. Louis, MO).

Confirmation

Confirmation of positive results was done with spray reagents such as H_2SO_4, by derivatization on TLC with trifluroacetic acid (TFA), by co-chromatography, or by 2-dimensional TLC (to avoid false-positives).

Results and Discussion

Aflatoxin B_1 was a major contaminant in all the positive samples, followed by aflatoxin B_2 (about 60% of positive samples), in smaller quantity. Aflatoxins G_1 and G_2 were found in only 4 samples of maize at average values of 212 and 30 ppb, respectively.

Cereals and Their Products

High incidence and levels of aflatoxins were found in maize (Tables 1–3). Of 862 samples of maize, 563 contained aflatoxin at levels ranging from 3 to 8260 ppb (the highest level); the average was 264 ppb. The level of contamination is much higher than those reported by others (6, 9, 10).

Of 244 samples of wheat, only 16 were positive (6.56%) for aflatoxins. This result indicates that wheat is less susceptible to aflatoxin contamination.

Of 5 samples of damaged rice, 2 contained aflatoxin (7 and 107 ppb).

Aflatoxin B_1 was also found in jowar at levels ranging from 6 to 145 pbb. However, the incidence and levels in jowar were less than for maize.

Out of 168 rice polish samples, 41 were contaminated with aflatoxins at levels ranging from 3 to 782 ppb. Among deoiled rice bran samples, 33.6% were contaminated with aflatoxin at levels ranging from 2 to 421 ppb (Tables 1–3).

Cakes

Among the cakes, groundnut cake (GNC) and deoiled groundnut cake (DGNC) were found to have the highest incidence (96%) and levels of aflatoxins (Tables 1 and 3).

The aflatoxin levels ranged from 8 to 6280 ppb for GNC and from 9 to 1623 ppb for DGNC. The average values of aflatoxins B_1 and B_2 for GNC were 450 and 80.4, respectively. For DGNC the average value of B_1 was 240 ppb and the average value of B_2 was 49.0 ppb (Table 2). Similar contamination has been previously reported (5, 6, 11). Negler (11) has pointed out that oilseed meals and groundnut meal in particular are most susceptible to aflatoxin contamination.

With soybean meals, the incidence and levels of aflatoxins were much lower than for maize and oilseed cakes, only 14.4% of samples analyzed were contaminated. The low incidence and levels of aflatoxin are consistent with the observation that soybeans are resistant to contamination even though they can support fungal growth (12, 13).

Poultry Feeds

Of 1368 poultry feed samples, 1108 (81%) were found to be contaminated with aflatoxins at levels ranging from 2 to 2410 ppb. The average aflatoxin B_1 content was 110.42 ppb, and the average aflatoxin B_2 content was 16.57 ppb (Table 2). These values are almost similar to those reported earlier by Patel et al. (2) and Johri et al. (6).

Cattle Feeds

Of the cattle feed analyzed, 76% were found to be contaminated with aflatoxin B_1 and B_2. The highest concentration was 1754 ppb (Tables 1 and 3). The average concentrations (Table 2) were 103.02 ppb for aflatoxin B_1 and 19.34 ppb for aflatoxin B_2. On an average feed intake of 3–4 kg/day, the amount of total aflatoxin (B_1 + B_2) ingested per day would be 489.36 ppb. That intake over a long period would have a cumulative effect that would result in secretion of aflatoxin M_1 in milk.

Other Animal Feeds

Of 3 samples of sheep feed (Tables 1 and 3), 2 contained aflatoxin (3 and 245.2 ppb). The average aflatoxin content was 223 ppb (Table 2). Continuous feeding of contaminated feed to sheep will result in aflatoxicosis.

Among pig feed samples, 90% were found to be contaminated with aflatoxins. The average aflatoxin content was 110 ppb (Table 2), and the range was 3–1231 ppb (Tables 1 and 3).

Rabbit feeds were also contaminated with aflatoxins B_1, with an average value and range of 155 and 37–381 ppb (Tables 1 and 2), respectively. The prawn feeds (Tables 1 and 3) were negative for aflatoxin.

Other Ingredients

Of 44 types of husks analyzed (Tables 1 and 3), only 8 were contaminated with aflatoxin at levels up to 99 ppb (Table 2).

Of 55 alfalfa samples, only 10.91% were positive for aflatoxin contamination.

Of 257 samples of fish meal (Tables 1 and 3), only 7% were positive, with an average value and range of 91.06 and 6–276 ppb, respectively.

Only one groundnut oil sample was analyzed; it had a total aflatoxin (B_1 and B_2) content of 113 ppb.

Other ingredients such as meat meal, liver meal, protoliv (sterilized meat meal), molasses, deoiled mustard cake, cottonseed, black sesame cake, and groundnut leaves were negative for aflatoxin B_1 contamination. With the exception of cotton seed, *A. flavus* and *A. parasiticus* rarely attack these feedstuffs.

Conclusions

The results from this study done over a 10-year period, clearly reveal that aflatoxin contamination in agricultural com-

modities, such as maize and groundnut cake, is very high and thus poses a serious problem to poultry and livestock industry in terms of production losses, morbidity, and mortality.

Rodricks and Stoloff (14) had also pointed out that aflatoxin contamination of the feeds of food-producing animals can result in residues of the ingested aflatoxin or its metabolites in edible tissues like meat, milk, and eggs.

Hence, feed manufacturers should strictly watch the quality of raw materials for compound feed, especially groundnut cake and maize.

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FOOD CHEMICAL CONTAMINANTS

Evaluation of a Postcolumn Electrochemical Reactor for Oxidation of Paralytic Shellfish Poison Toxins

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A liquid chromatographic method using a postcolumn electrochemical reactor that oxidizes paralytic shellfish poison toxins to fluorescent derivatives has been developed. Several experimental parameters, including pH and oxidation potential, were investigated. For nonhydroxylated toxins, the sensitivity improved with increasing pH and voltage. At optimum operating conditions, the sensitivity for saxitoxin and gonyautoxins 2 and 3 was an order of magnitude greater than that for neosaxitoxin and B1 and 2 orders of magnitude greater than that for B2. The limit of detection for saxitoxin was 0.10 ng (signal-to-noise ratio, 3:1). Electrochemical oxidation products were similar to those formed in the prechromatographic periodate oxidation method. Shellfish and plankton extracts were analyzed with the electrochemical system, and results agreed well with those obtained with established methods. Shellfish samples contaminated at the regulatory limit of 0.8 μg/g were readily analyzed by the method.

Paralytic shellfish poisoning (PSP) is a potentially deadly illness caused by ingestion of shellfish that have accumulated potent neurotoxins produced by marine algae such as those of the genus *Alexandrium* (1). Contamination of shellfish with PSP toxins has become a worldwide concern, with cases being reported in the South Pacific, Southeast Asia,

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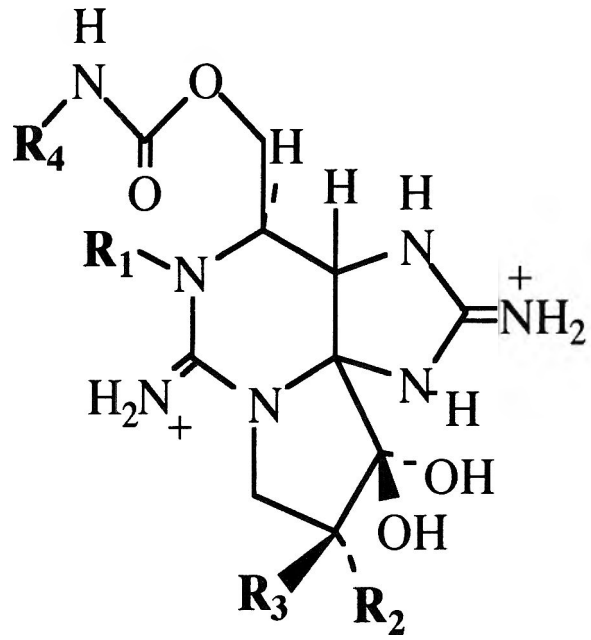
southern Australia, and both coasts of North America. Because the toxic algal blooms are unpredictable, protection of public health and of the fishing industry requires an effective monitoring program. Analytical methods must be capable of detecting at least 80 μg toxin in 100 g shellfish (0.8 μg/g), the current allowable limit in many countries. Because the choice method for monitoring PSP toxins is the mouse bioassay, which measures the toxicity of the shellfish extract, the allowable limit is based on toxicity equivalents relative to saxitoxin and does not specify the various PSP toxins.

The group of compounds known as PSP toxins is composed of saxitoxin and its derivatives, as shown in Figure 1. Because these compounds are nonvolatile, lack UV-absorbing chromophores, and are present in low concentrations in most samples, conventional analytical methods such as gas or liquid chromatography (LC) with UV–visible detection cannot be used. The most widely used method for monitoring PSP toxins is the mouse bioassay (2). The toxins are extracted from the shellfish with 0.1 M HCl and injected into a mouse. The time between injection and death is used as an indication of the amount of toxin present, and dilutions are made until the death time is between 5 and 7 min. Problems with this method include a variability of ± 20%, lack of sensitivity, interferences from high salt concentrations, and public pressure to stop mammalian bioassays.

Bates and Rapoport (3) devised a chemical assay in which saxitoxin is heated in dilute hydrogen peroxide under basic conditions to yield a fluorescent purine derivative. The assay is more sensitive than the mouse test but shows only modest agreement with the accepted method. Since then, LC methods using chemical oxidation in a postcolumn reactor have been developed (4, 5). The sensitivity of these methods is good, but the LC system is very sensitive to minor fluctuations in pumping rates of postcolumn reagents, and the reactor is prone to clogging and must be rinsed daily. Overall, the postcolumn method is very labor-intensive and impractical for monitoring purposes, especially when equipment may have to be set up and dismantled frequently.

A precolumn oxidation method was developed by Lawrence and Ménard (6) in which PSP toxins are chemically oxidized in a small vial and the products are then separated by LC. The main disadvantage of this method is that some toxins cannot be separated (e.g., neosaxitoxin and B2), because the (sulfo)carbamate group is cleaved during oxidation, and the final products are identical. Although this problem can be overcome by selective sample cleanup, which removes the B and C toxins (*see* Figure 1) from the carbamate toxins before the oxidation reaction, this requires some extra steps. Also, the isomers gonyautoxins 2 and 3, as well as gonyautoxins 1 and 4, are not separated after the oxidation reaction.

Recently, Janiszewski and Boyer (7) studied the feasibility of electrochemical oxidation of PSP toxins in a postcolumn reactor. Although their work was preliminary, they demonstrated that PSP toxins could indeed be oxidized electrochemically to fluorescent products. We have conducted similar studies, confirming these findings, and extended the work to optimize conditions for electrochemical oxidation of PSP toxins.



R_1	R_2	R_3	R_4	
H	H	H	H	STX
H	H	H	SO_3^-	B1
H	OSO_3^-	H	H	GTX2
H	OSO_3^-	H	SO_3^-	C1
H	H	OSO_3^-	H	GTX3
H	H	OSO_3^-	SO_3^-	C2
OH	H	H	H	NEO
OH	H	H	SO_3^-	B2
OH	OSO_3^-	H	H	GTX1
OH	OSO_3^-	H	SO_3^-	C3
OH	H	OSO_3^-	H	GTX4
OH	H	OSO_3^-	SO_3^-	C4

Figure 1. Structures of paralytic shellfish poison toxins: STX, saxitoxin; NEO, neosaxitoxin; GTX, gonyautoxin.

Experimental

Apparatus

(**a**) *LC system.*—Two pumps (Beckman Model 110B) with a gradient controller (Beckman Model 421A) and an injection port (Altex Model 210A) with a 50 μL loop (Beckman Instruments, Inc., San Ramon, CA).

(**b**) *LC column.*—PRP-1, 15 cm × 4.6 mm id, 10 μm (Hamilton Co., Reno, NV) heated to 45°C by LC column heater (BicRad Laboratories, Inc., Hercules, CA).

(**c**) *Electrochemical detector (used as a reactor).*—Coulochem controller (Model 5100A) equipped with analytical cell

(Model 5010) consisting of 2 porous carbon electrodes operating in coulometric mode (ESA, Inc., Bedford, MA). Operated at various voltages; an equilibration period of 30 min was allowed each time the potential was changed. An optimum potential of 0.75 V was chosen for analysis of PSP mixtures.

(**d**) *Fluorometric detector.*—Model 820-FP, operated at 330 nm for excitation and 400 nm for emission; gain, 100 (Jasco, Inc., Easton, MD). Used to monitor LC effluent.

(**e**) *Integrator.*—Model 3392A (BioRad). Connected to the fluorimetric detector.

(**f**) *Gradient.*—The PSP toxins were separated with a gradient similar to that described by Sullivan and Wekell (4). The mobile phase was modified as follows: mobile phase A consisted of 3.0 mM heptanesulfonate, 1.5 mM ammonium phosphate buffer (pH 7.0), and 1.0% (v/v) acetonitrile; mobile phase B contained 3.0 mM heptanesulfonate, 6.25 mM ammonium phosphate buffer (pH 7.0), and 25% (v/v) acetonitrile. The 2-part gradient ramped from 0 to 28% B in 10.5 min and then to 100% B in 7.5 min. Mobile phases were refrigerated when not in use and prepared every second day. Mobile phase flow rate was 1.0 mL/min.

(**g**) *Precolumn oxidation LC system.*—Used to identify oxidation products of PSP toxins. Assembled and operated as described by Lawrence and Ménard (6).

Reagents

All solvents and reagents were analytical or LC grade materials.

(**a**) *Water.*—Deionized distilled.

(**b**) *PSP standards.*—Saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins 2 and 3 (GTX 2/3) (National Research Council of Canada, Institute for Marine Biosciences, Halifax, NS, Canada).

(**c**) *Mixture of GTX 1–4.*—Received as gifts from Y. Oshima, Tohoku University, Sendai, Japan.

(**d**) *B1 and B2 toxins.*—Nonquantitative, received as gifts from S. Hall, Food and Drug Administration. Wahington, D.C.

Sample Cleanup

Extraction and cleanup of samples were performed as described for the standard mouse bioassay (2).

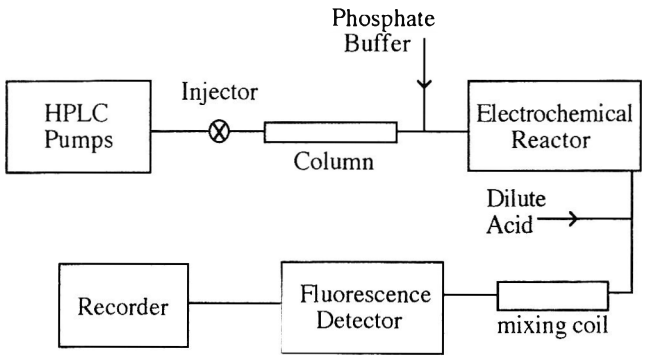


Figure 2. Schematic representation of the postcolumn electrochemical reactor for PSP toxins. The phosphate buffer, the dilute acid, and the mixing coil were used to modify the pH and were removed when the system was operated at pH 7.

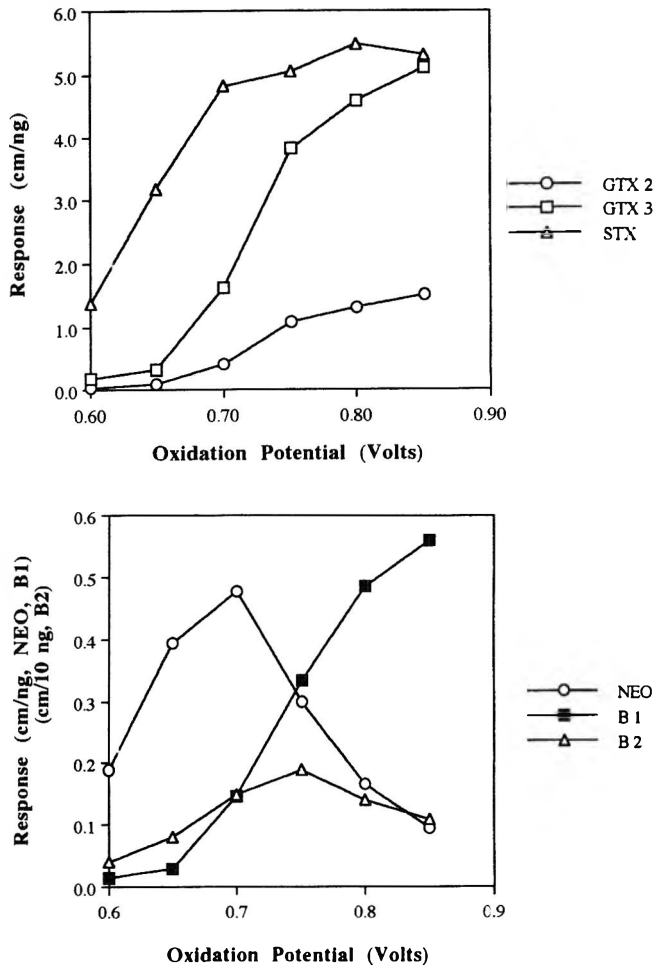


Figure 3. Fluorescence detector response as a function of oxidation potential for individual PSP toxins. The pH was held constant at 7. The graphs are plotted on 3 scales because of the different sensitivities for the toxins.

Results and Discussion

Just as the sensitivity of the postcolumn chemical reactor varies with pH, mobile phase composition, reagent flow rates, and temperature, the electrochemical reactor also was affected by changes in oxidation potential, pH, and composition and flow of the mobile phase. Unfortunately, no single set of optimum parameters was found for all PSP toxins; when oxidation conditions were best for STX, a decrease in sensitivity was observed for NEO. After an extensive study of reaction parameters, the operating conditions described in *Experimental* were chosen for routine analyses.

pH Studies

The effect of pH on oxidation yield was investigated. Because the chemical oxidation required basic conditions, it was thought that the electrochemical oxidation should also be carried out at pH >7. Previous studies in our laboratory had shown that the optimum pH for electrochemical detection of PSP toxins was 12. Two postcolumn pumps were installed and oper-

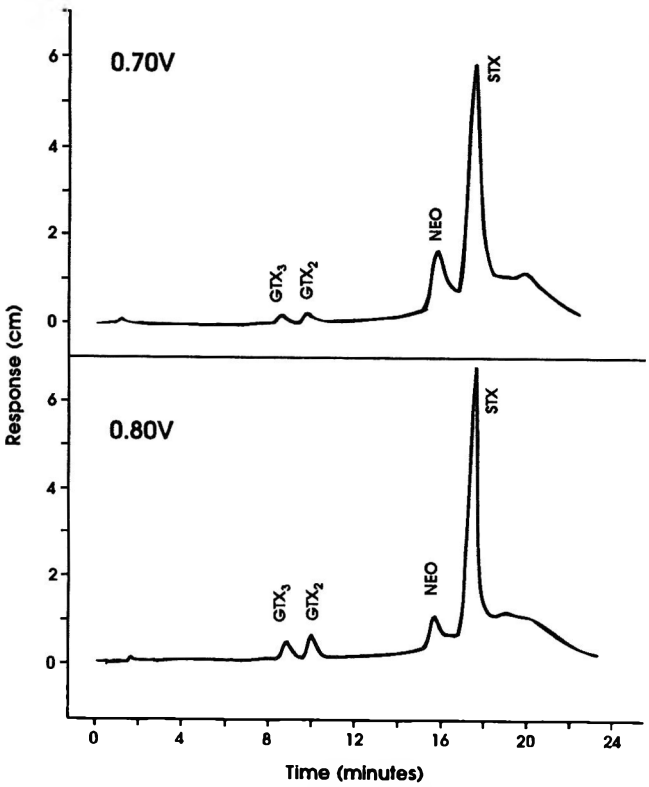


Figure 4. Verification of NEO peak by changing the oxidation potential. The 2 chromatograms were obtained at different oxidation potentials: 0.70 and 0.80 V. As the potential was increased, the peak height for nonhydroxylated toxins (STX, GTX 2/3) increased, while the peak height for NEO decreased.

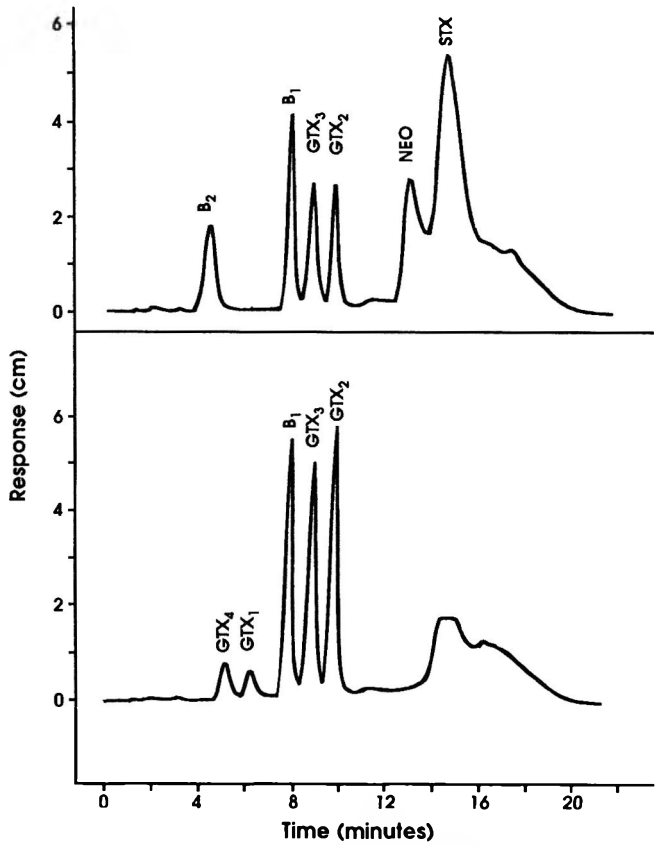


Figure 5. Typical chromatograms of mixtures of PSP standards. Oxidation parameters were pH 7 and 0.75 V. Amounts injected (ng) for top panel: B2, 90; B1, 11; GTX 3, 0.97; GTX 2, 4.0; NEO, 7.0; STX, 0.94. Amounts injected for bottom panel: GTX 4, 9.0; GTX 1, 26; B1, 11; GTX 3, 2.5; GTX 2, 7.5.

ated at flow rates of 0.3 mL/min, as shown in Figure 2. The first pump introduced a basic buffer solution (0.1M Na_2HPO_4 adjusted to pH 12.0 with KOH) to the effluent before the electrochemical cell, and the second pumped 0.1M HNO_3 to restore the effluent pH to neutral before entering the fluorescence detector. The pH of the solution entering the electrochemical reactor could be changed by adjusting the pH of the buffer. Our studies revealed that as the pH of the solution in the electrochemical cell increased from 9 to 12, the peak heights increased for a given amount of STX or GTX 2/3 injected. NEO could not be detected at basic pH levels, even with injected quantities as high as 100 ng.

Studies at pH 12 with GTX 2/3 and STX showed other trends. The peak area increased with decreased flow rate through the electrochemical cell probably because of a longer contact between analyte and electrode. Unfortunately, peak shape deteriorated as flow decreased. Switching one of 2 electrodes off in the electrochemical cell caused the peak area to be halved, the same effect induced by doubling the flow rate. An increase in oxidation potential led to higher sensitivity at pH 12. With optimum operating conditions at pH 12, the estimated limits of detection (at a 3:1 signal-to-noise ratio) for STX, GTX 2, and GTX 3 were 4, 0.1, and 0.07 ng, respectively. The actual detection limit for STX is probably lower than the figure given because the peak showed extensive tailing, a condition that improved when a new column was installed.

The major drawback of basic electrochemical oxidation was the noisy baseline signal, caused by fluctuations in flow from the postcolumn pumps and thus variation in the effluent composition. A 1 mL, narrow-bore delay coil was placed between the acid pump and the fluorescence detector to allow the acid to mix with the effluent. The noise was reduced, but the peaks were broadened by the additional dead volume. Other disadvantages of using postcolumn pumps were the dilution of samples by reagents and the effective increase of the flow rate through the electrochemical reactor, both of which would decrease the sensitivity of the system. If these last 2 effects were taken into account quantitatively, yield of fluorescent products would be greater at pH 12 than at pH 7 (i.e., no postcolumn pumps). In practical terms, however, the absolute sensitivity was better at pH 7 because of these combined effects. Consequently, further studies were performed at pH 7, especially because the LC system without additional pumps was simpler to operate and maintain.

Optimization of Oxidation Potential

To see how oxidation varied with voltage for each PSP toxin, a mixture of STX, NEO, GTX 2, GTX 3, B1, and B2 was

Table 1. Limits of detection of PSP toxins[a]

Toxin	Limit of detection, ng per injection
STX	0.10
NEO	0.84
GTX 1	2.1
GTX 2	0.090
GTX 3	0.050
GTX 4	0.70
B1	0.50
B2	15

[a] Values are estimated on the basis of a 3:1 signal-to-noise ratio; oxidation was at pH 7 and 0.75 V.

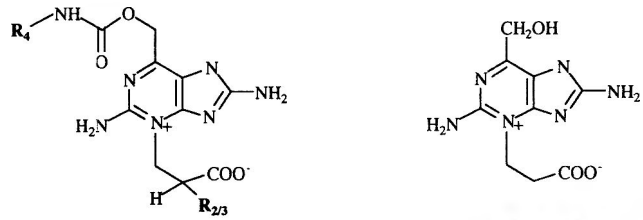
analyzed at various oxidation potentials. Figure 3 shows peak height as a function of potential for each toxin. The response varied greatly from toxin to toxin; the fluorescence sensitivities for STX and GTX 3 were 3 times greater than for GTX 2, 10 times greater than for NEO and B1, and 100 times greater than for B2. Similar relative sensitivities were observed in the post- and precolumn chemical oxidation systems. The graphs in Figure 3 also reveal that the fluorescence response of hydroxylated toxins (NEO and B2) reaches a maximum, after which the sensitivity decreases, whereas nonhydroxylated toxins show greater sensitivity with increasing voltage. The optimum potential for NEO corresponds roughly to the potential at which a plateau is observed in the electrochemical detector response (7). Perhaps NEO oxidation is a 2-step process. If this were true, it would appear that the product of the second oxidation step is nonfluorescent. This characteristic behavior of hydroxylated compounds can be used to verify peak identity.

Figure 4 shows 2 chromatograms of the same standard mixture at 2 different voltages (0.70 and 0.80 V). Because 0.80 V is beyond the optimum potential for NEO, the NEO peak is smaller than the one obtained at the lower voltage. The nonhydroxylated toxins, however, exhibit larger peaks at higher potentials. (The broader peaks in the first chromatogram are not related to the lower oxidation potential but are probably due to nonequilibrium column conditions at the time of injection.) Figure 5 shows typical chromatograms of standard PSP toxin mixtures under the optimum conditions outlined in *Experimental* and at 0.75 V. The estimated limits of detection are given in Table 1.

Table 2. Chemical and electrochemical oxidation products[a] of PSP toxins

Toxin	Chemical oxidation product(s)	Electrochemical oxidation product(s)
STX	A	A
NEO	A, D, E	A, (D)[b]
GTX 2	B	B
GTX 3	B	B
B1	C, (D)	C, (D)
B2	D, E, (A), (C)	C, (D)

[a] Refer to Figure 6 for structures of products.
[b] Letters in parentheses indicate minor products.



Product	R_4	$R_{2/3}$	Precursors
A	H	H	STX, NEO, (B1), (B2)
B	H	OSO_3H	GTX1-4, C1-4
C	SO_3H	H	B1, (B2)
D	—	—	dSTX[a], NEO, B2, (STX), (B1)
E	unidentified product		NEO, B2

( ) indicates minor product of periodate oxidation
a = decarbamoyl saxitoxin

Figure 6. Structures of fluorescent products of PSP toxins after precolumn periodate oxidation.

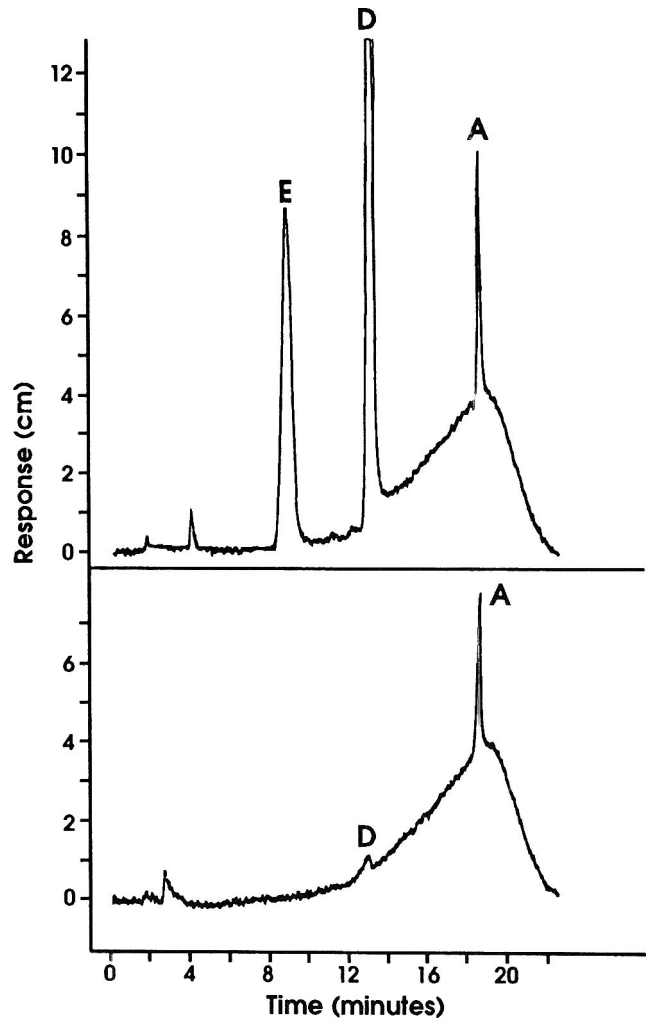


Figure 7. Chromatograms of products of periodate (top) and electrochemical (bottom) oxidation of NEO. Refer to Figure 6 for explanation of product letters.

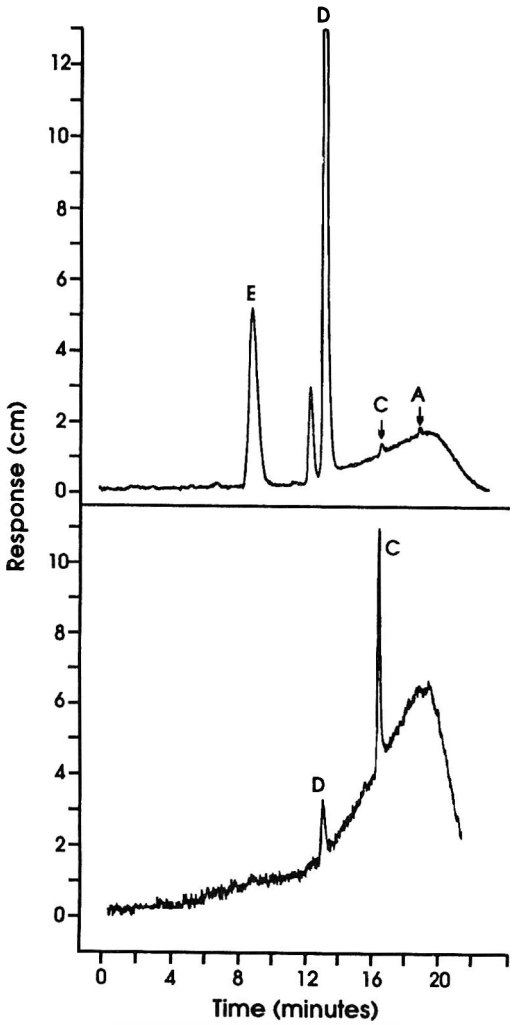


Figure 8. Chromatograms of products of periodate (top) and electrochemical (bottom) oxidation of B2. Refer to Figure 6 for explanation of product letters.

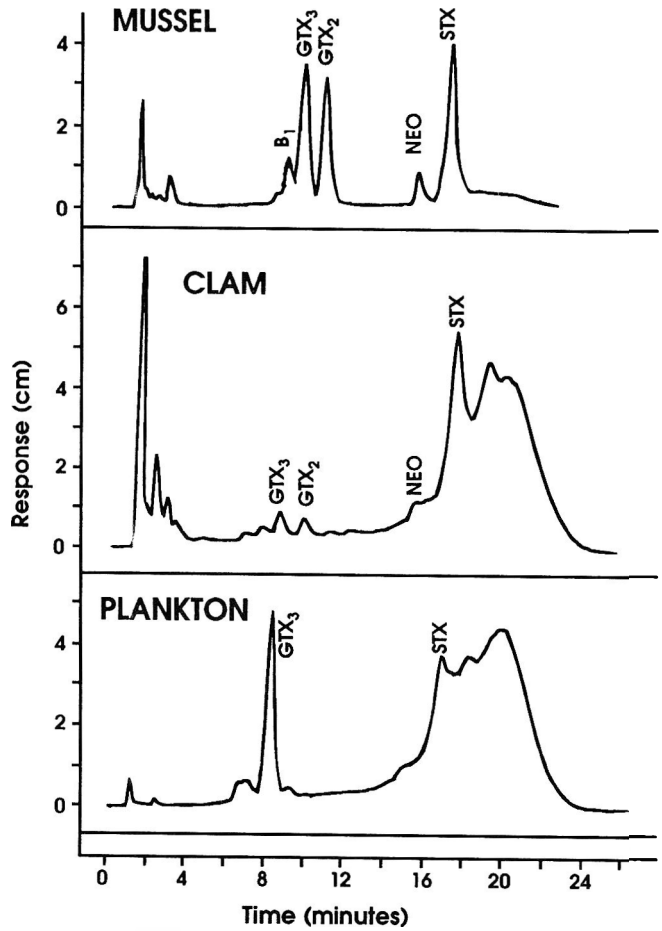


Figure 9. Chromatograms depicting separation of PSP toxins in various extracts. The mussel extract contained total PSP toxin at 25 μg/g, and the clam extract was contaminated at 1 μg/g (0.5 g tissue mL; dilution factor, 1/30 for both samples). Plankton chromatogram is qualitative only. Integrator attenuation was set at 8 for the mussel sample and at 5 for the clam and plankton samples.

Chromatography

No detailed studies were conducted to optimize chromatographic separation of PSP toxins, because this has been done previously (4, 5). However, STX and NEO exhibited rather broad peaks in the system studied. Some attempts were made to improve their peak shapes. Problems with extensive tailing in our studies were thought to occur partly because of adsorption of less polar oxidation products to the porous carbon electrode. Many attempts were made to improve peak shape, including heating the electrode, using more acetonitrile at the end of the gradient, and introducing an organic solvent (methanol, acetonitrile) into the stream before the electrode. None of these gave the desired change. The only condition that improved STX peak shape was concentration of ion-pairing agent in the mobile phase. At pH 7 and 12 (with postcolumn addition of basic buffer), the same trend was observed: The greater the concentration of heptanesulfonate, the better was the peak shape and thus the sensitivity for STX. Unfortunately, changing this parameter is not very practical because separation of various toxins is compromised at concentrations higher than the generally accepted 3 mM concentration of ion-pairing agents used in postcolumn chemical oxidation (4).

Identification of Electrochemical Oxidation Products

Standards of single PSP toxins were injected into the electrochemical LC system, and as the peak eluted, the effluent was collected from the outlet of the fluorescence detector and then analyzed by a second chromatographic system set up to identify the oxidation products (6). A precolumn periodate oxidation of each PSP toxin gave a qualitative standard with which to compare the electrochemical oxidation products. Products from precolumn chemical oxidation were recently identified by LC with mass spectrometry (8), and these structures are shown in Figure 6. The results are outlined in Table 2. Electrochemical oxidation at pH 7 and 0.65 V is milder than periodate oxidation. The products for STX and GTX 2/3 were the same for both methods, but NEO showed little cleaving of the carbamate group in electrochemical oxidation. Quilliam and coworkers (8) noticed that the longer the chemical oxidation was allowed to proceed, the greater the proportion of the cleaved product.

The same trend was noted for B2, which is the sulfocarbamoyl analogue of NEO. The main product of electrochemical oxidation has the sulfocarbamate intact, whereas periodate oxidation cleaves that group. Chromatograms of electrochemical and periodate oxidation products of NEO and B2 are compared in Figures 7 and 8.

Analysis of Shellfish Samples

To test method ruggedness, we analyzed extracts from different species of shellfish (mussels, clams, and oysters) and plankton containing various concentrations of PSP toxins (0.5–25 μg/g). Typical chromatograms of extracts are shown in Figure 9. Shellfish contaminated with 1 μg/g (total toxin), which is close to the regulatory limit, could be analyzed easily, and lower concentrations could be detected by decreasing the extract dilution factor of 30–60, which was used in our experiments. Precolumn periodate oxidation was performed on the same extracts to gauge the accuracy of the electrochemical method. Although no detailed comparison was carried out, the results of the 2 methods agreed reasonably well.

The electrochemical oxidation system gave results similar to those of postcolumn chemical oxidation. However, the electrochemical reactor is much more easily set up and maintained.

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FOOD CHEMICAL CONTAMINANTS

Immunoaffinity Column Coupled with Liquid Chromatography for Determination of Fumonisin B_1 in Canned and Frozen Sweet Corn

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A modified liquid chromatographic (LC) method for determining fumonisin B_1 (FB_1) in corn was applied to canned and frozen sweet corn. The corn is extracted with methanol–water (8 + 2), and the extract is filtered. The filtrate is diluted with water and passed through an immunoaffinity column. After the column is washed with water, FB_1 is eluted with methanol–water (8 + 2). The eluate is evaporated to dryness by using a vacuum concentrator, and the residue is dissolved in acetonitrile–water (1 + 1). FB_1 is derivatized with *o*-phthaldialdehyde. The derivative is separated on a reversed-phase C_{18} LC column using acetonitrile–water–acetic acid (50 + 50 + 1) and quantitated with a fluorescence detector. Recoveries of FB_1 from canned and frozen corn spiked over the range of 50–200 ng/g were 76–88%. The limit of determination was about 25 ng/g, and the limit of detection was about 4 ng/g. The method was applied to 97 commercial canned and frozen sweet corn samples collected from different areas of the United States. Sixty samples contained no FB_1. Low levels (trace–82 ng FB_1/g corn) were found in 35 samples; 235 ng FB_1/g was found in 1 canned corn sample, and 350 ng FB_1/g was found in 1 frozen corn sample.

Fumonisins are structurally related mycotoxins produced by *Fusarium moniliforme*, *F. proliferatum*, and related fusaria. Seven fumonisins have been isolated—fumonisins A_1, A_2, B_1, B_2, B_3, B_4 (1), and C_1 (2). Fumonisin B_1 (FB_1), commonly found in corn, is usually the most abundant. FB_1 causes equine leukoencephalomalacia (3), pulmonary edema in swine (4), and liver cancer in rats (5). *F. moniliforme* has been associated with high risk of human esophageal cancer in Transkei, South Africa (6).

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Scott (7) classified methods of analysis for fumonisin toxins into 4 categories: thin-layer chromatography, liquid chromatography (LC), posthydrolysis gas chromatography, and immunochemistry. Chromatographic methods require extraction, purification, and derivatization steps. The immunochemical methods include both direct and indirect competitive enzyme-linked immunosorbent assays as well as use of immunoaffinity columns.

FB_1 was reported recently in canned yellow corn (8). However, current methods for determination of fumonisins were developed primarily for analysis of dent corn. Therefore, additional studies are needed to investigate the occurrence of fumonisins in other types of corn products. We applied 3 published procedures—2 solid-phase extraction (SPE) methods and 1 immunoaffinity column method—to the determination of FB_1 in canned and frozen sweet corn. The methods used either strong-anion-exchange (SAX) (9, 10) or reversed-phase C_{18} (11) cartridges for cleanup, *o*-phthaldialdehyde–mercaptoethanol (OPA/MCE) derivatization, reversed-phase LC separation, and fluorescence detection. Recoveries of FB_1 from corn spiked at 1000 ng/g by the SPE methods were 10–40%. The use of immunoaffinity column cleanup (12), vacuum evaporation, derivatization, and quantitation steps (10), however, gave consistent recoveries of >70%. The limit of determination was about 25 ng/g, and the limit of detection was about 4 ng/g. This method was used to analyze 97 canned and frozen corn samples.

METHOD

Sample Collection

Canned and frozen sweet corn samples were collected by 10 U.S. Food and Drug Administration (FDA) district offices from retail establishments within their areas during the spring of 1993. The samples represented as many different manufacturers and distributors as possible. Each district was assigned to collect 10 domestically produced sweet corn samples (7 canned and 3 frozen).

Apparatus

(a) *Explosion-proof blender.*—Waring Model EP-1 with 500 mL jar and cover.

(**b**) *Immunoaffinity column.*—Fumonitest column (Vicam, Somerville, MA).

(**c**) *Filter paper.*—18 cm, prefolded (Whatman 2V performed satisfactorily; Whatman, Inc., Clifton, NJ).

(**d**) *Glass microfiber filter paper.*—11 cm (Whatman 934AH performed satisfactorily).

(**e**) *Column reservoir.*—15 mL, polypropylene (Alltech Associates, Deerfield, IL).

(**f**) *SPE manifold.*—12 ports (Alltech Associates).

(**g**) *Vacuum concentrator.*—Savant Instruments (Farmingdale, NY).

(**h**) *LC column.*—μBondapak C_{18}, 300 × 3.9 mm (Millipore-Waters, Milford, MA).

(**i**) *LC system.*—Model 510 pump, U6K injector, Model 540 fluorescence detector, and Millenium data system (Millipore-Waters). Operating conditions: flow rate, 1.0 mL/min; excitation, 335 nm; emission, 440 nm.

Reagents

(**a**) *Solvents and reagents.*—Distilled-in-glass methanol; LC grade acetonitrile and methanol; ACS grade acetic acid, sodium bicarbonate, sodium chloride, sodium tetraborate, and Tween 20; OPA and MCE (Sigma Chemical Co., St. Louis, MO); and Milli-Q water (Millipore Corp., Bedford, MA).

(**b**) *Diluting solution.*—Dissolve 12.5 g sodium chloride, 2.5 g sodium bicarbonate, and 0.05 mL (ca 1 drop) Tween 20 in 500 mL water.

(**c**) *OPA derivatization reagent.*—Dissolve 40 mg OPA in 1 mL methanol in 16 mL amber vial; add 5 mL filtered 0.1M sodium tetraborate and 50 μL MCE. Reagent is stable for ca 5 days.

(**d**) *FB_1 standard solutions.*—Obtain standard from Sigma Chemical Co. (*1*) *FB_1 stock standard solution.*—100 μg/mL. Dissolve 1 mg FB_1 in 10 mL acetonitrile–water (1 + 1). (*2*) *Working standard solutions.*—Transfer 50 μL FB_1 stock standard solution to 4 mL vial and add 2450 μL acetonitrile–water (1 + 1) to prepare FB_1 solution at 2 ng/μL; make serial dilutions with same solvent to prepare FB_1 solutions at 1, 0.5, and 0.25 ng/μL. Prepare all working standard solutions weekly except 0.25 ng/μL solution, which should be prepared daily.

(**e**) *LC mobile phase.*—Water–acetonitrile–acetic acid (50 + 50 + 1), degassed.

Extraction of Corn

Weigh 25 g frozen corn or drained canned corn and 2.5 g sodium chloride in blender jar. Add 50 mL methanol–water (8 + 2) and blend 2 min at high speed. Filter through prefolded paper. Pipet 10 mL filtrate into 125 mL Erlenmeyer flask. Add 40 mL diluting solution, mix, and filter through glass microfiber paper. If filtrate is not clear, refilter. Proceed immediately with immunoaffinity column chromatography.

Immunoaffinity Column Chromatography

Insert 12-gauge needle into top cap of affinity column. Remove top cap from column. Cut off tip and use cap as connector between column and reservoir. Remove end cap from column. Immediately pipet 10 mL second filtrate (equivalent to 0.71 g test portion) into reservoir. Let filtrate flow through column. If flow stops, use hand pump to restart. Wash column with 5 mL diluting solution followed by 5 mL water. Remove column from manifold. Place 15 mL polypropylene test tube under column. Add 0.8 mL methanol–water (8 + 2) to column. Use syringe to push solution slowly through column (dropwise). Add additional 0.8 mL methanol–water (8 + 2) to column and push through column in same manner. Evaporate eluate to dryness in vacuum concentrator (ca 2 h). Proceed with LC analysis or cap test tube and store at 4°C.

Derivatization and LC Analysis

Dissolve residue in 100 μL acetonitrile–water (1 + 1). Mix 1 min. Pipet 200 μL OPA derivatization reagent into separate 1 mL glass vials. Add 50 μL working standard solution or 50 μL test solution containing dissolved residue to vial. Mix 30 s. Inject 50 μL derivatization mixture into liquid chromatograph 1 min after initiation of derivatization reaction. FB_1 derivative elutes in 10.5 min. The 50 μL aliquots of derivatized working standard solutions contain 20, 10, 5, and 2.5 ng FB_1. Construct standard curve. Determine quantity of FB_1 in injected aliquot of derivatized test solution by comparing area of chromatographic peak for derivatized test solution with that for derivatized standard solution.

Calculations

Calculate C, concentration of FB_1 in frozen and canned corn (ng/g), with the following equation:

$$C\ (\text{ng/g}) = A \times (5/W)$$

where A is the amount of FB_1 (ng) in 50 μL aliquot of derivatized test solution injected into liquid chromatograph (from standard curve), 5 is the dilution factor (250 μL/50 μL), and W is the wet weight of corn (g) represented by 50 μL test solution. Calculate W for a 25 g test portion with the following equation:

$$W = 25\ \text{g} \times \frac{10\ \text{mL}}{(50\ \text{mL} + 20\ \text{mL})} \times \frac{10\ \text{mL}}{50\ \text{mL}} \times \frac{50\ \mu\text{L}}{100\ \mu\text{L}}$$

$$= 0.36\ g$$

(Because corn contains about 80% water, a 25 g test portion would contain 20 mL water. The water content of frozen corn was determined gravimetrically after drying at 105°C overnight. This determination can give only approximate results because frozen corn often contains ice crystals on the surface. The value for the water content of frozen corn was also used for canned corn.)

Results and Discussion

Two SPE methods (9–11) were used to analyze canned corn spiked with FB_1 at 1000 ng/g. Recoveries of FB_1 were inconsistent for both methods, ranging from 10 to 40%. These low recoveries may be due to matrix effects, which can cause in-

complete or irreversible adsorption of the toxin on the solid phase. The immunoaffinity column method (12) showed no matrix interferences in the liquid chromatogram. However, the limit of determination of the method was about 100 ng FB_1/g. The method was modified as follows: Before the top cap of the column was removed, a large needle was used to introduce air above the affinity column to prevent cracking of the column packing. The corn was extracted with methanol–water. Because corn contains about 80% water and FB_1 is quite water soluble, the methanol–water system was thought to be an appropriate solvent for extraction. Therefore, no modifications in solvent composition or extractions with other solvents were explored. The amount of diluted and filtered extract applied to the affinity column was increased 5-fold over the quantity used in the analysis of dent corn. This amount seems to be the maximum volume of extract that can be applied to the column. When the volume was increased 8-fold, recoveries of FB_1 from corn spiked at 1000 ng/g decreased from 90% to less than 20%. The maximum FB_1 binding for the column is about 1000 ng. However, the methanol in the extract affects the antibody–antigen binding. The larger the extract volume applied to the column, the less FB_1 binds to the column. FB_1 was eluted with 1.6 mL methanol–water (8 + 2) instead of methanol to improve FB_1 solubility. The final extract was evaporated to dryness by using a vacuum concentrator, and the residue was redissolved in a small volume of acetonitrile–water (1 + 1). The derivatization reagent was prepared as described by Sydenham et al. (9). This reagent is more stable than the one used in the original method (12).

The fluorescence intensity of the OPA derivative is time dependent. The maximum intensity is obtained within 1 min after addition of the extract to the OPA reagent, as shown in Figure 1. FB_1 (100 ng) was derivatized, and aliquots of the reaction solution were injected in triplicate at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 60 min. The fluorescence intensity response decreased 15% after 10 min and 30% after 1 h (data not shown). Although the OPA reagent is somewhat unstable, it can be stored at room temperature in amber bottles for about 6 days. The same OPA reagent was used over an 8-day period. New standard solutions were prepared from stock standard solution each day. The day-to-day variability of the fluorescence intensity of the OPA derivative was about 10%. Within the same day, the variation of fluorescence intensity of the OPA derivative of the same standard solution was about 3%. These differences may be due to changes in room temperature, variability or change in mobile phase composition, or other factors affecting the derivatization reaction. When the same OPA reagent was used after 8 days, the fluorescence intensity of the OPA derivatives declined more than 20% compared with that of OPA derivatives prepared with freshly made reagent.

FB_1 standard solutions (2, 1, and 0.5 ng/μL) were stable for about 4 days at room temperature in amber bottles. However, the 0.25 ng/μL solution should be prepared daily from the 0.5 ng/μL solution because of toxin degradation at this concentration. When the same 4 standard solutions (2, 1, 0.5, and 0.25 ng/μL) were used over a 5-day period, the standard curve showed a linear relationship between concentrations and responses on the first day. On the following 4 days, only the first 3 standard solutions gave responses similar to those obtained on the first day. On the second day, the response of the standard solution of lowest concentration was less than half of the response obtained on the first day. The stability of the standard solutions was not determined beyond 5 days.

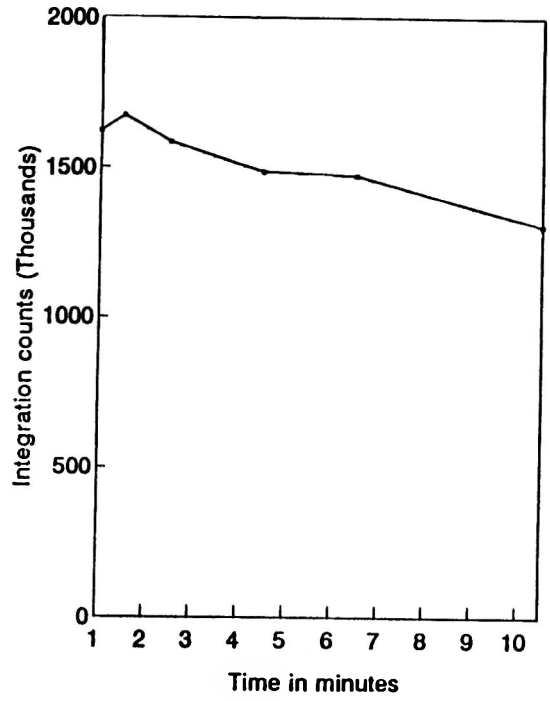

Figure 1. Stability of the FB_1 OPA derivative (20 ng standard).

Average recoveries of FB_1 added to canned corn, liquid from canned corn, and frozen corn were >70%. Recoveries based on an 80% moisture content for canned and frozen corn kernels are given in Table 1. Because of the large corn:solvent ratio, the water content of the corn increases the final volume of the mixture. To avoid complicating this step by dilution to volume or to weight, an allowance is made for the resulting change in volume. Average recoveries from canned corn con-

Table 1. Recovery of fumonisin B_1 added to canned and frozen sweet corn

Product	FB_1 added, ng/g	Recovery, % (n = 3)	SD	RSD, %
Canned corn	0	0	—	—
	50	76.7	9.1	11.9
	100	77.0	5.6	7.3
	200	81.3	4.0	4.9
Frozen corn	0	0	—	—
	50	88.3	12.0	13.6
	100	75.8	6.2	8.2
	200	79.3	7.3	9.2

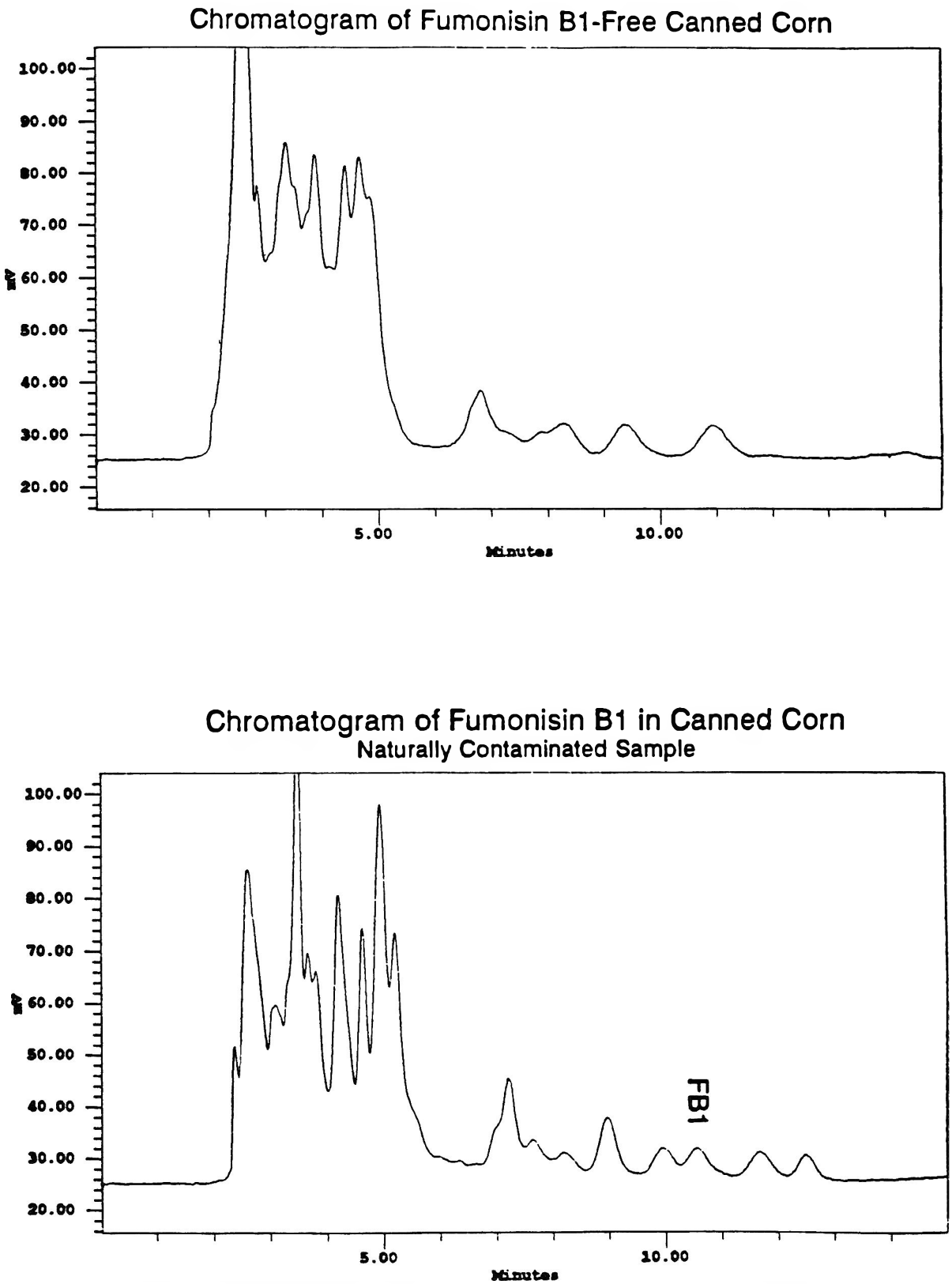

Figure 2. Chromatograms from analysis of FB_1-free (top) and naturally contaminated (bottom) canned corn.

taining FB_1 at 50–200 ng/g were 77–81%, and standard deviations were 4.0–9.1 ($n = 3$). Average recoveries from frozen corn containing FB_1 at 50–200 ng/g were 76–88%, and standard deviations were 7.3–12.0. Average recoveries from both products containing FB_1 at 25 ng/g (not shown in Table 1) were 75–111%, and standard deviations were 30–40. The limit of determination of the method was about 25 ng/g. The limit of detection was 4 ng/g (signal-to-noise ratio, 5:1).

Chromatograms from analysis of FB_1-free and naturally contaminated canned and frozen corn are shown in Figures 2

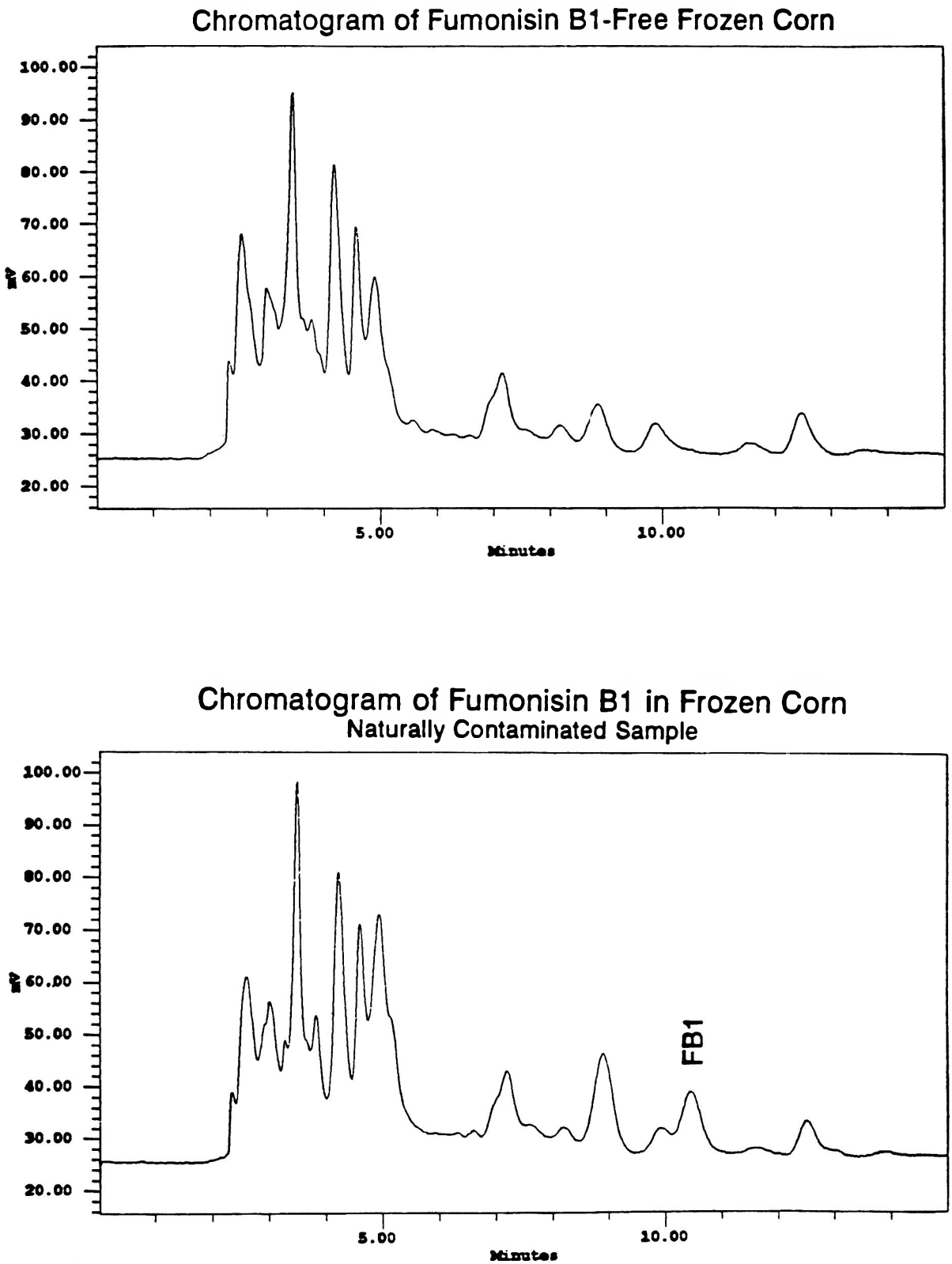

Figure 3. Chromatograms from analysis of FB_1-free (top) and naturally contaminated (bottom) frozen corn.

and 3, respectively. FB_1 is sufficiently separated from background fluorescent materials to permit quantitation. Other fumonisins (FB_2, FB_3, and FB_4) would most likely be eluted after FB_1. In these analyses, no attempt was made to detect these compounds.

The method was used to survey FB_1 in 70 canned corn and 27 frozen corn samples collected from 10 FDA districts. Be-

Table 2. Fumonisin B_1 in canned and frozen sweet corn sampled in 1993

Product	No. of samples	FB_1 concentration, ng/g	
		Range	Average
Canned corn (kernels)	42	<4	<4
	16	4–19	8
	8	20–33	27
	3	65–82	75
	1	235	235
Canned corn (liquid)	17	<4	<4
	2	4–10	6
	1	26	26
Frozen corn	18	<4	<4
	6	8–18	14
	2	21–25	23
	1	350	350

cause FB_1 is water soluble, the liquid and kernels of 21 canned corn samples were analyzed separately. One test portion each of liquid and corn kernels contained about 20 ng FB_1/g; 2 liquid and 3 kernel test portions contained only trace amounts (<10 ng FB_1/g). Because the occurrence and levels of FB_1 in both liquid and kernels were so low, only kernels in the remaining samples were analyzed for FB_1.

Table 2 shows results of analyses of sweet corn. Of the 70 canned corn samples analyzed, 42 were negative and 28 were positive for FB_1 contamination. Levels in 27 samples ranged from 4 to 82 ng/g; 1 sample contained 235 ng/g. Of the 27 frozen corn samples, 18 were negative and 9 were positive. FB_1 levels in 8 samples ranged from 8 to 25 ng/g; 1 sample contained 350 ng/g. The remaining extract of the canned corn containing FB_1 at 235 ng/g was analyzed by secondary ion mass spectrometry; the presence of the molecular ion at *m/z* 722 confirmed the identity of FB_1. These data demonstrate that FB_1 is present in canned or frozen sweet corn destined for human consumption. For the 1993 crop year, sweet corn contained a moderate amount (36%) of low-level FB_1 contamination. Additional studies are required to determine year-to-year variations.

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FOOD COMPOSITION AND ADDITIVES

Liquid Chromatographic Determination of Residual Isocyanate Monomers in Plastics Intended for Food Contact Use

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A liquid chromatographic (LC) method was developed for the analysis of 10 isocyanates in polyurethane articles and laminates intended for food use. Residual isocyanates are extracted by dichloromethane with concurrent derivatization by 9-(methylaminomethyl)anthracene. The resultant derivatives are analyzed by reversed-phase LC with fluorescence detection. Separation of the isocyanates was studied and optimized. Quantitation uses 1-naphthyl isocyanate as internal standard and standard addition to the food package. Validation demonstrated the method to have good precision (± 2–5%) and recovery (83–95%) for samples spiked with isocyanates at 0.1 mg/kg. The limit of detection was 0.03 mg/kg. Analysis of 19 commercial polyurethane or laminate food packages demonstrated that the method was not prone to interferences. Residues of diphenylmethane-4,4′-diisocyanate were detected in 5 packages and ranged from 0.14 to 1.08 mg/kg.

Organic isocyanates are chemicals in which the electrophilic character of the –NCO group permits easy reaction with molecules containing a nucleophilic center, e.g., water, amines, and alcohols or diols. Urethanes are formed in the reaction with alcohols and diols. If di- or polyisocyanates take part in the reaction, polyurethanes are formed (*see* Scheme 1).

Polyurethane polymers have important industrial applications, e.g., flexible and rigid polyurethane foam coatings, adhesives, and elastomers. Within the food packaging industry isocyanates are used in polyurethane polymers and adhesives. Adhesives are used in polyester or paperboard laminates [e.g., metallized poly(ethylene terephthalate) film laminated to paper as a microwave-interactive "susceptor" material], multilayer high-barrier plastics laminates (e.g., "shelf stable" products), and "boil-in-the bag" laminates. Polyurethane polymers are used for items such as conveyor belts. During manufacture residual unpolymerized isocyanate monomer can remain in the polymer and may migrate into food that subsequently comes into contact with the polymer. Isocyanates are toxic compounds and their health effects are well-documented (1–3). In this paper, we use the term isocyanates to refer collectively to both isocyanates and diisocyanates.

Within the European Community (EC) isocyanates used in the manufacture of plastics materials and articles intended to come into contact with foods are regulated by EC Directive 90/128/EEC and amendments (4, 5). Residual levels in the finished plastic must not exceed 1.0 mg/kg expressed as –NCO. Twelve isocyanates are currently permitted for use in food-contact materials (Table 1). Only 9 of the 12 isocyanates permitted on the EC positive list are commercially available within the EC. Consequently, methodology was developed for the isocyanates available and also for isopherone diisocyanate, which is not on the positive list but does find use in polyurethanes for nonfood applications. We are not aware of any existing method which analyzes combinations of these residual isocyanates in plastics.

The majority of scientific literature concerning isocyanate analysis covers the determination of isocyanates in air in industrial environments (6–11). These methods rely on impingers, coated with a derivatization reagent, to collect isocyanate residues. Derivatization reagents that were used include *N*-(*p*-nitrobenzyl)-*N*′-propylamine (6), 1-naphthalenemethylamine (7), 1-(2-pyridyl)piperazine (8), 1-(2-methoxyphenyl)piperazine (9), 9-(*N*-methylaminomethyl)anthracene (MAMA) (10), and tryptamine (11). Once collected, the final determinative step may be either colorimetric, chromatographic, or polarographic. The use of impingers makes these methods unsuitable for analysis of isocyanates in plastics materials.

Numerous methods were published that detail isocyanate analysis in polyurethane prepolymers (12–14). These methods rely on various derivatization reagents to improve chromatographic resolution and provide a convenient chromophore. One of the most successful derivatization reagents is MAMA (*see* Scheme 2).

The urea derivatives formed with MAMA are fluorescent and have a strong UV chromophore (molar absorptivity, 4×10^5 M/cm at 254 nm). Rastogi (15) developed methodology for the analysis of residual isocyanate monomers in chemical products containing polyurethane or prepolymer diisocyanate. MAMA was used as the derivatizing reagent and detection lim-

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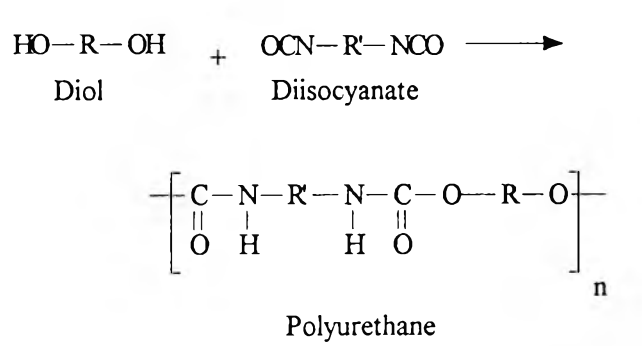


Scheme 1. Reaction of diol with diisocyanate to form a polyurethane of chain length *n*.

its for diphenylmethane diisocyanate (MDI) and toluene diisocyanate (TDI) were 50 and 5 µg/kg, respectively. We report a method applicable for the determination of 10 target isocyanates in plastic materials and laminates by reversed-phase liquid chromatography (LC) following derivatization by MAMA.

Experimental

An instrument or piece of apparatus is mentioned only if it is special or made to particular specifications. Usual laboratory equipment is assumed to be available. All laboratory glassware should be rinsed with dichloromethane (DCM) and baked at 105°C overnight before use. To avoid condensation, after baking vials should be placed in a desiccator to cool until required. The MAMA–isocyanate derivatives are not sensitive to moisture, and so glassware used for operations involving the derivatives need not be so efficiently dried before use.

Apparatus

(**a**) *Liquid chromatograph.*—Model 305 pump, Model 805 manometric module (Gilson, Anachem, Luton, UK). Model ISS-100 autosampler, Model LS-4 fluorescence detector (Perkin Elmer, Beaconsfield. UK) set at 254 nm (excitation) and 412 nm (emission). Slit widths were set at 10 nm for both excitation and emission.

(**b**) *LC columns.*—Spherisorb S5ODS2 (25 cm × 4.6 mm, 5 µm), Spherisorb S5ODS1 (25 cm × 4.6 mm, 5 µm), Spherisorb C8 (25 cm × 4.6 mm, 5 µm), Partisil ODSIII (25 cm × 4.6 mm, 5 µm), Zorbax ZODS (25 cm × 4.6 mm, 5 µm), Nucleosil 120 5C18 (25 cm × 4.6 mm, 5 µm), LiChrosorb RP-B (25 cm × 4.6 mm, 5 µm) (Hichrom, Theale, UK). In-line solvent filter, 2 µm (Anachem, Luton, UK). Solvent filter was connected to the analytical column. The analytical column and solvent filter were placed in a column heater (Jones Chromatography, Hengoed, UK) set at 45°C.

(**c**) *Glass vials (20 mL).*—Chromacol 20-CV (Chromacol Ltd, London, UK). Vials must be rinsed with DCM and baked at 105°C overnight before use.

(**d**) *Glass amber LC vials (2 mL).*—Chromacol 2-SV(A) (Chromacol Ltd). Vials must be rinsed with DCM and baked at 105°C overnight before use.

(**e**) *PTFE-faced silicone rubber septa and aluminum crimp caps.*—For 20 mL vials (Chromacol Ltd).

Table 1. Isocyanates on the positive list permitted for use in food contact materials within the European Community

Substance	Abbreviation	Conversion factor[a]
2,6-Toluene diisocyanate	2,6-TDI	0.483
2,4-Toluene diisocyanate	2,4-TDI	0.483
2,4-Toluene diisocyanate dimer	DIMER	0.483
Hexamethylene diisocyanate	HDI	0.500
Cyclohexyl isocyanate	CHI	0.336
Diphenylmethane-4,4′-diisocyanate	4,4′-MDI	0.336
Diphenylmethane-2,4′-diisocyanate	2,4′-MDI	0.336
Diphenylether-4,4′-diisocyanate	DPDI	0.321
1,5-Naphthalene diisocyanate	1,5-NI	0.400
Phenyl isocyanate	PI	0.353
Octadecyl isocyanate	ODI	0.142
3,3′-dimethyl-4,4′-diisocyanato-biphenyl	DIBP	0.318
Isopherone diisocyanate[b]	IPDI	0.378

[a] Factor to convert isocyanates to NCO equivalents (*see* text).
[b] IPDI is not on the positive list but is commonly used for nonfood contact applications.

(**f**) *PTFE-faced silicone rubber septa and screw caps.*—For LC vials (Chromacol Ltd).

(**g**) *Crimping device.*—For sealing 20 mL vials (Chromacol Ltd).

(**h**) *Evaporation unit.*—9 Port Reacti-Vap evaporator with 50 mL/min oxygen free nitrogen flowing through each port, single block Reacti-Therm heating module set at 45°C, and Reacti-Blocks Q-1 and C-1 to hold 20 mL and LC vials (Pierce and Warriner, Chester, UK).

(**i**) *Glass syringes.*—Type A, graduated (10, 50, 100, and 1000 µL) (SGE, Milton Keynes, UK).

(**j**) *Laboratory fan-assisted oven.*—Temperature-controlled at 105°C (Gallenkamp, Fisons Scientific Equipment, Loughborough, UK).

(**k**) *Orbital shaker.*—Model R100 (Luckham, Burgiss Hill, UK).

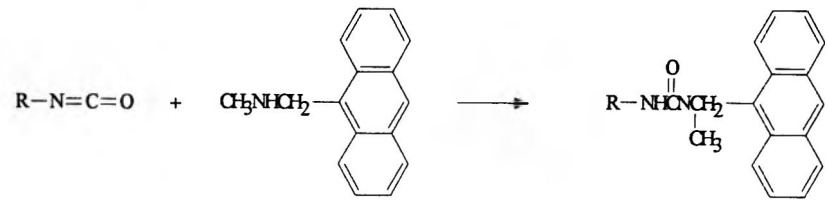
(**l**) *pH meter and electrode.*—Model PW9418 (Pye Unicam, Cambridge, UK).

Reagents

Caution: Isocyanates are toxic substances. Handling and preparation of standard solutions should be undertaken in a fume hood. Skin and eye contact with isocyanates and inhalation of vapor, should be strictly avoided. Isocyanates react rapidly with moisture. Isocyanate standards should be protected from moisture and stored at 20°C. Precautions should be taken to ensure all glassware is dry.

(**a**) *Solvents.*—*N,N*-Dimethylformamide (DMF), acetonitrile, and acetone (all LC grade) (Rathburn, Walkerburn, UK). DCM (<20 ppm H_2O) (Rathburn), dried over a bed of 4 Å molecular sieve for 24 h before use; and triethylamine (99%) (Aldrich, Gillingham, UK).

(**b**) *Standards.*—1-Naphthyl isocyanate (1-NI) (98%), diphenylmethane-4,4′-diisocyanate (98%), hexamethylene di-



Scheme 2. Isocyanate derivatization by MAMA.

isocyanate (98%), cyclohexyl isocyanate (98%), phenyl isocyanate (98%), and isopherone diisocyanate (95%) (Aldrich); 2,6-toluene diisocyanate (99%), 2,4-toluene diisocyanate (99%), and 2,4-toluene diisocyanate dimer (98%) (Bayer, Leverkusen, Germany); diphenylmethane-2,4′-diisocyanate (99%) (Kodak, U.S.); 1,5-naphthalene diisocyanate (98%) (Pfaltz and Bauer, Germany). For other abbreviations, *see* Table 1.

(**c**) *Molecular sieve.*—4 Å, 1/16 in., 8–12 mesh (Aldrich).

(**d**) *Orthophosphoric acid.*—1.75 specific gravity (BDH, Poole, UK).

(**e**) *9-(Methylaminomethyl)anthracene.*—99% (Aldrich).

(**f**) *LC mobile phase A.*—Acetonitrile–3% triethylamine in water (7 + 3, v/v), adjusted to pH 3.0 with orthophosphoric acid.

(**g**) *LC mobile phase B.*—Acetonitrile–3% triethylamine in water (3 + 1, v/v), adjusted to pH 3.0 with orthophosphoric acid.

(**h**) *LC mobile phase C.*—Acetonitrile–3% triethylamine in water (4 + 1, v/v), adjusted to pH 3.0 with orthophosphoric acid.

(**i**) *LC mobile phase D.*—Acetonitrile–3% triethylamine in water (85 + 15, v/v), adjusted to pH 3.0 with orthophosphoric acid.

(**j**) *Precipitate dissolution solvent.*—DMF–LC mobile phase C (1 + 1, v/v).

(**k**) *Polyurethane or laminate samples.*—Samples were obtained from various European suppliers as representative of polyurethane materials for food packaging.

Method Development

Derivatization of standards and LC peak assignment.—Solutions of 1 μg/mL PI; 2,4-TDI; 2,6-TDI; 2,4′-MDI; 4,4′-MDI; HDI; IPDI; PI; 1,5-NI; CHI; and 1-NI (internal standard) were prepared in DCM as working standards. Working standards were protected from moisture and stored at 20°C when not in use. A derivatization solution of 0.24 mg/mL MAMA in DCM was prepared daily because of the photo-instability of MAMA and stored with the exclusion of light. Into 20 mL vials, 100 μL individual working standard and 100 μL derivatization solution was dispensed and vials were capped immediately. Derivatization was allowed to proceed in the dark at ambient temperature for 60 min. The derivatized solution was evaporated to dryness at 45°C under a stream of nitrogen, 10 mL precipitate dissolution solvent was added, and the vial was recapped and shaken vigorously to redissolve the precipitate. A subsample of this solution (1000 μL) was dispensed into an LC sample vial.

Effect of column choice and mobile phase composition.—Derivatized working solutions of 7 isocyanates (PI; CHI; 2,6-TDI; 2,4-TDI; HDI; 2,4′-MDI; and 4,4′-MDI) were analyzed by LC using a variety of columns and LC mobile phases A–D at a flow rate of 1 mL/min. Capacity factors (k) were calculated for each isocyanate derivative peak to determine which column and mobile phase gave the best peak-to-peak resolution.

Effect of derivatization time.—Aliquots (100 μL) of 7 isocyanate working standards (PI; CHI; 2,6-TDI; 2,4-TDI; HDI; 2,4′-MDI; and 4,4′-MDI) and derivatization solution (100 μL) were dispensed into seven 20 mL vials and capped immediately. Individual vials were set aside for 10, 30, 60, 90, 150, 180, and 240 min in the dark. Solutions were prepared for LC as detailed previously and analyzed using a Spherisorb S5ODS1 column and mobile phase C at 1 mL/min.

Effect of extraction time.—Subsamples (1 g) of packaging film NCO19 were cut into 0.25 cm^2 pieces, weighed to an accuracy of 5 mg, and placed into 20 mL vials. To each vial, 15 mL DCM, 200 μL 1-NI working solution, and 1000 μL derivatizing reagent were added. Vials were capped immediately and shaken in the dark for 1, 3, 6, 14, and 24 h on an orbital shaker. Undissolved pieces of film were removed from the vial, and the contents were evaporated to dryness at 45°C under a stream of nitrogen. The evaporated samples were then prepared for LC analysis as described previously and analyzed using a Spherisorb S5ODS1 column and mobile phase C at 1 mL/min.

All extraction time experiments were undertaken in duplicate, and a graph was plotted of isocyanate derivative peak area versus extraction time.

Optimized Procedure

Identification of isocyanates present in test samples.—A 1 g portion of test sample was accurately weighed to the nearest 5 mg, and then cut into 0.25 cm^2 pieces. Pieces were placed into a 20 mL vial, and 15 mL DCM, 200 μL 1-NI working standard, and 1000 μL derivatizing solution were added. The vial was capped immediately. Vials were shaken gently on an orbital shaker for 12 h in the dark. Undissolved pieces of sample were removed from the vial, and the contents were evaporated to dryness at 45°C under a stream of nitrogen. A 10 mL aliquot of precipitate dissolution solvent was added and mixed thoroughly. Ultrasonication may be used to aid dissolution. The solution was filtered through a 0.45 μm syringe filter (prepurged with 2 mL LC mobile phase C) and transferred to an LC sample vial.

Preparation of reagent blank sample.—15 mL DCM, 200 µL 1-NI working standard, and 1000 µL derivatizing reagent were dispensed into a 20 mL vial. The vial was capped and treated as above, leading to sample in an LC vial.

Preparation of internal standard check sample.—A representative 1 g sample was weighed to an accuracy of 5 mg, cut into small pieces where possible, and placed into a 20 mL vial. A 15 mL aliquot of DCM and 1000 µL derivatizing reagent were added. The vial was capped and treated as for test samples, leading to sample in an LC vial.

Chromatographic determination.—Because of the response characteristics of many fluorescence detectors, obtaining a linear response for all calibration solutions may not be possible. In this case the detector must be optimized by decreasing the injection volume or adjusting the slit widths so that the detector is linear over the desired range.

The LC system was equilibrated using a Spherisorb S5ODS1 column and mobile phase C at 1 mL/min. To establish retention times of analytes and the internal standard derivative, 20 µL of each individual isocyanate derivative was injected.

A 20 µL aliquot of sample extract was injected, and the presence of one or more of the 10 isocyanate derivatives was established from retention times. Any isocyanate identified must be used for the standard addition solution preparation. The signal-to-noise ratio for the internal standard derivative must exceed 3:1 to indicate that the derivatization and analysis was successful.

The reagent blank sample (20 µL) and the internal standard check sample (20 µL) were injected. If peaks coelute with those of the isocyanate derivatives, the area of the derivatives should be adjusted accordingly in the final calculation.

Standard addition.—If the presence of isocyanate was indicated, quantitation was carried out by standard addition. A 0.01 g portion of any isocyanate standard(s) identified by screening was weighed to an accuracy of 0.1 mg into a 100 mL volumetric flask. The flask was rapidly made up to the mark with DCM and shaken thoroughly. Ultrasonication may be used to aid dissolution. Into each of seven 1000 µL volumetric flasks, 0, 5, 10, 50, 100, 250, and 500 µL of individual standard addition stock solutions of the isocyanates identified were accurately dispensed. The flask was made up to the mark with DCM and mixed thoroughly.

One-gram portions of a representative sample of the test material or article were weighed to an accuracy of 5 mg and placed into each of seven 20 mL vials after being cut into 0.25 cm^2 pieces, where possible. To each vial, 15 mL DCM, 200 µL internal standard solution, 1000 µL derivatizing reagent, and 1000 µL of each diluted standard addition solution were added. Vials were capped and treated as for test samples leading to the sample in an LC vial. LC was used to analyze 20 µL injections of each extract, to identify the isocyanate derivatives and internal standard derivative peaks on the basis of their retention times, and to measure the respective peak areas.

Data analysis.—Each sample must be determined at least in duplicate. The test sample solution and the samples fortified with isocyanates were used to construct a calibration graph. The graph was a plot of the isocyanate derivative and 1-naphthyl isocyanate derivative peak-area ratio obtained from the standard addition solutions versus the isocyanate concentration added to the test material (mg/kg). Outliers were identified. The isocyanate concentration of the test sample solution was read from the calibration graph by back-extrapolation to the x-axis, where the magnitude of the intercept was equal to the isocyanate concentration. Alternatively, the isocyanate concentration of the test sample solution could be determined mathematically by least-squares regression.

If the internal standard check sample shows an interference in the internal standard region of the chromatogram that exceeds 10% of the area of the internal standard in the calibration samples, and if the analysis of replicate control samples reveals that this interference varies by more than ± 20% in absolute size, then quantitation by external calibration must be used.

If the reagent blank sample shows a peak eluting at the same retention time as the isocyanate derivative, the peak must be quantitated by standard addition omitting the test sample and subtracted from the test sample value that was determined by standard addition.

The concentrations of individual isocyanates should be converted to NCO equivalents by multiplication with the appropriate factor indicated in Table 1. Add NCO values for each individual isocyanate to give total NCO content. This procedure directly yields the isocyanate concentration in the test sample (mg NCO/kg polymer).

Method validation.—A standard mixture (1 µg/mL) of PI; 2,4-TDI; 2,6-TDI; 2,4′-MDI; 4,4′-MDI; HDI; IPDI; 1,5-NI; DIMER; and CHI was prepared in DCM. Exactly 1 g test material was weighed to an accuracy of 5 mg. Ten 1 g portions were placed into separate 20 mL vials, and pieces were cut into 0.25 cm^2 sections where appropriate. Five vials were spiked with 100 µL test mixture, and the remaining 5 vials were spiked with 2.5 mL test mixture. Isocyanates were analyzed as previously described and percentage recoveries were calculated.

Results and Discussion

Method Development

The 7 isocyanates used during method development were chosen to represent the range of isocyanates on the positive list.

Effect of column choice and mobile phase composition.—Figure 1 (I–VI) shows the elution characteristics of the 7 isocyanate derivatives obtained by using commercial LC packings. All the phases examined resulted in the clean separation of the reagent amine and the isocyanate derivatives. Resolution varied greatly depending on the stationary phase. The most variable isocyanate derivative was that of HDI. Using Spherisorb S5ODS1 and S5ODS2 columns the HDI derivative eluted last, whereas elutions using Zorbax ZODS and Nucleosil 120 5C18 columns showed the HDI derivative to elute third and forth, respectively. Elutions using Partisil ODSIII or Zorbax ZODS showed the 2,6-TDI derivative to elute before the 2,4-TDI derivative, whereas elutions using the remaining 4 columns showed the 2,4-TDI derivative to elute before the 2,6-TDI derivative. Using a Nucleosil 120 5C18 column the HDI deriva-

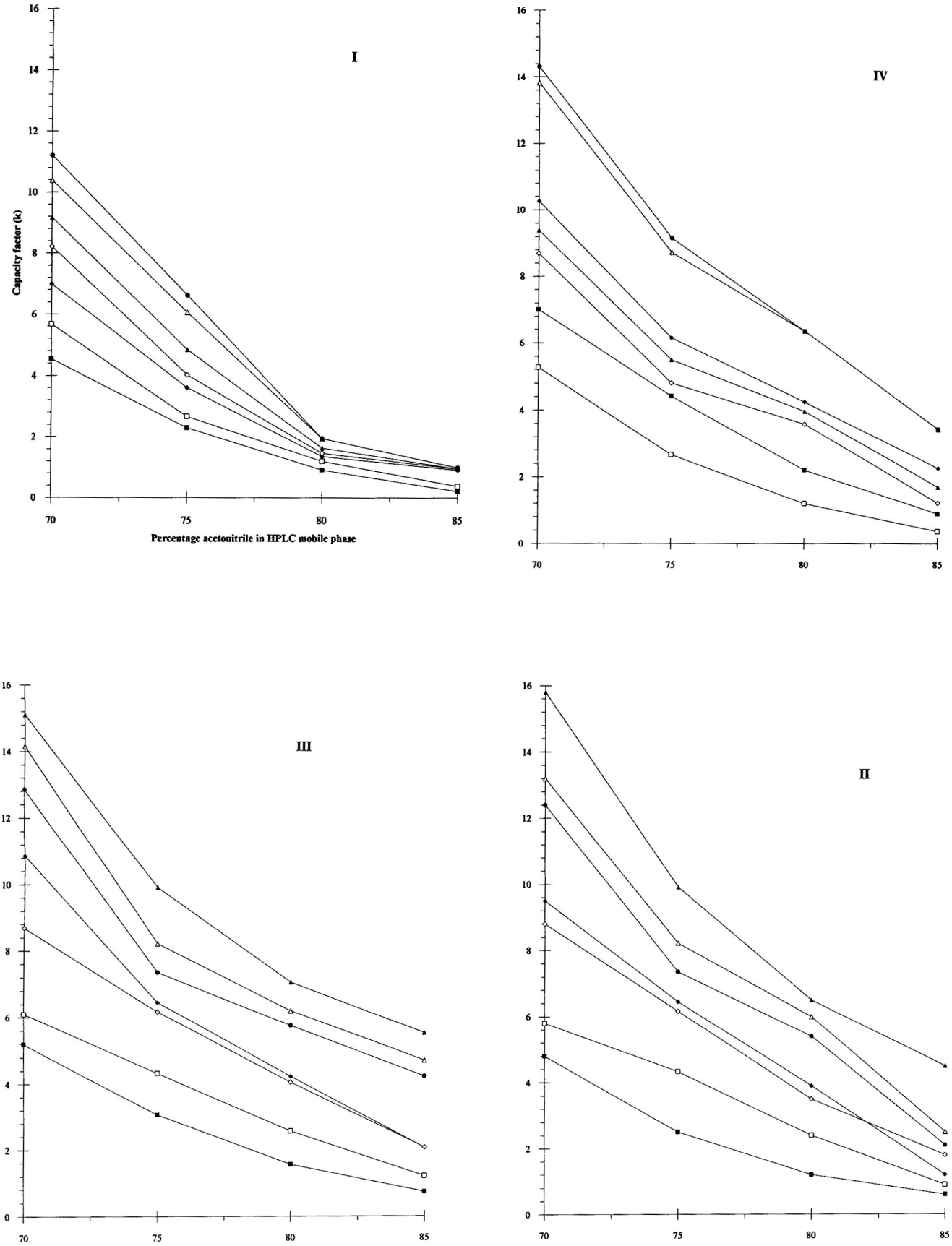


Figure 1. Effect of mobile phase composition on the elution of isocyanate derivatives on various LC stationary phases: I, Spherisorb C8; II, Spherisorb S5ODS1; III, Spherisorb S5ODS2; IV, Nucleosil 120 5C18; V, Partisil ODS3; and VI, Zorbax ZODS.

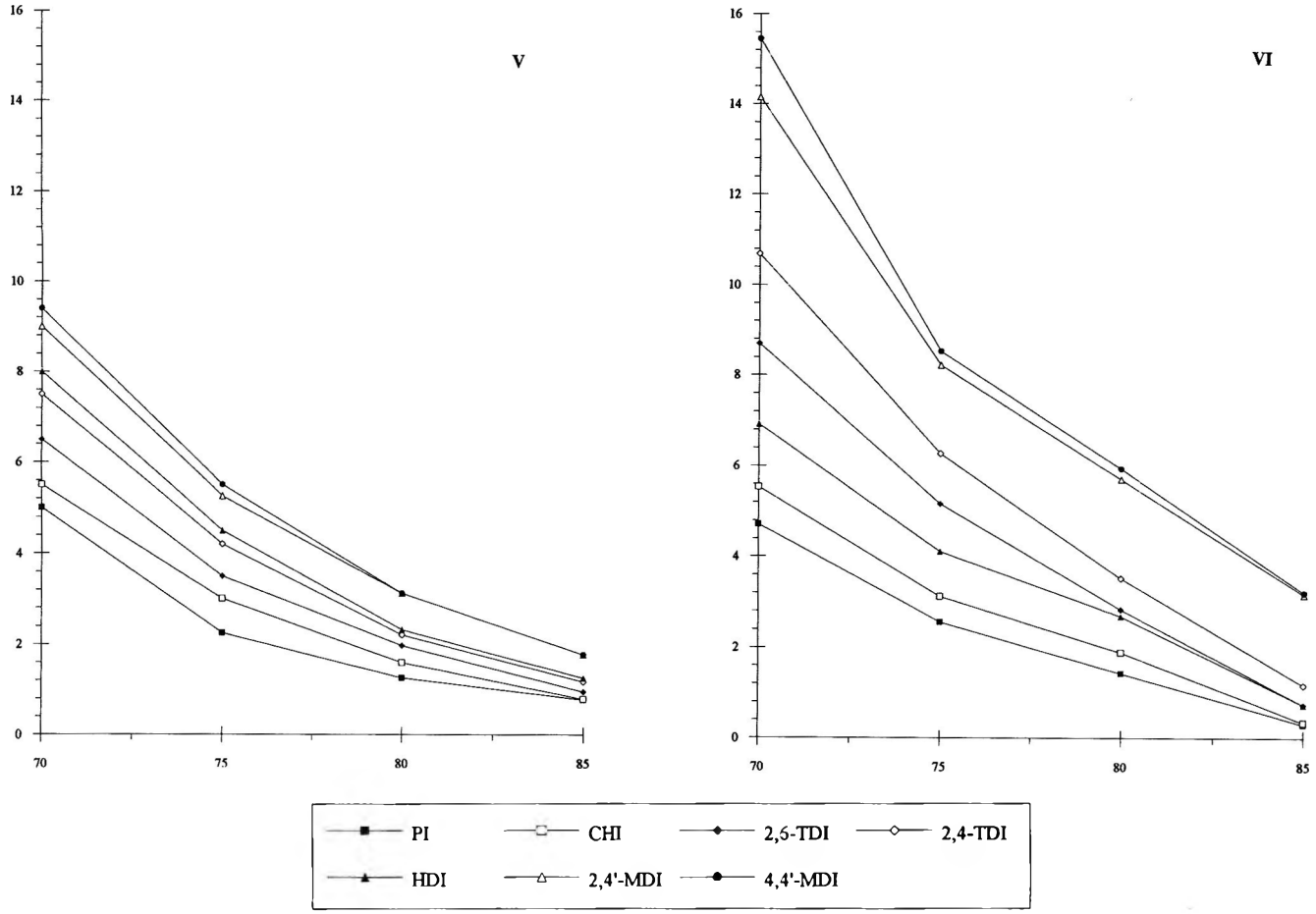


Figure 1. ***(Continued)***

Table 2. Method validation recovery

Sample	PI	CHI	1,5-NI	2,6-TDI	2,4-TDI	2,4′-MDI	4,4′-MDI	IPDI	DIMER	HDI
					NCO19, 2.5 mg/kg spike					
Mean, %	89	92	93	87	89	87	91	90	87	88
STD, %	2.3	3.1	2.8	2.9	3.1	1.9	3.4	3.8	2.8	2.1
RSD, %	2.6	3.4	3.0	3.3	3.5	2.2	3.7	4.2	3.2	2.4
					NCO19, 0.1 mg/kg spike					
Mean, %	91	93	91	90	87	86	87	88	88	87
STD, %	2.8	2.9	2.4	3.5	4.0	2.4	2.8	3.1	2.8	2.7
RSD, %	3.1	3.1	2.6	3.9	4.6	2.8	3.2	3.5	3.2	3.1
					NCO20, 2.5 mg/kg spike					
Mean, %	92	84	86	87	92	91	94	86	88	87
STD, %	2.8	3.4	2.9	3.1	3.5	4.1	2.5	3.6	3.1	2.9
RSD, %	3.0	4.0	3.4	3.6	3.8	4.5	2.7	4.2	3.5	3.3
					NCO20, 0.1 mg/kg spike					
Mean, %	92	93	95	83	87	86	84	88	87	89
STD, %	3.5	3.6	2.9	3.8	3.4	3.6	3.1	3.5	3.3	3.5
RSD, %	3.8	3.9	3.1	4.6	3.9	4.2	3.7	4.0	3.8	3.9

Table 3. Polyurethane or laminate samples used for method development and validation[a]

Sample code	Manufacturer	Isocyanate found	Isocyanate, mg/kg	NCO, mg/kg
NCO11	C	4,4′-MDI	0.68	0.23
NCO12	C	4,4′-MDI	0.58	0.19
NCO13	C	4,4′-MDI	1.08	0.36
NCO14	C	4,4′-MDI	0.94	0.32
NCO19	E	4,4′-MDI	0.14	0.05
NCO20[b]	F	IPDI	0.48	0.18

[a] A selection of 20 samples (coded NCO1–NCO20) from 6 manufacturers were analyzed. Fourteen samples contained no detectable isocyanates. Results for the remaining 6 are shown.
[b] NCO20 is not for food use.

tive eluted between the 2 TDI derivatives. It is reasonable to assume that the observed effects reflect differences in the properties of the silica matrix and in the methods of bonding the octadecyl silyl group to the matrix. The type of chlorosilane used, e.g., octadecyltrichlorosilane or an octadecyl silane in which one or 2 of the chlorine atoms have been exchanged for methyl groups, could also influence the properties of the phase, especially the number of remaining silanol groups, which are known to contribute to the chromatographic retention (10).

Standard chromatographic theory stipulates that for optimum resolution, peak symmetry, and analysis time, peak capacity factors (k) should be $1 < k < 10$. Of the stationary phases and mobile phases examined, optimal resolution was obtained with the Spherisorb S5ODS1 column and mobile phase C. Under these conditions, all the derivatives were fully resolved, and capacity factors ranged from 1.8 to 7.6. Full resolution was obtainable with any of the other columns when using mobile phases with lower concentrations of acetonitrile; however, a concurrent increase in capacity factor values and hence lengthy analysis times with isocratic elution resulted.

Effect of derivatization time.—Derivatization time for the 7 isocyanates tested was varied from 10 to 240 min. Derivative peak areas for each isocyanate did not change significantly over the time periods examined. Relative standard deviations (RSDs) over the test periods ranged from 1 to 6%. Higher RSDs were obtained for the PI and CHI derivatives because these derivatives eluted slightly on the tail of the MAMA peak. Because of this tailing, the PI and CHI derivatives were not optimally integrated. Nevertheless, the values were within acceptable analytical limits and indicated good precision. The derivatization is rapid and is complete within 10 min. The isocyanate–MAMA derivatives were stable. Degradation was approximately 1–2% over 3 months when stored in LC mobile phase C, in the absence of light, at 20°C.

Effect of extraction time.—Preliminary analysis of food package NCO19 indicated the presence of 4,4′-MDI residues, so this sample was used in tests to optimize the extraction efficiency. Maximum extraction was obtained after 9–14 h, and between 14–24 h no increase occurred in the amount of 4,4′-MDI extracted. Sample NCO19 was a laminate; hence, the polyurethane–isocyanate, used as an adhesive, was protected by the outer layers of material. An extraction time of 12 h was considered to be sufficient to extract residual isocyanate monomer from laminate materials. For packaging materials in other forms, such as solid polyurethane sheets, we recommend optimization of extraction times as previously mentioned.

Method validation.—Validation data are shown in Table 2. Recoveries from laminate food package NCO19 spiked at 2.5 mg/kg were 87–91%, and RSDs were 2.2–4.4% (n = 3).

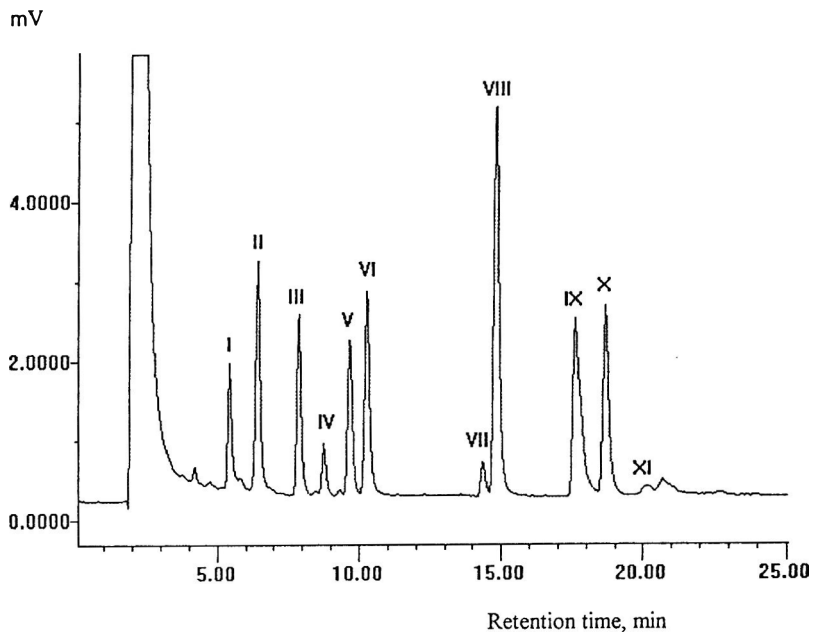


Figure 2. Example liquid trace chromatogram for standard isocyanate derivatives: I, PI; II, CHI; III, 2,4-TDI; IV, 2,6-TDI; V, 1,5-NI; VI, 1-NI (internal standard); VII, 2,4′-MDI; VIII, IPDI; IX, 4,4′-MDI; X, HDI; XI, dimer.

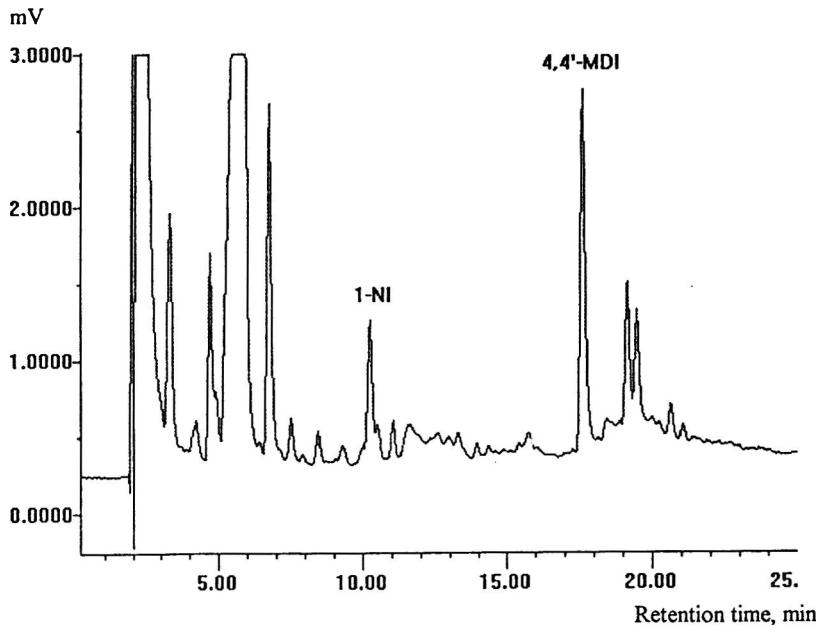


Figure 3. Example of liquid chromatogram for sample NCO13 containing 1.08 mg/kg 4,4′-MDI.

Recoveries from the same sample spiked at 0.1 mg/kg were 86–93%, and RSDs were 2.6–4.6%.

Recoveries from polyurethane sample NCO20 spiked at 2.5 mg/kg were 84–94%, and RSDs were 2.7–4.5%. Recoveries from the same sample spiked at 0.1 mg/kg were 83–95%, and RSDs were 3.1–4.6%. The validation results clearly show that the method will quantitatively determine residual isocyanates effectively extracted from polyurethanes and laminates. The low RSDs indicate good method precision. The mean limit of detection for each of the 10 isocyanate derivatives was 0.03 mg/kg.

Analysis of Commercial Food Packages

The method was applied to 19 commercial food packages (Table 3). The packages were plastic laminates that typically contain a polyurethane adhesive. Five samples contained 4,4′-MDI ranging from 0.05 to 0.36 mg/kg NCO. This range is well below the legislative limit of 1 mg/kg NCO. We also demonstrated that the method could detect isocyanates not on the approved positive list. For this purpose a sample of polyurethane sheet of 2 mm thickness was obtained. This sheeting was not food-contact grade plastic, and IPDI was found at 0.18 mg/kg NCO. IPDI is a common ingredient of polyurethanes outside food contact applications.

Figures 2 and 3 show specimen chromatograms of an isocyanate standard mixture and the extract obtained from food package NCO13, respectively. These results indicate that the method can be applied successfully to commercial food packaging materials without interference.

Conclusions

A method was developed for the analysis of residual isocyanates in food contact materials. Validation demonstrated good method precision. Recoveries from spiked samples ranged from 84 to 95% for the isocyanates analyzed. The isocyanate derivatives formed during analysis exhibited minimal decomposition over a 3-month period. This finding may be useful for enforcement procedures, enabling extract reanalysis by a secondary laboratory or confirmation by reanalysis using different chromatographic conditions. Method development showed that LC column choice is critical if isocyanate derivatives are to be fully resolved from each other in a reasonable time. Spherisorb S5ODS1 columns gave optimum resolution. Derivatization times are not critical. However, we recommend that extraction times of at least 12 h should be used to be certain that all available isocyanate is extracted from the test plastic. Analysis of a small number of polyurethane or laminate materials showed that the method could be applied readily to commercial materials. Residues of 4,4′-MDI were detected in 5 samples and ranged from 0.05 to 0.36 mg/kg NCO.

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FOOD COMPOSITION AND ADDITIVES

Liquid Chromatographic Determination of Vitamin K_1 in Infant Formulas and Milk

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Vitamin K_1 in infant formulas and milk products is determined by reversed-phase liquid chromatography (LC) with UV detection. The sample is hydrolyzed enzymatically, and the vitamin is extracted with hexane. Fractionation by normal-phase semi-preparative LC is followed by analytical LC, with quantitation by the internal standard technique. Recovery of the analyte was 97.4 ± 2.8%. Linearity was established between 0.05 and 4.0 μg/mL. The limit of quantitation is 0.5 μg/100 g for milk powder, which allows the method to quantitate endogenous levels of vitamin K_1.

Vitamin K_1 (phylloquinone) is a cofactor in posttranslational modification of calcium-binding proteins involved in antihemorrhagic activity and, more speculatively, in calcium homeostasis (1, 2). Supplementation of infant formulas with vitamin K_1 is intended to protect newborns against hemorrhagic disease. Fortification levels remain controversial, because they significantly exceed typical concentrations of the vitamin in human breast milk.

The physicochemical properties and low levels of vitamin K_1 in biological fluids, combined with the high concentration of lipids in milk, make analysis of this micronutrient in milk challenging. The occurrence of the *cis* isomer in infant formula milks may also be a complication. Earlier data for foods were based on time-consuming biological assays, as well as thin layer and gas–liquid chromatography. These methods have been reviewed comprehensively (3–5) and generally assessed as nonspecific and imprecise.

Liquid chromatography (LC) has superseded other techniques. Several procedures of varying complexity are available for estimation of phylloquinone in milk and supplemented infant formulas (6–15). Initial extraction of vitamin K_1 has been achieved either by exhaustive total lipid partition (7, 10, 11, 13, 14) or after enzymatic hydrolysis of milk triglycerides (6, 8, 9, 12, 15). Further chromatographic purification, including open-column LC or solid-phase extraction (SPE), were required irrespective of the final analytical LC detection technique. LC analysis of infant formulas after enzymatic removal of bulk lipids was successful with UV (8) or fluorescence (9) detection. Further fractionation steps are, however, recommended for analysis of the vitamin in human milk at endogenous levels, prior to analytical LC with more sensitive electrochemical (12) or fluorescence (15) detection.

Highly manipulative extractions and sophisticated detection strategies are successful in small-scale clinical studies, but they are impractical for quality control (QC) of infant formulas. The Association of Official Analytical Chemists has adopted as first action the method of Hwang (10), despite difficulties associated with routine, open-column LC cleanup, absence of internal standard, and formulas containing corn oil (16). For these reasons, variations of the enzymatic procedure have been established (6, 8, 9). Enzymatic hydrolysis may be the easiest procedure, combining minimal sample preparation, instrumental simplicity, and robust UV detection. However, our experience

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is that it fails for complex formulations. The method presented incorporates an improvement: semipreparative LC fractionation of enzymatic hydrolysate before LC analysis with UV detection. The technique was successful for all infant formulas we encountered, and it allowed analysis of the vitamin at endogenous levels in milk.

METHOD

Apparatus

(a) *Semipreparative LC system.*—A 510 pump, U6K injector, and 745 integrator (Waters, Milford, MA) was coupled with a PU 4025 variable-wavelength detector (Philips, Cambridge, UK); 5 μm silica Resolve column in an 8 × 10 radial compression module at ambient temperature was preceded by a Guard-Pak silica insert (Waters). The mobile phase was hexane–isopropyl alcohol (99.9 + 0.1).

(b) *Analytical LC system.*—A 6000 pump and 490 multichannel detector (Waters) was coupled with a Rheodyne 7125 injector and a D-2500 integrator (Hitachi, Tokyo, Japan); 5 μm Resolve C_{18} column in an 8 × 10 radial compression module at ambient temperature was preceded by a Guard-Pak C_{18} insert (Waters). An HP1040A diode-array detector (Hewlett-Packard, Palo Alta, CA) was used for validation studies. The mobile phase was methanol–isopropyl alcohol–ethyl acetate–water (450 + 350 + 145 + 135).

(c) *Spectrophotometer.*—Model UV-160 dual-beam spectrophotometer (Shimadzu, Tokyo, Japan) and matched quartz cuvettes.

(d) *Water bath.*—Ultrasonic water bath (Bransonic 52, Branson, Shelton, CT) capable of maintaining 37° ± 1°C.

(e) *Rotary evaporator.*—Buchi RE111 with 50 mL pear-shaped flasks (Flawil, Switzerland).

(f) *System for nitrogen evaporation.*—A 6-stem glass manifold of laboratory design.

(g) *Horizontal shaker.*—Chiltern orbital shaker SS70 (Whangarei, New Zealand).

(h) *Centrifuge.*—8-place Damon IEC centrifuge (Needham, MA).

(i) *Reaction tubes.*—Glass boiling tubes (200 × 24 mm) fitted with ground-glass stoppers.

Reagents

(a) *Solvents.*—*n*-Hexane, isopropyl alcohol, methanol, ethyl acetate; all LC grade (BDH or equivalent). Ethanol, absolute.

(b) *Reagent alcohol.*—Ethanol–methanol (95 + 5).

(c) *Lipase.*—From *Candida cylindracea* (Type VII, 700–1500 units/mg, Sigma, St. Louis, MO).

(d) *Phosphate buffer.*—Potassium phosphate, monobasic, 0.8M adjusted to pH 8.0 with NaOH.

(e) *Potassium carbonate.*—Reagent grade.

(f) *Vitamin K_1 reference material.*—USP grade phylloquinone (Serva, Heidelberg, Germany). Standard solutions are stored under nitrogen at 4°C in the absence of light. (*1*) *Stock standard solution.*—Accurately weigh about 100 mg vitamin K_1 into a volumetric flask (100 mL) and make to volume with isopropyl alcohol. Discard after 3 months. (*2*) *Intermediate standard solution.*—Dilute 5.0 mL stock to 100 mL with isopropyl alcohol. Discard after 1 month. (*3*) *Working standard solution (ca 2.5 μg/mL).*—Dilute 5.0 mL intermediate standard solution to 100 mL with isopropyl alcohol. Prepare fresh daily. Determine concentration of the working standard solution after recording absorbance (248 nm) of the 5.0 mL intermediate standard solution, evaporated under nitrogen and redissolved in 25 mL hexane ($E^{1\%}_{1\ cm}$ = 419) (4).

(g) *Cholesteryl phenylacetate (CPA).*—Sigma. (*1*) *Internal standard solution (ca 1.0 mg/mL).*—Accurately weigh about 50 mg into a volumetric flask (50 mL), dissolve, and make to volume in hexane. For analysis of vitamin K_1 at natural levels, dilute 10-fold in hexane (ca 0.1 mg/mL). Discard after 3 months. (*2*) *Calibration standard solution.*—Pipette 1.0 mL CPA internal standard solution into a vial (1.5–2.0 mL, screw capped), evaporate to dryness under nitrogen, and redissolve in 1.0 mL vitamin K_1 working standard solution.

(h) *Vitamin $K_{1(15)}$ and $K_{1(25)}$.*—Gifts from Hoffman La Roche (Switzerland).

Digestion and Extraction

Procedures are done under subdued light to minimize vitamin degradation.

Accurately weigh ca 3.0 g infant formula powder or milk powder or 15.0 g ready-to-use formula, into a boiling tube. Dissolve powders in 15 mL warm water with thorough mixing. Add 5.0 mL phosphate buffer and 1.0 g lipase. A control is included in each sample set to monitor analytical performance. Stopper the tubes and shake mechanically for 5 min. Transfer loosely stoppered tubes to water bath at 37°C and incubate, with ultrasound, for 120 min. During this period, shake tubes vigorously about every 20 min. Cool to ambient temperature.

Add 10 mL reagent alcohol and 1.0 g potassium carbonate to each tube. Add 1.0 mL CPA internal standard solution and extract with 15.0 mL hexane by mechanical shaking for 7 min and centrifugation for 5 min. Collect most of the upper layer with a Pasteur pipette into a conical flask, reextract aqueous layer with 15.0 mL hexane, and pool the extracts. (Alternatively, a more convenient single extraction with 30.0 mL hexane gives near-quantitative recovery and may be used for routine QC assays.)

Transfer 25.0 mL hexane extract to a 50 mL pear-shaped flask and reduce to near dryness at 40°C on a rotary evaporator. Transfer oily residue with a Pasteur pipette and small amounts of hexane into a low-volume vial (1.5–2.0 mL). Capped vials may be stored for 24 h at 4°C. (If white precipitate separates at this point, transfer only the clear supernatant.)

Semipreparative LC Fractionation

Establish stable LC conditions by prior overnight equilibration with mobile phase at low flow rate. Operate at 2.0 mL/min and 269 nm (0.1–0.2 absorbance units full scale [aufs]). Prepare a mixed standard in hexane by evaporating 100 μL vitamin K_1 working standard under nitrogen and redissolving in 100 μL CPA standard solution. Inject 5 μL; ensure that reten-

tion times are reproducible (ca 3.4 min for CPA and *cis*-vitamin K_1 and ca 4.0 min for *trans*-vitamin K_1). Ensure absence of carryover by injecting 100 µL hexane prior to sample. Evaporate sample extract to dryness under nitrogen and redissolve in 100 µL hexane with agitation on a Vortex mixer.

Manually inject total sample extract (100–150 µL) under the same operating conditions; collect fraction between 2.0 and 4.5 min into a vial. This relatively wide window is needed to avoid analyte losses. After collection, elute remaining components at a flow rate of 8 mL/min until the baseline stabilizes (ca 30 min). Ensure that system pressure remains below 2000 psi (1000 bar). Adjust flow rate to 2.0 mL/min and inject next sample extract.

Evaporate collected fraction to dryness under nitrogen and redissolve in 500 µL isopropyl alcohol (200 µL for samples containing vitamin K_1 at natural levels). Vials may be stored for 24 h at 4°C.

Analytical LC

Establish LC conditions with the quaternary mobile phase until the baseline stabilizes. Normal operating parameters are flow rate, 2.0 mL/min; dual wavelength, 269 and 277 nm; attenuation, 0.005 aufs (0.002 for analysis at natural levels); and injection volume, 20–50 µL. Retention times for vitamin K_1 and CPA are ca 26 and 43 min, respectively.

Inject calibration standard at beginning and end of each run or after 6–10 samples. After elution of internal standard peak in each sample extract, purge column with methanol–ethyl acetate (50 + 50) for 10 min, return to analytical mobile phase until a stable baseline is obtained (ca 5 min), and inject the next sample.

Calculate vitamin K_1 content by using internal standard methodology with peak area (or height) as follows:

$$\text{Vitamin } K_1 \text{ (µg/100 g sample)} = \frac{RF \times A_{Ks} \times C_{CPAs} \times 100}{A_{CPAs} \times W_s}$$

where A_{Ks} and A_{CPAs} are peak areas of vitamin K_1 and internal standard in sample extract, respectively; C_{CPAs} is amount (µg) of internal standard in sample extract; W_s is amount (g) of sample powder; and RF is response factor calculated with the following formula:

$$RF = \frac{A_{CPAcs} \times C_{Kcs}}{A_{Kcs} \times C_{CPAcs}}$$

where all terms are areas or concentrations (µg/mL) of calibration standard.

Results and Discussion

Extraction

Enzymatic hydrolysis and extraction were modifications of an earlier method (8). Ultrasonic treatment was incorporated as a precaution against the potentially inhibiting effect of the lipid membrane on lipase activity. Sonication facilitates quantitative release of phylloquinone from human milk (15). Sample amounts and conditions of hydrolysis were optimized to minimize the mass of residual lipids, thereby allowing dissolution in small volumes of hexane.

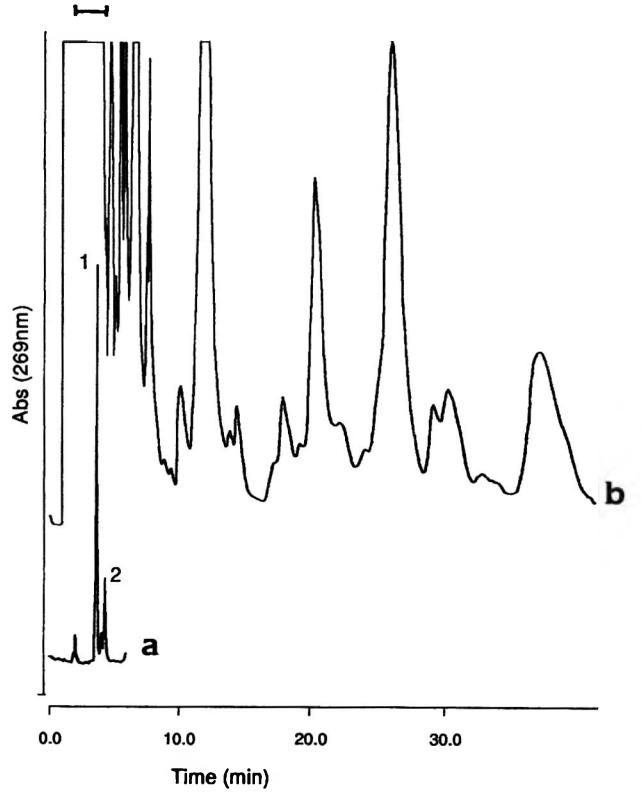

Figure 1. Semipreparative chromatogram of standards (a) and a typical infant formula (b). Conditions: column, 5 µm silica Resolve; mobile phase, hexane–isopropyl alcohol (99.9 + 0.1); flow rate, 2.0 mL/min rising to 8.0 mL/min after fraction collection; detector, 269 nm (0.16 aufs). Peak 1, cholesteryl phenylacetate + *cis*-vitamin K_1; peak 2, *trans*-vitamin K_1; ⟼, collection window.

LC Fractionation

Figure 1 shows a semipreparative chromatogram, under normal-phase conditions, of vitamin K_1 and CPA standards and an extract from a typical infant formula.

Fraction collection is timed to allow for the slightly earlier elution of target analytes in the sample extract compared to standards.

LC Analysis

Figure 2 shows chromatograms after analytical reversed-phase, single-wavelength (269 nm) chromatography of the calibration standard, overlaid with extracts from reagent blank, skim milk, whole milk, and vitamin K-supplemented infant formula after semipreparative LC fractionation. Significant absorption at 248 nm by the mobile phase precludes optimal detection at the most pronounced λ_{max} of phylloquinone.

The identity and purity of phylloquinone and CPA in samples were verified by comparison of retention times and absorbance ratios (269:277 nm) against standards. Extracts from reagent blanks and skim milk did not show interferences; the

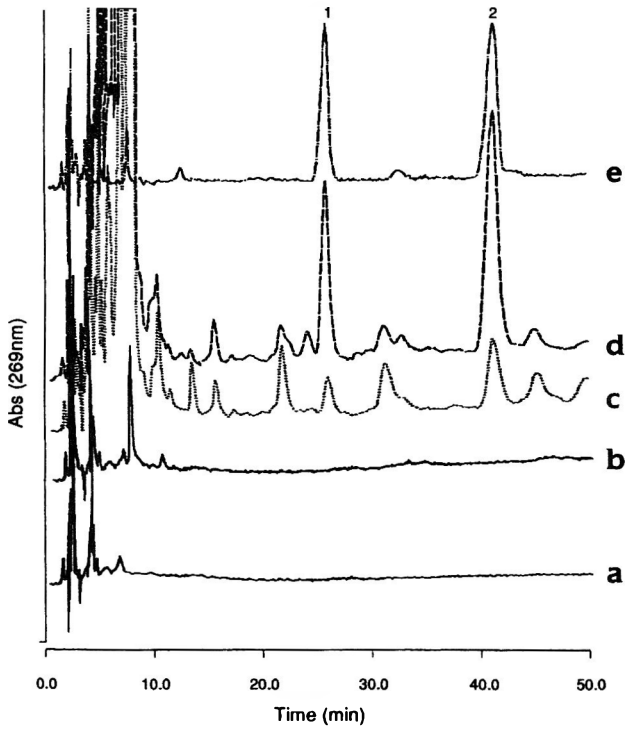

Figure 2. Analytical reversed-phase chromatograms of extracts of reagent blank (a), skim milk (b), whole milk (c), infant formula (d), and standards (e). Conditions: column, 5 μm C_{18} Resolve; mobile phase, methanol–isopropyl alcohol–ethylacetate–water (450 + 350 + 145 + 135); flow rate, 2.0 mL/min; detection, 269 nm (0.005 aufs; 0.002 aufs for whole milk extract). Peak 1, vitamin K_1 (*cis* + *trans*); peak 2, cholesteryl phenylacetate.

latter contains no lipids. The internal standard is uncompromised by underlying artifacts, as demonstrated for samples assayed without CPA. Confirmation was made through diode-array spectral comparison against authentic standards. Spectral similarity factors (defined instrumentally as $1000 \times r^2$, where r^2 is the least-square-fit coefficient of all absorbances at the same wavelength) for vitamin K1 and CPA spectra both measured 994 in a typical infant formula and 993 and 981, respectively, in unsupplemented whole milk.

Method Performance

The detector gave a linear response from 0.05 to 4.0 μg vitamin K_1 per mL ($r = 0.9999$). The regression for calibration standards containing a constant level of CPA (1.0 mg/mL) and variable amounts of vitamin K_1 (0.4–4.0 μg/mL) was linear ($y = 1.69x - 0.09$, $r = 0.9981$). Therefore, a single-point calibration standard was adequate for routine analysis.

The detection limit (signal-to-noise ratio, 3) for vitamin K_1 was estimated to be 1 ng on column (20 μL injection). The limit allows quantitation at levels of 0.5 μg/100 g in milk powder or 0.1 μg/100 g in fluid milk. Sensitivity was therefore sufficient for determining endogenous levels of vitamin K_1 in milk. This detection performance is consistent with previous reports for photometric detectors, which also showed that electrochemical and fluorescence detection are up to one order of magnitude more sensitive (10, 17–19).

Recoveries of vitamin K_1 and CPA were similar (88.9 ± 1.0% and 93.9 ± 0.4%, respectively), as estimated after spiking unsupplemented whole milk powder. The mean overall recovery of vitamin K_1 was 97.4 ± 3.0% ($n = 5$) when calculated against internal standard. When a single hexane extraction was used, such as during routine QC analysis, recovery was 92.5%.

Overall method precision was evaluated by within-day and between-days replicate analyses of control infant formula powder. The relative standard deviation for repeatability (RSD_r) was 1.5% ($\bar{x} = 61.9$ μg/100 g, $n = 5$); for reproducibility (RSD_R) it was 2.8% ($\bar{x} = 63.0$ μg/100 g, $n = 10$). RSD_R at endogenous levels in unsupplemented whole milk powder was 5.9% ($\bar{x} = 4.5$ μg/100 g, $n = 7$).

Method ruggedness was evaluated with the control sample by 2 analysts and LC instrument combinations. Triplicate means of 57.8 (± 0.7) and 59.8 (± 1.5) μg/100 g were obtained, with a difference of 3.5%.

Vitamin K-supplemented infant formula powders were analyzed by the proposed method (Table 1). Also included in Table 1 are published data from a clinical method using reversed-phase LC with dual electrochemical detection (LC–ECD) after silica SPE cleanup (20). An unsupplemented whole milk powder analyzed at endogenous levels by the proposed method gave a value of 2.9 μg/100 g. This result agrees well with the 2.7 μg/100 g found by the LC–ECD method (20) for a closely related sample.

Theoretical and observed values for infant formula milks agree well. Theoretical contents are calculated from the amount of synthetic phylloquinone added during production. The difference between theoretical and measured values is generally explained by endogenous levels of vitamin K_1 in base materials. This difference is small for formulas containing mainly

Table 1. Observed and theoretical vitamin K_1 values for supplemented infant formula powders

Sample[a]	Vitamin K_1, μg/100 g		
	Found	Theoretical	LC–ECD[b]
1	59.8 (1.9)[c]	51	ND[d]
2	71.2 (3.5)	68	ND
3	46.8 (3.3)	40	47.3 (1.8)
4	53.0 (3.9)	38	57.4 (2.3)
5	50.5 (3.5)	30	47.7 (0.5)
6	60.6 (4.0)	69	ND
7	138.4 (8.2)	133	ND
8	51.0 (2.3)	43	ND
9	71.5 (5.6)	50	75.2 (4.2)
10	40.9 (2.5)	30	ND
11	76.2 (2.8)	72	ND

[a] Samples 1–3, whey-based, partially oil-filled; samples 4 and 5, whey-based, fully oil filled; samples 6–9, milk-based, fully oil-filled; samples 10 and 11, goat-milk-based, partially oil-filled.
[b] Method as described by Hart et al. (20).
[c] Values are means (standard deviations) of triplicate analyses.
[d] ND, not determined.

milk fat but significant for formulas based on vegetable oil if soybean oil is the major vegetable oil (7, 9, 21). Vitamin K_1 contents of infant formulas found by the proposed method agree with other reported data (7, 8, 10, 13, 16).

Among samples surveyed in Table 1 are those consistently failing quantitation in absence of the semipreparative step. Reversed-phase LC reveals that phylloquinone is masked by large and ill-defined chromatographic artifacts, which are successfully removed during the described normal-phase fractionation.

The estimates of natural levels in liquid whole milks (0.4–0.9 μg/100 g) are in excellent agreement with previously reported data (0.1–0.9 μg/100 g) (5, 7, 9, 12, 17), further evidence of analytical accuracy. These levels are at least one order of magnitude lower than those in infant formulas, confirming the reliability and versatility of the method.

Some studies have relied on an external standard for quantitation (10, 13, 16), but the potential for losses during sample preparation and cleanup makes use of an internal standard preferable, as emphasized previously by others (2, 6, 7, 9, 15). Three candidate compounds were evaluated as internal standards, including 2 analogues of phylloquinone, $K_{1(15)}$ and $K_{1(25)}$, containing one less and one more isoprene unit, respectively, in the phytyl side chain, and CPA. All 3 satisfied the criteria, eluting with retention times similar to vitamin $K_{1(20)}$ during normal-phase LC and therefore facilitating collection of a relatively narrow fraction. Unlike $K_{1(25)}$ and CPA, $K_{1(15)}$ eluted unfavorably during analytical reversed-phase LC. However, $K_{1(25)}$ was unavailable in adequate amounts or purity, while CPA was commercially available with high purity and was satisfactory, even though relatively high levels were necessary to compensate for its poor spectral absorptivity relative to phylloquinone.

Reversed-phase LC does not discriminate the *cis* and *trans* forms of vitamin K_1. The method therefore estimates total phylloquinone including the nonbioactive *cis* congener present in synthetic preparations (about 10–20%) added to infant formulas. Because production compliance is currently monitored with reference to total vitamin K_1, the procedure is appropriate.

Conclusions

The method incorporates enzymatic digestion of bulk lipid and simple and robust analytical LC with UV detection. Use of an LC cleanup and an internal standard technique provides higher analytical confidence during QC compliance monitoring of infant formulas and allows analysis of endogenous levels in milk and dairy products.

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FOOD COMPOSITION AND ADDITIVES

Determination of Soluble and Insoluble Dietary Fiber in Psyllium-Containing Cereal Products

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A method for soluble and insoluble dietary fiber determinations was developed for psyllium-containing food products, which are highly viscous in aqueous solutions. The assay is based on a modification of the AOAC soluble and insoluble dietary fiber method (991.43), which was recommended for nutrition labeling in the final U.S. food labeling regulations. We found that method 991.43 and other existing dietary fiber methods could not be applied to psyllium food products, which exhibit high viscosity in aqueous solutions, because highly viscous solutions could not be filtered easily. In this study, we modified AOAC method 991.43 to accommodate the filtration process of viscous sample solutions. Sonication followed by high-speed centrifugation was used before filtration. The principles of the method are similar to those for AOAC method 991.43, including the use of the same 3 enzymes (heat-stable α-amylase, protease, and amyloglucosidase) as well as similar enzyme incubation conditions. The modification using sonication and high-speed centrifugation did not alter the method performance for analytically normal products such as wheat bran, oat bran, and soy fiber. Yet, the modification allowed the separation of soluble dietary fiber fractions from insoluble fractions for psyllium products with satisfactory precision. This method for psyllium dietary fiber determinations may be applied to other food products that exhibit high viscosity in aqueous solutions.

The final food labeling regulations in the United States require mandatory labeling of total dietary fiber (TDF) with optional breakdown into soluble and insoluble dietary fiber (SDF/IDF) fractions (1, 2). To meet the needs for nutrition labeling, we reported methods **985.29** and **991.43** for determination of total, soluble, and insoluble dietary fiber in foods such as grain products, fruits, and vegetables (3, 4).

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Methods **985.29** and **911.43** and other existing dietary fiber methods could not be applied to products that are highly viscous in aqueous solutions, such as psyllium and flaxseed. Highly viscous solutions contribute to filtration problems and resulting assay precision problems (4, 5). Thus, we used sonication techniques to effectively reduce viscosity of psyllium sample solutions without destruction of DF and to accommodate the filtration process. In the procedure described in this paper, SDF residues from psyllium-containing cereals are suspended in buffer; incubated with Termamyl (heat-stable α-amylase). protease, and amyloglucosidase; sonicated; centrifuged; and filtered to obtain a liquid fraction containing SDF. SDF is precipitated by the addition of ethanol. DF residue is filtered and dried as described in **991.43** (6).

We investigated the effect of sonication on DF values by using analytically normal samples such as wheat bran, oat bran, and soy fiber as well as psyllium products. Results of assay validation by using optimized analytical conditions are reported for psyllium and psyllium-containing cereal products.

METHOD

Apparatus

(a) *Centrifuge tube.*—50 mL.

(b) *Filtering crucible.*—With fritted disk, coarse, ASTM 40–60 μm pore size, Pyrex 60 mL (Corning No. 36060 Buchner, or equivalent). Prepare as follows: Ash overnight at 495°C in muffle furnace. Let furnace temperature fall below 130°C before removing crucibles. Vacuum clean crucibles once they cool to room temperature. Rinse crucibles with H_2O (3 times); dry crucibles in oven (130°C). Add ca 1.0 g Celite to dry crucibles, and dry at 130°C to constant weight. Cool crucible ca 1 h in desiccator, and record weight to nearest 0.1 mg of crucible plus Celite.

(c) *Vacuum system.*—Vacuum pump or aspirator with regulating device. Heavy walled filtering flask, 1 L, with side arm. Rubber ring adapters, for use with filtering flasks.

(**d**) *Shaking water baths.*—(*1*) Capable of maintaining 98° ± 2°C and with automatic on-and-off timer. (*2*) Constant temperature, adjustable to 60°C.

(**e**) *Balance.*—Analytical, sensitivity ± 0.1 mg.

(**f**) *Muffle furnace.*—Capable of maintaining 525° ± 5°C.

(**g**) *Oven.*—Capable of maintaining 105°C and 130° ± 3°C.

(**h**) *Desiccator.*—With SiO_2 or equivalent desiccant. Dry desiccant overnight at 130°C twice a week.

(**i**) *pH meter.*—Temperature compensated, standardized with pH 4.0, 7.0, and 10.0.

(**j**) *Pipettors.*—With disposable tips, 50–300 µL and 10 mL capacity.

(**k**) *Dispensers.*—Capable of dispensing 15 ± 0.5 mL for 78% EtOH, 95% EtOH, and acetone; 40 ± 0.5 mL for buffer.

(**l**) *Shaker.*—Horizontal, reciprocating (KKA-HS 501), with separating funnel attachments (KA-HS 70081.01).

(**m**) *Sonicator.*—Sonifer 250 (Branson Ultrasonic Corp., Dansbury, CT), or equivalent.

(**n**) *Centrifuge and rotor.*—Sorvall RC-5B Superspeed Refrigerated Centrifuge (Dupont No. 50253), or equivalent.

(**o**) *Beaker.*—400 mL.

Reagents

Use deionized water throughout.

(**a**) *Ethanol solutions.*—(*1*) *85%.*—Place 895 mL 95% ethanol into a 1 L volumetric flask and dilute to volume with H_2O. (*2*) *78%.*—Place 821 mL 95% ethanol into a 1 L volumetric flask and dilute to volume with H_2O.

(**b**) Heat-stable *α-amylase solution.*—Cat. No. A 3306 (Sigma Chemical Co., St. Louis, MO), Termamyl 300L, Cat. No. 361-6282 (Novo-Nordisk, Bagsvaerd, Denmark), or equivalent. Store at 0°–5°C.

(**c**) *Protease.*—No. P3910 (Sigma Chemical Co.), or equivalent. Prepare 50 mg/mL enzyme solution in 2-(*N*-morpholino)ethanesulfonic acid (MES)–tris(hydroxymethyl)aminomethane (TRIS) buffer fresh daily. Store at 0°–5°C.

(**d**) *Amyloglucosidase (AMG) solution.*—Cat. No. AMG 200 L (Novo-Nordisk, Bagsvaerd, Denmark). Store at 5°C.

(**e**) *Diatomaceous earth.*—Acid-washed Celite 545 AW, No. C8656 (Sigma Chemical Co.), or equivalent.

(**f**) *Cleaning solution.*—Liquid surfactant-type laboratory cleaner, designed for critical cleaning (Micro, International Products Corp., Trenton, NJ), or equivalent. Prepare 2% solution in H_2O.

(**g**) *MES.*—No. M-8250 (Sigma Chemical Co.), or equivalent.

(**h**) *TRIS.*—No. T-1503 (Sigma Chemical Co.), or equivalent.

(**i**) *MES–TRIS buffer solution.*—0.05M MES–0.05M TRIS, pH 8.2 at 24°C. Dissolve 19.52 g MES and 12.2 g TRIS in 1.7 L H_2O. Adjust pH to 8.2 with 6N NaOH, and dilute to 2 L with H_2O. (*Note*: It is important to adjust pH to 8.2 at 24°C because pKa of organic buffer changes with temperature. However, if buffer temperature is 20°C, adjust pH to 8.3; if temperature is 28°C, adjust pH to 8.1. For deviations between 20° and 28°C, adjust by interpolation.)

(**j**) *Hydrochloric acid solution.*—0.561N. Add 93.5 mL 6N HCl to ca 700 mL H_2O in a 1 L volumetric flask. Dilute to 1 L with H_2O.

Preparation of Analytical Samples

Grind dried food samples in a Wiley mill with a 0.5 mm screen. If the fat content of any food exceeds 10%, defat food with petroleum ether (3 rinses with 25 mL/g food) before milling. (*Note*: High amounts of fat [>10%] in sample may interfere with DF determinations.)

Enzyme Purity

Enzyme purity and activity is critical to this assay. Standards listed in Table **991.3B** (6) should be run each time the enzyme lot changes or at a maximum interval of every 6 months.

Sample Preparation and Digestion

Run 2 blanks per assay with samples to measure any contribution from reagents to residue. Weigh duplicate 0.500 ± 0.005 g samples (M_1 and M_2), accurate to 0.1 mg, into 50 mL centrifuge tubes. For samples containing 20–100% psyllium, use 0.250 ± 0.005 g. Add 25 mL MES–TRIS buffer solution, pH 8.2, to each. Shake on horizontal shaker for 1 min until sample is completely dispersed to prevent lump formation, which would make test material inaccessible to enzymes. Add 50 µL heat-stable α-amylase solution while stirring at low speed. Incubate in 95°–100°C water bath for 15 min with continuous agitation. Start timing when bath temperature reaches 95°C.

Remove all tubes from bath and cool to 60°C. Scrape any ring from inside of tube and disperse gel in bottom of beaker with spatula. Rinse tube walls and spatula with 7.5 mL H_2O.

Add 100 µL protease solution to each tube. Incubate 30 min at 60° ± 1°C with continuous agitation. Start timing when bath temperature reaches 60°C.

Dispense 3.5 mL 0.561N HCl into tube while stirring. Adjust pH to 4.0–4.7 at 60°C by adding 1N NaOH solution or 1N HCl solution. It is important to check and adjust pH while solutions are at 60°C because pH will increase at lower temperatures. Most cereal, grain, and vegetable products do not require pH adjustment. Once verified for each laboratory, pH checking procedure can be omitted. As a precaution, check pH of blank routinely. If pH of blank is outside desirable range, adjust blank and samples as necessary.

Add 300 µL amyloglucosidase solution while stirring. Incubate 30 min at 60 ± 1°C with constant agitation. Start timing when bath reaches 60°C.

Separation of Soluble and Insoluble Dietary Fiber Fractions

Sonicate each sample after 3 enzymatic digestion steps under the following conditions: power output, 43 W; cycle time, 75%; sonication time, 3 min (*see* Figure 1). Centrifuge samples at 20 000 rpm (47 800 × *g*) and 22°C for 30 min. (*Note*: An expression of power output in watts might be more universally adopted.)

Output control "3" and cycle time "75%" correspond to 43 W output by Sonifier 250. Different output control numbers and cycle combinations can also produce 43 W power output. Different sonicators might use different output control numbers and cycle times to reach 43 W power output.

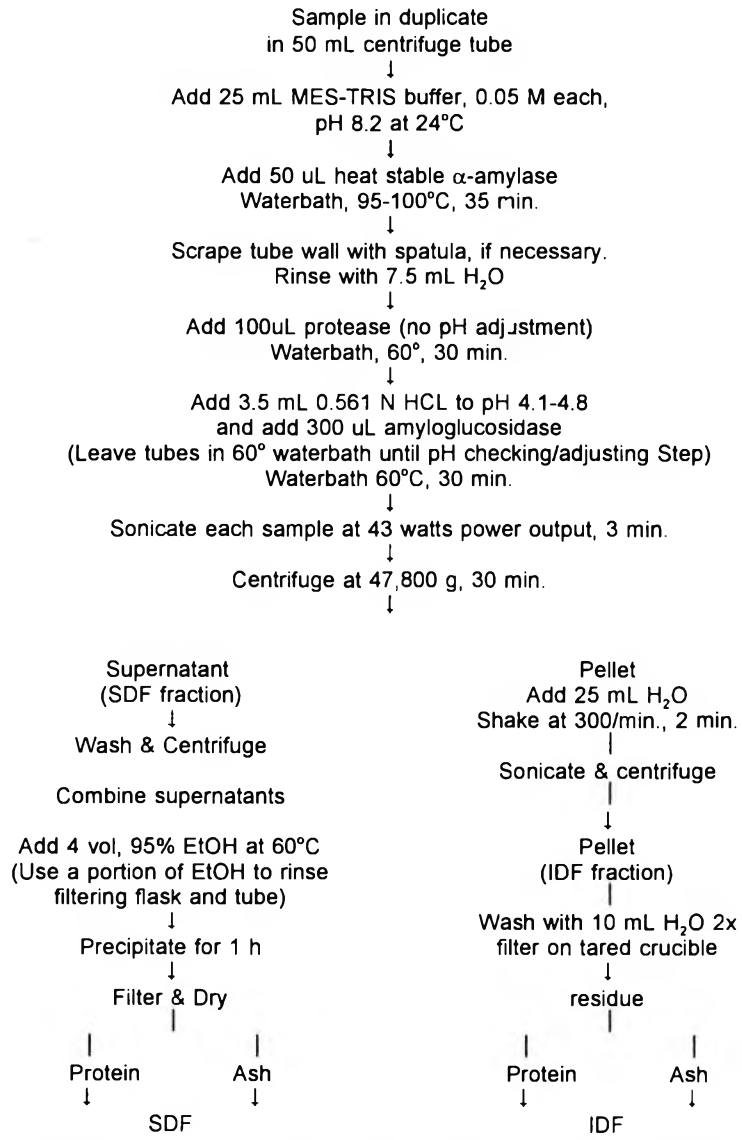

Figure 1. Flow diagram of SDF and IDF determinations for psyllium-containing products.

Carefully pour each supernatant into prelabeled tall-form 400 mL tared beakers. Do not disturb the IDF pellet. Add 25 mL H_2O to each pellet in centrifuge tube. Cap and shake on horizontal shaker for 2 min at 300 shakes/min to break up the IDF pellet. Repeat sonication and centrifugation steps for each sample.

Pool the resulting wash supernatant into the appropriately labeled 400 mL tall-form beakers mentioned previously. Weight beakers with combined solution of pooled supernatants and estimate volumes (ca 70–75 mL).

Determination of Insoluble Dietary Fiber

Wet and redistribute Celite bed in previously tared crucible by using ca 3 mL H_2O. Apply suction to crucible to draw Celite into even mat.

Quantitatively transfer resultant pellet in centrifuge tubes into Celite and sintered glass crucible under vacuum regulated at 5 ± 2 in. Hg. Rinse beaker and then wash residue 2 times with 10 mL 70°C H_2O.

Using vacuum, wash residue 2 times each with 15 mL portions of 78% EtOH, 95% EtOH, and acetone. (*Note*: Delay in washing IDF residues with 78% EtOH, 95% EtOH, and acetone may cause inflated IDF values.)

Dry crucible containing residue overnight in 105°C oven. Cool crucible in desiccator ca 1 h. Weigh crucible containing dietary fiber residue and Celite to nearest 0.1 mg, and calculate residue weight by subtracting weight of dry crucible with Celite.

Use one duplicate from each sample to determine protein by method **960.52** (6), using $N \times 6.25$ as the conversion factor. For ash analysis, incinerate second duplicate for 5 h at 495°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract weight of crucible and Celite to determine ash weight.

Table 1. Effect of sonication on dietary fiber values (Av. ± SD, %) for psyllium products

Sample	Sonication power output[a] 0 W	43 W	47 W
	SDF		
Psyllium	20.8 ± 1.71 (8.2%)	51.0 ± 10.85 (21.0%)	73.3 ± 1.86 (2.5%)
Psyllium cereal	9.3 ± 1.66 (18.4%)	17.0 ± 1.76 (10.4%)	19.1 ± 0.94 (4.9%)
	IDF		
Psyllium	69.4 ± 2.35 (3.4%)	32.9 ± 8.91 (27.1%)	11.5 ± 0.50 (4.3%)
Psyllium cereal	18.2 ± 1.70 (9.3%)	7.0 ± 1.05 (15.0%)	6.3 ± 0.52 (8.2%)
	TDF		
Psyllium	90.2 ± 1.85 (2.0%)	84.0 ± 2.41 (2.9%)	85.5 ± 2.81 (2.4%)
Psyllium cereal	27.2 ± 1.05 (3.8%)	24.0 ± 1.23 (5.1%)	25.4 ± 0.81 (3.2%)

[a] Numbers in parenthesis indicate CV, %; $n = 6$; as-is basis.

Determination of Soluble Dietary Fiber

Add 4 volumes of 95% EtOH, preheated to 60°C, to supernatant from separation of soluble and insoluble dietary fiber fractions.

Determination of Insoluble Dietary Fiber.

Wet and redistribute Celite bed in tared crucible using 15 mL 78% EtOH from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat.

Filter alcohol-treated enzyme digestate through crucible. Using wash bottle with 78% EtOH and rubber spatula, quantitatively transfer all remaining particles to crucible. If some samples form a gum and trap the liquid, break film with spatula.

Using vacuum, wash residue 2 times each with 15 mL portions of 78% EtOH, 95% EtOH, and acetone.

Follow *Determination of Insoluble Dietary Fiber* from "Dry crucible..."

Calculations

Determine blank (B) in milligrams with the following equation:

$$B = \frac{BR_1 + BR_2}{2} - P_B - A_B$$

where BR_1 and BR_2 are residue weights (mg) for duplicate blank determinations, and P_B and A_B are weights (mg) of protein and ash, respectively, determined on first and second blank residues.

Determine DF (g/100 g) with the following equation:

Table 2. Effect of sonication on dietary fiber values (Av. ± SD, %) for various samples

Sample	Sonication power output[a] 0 W	43 W	47 W
	SDF		
Soy fiber	5.5 ± 0.35 (6.4%)	5.4 ± 0.25 (4.7%)	5.4 ± 0.31 (5.7%)
Wheat bran	3.6 ± 0.16 (4.6%)	3.6 ± 0.37 (10.5%)	3.1 ± 0.71 (22.9%)
Oat bran	7.6 ± 0.52 (6.9%)	8.3 ± 0.66 (7.9%)	6.8 ± 0.96 (14.1%)
Oat bran[b]	3.7 ± 1.98 (53.5%)	2.6 ± 1.17 (45.0%)	3.8 ± 0.34 (8.9%)
Prune	18.2 ± 1.48 (8.1%)	NA[c]	19.4 ± 1.31 (7.2%)
Raisin	12.9 ± 2.02 (15.6%)	NA	14.6 ± 1.94 (13.3%)
	IDF		
Soy fiber	71.3 ± 0.62 (0.87%)	72.1 ± 1.60 (2.2%)	72.5 ± 1.43 (2.0%)
Wheat bran	39.5 ± 0.43 (1.1%)	39.6 ± 0.19 (0.5%)	39.8 ± 0.71 (1.8%)
Oat bran	9.7 ± 0.55 (5.8%)	10.1 ± 0.68 (6.7%)	8.4 ± 0.40 (4.8%)
Oat bran[b]	9.0 ± 0.68 (7.4%)	7.9 ± 1.75 (22.1%)	7.7 ± 0.09 (1.2%)
Prune	13.0 ± 0.33 (2.5%)	NA	12.8 ± 1.14 (8.9%)
Raisin	34.7 ± 0.46 (1.3%)	NA	32.9 ± 0.72 (2.2%)

[a] Numbers in parenthesis indicate CV, %; $n = 6$, except prune and raisin, 0% ($n = 3$). All experiments were done with 200L Novo amyloglucosidase unless otherwise specified.
[b] Oat bran with Sigma AMG A 9913.
[c] NA, not analyzed.

$$DF = \frac{\frac{BR_1 + BR_2}{2} - P_B - A_B}{\frac{M_1 + M_2}{2}} \times 100$$

where R_1 and R_2 are residue weights (mg) for duplicate samples; P and A are weights (mg) for protein and ash, respectively, determined on first and second residues; B is blank weight (mg); and M_1 and M_2 are weights (mg) for samples.

Determine total dietary fiber by summing IDF and SDF.

Results and Discussion

The preparation and determination of SDF of a psyllium-containing product by the enzymatic–gravimetric method can lead to several problems. In an earlier collaborative study (4), Fabulous Fiber (Lewis Laboratories International, Ltd, Westport, CT), a mixture of maltodextrin, whey, psyllium hulls, guar

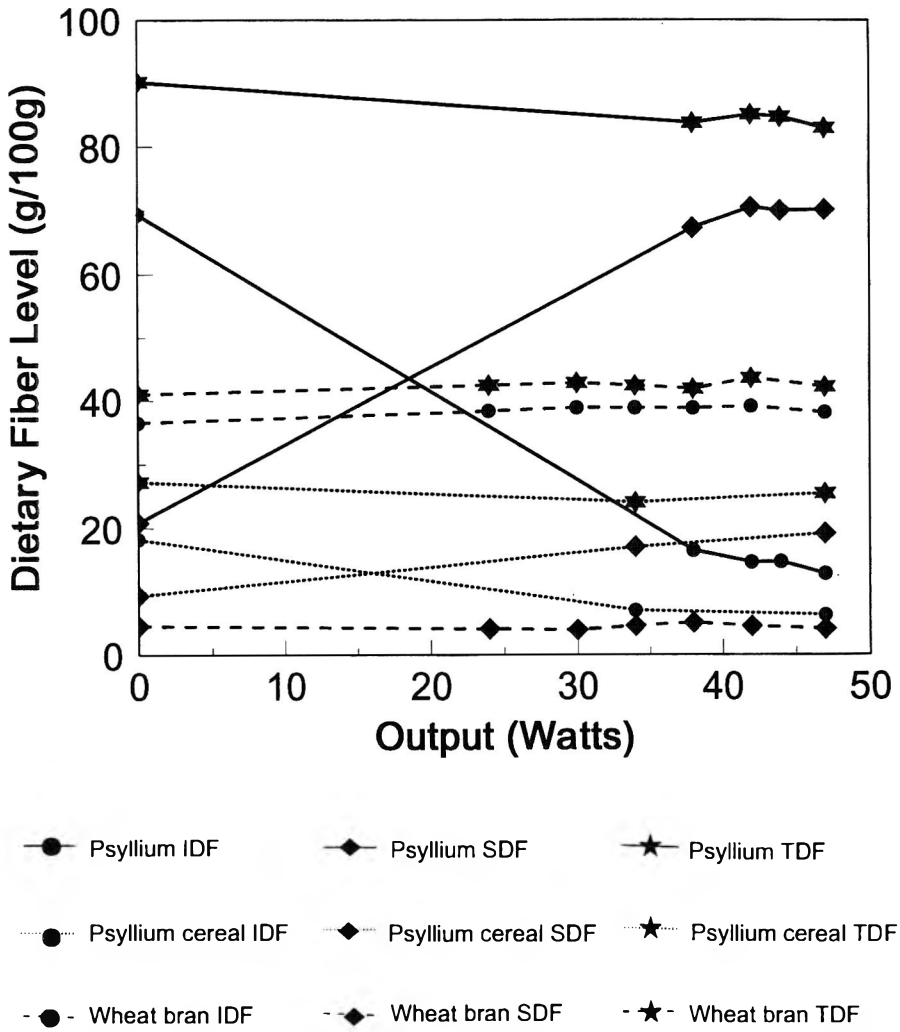

Figure 2. Effect of sonication cycle time on SDF and IDF.

gum, pectin, vitamins, and minerals was analyzed for SDF and IDF. Several collaborators reported that they were unable to filter the soluble, viscous, and thixotropic food samples so they resorted to centrifugation before weighing the residue. This problem was due to the psyllium in the product. We, therefore, sought an alternative way of analyzing for psyllium.

Initially, we used centrifugation techniques to spin down IDF exclusively, so that a clean separation of SDF and IDF could be obtained. However, with high-speed centrifugation only (without sonication), soluble psyllium gels were also spun down and 3 layers were left in a tube: lower IDF layer, middle SDF gels, and upper clear solution. Separation of SDF gels from the bottom IDF layer was technically difficult because both dietary fibers mingled together, especially at the interface. When only the upper solutions were taken and precipitated with 4 volumes of ethanol, 20.8 and 9.3% SDF were reported for psyllium and psyllium-containing cereal, respectively (Table 1). IDF values appeared to be higher than SDF values for these samples when all the precipitates, including middle soluble gel layers, were treated as IDF, although a main component of psyllium is SDF. Reported IDF values for psyllium and psyllium cereal were 69.4 and 18.2%, respectively.

For accurate and clean separation of the solution containing SDF from the IDF pellet, SDF gels must stay dispersed when reducing the viscosity of the solution. Thus, various sonication levels were tested to study the effect of sonication on SDF and IDF recovery. As shown in Figure 2 and Tables 1 and 2, sonication significantly improved the recovery of SDF from psyllium products. At or above 38 W sonication power output, the SDF values started to level off and the corresponding IDF values became constant as well. Visually, there was little SDF gel and IDF mixture layer at or above 38 W sonication power output.

Because the different sonication levels produced different SDF/IDF distribution ratios, the accuracy of SDF/IDF values for psyllium was tested. Analytically normal products such as wheat bran, soy fiber, prunes, raisins, and oat bran were sonicated to evaluate if sonication alters SDF/IDF values of these samples. The sonication levels, which do not alter the ratio of SDF/IDF values for these products, were considered adequate. Sonication did not affect the recovery of both SDF and IDF for analytically normal products such as wheat bran, soy fiber, prunes, and raisins, even when the sonication level was as high as 47 W. In the case of oat bran, some of the DF components

Table 3. Assay validation for psyllium products[a,b]

Sample	Soluble DF, Av. ± SD, %	Insoluble DF, Av. ± SD, %	Total DF, Av. ± SD, %
Cereal A containing psyllium	22.5 ± 0.24 (1.1%)	7.3 ± 0.46 (6.2%)	29.8 ± 0.47 (1.6%)
Cereal B containing psyllium	23.4 ± 0.68 (2.9%)	7.8 ± 0.57 (7.4%)	31.2 ± 0.68 (2.2%)
Cereal C containing psyllium	10.7 ± 0.31 (2.9%)	6.6 ± 0.20 (3.1%)	17.3 ± 0.48 (2.8%)
Psyllium	71.1 ± 2.13 (3.0%)	14.6 ± 0.82 (5.6%)	86.2 ± 1.836 (2.1%)

[a] Enzyme digestates were sonicated at 43 W power output before centrifugation and filtration.
[b] Numbers in parenthesis indicate CV, %; $n = 3$ for psyllium cereals A and B; otherwise, $n = 6$.

were destroyed with harsh sonication at 47 W, although 43 W sonication did not show the evidence of degradation of DF to alcohol-soluble fragments. Thus, sonication level was optimized to 43 W for more thorough assay validation for psyllium products containing other grains (Table 3). Coefficients of variation of the assay ranged from 1.1 to 3.0% for SDF, 3.1 to 14.6% for IDF, and 1.6 to 2.8% for TDF with the psyllium products.

High-energy sonication at ultra high frequencies reduces the molecular size of polysaccharides by a random scission process (7). Reduction in chain length significantly reduces the viscosity of the gel solution (8). This reduction may accommodate easier filtration of the solution containing SDF. Optimization of the degree of sonication is important so that dietary fiber components are not destroyed during the sonication process. If polysaccharides are reduced to chain length of below 10, they are not likely to be recovered as DF in 78% ethanol precipitation (9, 10). The decrease in DF content with sonication could be evidence that the DF polysaccharide chains are chopped down to a degree that a complete recovery of DF is not achieved. In all the samples except oat bran, sonication did not affect the recovery of DF. For samples containing oat bran, care should be taken not to destroy DF polysaccharides during the sonication process.

Use of Sigma amyloglucosidase A 9913 significantly reduced the recovery of oat bran DF at all sonication levels (Table 2). Use of Novo amyloglucosidase 200L overcame the problem and allowed a full recovery of DF at 43 W sonication level. This difference probably occurred because this procedure allows long incubation time of sample with amyloglucosidase during the repeated centrifugation process. Some lots of Sigma AMG A9913 preparation were reported to have a contaminated β-glucanase activity (Zygmunt and McCleary, personal communication, 1994), which continued to act on β-glucan during the centrifugation and filtration steps. Novo amyloglucosidase 200L has a negligible β-glucanase activity (Zygmunt, Quaker Oats, personal communication, 1994). Amyloglucosidase from Megazyme, Australia, also contains less than 0.01% β-glucanase activity and almost 100% of the β-glucan is recovered (McCleary, Megazyme, personal communication, 1994). The employment of more purified enzyme did not lower the DF recovery. One way to overcome the problems associated with contaminated activities might be to boil the sample after the enzyme incubation. However, this procedure is not practical, because it will add a step that may contribute to additional sources of errors. In previous collaborative studies (3), the reproducibility for oat bran dietary fiber analysis was poorer than the other samples tested. This poor reproducibility may have occurred because A 9913 preparation was used in those studies, and the total length of AMG incubation and the filtration times may have differed from laboratory to laboratory. The actions of AMG and other contaminant enzymes continued during the filtration process. The performance of DF analytical methods needs to be reevaluated by using highly purified enzymes for samples containing high amounts of β-glucan, such as oats.

Table 3 lists the data for psyllium products that were obtained by using the optimized sonication and centrifuge technique. The method showed good repeatability and had a 1.1–7.4% coefficient of variation range. As a next step, this optimized method for psyllium DF determinations is recommended for a collaborative study to evaluate the method performance among various laboratories.

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FOOD COMPOSITION AND ADDITIVES

Determination of Six Common Phthalate Plasticizers in Grain Neutral Spirits and Vodka

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Two direct sample injection methods using liquid chromatography (LC) and gas chromatography coupled with mass spectrometry (GC/MS) were used to determine phthalate residues in grain neutral spirits and vodka. Six reported phthalates were quantitated at concentrations as low as 20 μg/L (20 ppb) with no sample preparation or sample enrichment.

Phthalates are necessary components of plastics and coating materials used in various food and beverage packaging. Contact with the food or beverage can lead to extraction of phthalates, resulting in phthalate residues in the product. Many methods to detect and quantitate phthalate plasticizers in foods, food packaging materials, and the environment have been reported (1–12). No reported method, however, can directly detect and quantitate phthalate residues without sample preparation. Recent public concern with the levels of some common phthalates, specifically di-*n*-butyl and di-*n*-octyl phthalate, in certain vodkas and grain neutral spirits led to the development of direct sample injection liquid chromatography (LC) and gas chromatography/mass spectrometry (GC/MS) methods. These 2 methods are complementary and can be used to quantitate the 6 phthalates shown in Figure 1 at concentrations as low as 20 μg/L.

This study revealed that concern with the levels of the common phthalates in vodkas and grain neutral spirits has no basis. The detected levels were insignificant compared with the reported limit of 15 mg/L (15 ppm) for long-term exposure to di-*n*-butyl phthalate in vodka (13). The tolerance levels of the other reported phthalates are similar (13–16).

METHOD

Reagents

(a) *Standards and solvents.*—Acetonitrile (ACN), LC grade (Fisher A 998-4, or equivalent); benzyl butyl phthalate (BBP), reagent grade; bis(2-ethylhexyl)phthalate (DEHP), reagent grade; water, LC grade (Fisher W5-4, or equivalent); diethyl phthalate (DEP), reagent grade; dimethyl phthalate (DMP), reagent grade; di-*n*-butyl phthalate (DBP), reagent grade; di-*n*-octyl phthalate (DOP), reagent grade; ethanol–water solution, 40% ethanol by volume (transfer 200 mL dehydrated 200 proof ethyl alcohol, USP, into a 500 mL volumetric flask; dilute to volume with LC water); methanol, GC/MS grade (Burdick & Jackson GC 60500, or equivalent); and phthalates mix (1000 μg/mL) C-146-01, AccuStandard, Inc.

(b) *Phthalate standard(s) preparation.*—(*1*) *Phthalates mix stock solution (10 mg/L).*—Transfer 1 mL phthalates mix C-146-01 standard into a 100 mL volumetric flask. Dilute to volume with methanol. (2) *Phthalates mix working standard.*—To prepare a 1 mg/L phthalates mix working standard, transfer 5 mL of 10 mg/L phthalates mix stock solution into a 50 mL volumetric flask. Dilute to volume with ethanol–water solution. Using this working standard, prepare additional working standard(s), as appropriate. (*3*) *Individual phthalate standards.*—Weigh 10 mg of the desired phthalate into a 10 mL volumetric flask and dilute to volume with ethanol–water solution (40% ethanol by volume). Prepare individual phthalate stock and working standard solution as specified above for the phthalates mix standard.

(c) *LC mobile phase.*—Solvent A. LC grade water; solvent B, ACN–methanol (99 + 1).

Equipment

(a) *LC system.*—Hewlett-Packard HP1090 (or equivalent), with ternary pump, helium sparge, autosampler, diode array detector, computer data station, and gradient capabilities.

(b) *LC column.*—HP Spherisorb ODS-2, 5 μm particle size, 250 mm × 4 mm (or equivalent) with guard column.

(c) *GC/MS system.*—Fisons 8000 series GC with MD 800 MSD (or equivalent).

(d) *GC column.*—DB-5, 15 m, 0.32 mm id, with 0.25 mm film thickness (or equivalent).

(e) *Weighing balance.*—Sartorius A 200 S (or equivalent).

LC Procedure

Transfer 1–2 mL vodka or grain neutral spirits sample and appropriate working standard(s) into autosampler vials. Analyze, using the following parameters: gradient elution, 37.5% solvent A and 62.5% solvent B for 3 min, ramp to 100% B from 3 to 8 min, and hold at 100% B for 8 min; flow rate,

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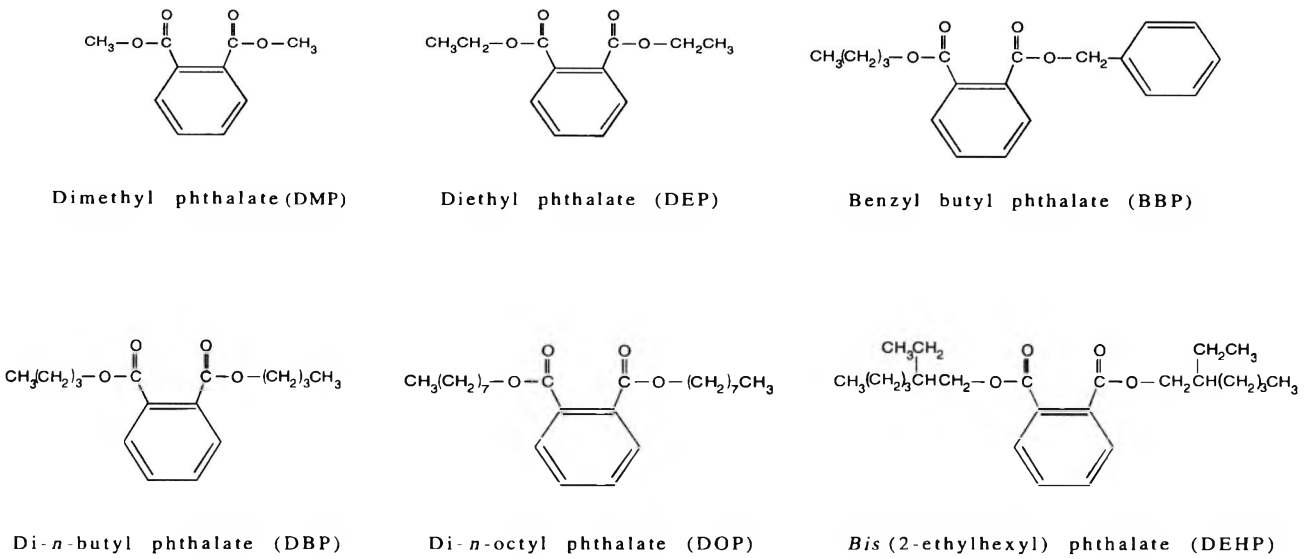

Figure 1. Structures of 6 common phthalates.

1.0 mL/min; injection volume, 100 µL; detection, diode array (2 nm slit) at 225 nm, 4 nm bandwidth. Use the following retention times: DMP, 3.4 min; DEP, 4.8 min; BBP, 8.6 min; DBP, 9.0 min; DEHP, 13.4 min; DOP, 14.1 min.

Visually, all target phthalates are well resolved from each other, with approximate minimum detection and/or quantitation limits of 20 µg/L. Figure 2 shows the liquid chromatogram of a 1.0 mg/L phthalates mix standard, Figure 3, the chromatogram of a 20 µg/L phthalates mix standard, and Figure 4, the chromatogram of a vodka sample containing DBP at 34 µg/L and DEHP at 320 µg/L.

GC/MS Procedure

Transfer 1–2 mL vodka and/or grain neutral spirits sample and appropriate working standard(s) into autosampler vials. Analyze, using the following parameters: carrier, helium at a head pressure of 1 psi; injection, 1 µL, splitless for 1 min; in-

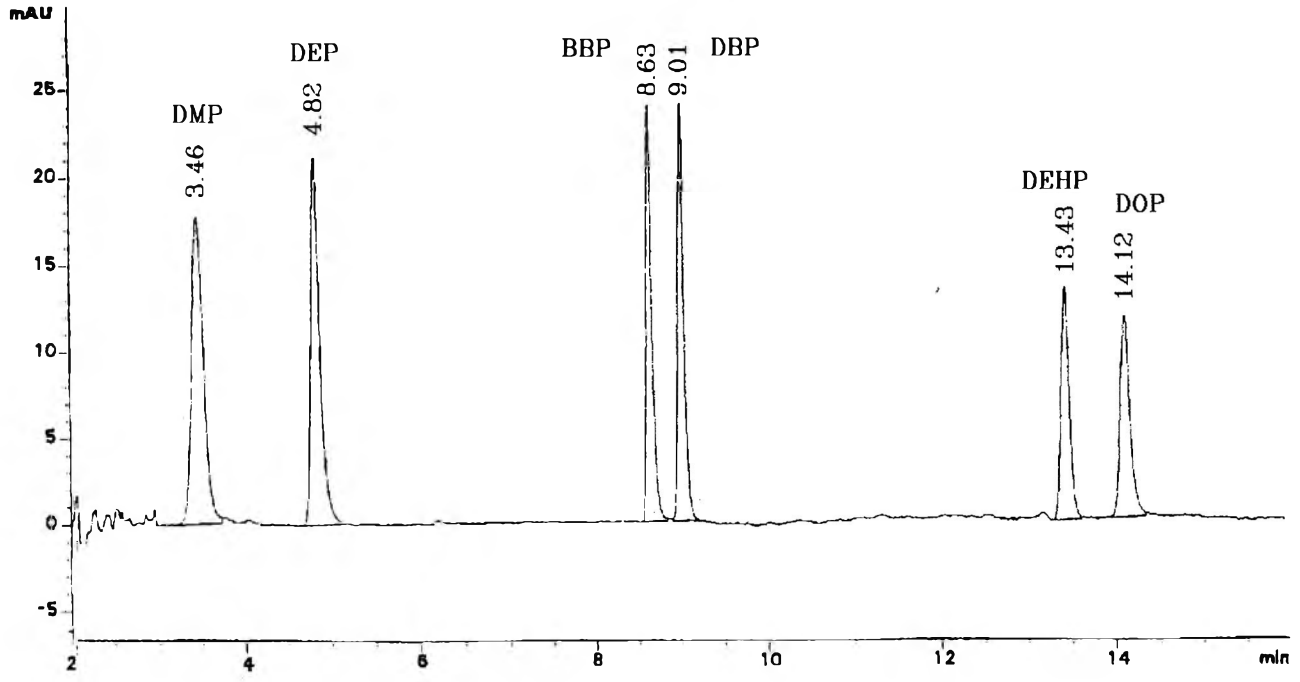

Figure 2. Liquid chromatogram of a 1.0 mg/L phthalates mix standard.

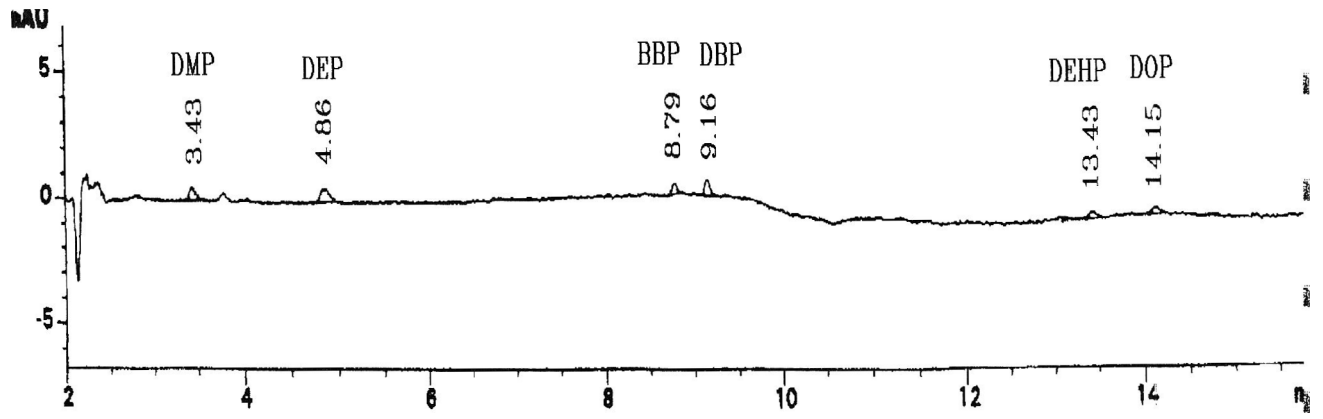

Figure 3. Liquid chromatogram of a 20 μg/L phthalates mix standard.

jector temperature, 225°C; detector, single-ion recording (SIR) at *m/z* 149 and 163; detector temperature: source, 200°C; interface 250°C. Program temperature as follows: 80°C, hold for 2 min, ramp at 20°C/min to 280°C, and hold at 280°C for 3 min. Use the following retention times: DMP, 5.3 min; DEP, 6.2 min; DBP, 8.3 min; BBP, 10.2 min; DEHP, 11.2 min; DOP, 12.1 min.

Visually, all target phthalates are well resolved from each other, with approximate minimum detection and/or quantitation limits of 20 μg/L. Figure 5 shows the gas chromatogram of a 500 μg/L phthalates mix standard, Figure 6, the chromatogram of a 20 μg/L phthalates mix standard, and Figure 7, the chromatogram of a vodka sample containing DBP at 34 μg/L and DEHP at 320 μg/L.

Calculations

The Fisons GC/MS system is equipped with LAB-Base software, configured to supply area over each peak detected.

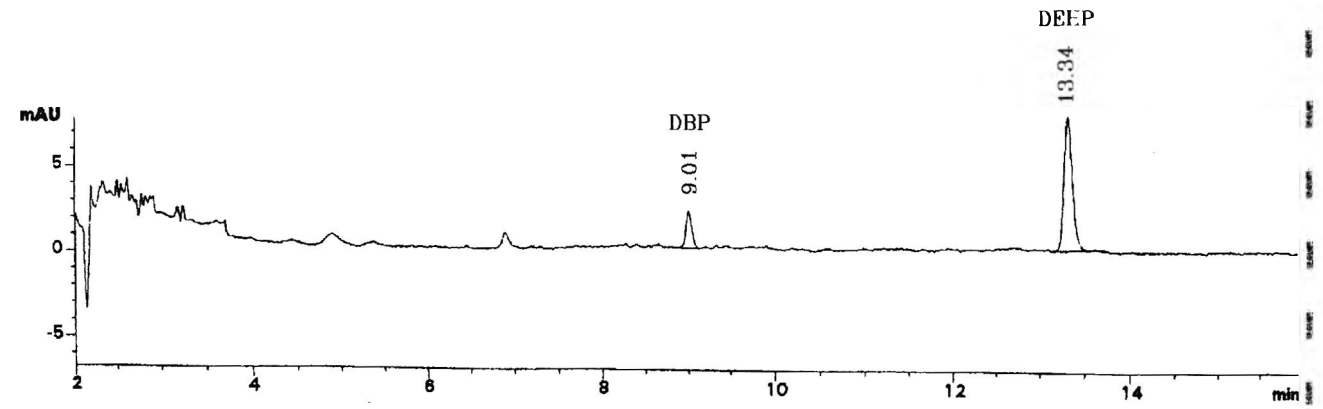

Figure 4. Liquid chromatogram of vodka sample containing DBP at 34 μg/L and DEHP at 320 μg/L.

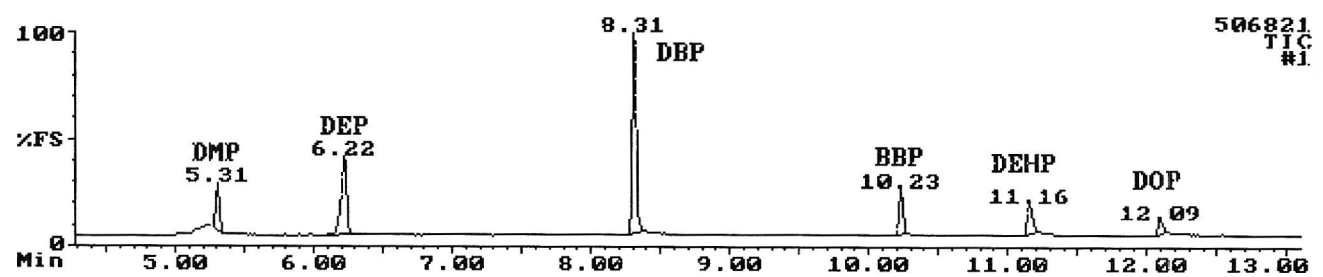

Figure 5. GC/MS chromatogram of a 500 μg/L phthalates mix standard.

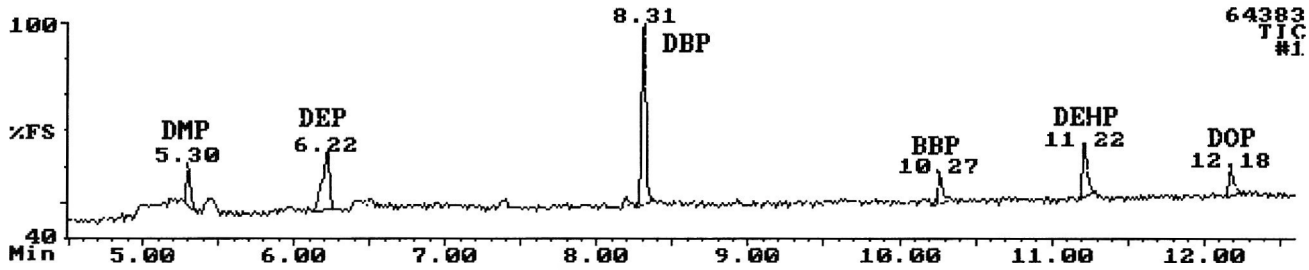

Figure 6. GC/MS chromatogram of a 20 μg/L phthalates mix standard.

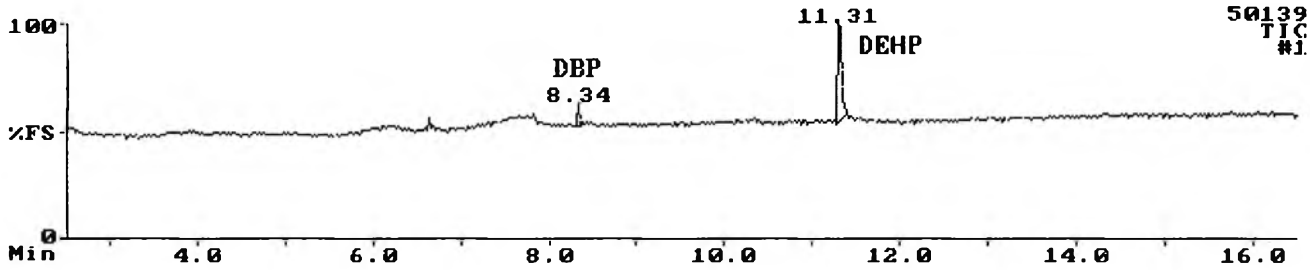

Figure 7. GC/MS chromatogram of vodka sample containing DBP at 34 μg/L and DEHP at 320 μg/L.

Table 1. Recoveries of DBP in spiked vodka

Method	Amount spiked, ppb	Amount found, ppb					Recovery, %
		1	2	3	4	Mean	
GC/MS	25	24.8	24.4	24.5	24.9	24.7	98.8
	250	249.9	249.4	250.2	249.6	249.8	99.9
LC	25	22.7	24	24.4	26.1	24.3	97.2
	250	220.5	265	239.7	233.8	239.8	95.9

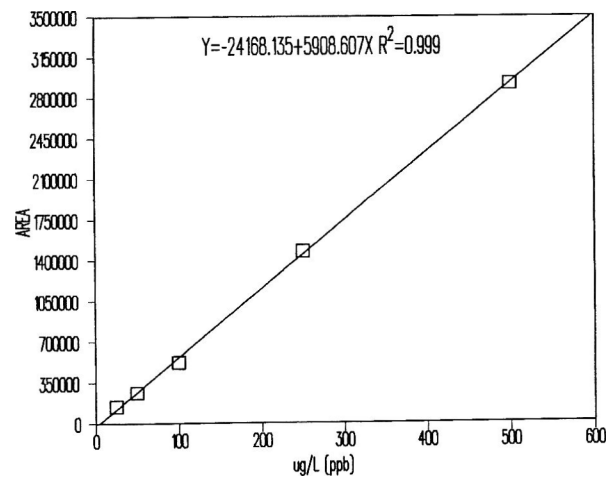

Figure 8. Standard curve of DBP by GC/MS.

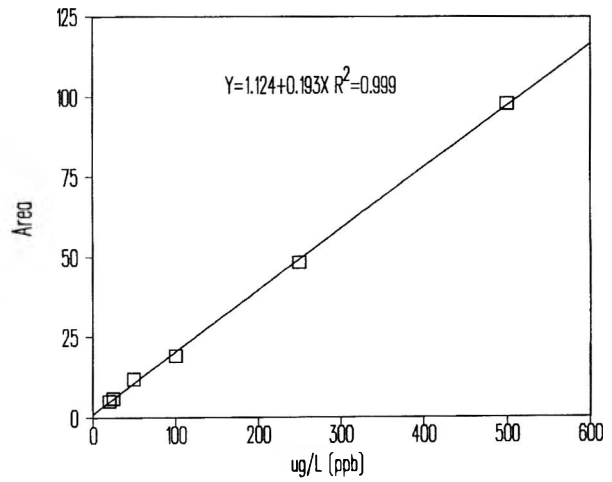

Figure 9. Standard curve of DBP by LC.

Table 2. Phthalates in vodka determined by GC/MS[a]

Label proof	Amount found, ppb		
	DBP	DEHP	DOP
80	83	149	ND
80	105	134	ND
80	ND	182	ND
80	ND	154	ND
80	45	149	ND
80	175	150	ND
80	22	164	ND
80	ND	169	ND
80	ND	212	131
100	34	320	ND
100	ND	157	57
100	ND	326	ND
100	ND	317	ND
100	ND	161	ND
100	20	251	ND
100	50	187	ND
100	ND	144	ND
80	ND	160	ND
80	ND	180	ND
80	ND	125	ND
80	ND	62	ND
80	ND	161	ND
80	28	112	ND
80	ND	139	ND

[a] ND, not detected at or above the detection and/or quantitation limit of 20 µg/L (20 ppb). DMP, DEP, and BBP were not detected in any vodka sample.

Table 3. Phthalates in flavored vodka determined by GC/MS[a]

Label proof	Amount found, ppb		
	DBP	DEHP	DOP
70	ND	196	ND
70	ND	299	ND
70	ND	163	ND
70	204	ND	ND
70	42	190	ND
70	ND	346	ND
70	ND	128	ND
70	ND	193	ND
70	ND	142	ND
70	ND	329	ND
70	178	ND	ND
70	39	192	ND
80	ND	252	75
80	149	ND	ND
80	22	492	ND
80	ND	285	123
80	156	184	ND
80	ND	140	ND
80	ND	159	ND
80	122	186	ND
80	34	185	ND
80	ND	232	ND
90	ND	184	ND
90	ND	334	ND
90	ND	320	ND
90	ND	189	ND

[a] ND, not detected at or above the detection and/or quantitation limit of 20 µg/L (20 ppb). DMP, DEP, and BBP were not detected in any flavored vodka sample.

Quantitation masses of *m/z* 163 for DMP and *m/z* 149 for the other 5 phthalates are recommended. The HP1090 LC Chemstation software also supplies area counts for each peak. For either instrument, integration parameters are optimized by using a 1.0 mg/L working standard, and then the same parameters are used when analyzing samples. After establishing standard linearity, sample concentration can be determined using the QUAN function in LAB-Base, the calibration feature in Chemstation, or the following equation:

$$\frac{CN_{std} \times PKA_{smp}}{PKA_{std}} = CN_{smp}$$

where CN_{std}, concentration of standard; PKA_{smp}, peak area of sample; PKA_{std}, peak area of standard; CN_{smp}, concentration of sample.

Discussion

The LC and GC/MS methods require no sample preparation and allow efficient analysis of 6 phthalates in grain neutral spirits and vodka with an approximate minimum detection limit of 20 µg/L. One method can be used to confirm the identity of phthalates detected by the other, or confirmation can be done by GC/MS with detection of 3 ions from the phthalate of interest. Both methods were thoroughly evaluated for linearity for all 6 phthalates at concentrations ranging from 20 to 1000 µg/L. Recoveries of the 6 phthalates spiked at 25 and 250 µg/L were greater than 95% with both methods. Recovery results for DBP are presented in Table 1. Recoveries of other phthalates were similar. Solvents and control samples were evaluated for phthalate contamination by both methods prior to use. Because phthalates are ubiquitous materials, solvents and controls must be analyzed prior to sample analysis to ensure that there is no carryover or contamination. The phthalate standard prepared in 35–40% alcohol closely resembles the sample matrix and also improves peak shape, particularly for LC analysis. Also, 1% methanol in acetonitrile is suggested as a mobile phase modifier for LC. Without this modifier, DEHP and DOP tend to coelute. To ensure reproducibility, new GC columns must be conditioned by making several injections of the 1.0 mg/L phthalates mix standard prior to sample analysis.

Selected vodkas were analyzed by the 2 methods for DBP and other common phthalates. These methods were also used to determine phthalate levels in grain neutral spirits packaged in plastic bottles. With a linear dynamic range of 20 to

Table 4. DEHP in grain neutral spirits in plastic containers determined by GC/MS[a]

Label proof	DEHP, ppb
190	108
190	ND
192	144
192	ND
80	ND
80	ND
80	ND

[a] ND, not detected at or above the detection and/or quantitation limit of 20 µg/L (20 ppb). DBP, DOP, DMP, DEP, and BBP were not detected in any flavored vodka sample.

1000 µg/L established for all reported phthalates, samples were analyzed by direct injection with an appropriate calibration standard to maintain calibration accuracy. For example, standard curves for DBP by GC/MS and LC in the working range of 20 to 500 µg/L are shown in Figures 8 and 9, respectively. Tables 2 and 3 show phthalate residues detected in 24 vodkas and 26 flavored vodkas, respectively, by GC/MS. LC results were consistent with those obtained by GC/MS. However, some flavored vodkas showed interferences in LC analysis. When interference is noted, GC/MS should be the method of choice.

Of 50 samples analyzed, 18 contained very low levels of DBP and 4 contained very low levels of DOP. However, 47 samples contained various amounts of DEHP. None of the grain neutral spirits contained DBP or DOP. However, low levels of DEHP were detected in 2 of 7 samples of grain neutral spirits (Table 4). The concentrations of phthalates in these samples were considered biologically insignificant (13–16).

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FOOD COMPOSITION AND ADDITIVES

Determination of Amino Acids in Food and Feed by Derivatization with 6-Aminoquinolyl-*N*-Hydroxysuccinimidyl Carbamate and Reversed-Phase Liquid Chromatographic Separation

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A study of a new amino acid analysis method using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as a precolumn derivatization reagent for the analysis of food and feed is described. All amino acids, including methionine sulfone and cysteic acid, were well separated on a liquid chromatographic system using the optimized chromatographic conditions. Salts in food and feed interfered very slightly with the derivatization yields of all amino acids. Several typical agricultural products and animal feeds, including 2 AOAC test samples, were analyzed with the method. The results agreed well with the data generated by using the classical postcolumn method with ion-exchange chromatography. The average relative standard deviations for corn and broiler starter feed were 0.74 and 0.70%, respectively. Good recoveries of all amino acids were demonstrated (average, 101%), even for a sample with a very complex matrix.

Because amino acids are the basic structural units of proteins, which are nutritionally important for human and animal health, qualification and quantitation of amino acids in food and feed are necessary. Although the most reliable determination of amino acids in food and feed can be conducted by using ion-exchange chromatography (IEC) followed by derivatization of amino acids with reagents such as ninhydrin (1, 2), precolumn derivatization has often been used as a faster, more sensitive, and less costly analysis of amino acids. Precolumn methods are based on the derivatization of amino acids with reagents that are strongly absorbent or fluorescent, such as phenylisothiocyanate (3–7), *o*-phthalaldehyde (8–11), 9-fluorenylmethylchloroformate (12, 13), and 4-dimethylaminoazobenzenesulfonylchloride (14, 15). The derivatized amino acids are then separated with a reversed-phase liquid chromatographic (LC) system. Although successful applications were reported for the analysis of amino acids in many kinds of samples, precolumn methods have various shortcomings with each of the previously mentioned reagents. Therefore, postcolumn methods are still widely regarded as less reliable than classical IEC.

Recently, a novel derivatization reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), was synthesized (16) and was used for the analysis of amino acids in proteins, peptides, and other samples when using fluorescence (16, 17) or absorbance (18) detection. AQC can react in seconds with all primary and secondary amino acids without appreciable matrix interference to form single, quantitative, and very stable derivatives. In addition, the excessive reagent does not interfere with amino acid separation, and the derivatization procedure is very simple. All these features make this new method ideal for routine amino acid analyses (16).

The application of this method, like many other precolumn protocols, was focused mainly on the analysis of biological samples. We applied the AQC method to the analysis of amino acids including sulfur-containing amino acids in

Table 1. Gradient table for AQC derivatized amino acid separation[a]

	Common amino acids		Sulfur-containing amino acids	
Time, min	A, %	B, %	A, %	B, %
0	100	0	100	0
17	93	7	92	8
21	90	10	83	17
32	66	34	73	27
34	66	34	50	50
35	0	100	50	50
37	0	100	0	100
38	100	0	100	0
45	100	0	100	0

[a] All gradient segments were linear.

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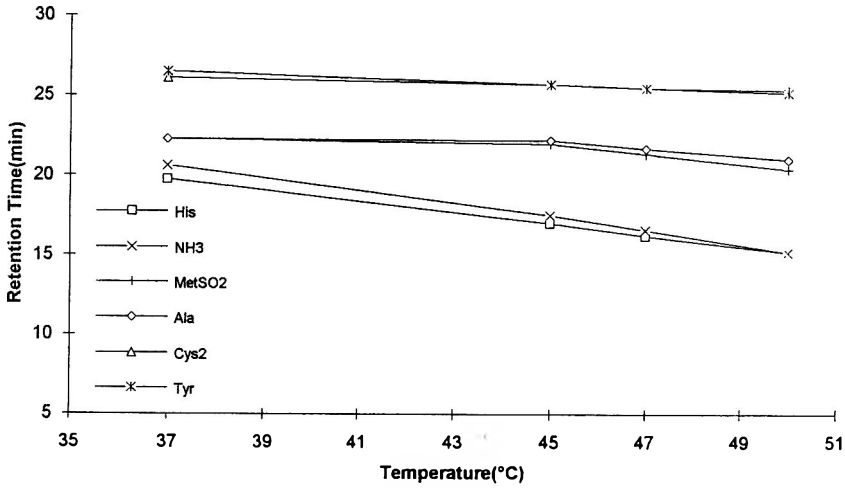

Figure 1. Effect of column temperature on separation of derivatized sulfur-containing amino acids and other amino acids: pH of eluent A, 4.95; gradient conditions, 100% A at initial time, 93% A at 17 min, 80% A at 21 min, and 60% A at 32 min (all curves linear).

other kinds of samples, namely agricultural products and animal feed by modifying the LC conditions reported before (16, 18).

Experimental

Materials and Reagents

(**a**) *Derivatization reagent kit.*—AQC, 0.2M borate buffer, and DNA grade acetonitrile (Waters Corp., Milford, MA).

(**b**) *Phosphoric and hydrochloric acid.*—Guaranteed reagent grades (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

(**c**) *α-Aminobutyric acid (AABA).*—Riedel de Haën (Seelze, Germany).

(**d**) *Acetonitrile.*—LC grade (Chang Hua Fine Chemical Factory, Beijing, China).

(**e**) *Ultrapure water.*—Supplied by a Milli-Q purification system (Millipore Corp., Bedford, MA).

(**f**) *All other reagents.*—Analytical reagent grade (Wako Pure Chemical Industries, Ltd).

(**g**) *Corn powder and broiler starter mixing feed.*—Degussa Corp. (Allendale, NJ). These were the same samples that were used as test samples for the 1992 AOAC worldwide collaborative study on amino acid analysis using the postcolumn IEC method.

(**h**) *Amino acid standards.*—Degussa Corp.

(**i**) *Shrimp feed.*—Chinese Academy of Agricultural Science (Beijing, China).

(**j**) *Vitalyte premixed animal nutrition.*—Approximately 1% amino acids and 40% salts and containing 10 vitamins (Anglian Nutrition Products Company, Ipswich, UK).

Sample Hydrolysis

Sample hydrolysis proceeded as previously described (19). Standard HCl hydrolysis was used for all amino acids except cystine (Cys), methionine (Met) for which performic acid oxidation followed by HCl hydrolysis was used. Test samples were ground and passed through a 60 mesh sieve before hydrolysis or oxidation.

(**a**) *Acid hydrolysis.*—Approximately 100 mg sample was accurately weighed and transferred to a hydrolysis tube, then 10 ml 6N HCl was added to the tube. The tube was then sealed under vacuum after freezing with liquid nitrogen. The sealed sample was placed in an electric oven for 22 h at 110° ± 2°C.

(**b**) *Performic acid oxidation and acid hydrolysis.*—Chilled performic acid (2 mL) was pipetted into a hydrolysis tube containing 50–70 mg sample. The tube was then placed in an ice bath (0°C) for 16 h. A 0.3 mL aliquot of 48% HBr was added to the tube, and the tube was allowed to stand in the ice bath for 15 min. The contents of the tube were dried on a rotary evaporator at ≤60°C. The sample hydrolysis procedure using 6N HCl was then performed.

Sample Preparation After Hydrolysis

The sample was cooled to room temperature before further preparation. Filtered hydrolysate (1–2 mL depending on the protein amount in the sample) was pipetted into an evaporator tube. The sample was dried on a rotary evaporator set at ≤50°C. AABA internal standard solution (2.5 μmol/mL) and Milli-Q water were added to the dried sample to make up a diluted hydrolysate with <13 μmol/mL total amino acids. The concentration of AABA was kept at

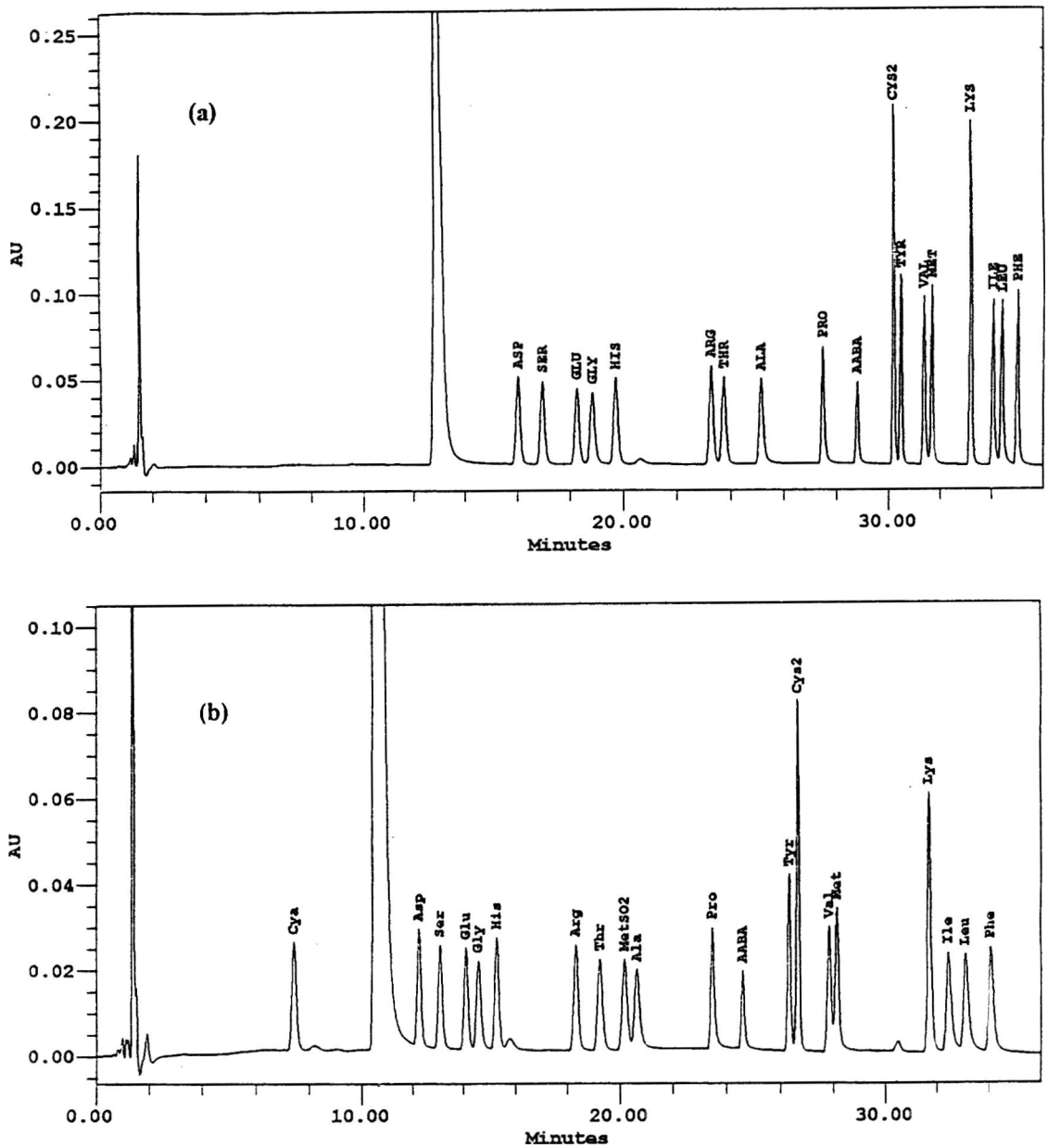

Figure 2. Chromatography of derivatized amino acid standard mixtures for (a) common amino acids (255 pmol) and (b) common and sulfur-containing amino acids (100 pmol). Separation conditions are described in *Experimental*.

250 nmol/ml in all the standard solutions and hydrolysates throughout the study. After thorough mixing on a Vortex mixer, the reconstituted hydrolysate was filtered through a MILLEX-HV 0.45 µm filter unit (Millipore).

The Vitalyte powder was first dissolved in Milli-Q water. After addition of AABA standard solution, the sample was filtered through a 0.45 µm filter unit.

Derivatization

Standards or filtered samples (10 µL each) were pipetted into the bottom of a derivatization tube. To the tube, 70 µL 0.2M borate buffer (pH 8.8) were added, and the solution was agitated on a Vortex mixer for 10 s. A 20 µL aliquot of AQC solution (3 mg/mL in acetonitrile) was added and the solution was immediately agitated on a Vortex mixer. The tube was sealed and heated in a reaction block for 10 min at 50°C.

Chromatography

The LC system consisted of two M510 pumps, a 717 autosampler, a 486 tunable absorbance detector, and a temperature control module (Waters). A Waters Millennium 2010 chromatography manager was used to control the system and collect data. Eluent A was 140 mM sodium acetate and 17 mM triethylamine (TEA) titrated to pH 4.95 with phosphoric acid. Sodium azide (0.1 g) was added to 1000 mL eluent A to protect the buffer from bacterial growth. Eluent B was 60% acetonitrile in water (v/v) containing 0.01% acetone. All separations were carried out on a 4 µm AccQ-Tag^{TM} C_{18} column (150 × 3.9 mm) supplied by Waters Corp. The column temperature was control-

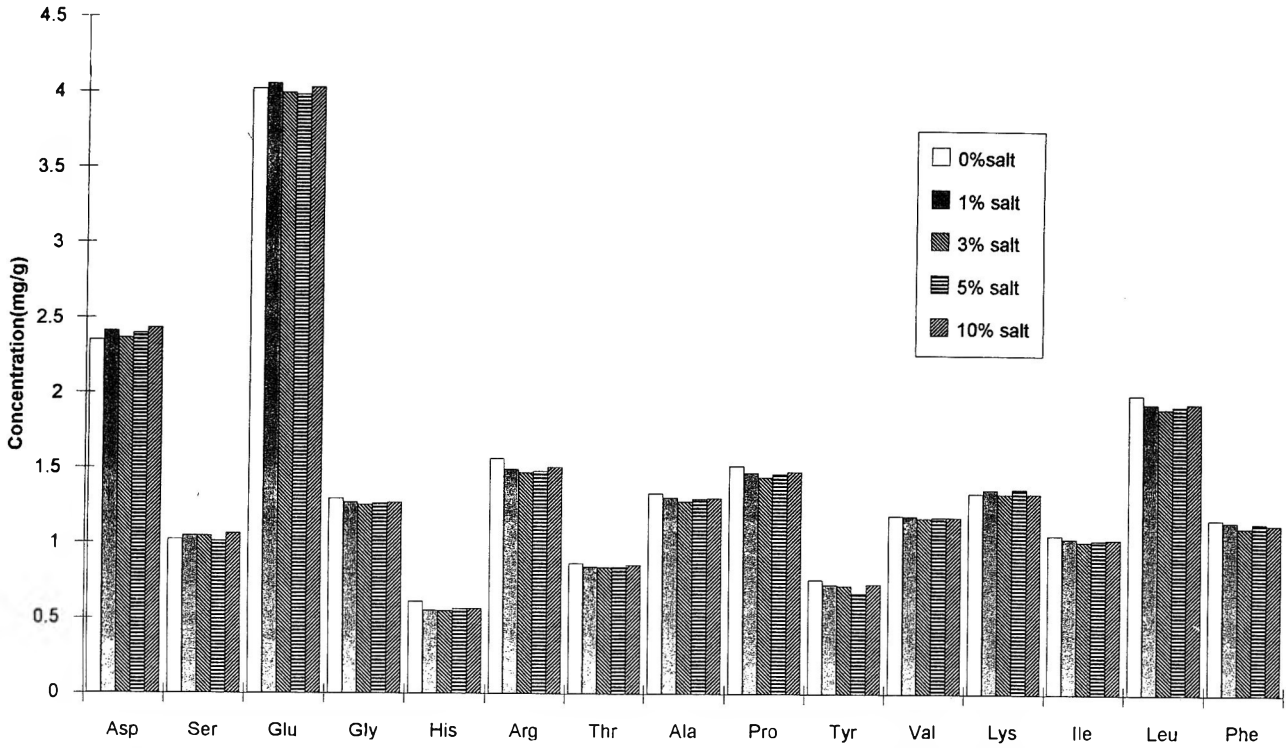

Figure 3. Effect of salt added to hydrolyzed broiler starter on amino acid derivative yields. Yields are expressed as the calculated amount of amino acids in the feed (%).

led at 37°C (for acid hydrolysates) or 47°C (for performic acid oxidation hydrolysates). The flow rate was set at 1.0 mL/min. Gradient conditions for both acid hydrolysis samples and performic acid oxidation samples are listed in Table 1. Derivatives (4 μL) were injected for separation. The UV detector was set at 248 nm.

Results and Discussion

Chromatography of Derivatized Sulfur-Containing Amino Acids

The chromatographic conditions for isolation of most common amino acid derivatives using fluorescence or UV detection were reported (16, 18). However, if methionine sulfone ($MetSO_2$) and cysteic acid (Cya) (converted from Met and Cys by performic acid oxidation) were included in the amino acid mixture, the reported conditions would not be applicable for the analysis of the derivatives of the mixture because of coelution of derivatized $MetSO_2$ and alanine (Ala). We attempted to separate this pair of amino acids by optimizing the pH value of eluent A, modifying the gradient conditions, and changing the column temperature. The most notable influence on the separation of $MetSO_2$–Ala was the column temperature (Figure 1): the higher the temperature, the bigger the resolution. Nevertheless, if the temperature were too high, the separation of other peak pairs such as His–NH_3, AMQ–Asp, Glu–Gly, Tyr–Cys, and Val–Met (Figure 2) would not be adequate. As a compromise, 47°C was chosen as the final column temperature for analysis of performic acid oxidation hydrolysates. However, with the original gradient conditions, the resolution of His–NH_3 and Cys–Tyr peak pairs became unacceptable at 47°C (Figure 1). Modification of gradient conditions was done to improve the resolution of these 2 pairs of derivatives. Under the final conditions described in *Experimental*, all peaks were separated with baseline or near-baseline resolution in approximately 35 min; all derivatized amino acid standards with and without $MetSO_2$ and Cya were resolved with the same set of eluents (Figure 2). The elution order of Cys–Tyr pair was reversed with the 2 different elution conditions.

Effects of Salts in Food and Feed on Quantitative Analysis

Many kinds of food and feed have relatively high salt content, and this characteristic is one of the factors affecting derivatization yields of amino acids and making the derivatization efficiency in the hydrolysates lower than that in amino acid standards. Salts can have significant effects on derivative yields with some precolumn reagents (7, 20). To investigate the effect of salts in food and feed on the derivatization yields of amino acids with AQC, a known amount of NaCl was added to the hydrolyzed broiler starter to vary the percentage of NaCl in the feed. The samples containing different amounts of NaCl were then derivatized with AQC, and the resulting derivatives were analyzed under the standard chromatographic conditions previously described. The yields of all amino acids were essentially unaffected by up to 10% (by weight) of added salt in the sample (Figure 3).

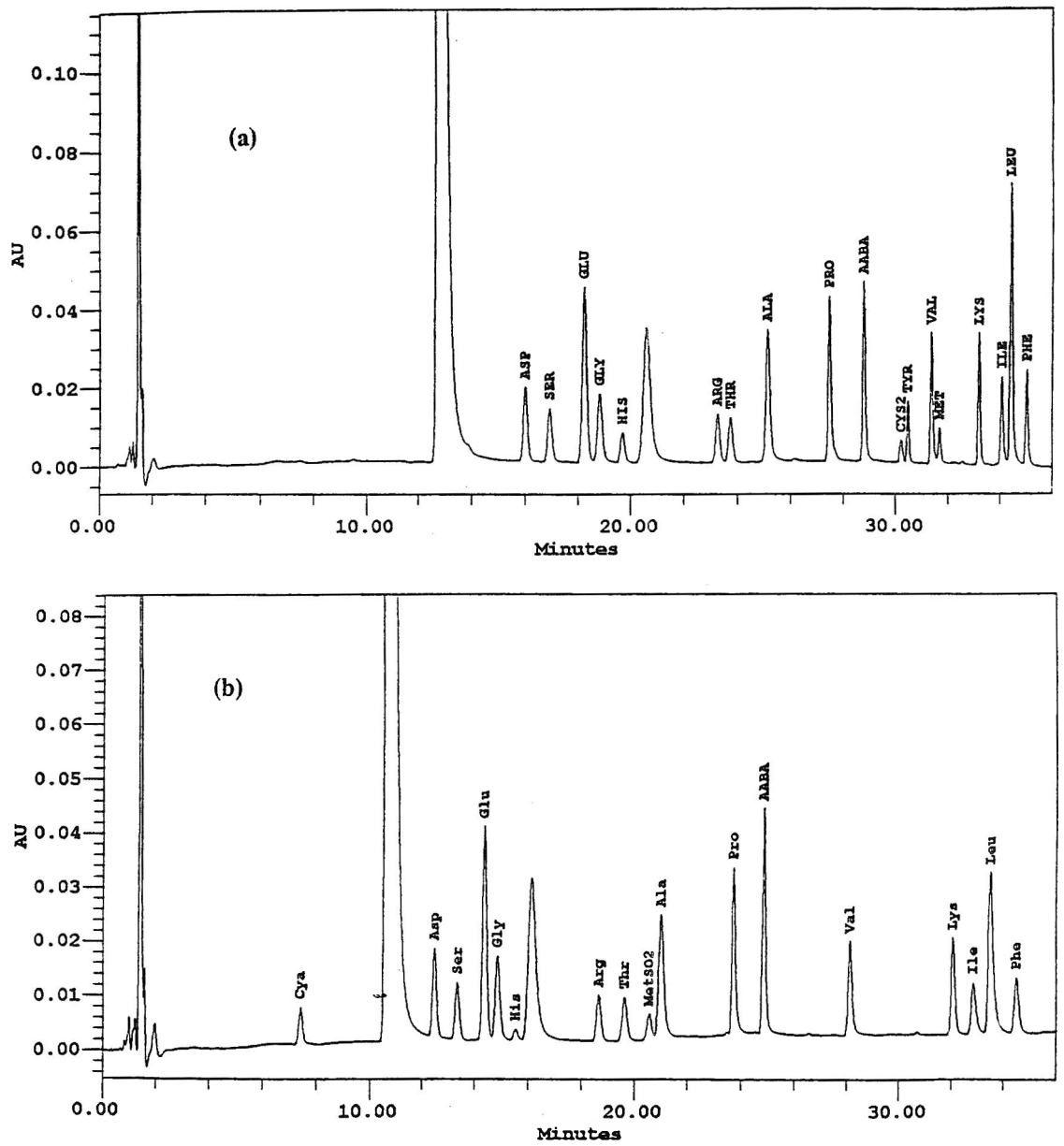

Figure 4. Chromatogram of amino acid derivatives in (a) acid hydrolyzed corn sample and (b) performic acid oxidized corn hydrolysate.

Analysis of Amino Acids in AOAC Test Samples

Chromatograms of derivatized amino acids in hydrolysates of corn and broiler starter samples are shown in Figures 4 and 5, respectively. All components in the derivatized samples were resolved completely. AABA was used as an internal standard because its derivative eluted in the middle of chromatograms with no other derivative or impurity peaks. AQC results for all amino acids in corn (Table 2) and broiler starter (Table 3) samples agreed very well with AOAC IEC results for the exact same test samples. (AOAC IEC data are from the collaborative study for the assay of amino acids in food and feed [21]). The "Data by IEC" column in Tables 2 and 3 were generated for the same hydrolysates as those used for the current method. Tyr, which was not determined in the collaborative study, was assayed with the current method, and the results agreed well with the IEC method. Good agreement for isoleucine (Ile) was observed for data obtained with the AQC and IEC methods for corn (0.32 and 0.34 mg/g, respectively) and broiler starter (1.07 and 1.05 mg/g, respectively) hydrolysates. Comparison of the AQC results for Ile with the AOAC data was good (Tables 2 and 3), although the difference was greater than the results for the same hydrolysates. This finding suggests the influence of the slightly different hydrolysis procedures on the amount of amino acids recovered. Excellent repeatability of the current method was observed (Tables 2 and 3). Because of the stability of the derivatives, the current method is comparable to or better than any amino acid analysis technique, including the IEC method. The relative standard deviations (RSD) for corn and broiler starter were 0.01–2.8% (av-

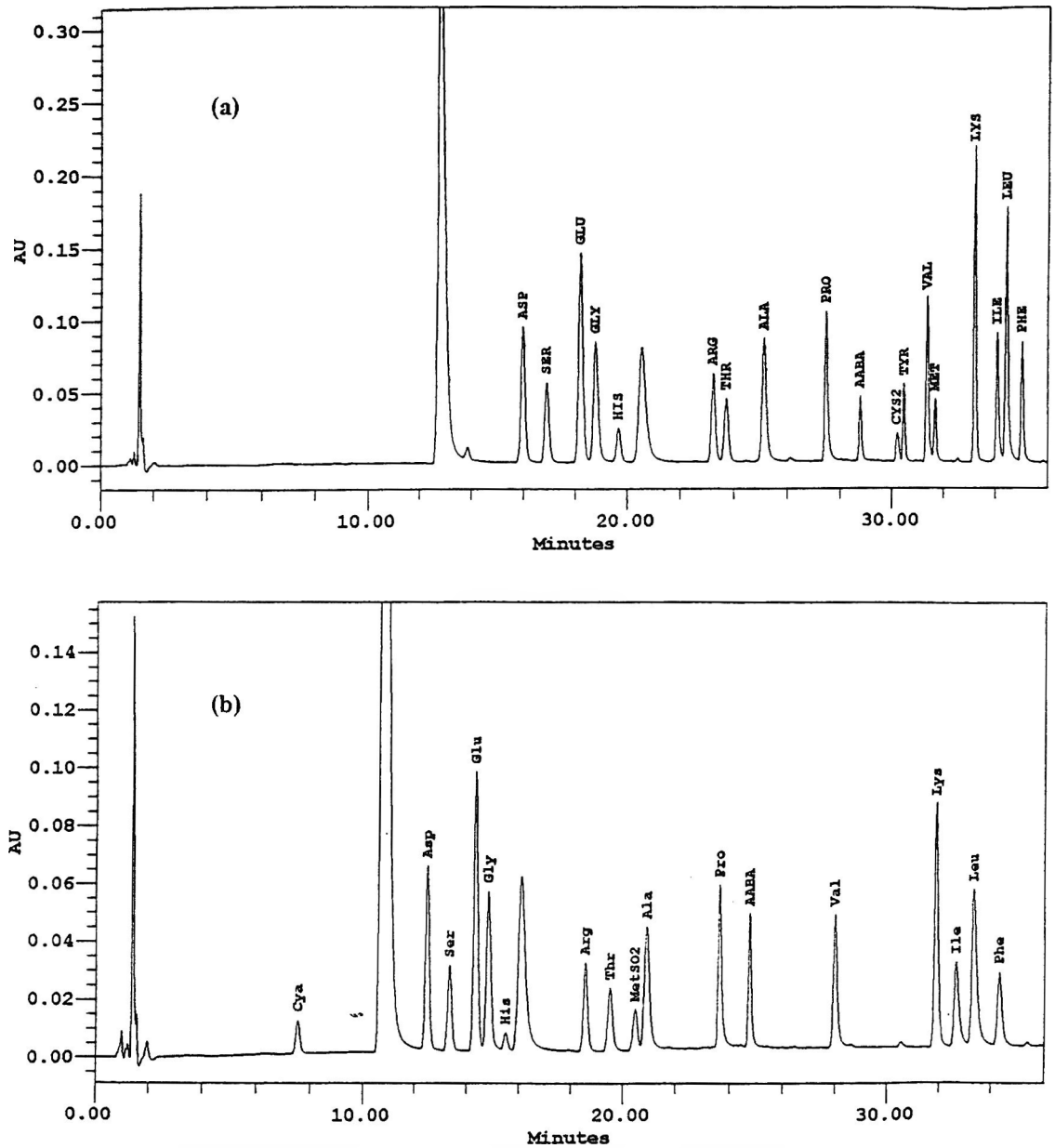

Figure 5. Chromatogram of amino acids derivatives in (a) acid hydrolyzed broiler starter sample and (b) performic acid oxidized broiler starter hydrolysate.

erage, 0.74%) and 0.01–2.9% (average, 0.70%), respectively. All of the AQC data were calculated using an internal standard (AABA). Accurate results were obtained by using an external standard calibration method, but the repeatability value increased slightly (average RSD of external calibration for the same corn hydrolysates was 2.0%).

Determination of Amino Acids in Other Samples

Shrimp feed, another kind of mixing feed, was also assayed in this study. Figure 6 shows the chromatogram of derivatives of amino acids in the performic acid oxidized hydrolysates. All amino acid derivatives were well resolved from each other as well as from the reagent peak. Data from the current method agreed well with data from the IEC method (Table 4). The analysis of amino acids in Vitalyte by IEC was complicated by the presence of much higher salt and vitamin levels compared with the amino acid content. We assayed the amino acids in the diluted sample. The chromatogram (Figure 7) shows that Met and lysine (Lys) can be isolated without any interference by other ingredients in the sample. Recoveries for the amino acids in Vitalyte were 98–106% (average, 101%; Table 5).

Conclusions

As previously described, our method has many advantages over other methods. All derivatized amino acids can be well resolved with shorter separation times and more sensitive detection than with IEC. Quantitation for all common kinds of feed can be done as reliably as with IEC. Also, unlike many other precolumn derivatization reagents, AQC can react with

Table 2. Comparison of amino acids recovery in corn hydrolysates by IEC and current method

Amino acid	AOAC data by IEC, mg/g[a]	Data by current method[b]		Data by IEC, mg/g[c]
		mg/g	RSD, %	
Asp	0.54	0.56	0.94	0.55
Thr	0.29	0.28	0.53	0.29
Ser	0.39	0.33	1.4	0.36
Glu	1.51	1.57	0.74	1.56
Pro	0.73	0.76	0.48	0.75
Gly	0.33	0.34	1	0.34
Ala	0.61	0.62	0.68	0.62
Cys	0.18	0.16	1.7	0.18
Val	0.38	0.41	0.38	0.43
Met	0.18	0.16	0.01	0.18
Ile	0.28	0.32	0.67	0.34
Leu	0.99	1.01	0.73	1.05
Tyr	ND[d]	0.27	0.38	0.3
Phe	0.38	0.41	2.8	0.5
Lys	0.26	0.26	0.69	0.3
His	0.27	0.25	1.4	0.27
Arg	0.4	0.38	0.56	0.41

[a] Cited from 1992 AOAC collaborative study for amino acid analysis. Samples used were acid hydrolysates oxidized by performic acid and with $Na_2S_2O_5$ as the reducer.
[b] All data were obtained from the analysis of acid hydrolysates of corn powder except the data for Met and Cys, which were from the analysis of performic acid oxidized hydrolysates (with HBr as the reducer). RSDs were calculated with data from 5 (2 for Met and Cys) replicate derivatizations of the same hydrolysates with AQC.
[c] Same hydrolysates were used for IEC method as for current method.
[d] ND, not determined.

Table 3. Comparison of amino acids recovery in broiler starter's hydrolysates by IEC and current method

Amino acid	AOAC data by IEC, mg/g[a]	Data by current method[b]		Data by IEC, mg/g[c]
		mg/g	RSD, %	
Asp	2.29	1.53	2.31	1
Thr	0.88	2.73	0.84	0.6
Ser	1.12	2.5	0.95	1.06
Glu	4.04	1.78	4	0.7
Pro	1.47	4.08	1.51	0.56
Gly	1.27	2.68	1.29	0.75
Ala	1.28	2.11	1.33	0.58
Cys	0.35	1.71	0.33	0.01
Val	1.11	1.71	1.2	0.46
Met	0.62	2.1	0.58	0.01
Ile	0.95	2	1.07	0.52
Leu	1.97	1.68	2	0.59
Tyr	ND[d]	ND	0.72	0.57
Phe	1.12	2.23	1.16	2.9
Lys	1.35	2.37	1.33	0.58
His	0.65	2.77	0.61	0.54
Arg	1.57	2.68	1.54	0.52

[a] Cited from 1992 AOAC collaborative study for amino acid analysis. Samples used were acid hydrolysates oxidized by performic acid and $Na_2S_2O_5$ as the reducer.
[b] All data were obtained from the analysis of acid hydrolysates of corn powder except the data of Met and Cys, which were from the analysis of performic acid oxidized hydrolysates (with HBr as the reducer). RSDs were calculated with data from 4 (2 for Met and Cys) replicate derivatizations of the same hydrolysates with AQC.
[c] Same hydrolysates were used for IEC method as for current method.
[d] ND, not determined.

Table 4. Comparison of amino acids recovery in shrimp feed hydrolysates by IEC and current method

Amino acid	Data by IEC,[a] % (w/w)	Data by current method,[b] % (w/w)
Asp	3.3	3.44
Thr	1.04	1.02
Ser	1.92	1.86
Glu	5.91	5.93
Pro	ND[c]	7.92
Gly	13.46	13.39
Ala	5.17	5.65
Cys	0.19	0.18
Val	1.65	1.9
Met	0.37	0.34
Ile	0.95	1
Leu	1.98	1.96
Tyr	0.43	ND
Phe	1.5	1.38
Lys	2.26	2.25
Arg	4.32	4.44

[a] All data were obtained from the analysis of acid hydrolysates except for Met and Cys, which were from performic acid oxidized samples.
[b] Same hydrolysates were used for current method as for IEC method.

Table 5. Recoveries of amino acids in Vitalyte

Amino acid	Calculated amount, μg/ml	Added amount, μg/ml	Recovery, %
Cya	20.37	19.18	106
Asp	12.33	12.28	100
Ser	8.488	8.32	102
Glu	11.688	11.64	100
Gly	6.069	5.94	102
His	12.616	12.28	103
Arg	13.556	13.81	98
Thr	9.476	9.44	100
$MetSO_2$	11.821	11.81	100
Ala	6.934	7.05	98
Pro	8.981	9.11	99
Tyr	14.303	14.34	100
Cys	19.637	19.18	102
Val	9.444	9.27	102
Met	11.95	11.81	101
Lys	12.21	11.57	106
Ile	10.874	10.38	105
Leu	10.809	10.38	104
Phe	13.42	13.07	102

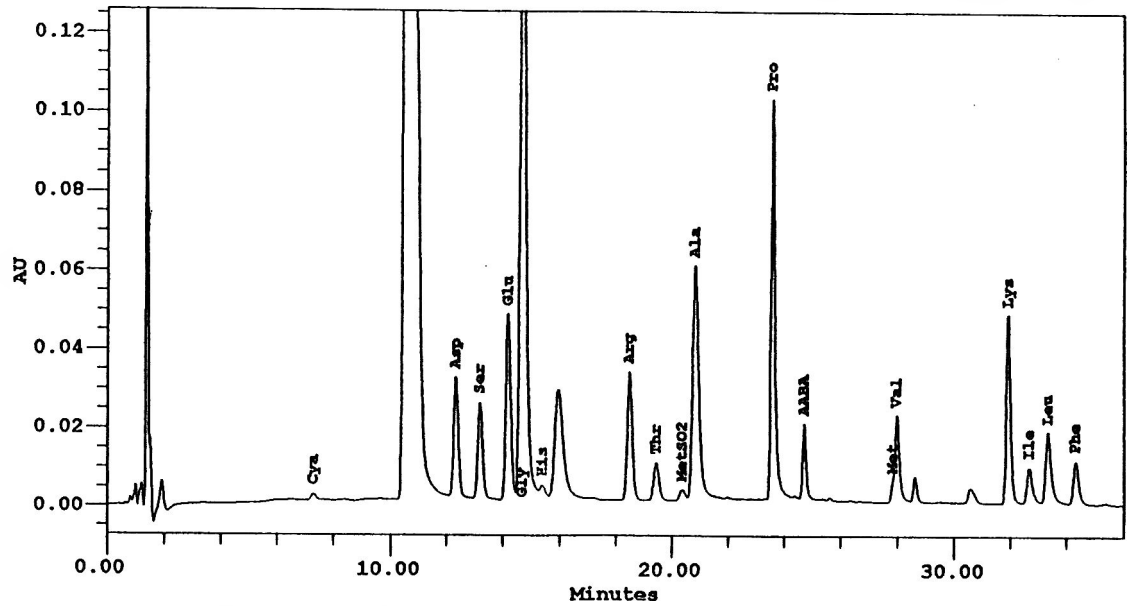

Figure 6. Chromatographic separation of amino acids in a performic acid oxidized hydrolysate of shrimp feed.

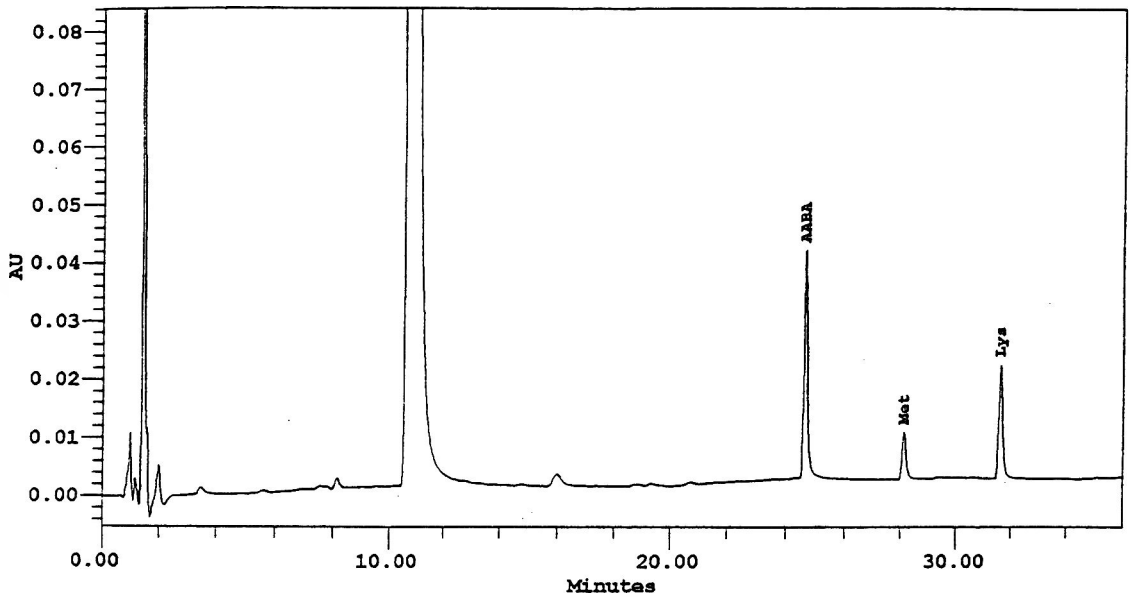

Figure 7. Chromatographic separation of amino acid derivatives in Vitalyte.

all amino acids quickly, quantitatively, and with little matrix interference. By analyzing sample hydrolysates with and without performic acid oxidation, all nutritionally important amino acids in the samples can be quantitated with excellent accuracy and precision.

Acknowledgment

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FOOD COMPOSITION AND ADDITIVES

Supercritical Fluid Extraction of *N*-Nitrosamines in Hams Processed in Elastic Rubber Nettings

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A method for analysing *N*-nitrosamines in hams processed in elastic rubber nettings by supercritical fluid extraction (SFE) is described. The study was carried out with the prototype of a commercial extractor with a silica gel adsorption cartridge integrally attached to the variable restrictor. The SFE method was compared with a solid-phase extraction procedure currently used for ham analysis. Both methods used the same gas chromatographic–chemiluminescence detection conditions. No significant difference ($p < 0.05$) was found between results obtained with the 2 methods. Repeatability standard deviation of the SFE method was 1.7 ppb, with a coefficient of variation (CV) of 2.7%, compared with 2.2 ppb, with a CV of 3.5%, for solid-phase extraction. SFE permits minimal use of solvent and more rapid analysis of nitrosamines.

Supercritical fluid extraction (SFE) is rapidly becoming the method of choice for isolation of certain analytes from sample matrixes. Compared with standard extraction techniques, SFE offers substantial time savings because of the unique properties of gases in their supercritical state (1). The polar and nonpolar characteristics of the supercritical fluid can be controlled by varying the pressure and temperature of the extraction system. This technique can be used both for sample extraction and analyte concentration. Carbon dioxide is the gas of choice because of its excellent physical properties in the supercritical state, low toxicity, and reasonable cost. The current emphasis on methods that use less solvent makes SFE an attractive alternative for the analysis of nitrosamines.

Since the discovery of carcinogenicity of *N*-nitrosodimethylamine (2), several hundred compounds containing the *N*-nitroso group have been found to be carcinogenic in a number of animal species. These compounds typically are isolated by distillation and/or solvent partition. However, only limited studies have been carried out on SFE of nitrosamines. Prokopczyk et al. (3) reported extraction efficiencies of 83 to 98% for the major nicotine-derived tobacco-specific nitrosamines in smokeless tobacco and snuff, with methanol-modified supercritical carbon dioxide. Recently, our group (4), using only carbon dioxide, obtained recoveries of 84 to 105% for 10 volatile nitrosamines, including aliphatic and alicyclic nitrosamines, in frankfurters fortified at 20 ppb. In these studies (3, 4), SFE was carried out with a self-assembled apparatus. In our study (4), a new design concept was necessary because of significant loss of nitrosamines at the restrictor–collector interface when these analytes were extracted with commercial SFE in-

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struments. For this reason, a unique integral micrometering valve–collector assembly was developed to trap nitrosamines on the sorbent bed of commercial solid-phase extraction (SPE) cartridges. This assembly design was described previously for isolation of 3 nitrobenzamide antimicrobial drug residues in chicken liver tissue (5). In this paper, we report the development of an SFE method for determination of nitrosamines in boneless hams processed in elastic rubber nettings. The method uses the prototype SFE with the integral metering valve–collector assembly. The SFE method also was compared with an SPE technique currently used for analysis of nitrosamines in hams.

METHOD

Caution: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

Materials

(**a**) *Ham samples.*—Samples were obtained from local retail outlets or producers, or from the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) and analyzed without further heating. The outer 1/4 in. of the ham was removed, ground through a 1/16 in. plate, and then thoroughly mixed. The comminuted sample was vacuum-packaged and stored at –20°C until analyzed.

(**b**) *Reagents.*—The sources and cleanup of Hydromatrix (Celite 566), Celite 545, anhydrous sodium sulfate, propyl gallate, silica gel, dichloromethane (DCM), pentane, and diethyl ether were described in detail elsewhere (4, 6, 7). Morpholine was doubly distilled before use and then checked for presence of *N*-nitrosomorpholine (NMOR) as a contaminant; none was found. Preparation of the SPE cartridge was described in detail elsewhere (4). Briefly, 1.0 g washed and sieved (70–150 mesh) silica gel was packed into an empty 6 mL SPE cartridge and then a frit was placed on top.

(**c**) *N-Nitrosodipropylamine (NDPA) internal standard solution.*—0.10 µg/mL in DCM.

(**d**) *Gas chromatographic working standard solution.*—Each 0.10 µg/mL in DCM: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), NDPA, *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), NMOR, and *N*-nitrosodibenzylamine (NDBzA). These nitrosamines were either purchased or synthesized from their corresponding amines and sodium nitrite according to a general procedure reported previously (8).

Apparatus

(**a**) *Supercritical fluid extractor.*—The extractor was a prototype of a commercial instrument developed jointly by our laboratory and Applied Separations (Allentown, PA) and now in commercial production. This instrument was configured for parallel extraction of 2 SFE vessels. Extraction vessels were connected to the system with hand-tightened, slip-free connectors (Keystone Scientific, Bellefonte, PA). The restrictors were micrometering valves (10 RMM2812, Autoclave Engineers, Inc., Erie, PA) encased in an aluminum block fitted with a cartridge heater and a thermocouple. The seat-retaining nuts of the micrometering valves, which connect the valve to other devices, were replaced by a redesigned retaining nut fabricated locally. This redesigned nut, referred to as the integral seat retainer–column nut, enables a commercial 6 mL SPE cartridge to be attached directly to the micrometering valve without fittings or connecting tubing. Components of this prototype instrument are shown in Figure 1. A detailed description of the metering valve–SPE interface has been reported elsewhere (5).

(**b**) *Gas chromatograph–thermal energy analyzer (GC–TEA).*—The instruments and operating conditions used for separation and quantitation of nitrosamines were described elsewhere (7).

(**c**) *Other glassware and equipment.*—All other items needed for SFE or SPE have been described elsewhere (4, 6).

Sample Preparation (SFE)

Weigh 5.0 g comminuted ham sample into a 100 mL beaker. Add 250 mg propyl gallate. Using a 0.5 mL transfer pipette, spike the sample with either 0.5 mL NDPA internal standard solution or 0.5 mL GC working standard solution. Add 5.0 g Hydromatrix. Stir the mixture with a glass rod until uniform in appearance (ca 1 min). Seal one end of the high pressure extraction vessel (66015 SFE vessel, 24 mL volume or capacity, Keystone Scientific) and label it as "top." Transfer the dry, free-flowing mixture into the extraction vessel prepacked with a

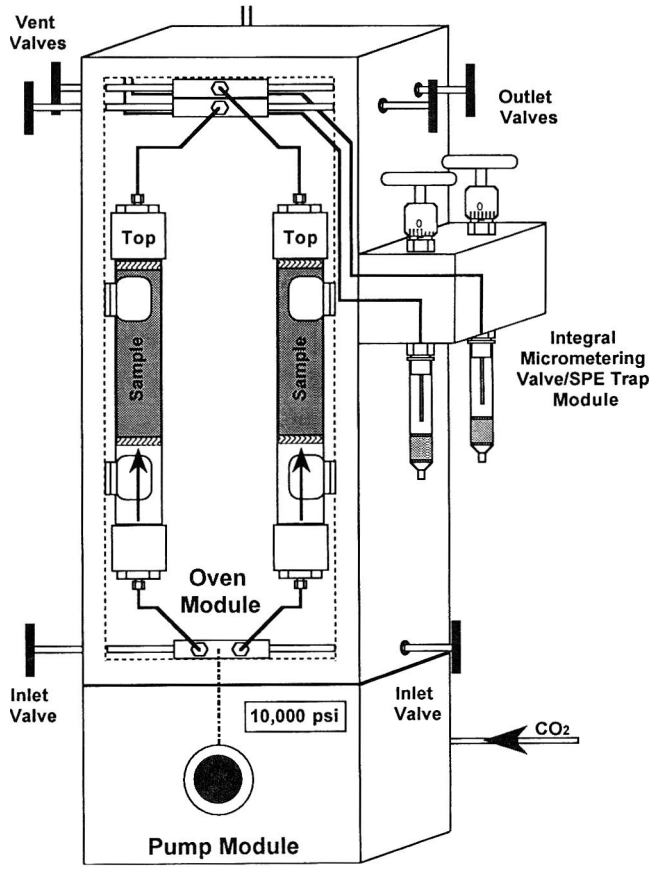

Figure 1. Schematic diagram of prototype SFE system.

plug of polypropylene wool (Aldrich Chemical Co., Milwaukee, WI). Tightly compress the mixture with a tamping rod that ensures uniform supercritical fluid flow. Finally, add a second plug of polypropylene wool to the extraction vessel and compress in place with the tamping rod. Tighten end fittings before the next step. The sample is now packed in the end labeled "top" (Figure 1).

SFE Procedure

Install the extraction vessels in the SFE as shown in Figure 1, with the ends labeled "top" connected to the upper fittings. Preheat the micrometering valves to 110°C. Close the oven shut-off and vent valves; open the inlet valves. Pressurize the SFE vessels with carbon dioxide to ca 9000 psi (612 bar); simultaneously set the oven temperature to 40°C and commence heating. Equilibrate the system by using a 10 min static holding period. When the system is equilibrated, adjust the pressure to a final setting of 10 000 psi (680 bar). During this period, pack empty SPE cartridges with silica gel and then attach the cartridges to the integral seat retainer–column nut of the micrometering valves (Figure 1). Attach the SPE cartridges with flexible tubing to a Floline SEF-51 flow meter–gas totalizer (Scott Specialty Gases, Plumsteadville, PA). The direction of fluid flow through the system is indicated by the arrow in Figure 1. After the 10 min heating period, open the outlet valves to direct flow to the micrometering valve module. Use these valves to adjust the flow of the expanded gas to 2.8 L/min through the SPE cartridges and maintain that rate throughout the experiment until 50 L are recorded on the gas totalizer. At that point, close the inlet and outlet valves and depressurize the SFE vessels by using the vent valves. During extraction, flow rates were kept between 2.7 and 2.9 L/min. Nitrosamine results indicated that this slight variation had no effect on analyte recoveries. Remove the extraction vessels from the oven module and attach Luer adapters to the upper slip-free connectors. Attach a filled syringe to each adapter and flush any trace residues of analyte–fat remaining in the discharge tube of the micrometering valves with 0.3 mL hexane. Remove the SPE cartridges containing the analyte–fat mixture from the seat retainer nut. Hold the cartridges below the seat retainer nuts and rinse the 1/16 in. stainless steel tubing of these assemblies with 0.1 mL hexane directly into the SPE cartridges to ensure quantitative recovery of nitrosamines.

Nitrosamine Recovery and Analysis (SFE)

Details of this procedure were described previously (4). Briefly, wash the SPE cartridge with two 4 mL portions of 25% DCM in pentane; discard the washes. Elute nitrosamines with two 4 mL portions of 30% ether in DCM. Concentrate to 1.0 mL and quantitate on the GC–TEA. The nitrosamine values of individual samples were corrected for recovery of NDPA internal standard. The minimum levels of reliable measurement were 0.5 ppb for NDMA and 1.0 ppb for the other nitrosamines.

Sample Preparation, Recovery, and Analysis (SPE)

The complete procedure for preparation, extraction, cleanup, and quantitation of ham samples by our SPE procedure were described in detail elsewhere (6, 7). A flow diagram is shown in Figure 2. The minimum levels of reliable measurement were 0.2 ppb for NDMA and NDEA, and 1.0 ppb for the other nitrosamines.

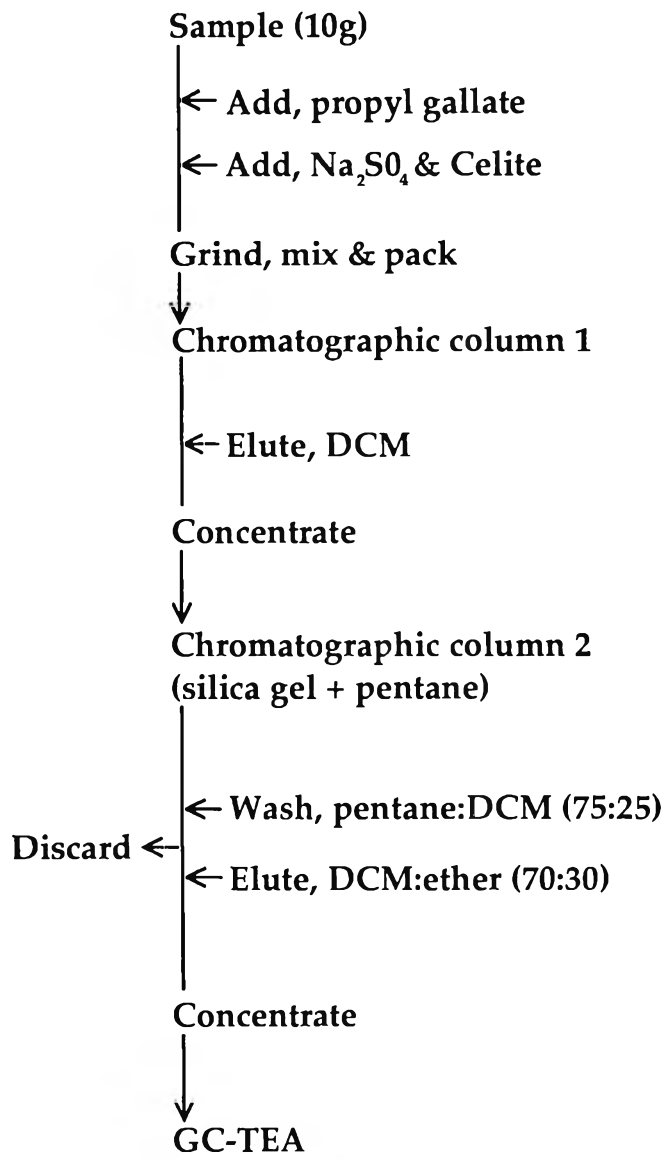

Figure 2. Flow diagram of SPE procedure.

Statistical Analysis

Data were analyzed by analysis of variance and means procedures of the Statistical Analysis System software distributed by SAS Institute, Inc. (9). These results were then interpreted according to the methods of Snedecor and Cochran (10).

Results and Discussion

An earlier SFE used for isolation of nitrosamines from frankfurters was assembled entirely in our laboratory (4). To obtain satisfactory recoveries, we fabricated an integral restrictor interface so that we could collect extracted analytes directly on sorbents in standard SPE cartridges (5) rather than from a cooled solvent, as with most laboratory-assem-

bled and commercially available SFE instruments. This interface adapter yielded excellent recoveries of nitrosamines and minimized post-SFE cleanup (4). We next attempted to modify a commercial multiport parallel SFE extractor, which used fixed restrictors vented into open refrigerated vials for analyte recovery, to a system where analytes are collected in SPE cartridges. We fabricated a retaining nut similar to that shown in Figure 3b (*see* reference 2) to retain the 6 mL SPE cartridges on the fixed restrictors of the commercial SFE. Although we were able to collect nitrosamines from samples with this modified system, we encountered several problems that we could not resolve. For example, the restrictors could not achieve the same flow rates that were used with the variable restrictors in the laboratory-assembled apparatus, partly because of the high fat content of the meat samples. Also, the extracted fat solidified in the SPE cartridges because of the inability of the restrictor heating block to keep the cartridges above freezing temperatures. As a result of the difficulties with the modified extractor, recoveries of nitrosamines were variable and low compared with those obtained with the laboratory-assembled apparatus. Experiments with this system were therefore discontinued. Instead, a Cooperative Research and Development Act (CRADA) agreement was signed with a commercial instrument manufacturer to build an SFE based on design concepts developed for the laboratory-assembled apparatus used in our previous studies (4, 5). The final prototype of this commercial SFE was used for all experiments described in this paper.

During our earlier investigation on use of SFE to analyze cured meat products for volatile nitrosamines, our sample size was 2.5 g (4) because we loosely packed the sample–Hydromatrix mixture, completely filling the extraction vessel. By changing to compressed packing without analyte loss, we increased sample size to 5.0 g, which resulted in increased sensitivity and without the need for more solvent to elute analytes from the SPE cartridges.

One problem initially encountered during development of the SFE method was artifactual nitrosamine formation (4). This occurred when the sample is heated (70°–80°C) in the presence of residual $NaNO_2$ prior to extraction with supercritical carbon dioxide. To eliminate this problem in analysis of frankfurters, we lowered the extraction temperature to 40°C and added propyl gallate, a nitrosamine inhibitor, to the samples. To determine whether nitrosamines could form artifactually in the ham samples, which have a lower fat content than frankfurters, morpholine, a rapidly nitrosated secondary amine precursor of NMOR, was added to several ham samples before SFE. No NMOR was detected in the SFE extract.

Recoveries of 7 volatile and 1 semivolatile nitrosamine (NDBzA) added to nitrosamine-free ham at 10 ppb and analyzed by SFE are shown in Table 1. The mean recovery of NDBzA, the nitrosamine found in ham processed in elastic rubber netting, was 96.1 ± 4.5%. The mean recovery of all other nitrosamines by SFE ranged from 95.2 to 103.5%. This compares favorably with the range of recoveries (92.4 to 97.8%, excluding NDBzA) we reported for SFE of 10 volatile nitrosamines in frankfurters fortified at 20 ppb (4). Analysis for NDBzA in hams by SPE gave a mean recovery of 95.6 ± 4.5% (7).

Table 1. SFE recovery of nitrosamines from hams fortified at 10 ppb

	Recovery, %			
N-Nitroso compound	Range	Mean (n = 8)	SD	CV
NDMA	91.8–102.0	96.4	4.1	4.2
NDEA	98.4–106.1	102.6	2.5	2.4
NDPA	96.3–109.4	100.9	5.6	5.5
NDBA	90.0–102.4	95.2	4.8	5.0
NPIP	96.4–109.1	103.5	4.5	4.4
NPYR	98.0–105.1	100.5	2.4	2.4
NMOR	97.3–107.5	102.8	3.7	3.6
NDBzA	88.4–101.2	96.1	4.5	4.6

The presence of nitrosamines in cured meat products processed in elastic rubber netting has been reported recently (6, 7, 11). Therefore, we chose this sample type for further investigation by SFE. Samples from the outer surface of 21 ham samples processed in elastic rubber netting were analyzed in duplicate for nitrosamines by both SFE and SPE. The outer ham surface has the maximum exposure to rubber in the netting and has the highest nitrosamine values. The SPE method was selected for comparison because it readily isolates volatile nitrosamines and NDBzA from the sample matrix. Raw data are compared in Table 2, and statistical results are given in Table 3. The samples analyzed contained nitrosamines in a wide range of concentrations, from none detected (ND) to 157 ppb. The internal standard for all extracted samples was NDPA. NDBzA was the only nitrosamine detected in the ham samples, reflecting the change in the formulation of the rubber netting, which previously yielded NDBA (6). As expected, highly significant differences (p <0.01) were found among random samples. No significant difference (p <0.05) in results was found for the 2 methods. The overall mean NDBzA from SFE-analyzed samples was 63.2 ppb, and the overall mean from the SPE-analyzed samples was 63.7 ppb. The repeatability standard deviation of the SFE method was 1.7 ppb, with a coefficient of variation (CV) of 2.7%, compared with 2.2 ppb and a CV of 3.5% for the SPE method. The overall mean recovery of NDPA from SFE-determined samples was 93.4%, and the overall mean from SPE samples was 85.0%. These results show that the SFE method is comparable with the SPE procedure for analysis of hams. GC–TEA chromatograms obtained from SFE-analyzed ham samples also appeared to be "cleaner" than those from the SPE-analyzed samples, even with the difference in sample size, 5 versus 10 g.

Although NDBzA was the only nitrosamine detected in ham samples, the presence of NDBA and, to a lesser extent NPIP, may still be possible. Their presence would be due to continued use of zinc dibutyldithiocarbamate and dipentamethylene thiuram tetrasulfide as vulcanizing agents in rubber formulation. A spiked recovery study showed that these 2 nitrosamines

Table 2. Determination of *N*-nitrosodibenzylamine (NDBzA) in netted hams by SFE and SPE

	SFE[a]		SPE[a]	
Sample	NDPA, %	NDBzA, ppb[b]	NDPA, %	NDBzA, ppb[b]
A	89.9	110.5	89.7	111.3
B	93.9	9.0	66.6	9.1
C	96.2	6.1	71.8	7.0
D	97.3	9.2	77.9	9.7
E	97.5	132.0	69.8	132.4
F	94.5	146.9	94.9	139.6
G	93.4	ND[c]	88.8	ND
H	97.7	118.1	100.0	115.5
I	81.9	21.1	94.5	28.5
J	89.6	58.2	91.9	66.7
K	88.5	27.4	98.2	31.0
L	128.1	157.3	91.1	157.3
M	79.5	100.8	95.2	99.8
N	82.1	146.6	85.5	143.8
O	88.7	43.7	84.6	43.3
P	83.2	25.8	91.6	28.4
Q	99.3	69.7	79.5	69.3
R	107.9	6.6	75.4	4.9
S	88.4	24.1	73.2	26.9
T	86.7	69.0	78.8	68.1
U	97.9	45.1	85.8	44.5

[a] Results are averages of duplicate determinations.
[b] Data corrected for recovery of the NDPA internal standard.
[c] ND, not determined.

could be isolated by SFE. However, to ensure that normally incurred NDBA and NPIP could be successfully extracted from ham by SFE, older ham samples previously found to contain these specific nitrosamines were analyzed. Again, the results showed no differences in NDBA and NPIP values between SFE and SPE methods.

Analysis for regulatory purposes requires use of standardized equipment and conditions to ensure good reproducibility of results. In this paper, we have reported a study conducted with a prototype commercial SFE instrument using an in-line nitrosamine collection system with an SPE cartridge, as shown in Figure 1. This SFE system avoided the use of a time consuming off-line transfer step and potential nitrosamine loss.

Conclusions

Although very little research has been done on the use of SFE to extract nitrosamines, this is a promising technique for extracting both volatile and semivolatile nitrosamines from complex food matrixes. SFE can extract various volatile nitrosamines, both aliphatic and alicyclic, and the semivolatile NDBzA from a low-fat cured meat product, ham. The minimal use of solvent, 16 mL compared with approximately 500 mL for SPE, will help laboratories in meeting new Environmental Protection Agency guidelines for solvent reduction (12). SFE also will reduce analysis times: 20–24 samples per day can be analyzed by SFE, compared with 8–10 samples by SPE. This new method will also meet the needs of regulatory agencies and others who analyze foods for carcinogenic *N*-nitrosamines.

Table 3. Analysis of variance of SFE and SPE data

Source	Degrees of freedom	Sum of squares	Mean square	*F* value
Sample	20	222947.24	11147.36	2852.89
Method	1	4.79	4.79	1.22
Sample × method	20	224.59	11.23	2.87
Error	42	164.11	3.91	
Total	83	223340.73		

Acknowledgments

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FOOD COMPOSITION AND ADDITIVES

Determination of Free and Total Carbohydrate Profile in Soluble Coffee

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A liquid chromatographic (LC) method was evaluated for the determination of free and total carbohydrates in soluble coffee. Samples are solubilized in water for free carbohydrates or hydrolyzed with 1.00N hydrochloric acid for total carbohydrates. An aliquot of the sample solution is analyzed on a pellicular anion-exchange polystyrene–divinylbenzene column using pure water as the mobile phase. Carbohydrates are quantitated by a pulsed amperometric detector. All major carbohydrates found in soluble coffee are determined in a single run. The technique allowed the detection of fraudulent addition of coffee husks or parchments as well as cereals or caramelized sugar for the 63 commercial products analyzed. High levels of free mannitol, free fructose, free glucose, sucrose, total glucose, and total xylose are a good indication of adulteration. Data were compared with those obtained from separate enzymatic determinations and from a different LC procedure. A close agreement among the methods was observed. However, the original method was superior in precision and was the only procedure that enabled the quantitation of all major carbohydrates. The technique is, therefore, a very powerful tool for routine analysis and for purity assessment of soluble coffee.

Differences in processing conditions as well as permitted or fraudulent addition of coffee substitutes greatly influence the free and total carbohydrate profile of soluble (instant) coffee (1–5). The liquid chromatographic (LC) (1–3, 6–8), thin-layer chromatographic (9), gas chromatographic (3, 10), enzymatic (4, 11), and spectrophotometric (12, 13) methods used so far for carbohydrate determination in soluble coffee are limited either by insufficient resolution, complex sample preparation, enzyme availability, or lack of specificity. Consequently, a complete carbohydrate profile is only obtained by combining the results of different techniques.

In this study, we evaluated the applicability of anion-exchange (AE) chromatography with pulsed amperometric detection (PAD) for the separation and quantitation of the major carbohydrates found in soluble coffee. A wide range of commercial products were analyzed, and the results were compared with those obtained by previously published LC (1–3) and enzymatic (4) procedures.

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Experimental

Materials

(a) *Soluble coffee.*—The 63 samples analyzed in this study were commercial products from different countries and manufacturers.

(b) *Coffee husks.*—The 11 samples analyzed in this study were sun-dried coffee husks (6 arabica and 5 robusta) from Brazil.

Analysis of Soluble Coffee

(a) *Free carbohydrates.*—The test portion was dissolved in water. A few milliliters of the solution were filtered through a C_{18} disposable cartridge and then through a 0.2 μm membrane filter. The filtered solution was injected into an LC system. Carbohydrates were separated on a pellicular AE column and detected by PAD. The identification and quantitation of carbohydrates was performed by comparison with standard solutions.

(b) *Total carbohydrates.*—The test portion was hydrolyzed with 1.00N HCl. The solution was then passed through a folded filter paper. A few milliliters of the filtrate were filtered through a disposable AE cartridge in the silver form to neutralize the solution and to eliminate the chloride anion. The neutralized solution was finally filtered through a 0.2 μm membrane filter before injection into the chromatograph. Identification and quantitation of carbohydrates were performed by comparison with standard solutions.

Apparatus

(a) *Balance.*—Analytical, sensitivity ± 0.1 mg.
(b) *Round-bottom flasks.*—250 mL.
(c) *Volumetric flasks.*—100 and 1000 mL.

(**d**) *Pipettors.*—With disposable tips, 200–1000 µL and 5 mL capacity.

(**e**) *Cylinders.*—Graduated, 50 and 1000 mL tall-form.

(**f**) *Funnels.*—Analytical, 60°.

(**g**) *Vacuum filtering system.*—Aspirator with regulating device; heavy walled filtering flask with ground cone neck, 1 L; funnel, 300 mL, with ground glass joint; aluminum assembly clip; connection with vacuum outlet; filter holder, 47 mm id, and membrane filters, low-water extractable, 0.2 µm, 0.47 mm id; other equivalent vacuum-filtering systems could also be used.

(**h**) *Filter papers.*—Qualitative, folded, medium fast, Model 597½, N311847 (Schleicher and Schüll, Feldbach, Switzerland).

(**i**) *C_{18} cartridges.*—Disposable, Sep-Pak C_{18} (Waters, Volketswil, Switzerland). Cartridges were conditioned with 5 mL methanol and 10 mL water.

(**j**) *AE cartridges.*—Disposable, OnGuard-Ag (Dionex, Olten, Switzerland). Cartridges were conditioned with 5 mL water.

(**k**) *Membrane filters.*—Disposable, 0.2 µm.

(**l**) *Water bath.*—Capable of maintaining 70° ± 2°C and 98° ± 2°C.

(**m**) *Liquid chromatograph.*—Metal-free, capable of resisting 300 mM NaOH, Model 4500 i (Dionex).

(**n**) *Pulsed amperometric detector.*—With gold electrode, Model PAD II (Dionex). Reference cell was filled with 300 mM NaOH.

(**o**) *Analytical column.*—CarboPac PA1 (Dionex), 4 × 250 mm, 10 µm thickness.

(**p**) *Guard column.*—CarboPac PA1 (Dionex).

(**q**) *Postcolumn delivery system.*—Capable of resisting 300 mM NaOH (Dionex).

(**r**) *Integrator.*—Computing integrator, Model AutoIon AI-450 (Dionex).

(**s**) *Autosampler.*—Model SP8875 (Spectra-Physics, Basel, Switzerland) fitted with a 20 µL loop.

Reagents

(**a**) *Solvents and chemicals.*—All chemicals were of analytical reagent grade. Demineralized water, 18 MΩ·cm, was obtained from a Milli-Q system (Millipore, Volketswil, Switzerland).

(**b**) *Sodium hydroxide.*—50% (w/w) aqueous solution, e.g., J.T. Baker No. 7067 (Basel, Switzerland). The reagent should contain a minimum amount of sodium carbonate and mercury. Do not shake or stir the solution before use.

(**c**) *Hydrochloric acid solution.*—1.00N standard volumetric solution.

(**d**) *Eluent A.*—18 MΩ·cm demineralized water was filtered through a 0.2 µm membrane filter and degassed by sparging with helium for 20–30 min.

(**e**) *Eluent B.*—300 mM NaOH; 15.6 mL 50% (w/w) NaOH was pipetted into 985 mL eluent A. It is extremely important to avoid the presence of carbon dioxide in the eluents. Carbonate acts as a strong "pusher" on the column and results in a drastic reduction in resolution.

(**f**) *Standard solutions.*—1 mg/mL aqueous stock solutions were made up for arabinose, fructose, fucose, galactose, glucose, mannose, rhamnose monohydrate, ribose, xylose, sucrose, and mannitol (all from Fluka, Buchs, Switzerland). Stock solutions were further diluted with water to reach carbohydrate concentrations similar to those found in nonhydrolyzed or hydrolyzed soluble coffee sample solutions. Mixed standard solutions were also prepared from separate stock solutions. The diluted standard solutions were passed through a 0.2 µm membrane filter before injection.

Determination

(**a**) *Free carbohydrates.*—Into a 100 mL volumetric flask, 300 mg sample was weighed to the nearest 0.1 mg. About 70 mL water was added and the flask was shaken until dissolution was complete. The solution was diluted to 100 mL with water and 5–10 mL was filtered through a C_{18} cartridge. The first milliliter was discarded, and the filtrate was passed through a 0.2 µm membrane filter before injection.

(**b**) *Total carbohydrates.*—Into a 100 mL volumetric flask, 300 mg sample was weighed to the nearest 0.1 mg; 50 mL 1.00N HCl was added. The flask was swirled and placed in a boiling water bath for 150 min. The level of the sample solution was always kept below that of the water in the bath. The flask was swirled by hand every 30 min. The solution was cooled to room temperature by passing the flask under tap water, diluted to 100 mL with water, and filtered through a folded filter paper. The filtrate (3 mL) was passed through an OnGuard-Ag cartridge. The first milliliter was discarded. The neutralized solution was filtered through a 0.2 µm membrane filter before injection.

Chromatographic Conditions

(**a**) *Mobile phase.*—Isocratic. Mobile phase conditions were as follows: 0 min, eluent A–eluent B (100 + 0, start acquisition); 50.0 min, A–B (100 + 0, stop acquisition); 50.1 min, A–B (0 + 100, start cleanup); 65.0 min, A–B (0 + 100, stop cleanup); 65.1 min, A–B (100 + 0, start reequilibration); 80.0 min, A–B (100 + 0, stop reequilibration).

(**b**) *Column temperature.*—Ambient.

(**c**) *Flow rate.*—1.0 mL/min.

(**d**) *Postcolumn addition.*—300 mM NaOH at a flow rate of 0.6 mL/min.

(**e**) *Detector settings.*—E_1 = +0.05 V (300 ms), E_2 = +0.60 V (120 ms), and E_3 = –0.80 V (300 ms).

Calculations

Duplicate 20 µL volumes of standard and sample solutions were injected. Results were calculated with the following formula:

$$\text{Carbohydrate content} = \frac{R_1}{R_2} \times \frac{C \times V}{W} \times 100$$

where R_1 = peak response of carbohydrate in sample solution; R_2 = peak response of carbohydrate in carbohydrate standard solution; C = concentration of carbohydrate in carbohydrate

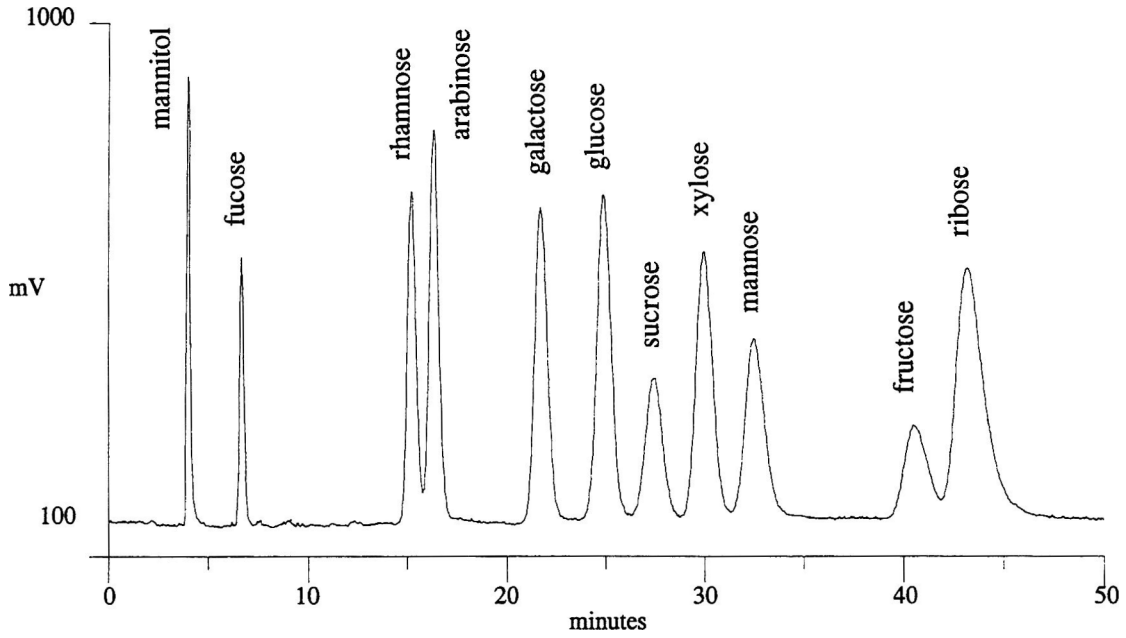

Figure 1. AE–PAD chromatogram of a mixed standard solution: mannitol, 15 μg/mL; fucose, 15 μg/mL; rhamnose, 35 μg/mL; arabinose, 40 μg/mL; galactose, 50 μg/mL; glucose, 55 μg/mL; sucrose, 45 μg/mL; xylose, 55 μg/mL; mannose 45 μg/mL; fructose, 90 μg/mL; and ribose, 90 μg/mL.

standard solution, mg/ml; V = volume of sample solution, mL; and W = weight of sample, mg.

After determination of moisture, results were expressed either in percent free or percent total carbohydrates on dry matter basis (db).

Analysis of Coffee Husks

The same procedure as for soluble coffee was applied with the exception of sample preparation.

Table 1. Retention time, relative response factor, linearity range, and detection limit of carbohydrates by AE–PAD

Carbohydrate	Retention time, min	Relative response factor[a]	Linearity range, μg/mL[b]	Detection limit, ng[c]
Mannitol	4.2	1.14	0.5–2000	10
Fucose	6.6	1.00	1.0–2000	20
Rhamnose	15.2	0.77	1.0–2000	20
Arabinose	16.1	1.06	1.0–2000	20
Galactose	21.2	1.01	1.0–2000	20
Glucose	24.3	1.00	1.0–2000	20
Sucrose	26.7	0.64	4.0–1000	80
Xylose	29.3	0.98	1.0–2000	20
Mannose	2.1	0.98	2.0–2000	40
Fructose	41.4	0.36	4.0–2000	80
Ribose	45.6	0.87	4.0–2000	80

[a] Response factor is relative to glucose.
[b] r = 0.9999 for all listed carbohydrates.
[c] Detection limit was measured on a standard solution and defined arbitrarily as the amount of carbohydrate on the column that produced a signal-to-noise ratio of 5.

(a) *Free carbohydrates.*—Into a 100 mL volumetric flask, 500 mg finely ground sample was weighed to the nearest 0.1 mg. About 70 mL water was added and carbohydrates were extracted at 70°C for 30 min. The solution was cooled to room temperature, diluted to 100 mL with water, and treated the same way as for the soluble coffee analysis.

(b) *Total carbohydrates.*—Procedure as for soluble coffee was followed except that a test portion of 500 mg finely ground sample was used with a hydrolysis time of 240 min. Because of a difference in polysaccharide susceptibility to hydrolysis (matrix effect) compared with soluble coffee, hydrolysis time of coffee husks was increased for optimal recovery of carbohydrates.

Results and Discussion

Method Evaluation

In the last few years, AE–PAD chromatography has been applied to the determination of carbohydrates in a wide range of foods. The basis of the technique was reviewed elsewhere (14–16). Briefly, the unique resolution capacity of a pellicular AE polystyrene–divinylbenzene column is combined with the sensitivity, specificity, and reliability of a PAD.

Carbohydrate analysis by AE–PAD chromatography is usually performed in an alkaline medium, using NaOH as mobile phase. Depending on the NaOH concentration, all important monosaccharides and some sugar alcohols (mannitol, sorbitol, and xylitol) can be completely separated (14–16). However, sucrose is poorly separated from xylose and mannose even when very dilute NaOH is used. At higher pH, sucrose is well-resolved, but the 2 monosaccharides coelute. These elution patterns are obviously not suitable to establish the carbohydrate

Table 2. Carbohydrate profile in soluble coffee by AE–PAD

Sample No.	Free carbohydrates, % db[a]							
	Mannitol	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose
1	0.02	0.98	0.55	0.09	0.00	0.00	0.56	0.14
2	0.03	1.47	0.35	0.04	0.08	0.00	0.14	0.00
3	0.01	1.28	0.33	0.03	0.04	0.00	0.14	0.00
4	0.06	0.91	0.44	0.16	0.19	0.00	0.39	0.33
5	0.03	1.26	0.57	0.14	0.07	0.00	0.65	0.15
6	0.07	1.14	0.51	0.08	0.12	0.00	0.43	0.20
7	0.07	0.60	0.28	0.11	0.39	0.00	0.11	0.24
8	0.10	0.66	0.37	0.17	0.08	0.00	0.80	0.37
9	0.01	0.95	0.35	0.05	0.09	0.00	0.21	0.00
10	0.03	1.07	0.34	0.06	0.07	0.00	0.16	0.00
11	0.06	0.63	0.36	0.17	0.15	0.00	0.96	0.40
12	0.05	0.91	0.67	0.12	0.00	0.00	0.95	0.26
13	0.08	0.98	0.77	0.15	0.04	0.00	1.04	0.31
14	0.06	1.04	0.72	0.16	0.05	0.00	1.04	0.29
15	0.13	0.63	0.50	0.17	0.04	0.00	1.23	0.45
16	0.09	0.60	0.43	0.15	0.00	0.00	1.49	0.38
17	0.06	0.85	0.51	0.13	0.11	0.00	0.56	0.23
18	0.06	1.09	0.70	0.15	0.06	0.00	1.02	0.29
19	0.02	1.06	0.46	0.05	0.06	0.00	0.39	0.00
20	0.06	1.06	0.38	0.04	0.08	0.00	0.30	0.04
21	0.02	0.77	0.29	0.03	0.04	0.00	0.21	0.00
22	0.05	1.25	0.55	0.05	0.07	0.00	0.38	0.06
23	0.05	1.21	0.43	0.06	0.21	0.00	0.33	0.08
24	0.06	0.73	0.47	0.08	0.00	0.00	0.58	0.00
25	0.04	1.04	0.37	0.00	0.00	0.00	0.20	0.00
26	0.09	1.05	0.43	0.00	0.00	0.00	0.34	0.00
27	0.10	0.71	0.63	0.26	0.32	0.05	0.65	0.36
28	0.09	0.89	0.54	0.11	0.08	0.04	0.65	0.32
29	0.03	1.31	0.49	0.15	0.00	0.00	0.31	0.00
30	0.03	1.17	0.56	0.08	0.15	0.04	0.35	0.00
31	0.10	1.02	0.48	0.25	0.26	0.00	0.58	0.00
32	0.03	0.68	0.38	0.10	0.07	0.00	0.37	0.16
33	0.01	0.62	0.31	0.03	0.08	0.01	0.15	0.00
34	0.02	0.66	0.15	0.03	0.16	0.00	0.09	0.04
35	0.03	1.38	0.35	0.12	0.38	0.00	0.20	0.28
36	0.05	0.68	0.56	0.09	0.05	0.00	0.57	0.21
37	0.26	1.02	0.41	0.05	0.00	0.00	0.32	0.00

Total carbohydrates, % db					
Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose
0.18	2.64	19.30	0.76	0.08	18.00
0.16	3.67	19.70	0.98	0.12	14.00
0.12	3.62	18.20	0.74	0.11	11.80
0.13	3.27	20.90	1.51	0.17	9.70
0.17	3.03	19.30	1.06	0.16	15.00
0.10	4.67	18.40	1.22	0.17	11.40
0.08	4.35	21.90	1.18	0.17	4.83
0.12	3.59	17.10	0.91	0.12	13.60
0.16	3.37	19.90	0.89	0.15	16.00
0.17	3.40	18.30	0.85	0.14	15.30
0.19	3.54	17.88	1.02	0.14	16.55
0.19	3.09	17.90	0.83	0.08	17.50
0.23	3.25	17.70	1.05	0.15	18.10
0.22	3.28	17.50	1.07	0.12	17.40
0.30	3.10	16.70	0.99	0.15	19.30
0.23	3.06	16.00	0.92	0.11	19.90
0.17	3.97	20.60	1.34	0.19	11.90
0.14	2.46	13.20	0.64	0.07	11.70
0.16	3.85	18.20	0.75	0.13	15.30
0.19	3.90	16.20	0.83	0.11	15.90
0.18	3.89	15.80	0.68	0.10	15.80
0.20	4.36	22.40	0.96	0.12	16.30
0.21	4.54	20.00	1.02	0.14	15.50
0.20	3.09	15.60	0.65	0.10	17.60
0.19	4.05	15.60	0.61	0.09	14.40
0.12	5.08	23.60	0.54	0.21	10.70
0.14	3.90	16.80	1.35	0.21	12.10
0.13	4.52	21.30	1.58	0.29	13.00
0.13	5.16	21.60	0.68	0.24	12.90
0.09	4.41	21.80	0.78	0.23	15.80
0.36	3.53	18.20	0.75	0.29	9.52
0.12	3.57	22.10	0.76	0.17	12.00
0.11	5.07	23.50	0.84	0.23	10.90
0.10	5.79	21.80	1.03	0.17	9.84
0.10	5.67	21.80	1.08	0.22	10.00
0.16	3.19	18.40	0.89	0.08	13.70
0.17	4.10	20.30	1.06	0.18	15.20

Table 2. ***(continued)***

Sample No.	Free carbohydrates, % db[a]							
	Mannitol	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose
38	0.07	0.74	0.49	0.19	0.08	0.00	0.50	0.62
39	0.03	0.10	0.11	0.03	0.02	0.00	0.06	0.07
40	0.11	0.87	0.56	0.28	0.00	0.00	0.49	0.30
41	0.12	0.88	0.64	0.28	0.06	0.00	0.55	0.61
42	0.20	1.37	0.76	0.28	0.26	0.00	0.55	0.49
43	0.10	1.90	1.10	0.19	0.00	0.00	0.71	0.29
44	0.14	1.24	0.71	0.29	0.28	0.00	0.51	0.47
45	0.16	1.31	0.81	0.30	0.37	0.03	0.52	0.78
46	0.17	1.28	0.77	0.30	0.57	0.19	0.51	0.76
47	0.41	0.87	0.53	1.17	0.25	0.00	0.56	2.28
48	0.34	0.42	0.25	0.46	0.14	0.00	0.17	0.83
49	0.41	0.99	0.60	0.79	0.00	0.00	0.40	1.25
50	0.52	1.01	0.62	0.86	0.00	0.00	0.43	1.45
51	0.54	0.95	0.61	0.77	0.00	0.00	0.41	1.26
52	0.51	1.01	0.61	0.80	0.00	0.00	0.41	1.29
53	0.87	1.18	0.71	0.95	0.24	0.00	0.57	1.66
54	1.16	1.42	0.87	1.01	0.24	0.00	0.56	1.50
55	0.72	0.78	0.40	1.64	0.76	0.00	0.32	3.66
56	0.35	0.59	0.47	0.17	0.14	0.03	0.29	0.18
57	0.73	1.22	0.46	1.01	0.90	0.04	0.50	2.04
58	1.17	0.85	0.46	0.73	0.18	0.10	0.26	0.87
59	0.07	0.13	0.09	0.68	3.58	0.00	0.15	0.19
60	0.14	0.56	0.34	1.72	3.94	0.00	0.74	1.61
61	0.08	0.59	0.38	2.11	4.23	0.00	0.19	1.04
62	0.09	0.86	0.33	2.20	2.48	0.00	0.60	0.85
63	1.55	0.48	0.15	1.99	1.29	0.07	0.42	4.95

[a] db, dry basis.

Total carbohydrates, % db					
Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose
0.12	4.00	18.80	1.33	0.17	10.60
0.17	3.30	19.20	1.03	0.41	10.90
0.14	4.22	18.20	1.88	0.39	9.48
0.16	4.16	17.60	1.72	0.37	9.41
0.25	4.41	18.40	1.49	0.40	9.39
0.18	4.43	29.60	1.27	0.39	18.90
0.26	4.44	18.80	1.54	0.44	9.61
0.26	4.68	19.30	1.67	0.57	10.10
0.25	4.51	18.90	1.66	0.51	10.00
0.46	4.33	18.54	2.79	0.71	8.68
0.64	4.66	15.60	3.40	0.61	6.47
0.52	4.97	21.00	2.38	0.67	11.00
0.61	5.05	20.60	2.34	0.64	10.80
0.62	5.07	21.00	2.24	0.64	10.50
0.60	4.85	20.00	2.16	0.62	10.00
0.98	3.98	16.30	2.46	0.56	7.89
1.06	3.96	15.40	2.63	0.64	7.35
0.79	3.43	16.40	3.90	0.66	5.33
0.34	3.28	18.70	2.01	1.83	7.51
0.77	5.55	15.10	3.28	1.73	5.67
1.13	4.13	10.30	3.10	3.15	4.34
0.11	1.66	8.82	42.50	0.17	4.29
0.13	2.41	12.90	16.10	0.13	8.92
0.10	2.01	10.50	31.20	0.11	7.28
0.12	2.93	14.30	22.70	0.14	7.13
1.69	3.64	8.02	16.20	1.62	2.60

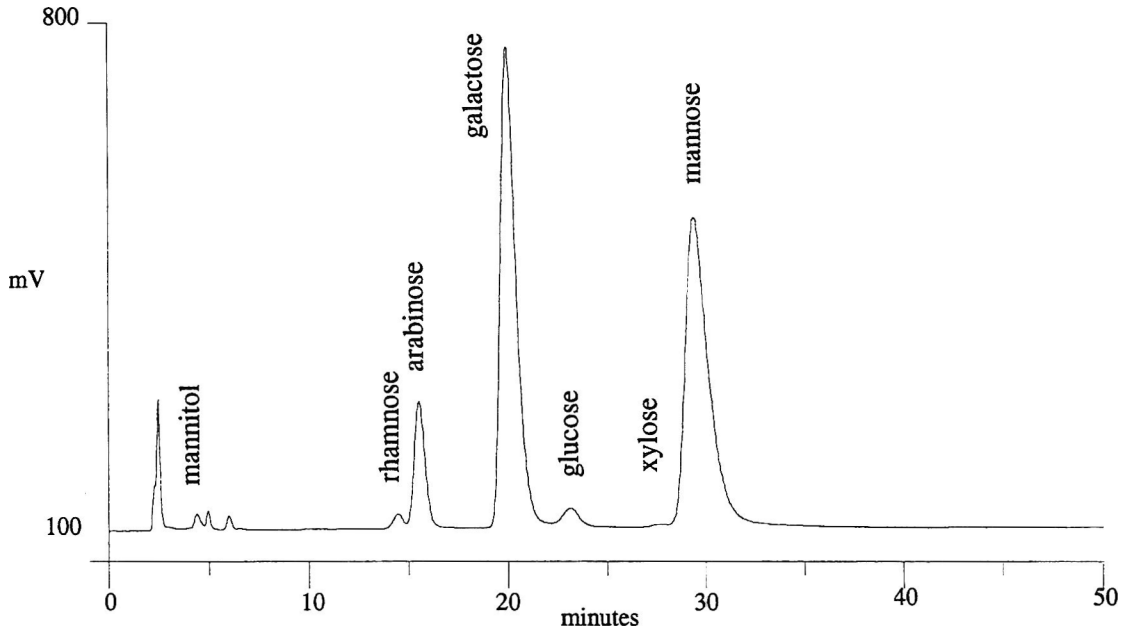

Figure 2. AE–PAD chromatogram of typical free carbohydrate profile of a pure soluble coffee (sample 1).

profile of products such as soluble coffee, in which all 3 sugars are present simultaneously.

We completely separated the major carbohydrates present in soluble coffee in less than 50 min by elution with pure water (Figure 1). Retention time, relative response factor, linearity range, and detection limit for each carbohydrate are given in Table 1.

A drift of retention times to lower values together with a significant loss of resolution were observed while analyzing soluble coffee samples. To assure stable retention times and reproducible resolution, column cleanup and reequilibration steps were introduced after each acquisition step.

Analysis of Soluble Coffee

The free and total carbohydrate profiles were determined according to the described AE–PAD chromatography method in 63 commercial soluble coffees of different origins. The free carbohydrate profile was obtained after a simple dissolution of the product in water and a cleanup of the solution through a C_{18} disposable cartridge. The total carbohydrate profile was measured after strong hydrolysis of the product with 1.00N HCl (4). Subsequent neutralization was performed by filtration of the hydrolyzate through an AE disposable cartridge. Results are given in Table 2. Because fructose is decomposed to a large

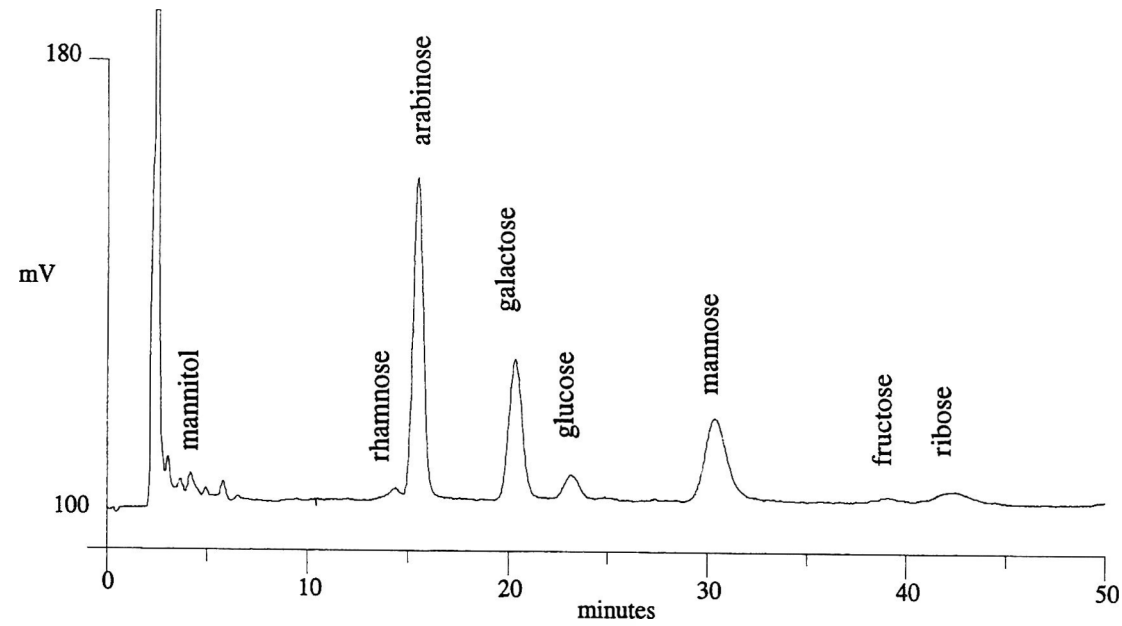

Figure 3. AE–PAD chromatogram of typical total carbohydrate profile of a pure soluble coffee (sample 1).

Table 3. Carbohydrate profile in Brazilian coffee husks/parchments by AE–PAD

Sample type	Sample No.	Free carbohydrates, % db[a]							Total carbohydrates, % db					
		Mannitol	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose
Arabica	1	—	—	—	—	—	—	—	—	1.86	4.51	2.40	6.64	5.93	1.00
	2	1.31	0.00	0.00	8.40	2.98	0.00	0.00	13.70	1.48	4.04	2.19	11.30	5.24	0.63
	3	1.17	0.00	0.00	8.60	3.08	0.00	0.00	14.00	1.53	4.29	2.26	11.50	4.67	0.63
	4	1.26	0.00	0.00	8.35	3.20	0.00	0.00	13.80	1.40	4.23	2.35	11.00	5.26	0.70
	5	1.43	0.00	0.00	8.97	3.33	0.00	0.00	14.70	1.52	4.16	2.13	11.40	4.97	0.52
	6	1.31	0.00	0.00	8.72	3.27	0.00	0.00	14.20	1.41	3.85	2.09	11.20	5.11	0.64
Robusta	7	0.19	0.02	0.02	2.40	0.27	0.00	0.02	3.29	0.21	3.74	3.39	3.43	5.26	0.61
	8	0.20	0.01	0.03	2.27	0.33	0.00	0.02	3.12	0.22	3.72	3.35	3.32	5.59	0.68
	9	0.18	0.02	0.02	2.15	0.25	0.00	0.03	3.04	0.21	3.72	3.47	3.34	4.87	0.77
	10	0.21	0.01	0.03	2.54	0.34	0.00	0.03	3.48	0.19	3.73	3.42	3.43	5.06	0.66
	11	0.18	0.01	0.03	2.72	0.34	0.00	0.03	3.69	0.19	3.49	3.05	3.59	4.96	0.52

[a] db, dry basis.

extent under the strong acidic conditions applied (4), no results were reported for total fructose. In certain chromatograms, rhamnose was not always clearly separated from arabinose. For that reason, the results reported for arabinose in Table 2 were in fact the sum of the 2 monosaccharides. However, the overestimation of arabinose is small because rhamnose is a minor carbohydrate in soluble coffee (5).

Four different types of soluble coffee could be clearly distinguished from the figures. Samples 1–38 were characterized by low free carbohydrate contents and high amounts of total galactose and mannose. These profiles are typical for pure soluble coffee (1, 3). Examples of chromatograms are given in Figures 2 and 3.

Samples 45–58 presented high levels of free mannitol, free fructose, free glucose, total glucose, and total xylose compared with pure soluble coffee. These results are typical for products obtained by coextraction of roasted coffee beans with coffee husks or parchments (1, 3). High levels of sucrose were also found in samples 46, 55, and 57. Separate analyses of arabica and robusta sun-dried coffee husks (Table 3) (1, 8), in which all these carbohydrates are present in high quantities, support this conclusion. Figures 4 and 5 show the chromatograms obtained for sample 58 before and after hydrolysis, respectively.

Intermediate levels of total glucose (1.03–1.88%) and total xylose (0.37–0.44%) were found in samples 39–44. These soluble coffees may also have been adulterated with coffee husks or parchments, but certainly to a lesser extent than those described above.

Samples 59–62 contained high amounts of free fructose (except sample 59), free glucose, and sucrose as well as huge quantities of total glucose. These products were obtained by coextraction of roasted coffee beans with, probably, starch-containing substitutes (cereals or malt) and caramelized sugar. The presence of such soluble coffee was already reported (1, 3). Free and total carbohydrate profiles of sample 61 are shown in Figures 6 and 7, respectively.

Finally, sample 63 exhibited unusually high levels of free mannitol, free fructose, free glucose, sucrose, total xylose, and very large amounts of total glucose (Figures 8 and 9). This finding indicates a fraudulent addition of coffee husks or parchments and cereals.

Comparison of Methods

Samples of each type of soluble coffee were analyzed additionally by an enzymatic procedure (4) and by a different LC method (1). For the LC method, carbohydrates were separated on an amino-bonded phase and spectrophotometrically detected after postcolumn derivatization with tetrazolium blue (LC–TTB). Table 4 compares the results of the 2 methods with those determined by AE–PAD chromatography.

Because only reducing sugars are derivatized with TTB, the determination of sucrose and mannitol is precluded by LC–TTB. On the other hand, the availability of pure, specific enzymes limits enzymatic determination to glucose, fructose, sucrose, and mannose only. However, the complete separation and detection of all these carbohydrates was achieved by AE–PAD chromatography.

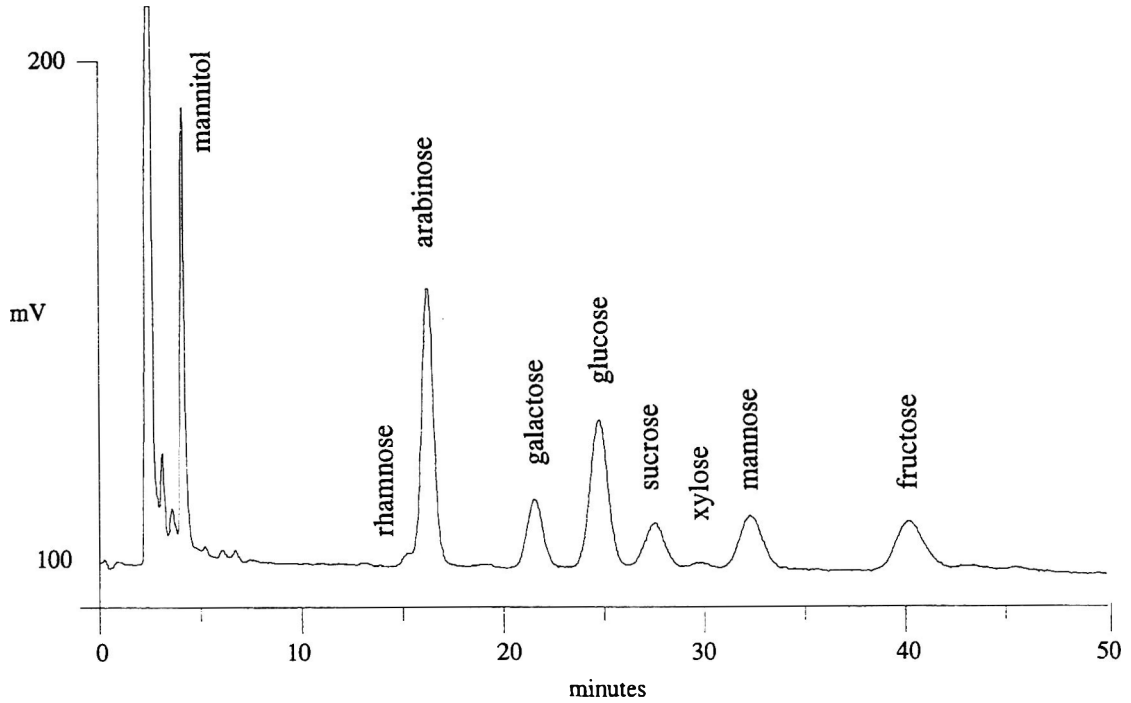

Figure 4. AE–PAD chromatogram of free carbohydrate profile of a soluble coffee containing coffee husks or parchments (sample 58).

For those carbohydrates where a comparison was possible, *t*-tests of the data showed that AE–PAD chromatography gave significantly higher results than LC–TTB for free arabinose and higher results than the enzymatic procedure for free mannose. However, the differences between the results were rather small and constant (0.10 and 0.05% for free arabinose and free mannose, respectively). In all other cases, the differences between AE–PAD chromatography and LC–TTB and between AE–PAD chromatography and enzymatic determinations were not significant at a 95% confidence level. The results of the 3 methods were, therefore, in close agreement even if different hydrolysis conditions were used in LC–TTB (1N H_2SO_4, 100°C, and 4 h).

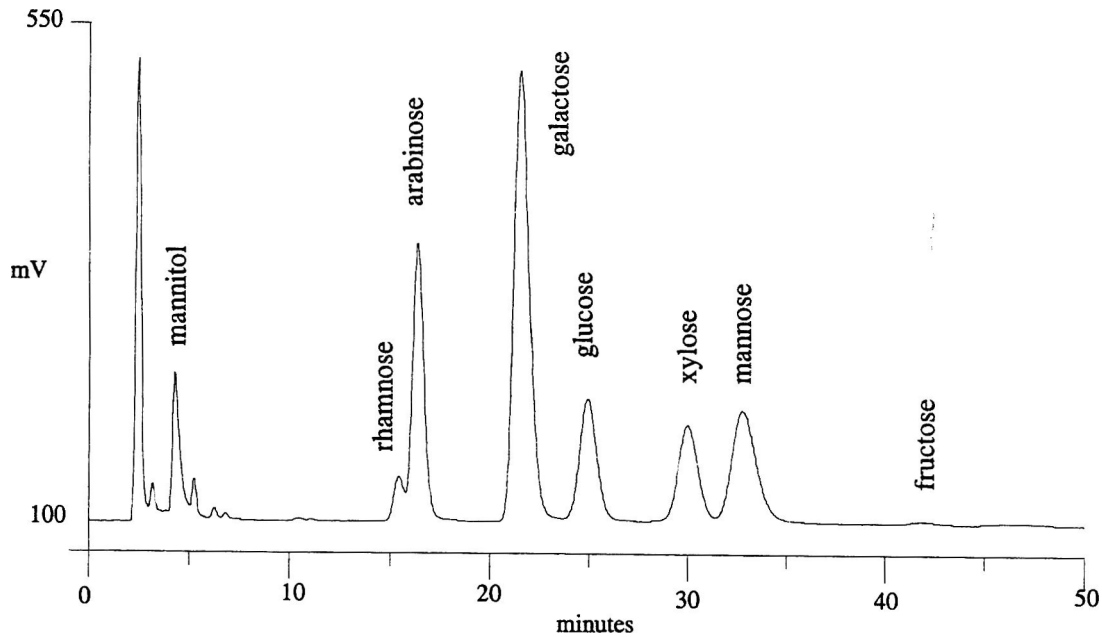

Figure 5. AE–PAD chromatogram of total carbohydrate profile of a soluble coffee containing coffee husks or parchments (sample 58).

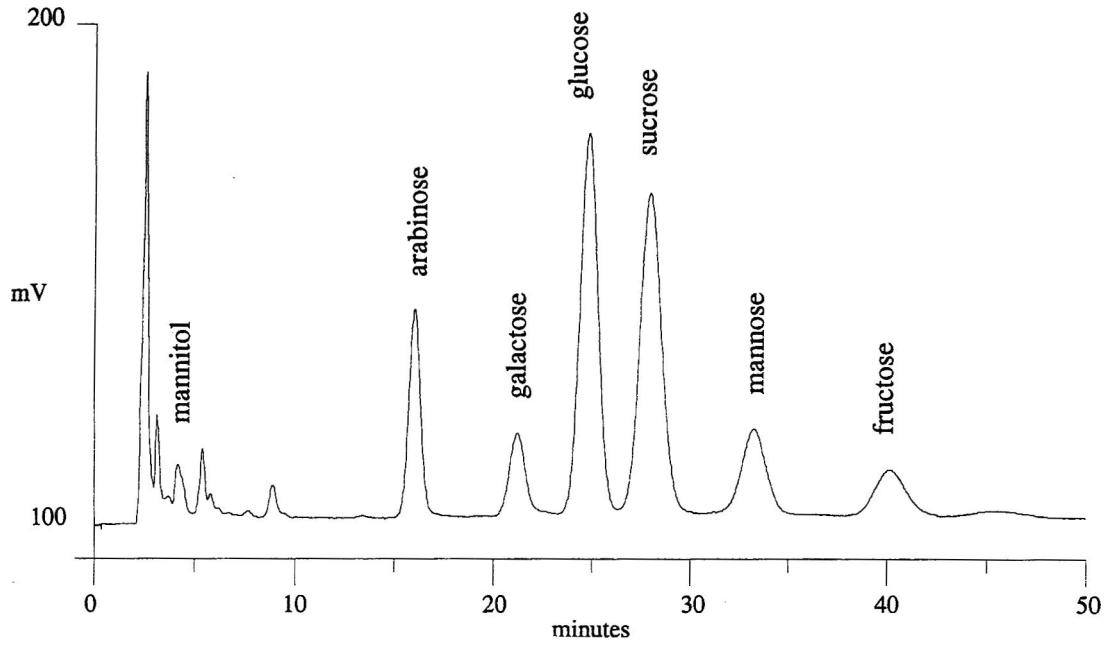

Figure 6. AE–PAD chromatogram of free carbohydrate profile of a soluble coffee containing cereals or caramelized sugar (sample 61).

The precision of AE–PAD chromatography was superior to that of the other 2 methods. The mean repeatabilities (r) of the measurements, calculated from the differences between the duplicates, were 0.03% for free carbohydrates and 0.11% for total carbohydrates, whereas the mean repeatability coefficients of variation (CV_r) were 2.23 and 1.70%, respectively. LC–TTB and enzymatic determinations were, respectively, 3 and 2 times less precise than AE–PAD chromatography.

Conclusions

The AE–PAD procedure allows the determination of all major carbohydrates present in soluble coffee in a single run. The technique is precise and sensitive. It enables the detection of fraudulent addition of cheaper coffee substitutes in commercial products. High levels of free mannitol and total xylose indicate the presence of coffee husks or parchments, whereas so-called pure soluble coffees containing cereals or caramelized sugar

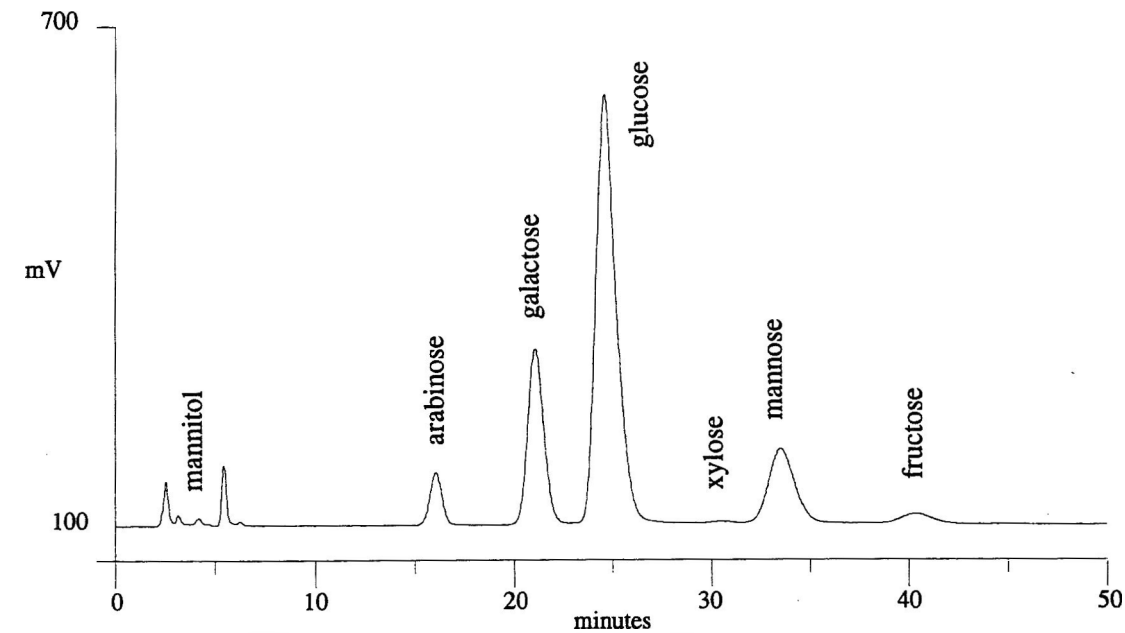

Figure 7. AE–PAD chromatogram of total carbohydrate profile of a soluble coffee containing cereals or caramelized sugar (sample 61).

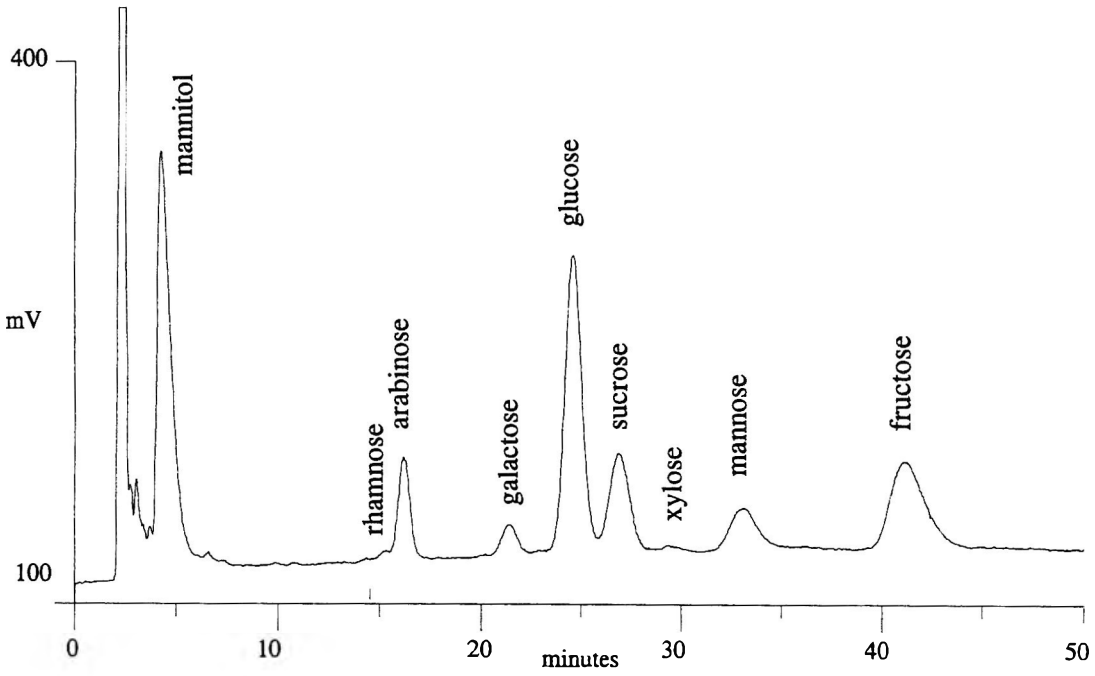

Figure 8. AE–PAD chromatogram of free carbohydrate profile of a soluble coffee containing coffee husks or parchments and cereals (sample 63).

show very large amounts of total glucose. Free fructose, free glucose, and sucrose are also good tracers of adulteration. AE–PAD chromatography is a very powerful tool for routine analysis and for purity assessment of soluble coffee.

Acknowledgments

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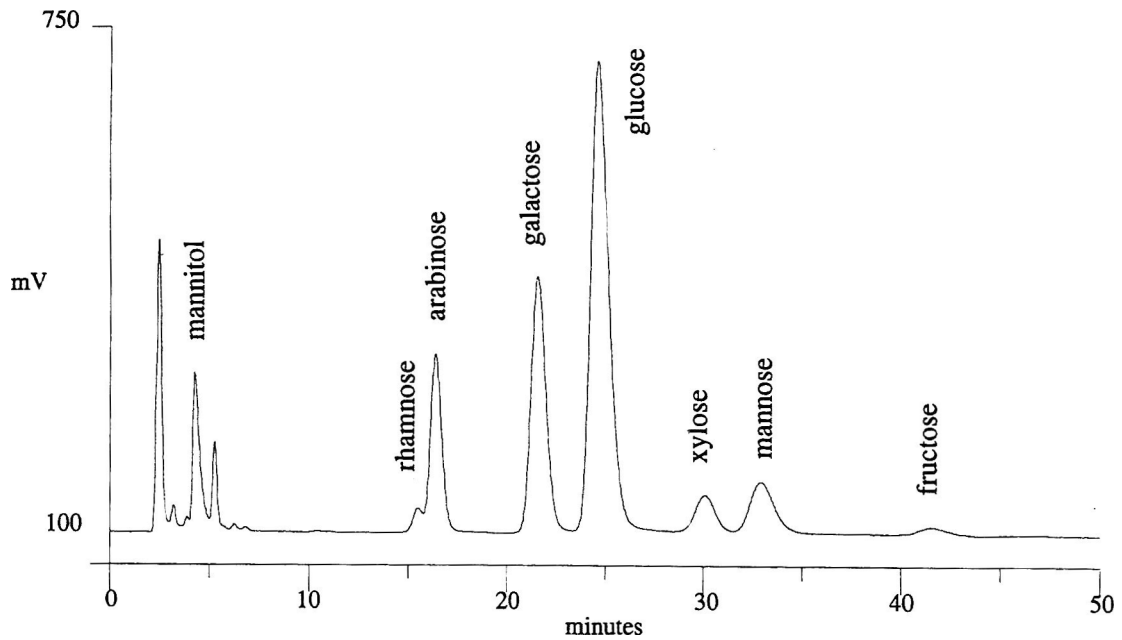

Figure 9. AE–PAD chromatogram of total carbohydrate profile of a soluble coffee containing coffee husks or parchments and cereals (sample 63).

Table 4. Carbohydrate profile in soluble coffee: comparison of AE–PAD, LC–TTB, and enzymatic methods

Sample	Method	Free carbohydrates, % db[a]								Total carbohydrates, % db					
		Mannitol	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose
1	AE–PAD	0.02	0.98	0.55	0.09	0.00	0.00	0.56	0.14	0.18	2.64	19.30	0.76	0.08	18.00
	LC–TTB	—	0.73	0.44	0.15	—	0.00	0.45	0.14	—	0.70	16.00	0.59	0.09	16.20
	Enzymatic	—	—	—	0.09	0.00	—	0.52	0.15	—	—	—	0.67	—	17.70
2	AE–PAD	0.03	1.47	0.35	0.04	0.08	0.00	0.14	0.00	0.16	3.67	19.70	0.98	0.12	14.00
	LC–TTB	—	1.23	0.31	0.13	—	0.00	0.16	0.07	—	4.36	16.90	0.81	0.13	13.40
	Enzymatic	—	—	—	0.03	0.06	—	0.13	0.04	—	—	—	0.83	—	13.30
3	AE–PAD	0.01	1.28	0.33	0.03	0.04	0.00	0.14	0.00	0.12	3.62	18.20	0.74	0.11	11.80
	LC–TTB	—	1.16	0.32	0.05	—	0.00	0.14	0.04	—	4.18	17.30	0.73	0.12	11.60
	Enzymatic	—	—	—	0.01	0.00	—	0.14	0.02	—	—	—	0.62	—	11.60
4	AE–PAD	0.06	0.91	0.44	0.16	0.19	0.00	0.39	0.33	0.13	3.27	20.90	1.51	0.17	9.70
	LC–TTB	—	0.82	0.43	0.21	—	0.00	0.38	0.39	—	3.52	18.20	1.31	0.15	9.25
	Enzymatic	—	—	—	0.14	0.24	—	0.32	0.38	—	—	—	1.27	—	8.76
5	AE–PAD	0.03	1.26	0.57	0.14	0.07	0.00	0.65	0.15	0.17	3.03	19.30	1.06	0.16	15.00
	LC–TTB	—	1.18	0.53	0.13	—	0.00	0.62	0.21	—	2.98	16.80	0.76	0.13	16.30
	Enzymatic	—	—	—	0.11	0.05	—	0.55	0.18	—	—	—	0.91	—	14.90
6	AE–PAD	0.07	1.14	0.51	0.08	0.12	0.00	0.43	0.20	0.10	4.67	18.40	1.22	0.17	11.40
	LC–TTB	—	1.21	0.49	0.08	—	0.00	0.43	0.25	—	4.24	20.70	1.35	0.22	11.10
	Enzymatic	—	—	—	0.04	0.22	—	0.34	0.18	—	—	—	1.27	—	11.00
7	AE–PAD	0.07	0.60	0.28	0.11	0.39	0.00	0.11	0.24	0.08	4.35	21.90	1.18	0.17	4.83
	LC–TTB	—	0.68	0.31	0.16	—	0.00	0.16	0.21	—	4.14	24.60	1.31	0.24	5.43
	Enzymatic	—	—	—	0.09	0.47	—	0.06	0.17	—	—	—	1.24	—	5.33
8	AE–PAD	0.10	0.66	0.37	0.17	0.08	0.00	0.80	0.37	0.12	3.59	17.10	0.91	0.12	13.60
	LC–TTB	—	0.72	0.37	0.16	—	0.00	0.82	0.39	—	3.13	18.80	0.96	0.18	14.40
	Enzymatic	—	—	—	0.14	0.18	—	0.73	0.37	—	—	—	0.93	—	14.70
9	AE–PAD	0.01	0.95	0.35	0.05	0.09	0.00	0.21	0.00	0.16	3.37	19.90	0.89	0.15	16.00
	LC–TTB	—	0.81	0.28	0.04	—	0.00	0.21	0.06	—	3.53	17.50	0.83	0.14	13.80
	Enzymatic	—	—	—	0.03	0.10	—	0.21	0.07	—	—	—	0.75	—	15.50
10	AE–PAD	0.03	1.07	0.34	0.06	0.07	0.00	0.16	0.00	0.17	3.40	18.30	0.85	0.14	15.30
	LC–TTB	—	0.94	0.31	0.07	—	0.00	0.20	0.05	—	3.75	17.60	0.77	0.12	14.20
	Enzymatic	—	—	—	0.03	0.06	—	0.15	0.08	—	—	—	0.74	—	14.20
43	AE–PAD	0.10	1.90	1.10	0.19	0.00	0.00	0.71	0.29	0.18	4.43	29.60	1.27	0.39	18.90
	LC–TTB	—	1.71	0.88	0.16	—	0.00	0.69	0.28	—	4.20	31.20	1.33	0.41	19.90
	Enzymatic	—	—	—	0.13	0.07	—	0.63	0.27	—		—	—	1.33	—
53	AE–PAD	0.87	1.18	0.71	0.95	0.24	0.00	0.57	1.66	0.98	3.98	16.30	2.46	0.56	7.89
	LC–TTB	—	1.23	0.77	1.14	—	0.00	0.58	1.67	—	3.84	18.10	2.95	0.68	7.86
	Enzymatic	—	—	—	0.89	0.29	—	0.44	1.46	—	—	—	2.47	—	7.52

Table 4. ***(continued)***

Sample	Method	Free carbohydrates, % db[a]								Total carbohydrates, % db					
		Mannitol	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose
54	AE–PAD	1.16	1.42	0.87	1.01	0.24	0.00	0.56	1.50	1.06	3.96	15.40	2.63	0.64	7.35
	LC–TTB	—	1.18	0.74	0.91	—	0.00	0.52	1.48	—	3.61	16.40	2.74	0.77	6.82
	Enzymatic	—	—	—	0.81	0.33	—	0.47	1.44	—	—	—	2.65	—	7.37
55	AE–PAD	0.72	0.78	0.40	1.64	0.76	0.00	0.32	3.66	0.79	3.43	16.40	3.90	0.66	5.33
	LC–TTB	—	0.68	0.43	1.57	—	0.00	0.31	2.85	—	3.20	15.30	4.09	0.43	4.47
	Enzymatic	—	—	—	1.70	0.75	—	0.26	2.55	—	—	—	3.89	—	5.15
56	AE–PAD	0.35	0.59	0.47	0.17	0.14	0.03	0.29	0.18	0.34	3.28	18.70	2.01	1.83	7.51
	LC–TTB	—	0.44	0.42	0.18	—	0.00	0.28	0.28	—	3.20	14.70	1.72	1.40	6.74
	Enzymatic	—	—	—	0.17	0.21	—	0.25	0.27	—	—	—	1.80	—	7.17
58	AE–PAD	1.17	0.85	0.46	0.73	0.18	0.10	0.26	0.87	1.13	4.13	10.30	3.10	3.15	4.34
	LC–TTB	—	0.62	0.37	0.59	—	0.00	0.25	0.91	—	3.90	8.75	3.02	2.21	3.59
	Enzymatic	—	—	—	0.82	0.03	—	0.30	1.11	—	—	—	2.86	—	3.82
59	AE–PAD	0.07	0.13	0.09	0.68	3.58	0.00	0.15	0.19	0.11	1.66	8.82	42.50	0.17	4.29
	LC–TTB	—	0.10	0.12	0.77	—	0.00	0.15	0.36	—	1.49	7.52	40.40	0.15	3.55
	Enzymatic	—	—	—	0.76	4.01	—	0.06	0.36	—	—	—	41.40	—	3.87
63	AE–PAD	1.55	0.48	0.15	1.99	1.29	0.07	0.42	4.95	1.69	3.64	8.02	16.20	1.62	2.60
	LC–TTB	—	0.42	0.21	1.96	—	0.00	0.43	3.53	—	3.78	7.43	17.80	1.25	2.41
	Enzymatic	—	—	—	2.04	1.84	—	0.44	3.71	—	—	—	16.60	—	2.36

[a] db, dry basis.

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FOOD COMPOSITION AND ADDITIVES

Adulteration of Soluble Coffee with Coffee Husks and Parchments

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Commercial soluble coffee can be adulterated with coffee husks or parchments. Xylose is a good tracer for this type of mispractice. The analysis of total xylose in a wide selection of green beans and the assessment of its fate during processing allowed the derivation of a maximum total xylose limit of 0.40%, above which a soluble coffee should be considered as adulterated. Out of the 700 commercial soluble coffees analyzed, 81 exhibited a total xylose level above this limit. Of the samples with total xylose level lower than the limit, 99% displayed concentrations in free mannitol and total glucose below 0.30 and 2.10%, respectively.

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A coffee cherry consists of 6 different components (Figure 1): the red (or yellow) "skin" or exocarp, the yellow-white "pulp" or mesocarp, the pale yellow "parchment" or endocarp, the yellow-white "mucilage," the silver spermoderm or "silverskin," and the brown-yellow to bluish-green "bean" or endosperm. Skin, pulp, and parchment constitute the pericarp. When dry, the pericarp and the silverskin parts are called "husks" or "hulls," and they represent about half of the total dry matter of the cherry. Furthermore, a coffee cherry may contain 1, 2, or, rarely, more beans.

The International Coffee Organization (ICO), an intergovernmental body formed by the coffee producing and consuming countries in close cooperation with the United Nations, defines "green coffee" as "all coffee in the naked bean form before roasting," "roasted coffee" as "green coffee roasted to any degree," and "soluble coffee" as "dried water-soluble solids derived from roasted coffee" (1). These definitions clearly

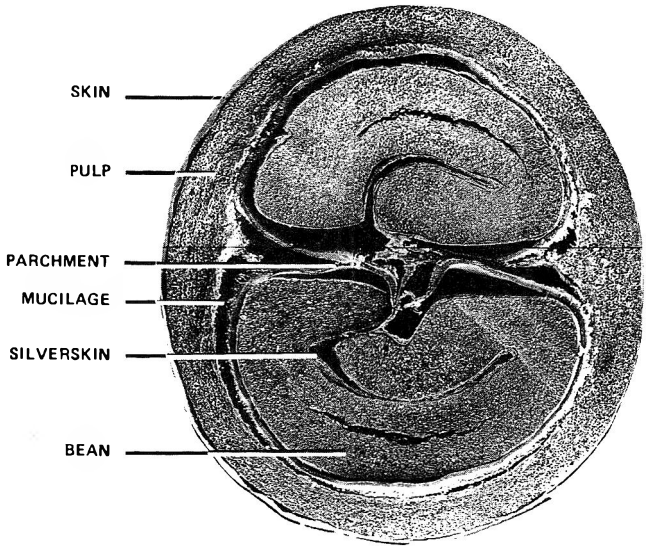

Figure 1. Transverse section of a coffee cherry containing 2 beans.

establish a direct link between naked green beans and soluble (instant) coffee.

Other international bodies, such as the International Organization for Standardization (ISO), have given equivalent definitions and codified the impurities or "defects" such as wood, sticks, husks, parchment, or whole cherries, that may be present (2). Most countries have stated the maximum amount of defects tolerated in commercial coffee (3).

Visual defect counting is traditionally used to assess the purity of green beans, but the technique is obviously inappropriate for soluble coffee. However, free and total (after strong or mild acidic hydrolysis) carbohydrate profiles of soluble coffee can be used for detecting and eventually characterizing fraudulent addition of coffee substitutes in commercial products (4–9). Possible adulterants include roasted or unroasted coffee husks or parchments, chicory, cereals, malt, starch, maltodextrins, glucose syrups, and caramelized sugar.

So far, liquid chromatography (LC), gas chromatography (GC), and enzymatic methods have been applied for determination of the carbohydrate profile of soluble coffee. However, anion-exchange chromatography with pulsed amperometric detection (AE–PAD) is certainly the simplest and most powerful technique (8, 9). For that reason, our method was submitted to a collaborative study (10) within the Association of Soluble Coffee Manufacturers of the European Community (AFCASOLE), with the participation of the main soluble coffee manufacturers in Europe and the United States. Results showed that all carbohydrates at a concentration level higher than 0.3% can be determined with good precision (10).

Because adulteration with coffee husks or parchments seems to be relatively widespread, the first aim of this study was to propose a limit for total xylose, the best tracer (4, 8, 9), above which a soluble coffee should be considered as adulterated. For this purpose, all total xylose results on green coffee, roasted coffee, and commercial soluble coffee obtained in 3 laboratories (all participants in the collaborative study [10]) with the above-mentioned AE–PAD procedure were gathered and discussed. Furthermore, the fate of xylose during the roasting and extraction steps was assessed.

Experimental

Materials

(a) *Green coffee.*—The 25 samples analyzed in this study were arabica and robusta from different countries.

(b) *Roasted coffee.*—The 21 samples analyzed in this study were arabica and robusta from different countries and were of different roasting degrees.

(c) *Soluble coffee.*—The 700 samples analyzed in this study were commercial products. All were sold as pure soluble coffee and covered almost the whole range of soluble coffee manufacturers and producing and consuming countries.

Roasting and Extraction

The conditions applied in the plant were as described in the Guggenheim and Stinchfield patent (11).

Analysis of Soluble Coffee

A detailed description of the AE–PAD method used for the determination of free and total carbohydrates is given elsewhere (10). Briefly, in the free carbohydrate procedure, a filtered aqueous extract of the sample is injected into an LC system. Carbohydrates are eluted on a pellicular anion-exchange column by using pure water as mobile phase and are detected by PAD. In the total carbohydrate procedure, the sample is hydrolyzed with 1.00N HCl at 100°C for 150 min.

Analysis of Green Coffee, Roasted Coffee, and Coffee Grounds

(a) *Free carbohydrates.*—Into a 100 mL volumetric flask, 500 mg finely ground sample was weighed to the nearest 0.1 mg. About 70 mL water was added and carbohydrates were extracted at 70°C for 30 min. The solution was cooled to room temperature, diluted to 100 mL with water, and treated the same way as in the soluble coffee analysis.

(b) *Total carbohydrates.*—Procedure was the same as for soluble coffee, except a test portion of 500 mg finely ground sample was used and the hydrolysis time for husks was increased to 240 min (9). The applied hydrolysis conditions (ca 1.00N HCl, 100°C, and 240 min) gave similar total xylose results to those obtained with more commonly used conditions in polysaccharide analysis (ca 72% H_2SO_4, 25°C, and 120 min followed by 2N H_2SO_4, 100°C, and 180 min) (12). Results are given in Table 1.

Color Measurement

Roasted coffee samples (30 g) were ground to a particle size between 400 and 600 μm. The color was measured on a Model Minolta II L100 Chroma Meter (3 filters; illuminant C; measuring diameter, 5 cm), calibrated with a white standard. The reported results are the average of triplicate measurements.

Table 1. Comparison of hydrolysis conditions for determination of total xylose

Sample	Total xylose, % db[a]	
	A	B
Green coffee		
Colombia	0.32	0.28
Kenya	0.24	0.30
Togo	0.31	0.27
Ivory Coast	0.25	0.23
Roasted coffee		
Brazil	0.24	0.23
Colombia	0.22	0.20
Ivory Coast	0.17	0.15
Coffee grounds		
Colombia	0.24	0.25

[a] db, dry basis. A, conditions used in our study (1N HCl, 100°C, and 240 min); B, conditions used in reference 12 (72% H_2SO_4, 25°C, and 120 min followed by 2N H_2SO_4, 100°C, and 180 min).

Results and Discussion

High levels of free fructose, free glucose, total fructose, total glucose, total xylose, sucrose, and mannitol are good indications of fraudulent addition of cheap coffee substitutes in soluble coffee (4, 6–9). In particular, total xylose is a very good tracer of the presence of coffee husks or parchments (4, 8, 9). To set up maximum limits for total xylose above which a soluble coffee should be considered as adulterated with coffee husks or parchments, xylose should ideally be analyzed in soluble coffees prepared from pure green coffee from different harvesting conditions, origins (geographical and botanical), and grades, under different roasting and extraction conditions. This sampling should be done to take into account all natural variations in green coffee composition and the respective influence of the different processing conditions currently applied within the coffee industry. Obviously, analysis of thousands of samples and a very large number of industrial trials would be required. Alternatively, we determined total xylose in a selection of green coffee. Furthermore, the fate of xylose during the roasting and extraction steps was separately assessed. The results allowed the derivation of a maximum total xylose limit of pure soluble coffee.

Analysis of Green Coffee

The total xylose content was determined according to the described AE–PAD method in 25 green coffees from different geographical and botanical origins. Results are presented in Table 2.

Levels of total xylose in green beans ranged from 0.18 to 0.35%. The average value, 0.26%, is very close to the 0.2% data reported by Bradbury and Halliday (13, 14). Presence of xylose traces can be attributed to very small amounts of coffee husk left on the beans after the cleaning process (6) and to unidentified endogenous polysaccharides (14). No difference was found in the levels of total xylose between arabica and robusta beans.

Table 2. Determination of total xylose in green coffee

Sample	Total xylose, % db[a]
Arabica	
Brazil 1, NY 2	0.32
Brazil 2, NY 2/3	0.23
Brazil 3, NY 4	0.27
Brazil 4, NY 4	0.23
Brazil 5, NY 4	0.25
Brazil 6, NY 6	0.25
Brazil 7, NY 6	0.25
Brazil 8, NY 8	0.29
Brazil 9, NY 8	0.23
Mexico 10	0.26
Mexico 11	0.28
Colombia 12	0.32
Colombia 13	0.23
Kenya 14	0.24
Robusta	
Brazil 15, grade 6	0.24
Brazil 16, grade 8	0.26
Brazil 17	0.24
Brazil 18	0.25
Brazil 19	0.24
Indonesia 20	0.30
Ouganda 21	0.31
Ivory Coast 22, grade 2	0.18
Ivory Coast 23	0.25
Togo 24	0.35
Togo 25	0.31

[a] db, dry basis; NY, New York grade.

Loss During Roasting

Arabica and robusta green beans were roasted to various degrees to determine the losses in total xylose. Results are shown in Table 3. Variations in roasting conditions were reflected by the organic loss (OL) values and by the color measurement results. An inverse correlation was observed between the 2 parameters. Results were all expressed on original, dry, green coffee basis to allow a true comparison between green and roasted coffee.

Xylose was fairly stable at a very light roast (OL <4%). However, up to 48% degradation was observed when beans were dark roasted. A wide range of roasting conditions are currently applied in the production of soluble coffee. The lighter ones usually correspond to OL values of about 5%. According to our figures, this value represents losses in total xylose of at least 20%.

This means that if a value of 0.40% is taken as the acceptable maximum content of total xylose in pure green coffee (0.05% above our highest figure; *see* Table 2), the maximum total xylose in roasted coffee should be 0.32% dry green coffee basis or 0.34% dry roasted coffee basis. The analysis of 21 pure

Table 3. Loss in total xylose during roasting

Sample	Form	Organic loss, %	Color, Minolta b*	Total xylose, % db green coffee[a]	Xylose loss, %
Arabica					
	Green			0.28	
Colombia	Roasted	6.71	16.0	0.20	28.6
	Green			0.32	
Brazil	Roasted	5.44	18.3	0.22	31.3
	Green			0.25	
Kenya	Roasted	3.80	20.1	0.23	8.0
	Green			0.23	
Colombia	Roasted	3.54	19.7	0.24	0
Robusta					
	Green			0.25	
Ivory Coast	Roasted	6.58	16.2	0.16	36.0
	Green			0.31	
Togo	Roasted	6.51	15.9	0.18	41.9
Arabica/robusta					
Guatemala/Togo	Green			0.27	
(60 + 40)	Roasted	8.25	7.7	0.14	48.1

[a] db, dry basis.

roasted coffees from different origins and roasting degrees (Table 4) gave credit to the proposed limits. Indeed the total xylose levels ranged from 0.15 to 0.33% dry basis roasted coffee and averaged 0.22%.

Table 4. Determination of total xylose in roasted coffee

Sample	Color, Minolta b*	Total xylose, % db[a]
Arabica		
Brazil 1, NY 4	21.0	0.16
Brazil 2, NY 6	21.7	0.19
Brazil 3, NY 8	21.4	0.28
Brazil 4	18.3	0.24
Brazil 5	8.5	0.22
Brazil 6	14.6	0.26
Brazil 7	10.0	0.24
Brazil 8	16.6	0.23
Brazil 9	13.5	0.19
Brazil 10	12.0	0.18
Brazil 11	10.7	0.25
Brazil 12	12.7	0.25
Brazil 13	11.7	0.27
Columbia 14	19.7	0.25
Kenya 15	20.1	0.24
Robusta		
Brazil 16, grade 6	21.3	0.33
Brazil 17, grade 8	22.6	0.18
Columbia 18	16.0	0.22
Togo 19	15.9	0.19
Ivory Coast 20	16.2	0.17
Arabica/robusta		
Guatemala/Togo (60 + 40) 21	7.7	0.15

[a] db, dry basis.

Extraction Recovery

Two samples of green coffee, one arabica and one arabica/robusta blend, were processed separately at the industrial level under different roasting and extraction conditions. Total xylose was analyzed at each stage of the process and in the coffee grounds to determine the extraction recovery in soluble coffee (Table 5). All results were expressed on original, dry, green coffee basis. Therefore, the sum of the total xylose content of the soluble coffee and that of the spent grounds should be equal to that of the corresponding roasted coffee, assuming that no degradation occurred during extraction. In our study, 35.0% (arabica) and 35.7% (arabica/robusta blend) of total xylose in roasted coffee was recovered in soluble coffee, and the rest was found in the spent grounds.

Table 5. Fate of total xylose during processing of soluble coffee

Sample	Organic loss, %	Extraction yield, %	Total xylose, % db green coffee[a]
Arabica			
Colombia			
Green	—	—	0.28
Roasted	6.71	—	0.20
Soluble	—	43.9	0.07
Spent grounds	—	56.1	0.13
Arabica/robusta			
Guatemala/Togo (60 + 40)			
Green	—	—	0.27
Roasted	8.25	—	0.14
Soluble	—	46.6	0.05
Spent grounds	—	53.4	0.11

[a] db, dry basis.

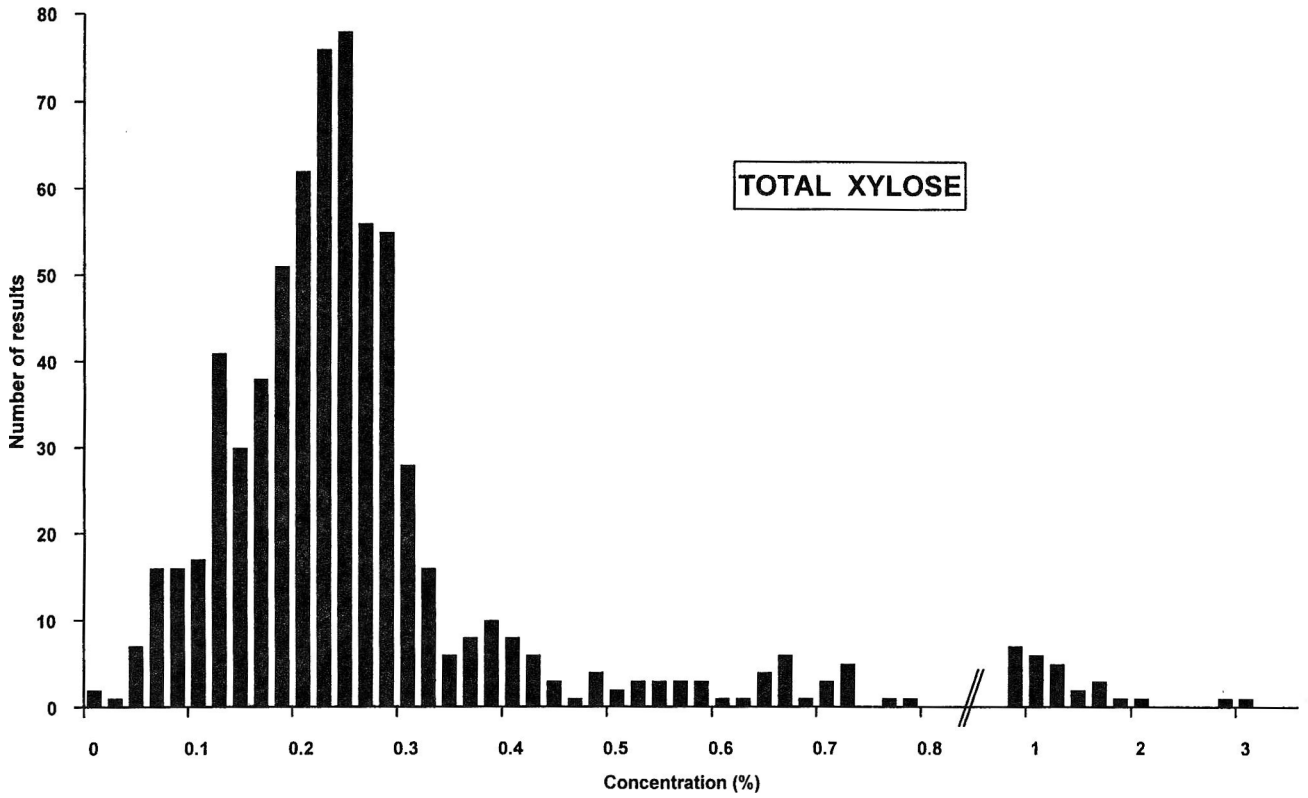

Figure 2. Determination of total xylose in commercial soluble coffee (700 results).

Limit for Total Xylose

Assuming that the maximum total xylose level in roasted coffee is 0.34% on dry roasted coffee basis, the extraction yield in the production of soluble coffee is at least 40%, and less than 40% total xylose is extracted in soluble coffee, the maximum content of total xylose in pure soluble coffee should be about 0.34% on dry soluble coffee basis. Because of the necessarily limited survey of coffee samples, some commercial, pure soluble coffees may have total xylose levels slightly higher than 0.34%. Therefore, allowing again for a small uncertainty margin, we propose that a value of 0.40% be adopted as an upper limit for total xylose, above which a soluble coffee should be considered as adulterated. Using another LC method and a smaller selection of samples, Blanc et al. (4) reported a maximum total xylose content of 0.3%.

Analysis of Commercial Soluble Coffee

A complete survey of the soluble coffee market was organized to evaluate the extent of adulteration practices. Total xylose was analyzed in 700 commercial samples, all of which were sold as pure soluble coffee. A histogram of the results is shown in Figure 2. Total xylose ranged from 0.01 to 3.15%, and most of the values were centered around 0.25%. Blanc et al. (4) reported a similar total xylose range of 0.11–1.95% after LC analysis of 122 commercial soluble coffee samples. Davis et al. (6) reported a 0–3.24% range for 145 samples. Both research groups used different LC procedures. Surprisingly, 81 samples exhibited a total xylose level exceeding the proposed limit and represented a large proportion (11.6%) of the total number of the soluble coffees analyzed.

Free mannitol was also reported to be a tracer for adulteration with coffee husks (6, 8, 9). Very low levels of the polyol, up to 0.05%, were detected in sound green coffee (6), whereas the polyol is present at relatively high concentrations, up to 2.03%, in dried coffee husks (6, 9). Mannitol was left undegraded under the roasting and extraction conditions normally used in soluble coffee processing. Free mannitol was, therefore, determined in the same set of 700 samples. Values ranged from 0.01 to 1.55%. A similar range of values, 0.02–1.85%, was obtained by Davis et al. (6) when analyzing a set of 145 commercial soluble coffees by a GC method. A histogram of the results is shown in Figure 3; the data obtained for the 81 samples with total xylose >0.40% were omitted. About 99% of the resulting 619 soluble coffees exhibited free mannitol levels below 0.30%. This finding gives additional credit to previous conclusions (6) that mannitol content exceeding 0.3% constitutes further evidence that a soluble coffee contains undeclared material. We suggest that this value should be adopted as an additional criterion for soluble coffee purity. Therefore, a soluble coffee should be considered as adulterated if either of the 2 limits for total xylose or mannitol is exceeded.

Finally, abnormally high concentrations of total glucose were generally associated with the presence of coffee husks or parchments (4, 6, 8, 9). However, maximum limits for total glucose cannot be set up with the same strategy as for total xylose. Indeed, glucose is a natural constituent of green coffee and is present at high concentrations, mainly under 2 hidden

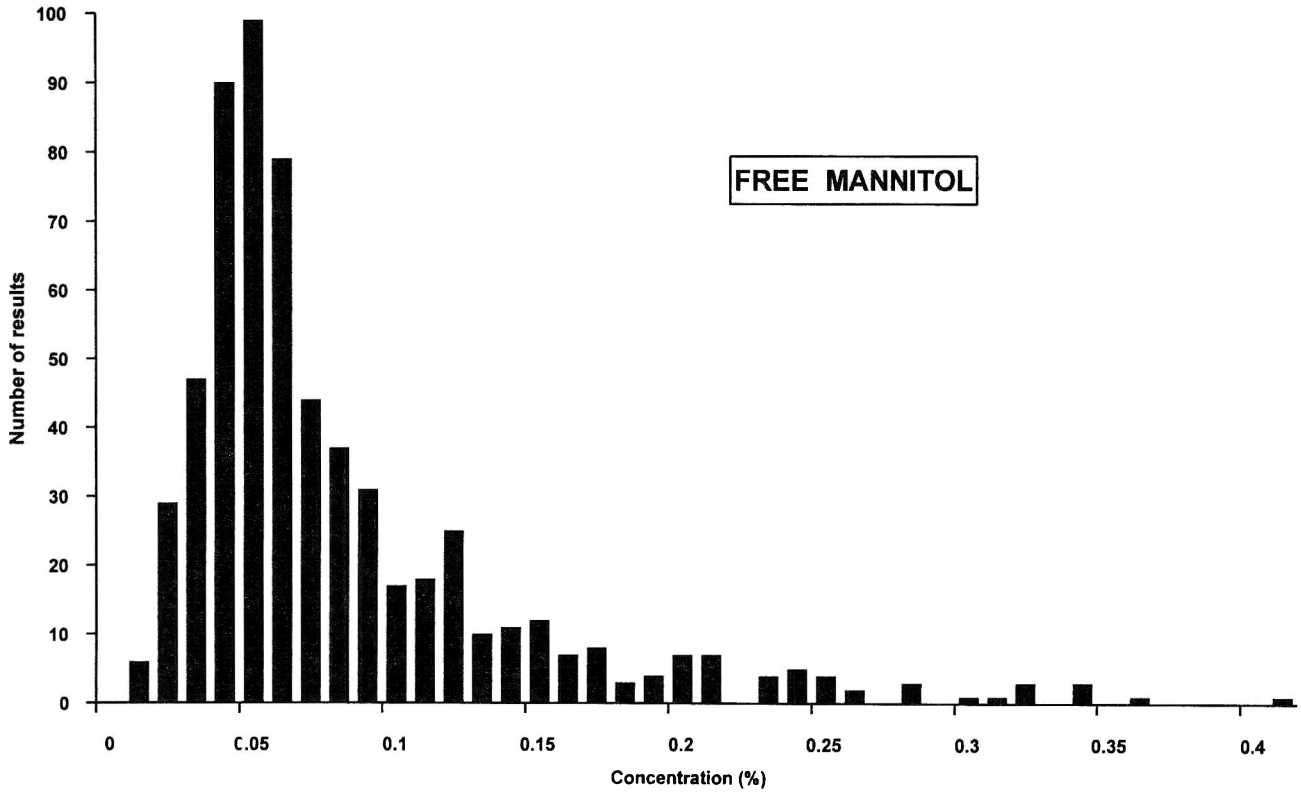

Figure 3. Determination of free mannitol in commercial soluble coffee (619 results). Results for products with total xylose levels >0.40% were omitted.

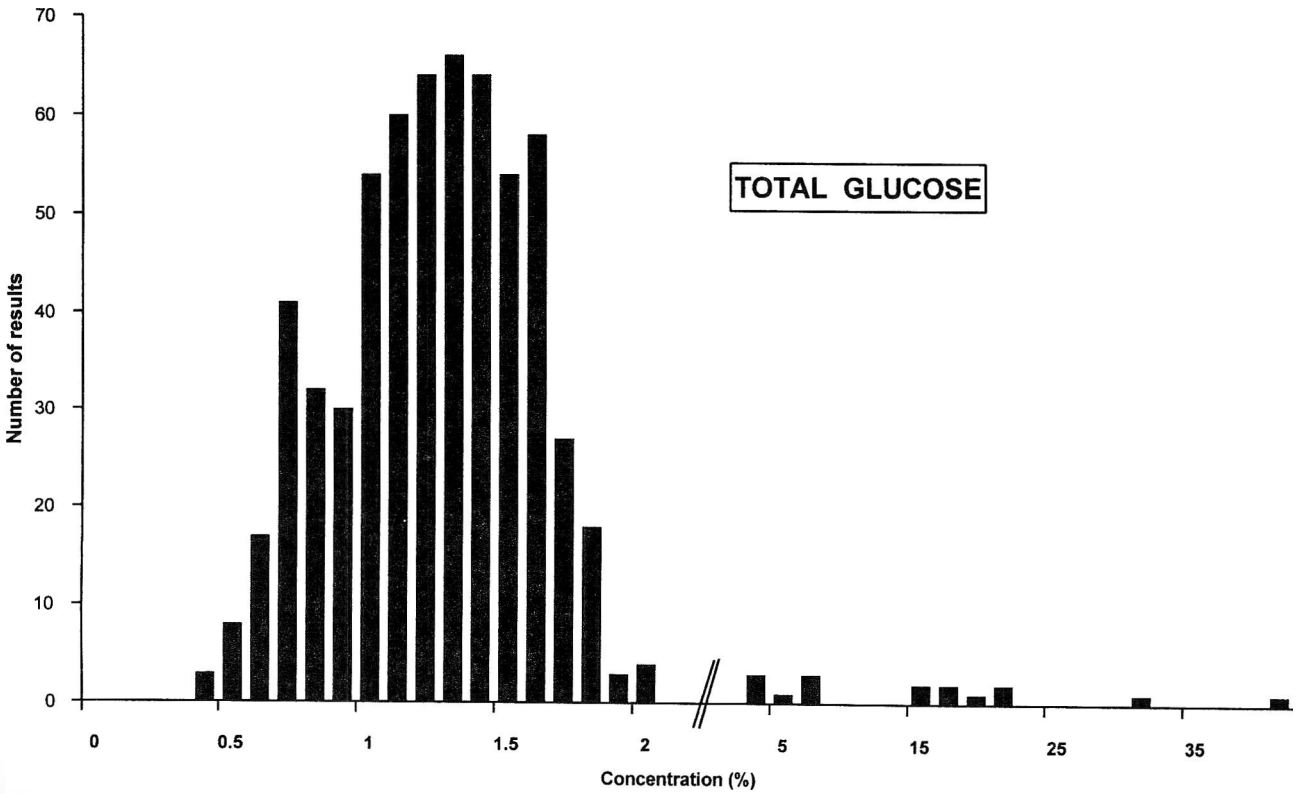

Figure 4. Determination of total glucose in commercial soluble coffee (619 results). Results for products with total xylose levels >0.40% were omitted.

forms: sucrose and cellulose. The potential level of total glucose in soluble coffee could, therefore, be very high if manufacturing conditions that avoided sucrose decomposition during roasting and that extracted all cellulosic material were used. However, in such a case, the total glucose concentration in soluble coffee would be extremely process-dependent. Nevertheless, total glucose was determined in the previously mentioned soluble coffees. Values ranged from 0.46 to 42.5%. A histogram of the results is shown in Figure 4; data obtained for the 81 samples with total xylose >0.40% were omitted. Total glucose concentration in 9 products was higher than 15.0%. These products were undoubtedly adulterated, most likely by coextraction of roasted coffee beans with starch-containing substitutes or caramelized sugar, or by addition of maltodextrins (4, 6–9). Total glucose level was lower than 2.10% in about 99% of the other products.

Conclusions

Fraudulent addition of coffee husks or parchments in soluble coffee can easily be detected by AE–PAD analysis of total xylose, free mannitol, and, to a certain extent, total glucose. Total xylose and free mannitol levels of 0.40 and 0.30%, respectively, are proposed as upper limits, above which a soluble coffee should be considered as adulterated. This adulteration practice is still rather widespread; about 12% of the 700 samples analyzed in this study were beyond the limits.

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FOOD COMPOSITION AND ADDITIVES

Determination of Carbohydrates in Soluble Coffee by Anion-Exchange Chromatography with Pulsed Amperometric Detection: Interlaboratory Study

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A collaborative study was conducted to validate a liquid chromatographic (LC) method to determine the free and total (after acid hydrolysis) carbohydrate profile of soluble coffee. Carbohydrates were separated on a pellicular anion-exchange column using pure water as mobile phase, and were detected by pulsed amperometry. Eleven collaborators were sent 6 test samples of commercial soluble coffee for duplicate analysis. They were also sent a practice sample with known levels of free and total carbohydrates and material for preparation of all standard solutions. The reproducibility relative standard deviations (RSD_R) were 9.9–59.5% for mannitol, 35.6–72.6% for fucose, 4.9–21.1% for arabinose, 4.1–13.0% for galactose, 6.1–24.3% for glucose, 10.0–41.6% for sucrose, 20.2–37.7% for xylose, 10.6–40.0% for mannose, 15.5–71.7% for fructose, and 17.8–97.9% for ribose. Precision in the determination of free and total carbohydrates was very similar. The average repeatability RSD_r and RSD_R values were 4.5 and 14.3%, respectively, for carbohydrate levels above 0.3%. The precision of the technique was considered good, regardless of the usual peak integration problems always encountered in LC, the low levels of free carbohydrates, the hydrolysis step, and the relative lack of experience of most participating laboratories. The method allows good and reproducible separation of all major carbohydrates found in soluble coffee and is, therefore, suitable for routine analysis.

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Anion-exchange chromatography with pulsed amperometric detection (AE–PAD) is a very powerful tool for determination of both free and total carbohydrates in soluble coffee (1, 2). The technique is simple and sensitive and allows a complete separation of all major carbohydrates in a single run. To evaluate the precision of the method, the Association of Soluble Coffee Manufacturers of the European Community (AFCASOLE) organized a collaborative study among 11 laboratories that were mainly from the food industry. We report the result of the study, which was designed according to international guidelines (3, 4).

Collaborative Study

The 11 collaborators participating in this study were analysts in food industry, research, and commercial laboratories representing 4 countries. Collaborators were sent 6 test samples of commercial soluble coffee from Japan, Korea, Australia, New Zealand, Switzerland, and the United Kingdom; 1 practice sample of soluble coffee with known levels of free and total carbohydrates; and material for the preparation of all carbohydrate standard solutions. The coffee powders and the carbohydrate standards were sent in air-tight containers, and the participants were advised to avoid prolonged exposure of these hygroscopic materials to the atmosphere.

The collaborators were first asked to familiarize themselves with the method on the practice sample. Once the target values were matched, they were further instructed to perform duplicate determinations of both free and total carbohydrates on each of the 6 test samples (4 analyses per sample). Results were

Table 1. Collaborative results (duplicate) of determination of carbohydrates in soluble coffee by AE–PAD

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
					Sample 1—free carbohydrates					
1	0.050	0.010	0.930	0.580	0.140	—	—	0.600	0.220	—
	0.050	—[b]	0.930	0.570	0.120	—	—	0.610	0.230	—
2	0.020	—	0.950	0.600	0.090	—	—	0.400	0.170	0.090
	0.020	—	0.960	0.610	0.100	—	—	0.390	0.180	0.080
3	—	—	0.773	0.551	0.110	—	—	0.561	0.169	0.063
	—	—	0.852	0.514	0.122	—	—	0.525	0.211	—
4	0.008	0.023	1.009	0.615	0.103	0.045	0.009	0.595	0.149	0.038[c]
	0.007	0.017	0.938	0.575	0.085	0.034	0.001	0.564	0.141	0.003[c]
5	0.050[d]	—	0.750	0.540	0.070	—	—	0.500	0.050	0.130
	0.060[d]	—	0.770	0.560	0.090	—	—	0.520	0.090	0.120
6	0.010	—	1.170	0.570	0.150	—	—	0.720	—	0.370[d]
	0.010	—	1.200	0.600	—	—	—	0.800	—	0.050[d]
7	0.022	0.002	0.807	0.544	0.100	—	0.001	0.902	0.226	0.018
	0.021	0.002	0.862	0.568	0.104	—	2.000	0.913	0.219	0.016
8	0.032	0.015	0.833	0.541	0.107	0.052	0.007	0.470	0.177	0.042
	0.032	0.012	0.786	0.524	0.083	0.030	0.006	0.457	0.153	0.045
9	—	—	0.910	0.530	0.080	—	—	0.540	—	—
	—	—	0.840	0.510	0.080	—	—	0.530	—	—
10	0.030	0.010	0.900	0.560	0.100	—	—	0.550	0.130	0.150
	0.030	0.010	0.890	0.560	0.100	—	—	0.550	0.140	0.150
11	0.063[d]	0.039	0.760	0.576	0.140	—	—	0.515	0.158	0.136[d]
	0.105[d]	—	0.783	0.557	0.126	—	—	0.605	0.261	0.252[d]
					Sample 1—total carbohydrates					
1	0.200	0.020	3.440	18.070	0.710	—	0.100[d]	17.450	0.380	0.080
	0.200	0.020	3.440	18.120	0.650	—	0.240[d]	17.690	0.250	0.050
2	0.190	0.020	3.450	17.520	0.800	—	0.130	21.040	—	—
	0.200	0.020	3.520	17.870	0.730	—	0.120	21.530	—	—
3	0.158	—	3.591	17.360	0.488	—	0.152	16.090	0.211	0.100
	0.132	—	3.233	17.430	0.421	—	0.154	15.290	0.276	—
4	0.127	—	3.160	16.466	0.640	—	0.084	18.258	0.294	0.066
	0.115	—	2.923	15.817	0.587	—	0.081	17.549	0.302	0.037
5	0.250	0.220	2.820	17.890	0.610	—	0.120	18.620	—	—
	0.260	0.240	2.840	17.930	0.670	—	0.120	18.670	—	—
6	0.010	0.010	4.450	19.970	0.870	—	0.100[c]	20.780	0.010	—
	0.010	0.010	4.700	19.540	0.730	—	0.020[c]	19.730	—	—
7	0.248	0.025	5.052	28.977[e]	0.780	—	0.045	28.666[e]	—	—
	0.242	0.016	5.345	30.066[e]	0.660	—	0.076	29.777[e]	—	—
8	0.140	0.017	2.290	13.789	0.514	—	0.073	13.084	0.133	0.042
	0.179	0.018	3.186	17.610	0.645	—	0.093	16.945	0.141	0.063
9	0.210	—	3.360	17.950	0.800	—	—	17.510	—	—
	0.190	—	3.340	18.600	0.810	—	—	19.000	—	—
10	0.150	0.010	3.330	17.850	0.760	—	0.090	17.720	0.040	0.030
	0.170	0.010	3.290	17.590	0.750	—	0.090	17.640	0.040	0.030
11	0.288	—	3.725	21.640	0.757	—	0.375[e]	17.520	—	—
	0.279	—	3.481	16.570	0.676	—	—[e]	16.150	—	—
					Sample 2—free carbohydrates					
1	0.040	0.010	1.420	0.340	0.040	0.150	0.010	0.150	0.040	0.080
	0.040	0.010	1.470	0.350	0.050	0.140	0.010	0.160	0.070	0.070
2	0.010	0.010	1.310	0.350	0.040	0.150	0.010	0.010	0.050	0.010
	0.010	—	1.360	0.360	0.040	0.140	—	—	—	0.040
3	0.079	—	1.456[d]	0.308	0.049	0.102	—	0.159	—	—
	0.080	—	1.304[d]	0.275	0.044	0.119	—	0.153	—	—
4	0.064	0.004	1.359	0.338	0.038	0.119	0.006	0.145	0.055	0.043
	0.062	0.003	1.347	0.341	0.038	0.121	0.005	0.142	0.048	0.027

Table 1. ***(continued)***

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
5	0.070	—	1.240	0.330	0.050	0.200	—	0.140	0.080	—
	0.070	—	1.280	0.380	0.050	0.190	—	0.170	0.070	—
6	0.040	—	1.630[e]	0.310	—	0.010	0.010	—	0.010	0.010
	0.040	—	1.720[e]	0.320	—	—	—	—	—	—
7	0.040	0.002	1.284	0.344	0.034	0.247	—	0.274	0.069	0.032
	0.041	0.002	1.289	0.357	0.038	0.234	—	0.250	—	—
8	0.066	0.013	1.335	0.347	0.049	0.141	0.007	0.136	0.039	0.026
	0.066	0.016	1.341	0.349	0.040	0.136	0.008	0.132	0.055	0.043
9	0.140	—	1.270	0.300	0.020	—	—	0.120	—	—
	0.140	—	1.300	0.310	0.030	—	—	0.120	—	—
10	0.050	0.010	1.400	0.340	0.050	0.110	—	0.140	0.060	0.080
	0.050	0.010	1.380	0.340	0.050	0.110	—	0.140	0.060	0.080
11	0.100[d]	—	1.239	0.379	0.079[e]	0.216	—	0.216	0.188[d]	0.205[e]
	0.123[d]	—	1.234	0.382	0.098[e]	0.204	—	0.181	0.112[d]	—[e]
					Sample 2—total carbohydrates					
1	0.180	0.020	4.480	16.540	0.730	—	0.150	12.220	—	—
	0.190	0.030	4.550	16.950	0.790	—	0.190	12.400	—	—
2	0.170	0.020	4.550	18.440	1.010	—	0.160	17.620	—	—
	0.170	0.020	4.650	18.600	1.040	—	0.160	17.780	—	—
3	0.129	—	4.509	18.600	0.638	—	0.131	12.500	0.201	0.101
	0.121	—	4.641	17.020	0.540	—	0.140	11.800	0.191	—
4	0.065	0.003	4.112	16.002	0.730	—	0.118	13.404	0.163	0.048
	0.082	0.005	4.552	16.742	0.822	—	0.120	14.677	0.180	0.064
5	0.210	0.160[e]	3.360	15.820	0.740	—	0.180	12.200	—	—
	0.170	0.150[e]	3.410	15.630	0.700	—	0.210	12.040	—	—
6	0.010	0.010	6.810	21.670	1.190[d]	—	0.140	17.380	—	—
	0.010	0.010	6.480	21.190	0.030[d]	—	0.120	17.760	—	—
7	0.163	0.019	5.876	22.796	0.568	—	0.178	17.066	—	—
	0.152	0.016	5.726	22.493	0.545	—	0.154	16.820	—	—
8	0.190	0.013	4.805	19.005	0.881	—	0.113	13.655	0.084	0.087
	0.181	0.018	4.775	18.793	0.867	—	0.122	13.507	0.102	0.111
9	0.120	—	4.920	18.600	0.970	—	—	15.140	—	—
	0.140	—	5.020	18.700	1.030	—	—	15.290	—	—
10	0.160	0.020	4.660	18.110	0.950	—	0.120	13.700	—	0.070
	0.150	0.020	4.620	17.910	0.940	—	0.120	13.540	—	0.060
11	0.284	—	5.129	21.870[d]	1.121	—	—	13.180	—	—
	0.272	—	4.692	16.610[d]	0.904	—	—	12.340	—	—
					Sample 3—free carbohydrates					
1	1.700	0.010	0.460	0.180	2.000	1.300	0.040	0.510	3.340	—
	1.610	0.010	0.450	0.210	1.890	1.290	0.050	0.500	3.170	—
2	1.040[d]	0.010	0.520	0.220	2.160	1.190[d]	—	0.360	4.790[d]	—
	1.370[d]	0.020	0.570	0.180	2.110	0.380[d]	—	0.400	5.720[d]	—
3	1.568	—	0.435	0.204	2.138	1.444	0.118	0.440	3.316	—
	1.638	—	0.456	0.217	1.998	1.523	0.074	0.442	3.512	—
4	1.524	0.017	0.523	0.217	1.932	1.280	0.079	0.465	3.035	0.023
	1.513	0.014	0.478	0.194	1.939	1.268	0.077	0.462	3.087	0.032
5	1.610	—	0.390	0.160	2.210	1.550	—	0.370	4.080	—
	1.610	—	0.380	0.200	2.300	1.540	—	0.370	4.130	—
6	1.620	0.010	0.500	0.180	3.840[e]	1.130	—	0.580	5.100	—
	1.600	0.010	0.480	0.180	3.690[e]	1.130	—	0.540	5.200	—
7	1.331	0.006	0.423	0.172	2.172	1.223	—	0.603	7.625[e]	—
	1.350	0.006	0.440	0.175	2.217	1.193	—	0.628	7.813[e]	—
8	1.593	0.029	0.433	0.180	1.963	1.284	0.099	0.415	2.892	0.015
	1.666	0.033	0.456	0.182	2.019	1.312	0.103	0.438	3.109	0.050
9	1.390	—	0.410	0.160	1.890	1.210	—	0.380	3.720	—

Table 1. ***(continued)***

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
	1.300	—	0.420	0.160	1.890	1.240	—	0.410	3.530	—
10	1.630	0.010	0.470	0.180	1.930	1.280	—	0.450	3.340	—
	1.660	0.010	0.470	0.180	1.950	1.300	—	0.460	3.400	—
11	1.918	—	0.515	0.209	2.027	1.420	—	0.536	3.534	—
	1.816	—	0.529	0.263	2.081	1.457	—	0.591	3.705	—
					Sample 3—total carbohydrates					
1	2.010	0.050	4.790	8.030	13.840	—	1.710	2.530	0.890	—
	2.070	0.050	4.920	8.260	14.960	—	1.640	2.560	0.950	—
2	1.690	0.070	5.400	8.860	20.790	—	2.480	3.360	0.800	—
	1.740	0.070	5.680	9.090	21.380	—	2.590	3.430	0.770	—
3	1.483	—	4.341	7.573	16.380	—	1.592	2.354	2.629	0.102
	1.459	—	4.134	7.866	13.570	—	1.663	2.426	2.705	0.107
4	1.684	0.027	4.400	8.052	14.480	—	1.841	2.323	3.982	0.023
	1.657	0.024	4.285	7.947	14.841	—	1.867	2.323	3.909	—
5	1.370	0.140	3.600	7.030	11.290	—	1.440	2.160	0.550	—
	1.330	0.130	3.540	7.060	11.350	—	1.420	2.100	0.560	—
6	1.810[d]	0.040	5.750	8.070	20.740	—	1.960	2.970	4.570	—
	1.480[d]	0.050	5.540	8.500	23.780	—	2.170	3.070	4.340	—
7	1.941	0.019	5.006	9.256	24.110	—	2.733	2.518	—	—
	1.903	0.022	4.938	9.141	23.946	—	2.716	2.477	—	—
8	2.007	0.043	4.733	8.252	15.994	—	1.578	2.597	1.837	0.046
	2.018	0.046	4.751	8.116	14.605	—	1.846	2.611	1.589	0.062
9	1.990	—	4.810	8.000	16.680	—	1.660	2.820	—	—
	2.050	—	5.060	8.500	16.640	—	1.530	2.710	—	—
10	1.830	0.040	4.510	7.380	14.660	—	1.510	2.440	0.820	—
	1.830	0.040	4.500	7.360	13.920	—	1.530	2.430	0.800	—
11	2.582	—	5.231	8.075	14.390	—	1.661	2.576	2.399	—
	2.444	—	4.771	7.458	13.940	—	1.684	2.431	2.076	—
					Sample 4—free carbohydrates					
1	0.650	0.010	0.750	0.420	1.610	0.790	—	0.320	2.960	—
	0.690	0.010	0.760	0.420	1.650	0.760	—	0.340	3.110	—
2	0.290	0.010	0.790	0.420	1.880	0.920[d]	0.020	0.230	4.220	—
	0.300	0.010	0.820	0.430	1.720	0.230[d]	0.010	0.190	4.270	—
3	0.785	—	0.767	0.401	1.616	0.827	—	0.289	2.667	—
	0.820	—	0.644	0.392	1.433	0.734	—	0.377	2.545	—
4	0.691	0.015	0.822	0.500	1.682	0.792	0.023	0.356	2.780	0.051
	0.659	0.007	0.800	0.491	1.619	0.748	0.021	0.342	2.716	0.048
5	0.460	—	0.570	0.440	1.690	0.870	—	0.240	3.240	0.080
	0.460	—	0.590	0.460	1.730	0.850	—	0.250	3.300	0.090
6	0.570	0.010	0.770	0.360	2.580[d]	0.570	0.030	0.400	3.830	—
	0.580	0.010	0.950	0.430	3.060[d]	0.680	—	0.370	4.090	—
7	0.526	0.003	0.719	0.426	1.818	0.611	—	0.427	6.120[e]	—
	0.506	0.003	0.689	0.410	1.775	0.571	—	0.411	6.085[e]	—
8	0.703	0.017	0.770	0.467	1.658	0.766	0.033	0.321	2.600	0.056
	0.685	0.017	0.741	0.456	1.616	0.745	0.049	0.319	2.418	0.052
9	0.680	—	0.750	0.430	1.600	0.710	—	0.310	3.130	—
	0.690	—	0.790	0.430	1.690	0.790	—	0.320	3.010	—
10	0.660	0.010	0.800	0.450	1.580	0.790	0.030	0.370	2.960	0.100
	0.650	0.010	0.800	0.480	1.570	0.760	0.030	0.370	2.900	0.110
11	0.825	—	0.723	0.508	1.664	0.844	—	0.349	2.874	—
	0.748	—	0.611	0.418	1.546	0.709	—	0.334	2.865	—
					Sample 4—total carbohydrates					
1	0.820	0.030	5.360	15.750	4.380	—	0.790	5.590	0.590	0.280
	0.830	0.030	5.320	15.810	4.330	—	0.730	5.570	0.640	—
2	0.510	0.030	4.500	16.560	5.270	—	0.700	6.420	0.280	—

Table 1. ***(continued)***

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
	0.490	0.030	4.690	16.970	5.870	—	0.720	6.610	0.420	—
3	0.866	—	4.394	15.880	3.713	—	0.641	5.174	1.813	0.130
	0.853	—	3.781	15.590	3.433	—	0.643	5.341	1.758	0.083
4	0.736	0.015	4.258	15.861	3.986	—	0.759	5.433	2.707	0.061
	0.734	—	4.232	15.616	3.898	—	0.738	5.414	2.651	0.050
5	0.580	0.100[f]	3.390	14.690	3.550	—	0.450	4.740	—	—
	0.560	0.080[f]	3.380	14.460	3.510	—	0.440	4.690	—	—
6	0.920	0.010	6.230	18.590	6.010	—	0.820	6.890	2.430	—
	0.990	0.030	6.410	18.230	5.920	—	0.800	7.000	2.630	—
7	0.871	—	5.393	21.797	6.320	—	1.156	7.221	—	—
	0.834	—	4.952	20.841	6.132	—	1.157	6.752	—	—
8	0.675	0.026	3.497	13.186	3.292	—	0.488	4.321	0.965	0.063
	0.738	0.026	3.872	14.536	3.615	—	0.573	4.789	0.857	0.070
9	0.700	—	4.350	16.050	4.100	—	0.980	5.560	—	—
	0.830	—	4.320	16.400	4.130	—	1.040	5.490	—	—
10	0.770	0.030	4.250	15.560	3.630	—	0.640	5.070	0.700	0.080
	0.780	0.030	4.250	15.630	3.660	—	0.650	5.120	0.710	0.080
11	1.062	0.215[e]	4.721	16.120	3.897	—	0.646	5.151	2.130[d]	—
	1.053	—[e]	4.304	13.790	3.828	—	0.622	4.813	1.460[d]	—
				Sample 5—free carbohydrates						
1	0.250	0.010	0.520	0.470	0.190	0.190	0.020	0.300	0.290	0.020
	0.250	0.010	0.540	0.470	0.190	0.200	0.030	0.300	0.270	0.020
2	0.050	0.010	0.520	0.470	0.190	0.210	0.030	0.060	0.220	0.080
	0.060	0.010	0.510	0.500	0.160	0.140	0.020	0.080	—	0.060
3	0.298	—	0.481	0.376[e]	0.174	0.205	0.028	0.276	0.351	—
	0.261	—	0.411	0.388[e]	0.189	0.254	0.041	0.294	0.291	—
4	0.184	0.006	0.572	0.483	0.182	0.178	0.015	0.298	0.218	0.022
	0.187	0.006	0.534	0.518	0.212	0.260	0.029	0.292	0.243	0.047
5	0.150	0.020[d]	0.450	0.460	0.230	0.230	—	0.240	0.420	0.210
	0.160	0.030[d]	0.490	0.480	0.220	0.230	—	0.250	0.400	0.170
6	0.150	0.010	0.580[d]	0.480	0.350[e]	0.020	0.030	0.500	0.050	0.010
	0.200	0.010	0.910[d]	0.490	0.470[e]	—	—	0.470	0.010	—
7	0.199	0.005	0.489	0.450	0.183	0.143	—	0.363	0.152	—
	0.196	—	0.514	0.460	0.176	0.135	—	0.364	—	—
8	0.206	0.010[c]	0.482	0.449	0.162	0.183	0.023	0.280	0.251	0.051
	0.206	0.012[c]	0.490	0.465	0.223	0.205	0.030	0.260	0.275	0.049
9	—	—	0.510	0.590[d]	0.110	0.060	—	0.150	—	—
	—	—	0.520	0.410[d]	0.080	0.050	—	0.170	—	—
10	0.230	0.020	0.550	0.470	0.190	0.160	0.040	0.290	0.390	0.170
	0.210	0.020	0.560	0.450	0.190	0.160	0.040	0.300	0.360	0.180
11	0.232[d]	—	0.465	0.481	0.230	0.289	—	0.299[d]	0.418	0.336
	0.343[d]	—	0.490	0.503	0.242	0.301	—	0.405[d]	0.466	—
				Sample 5—total carbohydrates						
1	0.280	0.030	4.190	18.070	1.960	—	1.770	7.370	0.220	0.090
	0.310	0.030	4.450	18.720	2.040	—	1.810	7.490	0.150	0.060
2	0.210	0.030	4.070	18.330	2.440	—	1.870	8.650	—	—
	0.220	0.030	4.120	18.260	2.530	—	2.000	9.160	—	—
3	0.314	—	3.884	17.410	1.796	—	1.754	6.802	0.381	0.091
	0.337	—	3.965	18.400	1.583	—	1.782	7.337	0.294	0.083
4	0.108	0.019	3.905	16.992	1.856	—	1.774	7.121	0.414	0.076
	0.106	0.020	3.759	16.581	1.820	—	2.004	7.023	0.345	0.055
5	0.250	—	2.910[e]	15.290[e]	1.600	—	1.250	6.450	—	—
	0.220	—	2.900[e]	15.330[e]	1.560	—	1.240	6.390	—	—
6	0.370	0.030	5.260[d]	18.180	2.260	—	1.950	8.650	0.020	—
	0.300	0.030	4.060[d]	18.500	1.870	—	1.900	8.860	0.030	—

Table 1. ***(continued)***

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
7	0.312	0.027	4.289	21.754[f]	2.076	—	2.875	8.569	0.473	0.257[d]
	0.290	0.025	4.386	21.806[f]	1.909	—	2.781	8.663	0.347	0.011[d]
8	0.339	0.026	4.079	18.630	2.040	—	1.739	7.598	0.221	0.102
	0.354	0.031	4.086	18.686	2.056	—	1.342	7.424	0.228	0.087
9	0.300	—	3.980	17.970	1.840	—	1.370	7.800	—	—
	0.290	—	3.890	17.920	1.930	—	1.540	7.910	—	—
10	0.300	0.030	3.990	17.910	1.910	—	1.850	7.230	0.160	0.080
	0.270	0.030	3.990	17.870	1.900	—	1.850	7.200	0.130	0.080
11	0.499	—	4.407	17.680[d]	2.044	—	2.037	7.620	—	—
	0.617	—	4.019	15.310[d]	1.908	—	1.681	7.054	—	—
					Sample 6—free carbohydrates					
1	0.100	—	0.620	0.350	0.160	0.140	0.010	0.990	0.510	0.020
	0.100	—	0.620	0.340	0.170	0.140	—	0.950	0.510	0.020
2	0.060	0.030	0.700	0.350	0.200	0.170[d]	—	0.800	0.490	0.190
	0.050	0.030	0.650	0.360	0.180	0.120[d]	—	0.760	—	—
3	0.061	0.021	0.602	0.315	0.231	0.175	—	0.875	0.267[d]	—
	0.062	—	0.548	0.268	0.164	—	—	0.793	0.112[d]	—
4	0.018	0.040	0.664	0.447	0.180	0.154	0.002	1.002	0.376	0.021
	0.017	0.039	0.631	0.455	0.173	0.150	0.002	0.953	0.398	0.049
5	—	—	0.590	0.370	0.150	0.200	—	0.970	0.380	0.240
	—	—	0.600	0.400	0.170	0.190	—	1.020	0.410	0.260
6	0.020	—	0.720	0.320	0.250	0.020	—	1.230	0.070[e]	—
	0.020	—	0.770	0.330	0.270	—	—	1.320	0.030[e]	—
7	0.078	0.013[c]	0.640	0.347	0.170	0.124	—	1.303	0.477	—
	0.090	0.021[c]	0.632	0.352	0.177	—	—	1.304	0.466	—
8	0.080	0.022	0.619	0.358	0.226	0.157	0.016	0.869	0.477	0.049
	0.074	0.020	0.577	0.329	0.229	0.156	0.020	0.807	0.440	0.046
9	—	—	0.680	0.380	0.090	—	—	1.040	—	—
	—	—	0.620	0.360	0.110	—	—	0.980	—	—
10	0.060	0.020	0.630	0.360	0.170	0.140		0.960	0.400	0.180
	0.060	0.020	0.630	0.350	0.170	0.150	—	0.960	0.400	0.180
11	0.183[d]	0.062[d]	0.552	0.391	0.209	0.228	0.089	0.972	0.549	0.189
	0.130[d]	0.032[d]	0.545	0.440	0.242	0.239	—	0.943	0.620	—
					Sample 6—total carbohydrates					
1	0.210	0.010	4.100	19.000	1.030	—	0.170	17.260	0.240	0.110
	0.170	0.010	3.830	18.470	0.940	—	0.130	17.050	0.250	0.150
2	0.220	0.020	3.930	18.340	1.100	—	0.170	20.960	0.090	—
	0.200	0.010	3.720	18.130	1.280	—	0.170	21.080	0.020	—
3	0.143	—	3.443	18.030	0.867	—	0.137	16.480	0.474	0.102
	0.167	—	3.429	18.560	0.795	—	0.123	17.450	0.487	0.084
4	0.118	0.008	3.607	16.960	0.974	—	0.098	18.994	0.746	—
	0.119	0.007	3.658	16.928	0.950	—	0.097	19.142	0.777	—
5	0.021	—	2.550	15.220	0.870	—	0.150	14.990	—	—
	0.000	—	2.510	15.140	0.850	—	0.150	14.920	—	—
6	0.210	0.010	5.270	20.420	1.320	—	0.170	23.540	0.430[d]	0.030
	0.180	0.010	5.040	20.110	1.180	—	0.150	23.880	1.260[d]	—
7	0.225	—	4.935	26.856[e]	1.063	—	0.109	27.399	—	—
	0.237	—	5.247	28.537[e]	1.096	—	0.058	28.755	—	—
8	0.140	0.013	3.757	19.032	1.057	—	0.136	17.525[d]	0.438	0.105
	0.130	0.012	2.929	16.225	0.857	—	0.096	12.346[d]	0.390	0.061
9	—	—	3.500	17.970	1.030	—	—	17.900	—	—
	—	—	3.410	17.200	1.200	—	—	17.720	—	—
10	0.190	0.010	3.490	17.770	1.010	—	0.140	16.500	0.220	0.070
	0.190	0.010	3.590	17.980	1.030	—	0.140	16.590	0.220	0.070
11	0.382	—	3.791	16.890	1.068	—	—	15.750	—	—

Table 1. *(continued)*

Lab.	Mannitol	Fucose	Arabinose[a]	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
	0.337	—	3.562	15.360	0.974	—	—	14.970	—	—

[a] Rhamnose and arabinose, coelution, *see* text.
[b] No results reported.
[c] Cochran test outlier not removed because of the 2/9 rule; *see* text.
[d] Cochran test outlier.
[e] Grubbs test outlier.
[f] Grubbs test outlier not removed because of the 2/9 rule; *see* text.

expressed in percent (w/w) carbohydrate on an as-is basis to 4 significant figures and reported on the data sheets provided (Table 1).

METHOD

Method Performance:

See Tables 2–13 for method performance data.

A. Principle

(**a**) *Free carbohydrates.*—The test portion was dissolved in water. A few milliliters of the solution were filtered through a C_{18} disposable cartridge and then through a 0.2 μm membrane filter. The filtered solution was injected into a liquid chromatograph (LC) system. Carbohydrates were separated on a pellicular anion-exchange (AE) column and detected by PAD. The identification and quantitation of carbohydrates was performed by comparison with standard solutions.

(**b**) *Total carbohydrates.*—The test portion was hydrolyzed with 1.00N HCl. The solution was then passed through a folded filter paper. A few milliliters of the filtrate were filtered through an AE disposable cartridge in the silver form to neutralize the solution and to eliminate the chloride anion. The neutralized solution was finally filtered through a 0.2 μm membrane filter before injection into the chromatograph.

B. Apparatus

(**a**) *Balance.*—Analytical, sensitivity ± 0.1 mg.

(**b**) *Round-bottom flasks.*—250 mL.

(**c**) *Volumetric flasks.*—100 and 1000 mL.

(**d**) *Pipettors.*—With disposable tips, 200–1000 μL and 5 mL capacity.

(**e**) *Cylinders.*—Graduated, 50 and 1000 mL tall-form.

(**f**) *Funnels.*—Analytical, 60°.

(**g**) *Vacuum filtering system.*—Aspirator with regulating device; heavy walled filtering flask with ground cone neck, 1 L; funnel, 300 mL, with ground glass joint; aluminum assembly clip; connection with vacuum outlet; filter holder, 47 mm id, and membrane filters, low-water extractable, 0.2 μm and

Table 2. Method performance for determination of free carbohydrates in soluble coffee sample 1 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Mannose	Fructose	Ribose
No. participating laboratories	9	11	11	11	11	9	9
No. retained laboratories	7	11	11	11	11	9	7
No. outlying laboratories removed	2	0	0	0	0	0	2
Raw data[a]							
M	0.034	0.891	0.562	0.105	0.583	0.171	0.103
s_r	0.010	0.033	0.017	0.010	0.028	0.029	0.086
r	0.029	0.092	0.047	0.029	0.080	0.082	0.242
RSD_r	29.560	3.664	2.964	9.911	4.875	16.882	83.032
s_R	0.026	0.123	0.030	0.022	0.142	0.054	0.094
R	0.073	0.349	0.084	0.062	0.402	0.152	0.267
RSD_R	74.447	13.832	5.261	21.057	24.373	31.370	91.591
Data without outliers							
M	0.024	0.891	0.562	0.105	0.583	0.171	0.073
s_r	0.000	0.033	0.017	0.010	0.028	0.029	0.011
r	0.001	0.092	0.047	0.029	0.080	0.082	0.031
RSD_r	1.547	3.664	2.964	9.911	4.875	16.882	15.059
s_R	0.015	0.123	0.030	0.022	0.142	0.054	0.054
R	0.041	0.349	0.084	0.062	0.402	0.152	0.152
RSD_R	59.454	13.832	5.261	21.057	24.373	31.370	73.692

[a] M is mean, s is standard deviation, r is repeatability value, RSD is relative standard deviation, and R is reproducibility.

Table 3. Method performance for determination of total carbohydrates in soluble coffee sample 1 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose	Fructose
No. participating laboratories	11	11	11	11	10	11	6
No. retained laboratories	11	11	10	11	8	10	6
No. outlying laboratories removed	0	0	1	0	2	1	0
Raw data							
M	0.179	3.544	18.847	0.684	0.119	18.941	0.189
s_r	0.013	0.234	1.393	0.059	0.039	1.018	0.046
r	0.035	0.662	3.944	0.168	0.111	2.880	0.130
RSD_r	6.986	6.597	7.394	8.661	32.802	5.373	24.416
s_R	0.075	0.749	3.850	0.114	0.079	3.919	0.129
R	0.213	2.120	10.896	0.323	0.223	11.092	0.366
RSD_R	41.996	21.141	20.430	16.667	66.245	20.692	68.482
Data without outliers							
M	0.179	3.544	17.779	0.684	0.097	17.913	0.189
s_r	0.013	0.234	1.441	0.059	0.022	1.038	0.046
r	0.035	0.662	4.078	0.168	0.063	2.938	0.130
RSD_r	6.986	6.597	8.105	8.661	22.929	5.796	24.416
s_R	0.075	0.749	1.582	0.114	0.037	2.029	0.129
R	0.213	2.120	4.478	0.323	0.103	5.741	0.366
RSD_R	41.996	21.141	8.900	16.667	37.730	11.324	68.482

[a] For abbreviations, *see* Table 2.

0.47 mm id; other equivalent vacuum-filtering systems could also be used.

(**h**) *Filter papers.*—Qualitative, folded, medium fast.

(**i**) C_{18} cartridges.—Disposable, e.g., Sep-Pak C_{18} (Waters), Supelclean LC-18 (Supelco, Gland, Switzerland), or equivalent. Condition and use cartridges according to the manufacturer's instructions.

(**j**) *Anion-exchange cartridges.*—Disposable, OnGuard-Ag (Dionex). Condition and use according to the manufacturer's instructions.

(**k**) *Membrane filters.*—Disposable, 0.2 μm, polypropylene.

(**l**) *Water bath.*—Capable of maintaining 98° ± 2°C.

(**m**) *Liquid chromatograph.*—Metal free, compatible with 300 mM NaOH, e.g., Model 4500 i (Dionex), or equivalent.

Table 4. Method performance for determination of free carbohydrates in soluble coffee sample 2 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Sucrose	Mannose	Fructose	Ribose
No. participating laboratories	11	11	11	10	10	10	9	8
No. retained laboratories	10	9	11	9	10	10	8	7
No. outlying laboratories removed	1	2	0	1	0	0	1	1
Raw data								
M	0.065	1.362	0.339	0.046	0.149	0.155	0.067	0.057
s_r	0.005	0.042	0.014	0.006	0.007	0.013	0.024	0.012
r	0.014	0.120	0.039	0.017	0.020	0.036	0.069	0.035
RSD_r	7.635	3.104	4.070	12.608	4.813	8.155	36.220	21.661
s_R	0.036	0.124	0.027	0.017	0.057	0.056	0.041	0.053
R	0.103	0.351	0.077	0.048	0.162	0.158	0.116	0.149
RSD_R	56.350	9.112	8.045	36.918	38.395	36.065	61.124	91.738
Data without outliers								
M	0.060	1.325	0.339	0.042	0.149	0.155	0.054	0.045
s_r	0.001	0.021	0.014	0.004	0.007	0.013	0.011	0.012
r	0.002	0.060	0.039	0.012	0.020	0.036	0.032	0.035
RSD_r	0.914	1.603	4.070	10.151	4.813	8.155	21.035	27.571
s_R	0.034	0.068	0.027	0.009	0.057	0.056	0.018	0.027
R	0.098	0.191	0.077	0.024	0.162	0.158	0.052	0.077
RSD_R	57.581	5.105	8.045	20.419	38.395	36.065	33.846	60.278

[a] For abbreviations, *see* Table 2.

Table 5. Method performance for determination of total carbohydrates in soluble coffee sample 2 (results are in %)

Parameter[a]	Mannitol	Fucose	Arabinose	Galactose	Glucose	Xylose	Mannose
No. participating laboratories	11	8	11	11	11	9	11
No. retained laboratories	11	7	11	10	10	9	11
No. outlying laboratories removed	0	1	0	1	1	0	0
Raw data							
M	0.151	0.033	4.833	18.550	0.806	0.146	14.365
s_r	0.011	0.004	0.160	1.194	0.254	0.014	0.380
r	0.032	0.011	0.453	3.379	0.719	0.040	1.075
RSD_r	7.609	11.556	3.311	6.436	31.528	9.750	2.645
s_R	0.069	0.050	0.836	2.227	0.254	0.030	2.180
R	0.196	0.140	2.365	6.304	0.719	0.084	6.168
RSD_R	45.961	148.754	17.289	12.008	31.528	20.226	15.173
Data without outliers							
M	0.151	0.016	4.833	18.481	0.826	0.146	14.365
s_r	0.011	0.003	0.160	0.430	0.062	0.014	0.380
r	0.032	0.009	0.453	1.216	0.174	0.040	1.075
RSD_r	7.609	19.623	3.311	2.325	7.449	9.750	2.645
s_R	0.069	0.007	0.836	2.181	0.178	0.030	2.180
R	0.196	0.021	2.365	6.171	0.504	0.084	6.168
RSD_R	45.961	46.004	17.289	11.800	21.551	20.226	15.173

[a] For abbreviations, *see* Table 2.

(**n**) *Pulsed amperometric detector.*—With gold electrode, e.g., Model PAD II (Dionex), Model PED (Dionex), or equivalent. Fill reference cell with 300 mM NaOH. Select the detector range to avoid saturation of the major peak in the chromatogram.

(**o**) *Analytical column.*—CarboPac PA1 (Dionex), 4 × 250 mm, 10 µm thickness.

(**p**) *Guard column.*—CarboPac PA1 (Dionex).

(**q**) *Postcolumn solvent delivery system.*—Compatible with 300 mM NaOH.

(**r**) *Integrator.*—Computing integrator, e.g., Model 450 (Dionex).

C. Reagents

Use 18 MΩ-cm demineralized water throughout.

Table 6. Method performance for determination of free carbohydrates in soluble coffee sample 3 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Sucrose	Mannose	Fructose
No. participating laboratories	11	11	11	11	11	11	11
No. retained laboratories	10	11	11	10	10	11	9
No. outlying laboratories removed	1	0	0	1	1	0	2
Raw data							
M	1.548	0.464	0.191	2.198	1.270	0.470	4.143
s_r	0.082	0.017	0.019	0.058	0.174	0.020	0.223
r	0.232	0.049	0.053	0.163	0.493	0.056	0.632
RSD_r	5.290	3.755	9.810	2.627	13.709	4.176	5.394
s_R	0.194	0.049	0.025	0.534	0.237	0.082	1.422
R	0.549	0.139	0.070	1.512	0.672	0.233	4.025
RSD_R	12.531	10.556	13.032	24.311	18.692	17.514	34.326
Data without outliers							
M	1.582	0.464	0.191	2.041	1.319	0.470	3.622
s_r	0.044	0.017	0.019	0.050	0.023	0.020	0.105
r	0.124	0.049	0.053	0.143	0.066	0.056	0.297
RSD_r	2.777	3.755	9.810	2.470	1.773	4.176	2.900
s_R	0.157	0.049	0.025	0.128	0.132	0.082	0.667
R	0.444	0.139	0.070	0.361	0.372	0.233	1.886
RSD_R	9.925	10.556	13.032	6.249	9.972	17.514	18.403

[a] For abbreviations, *see* Table 2.

Table 7. Method performance for determination of total carbohydrates in soluble coffee sample 3 (results are in %)

Parameter[a]	Mannitol	Fucose	Arabinose	Galactose	Glucose	Xylose	Mannose	Fructose
No. participating laboratories	11	8	11	11	11	11	11	9
No. retained laboratories	10	8	11	11	11	11	11	9
No. outlying laboratories removed	1	0	0	0	0	0	0	0
Raw data								
M	1.835	0.054	4.759	8.085	16.649	1.856	2.601	2.010
s_r	0.080	0.004	0.147	0.219	0.990	0.085	0.052	0.114
r	0.227	0.011	0.417	0.619	2.803	0.239	0.147	0.323
RSD_r	4.379	7.000	3.098	2.704	5.949	4.555	2.003	5.683
s_R	0.324	0.036	0.598	0.647	4.053	0.424	0.352	1.440
R	0.918	0.103	1.691	1.831	11.471	1.200	0.997	4.076
RSD_R	17.677	67.311	12.559	8.003	24.345	22.844	13.546	71.660
Data without outliers								
M	1.854	0.054	4.759	8.085	16.649	1.856	2.601	2.010
s_r	0.041	0.004	0.147	0.219	0.990	0.085	0.052	0.114
r	0.115	0.011	0.417	0.619	2.803	0.239	0.147	0.323
RSD_r	2.198	7.000	3.098	2.704	5.949	4.555	2.003	5.683
s_R	0.331	0.036	0.598	0.647	4.053	0.424	0.352	1.440
R	0.938	0.103	1.691	1.831	11.471	1.200	0.997	4.076
RSD_R	17.866	67.311	12.559	8.003	24.345	22.844	13.546	71.660

[a] For abbreviations, *see* Table 2.

(**a**) *Sodium hydroxide.*—50% (w/w) aqueous solution, e.g., J.T. Baker No. 7067. The reagent should contain a minimum amount of sodium carbonate and mercury. Do not shake or stir the solution before use.

(**b**) *Hydrochloric acid.*—1.00N standard volumetric solution.

(**c**) *Eluent A.*—18 MΩ·cm demineralized water. Filter through a 0.2 μm membrane filter. Degas by sparging with helium for 20–30 min.

(**d**) *Eluent B.*—300 mM NaOH. Pipet 15.6 mL 50% (w/w) NaOH to 985 mL eluent A. It is extremely important to remove dissolved carbon dioxide from the eluents. Carbonate acts as a

Table 8. Method performance for determination of free carbohydrates in soluble coffee sample 4 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Sucrose	Mannose	Fructose
No. participating laboratories	11	11	11	11	11	11	11
No. retained laboratories	11	11	11	10	10	11	10
No. outlying laboratories removed	0	0	0	1	1	0	1
Raw data							
M	0.619	0.747	0.438	1.763	0.730	0.329	3.395
s_r	0.022	0.054	0.026	0.121	0.155	0.023	0.087
r	0.063	0.154	0.074	0.343	0.438	0.065	0.247
RSD_r	3.594	7.297	5.948	6.866	21.206	6.960	2.571
s_R	0.150	0.087	0.036	0.371	0.155	0.061	1.048
R	0.424	0.247	0.103	1.049	0.438	0.174	2.965
RSD_R	24.162	11.707	8.317	21.018	21.206	18.647	30.864
Data without outliers							
M	0.619	0.747	0.438	1.657	0.746	0.329	3.124
s_r	0.022	0.054	0.026	0.068	0.051	0.023	0.091
r	0.063	0.154	0.074	0.192	0.144	0.065	0.258
RSD_r	3.594	7.297	5.948	4.092	6.807	6.960	2.919
s_R	0.150	0.087	0.036	0.101	0.087	0.061	0.569
R	0.424	0.247	0.103	0.286	0.246	0.174	1.611
RSD_R	24.162	11.707	8.317	6.091	11.632	18.647	18.217

[a] For abbreviations, *see* Table 2.

Table 9. Method performance for determination of total carbohydrates in soluble coffee samples 4 (results are in %)

Parameter[a]	Mannitol	Fucose	Arabinose	Galactose	Glucose	Xylose	Mannose	Fructose
No. participating laboratories	11	8	11	11	11	11	11	8
No. retained laboratories	11	7	11	11	11	11	11	7
No. outlying laboratories removed	0	1	0	0	0	0	0	1
Raw data								
M	0.782	0.049	4.539	16.269	4.385	0.736	5.598	1.421
s_r	0.036	0.008	0.209	0.632	0.166	0.027	0.171	0.182
r	0.102	0.023	0.590	1.789	0.469	0.077	0.483	0.515
RSD_r	4.594	16.761	4.594	3.886	3.780	3.717	3.047	12.792
s_R	0.161	0.055	0.831	2.097	1.038	0.204	0.859	0.908
R	0.454	0.157	2.353	5.935	2.938	0.578	2.431	2.569
RSD_R	20.535	113.806	18.319	12.891	23.672	27.759	15.346	63.879
Data without outliers								
M	0.782	0.036	4.539	16.269	4.385	0.736	5.598	1.368
s_r	0.036	0.008	0.209	0.632	0.166	0.027	0.171	0.076
r	0.102	0.023	0.590	1.789	0.469	0.077	0.483	0.214
RSD_r	4.594	22.729	4.594	3.886	3.780	3.717	3.047	5.527
s_R	0.161	0.026	0.831	2.097	1.038	0.204	0.859	0.958
R	0.454	0.074	2.353	5.935	2.938	0.578	2.431	2.713
RSD_R	20.535	72.625	18.319	12.891	23.672	27.759	15.346	70.068

[a] For abbreviations, *see* Table 2.

strong "pusher" on the column and results in a drastic reduction in resolution.

(e) *Standard solutions.*—1 mg/mL aqueous stock solutions for arabinose, fructose, fucose, galactose, glucose, mannose, rhamnose monohydrate, ribose, xylose, sucrose, and mannitol. Weigh, to the nearest 0.1 mg, 100 mg of each carbohydrate in separate 100 mL volumetric flasks. Dissolve and dilute to 100 mL with water. Further dilute to reach carbohydrate concentrations similar to those found in nonhydrolyzed or hydrolyzed soluble coffee sample solutions. Mixed standard solutions can also be prepared from separate stock solutions. The resolution of rhamnose from arabinose is sometimes difficult to achieve. In that case, do not add rhamnose in a mixed standard solution. Pass the diluted standard solution through a 0.2 µm membrane filter before injection.

Table 10. Method performance for determination of free carbohydrates in soluble coffee sample 5 (results are in %)

Parameter[a]	Mannitol	Fucose	Arabinose	Galactose	Glucose	Sucrose	Xylose	Mannose	Fructose	Ribose
No. participating laboratories	10	8	11	11	11	11	7	11	10	8
No. retained laboratories	9	7	10	9	10	11	7	10	10	8
No. outlying laboratories removed	1	1	1	2	1	0	0	1	0	0
Raw data										
M	0.201	0.013	0.527	0.469	0.207	0.181	0.029	0.284	0.282	0.102
s_r	0.029	0.003	0.074	0.041	0.031	0.027	0.007	0.025	0.026	0.015
r	0.082	0.008	0.208	0.115	0.088	0.077	0.020	0.071	0.072	0.043
RSD_r	14.416	21.631	13.955	8.678	15.082	15.081	24.731	8.860	9.045	14.816
s_R	0.070	0.007	0.095	0.044	0.079	0.075	0.008	0.109	0.128	0.100
R	0.199	0.020	0.270	0.124	0.225	0.213	0.023	0.307	0.361	0.282
RSD_R	34.995	56.091	18.099	9.362	38.463	41.584	28.215	38.274	45.292	97.890
Data without outliers										
M	0.192	0.011	0.505	0.475	0.186	0.181	0.029	0.277	0.282	0.102
s_r	0.016	0.001	0.022	0.015	0.019	0.027	0.007	0.012	0.026	0.015
r	0.045	0.002	0.063	0.041	0.053	0.077	0.020	0.033	0.072	0.043
RSD_r	8.245	5.400	4.419	3.085	10.006	15.081	24.731	4.166	9.045	14.816
s_R	0.065	0.005	0.039	0.019	0.040	0.075	0.008	0.111	0.128	0.100
R	0.183	0.014	0.110	0.055	0.113	0.213	0.023	0.313	0.361	0.282
RSD_R	33.789	44.826	7.729	4.075	21.370	41.584	28.215	39.969	45.292	97.890

[a] For abbreviations, *see* Table 2.

Table 11. Method performance for determination of total carbohydrates in soluble coffee sample 5 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose	Fructose	Ribose
No. participating laboratories	11	11	11	11	11	11	7	6
No. retained laboratories	11	9	9	11	11	11	7	5
No. outlying laboratories removed	0	2	2	0	0	0	0	1
Raw data								
M	0.300	4.027	17.982	1.951	1.826	7.653	0.244	0.089
s_r	0.032	0.278	0.576	0.111	0.134	0.212	0.049	0.072
r	0.091	0.788	1.631	0.313	0.380	0.600	0.140	0.204
RSD_r	10.776	6.916	3.205	5.674	7.356	2.772	20.261	80.557
s_R	0.112	0.483	1.665	0.245	0.410	0.808	0.144	0.072
R	0.316	1.366	4.713	0.693	1.161	2.288	0.406	0.204
RSD_R	37.260	11.988	9.261	12.541	22.468	10.563	58.874	80.557
Data without outliers								
M	0.300	4.081	18.444	1.951	1.826	7.653	0.244	0.080
s_r	0.032	0.122	0.306	0.111	0.134	0.212	0.049	0.013
r	0.091	0.344	0.867	0.313	0.380	0.600	0.140	0.036
RSD_r	10.776	2.979	1.661	5.674	7.356	2.772	20.261	15.880
s_R	0.112	0.199	1.377	0.245	0.410	0.808	0.144	0.014
R	0.316	0.562	3.898	0.693	1.161	2.288	0.406	0.041
RSD_R	37.260	4.868	7.468	12.541	22.468	10.563	58.874	17.805

[a] For abbreviations, *see* Table 2.

D. Sample Preparation

Use the sample as is, without grinding or homogenization.

E. Determination

(a) *Free carbohydrates.*—Weigh, to the nearest 0.1 mg, 300 mg sample into a 100 mL volumetric flask. Add 70 mL water and shake until dissolution is complete. Dilute to 100 mL with water. Filter 5–10 mL solution through a C_{18} cartridge. Discard the first milliliter. Pass filtrate through a 0.2 μm membrane filter before injection.

(b) *Total carbohydrates.*—Weigh, to the nearest 0.1 mg, 300 mg sample into a 100 mL volumetric flask. Add 50 mL 1.00N HCl and swirl. Place flask in a boiling water bath for 150 min. Always keep the level of the water in the bath above that of the sample solution. Swirl by hand every 30 min. Cool

Table 12. Method performance for determination of free carbohydrates in soluble coffee sample 6 (results are in %)

Parameter[a]	Mannitol	Fucose	Arabinose	Galactose	Glucose	Sucrose	Mannose	Fructose	Ribose
No. participating laboratories	9	7	11	11	11	10	11	10	7
No. retained laboratories	8	6	11	11	11	9	11	8	7
No. outlying laboratories removed	1	1	0	0	0	1	0	2	0
Raw data									
M	0.070	0.028	0.629	0.362	0.186	0.156	0.991	0.389	0.120
s_r	0.013	0.009	0.026	0.018	0.018	0.014	0.038	0.043	0.011
r	0.037	0.025	0.073	0.051	0.052	0.040	0.107	0.122	0.031
RSD_r	18.676	31.573	4.092	5.006	9.853	9.123	3.801	11.110	9.077
s_R	0.043	0.013	0.056	0.045	0.044	0.050	0.168	0.165	0.097
R	0.122	0.037	0.158	0.127	0.125	0.140	0.474	0.467	0.275
RSD_R	61.645	46.242	8.895	12.417	23.808	31.732	16.918	42.455	80.803
Data without outliers									
M	0.059	0.025	0.629	0.362	0.186	0.158	0.991	0.460	0.120
s_r	0.004	0.003	0.026	0.018	0.018	0.005	0.038	0.024	0.011
r	0.012	0.007	0.073	0.051	0.052	0.015	0.107	0.067	0.031
RSD_r	7.071	10.469	4.092	5.006	9.853	3.369	3.801	5.167	9.077
s_R	0.029	0.009	0.056	0.045	0.044	0.052	0.168	0.072	0.097
R	0.083	0.025	0.158	0.127	0.125	0.147	0.474	0.203	0.275
RSD_R	49.132	35.583	8.895	12.417	23.808	33.074	16.918	15.549	80.803

[a] For abbreviations, *see* Table 2.

Table 13. Method performance for determination of total carbohydrates in soluble coffee sample 6 (results are in %)

Parameter[a]	Mannitol	Arabinose	Galactose	Glucose	Xylose	Mannose	Fructose
No. participating laboratories	10	11	11	11	9	11	7
No. retained laboratories	10	11	10	11	9	10	6
No. outlying laboratories removed	0	0	1	0	0	1	1
Raw data							
M	0.179	3.786	18.597	1.025	0.133	18.691	0.432
s_r	0.018	0.216	0.809	0.081	0.019	1.177	0.223
r	0.050	0.612	2.289	0.230	0.053	3.330	0.632
RSD_r	9.801	5.709	4.349	7.937	14.175	6.295	51.713
s_R	0.090	0.771	3.348	0.140	0.032	4.191	0.330
R	0.254	2.182	9.475	0.397	0.090	11.861	0.934
RSD_R	50.084	20.366	8.004	13.702	24.006	22.423	76.458
Data without outliers							
M	0.179	3.786	17.687	1.025	0.133	19.067	0.363
s_r	0.018	0.216	0.760	0.081	0.019	0.426	0.027
r	0.050	0.612	2.152	0.230	0.053	1.206	0.075
RSD_r	9.801	5.709	4.300	7.937	14.175	2.234	7.310
s_R	0.090	0.771	1.503	0.140	0.032	4.137	0.247
R	0.254	2.182	4.255	0.397	0.090	11.708	0.700
RSD_R	50.084	20.366	8.500	13.702	24.006	21.698	68.236

[a] For abbreviations, *see* Table 2.

to room temperature by passing the flask under tap water. Dilute to 100 mL with water and filter solution through a folded filter paper. Pass 3 mL filtrate through an OnGuard-Ag cartridge. Discard the first milliliter. Filter the neutralized solution through a 0.2 μm membrane filter before injection.

F. Chromatographic Conditions

(**a**) *Mobile phase.*—Isocratic. Mobile phase conditions are as follows: 0 min, eluent A–eluent B (100 + 0, start acquisition); 50.0 min, A–B (100 + 0, stop acquisition); 50.1 min, A–B (0 + 100, start cleanup); 65.0 min, A–B (0 + 100, stop cleanup); 65.1 min, A–B (100 + 0, start reequilibration); 80.0 min, A–B (100 + 0, stop reequilibration).

Note: Retention times and resolution tend to vary from column to column. Start cleanup only when the last monosaccharide (ribose) has been eluted. It may be necessary to perform 2 to 3 injections of standard solution or to increase the reequilibration time to achieve good separation of glucose, sucrose, and xylose. Under normal conditions, approximate retention times are as follows: mannitol, 4 min; fucose, 7 min; rhamnose, 15 min; arabinose, 16 min; galactose, 22 min; glucose, 25 min; sucrose, 27 min; xylose, 30 min; mannose, 32 min; fructose, 40 min; and ribose, 43 min.

(**b**) *Column temperature.*—Ambient.

(**c**) *Flow rate.*—1.0 mL/min.

(**d**) *Postcolumn solvent.*—300 mM NaOH at a flow rate of 0.6 mL/min.

(**e**) *Detector settings.*—Use the optimum parameters as given by the manufacturer.

G. Calculations

Inject equal volumes, 10–20 μL, of standard and sample solutions. Calculate the carbohydrate content with the following formula:

$$\text{Carbohydrate content} = \frac{R}{R'} \times \frac{C'}{W} \times 100$$

where R and R' are peak response of carbohydrate in sample solution and standard solution, respectively; C' is concentration of carbohydrate in standard solution (mg/mL); and W is weight of sample taken (mg).

Express results either in % free or % total carbohydrates (as is).

Results and Discussion

The results of individual determinations of free and total carbohydrates in the 6 test samples are presented in Table 1. Most participants reported difficulties in separating the pairs glucose–sucrose, sucrose–xylose, and rhamnose–arabinose. Nevertheless, a complete resolution of glucose, sucrose, and xylose was achieved after 2–3 injections of standard solution. However, collaborators 1 and 10 only succeeded in separating rhamnose from arabinose. Therefore, the results reported for arabinose in Table 1 are the sum of the 2 monosaccharides. Rhamnose in soluble coffee is found in traces, <0.05%, as a free carbohydrate and represents approximately 10–15% of the total arabinose level in the hydrolyzed coffee (1). Consequently, the overestimation of arabinose is very little.

Raw data were treated statistically as recommended by official guidelines (3, 4). The statistical evaluation was not performed for carbohydrates for which less than 6 different duplicate values were reported. Some participants reported data that were detected as outlying either by Cochran or Grubbs test, but these data could not be removed because more than 2/9 of the data from one sample must not be rejected (4). The precision parameters calculated with and without outlying results are given for each sample and each carbohydrate in Tables 2–13.

The reproducibility relative standard deviations (RSD_R) were 9.9–59.5% for mannitol, 35.6–72.6% for fucose, 4.9–21.1% for arabinose, 4.1–13.0% for galactose, 6.1–24.3% for glucose, 10.0–41.6% for sucrose, 20.2–37.7% for xylose, 10.6–40.0% for mannose, 15.5–71.7% for fructose, and 17.8–97.9% for ribose.

Precision as a Function of Carbohydrate Type

Figure 1 shows the precision of the individual carbohydrates determination in the study. Each point corresponds to the mean repeatability RSD (RSD_r) and the mean RSD_R for one carbohydrate and was calculated from the results in Table 2.

Precision in the determination of free and total carbohydrates was very similar. The major monosaccharides present in soluble coffee, i.e., arabinose, galactose, and mannose, were clearly those analyzed with the highest precision. On the other hand, less precise results were obtained for minor carbohydrates such as fucose, ribose, and free xylose. Because the strong acidic conditions used in the hydrolysis step led to an intense degradation of fructose (1, 2), the observed RSD_R value for total fructose was not surprisingly high. Finally, the analysis of both free and total mannitol suffered from an important interlaboratory variability (RSD_R, approximately 40%). Mannitol eluted very early in the chromatogram with other unidentified compounds. This phenomenon certainly precluded a clear definition of the integration limits, particularly when the level of mannitol was low (<0.2%).

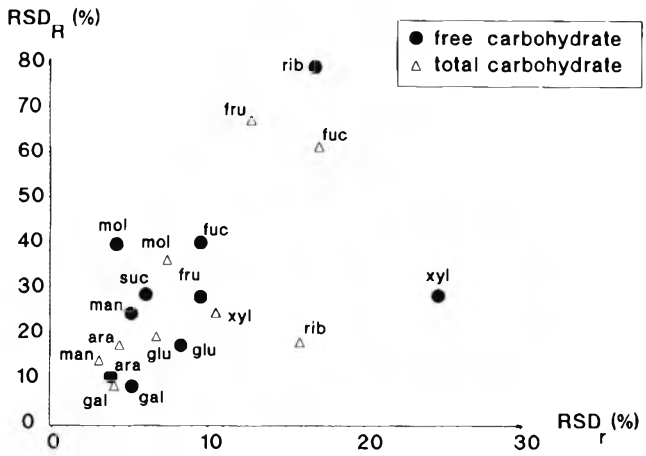

Figure 1. Precision of the method as a function of carbohydrate type: mol, mannitol; fuc, fucose; ara, arabinose; gal, galactose; glu, glucose; suc, sucrose; xyl, xylose; man, mannose; fru, fructose; rib, ribose.

With the exception of minor carbohydrates, the repeatability of the method can be considered good (RSD_r <10%). A more detailed analysis of the individual values in Figure 1 shows that average RSD_r values were 5.8 and 4.5% when the levels of free and total carbohydrates were above 0.1 and 0.3%, respectively.

Precision as a Function of Carbohydrate Concentration

Horwitz (5) demonstrated that the precision of methods is related exponentially to the concentration, independent of analyte, matrix, or method (5). However, most collaboratively tested methods reported for the determination of carbohydrates in foods showed high variability, and RSD_R values were far above those predicted by the Horwitz equation (6). Figure 2 gathers all RSD_R values observed in this study as a function of the decimal logarithm of the carbohydrate concentration (C).

A clear discontinuity in precision exists at $-\log C = 2.5$. This value corresponds to $C = 0.3\%$. Above this concentration, the RSD_R values ranged from 4.1 to 27.8% (without total fructose data) and averaged 14.3%. This variability can be considered satisfactory by taking into account the usual problems associated with peak integration in LC methodology, the generally low levels of free carbohydrates found in soluble coffee, the extra variability introduced by the hydrolysis step, and the lack of experience of most laboratories with the technique.

The average precision parameters were very close to those reported by Bugner and Feinberg (7) for LC determination of carbohydrates in foods on an amino-bonded silica column with refractive index detection. However, the observed variability was much lower than the average RSD_R values (24%) calculated by Horwitz from 91 collaborative assays (6).

Collaborators' Comments

Laboratory 1.—The optimal separation of the saccharides was achieved when the temperature of the column was kept between 28 and 30°C. At column temperatures lower than 28°C, we obtained a poor separation of rhamnose–arabinose and sucrose–xylose. At temperatures higher than 30°C, rhamnose was separated very well from arabinose. In contrast, the resolution of glucose–sucrose and fructose–ribose became worse.

Laboratory 2.—Addition of 70 mL water to the test portion produced excessive sample foaming, and the structure of the foam did not break down even after several hours. Gentle swirling of the sample while immersed in an ultrasonic bath prevented foam formation. Difficulties were encountered in separating the sucrose and glucose peaks. Allowing the chromatograph to complete several cycles before analysis or increasing reequilibrium time did not improve the separation.

Laboratory 3.—At the beginning of each day of analysis, the separation of glucose and sucrose was sufficient, but during the day, the separation became worse.

Laboratory 11.—The following equipment was used: liquid chromatograph, Model 600–MS (Waters); postcolumn delivery system, Model 501 (Waters); and PAD system (E.G. & G., Princeton Applied Research). Use of this equipment resulted in

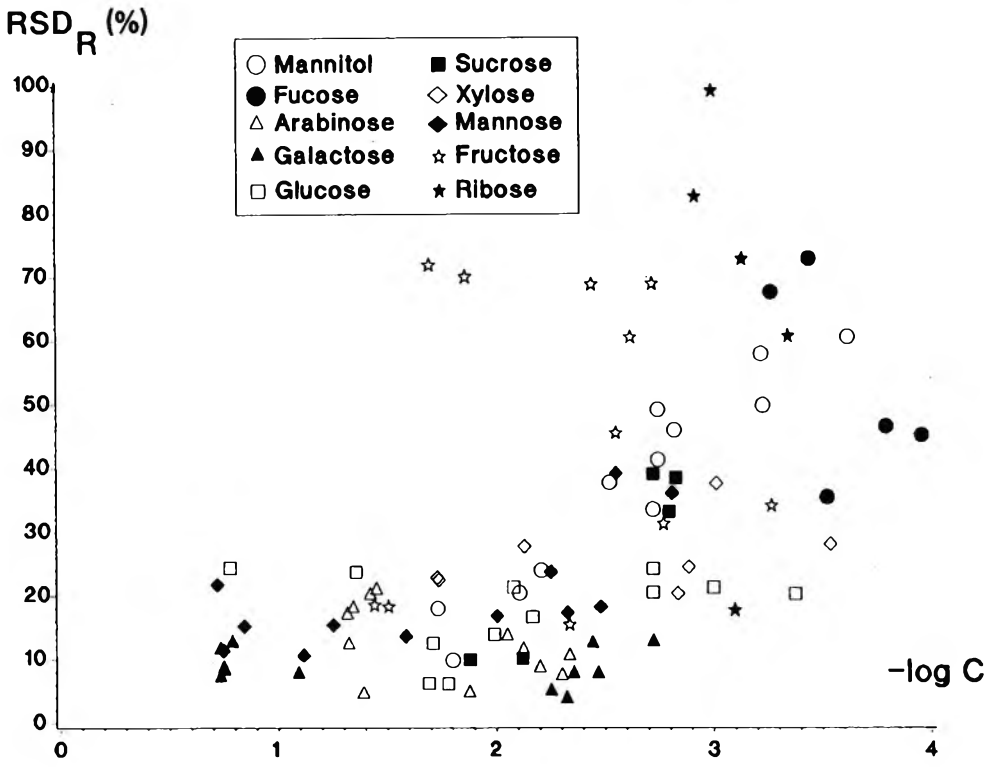

Figure 2. Precision parameters (RSD_R) for carbohydrate as a function of concentration (–log *C*).

a signal-to-noise ratio that was lower than that obtained by the Dionex 4500 i. As a result, the injection volume was increased from 20 to 50 µL. For free sugar analysis only, the test portion had to be doubled (600 mg rather than 300 mg). Rhamnose and arabinose elution order was reversed on our system. Quantitation of total arabinose, total galactose, and total mannose was performed by linear extrapolation outside the calibration range, and therefore, may not be reliable.

Conclusions

The proposed method allows a good and reproducible separation of all major carbohydrates found in soluble coffee. Precision was similar for the determination of both free and total carbohydrates. When the carbohydrate levels in the sample were >0.3%, the precision of the method was considered high. Therefore, the technique is suitable for routine analysis of soluble coffee.

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FOOD COMPOSITION AND ADDITIVES

Determination of *trans* Unsaturation by Infrared Spectrophotometry and Determination of Fatty Acid Composition of Partially Hydrogenated Vegetable Oils and Animal Fats by Gas Chromatography/Infrared Spectrophotometry: Collaborative Study

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An infrared spectrophotometric (IR) method for the determination of total *trans* unsaturated fatty acid (*trans*) content and a combined gas–liquid chromatographic/infrared spectrophotometric (GC/IR) method for determination of fatty acid composition of partially hydrogenated vegetable oils (PHVO) were studied collaboratively in 12 laboratories using 7 PHVO samples, including 1 pair of blind duplicates. The test samples were methylated and analyzed for total *trans* content by IR and for fatty acid composition by GC/IR using a capillary column coated with SP-2560 or another suitable cyanoalkylsiloxane stationary phase. From the measured IR absorption, the isolated *trans* content was calculated using a calibration curve of absorption versus *trans* content developed with 2-component calibration standard mixtures of methyl elaidate and oleate. The GC provided the levels of mono-*trans*-octadecadienoates (18:2*t*), di-*trans*-octadecadienoates (18:2*tt*) and mono-*trans*-octadecatrienoates (18:3*t*). The *trans*-octadecenoate (18:1*t*) content was calculated with the formula: 18:1*t* = IR *trans* − 0.84 × (18:2*t* + 18:3*t*) − 1.74 × 18:2*tt*. The *cis*-octadecenoate (18:1*c*) content was obtained as the difference between total octadecenoates (18:1) and 18:1*t*. Reproducibility relative standard deviations (RSD_R) for 15 to 35% *trans* content determined by IR were in the range of 8.8–11.7%, whereas RSD_R for the test sample with 5% *trans* content was 34.6%. RSD_R values for 18:1*t* by the GC/IR followed the same pattern as that of IR *trans* values: 36.4% for the test sample with 4.9% 18:1*t* versus 7.8–12.5% for test samples with 14.9 to 32.6% 18:1*t*. The content of 18:1*c* in the test samples varied from 24.7 to 34.5% and their RSD_R values ranged from 3.8 to 10.5%. The mean values for 18:1*t* and 18:1*c* compared favorably with the absolute levels determined by a silver nitrate-thin layer chromatography/GC procedure. The IR and GC/IR methods are recommended for determination of *trans* content and fatty acid composition, respectively, of partially hydrogenated fats derived from vegetable oils, terrestrial animal fats or such oils and fats isolated from food products containing >5% *trans* fatty acids. For samples containing ≤5% *trans* fatty acids, a direct GC method (American Oil Chemists' Society Official Method Ce 1c-89) is available for determination of both *trans* content and fatty acid composition, because at lower *trans* levels, overlap of 18:1 *cis* and *trans* isomers on GC with very polar capillary columns is negligible. The IR method for determination of isolated *trans* unsaturated fatty acid content in partially hydrogenated fats and the capillary GC/IR method for determination of total *cis*- and *trans*-octadecenoic isomers and general fatty acid composition in hydrogenated vegetable oils and animal fats have been adopted first action by AOAC INTERNATIONAL.

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The recommendation was approved by the Committee on Food Nutrition and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1994) *J. AOAC Int.* **77**, 203A, and "Official Methods Board Actions" (1994) *The Referee* **18**, October issue.

The widespread use of partially hydrogenated vegetable oils (PHVO) in many common foods and the widely publicized adverse health effects of dietary *trans* fatty acids (1–5) create a need for accurate determination of total *trans* unsaturation and detailed fatty acid composition, including levels of *cis*- and *trans*-monounsaturates in PHVO and die-

tary fats made from PHVO. In Canada (6) and the United States (7) voluntary labeling regulations of foods require that only monounsaturates of the *cis* configuration be declared on the nutrition label and polyunsaturates are restricted to all *cis*-methylene-interrupted structures. These labeling regulations also require accurate determination of fatty acid composition in dietary fats of PHVO origin.

The current American Oil Chemists' Society (AOCS) Official Method Ce 1c-89 (revised 1990) has been designed to evaluate the general fatty acid composition, including the levels of 18:1*c* and 18:1*t* isomers, and the total *trans* unsaturated fatty acid (*trans*) content in hydrogenated and unhydrogenated vegetable oils, by a direct, one-step capillary gas chromatographic (GC) procedure, using a 60 m × 0.25 mm id fused silica capillary column coated with SP-2340 stationary phase (8). AOAC INTERNATIONAL recommended the first action adoption of the same direct GC procedure (9). This direct GC method was based on the assumption that *cis* and *trans* isomers of 18:1 fatty acid are completely separable on the SP-2340 column. However, because of the complexity of isomers present in PHVO, a satisfactory separation of 18:1*t* isomers as a group from that of *cis* isomers is not feasible on SP-2340 or any other currently available GC stationary phases (10, 11). On SP-2340 and other polar columns, 18:1*t* isomers with Δ values lower than 11 are well separated from the *cis* isomers, but the isomers with high Δ values (18:1Δ12 – 16*t*) overlap with *cis* isomers. Factors such as extent of hydrogenation, level of total *trans* content, and amount of sample applied onto GC column could also influence isomer separation. Because of these overlaps, the direct GC method gives lower values for 18:1*t* isomer and consequently higher values for 18:1*c* (10–12).

Ratnayake et al. (11) proposed use of a combined capillary GC and infrared spectrophotometric (IR) method for accurate determination of 18:1*t* and 18:1*c* isomers in PHVO. Total *trans* unsaturation determined by IR was related to the capillary GC weight percentages of the component *trans* fatty acid methyl esters by a mathematical formula:

$$\text{IR } trans = 18{:}1t + 0.84 \times 18{:}2t + 1.74 \times 18{:}2tt + 0.84 \times 18{:}3t$$

where 0.84, 1.74, and 0.84 are correction factors relating GC weight percentages to the IR *trans* equivalents for 18:2*t*, 18:2*tt*, and 18:3*t*, respectively. This formula is the basis for determining total 18:1*t* and 18:1*c* isomers and hence the general fatty acid composition of PHVO. In capillary columns coated with polar cyanoalkylsiloxane stationary phases such as SP-2340 and SP-2560, 18:2*t*, 18:2*tt*, and 18:3*t* are separated as distinct groups without any serious interferences or overlaps (11, Figure **994.15**) and levels of these *trans* polyunsaturates are obtained directly by GC analysis. IR method provides *trans* unsaturation and, therefore, total 18:1*t* is calculated from the mathematical formula. The content of 18:1*c* is then calculated as the difference between total 18:1 fatty acid methyl esters, which is the sum of all 18:1 isomer peaks in GC and 18:1*t*.

Because in the above GC/IR method 18:1*t* and IR *trans* unsaturation are linearly related, accuracy of 18:1*t* determination is dependent solely on the accuracy of total *trans* unsaturation determination by IR spectroscopy. During the development of the GC/IR procedure, Ratnayake et al. (11) used AOCS official IR method Cd 14-61 (13) for determination of total *trans* unsaturation of fatty acid methyl esters. However, the AOCS method, despite using the baseline technique to correct for any background absorption, suffers from a few drawbacks (14). A major problem is that samples analyzed as methyl esters produce *trans* levels which are 1.5–3% lower for *trans* values from 1 to 15% (15). AOAC Official Method **965.34** prescribes incorporation of correction factors to compensate the lower absorption of methyl esters (16). Another problem is that conjugated *trans* double bonds absorb very close to the isolated *trans* bond and can interfere with the isolated *trans* measurement (17). Because of this interference, AOCS official method is applicable only to samples containing less than 5% conjugated fatty acids.

Madison et al. (18) proposed a 2-component calibration procedure to overcome some of the drawbacks of the AOCS official IR methods. *Trans* content was calculated using a calibration curve of absorption versus percentage isolated *trans* unsaturation developed using a series of carbon disulfide solutions containing different ratios of methyl elaidate and methyl linoleate. Calibration and test solutions are scanned from 900 to 1500 cm^{-1} against a carbon disulfide blank. A baseline is drawn between peak minima at about 935 and 1020 cm^{-1}, and the baseline-corrected absorbance of the *trans* peak (967 cm^{-1}) is obtained. Baseline for the test sample spectrum is drawn exactly as the baseline in the standard spectrum, by overlaying the 2 spectra. This procedure allows to analyze *trans* contents in the 0.5–36% range with increased accuracy. The 2-component calibration procedure suggested by Madison et al. (18) compensates for the low bias of the AOCS method (13) for methyl esters, and eliminates the need for calculation of correction factors in AOAC Official Method **965.34** (16).

The present international collaborative study had 2 objectives. One was to evaluate the combined capillary GC/IR procedure of Ratnayake et al. (11) for determination of 18:1*t*, 18:1*c*, as well as general fatty acid composition of partially hydrogenated fats. Since the IR procedure proposed by Madison et al. (18) is an improvement over the current official methods of AOAC (16) and AOCS (13), the evaluation of an IR procedure similar to that described by Madison et al. was the second objective of the present collaborative study. Madison et al. specified a mixture of methyl elaidate and methyl linoleate for the development of calibration curves, but in the collaborative study methyl linoleate was replaced by methyl oleate because of its greater oxidative stability over methyl linoleate and its availability.

Collaborative Study

Six fat samples (A, B, C1, C2, D, and E), prepared at the Proctor and Gamble Co., Cincinnati, OH, were used in the collaborative study. Samples C1 and C2 were blind duplicates. Samples A, B, C1, and C2 were prepared by blending various ratios of fat extracted from 2 retail samples of margarine (par-

tially hydrogenated soybean, liquid soybean oil, and cottonseed oil-based margarines) and diluting them with unhydrogenated corn oil. Samples D and E were prepared by blending the fat extracted from the 2 retail margarines with partially hydrogenated canola oil (hard stock). All samples were blind coded (each laboratory had a unique number code) and sent to 19 collaborators. Samples A, B, D, and E were distributed to all participating collaborators. However, due to limited availability, the blind duplicate samples (C1 and C2) were distributed only to 15 collaborators. Each collaborator was provided with instructions, study protocols, and data report forms. In addition, each collaborator was provided a reference sample (R, partially hydrogenated soybean oil) with a labeled GC scan of the fatty acid methyl esters to be analyzed prior to analysis of test samples. Each collaborator was also provided with authentic standards of methyl oleate and elaidate for construction of the calibration curve of IR absorption versus *trans* content. Collaborators were instructed to prepare methyl esters from the partially hydrogenated fat samples according to the AOAC Official Method **963.33** that uses boron trifluoride (19). The collaborators were requested to analyze each sample twice.

Levels of 18:1*t* and 18:1*c* isomers in the test samples were estimated in the author's laboratory by a procedure different from that of the GC/IR. A known amount (ca 15 mg) of fatty acid methyl esters of the test samples was fractionated on silver nitrate-thin layer chromatography ($AgNO_3$-TLC) with development in toluene at –25°C (20). The separated 18:1*t* and 18:1*c* bands were extracted quantitatively, then methyl heptadecanoate (internal standard) was added and extracts were analyzed on a Hewlett-Packard 5890 Series II GC system (Hewlett-Packard Co., Palo Alto, CA) using a SP-2560 flexible fused silica capillary column (100 m × 0.25 mm id, 20 μm). The column oven temperature was programmed at a rate of 1.5°C from 150° to 200°C. From GC peak areas, amounts of 18:1*t* and 18:1*c* were calculated with respect to the internal standard.

Levels of 18:1*c* and 18:1*t* were also determined in author's laboratory according to the current AOCS official method Ce 1-89 (8) for determination of fatty acid composition in hydrogenated and unhydrogenated vegetable oils.

Statistical Analyses

Statistical evaluation of collaborative study results was performed with the computer program AOAC BUBR, which was developed by the AOAC Statistics Committee. The program calculates the performance parameters according to AOAC guidelines for collaborative studies (21).

994.14 Isolated *trans* Unsaturated Fatty Acid Content, in Partially Hydrogenated Fats

Infrared Spectrophotometric Method First Action 1994

(Applicable to determination of total isolated [i.e., non-conjugated] *trans* content in fats and oils containing >5% *trans* fatty acids.

Not applicable to samples containing >5% conjugated unsaturation [e.g., tung oil] materials containing functional groups which modify absorption of C-H deformation around *trans* bond [e.g., castor oil containing ricinoleic or ricinelaidic acids], or any materials where specific groups may absorb close to 967 cm^{-1} [10.3 μm].)

Caution: See Appendix: Laboratory Safety "Safe Handling of Special Chemical Hazards"—carbon disulfide. Dispose of carbon disulfide in an appropriate manner compatible with environmental rules and regulations.

Method Performance:

See Table **994.14** for method performance data.

A. Principle

Isolated *trans* double bonds (predominant *trans* configuration in partially hydrogenated fats) show absorption at ca 967 cm^{-1} (10.3 μm) deriving from C-H deformation about *trans* bond. Isolated *trans* content is determined by measurement of absorption intensity. Triglycerides or fatty acids are converted to methyl esters before making IR measurements. Total isolated *trans* content is calculated using calibration curve of absorption versus *trans* content of calibration solutions.

Table 994.14. Method performance for infrared determination of isolated *trans* unsaturated fatty acids in partially hydrogenated vegetable oils

Sample[a]	$\bar{x}$, % *trans*	RSD_r, %	RSD_R, %	s_r	s_R	r	R
A	5.2	4.8	34.6	0.3	1.8	0.84	5.04
B	15.5	4.2	11.3	0.7	1.8	1.96	5.04
C1	18.9	4.6	11.7	0.9	2.2	2.52	7.06
C2	19.1	3.7	10.3	0.7	2.0	1.96	5.49
C1 and C2[b]	19.0	5.8	10.5	1.1	2.0	3.07	5.61
D	30.1	3.0	9.0	0.9	2.7	2.52	7.06
E	34.6	1.0	11.3	0.3	3.9	0.84	5.04
R	21.6	4.1	8.8	0.9	1.9	2.52	7.06

[a] A–C2 = blends of various ratios of fat extracted from 2 retail samples of margarine (partially hydrogenated soybean, liquid soybean oil, and cottonseed oil based margarines) diluted with unhydrogenated corn oil; D–E = blends of fat extracted from 2 retail margarines with partially hydrogenated canola oil; R = reference sample (partially hydrogenated soybean soil).

[b] Blind duplicates.

B. Apparatus

Infrared spectrophotometer (IR).—Double-beam IR or Fourier Transform IR (FTIR); capable of quantitative measurements at 1050–900 cm^{-1}, with scale readable to 1 cm^{-1}; holding fixed thickness cells, 0.1–1.0 mm, with NaCl or KBr windows. All instruments must be checked for wavelength and photometric scale accuracy according to manufacture's instructions. Chart paper must be linear in either wavelength or wave number, and calibrated in either transmission, *T*, or absorbance, *A*.

C. Reagents

(**a**) *Carbon disulfide (CS_2).*—Dry, ACS grade.

(**b**) *Methyl elaidate stock solution.*—20 mg/mL. Accurately weigh ca 2000 mg methyl elaidate (purity >99%) to the nearest 0.1 mg into 100 mL volumetric flask, dilute to volume with CS_2 solution, and mix thoroughly.

(**c**) *Methyl oleate stock solution.*—20 mg/mL. Prepare as in (**b**) using methyl oleate (purity >99%) instead of methyl elaidate.

(**d**) *Calibration solutions.*—0.8, 1.6, 4, 8, 12, 16, and 20 mg methyl elaidate/mL in 19.2, 18.4, 16, 12, 8, 4, and 0 mg/mL methyl oleate–CS_2 solution, respectively. Accurately add 1, 2, 5, 10, 15, 20, and 25 mL methyl elaidate stock solution, (**b**), into separate 25 mL volumetric flasks using pipets. Dilute contents of flasks to volume with methyl oleate stock solution, (**d**). Verify weight percentages of methyl elaidate and methyl oleate by capillary GC analysis as in **994.15**. If weight percentages of methyl elaidate differ from expected values by >5% (for samples containing <10% methyl elaidate) or >2% (for samples containing >10% methyl elaidate) prepare fresh calibration solutions. Perform step **E** immediately because of high volatility of CS_2.

D. Preparation of Test Sample

Melt solid fats or free fatty acids on steam bath or in oven at temperature 10°C above melting point. If melted fat is cloudy filter through filter paper. Using ca 400–500 mg sample prepare methyl esters as in **969.33**. (*Note:* Accurate results depend on purity of fatty acid methyl esters. Before IR analysis remove excessive levels of impurities [e.g., non-saponifiable matter, polymers] using suitable cleanup procedure [e.g., saponification followed by extraction of non-saponifiable matter, thin-layer or column chromatography].) Accurately weigh ca 400–500 mg undiluted fatty acid methyl esters (M_1) to the nearest 0.1 mg into 25 mL volumetric flask. Dilute to volume with CS_2 solution. Perform step **E** immediately because of high volatility of CS_2.

E. Infrared Determination

Fill cell with CS_2 solution, and matching cell with test sample from **D** or calibration solution from **C(d)**. Use hypodermic syringe with blunted needle, and cell upright, inject from bottom so bubbles pass up through cell. When using double-beam IR place cell with CS_2 in reference beam. Place cell with test sample or calibration solution in sample beam. Scan spectrum (*T* or *A*) from 1050 to 900 cm^{-1} at optimal instrument settings. When using FTIR, initially scan CS_2 reference from 1050 to 900 cm^{-1} (background spectrum) and store it in memory of instrument data handling system. Scan test sample or calibration solution in same range as that for reference. Ratio obtained spectrum against background spectrum to obtain true *T* or *A*. Measure spectra of calibration solutions in order of increasing concentrations.

F. Calculations

For each spectrum draw baseline tangent to peak minima adjacent to 967 cm^{-1} (*see* Figure **994.14**). (*Note*: It is important to draw correct baseline because of measurement of baseline corrected absorption. Absorption minima might vary slightly between samples. Concentration and amount of *trans* unsaturation may influence position of absorption minima. Best results are obtained when baseline for sample is drawn exactly as baseline in spectrum of one of calibration standards having approximately same intensity of absorption at 967 cm^{-1}. This can be obtained by superimposing 2 spectra to draw baseline.)

another straight line parallel to ordinate and passing through apex of analytical band as in Figure **994.14**. (Line meets zero line of chart at point *Z*, apex at *Y*, and baseline tangent at *X*.)

For transmission spectrum measure distances *XZ* and *YZ*. Calculate absorption of calibration solution, A_i:

$$A_i = \log \frac{XZ}{YZ}$$

To calculate absorption of calibration solution, A_i, read A_X at *X*, and A_Y at *Y*:

$$A_i = A_Y - A_X$$

Plot mg methyl elaidate/mL calibration solution as abscissae versus corresponding A_i values as ordinate. Draw best straight line through 7 points plotted. For better accuracy deter-

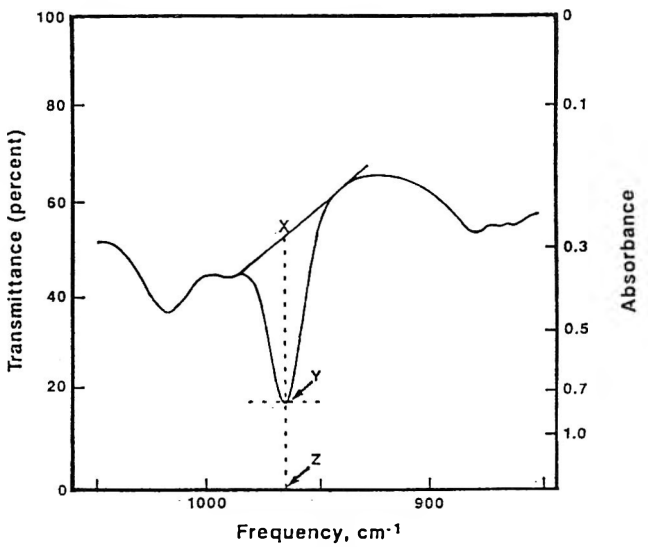

Figure 994.14. IR spectrum of partially hydrogenated canola oil methyl esters (2% solution in CS_2).

mine linear regression equation to fit data. (*Note:* Once obtained, calibration curve does not have to be repeated as long as instrument settings, parts, or cells have not been changed.)

Determine absorption of test sample, A_s, using same procedure as for calibration solutions. Using calibration curve or linear regression equation determine amount of methyl elaidate (mg M_2)/mL test sample solution, **D**, corresponding to A_s. Calculate percent *trans* unsaturated fatty acid content as methyl elaidate in test sample:

$$\%\ trans \text{ unsaturated fatty acid content (as methyl elaidate)} = 100 \times \frac{M_2}{M_1}$$

where M_1 = amount of fatty acid methyl esters in test sample solution, mg.

Ref.: *J. AOAC Int.* **78**, 783 (1995); *J. Chromatogr. Sci.* **28**, 633 (1990); *JAOCS* **67**, 804 (1990); *JAOCS* **69**, 95 (1992).

994.15 Total *cis*- and *trans*-Octadecenoic Isomers and General Fatty Acid Composition in Hydrogenated Vegetable Oils and Animal Fats

Capillary Gas Chromatographic/Infrared Spectrophotometric Method First Action 1994

(Applicable to partially hydrogenated vegetable oils and terrestrial animal fats containing >5% *trans* fatty acids. Not applicable to hydrogenated marine oils and partially hydrogenated fish oils, which contain large levels of *cis* and *trans* isomers of C_{16}, C_{18}, C_{20}, and C_{22} chain lengths.)

Method Performance:

See Table **994.15** for method performance data.

A. Principle

Total *trans* isomer content consists of *trans* fatty acids (*trans*-octadecenoate [18:1*t*]; mono-*trans*-octadecadienoate [18:2*ct* or *tc*, described as 18:2*t*]; *trans,trans*-octadecadienoate [18:2*tt*]; and mono-*trans*-octadecatrienoate [18:3*cct*, *ctc*, and *tcc*, described as 18:3*t*]), which occur in hydrogenated vegetable oils and terrestrial animal fats. Total *trans* content is determined by infrared spectrophotometry (IR) using methyl elaidate as external standard. Various isomers of 18:2*tt*, 18:2*t*, and 18:3*t* are resolved; their weight percentages are determined by GC. Based on IR determination, weight percentage of 18:1*t* is determined as described in *Calculations*. The difference between total methyl octadecenoate (18:1, as sum of all 18:1 peaks in GC) and calculated 18:1*t* gives weight percentage of *cis*-octadecenoate (18:1*c*).

B. Apparatus

(**a**) *Gas chromatograph (GC).*—With flame ionization detector, capillary column injection system (split ratio, 1:100). Operating conditions: injection port, 225°C; detector, 250°C. Temperature program: initial, 150°C; program rate, 1.0°C/min; final, 200°C; final hold, 20 min. (*Note:* Operator may change operating conditions to obtain optimum separation of isomeric fatty acid methyl esters.) Carrier gas, helium or hydrogen (≥99.99% purity), with oxygen scrubber in line.

(**b**) *GC column.*—100 m × 0.25 mm fused silica capillary column coated with SP-2560 (Supelco, Inc., Bellefonte, PA) or other suitable capillary column coated with cyanoalkylpolysiloxane (e.g., SP-2340, CP SIL-88) that provides same elution pattern as in Figure **994.15**.

(**c**) *GC syringe.*—Maximum volume 10 µL, graduated to 0.1 µL.

(**d**) *Infrared spectrophotometer (IR).*—Double-beam IR or Fourier Transform IR (FTIR); capable of quantitative measurements at 1050–900 cm^{-1}, with scale readable to 1 cm^{-1}; holding fixed thickness cells, 0.1–1.0 mm with NaCl or KBr windows. All instruments must be checked for wavelength and photometric scale accuracy according to manufacturer's instructions. Chart paper must be linear in either wavelength or wave number, and calibrated in either transmission, *T*, or absorbance, *A*.

C. Reagents

(**a**) *GC reference standards.*—Mixture of *cis* and *trans* isomers of known composition (Nu Chek Prep, Inc., Elysian, MN, or Supelco, Inc., Bellefonte, PA).

D. Preparation of Methyl Esters

Melt solid fats or free fatty acids at temperature ≤10°C above melting point and mix. If cloudy, filter through filter paper. If diluted sample is cloudy due to H_2O, add small portion of anhydrous Na_2SO_4 to melted sample, mix, and let settle before taking portion for methylation. Using ca 400–500 mg fat, prepare methyl esters as in **969.33**.

E. GC Analysis of Fatty Acid Composition

(**a**) *GC performance specifications.*—Inject 1–2 µL methyl esters from GC reference standards, **C(a)**, into GC. Select GC conditions to obtain resolution of methyl esters at least equivalent to that in Figure **994.15**.

(**b**) *GC determination.*—Inject 1–2 µL methyl esters (in hexane or heptane solution) from test sample into GC. Compare retention times of test sample with those of GC reference standards (*see* Figure **994.15**).

F. IR Determination of Total trans Content

Perform as in **994.14**.

G. Calculations

Calculate weight percentages of fatty acid methyl esters, W_X, assuming unity response factor for each component:

$$\%W_X = \frac{P_X}{P_T} \times 100$$

where P_X = GC area counts of specific methyl ester peak; P_T = total area counts of all fatty acid methyl ester peaks in chromatogram.

Table 994.15. Method performance for gas chromatographic/infrared determination of fatty acid composition of partially hydrogenated vegetable oils

Sample[a]	$\bar{x}$, %	RSD_r, %	RSD_R, %	s_r	s_R	r	R
			16:0				
A	10.3	0.3	2.4	0.03	0.25	0.08	0.7
B	9.1	1.4	2.8	0.13	0.25	0.36	0.7
C1	9.7	1.1	3.5	0.11	0.34	0.31	0.95
C2	9.7	1.2	4.0	0.21	0.39	0.59	1.09
C1 and C2[b]	9.7	1.0	4.0	0.11	0.39	0.31	0.10
D	10.7	1.0	3.2	0.10	0.34	0.28	0.95
E	9.7	3.7	3.7	0.13	0.36	0.36	1.01
R	10.8	1.3	2.6	0.02	0.28	0.06	0.78
			18:0				
A	3.8	0.8	1.9	0.03	0.07	0.08	0.20
B	7.0	1.6	3.3	0.11	0.23	0.31	0.64
C1	6.7	1.2	2.4	0.08	0.16	0.22	0.45
C2	6.7	1.2	3.9	0.08	0.26	0.22	0.73
C1 and C2[b]	6.6	2.6	4.8	0.17	0.32	0.48	0.88
D	7.3	0.6	1.2	0.04	0.08	0.11	0.22
E	6.9	2.9	3.6	0.20	0.25	0.56	0.7
R	5.8	1.8	4.4	0.10	0.25	0.28	0.7
			Total saturated fatty acids				
A	14.9	0.6	2.5	0.09	0.37	0.25	1.04
B	17.2	0.7	1.6	0.11	0.27	0.31	0.76
C1	17.6	2.0	2.9	0.35	0.51	0.98	1.43
C2	17.3	1.0	4.6	0.17	0.79	0.48	2.21
C1 and C2[b]	17.6	1.1	2.3	0.19	0.40	0.53	1.12
D	19.0	0.8	1.7	0.15	0.32	0.42	0.90
E	17.5	2.8	3.4	0.50	0.60	1.4	1.68
R	17.4	1.4	3.4	0.23	0.58	0.64	1.62
			18:1*t* Isomers				
A	4.9	5.2	36.4	0.25	1.77	0.7	4.96
B	14.9	2.1	9.5	0.32	1.41	0.90	3.95
C1	17.4	5.3	12.5	0.91	2.18	2.55	6.10
C2	17.5	4.2	10.3	0.73	1.81	2.04	5.07
C1 and C2[b]	17.4	6.9	10.9	1.20	1.90	3.37	5.31
D	26.6	3.6	9.6	0.96	2.55	2.69	7.14
E	32.6	1.9	7.8	0.61	2.53	1.71	7.08
R	19.4	4.3	9.7	0.83	1.87	2.32	5.24
			18:1*c* Isomers				
A	24.9	1.1	3.8	0.28	0.95	0.78	2.66
B	24.7	2.4	7.1	0.59	1.75	1.65	4.9
C1	28.1	3.2	6.9	0.88	1.94	2.46	5.43
C2	28.2	3.3	7.1	0.93	2.01	2.60	5.63
C1 and C2[b]	28.3	4.2	6.5	1.19	1.84	3.32	5.16
D	34.3	2.6	6.1	1.02	2.11	2.86	5.91
E	34.3	1.9	10.5	0.66	3.61	1.85	10.11
R	32.2	1.9	6.5	0.61	2.10	1.71	5.88

Table 994.15. ***(continued)***

Sample[a]	$\bar{x}$, %	RSD_r, %	RSD_R, %	s_r	s_R	r	R
			18:2(*n*-6)				
A	53.0	0.2	1.0	0.10	0.53	0.28	1.48
B	41.5	1.0	1.4	0.40	0.59	1.12	1.65
C1	33.6	0.7	1.7	0.22	0.57	0.62	1.60
C2	33.5	0.6	1.1	0.19	0.38	0.53	1.06
C1 and C2[b]	33.6	1.8	2.0	0.59	0.66	1.66	1.86
D	13.6	1.2	2.2	0.16	0.30	0.45	0.84
E	11.2	2.0	2.3	0.23	0.26	0.64	0.73
R	26.5	0.8	2.5	0.22	0.66	0.62	1.85
			18:3(*n*-3)				
A	0.9	7.8	9.6	0.07	0.09	0.20	0.25
B	0.8	11.8	20.2	0.09	0.50	0.25	1.4
C1	0.8	5.6	8.5	0.05	0.07	0.14	0.20
C2	0.8	5.2	8.7	0.04	0.07	0.11	0.20
C1 and C2[b]	0.8	7.7	19.9	0.06	0.16	0.18	0.46
D	0.9	2.5	8.6	0.02	0.08	0.06	0.22
E	0.8	6.8	17.5	0.05	0.14	0.14	0.39
R	1.0	3.1	8.4	0.03	0.08	0.08	0.22
			18:2*tt* Isomers				
A	ND[c]	NA[d]	NA	NA	NA	NA	NA
B	0.1	40.2	92.1	0.03	0.07	0.08	0.20
C1	0.2	19.8	96.3	0.03	0.15	0.08	0.42
C2	0.01	25.0	78.9	0.02	0.07	0.06	0.20
C1 and C2[b]	0.2	79.3	101.7	0.12	0.16	0.34	0.43
D	0.3	25.8	100.5	0.07	0.26	0.20	0.73
E	0.03	28.5	66.9	0.08	0.20	0.22	0.56
R	0.1	80.0	109.7	0.09	0.12	0.25	0.34
			18:2*t* Isomers				
A	0.3	25.8	49.8	0.08	0.16	0.22	0.45
B	0.7	33.1	55.0	0.24	0.39	0.67	1.09
C1	1.5	17.2	34.5	0.25	0.51	0.7	1.43
C2	1.6	2.1	35.5	0.03	0.56	0.08	1.57
C1 and C2[b]	1.5	6.0	34.2	0.09	0.51	0.25	1.43
D	3.3	5.1	22.6	0.17	0.75	0.48	2.1
E	3.1	2.6	27.3	0.08	0.84	0.22	2.35
R	2.3	4.3	20.8	0.10	0.48	0.28	1.34

Calculate weight percentage of 18:1*t* isomers, $W_{18:1t}$:

$$\% W_{18:1t} = W_{trans} - (1.74 \times W_{18:2tt}) - 0.84(W_{18:2t} + W_{18:3t})$$

where W_{trans} = total *trans* content determined by IR; $W_{18:2tt}$ = total weight percentage of all 18:2*tt* isomer peaks in GC; $W_{18:2t}$ = total weight percentage of all 18:2*t* isomer peaks in GC; $W_{18:3t}$ = total weight percentage of all 18:3*t* isomer peaks in GC; 1.74 and 0.84 = correction factors for *trans,trans* fatty acids and mono-*trans* fatty acids, respectively.

Calculate weight percentage of 18:1*c* isomer, $W_{18:1c}$:

$$\%W_{18:1c} = W_{18:1} - W_{18:1t}$$

where $W_{18:1}$ = total weight percentage of all the 18:1 isomer peaks in GC.

Ref: *J. AOAC Int.* **78**, 783 (1995); *J. Chromatogr. Sci.* **28**, 633(1990); *JAOCS* **67**, 804 (1990); *JAOCS* **69**, 95 (1992)

Table 994.15. ***(continued)***

Sample[a]	$\bar{x}$, %	RSD_r, %	RSD_R, %	s_r	s_R	r	R
			18:3*t* Isomers				
A	ND[c]	NA[d]	NA	NA	NA	NA	NA
B	0.03	152.3	193.7	0.05	0.06	0.14	0.17
C1	0.1	86.1	136.6	0.04	0.06	0.11	0.17
C2	0.04	75.0	141.6	0.03	0.06	0.08	0.17
C1 and C2[b]	0.04	93.81	133.8	0.04	0.06	0.11	0.16
D	0.2	55.4	76.9	0.12	0.16	0.34	0.45
E	0.2	31.7	79.7	0.06	0.15	0.17	0.42
R	0.1	0.0	150.4	0.00	0.08	0.00	0.22

[a] A–C2 = blends of various ratios of fat extracted from 2 retail samples of margarine (partially hydrogenated soybean, liquid soybean oil, and cottonseed oil based margarines) diluted with unhydrogenated corn oil; D–E = blends of fat extracted from 2 retail margarines with partially hydrogenated canola oil; R = reference sample (partially hydrogenated soybean soil).

[b] Blind duplicates.

[c] ND = not detected (content of total fatty acids <0.01%).

[d] NA = not applicable.

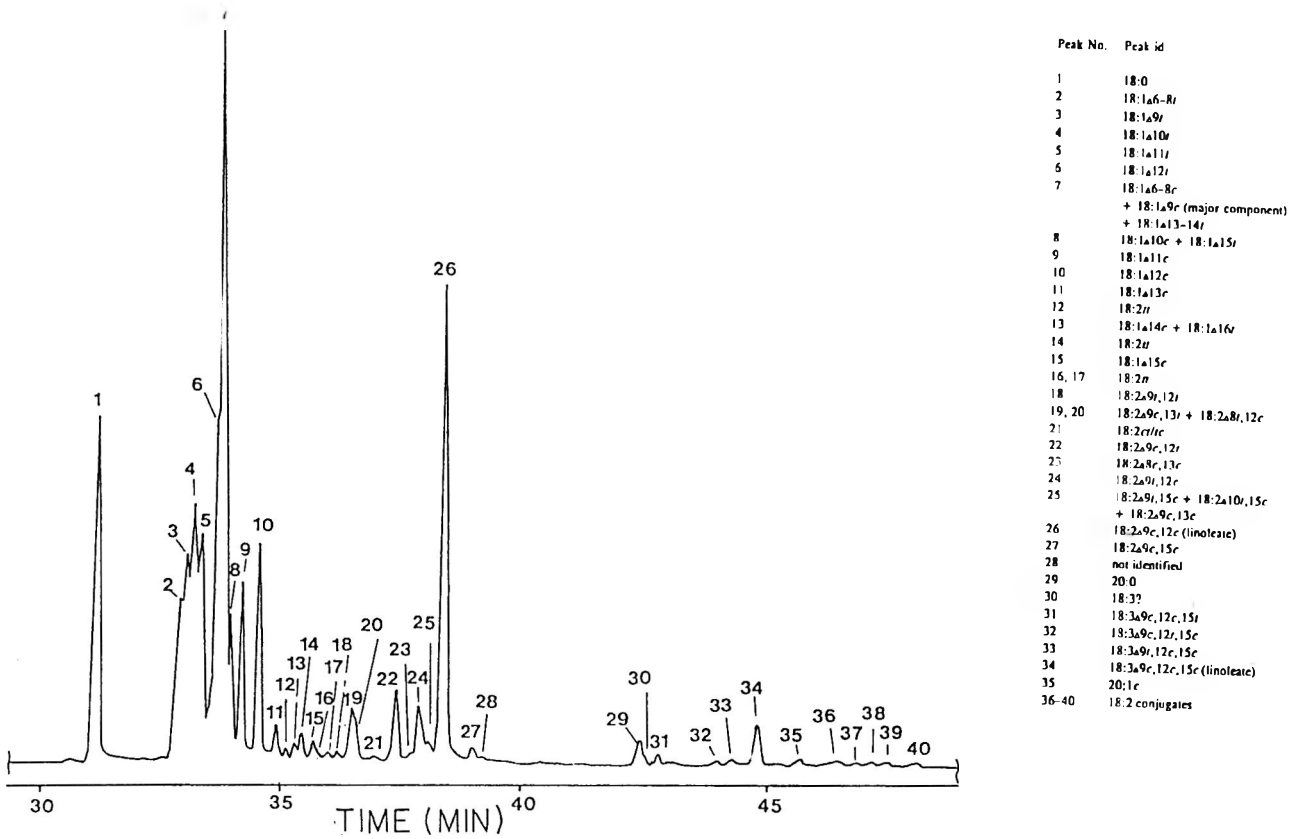

Figure 994.15. C_{18} **region of the gas chromatogram of the fatty acid methyl esters from partially hydrogenated soybean oil, using 100 m × 0.25 mm fused silica capillary column coated with SP2560.**

Table 1. Instrumentation and GC operating parameters reported by collaborating laboratories

Lab.	Instrument[a]		Column		Conditions		
	IR	GC	Liquid phase	Dimensions, m × mm	Carrier, gas, mL/min	Column temp., °C	Program
1	Bruker FTIR	HP-5890II	SP-2560	100 × 0.25	He, 0.49	150–200	1°C/min to 200°C, hold 25 min
2	Mattson FTIR	PE Sigma 300	SP-2560	100 × 0.25	He	150–200	hold 10 min, then 2°C/min to 200°C
3	PE16PC FTIR	HP-5890II	SP-2560	100 × 0.25	H_2, 0.70	150–200	1°C/min to 200°C, hold 10 min
4	Nicolet FTIR	HP-5890A	SP-2340	60 × 0.25	He	150–200	1°C/min to 185°C, then 10°C/min to 225°C, hold 10 min
5	PE-298, D	HP	CP-SIL-88	50 × 0.25	He, 0.05	150–200	hold 8 min, then 1°C/min to 210°C, hold 1 min
6	Pye Unicam, D	HP-5890	SP-2560	100 × 0.25	H_2, 1.3	125–220	hold 1 min, then 1°C/min to 175°C then, 15°C/min to 220°C
7	PE-597, D	Varian, 3400	SP-2380	30 × 0.25	He, 0.4	150–200	1°C/min
8	PE-1600 FTIR	Varian	SP-2560	100 × 0.25	H_2, 1.8	150–200	1°C/min
9	Nicolet FTIR	HP-5890II	SP-2560	100 × 0.25	H_2, 0.7	150–220	1°C/min
10	Nicolet FTIR	HP-5890II	SP-2560	100 × 0.25	He, 0.33	165–200	hold 65 min, then 5°C/min to 220°C, hold 25 min
11	Nicolet FTIR	HP-5890	SP-2560	100 × 0.25	He, 0.33	165–200	hold 75 min, then 7°C/min to 220°C, hold 40 min
12	PE-1600 FTIR	HP-5890II	SP-2560	100 × 0.25	H_2, 0.7	150–200	1°C/min to 200°C, hold 10 min

[a] PE = Perkin Elmer, HP = Hewlett Packard, FTIR = Fourier transform infrared spectrophotometer, D = dispersive spectrophotometer.

Results and Discussion

Analytical results were received from 12 of the 19 laboratories. Details of equipment and some of the operating parameters reported by the collaborators are listed in Table 1. Nine of the 12 collaborators reported using FTIR; 3 collaborators used conventional dispersive IRs. Nine collaborators reported using SP-2560 flexible fused silica capillary columns for GC analysis. Others used either SP-2340, SP-2380, or CP-Sil-88. It appears from the data submitted by the collaborators that use of either FTIR or conventional dispersive IR instrumentation did not affect the *trans* unsaturation results. Similarly, the type of cyanoalkylsiloxane column had no affect on the fatty acid composition data.

The raw data submitted by the collaborators for the 6 test samples and the reference sample, the statistical calculations of mean, within laboratory variation, and reproducibility are listed in Tables 2–9. Table 10 shows the statistical calculations for the blind duplicates C1 and C2. A summary of the mean values and the reproducibility relative standard deviations (RSD_R) of the 7 samples is given in Tables **994.14** (% *trans* data) and **994.15** (fatty acid composition data). Note that the duplicate values reported in Tables 2–9 are not blind duplicates, and hence do not provide a true measure of repeatability. (For the non-blind duplicates, new terms "within laboratory standard deviations" and "relative within laboratory standard deviations" with corresponding symbols S_w and RSD_w were introduced in Tables 2–9. The normal terms for performance parameters given in Harmonization Guidelines of AOAC [21] were used only with the true blind duplicates [Table 10].)

The *trans* content in the test samples, determined by IR, ranged from 5.2 to 34.6% and their RSD_R values ranged from 8.8 to 34.6% (Table **994.14**). Sample A, with the lowest level of *trans* unsaturation (5.2%) of all the samples, had the widest variation in reported IR *trans* values and the highest RSD_R (34.6%). All other samples tested had greater *trans* unsaturation, but their RSD_R values were much lower, ranging from 8.8 to 11.7%.

The similar RSD_R values for 18:1*t* (Table **994.15**) and IR *trans* unsaturation reflect the linear relationship between these 2 parameters. Sample A with lowest 18:1*t* content had the greatest RSD_R, while other samples with higher 18:1*t* contents (15–33%), had much lower RSD_R (7.8 to 12.5%, Table **994.15**).

RSD_R values for 18:1*c* ranged from 3.8 to 10.5% and were slightly lower than those for IR *trans* and 18:1*t*. For 16:0, 18:0, total saturates, and 18:2(*n*-6), the agreement between the laboratories was excellent, with RSD_R values less than 5%. For 18:3(*n*-3), however, reproducibility was somewhat less satisfactory; the RSD_R values ranged from 8.4 to 20.2%. These elevated values may be anticipated for 18:3(*n*-3), which rarely exceeds 1.0% in partially hydrogenated fats.

A very large variation was observed when analyzing *trans*-polyunsaturated fatty acids. This is to be expected, since the *trans*-polyunsaturated fatty acids are a complex mixture of several isomers, most of which constitute less than 0.1% of the total fatty acids.

The Cochran and Grubbs tests (21) identified a total of 53 outlier values among the 1080 values submitted by the collaborators (Tables 2–10). The reports of Collaborators 7 and 8 contained 26 and 13 outliers, respectively. No outliers occurred in the reports of 5 collaborators. In the remaining 4 collaborators' reports only 5 or fewer outliers were identified.

Overall, the IR and the GC/IR methods yielded reproducible results for *trans* content, saturated fatty acids, 18:1*t*, 18:1*c*, and *cis,cis*-polyunsaturated fatty acids. The variations obtained for both major and minor components are reasonable for a study of this kind. The excellent agreement between the pair of blind

Table 2. GC/IR collaborative study—% *trans* by IR

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
Sample	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
A	1.10	1.10	4.70	4.60	6.20	6.00	5.50	5.60	6.70	7.50	6.90	7.00	2.20	3.00	4.20	4.40	6.40	6.40	5.90	5.70	6.10	5.90	5.40	5.60
B	16.00	15.50	13.70	13.30	17.30	17.20	16.50	16.60	17.20	17.40	14.60	12.10	12.10	12.90	14.80	13.30	16.70	16.30	16.70	16.30	16.70	16.70	16.40	16.70
C1	15.90	15.80	20.60	18.30	20.90	20.90	20.40	20.30	20.70	21.30	18.50	19.90	15.40	17.00	14.70	16.90	19.80	20.30					20.40	20.30
C2	20.10	19.10	16.80	17.30	20.90	20.70	20.00	20.60	20.30	21.60	17.40	19.50	16.10	14.70	17.20	17.70	20.60	20.40					20.50	20.30
D	25.30	25.30	26.40	24.50	32.80	32.70	31.60	32.40	31.10	32.50	33.30	30.90	27.30	28.10	27.90	30.30	31.60	32.00	31.50	30.90	29.80	30.30	31.70	31.30
E	35.50	35.50	30.50	29.30	37.90	37.70	38.10	38.20	32.70	33.00	39.70	37.40	29.20	34.20	26.10	25.70	36.70	36.70	36.40	36.50	34.60	35.20	36.70	36.50
R	20.90	20.90	18.80	21.50	24.40	24.40	23.30	22.70	23.80	24.00	22.00	23.20	17.10	19.80	20.10	19.40	22.40	22.00	22.60	22.30	19.50	20.60	21.60	21.70

Statistical evaluation of % *trans* by IR

	Including outliers					Excluding outliers						
Sample	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
A	5.17	0.25	1.79	4.74	34.56	5.17	0.25	1.79	4.74	34.56	0/12	—
B	15.54	0.65	1.76	4.16	11.31	15.54	0.65	1.76	4.16	11.31	0/12	—
C1	18.92	0.87	2.21	4.62	11.69	18.92	0.87	2.21	4.62	11.69	0/10	—
C2	19.09	0.71	1.97	3.71	10.32	19.09	0.71	1.97	3.71	10.32	0/10	—
D	30.06	0.90	2.69	2.98	8.94	30.06	0.90	2.69	2.98	8.94	0/12	—
E	34.58	1.16	3.91	3.36	11.30	34.48	0.33	3.90	0.95	11.31	2/12	6,7
R	21.63	0.88	1.90	4.05	8.79	21.63	0.88	1.90	4.05	8.79	0/12	—

X1 = Data for experiment 1

X2 = Data for experiment 2

s_w = Within laboratory standard deviation

s_R = Reproducibility standard deviation

RSD_w = Relative within laboratory standard deviation

RSD_R = Reproducibility relative standard deviation

*Outlier laboratory, determined by Cochran and/or Grubbs tests

Table 3. GC/IR collaborative study results of PHVO sample A

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	10.40	10.40	10.60	10.60	10.70	10.70	10.50	10.50	9.90	9.90	10.10	10.10	10.00	11.20	10.10	10.10	10.40	10.40	10.00	10.10	10.30	10.30	10.40	10.50
18:0	3.80	3.80	3.60	3.60	3.70	3.70	3.80	3.80	3.80	3.80	3.70	3.70	2.80	2.90	3.50	3.60	3.80	3.70	3.80	3.80	3.80	3.70	3.80	3.80
SAT.	15.20	15.20	14.70	14.70	15.30	15.20	15.30	15.30	14.50	14.40	14.50	14.40	13.00	14.30	14.30	14.10	15.10	15.00	14.80	14.90	15.00	14.70	15.00	15.10
18:1*t*	0.90	0.90	4.20	4.30	5.90	5.70	5.10	5.20	6.30	7.20	6.80	6.90	2.10	2.80	3.80	3.80	6.10	6.10	5.70	5.50	5.70	5.50	5.10	5.40
18:1*c*	26.20	26.20	25.80	25.90	24.80	25.10	25.50	25.50	23.90	23.00	23.70	23.70	23.40	24.20	26.20	25.80	24.30	24.50	24.90	25.00	25.10	25.00	25.40	25.30
18:2(*n*-6)	52.30	52.30	53.40	53.30	52.50	52.50	52.40	52.40	53.30	53.50	53.40	53.40	58.90	56.80	53.90	54.10	52.60	52.70	53.00	53.10	52.80	53.10	53.00	52.80
18:3(*n*-3)	1.00	1.00	0.80	1.00	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.40	0.40	1.10	1.10	0.90	0.90	0.90	0.90	0.80	1.20	0.90	0.90
18:2*tt*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00
18:2*t*	0.20	0.20	0.20	0.30	0.40	0.30	0.40	0.40	0.50	0.40	0.10	0.20	0.10	0.20	0.50	0.80	0.30	0.40	0.20	0.20	0.30	0.40	0.30	0.20
18:3*t*	0.00	0.00	0.30	0.10	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00
IR-*trans*	1.10	1.10	4.70	4.60	6.20	6.00	5.50	5.60	6.70	7.50	6.90	7.00	2.20	3.00	4.20	4.40	6.40	6.40	5.90	5.70	6.10	5.90	5.40	5.60
CAL-*trans*	1.10	1.10	4.80	4.60	6.30	6.00	5.60	5.60	6.80	7.60	6.90	7.10	2.20	3.00	4.30	4.60	6.40	6.50	6.00	5.70	6.10	5.90	5.40	5.60

	Statistical evaluation of sample A											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	10.34	0.25	0.31	2.38	2.97	10.32	0.03	0.25	0.29	2.44	1/12	7
18:0	3.64	0.05	0.29	1.37	8.02	3.75	0.03	0.07	0.84	1.88	2/12	7,8
SAT.	14.75	0.28	0.53	1.90	3.59	14.85	0.09	0.37	0.63	2.50	1/12	7
18:1*t*	4.88	0.25	1.77	5.20	36.39	4.88	0.25	1.77	5.20	36.39	0/12	—
18:1*c*	24.93	0.28	0.95	1.14	3.79	24.93	0.28	0.95	1.14	3.79	0/12	—
18:2(*n*-6)	53.39	0.45	1.52	0.83	2.85	53.00	0.10	0.53	0.18	1.00	1/12	7
18:3(*n*-3)	0.90	0.07	0.18	7.86	19.90	0.90	0.07	0.09	7.78	9.56	1/12	11
18:2*tt*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0/12	—
18:2*t*	0.31	0.08	0.16	25.78	49.78	0.31	0.08	0.16	25.78	49.78	0/12	—
18:3*t*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1/12	2
IR-*trans*	5.17	0.25	1.79	4.74	34.56	5.17	0.25	1.79	4.74	34.56	0/12	—
CAL-*trans*	5.21	0.27	1.80	5.18	34.58	5.21	0.27	1.80	5.18	34.58	0/12	—

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 4. GC/IR collaborative study results of PHVO sample B

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	9.20	9.10	9.50	9.40	9.30	9.40	9.20	9.20	8.70	8.70	9.10	8.90	8.80	9.20	8.80	8.60	9.10	9.10	8.80	8.80	9.20	9.00	9.10	9.30
18:0	7.20	7.10	6.90	6.70	7.10	7.10	7.10	7.10	7.10	7.20	6.80	7.00	6.70	7.00	6.70	6.40	7.10	7.10	7.10	7.10	6.90	7.00	7.40	7.40
SAT.	17.40	17.30	17.20	17.10	17.60	17.60	17.60	17.50	16.70	16.90	16.90	16.90	15.70	16.40	16.30	15.70	17.40	17.40	17.20	17.20	17.10	17.00	17.10	17.50
18:1*t*	15.80	15.00	12.90	12.40	16.30	16.20	15.60	15.70	15.50	16.60	14.10	11.60	12.00	12.80	13.60	13.10	15.90	15.70	15.80	15.40	15.70	15.80	15.60	15.90
18:1c	27.90	27.50	25.90	26.00	23.20	23.20	24.00	24.00	22.80	22.00	25.40	27.70	26.50	27.40	25.00	25.50	23.70	23.50	23.70	24.00	23.00	23.50	24.20	23.70
18:2(*n*-6)	41.00	41.10	42.10	41.80	40.90	41.00	40.90	41.00	41.30	42.00	41.80	42.00	42.90	41.40	42.00	42.80	41.30	41.40	41.40	41.40	41.80	41.70	41.00	40.80
18:3(*n*-3)	0.80	0.80	0.80	1.10	0.70	0.70	0.80	0.80	0.70	0.70	0.70	0.70	0.50	0.30	1.00	0.80	0.70	0.70	0.80	0.80	0.90	0.80	0.80	0.80
18:2*tt*	0.00	0.00	0.10	0.10	0.20	0.20	0.10	0.10	0.00	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.10	0.00	0.10	0.10	0.10	0.20	0.10	0.10
18:2*t*	0.30	0.60	0.60	0.70	0.80	0.80	0.90	0.80	1.80	0.90	0.40	0.40	0.10	0.10	1.20	1.40	0.70	0.70	0.70	0.70	0.90	0.70	0.70	0.70
18:3*t*	0.00	0.00	0.10	0.20	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.20	0.00	0.10	0.00	0.10	0.10	0.00	0.00	0.00	0.00
IR-*trans*	16.00	15.50	13.70	13.30	17.30	17.20	16.50	16.60	17.20	17.40	14.60	12.10	12.10	12.90	14.80	13.30	16.70	16.30	16.70	16.30	16.70	16.70	16.40	16.70
CAL-*trans*	16.10	15.50	13.70	13.40	17.30	17.20	16.60	16.70	17.30	17.50	14.60	12.10	12.10	12.90	15.00	14.50	16.80	16.40	16.70	16.30	16.70	16.70	16.40	16.70

	Statistical evaluation of sample B											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	9.06	0.13	0.25	1.39	2.80	9.06	0.13	0.25	1.39	2.80	0/12	—
18:0	7.01	0.11	0.23	1.63	3.31	7.01	0.11	0.23	1.63	3.31	0/12	—
SAT.	17.04	0.17	0.63	1.01	3.73	17.23	0.11	0.27	0.66	1.60	2/12	7,8
18:1*t*	14.84	0.58	1.52	3.90	10.28	14.92	0.32	1.41	2.12	9.48	1/12	6
18:1*c*	24.70	0.59	1.75	2.39	7.08	24.70	0.59	1.75	2.39	7.08	0/12	—
18:2(*n*-6)	41.53	0.40	0.59	0.97	1.41	41.53	0.40	0.59	0.97	1.41	0/12	—
18:3(*n*-3)	0.76	0.09	0.15	11.82	20.21	0.76	0.09	0.50	11.82	20.21	0/12	—
18:2*tt*	0.07	0.03	0.07	40.20	92.11	0.07	0.03	0.07	40.20	92.11	0/12	—
18:2*t*	0.73	0.24	0.39	33.08	54.95	0.73	0.24	0.39	33.08	54.95	0/12	—
18:3*t*	0.03	0.05	0.06	152.26	193.65	0.03	0.05	0.06	152.26	193.65	0/12	—
IR-*trans*	15.54	0.65	1.76	4.16	11.31	15.54	0.65	1.76	4.16	11.31	0/12	—
CAL-*trans*	15.63	0.58	1.72	3.71	10.99	15.84	0.29	1.59	1.81	10.50	1/12	6

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 5. GC/IR collaborative study results of PHVO sample C1

Lab.	1		2		3		4		5		6		7		8		9		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	9.80	9.70	10.90	10.10	10.10	10.10	9.90	9.80	9.50	9.50	9.80	9.70	8.80	9.10	9.60	9.60	9.70	9.70	10.10	9.80
18:0	6.80	7.00	6.40	6.50	6.80	6.80	6.80	6.80	7.00	6.80	6.70	6.80	5.70	5.80	6.60	6.50	6.80	6.80	6.70	6.70
SAT.	17.80	18.00	18.10	17.40	18.00	18.00	18.00	17.80	17.40	17.00	17.40	17.40	14.70	15.00	17.00	16.80	17.60	17.60	17.70	17.40
18:1*t*	14.00	13.90	19.50	17.00	19.10	19.00	18.80	18.50	19.00	19.00	17.20	18.70	14.80	16.60	12.70	14.90	18.40	18.40	18.90	18.90
18:1c	31.00	31.30	26.50	29.20	26.70	26.70	27.10	27.40	25.80	25.40	28.60	27.10	29.40	28.80	32.30	30.00	27.20	27.20	27.20	27.20
18:2(*n*-6)	33.20	32.50	33.60	33.50	33.20	33.20	33.30	33.20	33.20	33.20	34.00	34.00	36.10	37.80	34.60	34.80	33.50	33.50	33.20	33.20
18:3(*n*-3)	0.80	0.90	0.90	1.00	0.80	0.80	0.80	0.80	0.90	0.80	0.80	0.80	0.50	0.40	0.90	1.00	0.80	0.80	0.80	0.80
18:2*tt*	0.50	0.50	0.10	0.00	0.20	0.30	0.20	0.20	0.00	0.00	0.10	0.10	0.00	0.00	0.30	0.30	0.20	0.10	0.10	0.10
18:2*t*	0.10	1.10	1.00	1.30	1.70	1.70	1.60	1.70	2.00	2.70	1.20	1.20	0.70	0.50	1.74	1.70	1.20	2.00	1.60	1.50
18:3*t*	0.00	0.00	0.10	0.20	0.00	0.00	0.00	0.10	0.00	0.10	0.10	0.10	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.00
IR-*trans*	15.90	15.80	20.60	18.30	21.00	20.90	20.40	20.30	20.70	21.30	18.50	19.90	15.40	17.00	14.70	16.90	19.80	20.30	20.40	20.30
CAL-*trans*	15.60	15.50	20.70	18.50	20.90	20.90	20.50	20.50	21.00	21.70	18.60	20.10	15.50	17.10	14.70	16.90	19.90	20.60	20.60	20.50

Statistical evaluation of sample C1

	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	9.77	0.21	0.43	2.11	4.39	9.68	0.11	0.34	1.12	3.48	1/10	2
18:0	6.65	0.08	0.35	1.21	5.26	6.74	0.08	0.16	1.21	2.40	1/10	7
SAT.	17.36	0.34	1.01	1.98	5.79	17.63	0.35	0.51	2.01	2.90	1/10	7
18;1*t*	17.37	0.91	2.18	5.26	12.53	17.37	0.91	2.18	5.26	12.53	0/10	—
18:1*c*	28.11	0.88	1.94	3.14	6.89	28.11	0.88	1.94	3.14	6.89	0.10	—
18:2(*n*-6)	33.93	0.43	1.22	1.28	3.60	33.59	0.22	0.57	0.66	1.70	1/10	5
18:3(*n*-3)	0.81	0.05	0.14	6.21	17.74	0.84	0.05	0.07	5.58	8.49	1/10	7
18:2*tt*	0.16	0.03	0.15	19.76	96.26	0.16	0.03	0.15	19.76	96.26	0/10	—
18:2*t*	1.46	0.25	0.51	17.20	34.51	1.46	0.25	0.51	17.20	34.51	0/10	—
18:3*t*	0.04	0.04	0.06	86.07	136.59	0.10	0.04	0.06	86.07	136.59	0/10	—
IR-*trans*	18.92	0.87	2.21	4.62	11.69	18.92	0.87	2.21	4.62	11.69	0/10	—
CAL-*trans*	19.02	0.88	2.33	4.63	12.26	19.02	0.88	2.33	4.63	12.26	0/10	—

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 6. GC/IR collaborative study results of PHVO sample C2

Lab.	1		2		3		4		5		6		7		8		9		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	9.90	9.90	10.20	10.40	10.00	10.00	9.90	9.90	9.20	9.10	9.70	9.60	9.00	9.20	9.30	9.70	9.80	9.80	10.00	9.90
18:0	6.80	6.90	6.60	6.30	6.80	6.80	6.80	6.80	6.80	6.80	6.70	6.80	6.10	6.20	6.30	6.30	6.80	6.80	6.80	6.70
SAT.	17.90	18.00	17.60	17.50	18.00	17.90	17.90	17.90	17.00	16.80	17.30	17.30	15.30	15.60	16.40	16.80	17.70	17.70	17.70	17.20
18:1*t*	19.00	18.00	15.50	16.20	19.10	18.90	18.30	19.00	18.10	19.40	16.00	18.20	15.60	14.20	14.60	14.90	18.90	18.60	19.00	19.00
18:1*c*	31.40	31.60	29.90	32.80	26.60	26.80	27.50	26.80	26.50	25.00	29.60	27.60	27.20	28.40	28.50	28.30	26.80	27.10	27.80	27.20
18:2(*n*-6)	33.20	32.90	34.30	37.10	33.20	33.40	33.40	33.30	33.90	33.70	34.10	34.10	38.30	37.00	33.50	34.10	33.50	33.40	33.10	33.40
18:3(*n*-3)	0.90	0.90	0.90	1.00	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.50	0.40	1.25	1.10	0.70	0.80	0.90	0.80
18:2*tt*	0.10	0.00	0.10	0.10	0.20	0.20	0.10	0.10	0.00	0.00	0.10	0.10	0.00	0.00	0.40	0.70	0.10	0.10	0.20	0.20
18:2*t*	1.10	1.10	1.30	0.90	1.60	1.60	1.70	1.60	2.60	2.60	1.40	1.20	0.60	0.60	1.90	1.90	1.80	1.80	1.60	1.60
18:3*t*	0.00	0.00	0.10	0.20	0.10	0.10	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.00	0.50	0.20	0.00	0.10	0.00	0.00
IR-*trans*	20.10	19.10	16.80	17.30	20.90	20.70	20.00	20.60	20.70	21.60	17.40	19.50	16.10	14.70	17.20	17.70	20.60	20.40	20.50	20.30
CAL-*trans*	20.20	19.10	17.00	17.40	21.00	20.80	20.20	20.70	20.70	22.00	17.60	19.60	16.20	14.80	17.30	17.50	20.80	20.60	20.80	20.30

	Statistical evaluation of sample C2											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	9.73	0.21	0.39	1.19	4.00	9.73	0.21	0.39	1.19	4.00	0/10	—
18:0	6.65	0.08	0.26	1.21	3.86	6.65	0.08	0.26	1.21	3.86	0/10	—
SAT.	17.26	0.17	0.79	0.98	4.58	17.26	0.17	0.79	0.98	4.58	0/10	—
18:1*t*	17.53	0.73	1.81	4.17	10.34	17.53	0.73	1.81	4.17	10.34	0/10	—
18:1*c*	28.17	0.93	2.01	3.29	7.14	28.17	0.93	2.01	3.29	7.14	0/10	—
18:2(*n*-6)	34.16	0.71	1.53	2.08	4.47	33.53	0.19	0.38	0.57	1.13	2/10	2,7
18:3(*n*-3)	0.83	0.06	0.19	7.62	23.04	0.83	0.04	0.07	5.21	8.66	2/10	7,8
18:2*tt*	0.14	0.07	0.17	50.51	119.40	0.09	0.02	0.07	24.96	78.92	1/10	8
18:2*t*	1.53	0.09	0.55	6.20	35.71	1.59	0.03	0.56	2.10	35.52	1/10	2
18:3*t*	0.08	0.07	0.12	98.88	163.90	0.04	0.03	0.06	75.00	141.56	1/10	8
IR-*trans*	19.09	0.71	1.97	3.71	10.32	19.09	0.71	1.97	3.71	10.32	0/10	—
CAL-*trans*	19.26	0.69	2.04	3.56	10.60	19.26	0.69	2.04	3.56	10.60	0/10	—

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1t, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 7. GC/IR collaborative study results of PHVO sample D

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	10.60	10.60	11.00	11.40	11.00	11.00	10.80	10.70	10.20	10.10	10.60	10.60	10.30	11.00	10.30	10.21	10.90	10.70	10.30	10.40	10.90	10.90	10.70	10.90
18:0	7.50	7.50	7.20	7.20	7.20	7.20	7.30	7.20	7.30	7.30	7.20	7.20	6.30	6.90	6.60	6.50	7.30	7.30	7.20	7.20	7.30	7.20	7.30	7.20
SAT.	19.20	19.10	19.00	19.40	19.40	19.20	19.30	19.10	18.40	18.20	18.70	18.70	16.80	18.60	17.50	17.30	19.20	19.00	18.70	18.80	19.40	18.90	18.90	18.80
18:1*t*	21.70	21.40	23.80	21.60	29.00	29.10	28.00	28.80	26.90	28.60	30.00	27.60	25.90	26.40	23.30	25.80	28.10	28.10	27.60	27.00	26.70	27.20	28.80	28.00
18:1*c*	39.00	38.80	38.50	36.60	32.70	32.70	33.60	32.60	33.00	31.70	31.90	34.20	34.70	33.60	36.10	32.80	34.10	33.60	34.10	34.60	35.00	35.10	32.70	33.40
18:2(*n*-6)	13.00	13.00	13.80	13.10	13.30	13.50	13.50	13.50	13.70	13.70	13.90	13.90	14.60	18.50	15.50	14.70	13.40	13.40	13.50	13.50	13.70	13.80	13.90	13.90
18:3(*n*-3)	0.80	0.90	1.00	1.00	0.80	0.80	0.90	0.90	0.90	0.90	0.90	0.90	0.40	0.50	1.30	1.50	0.80	0.80	0.90	0.90	1.00	1.00	1.00	1.00
18:2*tt*	0.90	0.90	0.10	0.10	0.40	0.30	0.20	0.20	0.00	0.00	0.20	0.20	0.00	0.00	0.60	0.50	0.20	0.30	0.40	0.40	0.00	0.20	0.00	0.20
18:2*t*	2.40	2.70	2.50	2.90	3.50	3.50	3.70	3.70	5.00	4.60	3.30	3.30	1.70	2.00	3.90	3.70	3.30	3.80	3.50	3.50	3.20	3.20	3.30	3.30
18:3*t*	0.10	0.10	0.30	0.30	0.20	0.20	0.20	0.20	0.10	0.10	0.20	0.20	0.00	0.00	0.30	0.70	0.20	0.20	0.30	0.30	0.50	0.10	0.20	0.20
IR-*trans*	25.30	25.30	26.40	24.50	32.80	32.70	31.60	32.40	31.10	32.50	33.30	30.90	27.30	28.10	27.90	30.30	31.60	32.00	31.50	30.90	29.80	30.30	31.70	31.30
CAL-*trans*	25.10	24.20	26.70	25.00	33.10	33.10	32.00	32.80	31.90	33.20	33.70	31.30	27.60	28.40	28.10	30.60	31.80	32.40	31.80	31.20	30.40	30.70	32.30	31.70

	Statistical evaluation of sample D											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	10.67	0.17	0.34	1.63	3.18	10.67	0.10	0.34	0.98	3.19	1/12	7
18:0	7.15	0.13	0.29	1.83	4.07	7.26	0.04	0.08	0.62	1.15	2/12	7,8
SAT.	18.72	0.39	0.67	2.10	3.60	18.95	0.15	0.32	0.78	1.69	2/12	7,8
18:1*t*	26.64	0.96	2.55	3.59	9.58	26.64	0.96	2.55	3.59	9.58	0/12	—
18:1*c*	34.38	1.02	2.11	2.95	6.14	34.38	1.02	2.11	2.59	6.14	0/12	—
18:2(*n*-6)	13.93	0.83	1.13	5.93	8.13	13.55	0.16	0.30	1.21	2.19	2/12	7,8
18:3(*n*-3)	0.91	0.05	0.22	5.50	23.89	0.91	0.02	0.08	2.47	8.60	2/12	7,8
18:2*tt*	0.26	0.07	0.26	25.79	100.52	0.26	0.07	0.26	25.79	100.52	0/12	—
18:2*t*	3.30	0.17	0.75	5.06	22.58	3.30	0.17	0.75	5.06	22.58	0/12	—
18:3*t*	0.21	0.12	0.16	55.43	76.92	0.21	0.12	0.16	55.43	76.92	0/12	—
IR-*trans*	30.06	0.90	2.69	2.98	8.94	30.06	0.90	2.69	2.98	8.94	0/12	—
CAL-*trans*	30.38	0.91	2.89	2.98	9.50	30.38	0.91	2.89	2.98	9.50	0/12	—

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 8. GC/IR collaborative study results of PHVO sample E

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	9.60	9.70	10.60	9.20	10.10	10.00	9.70	9.70	9.30	9.20	9.80	9.70	9.40	10.30	9.50	9.30	9.70	9.70	9.30	9.20	10.00	9.50	9.70	9.70
18:0	7.20	7.30	7.10	6.70	7.00	7.00	7.00	7.00	7.00	7.00	6.70	6.80	6.30	6.90	6.80	6.20	7.00	7.00	7.00	7.00	7.10	6.90	7.00	7.10
SAT.	18.00	18.10	18.40	17.10	18.10	18.10	17.90	17.90	17.40	17.40	17.60	16.90	15.90	17.40	17.10	16.20	17.80	17.80	17.50	17.40	18.10	17.30	17.60	17.70
18:1*t*	32.80	33.30	28.20	27.10	34.20	34.20	34.80	34.70	28.60	28.80	36.50	34.10	27.60	32.70	22.00	21.30	32.90	33.00	32.70	32.60	31.90	31.00	33.30	33.00
18:1*c*	38.60	38.70	38.20	36.50	31.50	31.70	30.40	30.80	34.80	34.40	29.60	32.20	33.20	32.60	42.60	42.70	32.50	32.40	32.90	32.90	34.10	34.00	32.60	32.70
18:2(*n*-6)	10.80	10.80	11.60	10.70	11.10	11.20	11.20	11.20	11.50	11.50	11.40	11.60	12.80	13.70	12.20	13.00	11.20	11.30	11.20	11.20	11.60	11.20	11.20	11.10
18:3(*n*-3)	0.80	0.70	0.80	0.90	0.70	0.70	0.90	0.90	0.70	0.70	0.80	0.80	0.50	0.50	1.00	1.10	0.80	0.80	0.80	0.80	0.80	1.00	0.80	0.80
18:2*tt*	0.40	0.10	0.10	0.10	0.50	0.40	0.20	0.30	0.00	0.10	0.30	0.30	0.00	0.00	0.60	0.60	0.40	0.50	0.50	0.50	0.10	0.30	0.40	0.40
18:2*t*	2.40	2.30	2.10	2.00	3.20	3.30	3.40	3.30	4.90	5.00	3.00	3.10	1.90	1.80	3.50	3.60	3.50	3.40	3.00	3.20	2.80	3.80	3.00	3.10
18:3*t*	0.10	0.10	0.30	0.40	0.10	0.00	0.10	0.10	0.00	0.00	0.20	0.20	0.00	0.00	0.20	0.40	0.20	0.10	0.40	0.40	0.30	0.30	0.20	0.20
IR-*trans*	35.50	35.50	30.50	29.30	37.90	37.70	38.10	38.20	32.70	33.00	39.70	37.40	29.20	34.20	26.10	25.70	36.70	36.70	36.40	36.50	34.60	35.20	36.70	36.50
CAL-*trans*	35.60	35.80	30.80	29.60	38.00	37.80	38.50	38.50	33.50	33.80	40.00	37.70	29.50	34.50	26.30	25.80	37.00	37.00	36.60	36.70	35.10	35.70	39.90	36.70

	Statistical evaluation of sample E											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	9.66	0.13	0.36	3.73	3.73	9.66	0.13	0.36	3.73	3.73	0/12	—
18:0	6.92	0.20	0.25	2.87	3.62	6.92	0.20	0.25	2.87	3.62	0/12	—
SAT.	17.53	0.50	0.60	2.83	3.40	17.53	0.50	0.60	2.83	3.40	0/12	—
18:1*t*	31.48	1.19	3.99	3.78	12.66	32.60	0.61	2.53	1.88	7.78	2/12	7,8
18:1*c*	34.28	0.66	3.61	1.92	10.52	34.28	0.66	3.61	1.92	10.52	0/12	—
18:2(*n*-6)	11.51	0.32	0.74	2.80	6.39	11.23	0.23	0.26	2.03	2.33	2/12	7,8
18:3(*n*-3)	0.80	0.05	0.14	6.79	17.50	0.80	0.05	0.14	6.79	17.50	0/12	—
18:2*tt*	0.30	0.08	0.20	28.45	66.93	0.30	0.08	0.20	28.45	66.93	0/12	—
18:2*t*	3.11	0.22	0.82	7.01	26.33	3.09	0.08	0.84	2.58	27.25	1/12	11
18:3*t*	0.18	0.06	0.15	31.49	79.33	0.18	0.06	0.15	31.49	79.73	0/12	—
IR-*trans*	34.58	1.16	3.91	3.36	11.30	34.48	0.33	3.90	0.95	11.31	2/12	6,7
CAL-*trans*	34.89	1.16	3.90	3.34	11.18	34.79	0.34	3.89	0.97	11.19	2/12	6,7

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 9. GC/IR collaborative study results of PHVO sample R

Lab.	1		2		3		4		5		6		7		8		9		10		11		12	
FA	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2
16:0	11.00	10.80	11.40	10.90	11.20	11.40	10.90	10.90	10.40	10.40	10.80	10.70	10.60	10.80	10.80	10.60	10.80	10.80	10.50	10.50	10.50	10.70	11.70	10.70
18:0	5.90	5.90	5.70	5.80	5.80	5.80	5.90	5.80	6.00	6.00	5.80	5.90	5.10	5.00	5.70	5.50	5.80	5.80	5.80	5.80	5.80	6.10	5.70	5.80
SAT.	17.90	17.80	17.70	17.10	18.00	18.10	17.90	17.80	17.30	17.20	17.40	17.40	15.90	16.00	16.90	16.50	17.60	17.60	17.40	17.40	17.10	17.60	17.70	17.00
18:1*t*	18.60	18.30	16.80	19.50	22.00	22.30	20.90	20.20	22.10	22.00	20.40	20.40	16.10	19.00	16.80	16.70	19.60	19.30	20.10	19.80	17.30	17.30	19.70	19.70
18:1*c*	35.90	35.70	33.60	31.70	29.60	29.20	30.70	31.30	28.90	28.40	31.30	31.40	31.40	32.70	33.00	34.30	31.90	32.20	31.50	31.80	35.20	34.10	32.20	33.10
18:2(*n*-6)	25.80	25.70	26.80	27.40	26.30	26.30	26.20	26.20	26.90	26.40	26.80	26.80	29.70	29.20	28.40	27.90	26.20	26.30	26.20	26.20	26.10	26.20	26.60	26.20
18:3(*n*-3)	1.00	1.00	0.90	0.90	0.90	0.90	1.00	0.90	0.90	1.00	0.90	0.90	0.50	0.50	1.10	1.10	0.90	0.90	1.00	1.00	1.10	1.10	0.90	1.00
18:2*tt*	0.10	0.30	0.00	0.10	0.20	0.00	0.10	0.10	0.00	0.00	0.10	0.10	0.00	0.00	0.53	0.20	0.10	0.10	0.20	0.20	0.10	0.10	0.00	0.00
18:2*t*	2.10	2.40	1.80	1.80	2.50	2.50	2.60	2.60	2.10	2.40	2.30	2.30	1.20	1.00	2.90	2.80	2.90	2.80	2.40	2.40	2.50	2.50	2.40	2.40
18:3*t*	0.00	0.00	0.60	0.40	0.00	0.00	0.10	0.10	0.00	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.20	0.20	0.20	0.20	0.00	0.00	0.00	0.00
IR-*trans*	20.90	20.90	18.80	21.50	24.40	24.40	23.30	22.70	23.80	24.00	22.00	23.20	17.10	19.80	20.10	19.40	22.40	22.00	22.60	22.30	19.50	20.60	21.60	21.70
CAL-*trans*	20.80	21.10	19.20	21.80	24.70	24.80	23.60	23.00	24.20	24.40	22.90	22.90	17.30	20.00	21.20	19.70	22.80	22.40	22.90	22.60	19.90	19.90	22.10	22.10

	Statistical evaluation of sample R											
	Including outliers					Excluding outliers						
FA	Mean	s_w	s_R	RSD_w	RSD_R	Mean	s_w	s_R	RSD_w	RSD_R	Outliers/ No. labs	Outlier lab.*
16:0	10.83	0.25	0.33	2.28	3.04	10.79	0.02	0.28	1.34	2.64	1/12	12
18:0	5.75	0.10	0.25	1.77	4.40	5.75	0.10	0.25	1.77	4.40	0/12	—
SAT.	17.35	0.23	0.58	1.35	3.37	17.35	0.23	0.58	1.35	3.37	0/12	—
18:1*t*	19.37	0.83	1.87	4.29	9.65	19.37	0.83	1.87	4.29	9.65	0/12	—
18:1*c*	32.16	0.61	2.10	1.89	6.53	32.16	0.61	2.10	1.89	6.53	0/12	—
18:2(*n*-6)	26.78	0.23	1.05	0.87	3.93	26.54	0.22	0.66	0.82	2.50	1/12	7
18:3(*n*-3)	0.93	0.03	0.15	3.12	16.69	0.96	0.03	0.08	3.13	8.37	1/12	7
18:2*tt*	0.11	0.09	0.12	79.94	109.72	0.11	0.09	0.12	79.94	109.72	0/12	—
18:2*t*	2.32	0.10	0.48	4.32	20.75	2.32	0.10	0.48	4.32	20.75	0/12	—
18:3*t*	0.09	0.04	0.15	44.54	167.18	0.05	0.00	0.08	0.00	150.37	1/12	2
IR-*trans*	21.63	0.88	1.90	4.05	8.79	21.63	0.88	1.90	4.05	8.79	0/12	—
CAL-*trans*	21.89	0.79	1.97	3.62	9.01	21.89	0.79	1.97	3.62	9.01	0/12	—

PHVO = Partially hydrogenated vegetable oil
X1 = Data for experiment 1
X2 = Data for experiment 2
s_w = Within laboratory standard deviation
s_R = Reproducibility standard deviation
RSD_w = Relative within laboratory standard deviation
RSD_R = Reproducibility relative standard deviation
CAL-*trans* = Sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*
*Outlier laboratory, determined by Cochran and/or Grubbs tests
SAT. = Sum of saturated fatty acids
IR-*trans* = Total *trans* unsaturation determined by IR
FA = fatty acid

Table 10. Pair of blind duplicates—samples C1 and C2[a]

	Including outliers					Excluding outliers						
FA[b]	Mean	s_r	s_R	RSD_r	RSD_R	Mean	s_r	s_R	RSD_r	RSD_R	Outliers/No. labs	Outlier lab.[c]
16:0	9.73	0.12	0.39	1.19	4.01	9.72	0.12	0.39	1.19	4.01	0/10	—
18:0	6.68	0.14	0.29	2.07	4.39	6.76	0.09	0.13	1.28	1.90	1/10	7
SAT.	17.30	0.16	0.86	0.95	4.97	17.54	0.10	0.43	0.57	2.43	1/10	7
18:1*t*	17.45	1.26	1.92	7.24	11.00	17.45	1.26	1.92	7.24	11.00	0/10	—
18:1*c*	28.16	1.12	1.87	3.97	6.65	28.16	1.12	1.87	3.97	6.05	0/10	—
18:2(*n*-6)	34.05	0.57	1.31	1.66	3.85	33.69	0.59	0.68	1.75	2.01	1/10	7
18:3(*n*-3)	0.82	0.07	0.15	8.23	17.85	0.83	0.00	0.05	0.00	5.61	2/10	7,8
18:2*tt*	0.15	0.11	0.15	73.03	97.88	0.15	0.11	0.15	73.02	97.88	0/10	—
18:2*t*	1.49	0.08	0.51	5.57	34.29	1.49	0.08	0.51	5.57	34.29	0/10	—
18:3*t*	0.07	0.04	0.10	61.75	147.30	0.04	0.04	0.05	109.26	132.09	1/10	8
IR-*trans*	19.04	1.22	2.04	6.42	10.72	19.04	1.22	2.04	6.42	10.72	0/10	—
CAL-*trans*	19.10	1.17	2.13	6.12	11.14	19.10	1.17	2.13	6.12	11.14	0/10	—

[a] Calculated using average values of samples C1 and C2 for each laboratory.
[b] FA = fatty acid; SAT. = sum of saturated fatty acids; s_r = repeatability standard deviation; s_R = reproducibility standard deviation; RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard ceviation; CAL-*trans* = sum of 18:1*t*, 18:2*tt*, 18:2*t*, and 18:3*t*; IR-*trans* = total *trans* unsaturation determined by IR.
[c] Outlier laboratory, determined by Cochran and/or Grubbs tests.

duplicate samples (Table 10) demonstrates that the IR and GC/IR methods are precise.

Although the Grubbs test did not identify any outliers for total *trans* by IR and 18:1*t* in sample A, the *trans* values reported by Collaborators 1 and 7 were considerably lower than those reported by other collaborators, and this large discrepancy could have contributed to the poor agreement between laboratories for total *trans* content and 18:1*t* in sample A. Nevertheless, this suggests that accurate determination of *trans* content by IR of samples containing low levels (≤5%) of *trans* unsaturation may be difficult. Direct GC analysis (e.g., AOCS Official Method Ce 1-89 [8]) is recommended for samples with *trans* content ≤5% when, relative to 18:1*c* isomers, the proportion of high Δ value 18:1*t* isomers is low and, consequently, the overlap of 18:1*c* and 18:1*t* isomers in GC cyanosilicone capillary columns is almost negligible.

Table 11 compares the values for 18:1*t* and 18:1*c* obtained from the collaborative study with those determined in the author's laboratory by 2 other independent methods: $AgNO_3$-TLC/GC and AOCS Official Method Ce 1c-89 (8). Mean values obtained in the collaborative study by GC/IR were equivalent to the absolute amounts determined by the tedious, combined procedure of $AgNO_3$-TLC and GC. This confirms the accuracy and reliability of the GC/IR method. The AOCS Official Method (direct GC method) gave substantially lower values for 18:1*t* and higher values for 18:1*c* than those of the other 2 methods, which is a consequence of ignoring the overlaps of *cis* and *trans* isomers (10, 11). The error in determining the 18:1*t* and 18:1*c* by the direct GC method was highest for samples containing high amounts of *trans* unsaturation and was low for sample A, which had the lowest *trans* content.

Table 12 compares the total *trans* unsaturation determined by the IR method described here, with *trans* unsaturation calculated by summing the 18:1*t* level determined by $AgNO_3$-TLC/GC method and the *trans* equivalents for 18:2*t*, 18:1*tt*, and 18:3*t*. The *trans* equivalents were calculated by multiply-

Table 11. Comparison of 18:1*t* and 18:1*c* levels of test samples determined by $AgNO_3$-TLC/GC, GC/IR, and direct GC

	18:1*t*, %			18:1*c*, %		
Sample	$AgNo_3$-TLC/GC[a]	GC/IR[b]	Direct GC[c]	$AgNO_3$-TLC/GC	GC/IR	Direct GC
A	5.1	4.9	4.4	24.7	24.9	25.9
B	15.2	14.9	12.3	24.1	24.7	26.0
C1	18.9	17.4	14.7	27.2	28.1	30.4
C2	18.9	17.5	14.7	27.2	28.2	30.4
D	26.1	26.6	19.6	35.0	34.4	41.8
E	31.9	32.6	23.4	33.0	34.3	41.6
R	19.9	19.4	16.8	31.0	32.2	36.0

[a] Values (n = 1) determined in author's laboratory. The 18:1*t* and 18:1*c* isomers isolated by $AgNO_3$-TLC were quantitatively analyzed by GC in the presence of 17:0 internal standard.
[b] Mean values (n = 12) from GC/IR collaborative study.
[c] Values (n = 1) determined in author's laboratory using AOCS Official Method Ce 1c-89.

Table 12. Comparison of total *trans* unsaturation calculated by combining $AgNO_3$-TLC/GC (18:1*t*) and GC (18:2*t* + 18:2*tt* + 18:3*t*) data obtained from IR determinations

Sample	*Trans* equivalents, %				Calculated *trans*[c], %	IR *trans*, %
	18:1*t*[a]	18:2*t*[b]	18:*tt*[b]	18:3*t*[b]		
A	5.1	0.3	0.0	0.0	5.4	5.2
B	15.2	0.6	0.1	0.0	15.9	15.5
C1	18.9	1.2	0.3	0.0	20.4	18.9
C2	18.9	1.3	0.2	0.1	20.5	19.1
D	26.1	2.8	0.4	0.2	29.5	30.1
E	31.9	2.6	0.5	0.1	35.1	34.6
R	19.9	1.9	0.2	0.1	22.1	21.6

[a] 18:1*t* values were determined in author's laboratory by $AgNO_3$-TLC/GC method (*see* Table 11). (*Note*: Correction factor for converting GC weight percentage data of 18:1*t* to IR-*trans* equivalent is 1.)

[b] Mean values (n = 12) of GC weight percentage data from GC/IR collaborative study (Tables 3–9) were converted to IR-*trans* equivalents using correction factors (0.84 for 18:2*t* and 18:3*t*, and 1.74 for 18:2*tt*).

[c] Sum of *trans* equivalents for 18:1*t*, 18:2*t*, 18:2*tt*, and 18:3*t*.

ing the GC mean weight percent data (Table **994.15**) for the above *trans* polyunsaturates with the appropriate correction factors. For mono-*trans* polyunsaturates, the experimentally determined correction factor correlating GC weight percent data to IR *trans* equivalents is 0.84, whereas for di-*trans* polyunsaturates the correction factor is 1.74 (11). Table 12 shows that IR *trans* values are in close agreement with calculated *trans* values for all test samples, except for the slightly higher calculated *trans* levels for the pair of blind duplicates, C1 and C2. This discrepancy could be attributed to higher 18:1*t* values obtained for C1 and C1 using the $AgNO_3$-TLC/GC method (Table 11).

Recommendations

The IR method is recommended for the determination of isolated *trans* unsaturated fatty acids and the GC/IR method is recommended for the determination of fatty acid composition, including the percentages of *cis* and *trans*-octadecenoates of partially hydrogenated fats derived from vegetable oils, terrestrial animal fats or such oils isolated from food products containing >5% *trans* unsaturation. For samples with ≤5% *trans* unsaturation, AOCS direct GC method Ce 1c-89 is available (combining the IR and GC data is unnecessary) for determination of 18:1*t* and 18:1*c*, as well as total *trans* content. This is possible because at lower *trans* levels, overlap of 18:1*t* and *c* isomers in GC analysis is almost negligible. The GC/IR method is not applicable to partially hydrogenated fish oils, because these fats contain a complex mixture of *cis* and *trans* isomers of mono- and polyunsaturated fatty acids with a wider range of chain lengths.

On the basis of the results of this study it is recommended that the IR method for determination of isolated *trans* unsaturated fatty acid content in partially hydrogenated fats and the capillary GC/IR method for determination of total *cis*- and *trans*-octadecenoic isomers and general fatty acid composition in hydrogenated vegetable oils and animal fats be adopted first action.

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FOOD COMPOSITION AND ADDITIVES

Determination of Fat, Protein, and Total Solids in Cheese by Near-Infrared Reflectance Spectroscopy

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Near-infrared reflectance (NIR) spectroscopy was used to analyze fat, protein, and total solids in cheese without any sample treatment. A set of 92 samples of cow's milk cheese was used for instrument calibration by principal components analysis and modified partial least-square regression. The following statistical values were obtained: standard error of calibration (SEC) = 0.388 and squared correlation coefficient (R^2) = 0.99 for fat, SEC = 0.397 and R^2 = 0.98 for protein, and SEC = 0.412 and R^2 = 0.99 for total solids. To validate the calibration, an independent set of 25 cheese samples of the same type was used. Standard errors of validation were 0.47, 0.50, and 0.61 for fat, protein, and total solids, respectively, and R^2 for the regression of measurements by reference methods versus measurements by NIR spectroscopy was 0.98 for the 3 components.

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Determining the major components of foodstuffs with classic analytical methods is slow and expensive. Over the past few years, near-infrared (NIR) spectroscopy has been used to analyze numerous foodstuffs (1–13) with the aim of obtaining results within a few minutes and at low cost.

NIR spectroscopy is very useful in the analysis of milk and whey powder (1, 6, 14–17), but reports of its application to cheese analysis are few (15, 18, 19), and in all the cases mentioned, the cheese was previously grated and only a few wavelengths were selected.

The objective of this study was to analyze the major components of cheese (fat, protein, and total solids) using NIR without

Table 1. Range of chemical composition and standard deviation (SD) of cheeses in the calibration and validation sets

	Calibration set		Validation set	
Component	Range, %	SD[a]	Range, %	SD
Fat	17.9–32.4	3.01	19.4–31.3	3.03
Protein	16.1–29.6	3.12	17.7–28.2	3.04
Total solids	45.2–61.7	4.12	46.3–59.7	4.15

[a] SD, standard deviation.

any prior sample manipulation. The work is based on the great development that has taken place over the past few years in calibration techniques based on multivariate analysis (20).

Experimental

Samples

A total of 117 cheese samples were divided into 2 groups: 92 samples for calibration and 25 samples for validation of the calibration (Table 1).

Samples of 3 cheese varieties (Tetilla, Arzúa, and Edam), all made with cow's milk and pressed curd and without molds for ripening, were supplied by 10 manufacturers. Samples were analyzed between 2 and 20 days after brining.

Reference Analysis

After NIR spectroscopic analysis, the same slice of cheese was analyzed by reference methods: oven drying for total solids (21), gravimetric extraction method for fat (22), and Kjeldahl method ($N \times 6.38$) for protein. All measurements were made in duplicate.

Apparatus

A wavelength-scanning instrument, Model NIR Systems 6500 (NIR Systems, Silver Spring, MD) with a scanning range from 400 to 2498 nm and wavelength increments of 2 nm was used. Every day before starting work, instrument checks recommended by the manufacturer were carried out.

Procedure

The cheese samples, at 8°–10°C, were cut into slices of ca 1 cm thick, wrapped in polyethylene film, and put into the measuring cell.

Because the sample does not have to be ground, the amount of work is reduced and the measurement can be taken quickly, hence avoiding heating of sample.

Reflectance measurements of monochromatic light were made from 400 to 2498 nm. An average of 25 spectral scans were taken for each sample; data were recorded as log $1/R$, where R is the reflectance energy.

Statistical Analysis

ISI software was used (24). Scatter correction was performed by standard normal variate transformation and detrend method (25). A general Mahalanobis distance (H statistic) was calculated from principal components analysis (PCA) scores; H values were standardized by dividing them by the average H value for the calibration file. If a new spectrum sample is more than 3.0 standardized units from the mean of the calibration file, the sample is defined as a global H outlier and may not have accurate predictions.

Calibration was performed by PCA and modified partial least-square (MPLS) regression (20). Three mathematical treatments were tested: (*1*) first derivative and a subtraction gap and smoothing segment of 4 data points: 1, 4, 4; (*2*) second derivative and a subtraction gap and smoothing segment of 6 data points: 2, 6, 6; (*3*) third derivative and a subtraction gap and smoothing segment of 10 data points: 3, 10, 10. Stepwise multiple linear regression (MLR) was also tested.

The optimum number of terms for calibration minimizing overfitting was based on the standard error of cross validation (SECV). The approach was as follows: 75% of samples from the calibration set were used for calibration, and with the remaining 25%, the standard error of prediction (SEP) was calculated. This operation was carried out 4 times, each time with a different group for calibration and prediction. SECV was calculated as the square root of the average of the squares of the 4 SEPs. The final calibration equation was developed on the total samples of the calibration set with the number of factors with the lowest SECV.

The critical T value for eliminating outliers was fixed at 2.5.

To check the calibration, the validation set was used. The standard error of validation (SEV) and R^2 (squared correlation coeficient) of values from reference analyses versus values from NIR spectroscopy were calculated.

Results and Discussion

Figure 1 shows the average spectra of cheeses in the calibration set. The following bands can be observed: 990 and 1450 nm, second and first overtone of the O–H stretch; 1940 nm, a combination O–H stretch and bend band; 1210 nm, the second overtone of the CH_2 group; at 1728 and 1765 nm, the first overtone of the CH_2 group; and peaks at 2310 and 2345 nm, stretch and bend combination band (1).

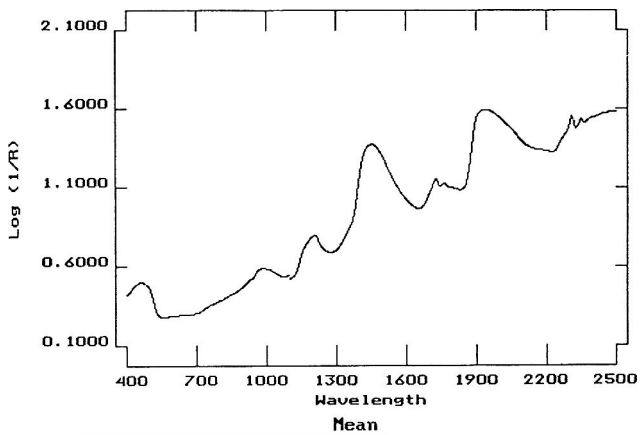

Figure 1. Mean spectra of calibration set samples.

Table 2. Repeatability of determinations of fat, protein, and total solids by reference and NIR methods

Method	Fat	Protein	Total solids
Reference	0.31	0.13	0.22
NIR	0.40	0.41	0.43

Repeatability

Table 2 shows the values of repeatability (S_r). For the reference methods, S_r was calculated according to the method of the International Dairy Federation (IDF; 26), using the 92 samples from the calibration set. For NIR spectroscopy, S_r was obtained by analyzing 2 slices of 16 cheese samples and using the same IDF test.

The higher S_r values from the NIR spectroscopy may be due to the large sample size (70–90 g) rather than to a lack of method precision. Differences in the composition of different cheese slices are due mainly to greater drying in parts near the surface. In the reference methods, small portions of cheese are taken from the same part and can be grated and mixed easily.

Calibration

The calibration set was selected with the aim of providing a strong calibration for the cheese varieties by maximizing variability among sample compositions and obtaining a wide range of spectra to avoid *H* outliers in the validation set.

To evaluate the results, the standard error of calibration (SEC), SECV, and R^2 for the calibration set and SEV and R^2 for the validation set were considered.

Table 3 shows SEC, SECV, and R^2 values for fat, protein, and total solids for the calibration set, obtained by MPLS and the 3 mathematical treatments. Very similar values were obtained with the different mathematical treatments used.

Table 3. Statistical data for calibration set

Component	No. of samples	PLS terms	SEC	SECV	R^2	Mathematical treatment
Fat	90	7	0.388	0.516	0.99	1
	91	6	0.396	0.537	0.98	2
	90	5	0.413	0.509	0.98	3
Protein	91	7	0.471	0.602	0.98	1
	90	8	0.397	0.561	0.98	2
	91	8	0.419	0.553	0.98	3
Total solids	92	5	0.613	0.767	0.98	1
	91	5	0.569	0.774	0.98	2
	89	8	0.412	0.621	0.99	3

For fat, the best statistical indicators corresponded to the calibration obtained with treatments 1, 4, 4, which uses 7 terms in the calibrations and eliminates 2 *T* outliers; the SEC and SECV values are lower than 1/8 and 1/6 of the standard deviation (SD) of the 92 calibration samples. With treatment 2, 6, 6, the values were very similar to the previous ones, and in this case, only one *T* outlier was eliminated.

For protein, the best calibrations were obtained with treatments 2, 6, 6 and 3, 10, 10, both with 8 terms in the calibration; SEC and SECV values were lower than 1/7 and 1/5 of the SD of the calibration samples.

Because the nonprotein nitrogen (NPN) contents of the cheeses are variable, errors in protein calibration may be due partly to the fact that the reference method includes true protein and NPN (crude protein). The behavior of NPN in NIR spectroscopy is different from that of true protein.

For total solids, the best values corresponded to treatment 3, 10, 10 with 8 terms in the calibration: the SEC and SECV values were lower than 1/10 and 1/6 of the SD of the calibration

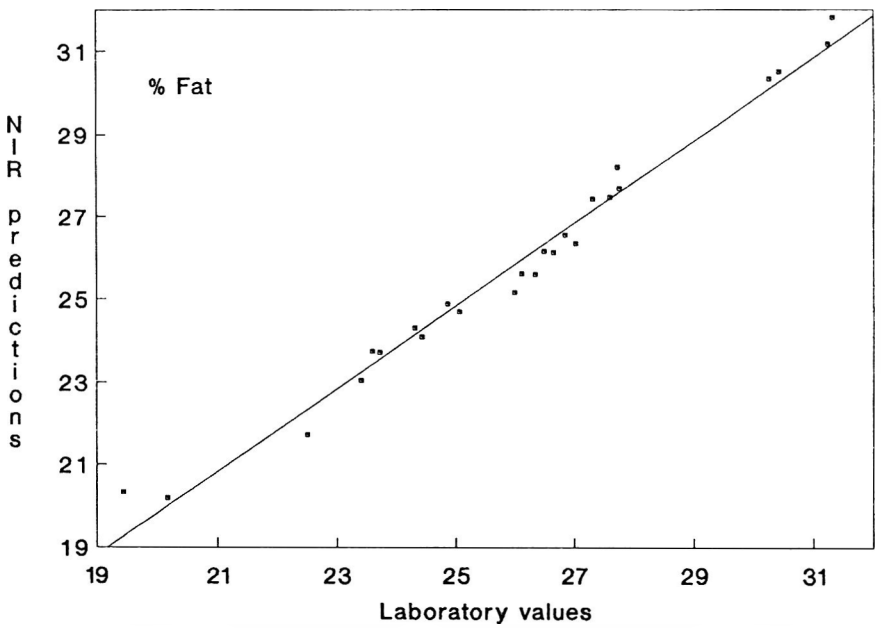

Figure 2. Relationship of fat reference method values versus NIR values for validation set.

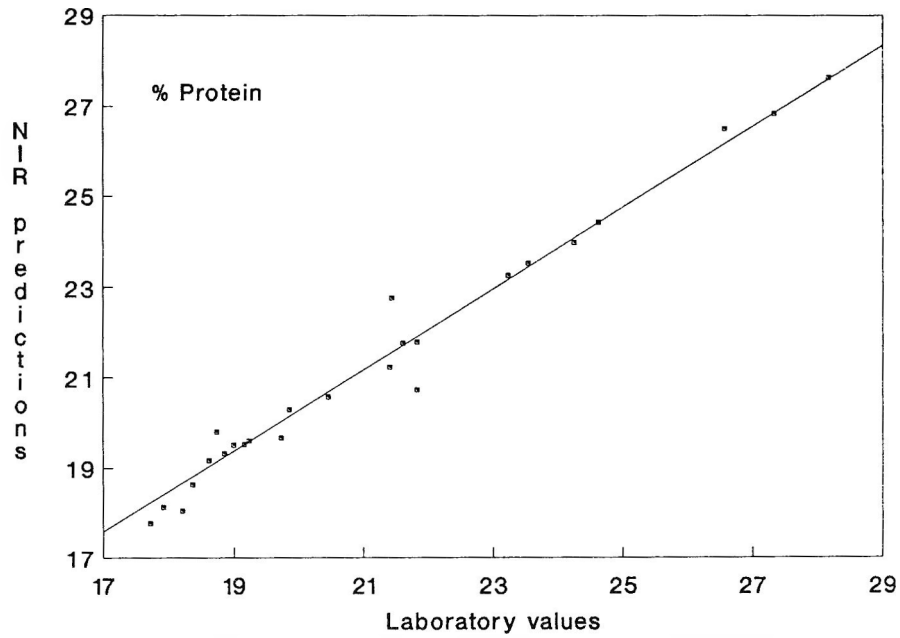

Figure 3. Relationship of protein reference method values versus NIR values for validation set.

samples. In this case, 3 T outliers were eliminated; in treatment 1, 4, 4, no outliers were eliminated, and in treatment 2, 6, 6, only one outlier was eliminated. The R^2 values were very high (0.98–0.99) in all cases.

When calibrations were applied to a set of independent samples (validation set), no H outliers were found. The spectra of the validation set did not differ from the spectra of the calibration set, indicating that the calibration set was wide enough for calibration of these cheese varieties.

To compare results obtained by the reference methods with those obtained by NIR spectroscopy for all 3 parameters, the paired t-test was applied (27). Because the calculated t value was less than the theoretical value ($p = 0.05$), the null hypothesis was retained: the methods do not give significantly different values.

Graphical comparisons between reference values and NIR-predicted values for the validation set are shown in Figures 2–4.

SEV values were similar to SECVs and only slightly higher than SECs, indicating that overfitting was not produced. Both SEV and R^2 values were very good for the 3 parameters studied (Table 4).

For fat, the lowest SEV corresponded to treatment 2, 6, 6, although there were no great differences among the 3 treat-

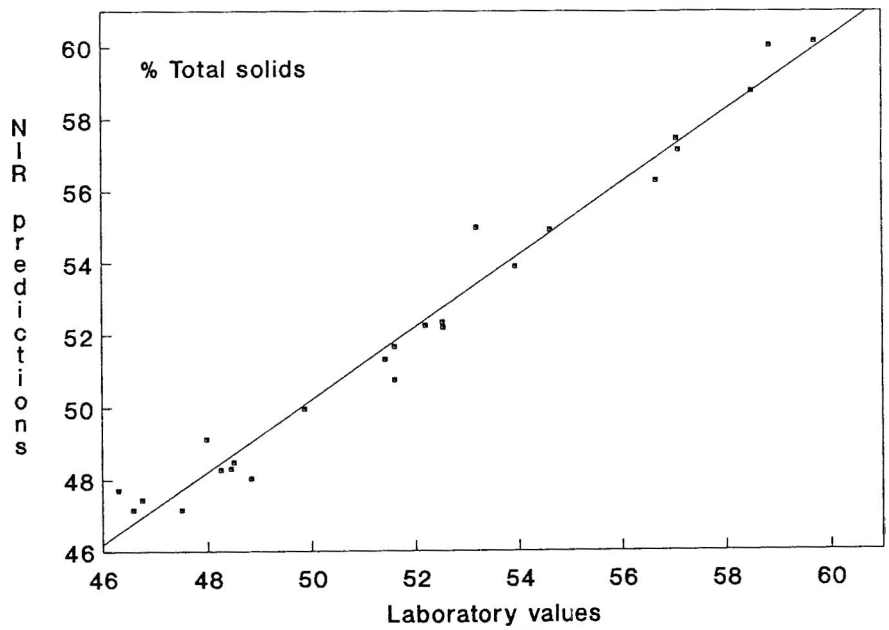

Figure 4. Relationship of total solids reference method values versus NIR values for validation set.

Table 4. Statistical data for validation set

Component	No. of samples	SEV	R^2	Mathematical treatment
Fat	25	0.47	0.98	1
	25	0.44	0.98	2
	25	0.53	0.97	3
Protein	25	0.55	0.97	1
	25	0.50	0.98	2
	25	0.47	0.98	3
Total solids	25	0.66	0.98	1
	25	0.75	0.97	2
	25	0.61	0.98	3

ments. For protein, the best SEV and R^2 values corresponded to treatments 2, 6, 6 and 3, 10, 10. For total solids, the best SEV and R^2 corresponded to treatments 1, 4, 4 and 3, 10, 10.

SEC, SEV, and R^2 values for both calibration set and validation set were less accurate when calibrated with MLR than with MPLS.

Conclusions

NIR spectroscopy is an adequate technique for analysis of cheese without any prior sample treatment (not even grating). The method requires that a group of cheeses of similar characteristics and with no H outliers but with a wide range of variation in chemical composition is used for calibration.

We recommend that the calibration be made by PCA and MPLS regression.

When the first, second, or third derivative is applied, no important differences are found. However, because the signal-to-noise ratio decreases when the order of the derivative increases, we do not recommend using the third derivative.

SECV is very effective in determining the maximum number of terms for calibration without overfitting, as proven by the validation set.

Acknowledgment

We thank Paz Peña for her technical assistance.

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FOOD COMPOSITION AND ADDITIVES

Enzyme Immunoassay for Carminic Acid in Foods

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A competitive enzyme immunoassay (EIA) for carminic acid was investigated. Monoclonal anti-carminic acid antibody was obtained from A/J mice immunized with carminic acid–human immunoglobulin G (IgG) conjugate. Carminic acid was extracted with distilled water from beverage, jelly, candy, pasta sauce, yogurt, or ice cream samples. Ham or fish paste samples were digested with pronase, then carminic acid was extracted from samples with sodium hydroxide solution. The extract was diluted more than 10-fold with 1% gelatin in borate buffer solution. Microtiter plates were coated with carminic acid–bovine serum albumin (BSA) conjugate or just BSA. Goat anti-mouse IgG(H+L)–peroxidase complex was used as a second antibody, and 3,3′,5,5′-tetramethylbenzidine was used as a substrate for the peroxidase. The working range for quantitative analysis was 0.3–10 ng/mL, and the detection limit was 0.2 μg/g original sample. Recoveries of carminic acid by this assay were >95% for milk beverage and jelly, and >85% for yogurt and fish paste. Carminic acid was detected in 7 of 26 red-colored commercial food products and ranged from 3.5 to 356 μg/g. This EIA system also responded to the structural analogue of carminic acid, iaccaic acid.

Food colors are classified mainly into 2 groups: artificially synthesized food colors such as coal–tar dyes, and natural food colors from sources such as flowers, plants, or insects. Public concern has led to an increase in the usage of natural food colors.

For the analysis of synthesized colors in foods, systematic procedures using thin-layer chromatography (TLC) (1–4) or liquid chromatography (LC) (5–7) were established. However, natural colors are difficult to systematically analyze by chromatography because they have various chemical structures.

We attempted to apply enzyme immunoassay (EIA) to the systematic analysis of natural food colors. Various substances can be analyzed by similar EIA procedures if their specific antibodies can be obtained.

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Carminic acid, the principal constituent of cochineal dye, is a red food color obtained from *Coccus cacti L.* The dye has superior stability against heat or light compared with other natural food colors and superior coloring ability similar to coal–tar dyes (8).

Carminic acid has been analyzed by TLC (9–11) and LC (12, 13), but we are unaware of any determinations by EIA. Thus, we attempted to apply EIA to the screening and semidetermination of carminic acid in foods.

Experimental

Apparatus

(a) *Autoplate washer.*—Model 1550 (Biorad, Tokyo, Japan).
(b) *Autoplate reader.*—Model 3550 (Biorad).
(c) *PD-10 column.*—Pharmacia (Uppsala, Sweden).

Chemicals and Materials

(a) *Carminic acid.*—Purchased from E. Merck (Darmstadt, Germany; ≥ 98% purity) for EIA and from Wako Pure Chemical Industries (Kyoto, Japan; chemical grade) for preparation of carminic acid–protein conjugate.

(b) *Complete Freund's adjuvant.*—Wako Pure Chemical Industries.

(c) *Goat anti-mouse immunoglobulin IgG(H+L)–peroxidase.*—Jackson Immuno Research (West Grove, PA).

(d) *3,3′,5,5′-Tetramethylbenzidine (TMBZ).*—Dojin Laboratories (Kumamoto, Japan).

(e) *N-Hydroxysuccinimide (HONSu) and N,N′-dicyclohexylcarbodiimide (DCC).*—Nacalai Tesque, Inc. (Kyoto, Japan).

(f) *Bovine serum albumin (BSA) and human IgG.*—Sigma Chemical Co. (Milwaukee, WI).

(g) *Pronase (6 units/mg).*—Boehringer Mannheim GmbH (Mannheim, Germany).

(h) *Other reagents.*—Analytical or chemical grade.

Preparation of Solutions

Substrate solution was prepared, just before assay, by mixing 100 μL TMBZ solution (10 mg TMBZ in 1 mL dimethylformamide [DMF]) and 1.5 μL 30% hydrogen peroxide in 9.9 mL 0.1M acetate buffer solution, pH 5.5. Acetate buffer solution was prepared by dissolving 8.2 g sodium acetate in 1 L distilled water and adjusting pH to 5.5 with acetic acid. Phosphate buffer solution (PBS), pH 7.4, was prepared by dissolving 8.77 g NaCl, 2.90 g $Na_2HPO_4 \cdot 12H_2O$, and 0.30 g

$NaH_2PO_4 \cdot 2H_2O$ in 1 L distilled water. Borate buffer solution (BBS), pH 8.0, was prepared by dissolving 10.3 g boric acid, 7.3 g NaCl, and 25.5 mL 1N NaOH in 1 L distilled water.

Preparation of Carminic Acid–Protein Conjugate

Carminic acid was coupled to BSA and human IgG by a modified HONSu method (14).

Carminic acid solution (24.5 mg/500 µL DMF) was mixed with 50 µL HONSu solution (12.7 mg/100 µL DMF), then 50 µL DCC solution (22.7 mg/100 µL) was added. The mixture was incubated for 20 h at room temperature. For convenience, this solution was called "CA-reactant."

(**a**) *Preparation of carminic acid–BSA.*—Aliquots (0.66 mL) of BSA solution (10 mg/mL in H_2O) and 0.12 mL 1M sodium hydrogen carbonate were added to 0.36 mL CA-reactant. After incubation for 1 h at 37°C, this mixture was centrifuged at 15 000 rpm for 5 min at 10°C. The supernatant was loaded onto the PD-10 column, which was equilibrated with PBS. The supernatant was eluted with PBS. The colored portion (2.15 mL) was collected.

(**b**) *Preparation of carminic acid–human IgG.*—Two milliliters of human IgG solution (7.5 mg/mL in H_2O) and 0.29 mL 1M sodium hydrogen carbonate were added to 0.6 mL CA-reactant. After incubation for 1 h at 37°C, the mixture was diluted 1:1 with PBS and then centrifuged at 15 000 rpm for 5 min at 10°C. The supernatant was precipitated with 5.78 mL saturated ammonium sulfate solution (pH was adjusted to 7.2 with 1M Tris–HCl buffer), allowed to stand for 1 h, and then centrifuged at 15 000 rpm for 5 min at 10°C. The supernatant was decanted, and the precipitate was dissolved with 4 mL PBS, and then centrifuged in the same way. The supernatant was precipitated with 4 mL saturated ammonium sulfate solution, allowed to stand for 5 min, and again centrifuged. After this precipitation procedure was repeated 3 times, the precipitate was redissolved in 4 mL PBS.

Immunization

Carminic acid–human IgG conjugate (2 mg/mL in PBS) was used as the immunogen by emulsification with an equal volume of complete Freund's adjuvant. For primary immunization, 200 µL/mouse of this emulsion was intraperitonealy injected to mice (female A/J mice, 8 weeks), and secondary immunization was given 4 months later by injection of this emulsion (200 µL/mouse) to produce a complete immunological response in the animal to carminic acid. Further, 10 months later, i.e., 3 days before fusion, the mice were given an intraperitoneal injection with the immunogen alone (2 mg/mL in PBS) at 100 µL/mouse to increase the number of circulating lymphocytes specific to the immunogen.

Fusion Protocol and Preparation of Monoclonal Antibody (15)

Spleen cells of immunized mice were fused to P3X63-Ag8,653 cells using polyethylene glycol. After fusion, the cells were suspended in Iscove's Medium supplement with 5% fetal calf serum, hypoxanthine, and thymidine at 2×10^6 spleen cells/mL and then transferred to 5 microtiter plates (96-well) at 100 µL/well. After 1 day culture at 37°C in a humidified 5% CO_2 atmosphere, the medium containing aminopterin (100 µL) was added to each well and further cultured for 10 days. Specific antibody-secreting hybridomas were selected by EIA for carminic acid and subsequently were cloned by the limiting dilution method. Each clone was ascertained to be a single cell under the microscope.

EIA Protocols

(**a**) *EIA.*—EIA was used to select the hybridomas and to examine the titer of anti-carminic acid antibodies. Microtiter plates were coated with 100 µL carminic acid–BSA solution (100 µg/mL in BBS) for 1 h. Plates were blocked with 250 µL 1% gelatin in BBS for 1 h. After washing 10 times with 300 µL distilled water, plates were incubated with 100 µL antibodies diluted with 1% gelatin in BBS for 1 h. Plates were then washed 3 times with 300 µL distilled water, and 100 µL goat anti-mouse IgG(H+L)–peroxidase solution (diluted to 1/5000 [v/v] with 1% gelatin in BBS) was added. Plates were incubated for 1 h and washed 10 times with 300 µL distilled water; then, 100 µL substrate solution was added and the plates were incubated for 10 min. The reaction was stopped with 100 µL 1N H_2SO_4, and absorbance was measured at 450 nm with the auto plate reader. The absorbance at 450 nm was corrected by subtracting the absorbance obtained by a nonspecific binding of both anti-carminic acid antibodies and the second antibody to the plate. To estimate this latter absorbance, the entire procedures were carried out in parallel using plates that were coated with BSA solution (100 µg/mL in BBS).

(**b**) *Competitive EIA.*—This assay was done to estimate the amount of carminic acid in foods. Carminic acid–BSA coated plates and BSA coated plates were prepared as described in (**a**). Monoclonal antibodies diluted with 1% gelatin in BBS were mixed with the same volume of carminic acid solution (dissolved with 1% gelatin in BBS) as standard or sample extract, and the solutions were incubated over 1 h. Aliquots of these mixtures (100 µL) were added to carminic acid–BSA coated plates or BSA coated plates, and the plates were incubated for 1 h. The determination was completed as described for EIA. All procedures in (**a**) and (**b**) were done at room temperature.

Sample Preparations

Beverage, yogurt, sauce, or ice cream samples (5 g) were homogenized with 80 mL distilled water. Jelly or candy samples (5 g) were dissolved in 80 mL hot distilled water (40°–50°C). Samples were centrifuged at 10 000 rpm for 5 min at 0°C, if necessary. The supernatant was filled to 100 mL with distilled water. This solution was diluted more than 10-fold with 1% gelatin in BBS and then analyzed by competitive EIA.

Ham or fish paste (5 g) was homogenized with 60 mL distilled water. The homogenate was incubated for 2 h at 37°C after addition of 50 mg pronase. If digestion was incomplete, the homogenate was incubated for 1 h more. It was then boiled in a water bath for 10 min; after cooling, 0.1 mL 1N NaOH was added, and the solution was centrifuged at 10 000 rpm for 5 min at 0°C. The supernatant was filled to 100 mL with dis-

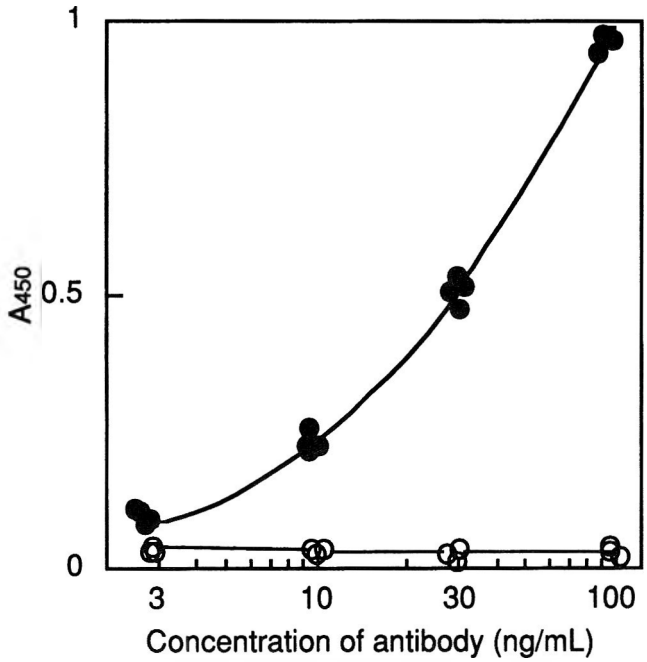

Figure 1. Binding curves of purified monoclonal antibody (C11–1) to carminic acid–BSA (●) and BSA (○) coated plates.

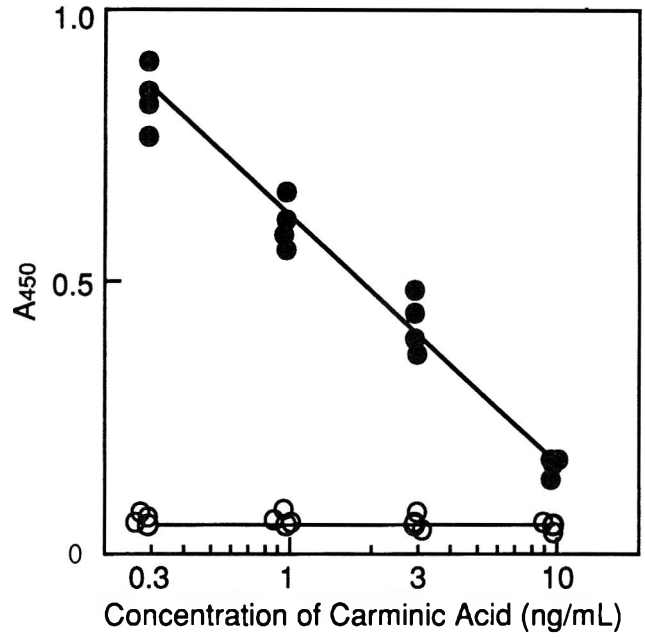

Figure 2. Binding inhibition curves of purified monoclonal antibody (C11–1) to carminic acid–BSA (●) and BSA (○) coated plates by carminic acid.

tilled water. This solution was diluted more than 10-fold with 1% gelatin in BBS and then assayed by competitive EIA.

Recovery Tests

Milk beverage, jelly, yogurt, or fish paste samples (5 g) were spiked with 25 μg, 100 μg (only yogurt), and 500 μg carminic acid (Merck) and then kept in a refrigerator for over 24 h. Concentrations of carminic acid in the samples were estimated by competitive EIA.

Results and Discussion

Because carminic acid has no immunogenic properties, carminic acid–human IgG conjugate was synthesized for the immunization of mice. The other hapten–protein conjugate, carminic acid–BSA, was synthesized for EIA. For selection of specific antibody-secreting hybridomas, the protein carrier used for EIA must be different than the protein carrier for mice immunization. The antibodies to the protein carrier were not reactive in EIA. Color development in EIA for the hybridoma supernatants was observed in 7 of the 480 wells. Each anti-carminic acid, antibody-secreting hybridoma was cloned by the limiting dilution method, and 7 clones (C1–2, C5–2, C7–30, C11–1, C15–20, C22–1, and C23–4) were obtained. The clone (C11–1) that showed the highest titer was selected out. The antibody was purified from the concentrated supernatant by precipitation with 50% saturated ammonium sulfate and gel filtration. The purified antibody was then used for the determination of carminic acid. The class of heavy and light chains of this antibody were IgG_1 and κ, respectively.

Figure 1 shows the binding curves of purified monoclonal antibody to carminic acid–BSA and BSA coated plates as the nonspecific binding. Absorbances at 450 nm by the nonspecific binding were about 0.015 at 3–100 ng/mL. This antibody was tested by the competitive EIA. In competitive EIA, the most appropriate absorbance was approximately 1.0 when competing carminic acid was not added. From this binding curve, the

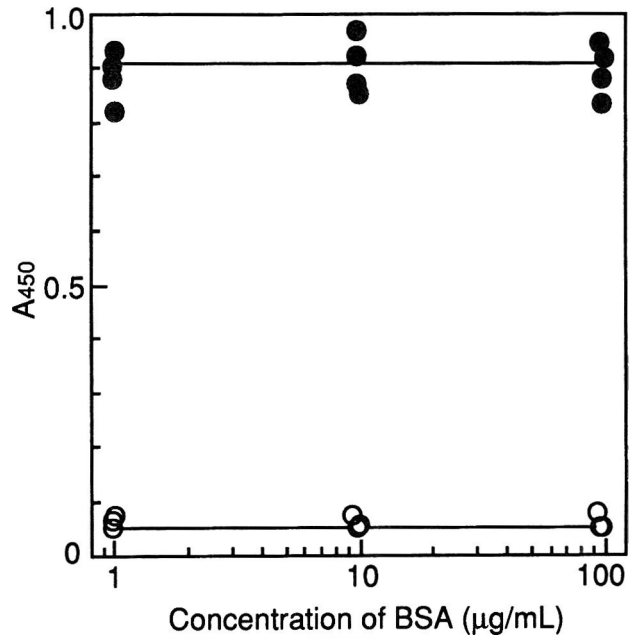

Figure 3. Binding inhibition curves of purified monoclonal antibody (C11–1) to carminic acid–BSA (●) and BSA (○) coated plates by BSA.

Table 1. Effect of sodium dodecyl sulfate on competitive EIA[a]

CA, ng/mL	SDS, μg/mL	A_{450}[b] CA–BSA plates	A_{450}[b] BSA plates
0	0	0.855 ± 0.047	0.048 ± 0.012
0	12.5	0.565 ± 0.031	0.069 ± 0.023
3	0	0.460 ± 0.095	0.050 ± 0.010
3	12.5	0.421 ± 0.089	0.086 ± 0.017

[a] CA, carminic acid; SDS, sodium dodecyl sulfate.
[b] Absorbance at 450 nm; values are means ± standard deviation of 4 examinations.

working concentration of the antibody was selected to be 100 ng/mL.

The dynamic range for the determination of carminic acid by competitive EIA was estimated (Figure 2). Linearity of the binding inhibition was observed from 0.3 to 10 ng/mL (final concentration), and the working range for the quantitative analysis of carminic acid in foods was 0.3–10 ng/mL. BSA did not inhibit the binding of the antibody to the plates even at 100 μg/mL (Figure 3).

Recovery of carminic acid in foods was tested. The extraction was performed with water only, because carminic acid is highly soluble in water. However, when carminic acid exists in a food matrix like ham or fish paste, enzymatic pretreatment to release carminic acid from foods is necessary (16). Pronase was used for digesting protein (13), after which it was inactivated by boiling for 10 min. Some investigators have recommended the use of sodium dodecyl sulfate (SDS) with pronase for thorough digestion and for easy extraction of carminic acid. Yamada et al. (13) used 10 mg pronase and 5 mg SDS to digest 1 g ham or sausage.

We examined the effect of the corresponding concentration of SDS on competitive EIA (Table 1). SDS appeared to disturb the competitive EIA by inhibiting the binding of the antibody

Table 2. Recovery tests of carminic acid in foods[a]

Foods	Added, μg/g	Found, μg/g	Recovery, %
Milk beverage	0	0	—
	5	4.76	95.2
	100	100	100
Jelly	0	0	—
	5	5.71	115
	100	102	102
Yogurt	0	0	—
	5	4.24	85.1
	20	19.4	97.0
	100	86.0	86.0
Fish paste	0	0	—
	5	4.35	87.0
	100	90.3	90.3

[a] Data are means of 3 examinations.

Table 3. Determination of carminic acid in commercial foods

Foods	Concentrations, μg/g[a]
Beverage	180, 96, 356, ND(8)[b]
Jelly	32, ND(2)
Candy	25, ND(3)
Ice cream	ND(2)
Pasta sauce	ND(2)
Ham	3.5, ND(1)
Fish paste	10, ND(1)

[a] Data are means of 3 examinations.
[b] Numbers of samples below detection limit (0.2 μg/g original sample). ND, not detected.

to carminic acid–BSA coated plates. Instead of adding SDS, the incubation time of pronase was prolonged because samples were digested incompletely, and the homogenate was made slightly basic with sodium hydroxide before the extraction procedure. The extract was diluted more than 10-fold for EIA. For dilution of the extract, 1% gelatin in BBS was used to avoid binding of diluted carminic acid to the surface of vessels.

Table 2 shows the results of recovery tests. In milk beverage, jelly, and fish paste samples, recoveries were satisfactory (87.0–115%). Some investigators mentioned that carminic acid is not sufficiently extracted from yogurt with only distilled water (12, 17) because carminic acid exists in the protein matrix of yogurt. However, our recoveries were satisfactory (85.1–97.0%). Extracted proteins from original foods do not affect the competitive EIA.

A total of 26 red-colored commercial foods were analyzed for carminic acid (Table 3). Carminic acid was detected in 7 samples and ranged from 3.5 to 356 μg/g. The detection limit was estimated to be 0.2 μg/g original sample. The detection limit of the LC methods (12, 13) is 0.1 μg/g. For LC methods, column cleanup and concentration procedures are necessary. On the other hand, our competitive EIA is simple and rapid. Therefore, our competitive EIA is considered applicable for screening and semidetermination of carminic acid.

Carmine, the lake of carminic acid, is banned as an additive to commercial food products in Japan. Although it was not detected by our competitive EIA as an aqueous solution, after boiling in the acidic solution, it was detectable by our EIA. Further investigation into the application of EIA to carmine is underway. This system also responded to the structural analogue of carminic acid, laccaic acid. The sensitivity of this antibody for laccaic acid and a separative determination system from carminic acid are being examined.

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RESIDUES AND TRACE ELEMENTS

Rapid Determination of Methyl Parathion and Methyl Paraoxon in Milk by Gas Chromatography with Solid-Phase Extraction and Flame Photometric Detection

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Methyl parathion (MPT; *O,O*-dimethyl-*O*-4-nitrophenyl phosphorothioate) and its active metabolite, methyl paraoxon (MPO; *O,O*-dimethyl-*O*-4-nitrophenyl phosphate), were isolated from raw milk by solid-phase extraction (SPE) and determined by gas chromatography with flame photometric detection. The SPE method was compared with a traditional liquid–liquid extraction (LLE) procedure to determine whether SPE had suitable sensitivity and better efficiency in extracting MPT and MPO from milk of cows and goats. Method detection limits were higher for SPE, but the differences were not significant (*t*-test). Recoveries of MPT and MPO from raw milk samples spiked at 0.05, 0.5, and 5.0 μg/mL ranged from 80.0 to 118%, and the coefficients of variation were usually less than 10% for both methods. LLE required more organic solvents and was more time consuming compared with SPE.

Methyl parathion (MPT; *O,O*-dimethyl-*O*-4-nitrophenyl phosphorothioate) is a widely used crop insecticide in the United States. During commercial aerial application of MPT, drift can occur, resulting in concern for human exposure (1). Because MPT drift could contaminate food animals through percutaneous absorption as well as ingestion of grass, the pesticide can enter the food chain through meat and dairy products derived from contaminated animals. Dietary exposure of wildlife to MPT is hazardous (2).

With most foods, especially milk, it is very difficult to quantitatively and rapidly remove all organophosphate (OP) residues and then isolate only those residues of interest in the presence of coextractant interferences, such as plant pigments and milk fats (3). Luke et al. (4) reported that these interferences can broaden peaks and increase tailing in the gas chromatogram and shorten the life of a capillary column. Efforts over the past decade have been directed to either minimizing cleanup steps or developing automated cleanup procedures, including liquid–liquid extraction (LLE) and solid-phase extraction (SPE) methods.

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LLE often requires large volumes of harmful organic solvents, for which containment and disposal may be a problem. LLE can be very time consuming, normally requiring multiple extractions of a matrix. For aqueous matrixes, emulsions are frequently produced, resulting in lower extraction efficiencies. Despite these shortcomings, Toyoda et al. (5) and Beroza and Bowman (6) have successfully isolated OPs such as parathion from milk by LLE.

Data from limited experimental studies suggest that SPE may aid removal of various compounds from water, milk, urine, and plasma. Long et al. (7) used C_{18} columns to extract chlorsulfuron (a selective pre- and postemergence sulfonyl urea herbicide) from milk. Swineford and Belisle (8) extracted MPT and MPO from pond water by using a solid-phase C_{18} column (Sep-Pak).

Liu et al. (9) used a simple and rapid SPE with a C_{18} column (Sep-Pak) to isolate 11 OP pesticides from urine and plasma. Samples were diluted and passed through the column, then the compounds of interest were eluted with chloroform–isopropyl alcohol (9 + 1). The chloroform–isopropyl alcohol eluate took less than 30 min to evaporate.

The objective of this study was to use SPE to isolate MPT and MPO from raw cow's and goat's milk and to evaluate the performance of SPE in comparison with that of more traditional LLE. Although polar metabolites such as dimethyl phosphate and dimethyl thiophosphate may require derivatization, direct extraction of the active metabolite, MPO, by SPE also was considered.

Experimental

Reagents

(a) *Solvents.*—Acetone, methylene chloride, and hexane (Baker Resi-analyzed pesticide grade); ethanol (Baker Analyzed ACS reagent); chloroform and acetonitrile (Baker Analyzed LC grade).

(b) *90% Chloroform–ethanol.*—90 + 10, v/v.

(c) *50% Acetonitrile–water.*—50 + 50, v/v.

(d) *Anhydrous sodium sulfate.*—Fisher S-421, heated at 500°C for 4 h.

(e) *Organophosphorus compounds.*—Methyl parathion, 43.3% (Helena Chemical Co., Nashville, TN); methyl paraoxon, 99% (Chem Service Co., Westchester, PA). Standards were prepared as acetone solutions, stored at 4°C, and used within 3 months.

(f) *C_{18} columns.*—Sorbent, 360 mg; void volume, 0.85 mL; Sep-Pak cartridges (Waters Associates, Milford, MA).

(g) *Milk.*—Raw cow's milk (RCM) and raw goat's milk (RGM) with 3.5 and 3.0% milk fat, respectively, were obtained directly from local dairy cow and goat herds within 6 h of milking, and assays were performed immediately. Pasteurized cow's skim milk (CSM) was obtained from a local supermarket.

Apparatus

(a) *Flash-evaporator.*—Buchler Instruments, Fort Lee, NJ.

(b) *Separatory funnels.*—125, 250, and 500 mL.

(c) *Flasks.*—500 mL Erlenmeyer flasks and 500 mL flash boiling flasks.

(d) *Gas chromatograph.*—Hewlett-Packard Model 5890 equipped with a flame photometric detector (Hewlett-Packard, Palo Alto, CA).

Liquid–Liquid Extraction

Fresh milk (50 mL) was placed in a 250 mL separatory flask and spiked to the desired concentration with MPT or MPO. Acetone (150 mL) was immediately added to the milk, and the flask was shaken manually periodically for 10 min. The entire contents were filtered through a Buchner funnel with filter paper (Whatman No. 1) and collected in a 500 mL Erlenmeyer flask. An additional 25 mL acetone was used to wash the filter cake in the Buchner funnel, and this was added to the filtrate. The filtrate was extracted first with 100 mL and then 50 mL methylene chloride. The extract was dried by passage through a column of anhydrous sodium sulfate before being concentrated to less than 1 mL on a flash evaporator using a water bath (55°–60°C). The concentrated residue was reconstituted to 10 mL with methylene chloride. One milliliter of this mixture was added to 10 mL hexane–acetonitrile (1 + 1). The acetonitrile layer was removed, and the remaining hexane phase was extracted once more with an additional 5 mL acetonitrile. The hexane layer was then discarded. The acetonitrile layers were combined and adjusted to 10 mL if required. Two milliliters of this mixture was then evaporated to almost dryness under a stream of nitrogen gas. Acetone (1 mL) was added to this residue, and then 1 µL of the acetone solution was injected into the gas chromatograph. This procedure was performed with 6 replicates of milk spiked with MPT and MPO at concentrations of 0.05, 0.5, and 5.0 µg/mL.

Solid-Phase Extraction

The C_{18} column was conditioned twice with 10 mL chloroform–ethanol (9 + 1), 10 mL acetonitrile, 10 mL acetonitrile–water (1 + 1), and 10 mL water. Spiked milk (1 mL) was mixed with 9 mL water and passed through the cartridge at a flow rate not greater than 5 mL/min. The cartridge was washed with 10 mL deionized water before 3 mL chloroform–ethanol (9 + 1) was passed through to elute MPT and MPO. The eluate consisted of an upper aqueous phase, which was discarded with a Pasteur pipet, and an organic layer, which was evaporated to dryness under a stream of nitrogen for 30 min. The residue was dissolved in 1 mL acetone, and 1 µL was injected into the gas chromatograph. This procedure was performed with 5 replicates of milk spiked with MPT and MPO at concentrations of 0.05, 0.5, and 5.0 µg/mL.

GC Analysis

MPT and MPO were analyzed with a Hewlett-Packard Model 5890 GC system equipped with a flame photometric detector and a phosphorus filter (526 nm). A 12 m, 0.2 mm id, HP-1 (100% methyl silicone), 0.33 µm, coated narrow-bore Hewlett-Packard capillary column was used. The GC conditions were as follows: helium carrier gas flow at 2 mL/min, air flow at 100 mL/min, hydrogen gas flow at 75 mL/min, nitrogen auxiliary gas flow at 10 mL/min, split injector at 200°C, oven at 110°C, and column program from 110° to 220°C (10°C/min), held for 5 min.

The method detection limit (MDL) was estimated on the basis of results for the 0.05 µg/mL concentrations of MPT and MPO. MDL was calculated with the following formula:

$$\text{MDL} = t_{(0.99)} \times \text{SD}$$

where $t_{(0.99)}$ is the Student's 1-tailed t value at the 99% confidence level and with $(n - 1)$ degrees of freedom and SD is the standard deviation of replicate analyses.

Peak areas of standards and extracts of spiked samples, run under identical conditions, were compared to determine percentage recoveries at each concentration. Precision was deter-

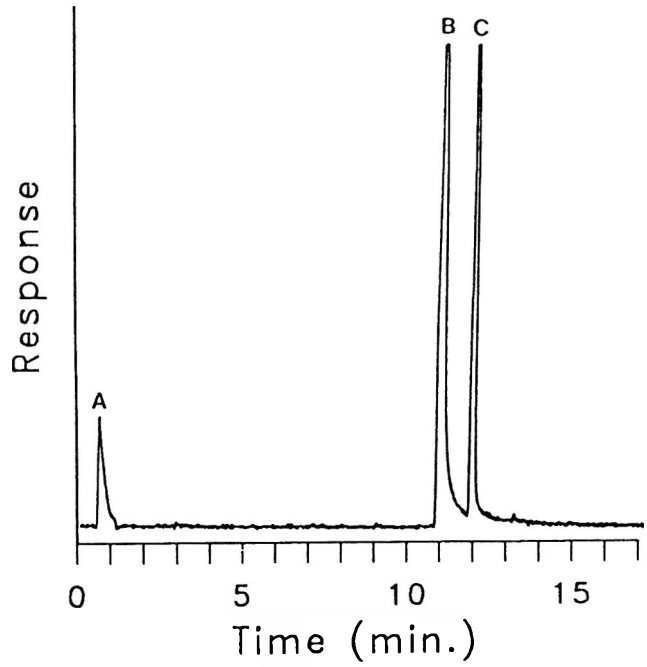

Figure 1. Chromatogram of an acetone extract of goat milk spiked with methyl paraoxon (MPO) and methyl parathion (MPT) at 5 µg/mL: acetone, A; MPO, B; MPT, C.

Table 1. Method detection limits[a] for methyl parathion (MPT) and methyl paraoxon (MPO) in raw cow's milk (RCM), raw goat's milk (RGM), and cow's skim milk (CSM) isolated by liquid–liquid extraction (LLE) (n = 6) and solid-phase extraction (SPE) (n = 5)

	MPT, μg/mL		MPO, μg/mL	
Matrix	LLE	SPE	LLE	SPE
RCM	0.007	0.006	0.010	0.012
RGM	0.009	0.011	0.010	0.015
CSM	0.005	0.013	0.013	0.013

[a] Concentration added, 0.05 μg/mL.

mined under the same conditions. Sample coefficient of variation (CV) was determined as the ratio of sample standard deviation to sample mean and expressed as a percentage.

Statistical Analysis

A 2-tailed t-test was used to determine the significance of differences between LLE and SPE in terms of MPT and MPO recoveries. Values were considered significantly different when $p < 0.05$.

Results

A typical gas chromatogram of MPT and MPO, shown in Figure 1, exhibits good resolution and minimal peak broadening and tailing. No interfering peaks with the same retention times were found in blank milk samples. MDL for MPT for both SPE and LLE and for different milk matrixes ranged from 0.006 to 0.013 μg/mL; for MPO, MDL for both methods ranged from 0.010 to 0.015 μg/mL (Table 1). MDLs for the 2 methods were not significantly different.

Table 2 shows results for recoveries of MPT and MPO from RCM, RGM, and CSM. There was no significant difference between the 2 methods in terms of MPT and MPO recoveries from the same milk matrix ($p > 0.05$). Recoveries of MPT by LLE ranged from 88.0 to 118%, with an average of 103%. CVs for these data ranged from 3.2 to 8.0%, with an average of 5.5%. Recoveries of MPT by SPE ranged from 90.0 to 116%, with an average of 98.0%. CVs for these data ranged from 2.0 to 11.5%, with an average of 6.1%.

Recoveries of MPO by LLE ranged from 80.0 to 118%, with an average of 104%. CVs for these data ranged from 3.7 to 12.1%, with an average of 6.4%. Recoveries of MPO by SPE ranged from 90.0 to 114%, with an average of 99.9%. CVs for these data ranged from 2.9 to 9.9%, with an average of 7.1%. Mean CV values exceeded 10% in only 2 of 36 analyses.

There was a tendency for an emulsion to form during mixing of aqueous and organic solvent phases in the LLE method. This method required more than 3 h for complete extraction of a sample, whereas SPE required less than 0.75 h. When the eluting solvent (chloroform–ethanol, 9 + 1) was used in volumes greater than 3 mL, coextractants were eluted.

Discussion

Both methods provided adequate recoveries independent of the milk matrix analyzed. LLE required larger sample and solvent volumes than did SPE. SPE yielded extracts with minimum interfering coextractants such as milk fats. Average CV values ranged from 5.5 to 7.1%; therefore, data precision is acceptable on the basis of Environmental Protection Agency (EPA) guidelines (10), which regard CV values less than 10% as acceptable. Average recoveries ranged from 80.0 to 118%, which are within the acceptable range established by EPA (10).

Beroza and Bowman (11) separated fenthion and its 5 polar metabolites from milk by an LLE procedure. For the present study, their method was modified by reducing by 50% the sample and solvent volumes for extraction. In addition, methylene chloride, rather than the more hazardous benzene, was used to dissolve the fatty residue after flash evaporation and cooling of residue. Consequently, further solvent partitioning with acetonitrile–hexane, rather than the more elaborate cleanup methods, was sufficient for extraction of MPT and particularly its metabolite, MPO, from the methylene chloride extract.

Liu et al. (9) described use of SPE to separate OPs from plasma and urine; however, these body fluids are less complex

Table 2. Recovery of methyl parathion (MPT) and methyl paraoxon (MPO) from raw cow's milk (RCM), raw goat's milk (RGM), and cow's skim milk (CSM) by liquid–liquid extraction (LLE) and solid-phase extraction (SPE)

		MPT recovery, %		MPO recovery, %	
Matrix	MPT or MPO added, μg/mL	LLE (n = 6)	SPE (n = 5)	LLE (n = 6)	SPE (n = 5)
RCM	5.00	109 (6.9)[a]	92.6 (2.0)	105 (5.5)	114 (2.9)
	0.50	106 (7.1)	96.0 (11.5)	118 (5.5)	104 (6.7)
	0.05	88.0 (4.9)	92.0 (3.6)	94.0 (6.2)	104 (6.1)
RGM	5.00	105 (6.0)	93.6 (8.1)	104 (4.6)	92.2 (9.9)
	0.50	96.0 (8.0)	90.0 (3.6)	102 (12.1)	92.0 (7.0)
	0.05	118 (4.6)	108 (5.5)	80.0 (7.5)	102 (7.8)
CSM	5.00	95.2 (5.7)	116 (9.3)	96.6 (5.5)	94.2 (8.0)
	0.50	112 (3.5)	104 (4.0)	118 (3.7)	106 (7.7)
	0.05	96.0 (3.2)	90.0 (7.6)	116 (6.8)	90.0 (7.8)

[a] Values in parentheses are coefficients of variation in percent.

and contain less fat than raw milk. Results of the present study demonstrate that the C_{18} column can be used to extract MPT and MPO from milk containing 3.0–3.5% milk fat. Lipids in milk may have associated with the lipophilic C_{18} polymer in the SPE cartridge and allowed selective elution of MPT and MPO. Apparently, the difference in polarity of the more polar active metabolite (MPO) and the parent compound (MPT) is not great and does not markedly alter MPO's affinity for the lipid–polymer complex. Various other widely used OPs and their common metabolites must be tested to determine the broad applicability of the proposed SPE method. The International Dairy Federation has expressed interest in the development of a simple method for the determination of OP pesticides in milk (5). The SPE method described in this paper has significant potential for simplification.

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RESIDUES AND TRACE ELEMENTS

Determination of Thiabendazole in Potatoes, Fruits, and Their Processed Products by Liquid Chromatography

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A liquid chromatographic method has been developed for analysis of the fungicide thiabendazole (TBZ) in fruits, potatoes, and their processed products. Extraction was performed in a 50 mL polyethylene conical centrifuge tube with a basic solution. TBZ was partitioned into methylene chloride with a polytron. No further cleanup was needed. Separation was carried out on a 5 μm Ultracarb 30 ODS column with fluorescent detection, excitation at 305 nm and emission at 345 nm. Total analysis time including extraction was 25 min per sample. Recoveries ranged from 77 to 135%. Average percent recovery for all sample types spiked from 5 ppb to 387 ppm was 94% with a coefficient of variation (CV) of 11%. Overall intra-assay CVs varied from 1.8 to 23%, and interassay CVs ranged from 1.8 to 15%. Of 194 commercial samples analyzed for TBZ, 129 were positive. TBZ in positive samples ranged from 1.1 ppb to 72.5 ppm. The limit of quantitation varied from 2.5 ppb for juices, 2.5 ppb for fruits and potatoes, and 4.5 ppb for bulk concentrates. Detection limits were 1.0 ppb for juice, fruits, and potatoes and 2 ppb for bulk concentrates. TBZ levels in the positive samples were corroborated by immunoassay.

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Thiabendazole (TBZ), a pre- and postemergence fungicide, has been used since 1968 (1). However, its primary use is for postharvest treatment of fruits and vegetables to protect from *Fusarium roseum, Colletotrichum musae, Verticullium theobromae, Thielaviopsis paradoxa, Botryodiplodia*

theobromae, Deightoniella torulosa, and *Nigrospora* spp. (2). Because of TBZ's frequent use and the quantities applied (3), and concerns about pesticides in diets of infants and children (4), TBZ must be determined in all types of foods.

Because of its high water solubility and fluorescence properties, TBZ is best quantitated by liquid chromatography (LC). Numerous LC methods have been developed that use either ultraviolet or fluorescent detection with partition and/or column cleanup (2, 5–13). However, these TBZ procedures either require much time and/or have not been tested on a wide range of foods.

We describe an LC method for determining TBZ in a wide variety of fresh and processed produce. The method involves microscale extraction and partition in one step. Further cleanup or multiple extractions are unnecessary, and the recovery, sensitivity, and reproducibility are excellent.

METHOD

Samples, Reagents, and Standards

(**a**) *Samples.*—Fruits, potatoes, and their processed products were purchased from supermarkets in Bangor, ME, except for bulk concentrates, which came from Coca-Cola Foods (Auburndale, FL) and Ocean Spray, Inc. (Lakeville-Middleboro, MA), and the extruded potato peel and potato peel cookie samples, which were from the Department of Food Science, University of Maine. Samples used for controls and fortifications were obtained from organic food stores in Bangor, ME, and Diamond Organics, Freedom, CA.

(**b**) *Reagents.*—All solvents were LC grade (VWR, Boston, MA).

(**c**) *TBZ reference standard.*—Purity, 97.9% (U.S. Environmental Protection Agency, Research Triangle Park, NC).

(**d**) *TBZ standard stock solution.*—Accurately weigh 22 mg TBZ into a 25 mL volumetric flask, dissolve, and dilute to volume with methanol–acetonitrile (90 + 10). Store at 4°C. Standard should be stable for 6 months if capped properly.

(e) *TBZ intermediate standard.*—Pipet a 50 µL aliquot of TBZ stock (**d**) and place into a 50 mL volumetric flask. Dilute to volume with LC mobile phase (**g**).

(**f**) *TBZ working standards.*—Remove aliquots (0.1, 0.25, 0.50, 1.0, and 2.0 mL) for standard curve determination from TBZ intermediate standard (**e**) and place into separate 10 mL volumetric flasks. Dilute to volume with LC mobile phase (**g**).

(**g**) *Mobile phase.*—Acetonitrile–methanol–water–monoethanolamine (260 + 70 + 500 + 0.1).

(**h**) *Sample extraction solvent.*—5 mL ethanol with 15 mL 2M ammonium chloride adjusted to pH 9.5 with 14.5N ammonium hydroxide.

Apparatus

(**a**) *Liquid chromatograph.*—Waters 510 pump (Waters Associates, Milford, MA), Valco pneumatic injector (VICI Instruments, Houston, TX), Waters 470 fluorescence detector, and a Hewlett-Packard 3396A integrator (Avondale, PA). Operating conditions: injection volume, 10 µL; flow rate, 1.0 mL/min; fluorescence, excitation at 305 nm and emission at 345 nm; attenuation, 8; gain, 100; filter, 1.5 s.

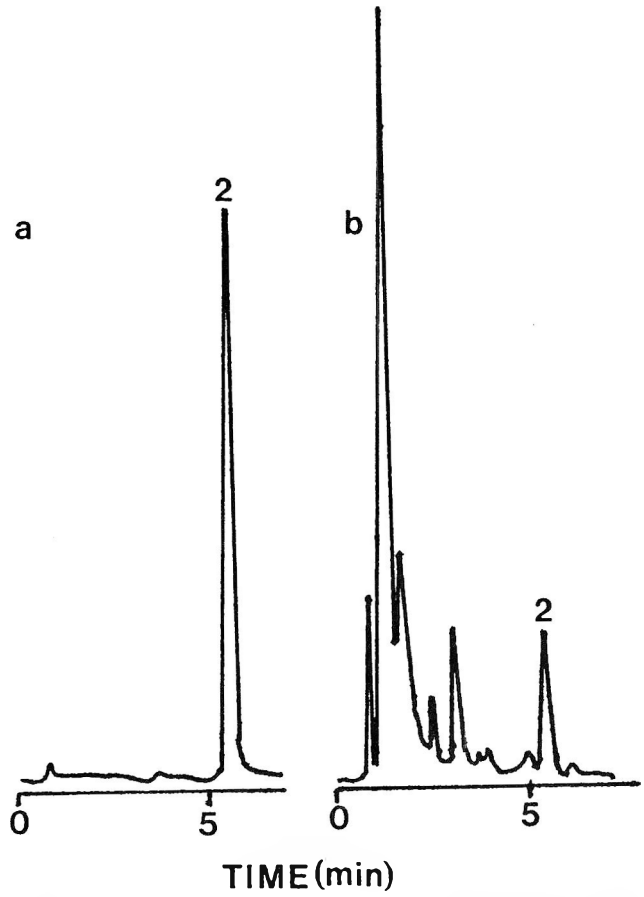

Figure 1. Liquid chromatogram of a TBZ standard (a) (peak 2 represents 0.84 ng TBZ injected) and an apple quencher sample (b) (peak 2 represents 0.21 ng TBZ injected).

(**b**) *Chromatographic column.*—Ultracarb 30 ODS, 5 µm, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Torrance, CA).

(**c**) *Immunoassay formats.*—EnviroGard (Millipore Corp., Bedford, MA) tube polyclonal immunoassay kits were used for all samples except juice and juice concentrates. Juices and bulk concentrates were analyzed by a monoclonal immunoassay developed and described by Brandon et al. (13).

Sample Preparation

Make sure that samples are homogeneous. Use a Hobart food processor for fruits and vegetables. Shake and/or stir juices and concentrates. Use a Wiley Mill for all other products.

Extraction

Weigh 5 g (juice, fresh fruits, and potatoes), 2 g (bulk juice concentrates) or 0.5 g (dried samples) of a homogeneous sample into a 50 mL polyethylene conical centrifuge tube. Add 20 mL extraction solvent (**h**) and 20 mL methylene chloride. Homogenize the mixture for 2 min at medium speed with a polytron (Brinkmann Instruments, Westbury, NY) and then shake rapidly by hand for 3 min. Centrifuge for 3 min at 5000 × *g*. Transfer bottom layer (methylene chloride) into a 20 mL

Table 1. Recovery of thiabendazole from fortified fruits, potatoes, and their processed products[a]

Sample[b]	TBZ added, ppb	TBZ found, ppb	Mean recovery, %	CV, %	Sample[b]	TBZ added, ppb	TBZ found, ppb	Mean recovery, %	CV, %
Banana P	50	40	80	6.2	Pear	50	48	96	1.5
Banana P[c]	200	172	86	3.7	Pear	200	202	101	5.0
Banana P[c]	1000	1100	110	4.7	Pear	1000	950	95	8.0
Banana P[c]	10000	8300	83	9.5	Pear	10000	8600	86	1.5
Banana P[c]	50000	43500	87	2.9	Pear	50000	42500	85	3.2
Banana F	50	43	85	3.9	Apple	50	68	135	1.3
Banana F	200	166	83	5.1	Apple	200	188	94	6.9
Banana F	1000	1000	100	11	Apple	1000	1010	101	3.1
Banana F	10000	7700	77	6.4	Apple	10000	7900	79	3.5
Banana F	50000	45500	91	4.6	Apple	50000	42500	85	4.9
Orange P[c]	200	240	120	7.4	Apple	387000	356040	92	10
Orange P	1000	1070	107	5.9	Potato	50	49	99	2.7
Orange P	10000	8200	82	8.6	Potato	200	190	95	9.1
Orange P	50000	47500	95	3.4	Potato	1000	780	78	6.3
Orange F	50	49	98	2.7	Potato	10000	9300	93	8.2
Orange F	200	186	93	2.4	Potato	50000	48500	97	3.0
Orange F	1000	870	87	3.4	Potato	387000	344430	89	8.5
Orange F	10000	9000	90	4.0	Fruit J[c]	5	4.8	96	21
Orange F	50000	44000	88	6.1	Fruit J[c]	25	23	90	13
Grapefruit P	50	46	91	5.7	Fruit J	50	49	98	9.7
Grapefruit P	1000	910	91	5.9	Fruit J	200	208	104	12
Grapefruit P[c]	10000	11600	116	8.0	Fruit J	1000	1030	103	7.3
Grapefruit P	50000	46500	93	3.2	Fruit C	5	4.5	89	9.8
Grapefruit F	50	48	95	3.2	Fruit C	25	22	86	7.1
Grapefruit F	1000	970	97	7.2	Fruit C	50	45	90	11
Grapefruit F	10000	9800	98	13	Fruit C	200	174	89	9.3
Grapefruit F	50000	57000	114	5.1	Fruit C	1000	910	91	2.8

[a] Means and coefficients of variations are based on 5 determinations.
[b] P, peel; F, flesh; J, juice. Juices were apple, apple grape, cranberry raspberry, apple sweet potato, apple cherry, and fruit punch; C, bulk juice concentrates: apple, orange, and raspberry.
[c] Only 4 determinations were made.

glass scintillation vial containing 0.5 g anhydrous sodium sulfate. Evaporate to dryness a 10 mL aliquot of methylene chloride under nitrogen. Dissolve residue in 1 mL mobile phase (**g**) and transfer to a 1.5 mL polyethylene centrifuge tube. Centrifuge for 5 min at 10 000 × *g*.

Determination

Inject 10 µL from the final 1 mL sample; use LC conditions described in *Apparatus* (**a**) and (**b**), and *Reagents* (**f**) and (**g**). Use peak height for quantitation, because it is linear from 0.04 to 1.7 ng TBZ injected.

Fortifications

Samples were analyzed by LC before spiking to ensure the absence of TBZ and/or interfering substances. Fruits and potatoes were fortified at 50 ppb to 387 ppm, and juices and bulk concentrates were spiked at 5 ppb to 1 ppm.

Reproducibility

Market samples containing TBZ were tested for intra- and interassay reproducibility based on different extractions. These samples were a good representation of concentrations and types.

Results and Discussion

LC chromatograms of the standard and a juice sample are shown in Figure 1. TBZ elutes a little after 5 min (Figure 1b) with complete resolution from other peaks. All samples had very similar chromatograms. Interfering peaks are absent, as further supported by analysis of organic controls. To corroborate this method, samples analyzed by LC were also analyzed by immunoassay, by either the method of Bushway et al. (14) or that of Brandon et al. (13). The correlation coefficient between immunoassay and LC for positive samples was 0.988, indicating that the LC TBZ peaks were free from interfering substances. These results also suggest that immunoassay is a good corroboration for TBZ analysis.

Peak height was used for quantitations. The linearity range was from 0.04 to 1.7 ng TBZ injected, with a correlation coefficient of 0.999. Because TBZ gave a linear response even at low concentrations, one standard (0.84 ng/10 µL) was injected (once every 3 samples) for quantitation. This approach enabled us not to have to inject the set of standards each day. The intraassay coefficient of variation (CV) based on 7 standard injections covering one day of sample analyses was 1.9%; the in-

terassay CV for 10 days representing 73 standards was 3.9%. These values indicate good reproducibility of the LC system for TBZ analysis.

An extensive recovery study was performed (Table 1), using 15 different fruits, potatoes, juices, and bulk juice concentrates. Fortification levels varied from 50 ppb to 387 ppm for fruits and potatoes; juices and concentrates were spiked from 5 ppb to 1 ppm. Before being used for spikes, samples were first shown to be free of detectable levels of TBZ and interfering substances. Average recovery values were based on 4 or 5 separate determinations (done on different days). Overall recovery was 94% (CV, 11%). Recoveries varied from 77 to 135%, and CVs ranged from 1.3 to 21%. Most recoveries were in the 85–99% range. Such recoveries point to the versatility of the method.

To test the true reproducibility of a method, it is best to use market samples that have been shown to contain TBZ. Table 2 shows intra- and interassay CVs for 21 different food products that were shown to have TBZ. These products cover a wide range of TBZ levels (4.2 to 80 100 ppb) and as can be seen, the CVs are excellent, with only 7 CVs above 10%.

This newly developed method was used on 194 food products from various sources including stores, food industries, and a university. Most samples were actual commercial products, except the extruded potato peels and potato peel cookies, which were experimental foods. Of the samples, 129 showed detectable amounts (detection limit, 1.0 ppb) of TBZ ranging from 1.1 to 72 471 ppb. If the experimental foods are excluded (6 samples), the TBZ amounts varied from 1.1 to 8633 ppb. The high value of 8633 ppb was from a grapefruit peel, which is not edible. Therefore, if only edible portions are included, the TBZ levels in these foods would vary from 1.1 to 3916 ppb, with most levels below 300 ppb.

Of the juices and juice concentrates, 46% were positive for TBZ at an average level of 76 ppb. Among fresh produce, 94% were positive for TBZ, with a mean level of 662 ppb.

TBZ does not seem to degrade during processing (unpublished results). The time of sampling or processing could be key to how much TBZ is present in samples, because TBZ is primarily used as a postharvest fungicide. If samples are tested before postharvest application of TBZ, then very little TBZ is expected. For this reason, fewer samples of juice and juice concentrate tested positive, and TBZ levels were lower. Also, during juice and concentrate processing, the peel is not included, and the peel is where most of the TBZ is found.

In this study, inedible peels of foods like bananas, oranges, limes, lemons, grapefruit, and ugli fruit were analyzed separately from the edible portion. Measurement of TBZ in inedible peels provides the best indication for presence of TBZ in a particular commodity, but a true measure of human dietary exposure must be made from analysis of edible portions. Table 3 illustrates that, in most cases, the peel contains much more TBZ. This finding is not surprising, because the postharvest treatment involves coating TBZ on the skin of produce.

This LC method for TBZ is versatile, sensitive, and reproducible. It is useful for gathering TBZ residue data to aid toxicologists and epidemiologists in their studies on toxic levels of TBZ. The primary differences between this method and others is a simplified extraction technique and a mobile phase and column that allows complete and rapid separation of TBZ from other substances.

Table 2. Reproducibility of the LC thiabendazole method for fruits, potatoes, and their processed products

Sample[a]	Thiabendazole, ppb	CV, %	
		Intra-assay[b]	Interassay[c]
Banana peel	468	5.6	11
Potato	180	4.0	1.8
Orange flesh	219	1.8	6.9
Orange peel	5520	1.9	3.0
Grapefruit peel	8242	2.3	3.7
Grapefruit flesh	380	6.6	4.2
Apple	2015	3.8	3.3
Pear	419	3.9	4.0
Extruded peel	80100	8.4	3.4
Cookie	1886	2.7	4.9
Apple cide-	159	4.6	13
Grapefruit J	119	11	5.0
Apple-pear J	4.2	11	6.0
Pear J	207	9.1	8.9
Apple cider	283	7.5	11
Apple C1	170	4.5	7.9
Apple C2	4.5	23	15
Apple C3	135	2.8	2.8
Apple C4	33	2.6	4.1
Apple C5	30	6.5	5.6
Orange C	15	2.8	3.2

[a] J, juice; C, bulk juice concentrate.
[b] CV based on 6 determinations in the same day except for juices and bulk concentrates, which were based on 4 determinations.
[c] CV based on 6 determinations performed on 6 different days except for juices and bulk juice concentrates.

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Table 3. Liquid chromatographic analysis of thiabendazole in commercial food samples

Sample	Thiabendazole, ppb	Sample	Thiabendazole, ppb
Extruded potato peel 1	63635	Lime juice 1	1.1
Extruded potato peel 2	61855	Grapefruit juice 3	23
Dried potato peel	63190	Apple juice 1	3.0
Ugli fruit peel 1	122	Apple grape juice 1	ND
Ugli fruit peel 2	34	Apple cherry juice 1	1.3
Grapefruit peel 1	8633	Apple cherry juice 2	3.6
Grapefruit flesh 1	223	Apple grape juice 2	5.5
Grapefruit peel 2	4895	Organic apple juice	ND
Grapefruit flesh 2	205	Natural apple juice	ND
MacIntosh apple 1	43	Grapefruit juice 4	188
Macintosh apple 2	24	Apple quencher juice	4.2
Red delicious apple 1	3916	Apple sweet potato juice	ND
Red delicious apple 2	3827	Fruit punch 1	72
Organic Bosc pear 1	475	Lime juice 2	1.5
Organic Bosc pear 2	507	Apple grape juice 3	16
Northern apple 1	10	Fruit punch 2	ND
Northern apple 2	12	Apple juice 2	ND
MacIntosh apple 3	8	Pear juice	220
MacIntosh apple 4	7	Lime juice 3	4.9
Potato 1	20	Raspberry concentrate 1	ND
Potato 2	10	Raspberry concentrate 2	ND
Potato 3	18	Raspberry concentrate 3	ND
Potato 4	15	Red grape concentrate 1	ND
Potato 5	112	Raspberry concentrate 4	ND
Potato 6	109	Peach concentrate 1	3.0
Potato 7	11	Peach concentrate 2	ND
Potato 8	11	Raspberry concentrate 5	ND
Potato 9	81	Apple concentrate 1	5.7
Potato 10	88	Cherry concentrate 1	ND
Potato 11	63	Apple concentrate 2	16
Potato 12	60	Apple concentrate 3	14
Potato 13	470	Apple concentrate 4	ND
Potato 14	638	Peach concentrate 3	4.7
Extruded potato peel 3	28226	Raspberry concentrate 6	ND
Extruded potato peel 4	51493	Apple cranberry concentrate 1	ND
Extruded potato peel 5	72471	Apple concentrate 5	118
Extruded potato peel 6	61029	Apple concentrate 6	ND
Raspberry 1	27	Apple concentrate 7	ND
Raspberry 2	2	Orange concentrate 1	ND
Sweet apple cider	4	Apple concentrate 8	172
Banana flesh 1	57	Raspberry concentrate 7	ND
Banana peel 1	282	Apple concentrate 8A	ND
Potato 15	259	Apple concentrate 9	ND
Potato 16	252	Apple concentrate 10	6.8
Potato 17	2.0	Apple concentrate 11	32
Potato 18	2.0	Orange concentrate 2	ND
Kiwi 1	2.0	Apple concentrate 12	133
Kiwi 2	3.0	Apple concentrate 13	ND
Bosc pear 1	725	Strawberry concentrate 1	ND
Bosc pear 2	610	Apple concentrate 14	ND
Lime peel	10	Apple concentrate 15	109
Lime flesh	ND[a]	Orange concentrate 3	3.0
Lemon peel	3	Apple concentrate 16	5.4
Lemon flesh	ND	Apple concentrate 17	ND
Orange peel	3433	Peach concentrate 4	ND

Table 3. (*continued*)

Sample	Thiabendazole, ppb	Sample	Thiabendazole, ppb
Orange flesh	328	Grape concentrate 1	ND
Golden delcious apple 1	412	Orange concentrate 4	ND
Golden delcious apple 2	354	Orange concentrate 5	9.3
Red delicious apple 3	1983	Apple concentrate 18	5.6
Red delicious apple 4	1484	Apple concentrate 19	ND
Anjou pear 1	3699	Apple concentrate 20	ND
Anjou pear 2	2285	White grape concentrate 1	ND
Keebler Tato Skins 1	38	Raspberry concentrate 8	ND
Keebler Tato Skins 2	46	Peach concentrate 5	ND
Potato chips 1	46	Raspberry concentrate 9	ND
Potato chips 2	23	Apple concentrate 21	3.0
Potato chips 3	31	Orange concentrate 6	ND
Potato chips 4	130	Raspberry concentrate 9	ND
Potato chips 5	310	Apple concentrate 22	35
Potato chips 6	317	Apple concentrate 23	4.4
Crackers 1	ND	Orange concentrate 7	ND
Crackers 2	ND	Grape concentrate 2	ND
Stuffed baked potatoes 1	20	Red grape concentrate 2	ND
Stuffed baked potatoes 2	1277	Apple concentrate 24	3.4
Baked potato skins 1	356	Apple concentrate 25	ND
Baked potato skins 2	267	Apple concentrate 26	4.0
Steak fries 1	8	Peach concentrate 6	ND
Steak fries 2	13	Apple concentrate 27	ND
Potato peel cookie raw	1780	Grape concentrate 3	ND
Potato peel cookie cooked	1691	Orange concentrate 8	ND
Banana peel 2	490	Peach concentrate 7	ND
Banana flesh 2	102	Apple concentrate 28	3.8
Sweet cider 1	179	Grape concentrate 4	ND
Sweet cider 2	281	Grape concentrate 5	ND
Sweet cider 3	284	Cranberry concentrate 1	ND
Sweet cider 4	363	Raspberry concentrate 10	ND
Sweet cider 5	292	Raspberry concentrate 11	ND
Sweet cider 6	336	Raspberry concentrate 12	ND
Sweet cider 7	298	Cranberry concentrate 2	3.1
Sweet cider 8	124	Apple concentrate 29	242
Apple raspberry juice	1.1	Peach concentrate 8	ND
Fruits A Plenty juice	ND	Strawberry concentrate 2	ND
Apple cranberry juice	11	Apple concentrate 30	84
Cranberry raspberry juice	ND	Apple concentrate 31	94
Grapefruit juice 1	18	Peach concentrate 8	ND
Grapefruit juice 2	15	Cranberry concentrate 3	ND
Lemon juice	17	Cranberry concentrate 4	ND

[a] ND, none detected at a detection limit of 1 ppb for fruits, potatoes, and juices and 2 ppb for bulk concentrates.

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RESIDUES AND TRACE ELEMENTS

Development of a Method of Analysis for 46 Pesticides in Fruits and Vegetables by Supercritical Fluid Extraction and Gas Chromatography/Ion Trap Mass Spectrometry

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A multiresidue method using supercritical fluid extraction (SFE) and gas chromatography/ion trap mass spectrometry (GC/ITMS) was developed for analysis of 46 pesticides in fruits and vegetables. The SFE procedure used 2 commercial instruments that trapped the extracts on solid-phase material. Silica gel chemically bound to octadecylsilane (ODS) collected the extracted pesticides efficiently, and elution of the trap with acetonitrile gave high recoveries. Extracts thus obtained were sufficiently clean for subsequent GC/ITMS analysis. The SFE conditions were 320 atm and 60°C (0.85 g/mL CO_2 density) and 1.6 mL/min CO_2 flow rate for 6 extraction vessel volumes. Trapping on 1 mL ODS occurred at 10°C, and a 0.4 mL/min flow rate of acetonitrile at 40°–50°C was used to elute the pesticides. Quantitative and qualitative analyses of the 46 pesticides were performed simultaneously by GC/ITMS. Studies of fortified samples gave >80% recoveries for 39 pesticides, and recoveries of >50% for the other pesticides, except methamidophos and omethoate. Grapes, carrots, potatoes, and broccoli were used as samples during method development, and a blind experiment involving incurred and fortified samples was used to test the approach. Results of the blind study compared satisfactorily with results from 7 laboratories using traditional GC detectors and solvent-based extractions.

Currently, analyses of pesticides in food are commonly performed with organic solvent extraction methods (1–3), which can be expensive, time consuming, and labor intensive and require much space and glassware as well as generate a large amount of hazardous waste. The Environmental Protection Agency has directed government agencies to reduce consumption of solvents, especially chlorinated solvents, in laboratories (4). The most commonly used multiresidue method for analysis of pesticides in fruits and vegetables uses 800 mL organic solvent (including 300 mL methylene chloride) per 100 g sample (1). Furthermore, because of large sample size, nonselective extraction conditions, and concentration of matrix interferants and organic solvent impurities, organic solvent extracts require extensive cleanup before analysis. The progress in chromatographic separation and detection of pesticides was not accompanied by an adequate improvement in sample preparation techniques (5). With technological advances in extraction methods and instrumentation, and heightened awareness of environmental and fiscal responsibilities, more efficient multiresidue methods for analysis of pesticides in produce are required.

Supercritical fluid extraction (SFE) offers an alternative to solvent-based extractions. It poses little threat to the environment, improves extraction selectivity, saves time and laboratory space, and lends itself to automation (6–9). Despite the great interest in SFE, however, not many studies describing applications of SFE in multiresidue analysis of pesticides in food have been published (10–23).

Gas chromatography/ion trap mass spectrometry (GC/ITMS) was selected as the universal method of detection because of its ability to perform simultaneous quantitative and qualitative analyses of different classes of pesticides at ultratrace concentrations. Reports have been published (23–26) describing the use of GC/ITMS in determining various components in complex matrixes.

This work combines commercialized SFE technologies with GC/ITMS to develop a method that will simultaneously monitor various pesticides in fruits and vegetables. The 46 pesticides chosen for analysis were based on compounds included in the Pesticide Data Program (27) that could be analyzed by GC. Many of these pesticides are commonly found at very low levels in produce (27). The 4 commodities tested–potatoes, carrots, broccoli, and grapes–had incurred pesticides or were fortified with pesticides. Also, the SFE and GC/ITMS procedure was used to analyze check samples as a quality assurance measure. Samples fortified with pesticides at concentrations unknown to the analysts were analyzed, and results were compared with those from 7 other laboratories that analyzed the same samples by traditional methods.

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Experimental

Apparatus

(a) *Supercritical fluid extractors.*—A Model 7680T (Hewlett-Packard, Little Falls, DE) and a Prepmaster (Suprex, Pittsburgh, PA), both equipped with automated variable restrictors, solvent modifier pumps, and solid sorbent collection systems, were used. For the 7680T, optimal instrumental parameters were: 320 atm extraction pressure and 60°C temperature (CO_2 density, 0.85 g/mL); 7 mL extraction vessel; 2 min static extraction followed by 42 mL CO_2 at a flow rate of 1.6 mL/min; 50°C restrictor temperature; collection on octadecylsilane (ODS) sorbent trap (1 mL) at 9°C; and elution with 1.5 mL acetonitrile at 0.4 mL/min and 50°C. The trap was rinsed to waste with 2 mL ethyl acetate followed by 2 mL acetonitrile at 2 mL/min to clean and regenerate the ODS between extractions. For the Prepmaster, instrument settings were the same except for the following: 10 mL vessel size and 60 mL CO_2 extraction volume; trap elution at 40°C with 6 mL acetonitrile; N_2 gas at 80 psi to blow trap dry between flushes. Total time for extraction–elution per sample was 36 min with the 7680T and 54 min with the Prepmaster. For experiments conducted under conditions other than those described, the settings are specified in the discussion of those experiments.

(b) *Gas chromatograph.*—A Model ITS40 GC/ITMS system (Finnigan MAT, San Jose, CA), consisting of a Varian 3300/3400 gas chromatograph and a CTC A200S autosampler, was used. Operating conditions for the GC/ITMS were: 1 μL injection volume into a Model 1093 (Varian, Walnut Creek, CA) septum-programmable injector (SPI); 3 s needle hold time in port before injection; 55°C injection port for 30 s followed by ramping to 250°C at 250°C/min; 5 psig He column head pressure; 55°C initial oven temperature for 30 s, ramped to 130°C at 50°C/min, then to 165°C at 1.5°C/min and to 250°C at 4°C/min, and held at 250°C until a total time of 65 min elapsed; 240°C transfer line temperature; and 215°C detector manifold temperature. Typical ITMS operating conditions (autotune calibration was performed before each injection sequence) were as follows: electron impact mode; 10 μA filament current; 1850 V electron multiplier tube; 1 ms ion time; and automatic gain control at 20 000.

(c) *Chromatographic columns.*—A DB-1701 capillary column (14%-cyanopropylphenyl)methylpolysiloxane, 30 m, 0.32 mm id, 0.25 μm film thickness (J&W Scientific, Folsom, CA), and 5 m phenylmethyl deactivated guard column (0.32 mm id) (Restek Corp., Bellefonte, PA).

(d) *Data collection.*—Data acquisition for mass spectra (70–425 *m/z*), was obtained from 5 to 65 min of the chromatogram. The GC/ITMS system had a Magnum version 2.4 software package loaded into a Gateway 2000 computer for data acquisition and processing and instrument control.

Reagents

(a) *Gases.*—SFC/SFE grade CO_2 (Air Products, Allentown, PA), with a He headspace of 1800 psi (for Prepmaster) or without He headspace (for 7680T), was used. Bone-dry grade CO_2 (for both instruments) and N_2 (for Prepmaster) were required for cryogenic cooling and drying of the trap, respectively. The septum-programmable injector on the GC/ITMS system also used CO_2 with dip-tube for cooling.

(b) *Solvents.*—Acetonitrile, methanol, ethyl acetate, acetone, and isooctane were pesticide grade (Fisher, Fair Lawn, NJ).

(c) *Solids.*—Hydromatrix (Varian, Harbor City, CA), a pelletized diatomaceous earth, was sieved (325 mesh) and washed with acetone before use to remove fine particles and contaminants. Its use in SFE has been described previously (11, 15, 17). The prepacked 30 μm Hypersil ODS (Hewlett-Packard) traps were provided with the 7680T; for the Prepmaster, 55–105 μm C_{18} (Waters, Milford, MA) and 80/100-mesh silanized glass beads (Suprex) were packed into the trap manually.

(d) *Pesticide standards.*—Pesticides were obtained from the U.S. Environmental Protection Agency (Research Park, NC, or Beltsville, MD), except for a duplicate standard of ethion (Niagara Chemical, Middleport, NY) used to double check accuracy of ethion results. Table 1 lists the pesticides, arranged by classification, included in this study. Chrysene-d_{12} and phenanthrene-d_{10} (Cambridge Isotope Laboratories, Woburn, MA) were used as internal standards. Individual stock solutions were prepared by weighing 10–12 mg amounts of standards, dissolving the pesticide with acetone and/or isooc-

Table 1. Pesticides included in the study and their chemical classes

Organochlorine (11)	Organophosphate (21)
Chlorothalonil	Azinphos-methyl
Dacthal	Chlorpyrifos
DDE	Diazinon
DDT	Dichlorvos
Endosulfan I	Dimethoate
Endosulfan II	Disulfoton
Hexachlorobenzene (HCB)	Ethion
Lindane	Ethoprop
Methoxychlor	Fenamiphos
Pentachlorobenzene (PCB)	Malathion
Pentachloronitrobenzene (PCNB)	Methamidophos
	Methidathion
Carbamate (3)	Mevinphos
Carbaryl	Omethoate
Carbofuran	Parathion
Chlorpropham	Parathion-methyl
	Phorate
Other (8)	Phosalone
Atrazine	Phosmet
Captan	Phosphamidon
Dicloran	Terbufos
Diphenylamine	
Iprodione	Pyrethroid (3)
Myclobutanil	Esfenvalerate
Propargite	Fenvalerate
Vinclozolin	*cis*-Permethrin

tane, and making up to 100 mL in volumetric flasks. Concentrations were corrected for the stated purities (typically >98%) of the standards. Working standard mixtures in acetone, containing 20 μg/mL for each pesticide, were used for spiking samples and preparing calibration standards.

Sample Preparation and Analysis

Commercially purchased potatoes, grapes, broccoli, and carrots served as blank or fortified samples. Incurred (contaminated) grape and carrot samples were provided by the State of Michigan Department of Agriculture, and fortified samples (containing unknown pesticides at concentrations unknown to the authors) of potato and broccoli were provided by the California Department of Food and Agriculture. The potato and broccoli samples were also analyzed 7 times by traditional methods (1, 2) by 6 state laboratories (California, Texas, Florida, New York, Michigan, and Washington) participating in the Pesticide Data Program (27), and the grape and carrot samples were analyzed solely by the Michigan laboratory.

For the store-bought samples, a 50 g portion of vegetables was shredded and mixed in a food processor, and a 3 g subsample was weighed into a tared beaker. Hydromatrix (2 g) was added to the beaker; a glass rod was used for mixing. The mixed samples were packed into the extraction vessels, and for fortified samples, the 20 μg/mL spiking solution was added to the sample in the middle of the vessel. A few minutes were allowed for the solvent to evaporate. Spiking levels varied from 0.1 to 1 μg/g in the samples; triplicate samples were extracted for analysis. For incurred grapes and carrots, which arrived precut and frozen, 3 g portions of frozen sample were mixed with 2 g Hydromatrix and packed into the vessels. The 100 g each of potato and broccoli check samples were mixed with 66.7 g Hydromatrix in the sample container, because chopped samples may not have been mixed thoroughly after fortification. In those cases, 5 g mixed sample–Hydromatrix was loaded into the vessels. To ensure instrument performance, a control spike of pentachlorobenzene at 0.2 μg/g was added to the vessels before extraction. The samples were extracted as described earlier. An internal standard (chrysene-d_{12} or phenanthrene-d_{10}) was added to the extracts before injection for quantitation by GC/ITMS.

For GC/ITMS calibration, the spiking solutions were diluted to make the calibration standards, and internal standard solution was added to the calibration standards in the same ratio as the extracts. For best quantitation, the calibration standards were prepared in SFE extracts from sample blanks of the same matrix. For samples of known fortification levels, 4 calibration concentrations varying from 4 times lower to 2 times higher than the fortification level were used. For samples of unknown concentration, 4–5 calibration standards ranging from 0.025 to 1.5 μg/g were used.

Calculations

(**a**) *Limits of detection.*—The ratio $3\sigma_{blank/sensitivity}$ was used to calculate limits of detection (LODs) where σ_{blank} is the standard deviation of blank measurements (or noise) and sensitivity is the slope of the linear calibration plot for each analyte. The average noise in the 60–100 s retention windows of the quantitation masses for each pesticide was calculated from the software-reported signal-to-noise (S/N) ratios for the calibration standards in potato. LODs were calculated by multiplying these noise levels by 3 and then dividing by the slope of a linear calibration curve generated from peak height data (both noise and signal were divided by the internal standard signal).

(**b**) *Pesticide concentrations.*—Integrated peak area data of selected masses versus the internal standard were used for quantitation. Table 2 lists masses chosen for quantitation of each pesticide. Calculations were done with a spreadsheet program or the instrument's software program. In most cases, the calibration curves were linear, and the linear least-squares calibration line was used for quantitation. In some cases, however, especially for the organophosphates, the calibration curve formed a distinctly quadratic relationship. In those cases, the best-fit quadratic curve was used for quantitation. A method of standard addition was also used to determine pesticide concentrations in the potato check sample.

(**c**) *Confirmation of pesticides.*—With GC/ITMS, the following criteria had to be met to confirm presence of a pesticide in the sample: retention time (t_r) difference of less than 10 s, S/N ratio >3, and mass spectrum match >90% versus the spectrum library for the pesticide (generated from pesticide standards). Only results for confirmed pesticides are presented in this paper.

Results and Discussion

Sampling

The 3 g sample size for SFE was much smaller than the 50–100 g sample sizes used in traditional methods of multiresidue analysis of pesticides in produce (1–3). For carrot and potato, reproducible results of several 3 g subsamples from a 50 g blended sample indicated that 3 g is sufficient to represent a larger sample (23).

For wet samples such as fruits and vegetables (80–95% water), moisture must be removed or absorbed before SFE. Addition of Hydromatrix to the sample (water–Hydromatrix, approximately 1 + 1) is an effective way to absorb water (11, 15, 23). For the potato and broccoli check samples, Hydromatrix was added to the entire 100 g sample and then homogenized because the fortified sample was not assumed to be homogeneous.

Development of SFE Procedure

In general, a new SFE method should be developed in 4 steps: (*a*) confirm quantitation accuracy and precision of the detection method, (*b*) ensure 100% collection and elution of analytes from SFE trap, (*c*) determine SFE conditions for highest recoveries, and (*d*) optimize the method for analysis of real samples. Each step may affect another, and care must be taken to maintain instrumentation in optimal operating conditions. This general outline was followed during development of the SFE procedure.

(**a**) *Elution of pesticides from SFE trap.*—Experiments to compare recoveries of pesticides from 3 sorbents (glass beads,

Table 2. Pesticide retention times (t_r), quantitation masses, and limits of detection (LODs) for potato SFE extracts analyzed by GC/ITMS

No.	Pesticide	t_r, min	Masses,[a] m/z	LOD,[b] ng/g	No.	Pesticide	t_r, min	Masses,[a] m/z	LOD,[b] ng/g
1	Dichlorvos	5.8	109* + 127 + 185	6	24	Phosphamidon	32.8	72 + 127* + 264	27
2	Methamidophos	7.7	94* + 95 + 141	14	25	Dacthal	33.0	299 + 301* + 303	0.4
3	PCB	9.9	248 + 250* + 252	2	26	Carbaryl	33.6	115 + 116 + 144*	5
4	Mevinphos	10.9	127* + 164 + 192	2	27	Malathion	33.8	125 + 127 + 173*	6
5	HCB	16.7	282 + 284* + 286	2	28	Parathion	34.8	97 + 109* + 291	18
6	Ethoprop	17.1	97 + 158* + 243	6	29	Endosulfan I	35.5	195 + 241* + 339	7
7	Diphenylamine	17.2	167 + 168 + 169*	3	30	DDE	37.1	246 + 316* + 318	17
8	Phorate	19.3	75* + 121 + 260	2	31	Captan	37.7	79*	10
9	Chlorpropham	20.0	127* + 171 + 213	5	32	Methidathion	38.1	85 + 93 + 145	9
10	PCNB	21.9	295 + 297 + 299	3	33	DDT	39.6	165 + 235* + 237	1
11	Omethoate	22.3	110* + 156 + 214	20	34	Fenamiphos	39.8	260 + 288 + 303	5
12	Terbufos	23.0	231*	3	35	Endosulfan II	41.2	195 + 241* + 339	8
13	Diazinon	24.3	137 + 179* + 304	2	36	Ethion	41.5	97 + 153 + 231*	6
14	Lindane	24.6	181* + 183 + 219	4	37	Myclobutanil	42.4	150 + 179* + 181	48
15	Disulfoton	25.4	88* + 89 + 97	4	38	Propargite	43.4	135* + 335 + 350	9
16	Dicloran	25.6	124* + 176 + 206	18	39	Methoxychlor	45.3	227*	3
17	Carbofuran	26.1	149 + 164*	2	40	Iprodione	46.9	314* + 316	5
18	Atrazine	26.1	200* + 215 + 216	4	41	Phosmet	47.1	160*	12
19	Dimethoate	28.6	87* + 93 + 125	4	42	Phosalone	48.8	182* + 184 + 367	17
20	Chorothalonil	30.6	264 + 266* + 268	2	43	Azinphos-methyl	49.2	132* + 160	150
21	Vinclozolin	31.3	198 + 212* + 285	4	44	*cis*-Permethrin	49.3	127 + 163 + 183*	13
22	Parathion-methyl	32.1	109 + 125 + 263*	6	45	Fenvalerate	61.3	125 + 225* + 419	29
23	Chlorpyrifos[c]	32.4	197 + 199 + 314	20	46	Esfenvalerate	63.2	125 + 225* + 419	13
IS[d]	Phenanthrene-d_{10}	22.7	188*		IS	Chrysene-d_{12}	46.7	240*	

[a] *, base peak.
[b] For the SFE method with the 7680T (1.5 mL final volume); LODs with the Prepmaster were 4 times higher (6 mL final volume).
[c] Potato matrix interfered spectrally.
[d] IS, internal standard.

alumina, and ODS) were planned. The first step was to ensure 100% elution from the traps. The trap materials were spiked with pesticide mixtures and rinsed with solvents to determine elution volumes. The pesticides studied completely eluted with 1–2 mL solvent from glass beads and ODS for all solvents tested (methanol, acetonitrile, acetone, and ethyl acetate), but many pesticides did not elute from alumina even with 10 mL solvent. A previous study found alumina useful for complete trapping, elution, and cleanup of organochlorine pesticides in vegetables (23), but alumina has limited use in a method involving a diverse mixture of pesticides.

(b) *Collection of pesticides on SFE trap.*—General extraction conditions were known for several analytes from previous studies (23), and were used to test the efficiency of collection of pesticides on the glass bead and ODS traps. In this study, pesticides were spiked onto Hydromatrix, CO_2 extraction density was 0.9 g/mL, and flow rate was 2.5 mL/min for 20 min. For both traps, collection temperatures were 10° and 25°C, and 1.5 mL methanol was used for elution at 0.5 mL/min and 25°C. Figure 1 compares recoveries of several pesticides (numbers refer to pesticides in Table 2) listed in order of increasing GC retention time (a trend of decreasing volatility). ODS trapped 100% of the pesticides tested, but no pesticide was trapped at 100% by the glass beads. For glass beads, the trend of increasing loss versus pesticide volatility indicated the importance of using a trap material that interacts with the analyte in the rapid stream of CO_2 and not to simply create a surface for analyte precipitation from the supercritical fluid. Slightly higher recoveries were obtained at 10°C than at 25°C in both cases, but the difference was not significant when considering the precision of the measurement. A lower temperature may improve recovery for glass beads, but a trap temperature below 0°C increases the likelihood of problems due to ice formation. Another advantage of ODS over glass beads is its potential for additional cleanup of SFE extracts.

(c) *Extraction conditions.*—In most cases, increasing CO_2 density increases SFE extraction capability (6–9). Experiments were performed to determine the effect of different CO_2 density on pesticide recovery: 0.3 g/mL (100 atm, 60°C), 0.5 g/mL (130 atm, 60°C), and 0.85 g/mL (320 atm, 60°C). SFE at a CO_2 density of 0.3 g/mL gave maximum recoveries for all pesticides tested except dimethoate, carbaryl, mevinphos, atrazine, dicloran, captan, and iprodione. Only a CO_2 density of 0.85 g/mL gave maximum recovery for those pesticides. A CO_2 density of 0.95 g/mL improved recovery slightly for a few analytes, but the benefits of slightly higher recoveries for only a few pesticides did not compensate for the cost of higher matrix interferences. An extraction pressure of 320 atm and a tempera-

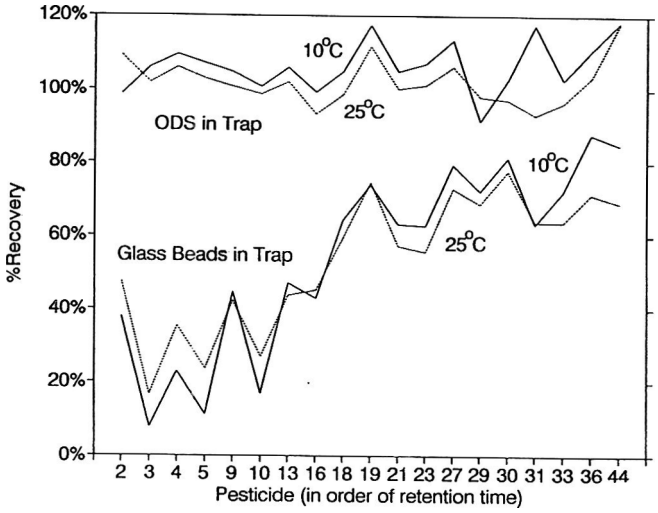

Figure 1. Comparison of SFE collection of pesticides on glass bead or ODS material in trap at 10° and 25°C. Pesticides are numbered as presented in Table 2 and ranked according to increasing retention time (a trend of decreasing volatility).

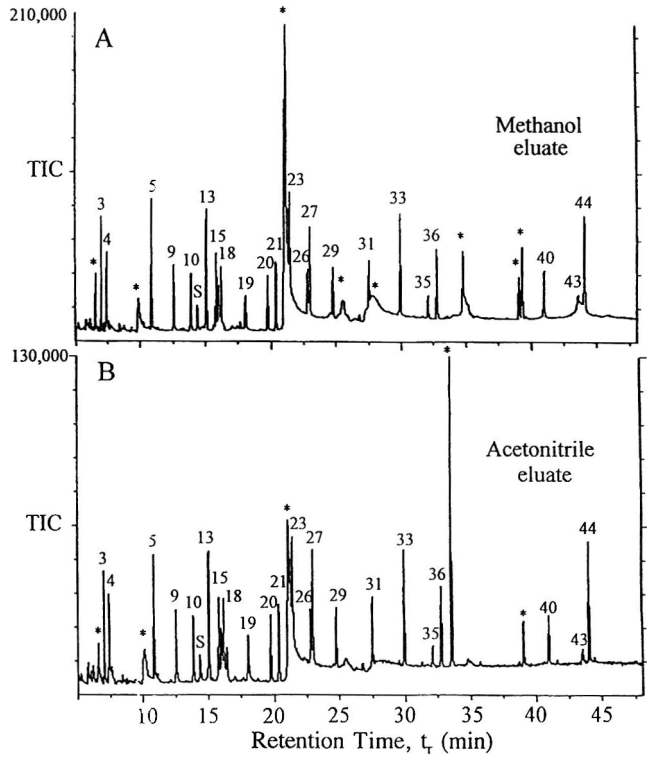

Figure 2. Comparison of GC/ITMS total-ion chromatograms of SFE potato extracts eluted from ODS with (A) methanol and (B) acetonitrile. Numbered peaks refer to the pesticides listed in Table 2; peaks designated with an asterisk were matrix components, and the peak labeled S was the internal standard, phenanthrene-d_{10}.

ture of 60°C (a CO_2 density of 0.85 g/mL) were chosen for subsequent extractions.

Extractions were conducted to optimize other SFE parameters. The length of static extraction steps made no difference. A flow rate of 1.6 mL/min gave a higher recovery of dichlorvos (72% versus 24%), the most volatile component, than did extraction at 2.5 mL/min. An amount of CO_2 equal to 6 empty vessel volumes gave slightly higher recoveries than if 5 vessel volumes were swept. Solvent modifiers were unnecessary, because satisfactory recoveries were achieved without them. However, modifier tests with acetone, methanol, and ethyl acetate were made; recoveries were not substantially different from results without modifiers, but matrix effects worsened.

(**d**) *Extract elution and cleanup.*—Elution of the ODS trap with methanol was compared with elution with acetonitrile. Both solvents gave similar recoveries, but acetonitrile extracts gave fewer matrix peaks and lower background levels during GC/ITMS than the methanol extracts. Figure 2 presents total ion chromatograms of SFE extracts of potato containing several pesticides at 5 μg/g eluted with methanol and acetonitrile. The methanol eluate gave a maximum background peak with total ion current (TIC) of 210 000, whereas the maximum background peak for the acetonitrile eluate was 130 000 under the same conditions. The peaks identified in chromatograms refer to the pesticides listed by number in Table 2, and peaks marked with an asterisk signify matrix components. Broad matrix peaks at ca 28, 35, and 44 min in the methanol eluate were not present in the acetonitrile eluate. Also, the higher boiling point of acetonitrile made it more compatible for septum-programmable injection. For the 7680T, 1.5 mL acetonitrile at 0.4 mL/min and 50°C was sufficient to remove all pesticides from the trap; no analytes were found in the ethyl acetate rinse afterwards. The C_{18} trap used with the Prepmaster required a larger volume of acetonitrile to elute the pesticides, but this was probably due to differences in sorbent material, elution temperature of 4C°C (instrument maximum), and manner of packing the trap.

GC/ITMS Analysis

Chromatographic separation of the 46 pesticides by GC/ITMS and analysis of SFE results in the presence of matrix interferences required careful attention to many details.

(**a**) *Injector.*—Initial experiments performed with a splitless injector gave good results for many stable pesticides, but many organophosphates, as well as captan, carbaryl, iprodione, and chlorothalonil, gave poor peak shapes and/or reduced responses. Injector temperature was varied from 100° to 250°C, but no significant difference in results was observed. For this reason, on-column injection with an SPI was investigated. The initial injector temperature was kept at 55°C (below the boiling point of the final extract solvent), and after 30 s, the temperature was ramped rapidly to 250°C. In this manner, the SPI rapidly transported the pesticides into the column at mild conditions, thereby minimizing losses to the walls of the glass injector liner. The peak shapes of many pesticides improved with the SPI, and losses of captan, chlorothalonil, iprodione, and carbaryl were reduced significantly.

(b) *Quantitation.*—In a complex sample matrix, GC/ITMS quantitation overestimated the concentrations of several pesticides when calibration curves were based on standards in pure solvent. Possible reasons for this systematic error in quantitation are: differences in injection conditions for samples containing matrix components versus calibration standards without matrix components and mass spectral overlaps due to coeluting matrix components. Mass spectral overlaps were noticeable in chromatograms. If overlap occurred, different quantitation masses were chosen to eliminate or reduce this source of error. However, in many instances, no spectral interferences were present but calculated concentrations of pesticides were still higher than the true concentrations. In those cases, it was believed that matrix components in the extracts filled active sites on the glass injection liner and analyte losses were reduced (28). But with standards, no matrix components were present and the incidence of analyte loss was higher. Use of the SPI reduced this error, but overestimation still occurred for several pesticides.

This source of quantitation error was virtually eliminated by preparing the calibration standards in blank sample extracts rather than in pure solvent. Table 3 compares the differences in quantitation of several pesticides with calibration standards in pure solvent or in blank potato SFE extracts. Most organochlorine pesticides, such as hexachlorobenzene (HCB), pentachlorobenzene (PCB), DDT, and DDE, were not affected by matrix components, but matrix effects were considerable for several other pesticides such as iprodione, captan, and carbaryl, and organophosphates such as dimethoate and mevinphos.

Table 3. Quantitation of pesticides fortified in potato (3 replicates), extracted by SFE, and analyzed by GC/ITMS versus calibration standards prepared in pure solvent or in blank potato extracts

Pesticide	Recovery, %	
	Calibration in pure solvent	Calibration in blank sample extracts
Mevinphos	161	88 ± 2
HCB	83	86 ± 7
Chlorpropham	145	97 ± 2
PCNB	90	101 ± 6
Diazinon	90	91 ± 2
Disulfoton	81	85 ± 6
Dicloran	98	90 ± 5
Atrazine	98	96 ± 3
Dimethoate	239	94 ± 6
Vinclozolin	110	86 ± 6
Chlorpyrifos	145	90 ± 3
Carbaryl	277	81 ± 2
Malathion	169	85 ± 2
Endosulfan I	87	88 ± 2
DDE	90	90 ± 2
Captan	207	38 ± 10
DDT	102	90 ± 4
Ethion	144	98 ± 4
Iprodione	230	94 ± 5
cis-Permethrin	93	93 ± 5

(c) *Method of standard addition.*—In SFE recovery studies, the same batch of fruit or vegetable served as the blank and fortified sample, and by controlling matrix effects, results were precise and accurate. However, in analyses of samples originating from a different source than the source of the control samples, results were satisfactory, but small differences in matrix effects were thought to affect results to a small extent. In general, the best way to control matrix effects is by the method of standard addition. In the analysis of a potato check sample, the check sample (3 g subsamples) was extracted by SFE 4 times. Then internal standard was added to each extract, followed by addition of 0.1, 0.2, or 0.5 µg/g of the 46 pesticides into 3 of the extracts. Figure 3 presents calibration curves obtained by the method of standard addition for iprodione and ethion (all other calibration curves passed through the zero point within the error in slope and y intercept). Three other subsamples of the potato were spiked with pesticides at 0.5 µg/g and analyzed. Figure 3 also includes analytical results for iprodione (recovery, 102 ± 28%) and ethion (recovery, 97 ± 16%) in the fortified samples.

(d) *Quadratic calibration curves.*—For analyses using standard addition, calibration curves for GC/ITMS were linear, but in other cases, calibration curves were quadratic. In such cases, the quadratic relation was used for quantitation. Figure 4 shows an example of how a quadratic calibration curve more closely fits the calibration of ethion (Figure 3 illustrates when a calibration curve was linear). Organochlorine pesticides nearly always followed linear slopes, and organophosphate pesticides sometimes presented quadratic relationships. The cause of this effect is unknown, but it is possibly due to partial losses of trace amounts of pesticide at particular GC conditions that become more significant at the picogram injection level.

(e) *Limits of detection.*—Table 2 lists the retention times (t_r), quantitation masses, and limits of detection (LODs) for the 46 pesticides analyzed by GC/ITMS. LODs are reported in ng/g for potato analyzed by the 7680T method (final volume, 1.5 mL). For the Prepmaster, the final volume was 6 mL, and LODs were 4 times higher. The reported values were typical of the GC/ITMS method, but S/N ratios fluctuated approximately 15%, depending on instrumental performance and matrix effects. Despite the 17-fold smaller sample size used in SFE, the LODs for the GC/ITMS method generally matched the method detection limits reported by regulatory laboratories using selective detection, such as electron capture, electrolytic conductivity (Hall), flame photometric and nitrogen–phosphorus detectors (27).

Sample Results

Table 4 presents recoveries of 46 pesticides fortified in potatoes at 0.5 µg/g. Recoveries were >80% for 39 pesticides and >55% for 44 pesticides; only omethoate and methamidophos gave recoveries of <50%. These results were typical of pesticide recoveries with either SFE instrument. The recovery of chlorpyrifos was probably higher than presented because, as shown in Figure 2, a potato matrix component chroma-

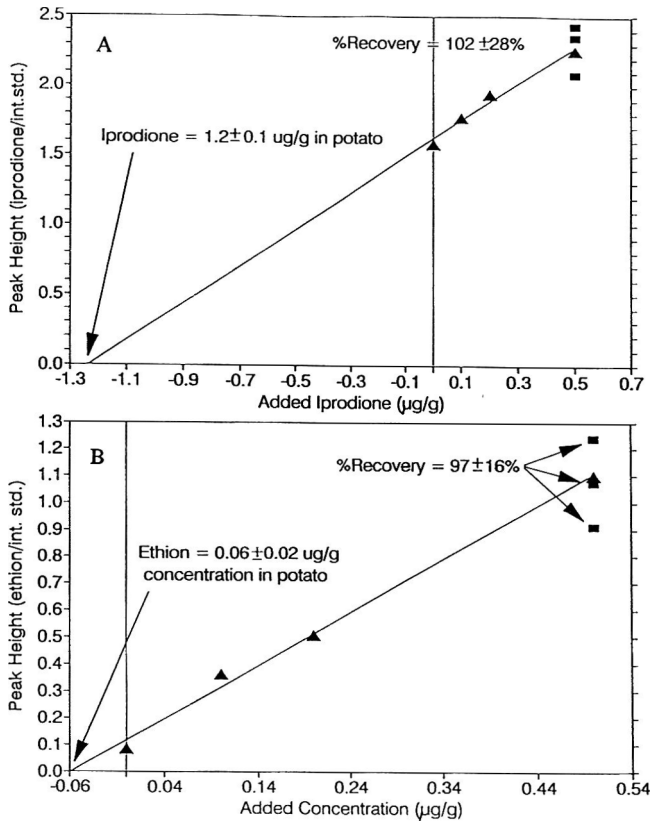

Figure 3. Results of method of standard addition for (A) iprodione and (B) ethion in potato check sample. Actual iprodione and ethion concentrations were 1.6 and 0.12 µg/g, respectively. Legend: (■), check samples fortified with pesticides at 0.5 µg/g before SFE, and (▲), fortified extracts used for calibration. Recoveries were 100% for the pesticides. The reason for the differences in experimental and expected ethion concentrations is unknown.

tographically overlapped with chlorpyrifos and affected quantitation (the background peak also coeluted with parathion-methyl, but less overlap of the chosen quantitation masses occurred). The lower recovery of dichlorvos was likely due to its high volatility. Decreasing trap temperature and/or CO_2 flow rate during SFE probably would have increased its recovery.

(a) *Troublesome pesticides.*—Of the 46 pesticides, disulfoton, captan, propargite, and especially omethoate and methamidophos, consistently gave lower recoveries compared with the others. These pesticides have unique traits. For example, an existing analytical method converts disulfoton to the more stable sulfone and sulfoxide forms before analysis (29). Captan, *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide, contains reactive structural moieties distinct from those in other pesticides. Propargite was the only pesticide analyzed containing an alkyne group. Omethoate and methamidophos, metabolic products of dimethoate and acephate, respectively, gave severely tailing peak shapes that made peak integration and quantitation difficult. Methamidophos was the only compound tested containing an unprotected phosphoramide, which also likely made SFE and elution from the ODS trap more difficult.

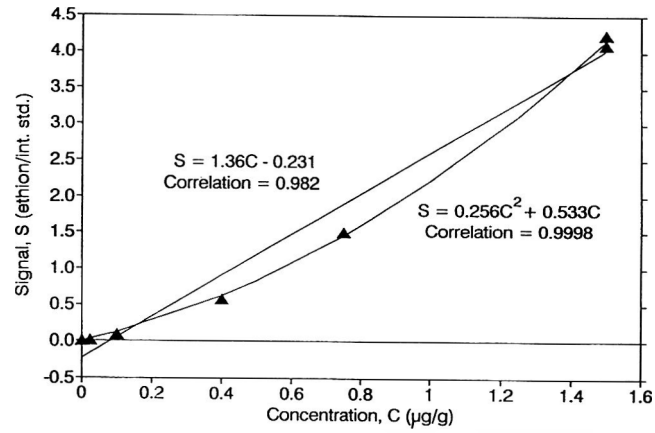

Figure 4. Calibration curve for ethion when quadratic relation was used for quantitation. Equations are the linear and quadratic best-fit functions for the data (and correlation coefficients), where S is peak area of the analyte divided by peak area of the chrysene-d_{12} internal standard, and C is analyte concentration.

(b) *Analyses of produce with incurred pesticides.*—An incurred carrot sample, previously analyzed by an SFE method developed for organochlorine pesticides (23), was extracted and reanalyzed by the multiresidue SFE and GC/ITMS procedure. HCB again was confirmed to be present at 8 ± 4 ng/g; additionally, DDE and iprodione were identified in the sample. Concentrations were calculated as 0.18 ± 0.01 µg/g for DDE and 0.19 ± 0.02 µg/g for iprodione, but comparison of results with those of a traditional approach was not possible because concentrations were below the LODs of a method performed by a regulatory laboratory.

(c) *Check sample results.*—A more detailed study to compare the results of the SFE and GC/ITMS method and traditional approaches was performed. Check samples of potato and broccoli, containing incurred and fortified pesticides at concentrations unknown to the analysts, were sent to 7 regulatory laboratories for analysis by validated traditional approaches (1, 2). Table 5 presents the results of interlaboratory and method comparisons for the potato check sample. The potato was analyzed in triplicate, 6 times on different days, with the 7680T and Prepmaster instruments. SFE and GC/ITMS results for iprodione compare favorably with results from the 7 laboratories. Average iprodione concentrations were 1.21 µg/g (RSD, 19%) by the SFE method and 1.27 µg/g (RSD, 20%) by the regulatory laboratories; the fortification level was 1.6 µg/g.

For ethion, the fortification level was 0.12 µg/g. The regulatory laboratories found 0.117 µg/g (RSD, 11%), whereas the SFE and GC/ITMS method determined only 0.057 µg/g (RSD, 22%). In all our previous SFE studies involving ethion, recoveries from fortified samples were consistently about 100%, and GC/ITMS results were accurate when appropriate calibration methods were used (as discussed previously). As Figures 3 and 4 show, extraction and quantitation results for ethion presented no indications of large error. Accuracy of the standard solutions was confirmed by comparison with a duplicate ethion standard.

Table 4. Recoveries of pesticides spiked at 0.5 μg/g in potatoes, by SFE and GC/ITMS method[a]

No.	Pesticide	Recovery, %	No.	Pesticide	Recovery, %
1	Dichlorvos	72 ± 9	24	Phosphamidon	91 ± 4
2	Methamidophos	0 ± 0	25	Dacthal	85 ± 5
3	PCB	91 ± 6	26	Carbaryl	91 ± 1
4	Mevinphos	92 ± 2	27	Malathion	87 ± 4
5	HCB	93 ± 2	28	Parathion	91 ± 3
6	Ethoprop	84 ± 3	29	Endosulfan I	93 ± 2
7	Diphenylamine	87 ± 2	30	DDE	91 ± 1
8	Phorate	82 ± 4	31	Captan	66 ± 4
9	Chlorpropham	91 ± 2	32	Methidathion	90 ± 4
10	PCNB	90 ± 4	33	DDT	93 ± 2
11	Omethoate	5 ± 8	34	Fenamiphos	83 ± 2
12	Terbufos	83 ± 5	35	Endosulfan II	114 ± 9
13	Diazinon	86 ± 5	36	Ethion	97 ± 16
14	Lindane	89 ± 2	37	Myclobutanil	83 ± 10
15	Disulfoton	78 ± 5	38	Propargite	57 ± 22
16	Dicloran	91 ± 4	39	Methoxychlor	90 ± 1
17	Carbofuran	90 ± 2	40	Iprodione	102 ± 28
18	Atrazine	92 ± 2	41	Phosmet	88 ± 4
19	Dimethoate	83 ± 8	42	Phosalone	86 ± 5
20	Chorothalonil	93 ± 2	43	Azinphos-methyl	94 ± 6
21	Vinclozolin	91 ± 2	44	*cis*-Permethrin	93 ± 3
22	Parathion-methyl	85 ± 6	45	Fenvalerate	93 ± 2
23	Chlorpyrifos	72 ± 5	46	Esfenvalerate	88 ± 2

[a] Data are means ± standard deviations of 3 replicate extractions.

The lower concentration of ethion obtained by the SFE and GC/ITMS method was probably due to degradation of ethion in the check sample. In Table 5, the SFE results are presented in the order that the experiments were performed. Twelve days elapsed from the first experiment (ethion at 0.077 μg/g), when the sample was first thawed and mixed in its entirety with Hydromatrix, to the 6th and final set of extractions (ethion at 0.04 μg/g). Table 5 shows a trend of lower result for each subsequent extraction and analysis (experiments 2 and 3 were performed on the same day, as were experiments 4 and 5). Another evidence to support sample degradation was that the sample was analyzed in this laboratory more than a month after analysis in the regulatory laboratories. The sample was stored at −40°C before shipment to this laboratory, where the sample was stored at −20°C before and after experiments. As the results indicate, the most degradation (from <0.12 to 0.077 μg/g) occurred when the sample was brought initially to room temperature and mixed thoroughly with Hydromatrix. In 12 days at −20°C, ethion concentration declined from 0.077 to 0.04 μg/g.

Table 6 presents results for the broccoli check sample. The fortification levels were 0.14 μg/g for dimethoate and 0.47 μg/g for propargite. The sample was analyzed only once (triplicate subsamples) with the 7680T. The SFE and GC/ITMS method found 0.102 ± 0 005 μg/g for dimethoate and 0.28 ± 0.01 μg/g for propargite. In previous studies, SFE recoveries (as shown in Table 4) were 83% for dimethoate and 57% for propargite. When the broccoli check sample results were corrected for the known recovery factors, the calculated concentrations (0.12 μg/g for dimethoate and 0.50 μg/g for propargite) closely agreed with the actual concentrations. The regulatory laboratories obtained results of 0.13 μg/g (RSD, 18%) for dimethoate and 0.50 μg/g (RSD, 29%) for propargite. None of the laboratories detected dacthal incurred in the sample, which was confirmed to be present and quantitated at 0.0011 μg/g by the SFE and GC/ITMS method.

(**d**) *SFE instruments.*—Similar recoveries and concentrations for incurred and fortified pesticides were obtained with both the 7680T and Prepmaster SFE instruments (as presented in Table 5). The SFE instruments were used interchangeably; the only difference was the 6 mL final extract volume for the Prepmaster versus the 1.5 mL volume for the 7680T. This difference was most likely due to the dissimilar ODS sorbents used in the traps and the manner in which they were packed. Research comparing SFE results obtained with different commercial SFE instruments (10) and a product review of different instruments (30) have been published.

Conclusions

This work's goal was to develop a method for multiresidue analysis of pesticides in fruits and vegetables by SFE and GC/ITMS. The method gave recoveries >80% for most pesticides in produce; methamidophos was the only pesticide of the 46 tested that was not recovered at all. Although the SFE and GC/ITMS method requires more study before implementation in regulatory laboratories, the results compared satisfactorily

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Table 5. Results of interlaboratory comparison of analysis of potato check sample

Lab. No.	Iprodione, µg/g		Ethion, µg/g	
	Solvent[a]	SFE[b]	Solvent[a]	SFE[b]
1	1.1	1.3 ± 0.1[c]	0.13	0.077 ± 0.006[c]
2	0.87	1.2 ± 0.1[c,d]	0.11	0.06 ± 0.02[c,d]
3	1.4	1.6 ± 0.2[e]	0.11	0.06 ± 0.02[e]
4	1.4	0.98 ± 0.04[c]	0.11	0.057 ± 0.002[c]
5	1.4	1.16 ± 0.05[e]	0.099	0.049 ± 0.004[e]
6	1.6	1.0 ± 0.1[c]	0.13	0.040 ± 0.008[c]
7	1.1	—	0.13	—
Average	1.27	1.21	0.117	0.057
Standard deviation	0.25	0.23	0.013	0.012
RSD,[f] %	20	19	11	22
Actual concentration	1.6 µg/g		0.12 µg/g	

[a] Regulatory solvent-based extraction method (1, 2).
[b] SFE using 2 different instruments; analyses were performed in triplicate on different days, and results are means ± standard deviations.
[c] SFE with 7680T.
[d] Result of method of standard addition.
[e] SFE with Prepmaster.
[f] Relative standard deviation.

with results of obtained using traditional approaches. LODs for the method were 20 ng/g or lower for 40 of the 46 pesticides, and in several instances, the SFE and GC/ITMS procedure confirmed the presence of pesticides not detected in samples analyzed by traditional approaches. The number of pesticides studied was limited to 46, but because several different classes of pesticides were represented, many other pesticides in the same classes could likely by simultaneously analyzed with only minor modifications.

The SFE and GC/ITMS approach has many advantages over the solvent-based extraction and GC/selective-detector methods currently used by regulatory laboratories. Speed of analysis is greatly increased. In approximately 2 h, a produce sample can be extracted by SFE and analyzed by GC/ITMS to simultaneously confirm the presence of and quantitate multiple pesticide residues at ultratrace levels. Using the automated instrumental techniques also reduces the amount of manual labor and laboratory space needed. SFE requires only small amounts of solvent and glassware, thereby reducing hazards to workers and amount of waste generated. SFE allows for a higher degree of selectivity in extraction compared with solvent-based methods, and use of solid-sorbent traps for SFE collection affords a rapid, single-step extraction and cleanup. Finally, the GC/ITMS method detects analytes in SFE extracts at ultratrace levels with a high degree of selectivity even in the presence of matrix components. With further research, the combination of SFE and GC/ITMS technologies may be able to supplant current inefficient approaches to multiresidue analysis of pesticides in food.

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Table 6. Results of interlaboratory comparison of analysis of broccoli check sample

Pesticide	Actual conc	Concentration, µg/g					
		Regulatory lab. results[a]			SFE and GC/ITMS result[b]		
		Av. ± SD	Low	High	Av. ± SD	Recovery, %	Corrected result
Dacthal	?[c]	ND[d]	—	—	0.0011 ± 0.0001	85	0.0013
Dimethoate	0.14	0.130 ± 0.024[e]	0.10	0.16	0.102 ± 0.005	83	0.12
Propargite	0.47	0.50 ± 0.14[f]	0.29	0.67	0.284 ± 0.012	57	0.50

[a] Regulatory solvent-based extraction method (1, 2).
[b] The 7680T was used; analyses were performed in triplicate on the same day.
[c] Unknown.
[d] Not detected.
[e] Results from 6 regulatory laboratories, one laboratory obtained 0.29 µg/g for dimethoate, which was not included.
[f] Results from 5 regulatory laboratories, 2 of which did not detect propargite.

ington for analyzing the samples; the State of Michigan Department of Agriculture for providing the incurred carrot and grape samples; and Hewlett-Packard for use of the 7680T SPE.

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RESIDUES AND TRACE ELEMENTS

Development of a Sample Preparation Technique for Supercritical Fluid Extraction for Multiresidue Analysis of Pesticides in Produce

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Supercritical fluid extraction (SFE) of fruits and vegetables poses unique sample preparation considerations because the sample size is small (1–3 g) and the analyte is distributed in a moist solid matrix. The goal of this research was to develop practical sample preparation procedures for SFE of pesticide residues in produce so that acceptable accuracy and precision are maintained. In this study, 130 extractions of potato, fortified with up to 40 pesticides, were performed with 2 commercial SFE instruments. Extracts were analyzed by gas chromatography with ion trap mass spectrometry or electron capture detection. Four sample preparation procedures were tested and Hydromatrix was used to control the amount of water in the sample. The highest recoveries and lowest standard deviations were obtained when 20–50 g samples were blended with an equal amount of Hydromatrix and dry ice was added to keep the samples frozen. The dry ice helped produce a homogeneous flowable powder and greatly reduced the degradation or vaporization of several pesticides. Recoveries of most pesticides from subsamples of <4 g with this procedure were 90–105%, with relative standard deviations of 1–6%. Only diphenylamine and disulfoton gave reduced recoveries with this procedure. When samples were extracted sequentially with an autosampler, certain pesticides were degraded in the extraction vessels over a period of several hours. To avoid losses of these pesticides, the sample in the extraction vessel was either purged with CO_2 to remove oxygen or kept frozen until extracted. Peach and orange check samples were analyzed with the method, and results were comparable with those from traditional analyses.

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Supercritical fluid extraction (SFE) is a new technology for extraction of a wide range of chemicals from many sample matrixes (1–4). An increasing number of publications on applications of SFE to analysis of pesticides in foods indicates the strong interest in and potential of this new technique (5–15). SFE is gaining acceptance as an alternative to solvent-based extraction methods but rarely has been applied for routine analysis. SFE offers an environmentally safer extraction; essentially obviates use of organic solvents; generates very little waste; reduces time, space, and glassware required for extraction; and enables automation.

Sampling and sample preparation for residue analysis—so that results are accurate, reproducible, and representative—have been evaluated thoroughly when current methods were being developed (16–18). Conventional sample preparation for multiresidue pesticide analysis involves chopping a frozen sample with a Hobart cutter and extracting subsamples of 50–100 g with organic solvents (19–21). With SFE, it is not practical to use a liquid solvent to disperse analytes in a homogenous solution before extraction, and a different sample homogenization approach must be developed. Water in the sample must be controlled so that extraction efficiency is not affected. Also, sample size for SFE is usually small (1–3 g plant material); therefore, to obtain a representative subsample of that size, homogenization of the larger sample is required. Moreover, without solvents, the analyte in the solid sample becomes more vulnerable to evaporation and degradation.

The objective of this study was to develop a novel sample preparation approach for SFE of pesticide residues that addresses the unique characteristics of SFE and still maintains the accuracy and precision of the current solvent-based extraction methods.

Experimental

Apparatus

(a) *Supercritical fluid extractors.*—A Model 7680T (Hewlett-Packard, Little Falls, DE) and a Prepmaster (Suprex, Pittsburgh, PA), both equipped with automated variable restrictors and solid sorbent collection systems, were used. The 7680T was automated so that 8 vessels could be loaded into a carousel and extracted in sequence. The Prepmaster was oper-

ated manually. For the 7680T, extraction parameters were as follows: extraction pressure, 320 atm; temperature, 60°C (CO_2 density, 0.85 g/mL); 7 mL extraction vessel, 2 min static extraction followed by 42 mL CO_2 at a flow rate of 1.6 mL/min; 50°C restrictor temperature; collection on (octadecylsilica) ODS sorbent trap (1 mL) at 9°C; and elution with 1.5 mL acetonitrile at 0.4 mL/min and 50°C. The trap was rinsed to waste with 2 mL ethyl acetate followed by 2 mL acetonitrile at 2 mL/min to clean and regenerate the ODS between extractions. For the Prepmaster, instrument settings were the same except for the following: vessel size, 5 or 10 mL (30 or 60 mL CO_2 extraction volume, respectively); C_{18} trap material mixed with Unibeads; trap elution at 40°C with 1.6 mL acetonitrile; N_2 gas at 50 psi to blow the trap dry; and 5 mL acetonitrile at 2 mL/min to flush the trap between extractions.

(**b**) *Gas chromatographs.*—A Model ITS40 gas chromatograph/ion trap mass spectrometer (GC/ITMS; Finnigan MAT, San Jose, CA), consisting of a Varian 3300/3400 gas chromatograph and a CTC A200S autosampler, and a Model 5890 gas chromatograph (Hewlett-Packard) equipped with a Model 7673 Hewlett-Packard autosampler, electron capture detection (ECD), and nitrogen-phosphorus detection (NPD) were used. To analyze a 40-pesticide mixture, the following operating conditions were used: 1 µL injection volume into a Model 1093 (Varian, Walnut Creek, CA) septum-programmable injector; 55°C injection port for 30 s followed by ramping to 250°C at 250°C/min; 6 psig He column head pressure; 55°C initial oven temperature for 30 s, ramped to 130°C at 50°C/min, then to 165°C at 1.5°C/min and to 250°C at 4°C/min, and held at 250°C until a total time of 60 min had elapsed; 240°C transfer line temperature; and 215°C detector manifold temperature. Conditions were the same for GC/ITMS analysis of chlorinated pesticides, except that the oven temperature program was 60°C to 130°C at 50°C/min and then to 250°C at 7.5°C/min and hold for 20 min. Typical ITMS operating conditions were as follows: electron impact mode; 10 µA filament current; 1500 V electron multiplier tube; 1 ms ion time; and automatic gain control at 20 000. The GC–ECD and GC–NPD conditions were: 1 µL splitless injection volume; 250°C injection port; 0.5 min purge delay; 21 psig He column head pressure (2.6 mL/min); 100°C initial oven temperature to 220°C at 3°C/min ramp rate; 300°C ECD temperature; 43 mL/min ECD makeup gas flow rate of 5% CH_4 in Ar; 260°C NPD temperature; 3.5 mL/min H_2, 100 mL/min air, and 30 mL/min He NPD gas flow rates.

(**c**) *Chromatographic columns.*—A DB-1701 or a DB-5ms (J&W Scientific, Folsom, CA), 30 m, 0.32 mm id, 0.25 µm film thickness capillary column and a 5 m phenylmethyl deactivated (Restek Corp., Bellefonte, PA) guard column (0.32 mm id) were used for GC/ITMS. For GC–ECD, a 100% dimethylpolysiloxane SPB-1 (Supelco, Bellefonte, PA) 30 m, 0.25 mm id, 0.25 µm film thickness capillary column was used, and for GC–NPD, a DB-17 (J&W Scientific) 30 m, 0.32 mm id, 0.25 µm film thickness capillary column was used.

(**d**) *Data collection.*—For GC/ITMS, a Magnum version 2.4 software package (provided with the instrument) loaded into a Gateway 2000 computer was used. For GC–ECD and GC–NPD, a Pascal version Chemstation software package loaded into a Hewlett-Packard 300 series computer was used for data collection and analysis and instrument control. For the ion trap, the data collection range was 65–425 *m/z* from 5 to 60 min for analysis of 40 pesticides and from 6 to 20 min for analysis of chlorinated pesticides only.

Reagents

(**a**) *Gases.*—SFC/SFE grade CO_2 (Air Products, Allentown, PA), with a He headspace of 1800 psi (for Prepmaster) or without He headspace (for 7680T), was used. Bone-dry grade CO_2 (for both instruments) and N_2 (for Prepmaster) were required for cryogenic cooling and drying of the trap, respectively. The septum-programmable injector on the GC/ITMS also used CO_2 with dip-tube for cooling.

(**b**) *Solvents.*—Acetonitrile and ethyl acetate, pesticide grade (Fisher, Fair Lawn, NJ).

(**c**) *Solids.*—Hydromatrix (HMX, Varian, Harbor City, CA), a pelletized diatomaceous earth, was washed with acetone before use to remove contaminants. Use of HMX in SFE has been described previously (8). The prepacked 30 µm Hypersil ODS (Hewlett-Packard) traps were provided with the 7680T; for the Prepmaster, 35 µm C_{18} mixed with Unibeads (Suprex) were contained in the trap.

(**d**) *Pesticide standards.*—Pesticides were obtained from the U.S. Environmental Protection Agency (Research Park, NC, or Beltsville, MD). Chrysene-d_{12} (Cambridge Isotope Laboratories, Woburn, MA) was used as internal standard for the 40 pesticides, and pentachloroanisole (PCAS) or aldrin was used as internal standard for the chlorinated pesticides. Individual stock solutions were prepared by weighing 10–12 mg amounts of standards, dissolving the pesticide in acetone and/or isooctane, and making up to 100 mL in volumetric flasks. Concentrations were corrected for the stated purities (typically >98%) of the standards. Working standard mixtures in acetone, containing 20 µg/mL for each pesticide, were used for spiking samples and preparing calibration standards.

Sample Preparation

Comercially purchased potatoes served as blank or fortified samples. An outline of experiments is given in Table 1. These experiments evolved from the need to produce a homogeneous, representative sample without use of liquids. The initial attempt (Experiment 1) was simply to prepare a homogeneous sample by using a Hobart cutter. The second experiment improved homogeneity by mixing a representative portion of the sample with HMX in a blender. In Experiment 3, the sample preparation procedure included 40 pesticides. In the last experiment, the procedure was refined by using dry ice during blending to improve mixing and reduce losses of certain pesticides.

Experiment 1

In this experiment, 19 potatoes (2.3 kg) were cut into quarters, and 19 pieces (25%) were randomly separated and spiked with hexachlorobenzene (HCB) at 0.41 µg/g and lindane at 0.43 µg/g (50 µL of 1000 µg/mL HCB and 184 µL of 286 µg/mL lindane on each piece). The spiking solvent was allowed to evaporate for 1 h, and then all 76 pieces were com-

Table 1. Procedures for sample preparation used in different experiments

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Initial sample size	2.3 kg	2.3 kg	50 g	50 g
Spiking level(s)	0.413 μg/g, HCB; 0.434 μg/g, lindane	0.413 μg/g, HCB; 0.434 μg/g, lindane	0.4 μg/g, 37 pesticides	0.2 or 0.4 μg/g, 40 pesticides
Sample processing	Chopped, frozen (100 g subsample); rechopped frozen (20 × 50 g subsamples)	50 g subsample from Experiment 1 blended with 100 g HMX	Blended with 100 g HMX	Blended with 50 g HMX + dry ice
Packing of extraction vessel (vessel volume)				
7680T	2.1 g potato + 1.4 g HMX (7 mL)	1.3 g potato + 2.7 g HMX + 1 mL H_2O (7 mL)	1.3 g potato + 2.7 g HMX + 1 mL H_2O (7 mL)	2 g potato + 2 g HMX (7 mL)
Prepmaster	3 g potato + 2 g HMX (10 mL)		1 g potato + 2 g HMX + 1 mL H_2O (5 mL)	1.5 g potato + 1.5 g HMX (5 mL)
No. of extractions				
7680T	48	9	14	15
Prepmaster	32		6	6
Method of analysis	GC/ITMS, GC–ECD	GC/ITMS, GC–ECD	GC/ITMS	GC/ITMS

bined and shredded with a Handi-Shortcut II food processor (Black & Decker, Shelton, CT). The shredded potatoes were stored overnight at –20°C. On the next day, 100 g sample was separated, and while still frozen, the remaining potato sample was chopped further with a Model 84142 cutter (Hobart, Troy, OH). The potato sample was divided into twenty 50 g portions and stored at –20°C until extraction. For SFE with the 7680T, 8 subsamples of 2.1 g each from the 100 g portion of potato and 4 subsamples (2.1 g each) from 10 of the 50 g portions (48 extractions total) were extracted in the course of 5 days (1.4 g HMX was mixed with each frozen 2.1 g subsample in a beaker with a glass rod before packing into a 7 mL extraction thimble). For SFE with the Prepmaster, three 3 g subsamples from 9 of the 50 g portions were extracted over the course of 3 days (2 g HMX was added to each sample before packing into a 10 mL extraction vessel). In all cases, 15 μL of 20 μg/mL pentachlorobenzene (PCB) was added to the sample in the vessel as an SFE matrix spike before extraction. An internal standard, aldrin or PCAS, was added to extracts at 0.5 μg/mL before analysis by GC/ITMS and GC–ECD.

Experiment 2

A 50 g portion of frozen potato from Experiment 1 was mixed with 100 g HMX in a blender (Waring, New York, NY). The sample became a flowable powder, and because the moisture content of the powder was lower than what had been used in previous studies (5–8), 1 mL water was added to the sample in the vessel before extraction. For SFE with the 7680T, eight 4 g subsamples (1.33 g potato and 2.67 g HMX) were extracted on the same day. As the control spike, 50 μL of 20 μg/mL PCB was added to the sample in the vessel before extraction. Aldrin was the internal standard for GC/ITMS and GC–ECD.

Experiment 3

Fresh potato (50 g) was sliced into 12 pieces with a knife, and 4 pieces were each spiked with 250 μL of a mixture of 40 pesticides in acetone containing 20 μg of each pesticide per mL (0.4 μg/g fortification level for the 50 g sample). The solvent was allowed to evaporate at room temperature for 1 h. The 12 pieces were blended with 80 g HMX for 5 min into a flowable powder, as in Experiment 2. For SFE with the 7680T, fourteen 4 g subsamples were extracted sequentially over the course of 2 days, and for the Prepmaster, six 3 g subsamples (in 5 mL vessels) were extracted on the same day. A 20 g potato sample blended with 40 g HMX served as a blank and control spiking matrix. Five 4 g subsamples were spiked in the extraction thimbles with 35 μL of the working standard mixture of 40 pesticides, and 4 other subsamples were extracted as blanks. The blank extracts were combined and used to prepare calibration standards for GC/ITMS.

Experiments 4A and 4B

Potato (50 g) was fortified with 20 pesticides at 0.2 μg/g each and the 20 other pesticides at 0.4 μg/g each. The fortified sample was placed in a precooled blender jar with dry ice and 50 g HMX. The sample was blended for 5 min and kept cold by addition of dry ice. In Experiment 4A, a portion of the sample was placed in the freezer and later loaded into 5 vessels and extracted individually; the sample was kept frozen until extracted by SFE. In Experiment 4B, 7 subsamples (4 g each) from the second portion were loaded into extraction vessels and

kept in the 7680T carousel at room temperature until analyzed in sequence. The first 5 samples were analyzed 1 h apart, and the 6th and 7th samples were analyzed 19 and 20 h after the first sample. The 6th thimble was kept in supercritical CO_2 from the 6th to the 19th hour. Experiment 4A was carried out also on the Prepmaster; six 3 g samples (1.5 g potato) in 5 mL thimbles were extracted in this manner.

Check Samples

Peach and orange check samples were provided by the California Department of Food and Agriculture (CDFA) as part of a quality assurance protocol for laboratories participating in the Pesticide Data Program (22). Samples consisted of unknown incurred and/or fortified pesticides at unknown concentrations. The pesticides were not evenly distributed when fortified into the sample by the CDFA. Frozen sample (100 g) was mixed with 100 g HMX and a small amount of dry ice in a blender, as in Experiment 4. Because of the larger sample size, about 25 g sample and HMX were blended at a time before mixing in another portion. In each case, 6 extraction vessels were loaded with 4 g subsamples (2 g sample) of the cold homogenate, and 15 µL of 25 µg/mL aldrin was added to each vessel. Two of the vessels were fortified with 50 µL of the 20 µg/mL working standard mixtures (equivalent to 0.5 µg/g for 40 pesticides in the sample). The capped vessels were purged with CO_2 gas before being loaded in the 7680T carousel. After extraction, 15 µL of 40 µg/mL chrysene-d_{12} was added to each 1.5 mL extract.

The method of standard additions was used for quantitation of check samples. Three of the 4 extracts were combined, and four 1 mL aliquots were added to autosampler vials with a syringe (the excess served as a second control extract). Microsyringes were used to add 2.5, 7.5, 22.5, and 62.5 µL of the 20 µg/mL pesticide standard mixture to give 0.05, 0.15, 0.45, and 1.35 µg/mL added standards which corresponded to 0.038, 0.112, 0.338, and 1.01 µg/g in the 2 g samples. For peaches, the controls and the 0.05 and 0.15 µg/mL added standards were analyzed twice; for all other cases, controls and standards were analyzed once.

Analysis

(a) *Calibration.*—For GC/ITMS calibration, the spiking solutions were diluted to make the calibration standards. For best quantitation, the calibration standards were prepared in SFE extracts from sample blanks of the same matrix. Calibration standards were 0.1, 0.2, 0.5, and 1.0 µg/mL for analysis of the 40 pesticides in potato. For GC/ITMS analysis of chlorpropham incurred in potato, 5.0, 2.5, 1.0, and 0.5 µg/mL calibration standards were prepared.

(b) *Data analysis.*—Integrated peak area data of selected masses versus the chrysene-d_{12} internal standard were used for GC/ITMS quantitation. Table 2 lists retention times (t_r) and masses chosen for quantitation of each pesticide (m/z = 240 amu for chrysene-d_{12}). Quantitation of GC–ECD data was by integrated peak area of selected masses versus the aldrin or PCAS internal standard. Calculations were done with a spreadsheet program or the instrument's software program. For fur-

Table 2. Retention times (t_r) of pesticides with the DB-5ms column and quantitation masses for GC/ITMS analysis

No.	Pesticide	t_r, min	Masses[a], *m/z*	No.	Pesticide	t_r, min	Masses[a], *m/z*
1	Dichlorvos	4.5	109* + 127 + 185	21	Vinclozolin	25.5	198 + 212* + 285
2	α-Mevinphos	7.4	127* + 164 + 192	22	Carbaryl	26.0	115 + 116 + 144*
3	PCB[b]	9.5	248 + 250* + 252	23	Malathion	29.4	125 + 127 + 173*
4	Diphenylamine	13.1	167 + 168 + 169*	24	Chlorpyrifos	29.4	197 + 199 + 314
5	Ethoprop	13.6	97 + 158* + 243	25	Dacthal	29.5	299 + 301* + 303
6	Chlorpropham	14.6	127* + 171 + 213	26	Parathion	30.0	97 + 109* + 291
7	HCB[c]	15.8	282 + 284* + 286	27	Methidathion	33.7	85 + 93 + 145
8	Phorate	15.9	75* + 121 + 260	28	Endosulfan I	34.1	195 + 241* + 339
9	Dicloran	17.0	124* + 176 + 206	29	DDE	36.0	246 + 316* + 318
10	Dimethoate	17.3	87* + 93 + 125	30	DDT	38.5	165 + 235* + 237
11	PCNB[d]	18.1	295 + 297 + 299	31	Ethion	38.6	97 + 153 + 231*
12	Carbofuran	18.2	149 + 164*	32	Propargite	41.6	135* + 173 + 350
13	Lindane	18.6	181* + 183 + 219	33	Phosmet	42.8	160*
14	Atrazine	18.7	200* + 215 + 216	34	Iprodione	42.9	314* + 316
15	Terbufos	19.7	231*	35	Methoxychlor	43.5	227*
16	Chlorothalonil	20.1	264 + 266* + 268	36	Phosalone	44.7	182* + 184 + 367
17	Diazinon	21.0	137 + 179* + 304	37	Azinphos-methyl	44.8	132* + 160
18	Disulfoton	21.3	88* + 89 + 97	38	*cis*-Permethrin	48.2	127 + 163 + 183*
19	Phosphamidon	24.6	72 + 127* + 264	39	Fenvalerate	55.4	125 + 225* + 419
20	Parathion-methyl	25.4	109 + 125 + 263*	40	Esfenvalerate	56.6	125 + 225* + 419

[a] *, base peak.
[b] Pentachlorobenzene.
[c] Hexachlorobenzene.
[d] Pentachloronitrobenzene.

ther comparison of results, a method of standard additions for GC/ITMS also was used to determine average pesticide concentrations in extracts from 8 potato subsamples.

(c) *Confirmation of pesticides.*—With GC/ITMS, the following criteria had to be met to confirm a pesticide in the sample: t_r difference of less than 10 s, signal-to-noise ratio greater than 3, and mass spectrum match greater than 90% versus the spectrum library for the pesticide (generated from pesticide standards). Only results for confirmed pesticides are presented in this paper.

Results and Discussion

Analysis

The SFE conditions and the GC/ITMS analysis used were similar to those in a previous study (7). Figure 1 shows a typical GC–ECD chromatogram (from Experiment 1) of a potato SFE extract containing PCB at 0.14 μg/g, HCB at 0.26 μg/g, lindane at 0.37 μg/g, and aldrin at 0.36 μg/g. As the figure shows, matrix interferences were negligible.

The extracts were analyzed by GC/ITMS. Table 2 lists the pesticides used, their retention times, and the quantitation masses. Figure 2 is a typical total-ion chromatogram of 40 pesticides fortified at 0.2 or 0.4 μg/g in potato and extracted by SFE (Experiment 4A). Detection limits for pesticides analyzed by these methods were reported previously (5, 7).

Experiments 1 and 2

Table 3 summarizes the results of Experiments 1 and 2. The experiments were done to determine whether a small (1–2 g) subsample accurately represents a large (2.3 kg) sample from which it was taken. After the 2.3 kg sample was chopped with the Hobart cutter, recoveries of HCB and lindane from 48 replicate extractions of 2.1 g subsamples had relative standard deviations of 11–14%. Recoveries from 4 replicate subsamples from each 50 g portion had RSDs of 10–13%. The results shown in Table 1 for HCB and lindane were obtained with GC–ECD. The same samples were analyzed also by GC/ITMS, and reproducibilities were similar (RSDs: 10.3% for HCB and 12.6% for lindane). Recoveries of chlorpropham, an incurred residue in potato that was analyzed by GC/ITMS, had an RSD of 11.4%. The calculated concentration of chlorproham, 4.2 μg/g, was corroborated by GC–NPD.

In Experiments 1 and 2, the amounts of HCB and lindane recovered from fortified potato were 63 and 85%, respectively. The losses, demonstrated by Experiments 3 and 4A, were due to evaporation during spiking, which was done at room temperature and in open air. When samples were frozen, losses were prevented, and recoveries became consistent.

In Experiment 2, a 50 g portion of potato from Experiment 1 was blended further with 100 g HMX. HMX enabled blending of the moist sample without use of a liquid, and the amount of HMX was adjusted to form a flowable material for proper mixing. The precision was greatly improved in Experiment 2, and

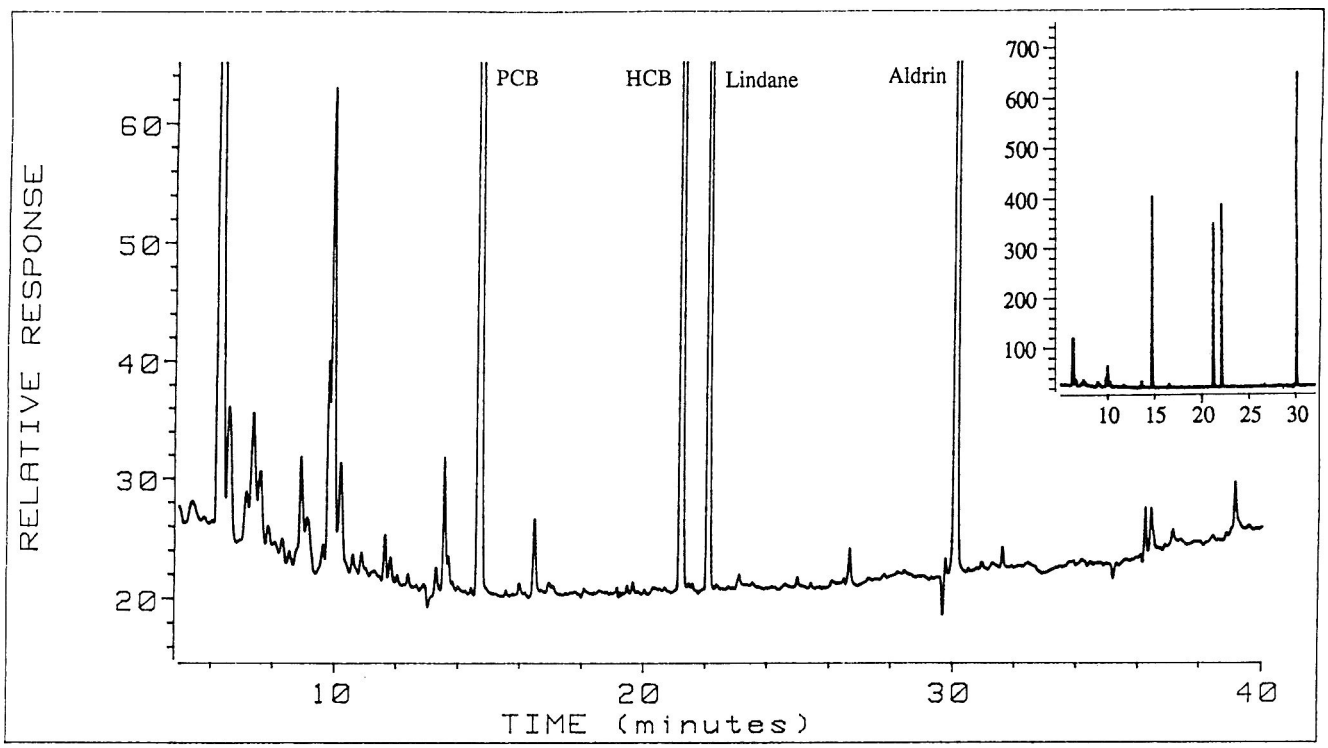

Figure 1. Typical GC–ECD chromatogram of potato extracted by SFE (Experiment 1). The full-scale chromatogram in the upper right shows the peak signals, and the larger chromatogram gives the noise levels of the potato extract, which was analyzed without additional cleanup. PCB, 0.14 μg/g; HCB, 0.26 μg/g; lindane, 0.37 μg/g; aldrin internal standard, 0.36 μg/g.

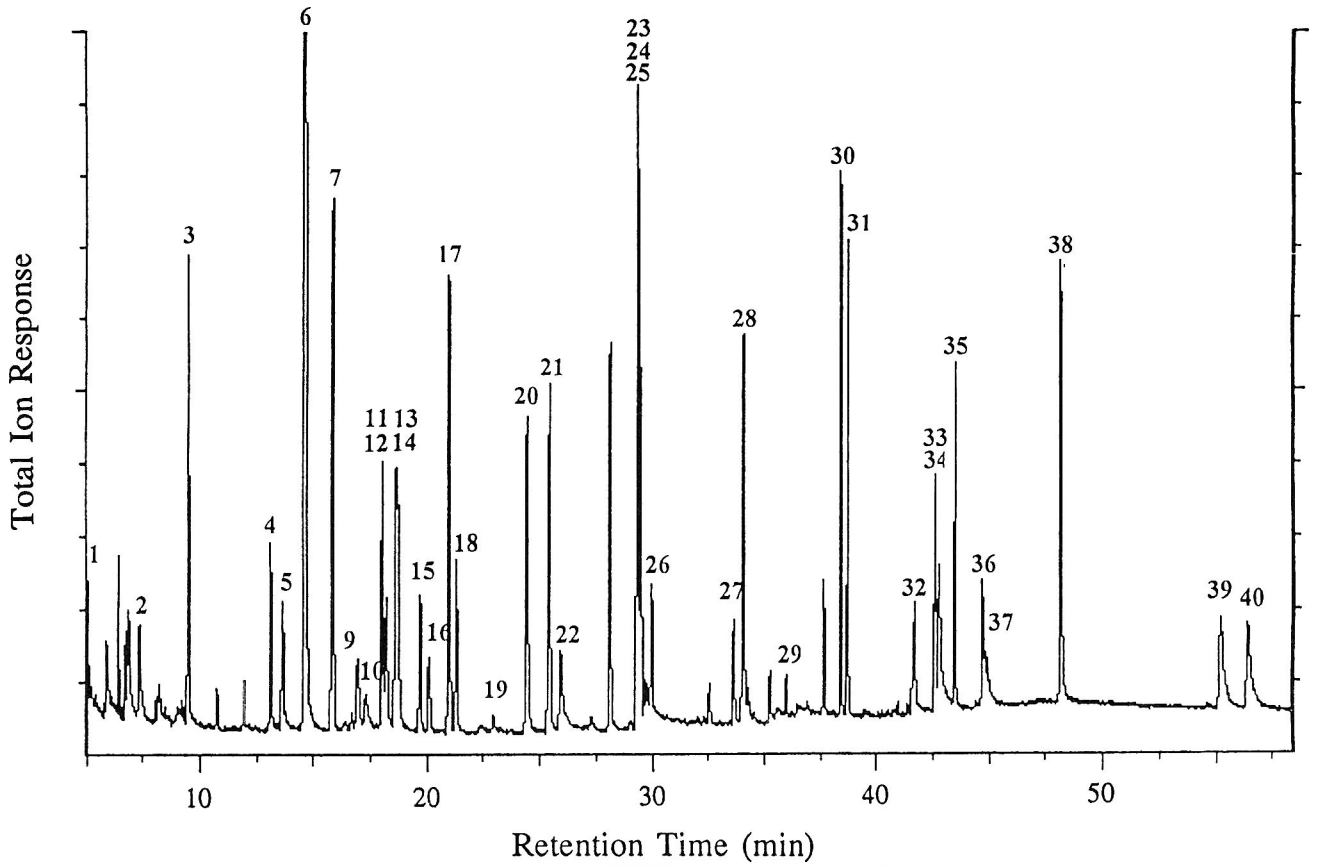

Figure 2. Typical GC/ITMS total-ion chromatogram of 40 pesticides fortified at 0.2 or 0.4 μg/g in potato and extracted by SFE (Experiment 4A). The numbered peaks refer to the pesticides listed in Table 2.

recoveries were the same as those in Experiment 1. GC–ECD gave RSDs of 2.8% for HCB and 2.9% for lindane, compared with 10.9 and 13.6% respectively, in Experiment 1. GC/ITMS analysis gave RSDs of 2.6% for both HCB and lindane. For incurred chlorpropham, GC/ITMS analysis gave an RSD of 2.1%.

This excellent precision was obtained from as little as 1.3 g potato. Thus, pesticide residues in a 50 g sample, the amount commonly used in a current solvent-based method (20), were accurately determined with a 1.3 g subsample using SFE. Furthermore, the improvement in precision from an RSD of 10–14% in Experiment 1, in which 2.1 g subsamples were individually chopped without HMX, to an RSD of 2–3% in Experiment 2, in which subsamples were blended with HMX, showed that greater sample homogeneity is achieved when samples are blended with HMX. Blending also may have created smaller sample particles, which are known to improve extraction by SFE (1–4).

Experiments 3 and 4

After Experiment 2, a similar experiment was carried out in which potato was spiked with a mixture of 40 pesticides. The blending procedure for 50 g potato was the same as in Experi-

Table 3. Reproducibility of repeated pesticide residue analyses of potatoes extracted by SFE (Experiments 1 and 2)

Pesticide	Overall sample size	Amount extracted by SFE, g	Number of replicates	Chopped without HMX		Blended with HMX	
				Conc., μg/g	RSD, %	Conc., μg/g	RSD, %
HCB	2.3 kg	2.1	48	0.26[a]	10.9[a]	—	—
HCB	50 g	1.3	9	—	—	0.28[a]	2.8[a]
Lindane	2.3 kg	2.1	48	0.37[a]	13.6[a]	—	—
Lindane	50 g	1.3	9	—	—	0.36[a]	2.9[a]
Chlorpropham	2.3 kg	2.1	48	4.21[b]	11.4[b]	—	—
Chlorpropham	50 g	1.3	9	—	—	4.49[c]	2.1[b]

[a] GC–ECD results. Analysis by GC/ITMS resulted in similar RSDs for samples prepared without and with HMX: 10.3 and 2.6 for HCB and 12.6 and 2.6 for lindane, respectively.
[b] GC/ITMS result.
[c] GC–NPD result.

Table 4. Effect of sample preparation procedures for SFE on recovery of 40 pesticides fortified in potato (Experiments 3 and 4A)

	Fortification in vessel (5 replicates)		Blended at room temp. (Experiment 3, 8 replicates)		Blended with dry ice (Experiment 4A, 5 replicates)	
Pesticide[a]	Recovery, %	RSD, %	Recovery, %	RSD, %	Recovery, %	RSD, %
			Category 1			
Atrazine	96	11	96	8.1	95	4.2
Carbaryl	94	24	69	13	116	3.4
Carbofuran	98	5.1	96	12.1	104	6.7
Chlorpyrifos	100	2.0	91	8.3	91	2.0
Dacthal	101	2.9	73	6.4	105	2.1
DDE	99	1.7	97	19	94	6.2
DDT	94	2.4	78	8.7	94	1.4
Diazinon	98	2.7	92	9.5	96	2.1
Dicloran	95	7.8	86	9.5	94	4.4
Dimethoate	98	14	77	17	113	5.0
Endosulfan I	100	1.3	95	10	95	2.0
Ethion	88	7.8	70	9.3	89	1.3
Ethoprop	92	4.6	85	3.6	103	3.8
Lindane	104	1.9	88	5.5	103	1.8
Methidathion	86	9.1	70	11	101	1.7
Methoxychlor	91	3.9	80	8.2	98	3.5
Parathion	81	19	72	7.5	83	9.1
Parathion-methyl	76	6.7	68	9.8	89	4.3
PCNB	95	1.6	63	6.8	81	3.4
cis-Permethrin	95	3.6	62	18	87	0.8
Vinclozolin	99	6.4	85	5.1	97	1.6
			Category 2			
Chlorothalonil	83	24	8	20	91	6.2
Dichlorvos	82	8.8	8	42	74	5.1
Diphenylamine	96	3.7	17	9.2	9	4.5
Disulfoton	93	4.9	20	29	36	3.2
HCB	99	2.9	56	8.7	90	4.3
Malathion	83	14	35	17	75	2.0
α-Mevinphos	68	24	52	11	104	1.7
PCB	97	3.9	13	41	89	7.1
Phorate	95	3.6	47	10	69	2.3
Phosalone	81	14	45	51	96	2.9
Phosmet	74	17	53	16	108	3.3
Phosphamidon	72	14	44	19	107	10
Terbufos	88	2.9	57	7.6	78	3.1
			Not categorized			
Azinphos-methyl	74	33	ND[b]	ND	107	5.1
Chlorpropham	Incurred	24	Incurred	8.4	Incurred	7.7
Esfenvalerate	80	12	ND	ND	90	3.8
Fenvalerate	91	14	ND	ND	90	3.5
Iprodione	81	26	ND	ND	102	2.3
Propargite	85	8.0	ND	ND	83	4.7

[a] PCNB, pentachloronitrobenzene; HCB, hexachlorobenzene; PCB, pentachlorobenzene.
[b] ND, not detected because of GC problems with pesticide.

Table 5. Losses of pesticides from fortified potato kept at room temperature for 5 h

Pesticide[a]	Recovery, %	
	Exposed to open environment	Stored in extraction vessel
HCB	55	95
PCNB	62	95
PCB	12	85
Phorate	48	72
Malathion	35	65
Chlorothalonil	8	52
Disulfoton	20	30
Dichlorvos	8	28

[a] HCB, hexachlorobenzene; PCNB, pentachloronitrobenzene; PCB, pentachlorobenzene.

ment 2; results are presented in Table 4. The first 2 columns show recovery of pesticides added directly to an extraction vessel packed with blank potato sample. This served as a control spike to determine the efficiency of SFE unrelated to sampling aspects. Even though calibration curves were prepared in blank potato extracts, which reduce matrix effects, accurate quantitation by capillary GC of a few pesticides (mainly organophosphorus insecticides, such as phosphamidon, azinphos-methyl, and parathion-methyl) still posed some difficulties.

The next 2 columns in Table 4 present results from Experiment 3, in which potatoes were spiked with the pesticides in open air and then allowed to stand for 1 h at room temperature before blending with HMX. Pesticides listed in Category 1, were relatively stable and nonvolatile, and consistently gave acceptable recoveries, although some losses occurred. Category 2 pesticides gave higher losses due to evaporation and/or degradation during sample preparation. Five of the 6 pesticides listed as "not categorized" were not detected in Experiment 3 because of a GC problem and could not be classified in Category 1 or 2. Recovery of chlorpropham was not determined because it was incurred.

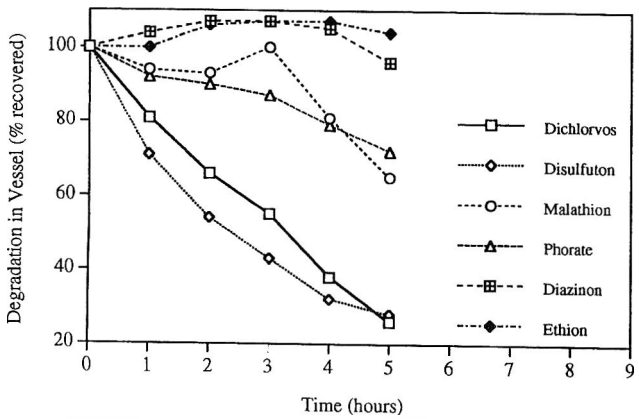

Figure 3. Degradation rates of 6 organophosphorus insecticides in potato samples kept in SFE vessels for 1–5 h at room temperature and exposed to air.

The final 2 columns present results of Experiment 4A, in which samples were kept cold with dry ice throughout sample preparation prior to SFE. Recoveries for most pesticides were 90–105%. Recoveries of many organophosphorus insecticides were more variable (75–116%) because of matrix effects when analyzed by capillary GC. Recoveries of repeated injections of these pesticides as standards in potato matrix solution fluctuated similarly. For most pesticides, recoveries in Experiment 4A were much better than those in Experiment 3, in which samples were not kept frozen throughout sample preparation.

The improved recoveries in Experiment 4A were also reflected in better precision. In Experiment 4A, RSDs varied from 0.8 to 10 % and typically were 3–4%, whereas in Experiment 3, RSDs fluctuated from 6 to 41%, mainly because of degradation or evaporation. Diphenylamine was the only pesticide tested that had lower recovery with addition of dry ice to potato. The recovery of disulfoton was slightly better but was still low (36%). Recoveries of other pesticides in Category 2, when compared with those in Experiment 3, were greatly improved.

Evaporation or Degradation

The experiments also provided data on losses of pesticides from the SFE vessel or during sample preparation in open air. Pesticides that were lost through volatilization could be separated from those lost through degradation. As shown in Table 5, the chlorinated pesticides PCB, HCB, and PCNB were lost only from samples exposed to air at room temperature. Once the samples were stored for the same period (5 h) in enclosed extraction vessels, losses were minimal. On the other hand, losses of pesticides such as dichlorvos, disulfoton, and malathion were due mainly to degradation. Degradation of these pesticides, as presented in Table 4 (Experiment 4A), was minimized by keeping samples frozen until extraction.

The results of Experiment 4B (Figure 3) show that degradation occurs in the extraction vessel over a period of several hours at room temperature as the vessels are awaiting extraction in sequence on the sample carousel. The organophosphorus insecticides, dichlorvos and disulfoton, were especially sensitive to degradation, with losses amounting to 70% of the original amount over a period of 5 h. Other pesticides like malathion and phorate degraded more slowly, whereas diazinon, ethion, and several others were not degraded. Except for disulfoton, the pesticides studied were kept from degrading by freezing the samples until they were extracted.

Pesticides in a sample kept for 13 h in the SFE chamber with CO_2 did not show any degradation. In a control sample, which was kept with air in the vessel for a similar period, some pesticides degraded completely. If a sequential automated sampler is being used for SFE, samples should either be kept frozen until extraction or stored in the vessel in the absence of oxygen.

Check Samples

The final sample preparation procedure was tested on peach and orange check samples. Table 6 compares results of the analyses with actual fortification levels and concentrations de-

Table 6. Analysis of peach and orange check samples

Sample	Pesticide	Actual conc., μg/g	Concentration detected, μg/g	
			SFE method	Traditional methods[a]
Peach	Dichlorvos	0.11	0.11 ± 0.021	0.10 ± 0.013
	α-Mevinphos	0.27[b]	0.26 ± 0.043	0.29 ± 0.091[b]
	Dicloran	Incurred	0.14 ± 0.013	0.12 ± 0.015
	Vinclozolin	0.095	0.098 ± 0.010	0.089 ± 0.014
	Iprodione	Incurred	0.68 ± 0.092	0.50 ± 0.12
Orange	Carbofuran	0.36	0.34 ± 0.013	0.31 ± 0.054
	Methoxychlor	0.19	0.14 ± 0.006	0.19 ± 0.048

[a] Traditional analyses (19, 20) were performed by 7 laboratories participating in the Pesticide Data Program.
[b] Fortification level was 0.39 μg of 70% α-mevinphos and 30% β-mevinphos per gram. Samples were analyzed for α-mevinphos only with the SFE method, and β-mevinphos results are not presented for the traditional methods.

termined by 7 laboratories participating in the Pesticide Data Program (22) using traditional methods (19, 20). Figure 4 presents the calibration curves for the method of standard additions for 3 of the 7 pesticides. The results compare well in all cases, except for methoxychlor in the orange check sample. This low result is puzzling, because recovery of methoxychlor from fortified samples was >90%. Recoveries of duplicate 0.5 μg/g fortification spikes in the vessels were 86% (RSD, 4%) for dichlorvos, 105% (RSD, 2%) for α-mevinphos, 105% (RSD, 3%) for dicloran, 105% (RSD, 4%) for vinclozolin, 117% (RSD, 0.5%) for iprodione, 128% (RSD, 1%) for carbofuran, and 94% (RSD, 1%) for methoxychlor. The check sample results further demonstrated the viability of the method for multiresidue analysis of pesticides in fruits and vegetables. The correct determination of dichlorvos in the peach sample showed that the sample preparation technique adequately controls losses of a volatile and unstable pesticide in the sample.

Conclusions

The goal of this study was to develop practical procedures for preparing fruit and vegetable samples for extraction by SFE in for multiresidue analysis of pesticides. Traditionally, an organic solvent distributes the analyte in a solution and helps to avoid losses from volatilization and degradation. With SFE, the amount of sample required is small (1–3 g) and the analyte is distributed in a moist solid matrix. Sample homogeneity was accomplished by blending the sample with HMX to form a flowable powder. A 2:1 HMX:sample ratio successfully reduced moisture to the point that the sample did not stick to itself or to the blender wall. With dry ice, a 1:1 HMX:sample ratio gave the same effect, thus increasing the amount of sample packed into a fixed-volume extraction vessel.

If a large (2.3 kg) frozen sample was chopped only with a Hobart cutter, without blending with HMX, results from ex-

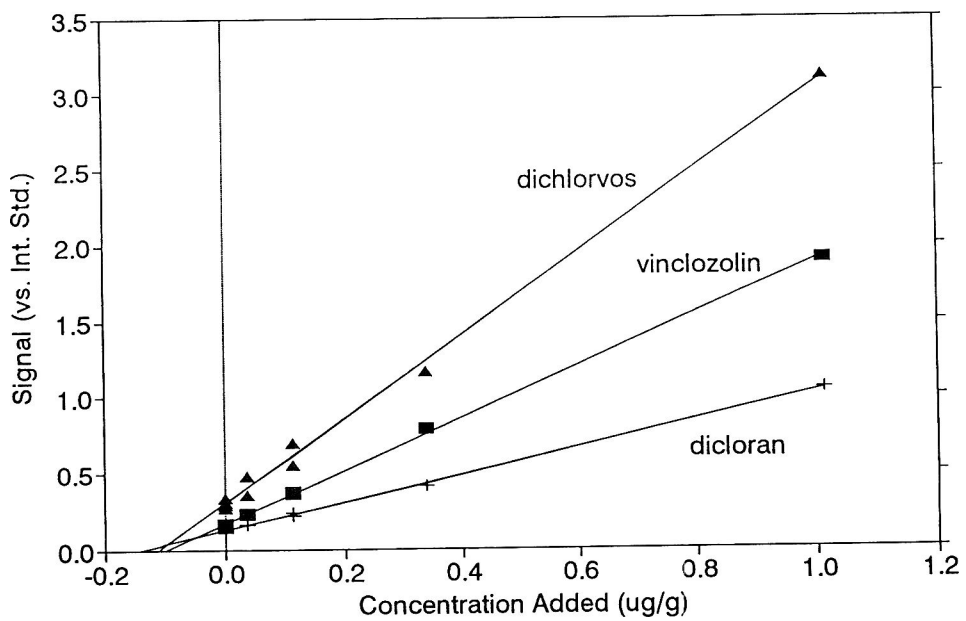

Figure 4. Determination of dichlorvos, vinclozolin, and dicloran in peach check sample using the method of standard additions.

tractions of 2.1 g subsamples gave an RSD of 10–14%. By adding HMX and forming a flowable material of a 50 g sample in a blender, the precision of the extraction of even smaller subsample amounts (1.3 g) was greatly improved (RSD, 1-6%). In most cases, high variations were related either to pesticide stability in the solid matrix or to capillary GC analysis and not to extraction efficiency. The precision of recovery results was usually limited by the reproducibility of the analytical method and not by sample preparation or SFE.

Some pesticides rapidly degraded and/or evaporated when exposed to air at room temperature. Volatilization was practically eliminated by keeping the sample frozen during sample preparation and by reducing sample exposure to an open environment. Once the sample was packed into an extraction vessel, evaporation was minimal. However, degradation of certain pesticides still occurred in the vessel as long as O_2 was present. When the sample vessels were loaded into the autosampling carousel for sequential extraction, several pesticides, especially some of the organophosphorus insecticides, degraded over a period of hours. By purging a packed vessel with gases such as N_2 or CO_2, oxidative degradation can be avoided, as shown by analysis of dichlorvos and α-mevinphos in the peach check sample and results for other pesticides in check samples being comparable with results from traditional analyses. The procedures offer a practical approach for sample preparation for SFE of plant materials for multiresidue analysis of pesticides.

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Liquid Chromatographic Determination of Bromide Ion in Cereals, Fruit, Vegetables, and Blood with a Silver Electrode in an Electrochemical Detector System

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A method is presented for rapid determination of bromide ion in commodities and blood by paired-ion liquid chromatography with electrochemical detection. The method involves extraction of samples with water and filtration. Blood is passed through a Sep-Pak C_{18} minicolumn. Recoveries are usually close to 100%, with satisfactory precision. The detection limit is 1 mg/kg. The method needs little labor and uses no noxious solvents or reagents.

Methyl bromide and ethylene dibromide are used for disinfection of vegetable cargo in international trade. They are used as fumigants and act by rapidly alkylating enzymes and nucleic acids in living species, leaving bromide ions as residue. The concentration of bromide ion is an indicator of the degree of treatment. Bromide ion also can be absorbed by vegetables grown in fumigated soil. The Food and Agriculture Organization–World Health Organization maximum residue limits for bromide ion in fruit, dried fruit, vegetables, and cereals vary between 20 and 300 mg/kg (1).

Methods for determination of bromide ion in foodstuffs include gas chromatography after ashing and derivatization with ethylene oxide (2–4) or propylene oxide (5) and after water extraction and head space gas chromatography of the ethylene oxide derivative (6), spectrophotometry after ashing (7), ion chromatography (8), use of bromide-selective electrode (9), x-ray fluorescence (10), or neutron activation (11). Earlier, we determined bromide ion residues by ion chromatography after ashing. Ashing was tedious and gave inconsistent results, especially for fatty samples, and other inorganic ions often interfered with the final determination.

Skelly (12) applied reversed-phase liquid chromatography (LC) to separate halides, using octylamine in the eluant for paired-ion separation. The analytes were monitored by UV detection at 205 nm. Rocklin and Johnson (13) used an electrochemical silver detector to determine halides after separation by ion chromatography (13). In the method presented here, paired-ion chromatographic separation was combined with electrochemical silver detection in an LC system for determination of bromide ion in commodities and blood. The method has been used in Swedish food-monitoring programs since 1983.

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METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Spectra-Physics Model 3500 with Valco 25 µL injector loop.

(b) *Detector.*—Bioanalytical Systems, Inc., LC-4 amperometric controller with a silver rod working electrode (the silver rod was purchased from a local goldsmith; now it is also available from Bioanalytical Systems), set at 0.5–1.0 V versus a Ag/AgCl reference electrode according to performance. The silver electrode is reactivated by polishing with household silver polish at intervals. The same electrode has been used since 1983.

(c) *Column.*—Chromspher pesticides (Chrompack, a C_{18} material), 5 µm, 20 cm × 3 mm id, with 1 cm × 3 mm id precolumn packed with the corresponding material.

(d) *Eluent.*—0.01M *n*-octylamine, 98% purity (Merck No. 806917), adjusted to pH 6.2 with phosphoric acid and fortified with 5% methanol.

(e) *Reference standard.*—Potassium bromide, AR. Bromide ion standard solutions of 0.1–3 mg/L were prepared in deionized water.

(f) *Water.*—Deionized.

Materials

(a) *Fresh and dried fruits and vegetables.*—Extract a representative portion of macerated sample or sample finely cut into about 5 × 5 mm pieces with 9 times the amount (w/w) of water in an ultrasonic bath for 15 min (or blend a macerated sample with water and leave in the refrigerator overnight). Centrifuge if needed. Filter an aliquot of extract through a 0.45 µm Teflon filter (e.g., Acrodisc LC PVDF).

(b) *Cereals.*—Mill grain and extract a portion with water as described for vegetables.

(c) *Blood.*—Dilute frozen blood 50 times with water. Draw an aliquot through a Sep-Pak C_{18} minicolumn (previously

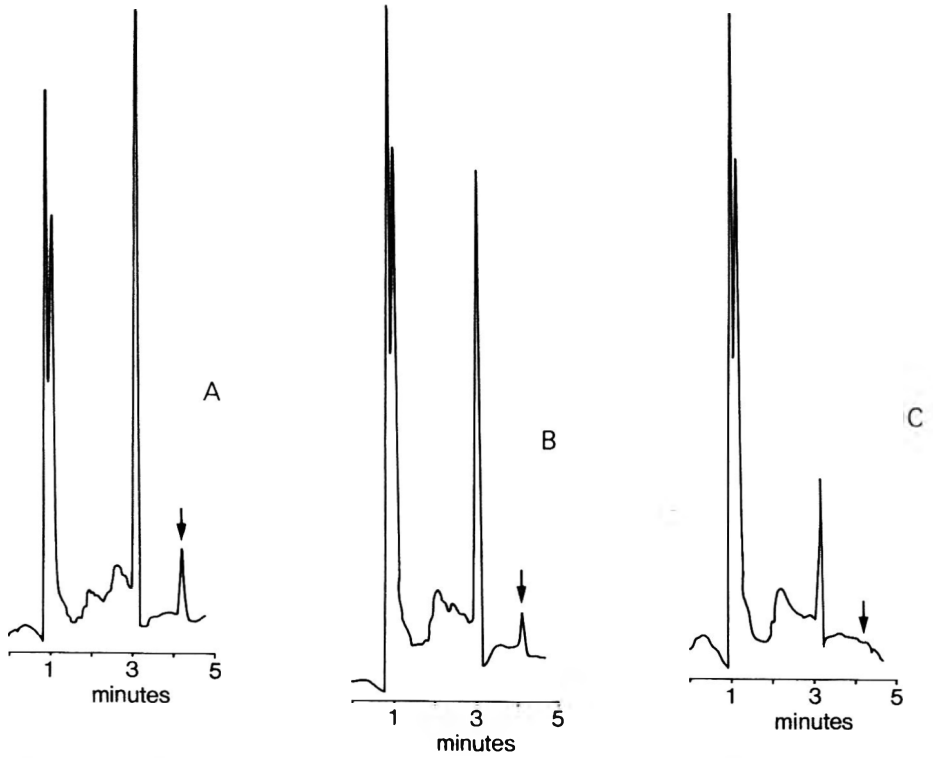

Figure 1. Liquid chromatograms of (A) wheat–flour extract, 3.5 mg bromide/kg; (B) lettuce extract, 1.7 mg bromide/kg; (C) and lettuce extract, bromide not detected. Arrow indicates retention time for bromide. The peak at 3 min is chloride.

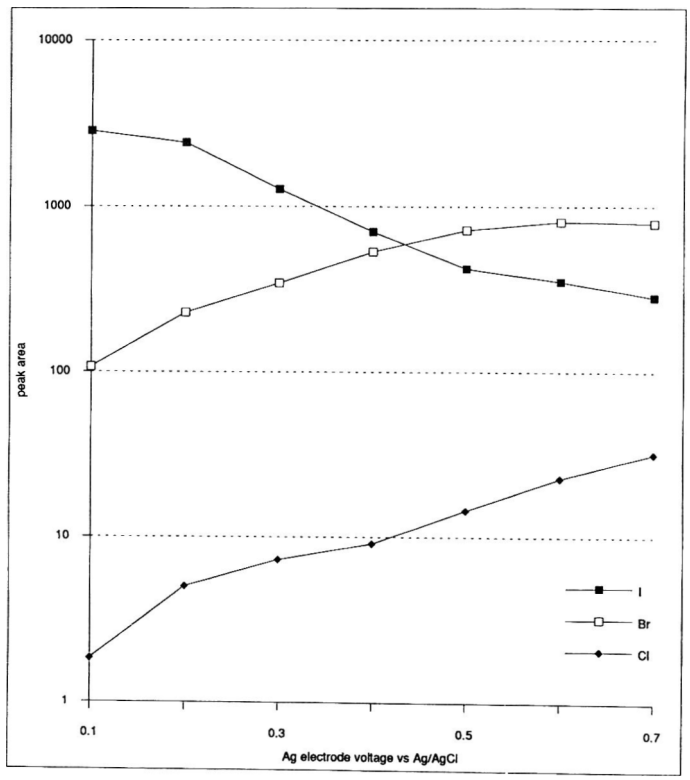

Figure 2. Relative responses of chloride, bromide, and iodide to the silver electrode at different potentials.

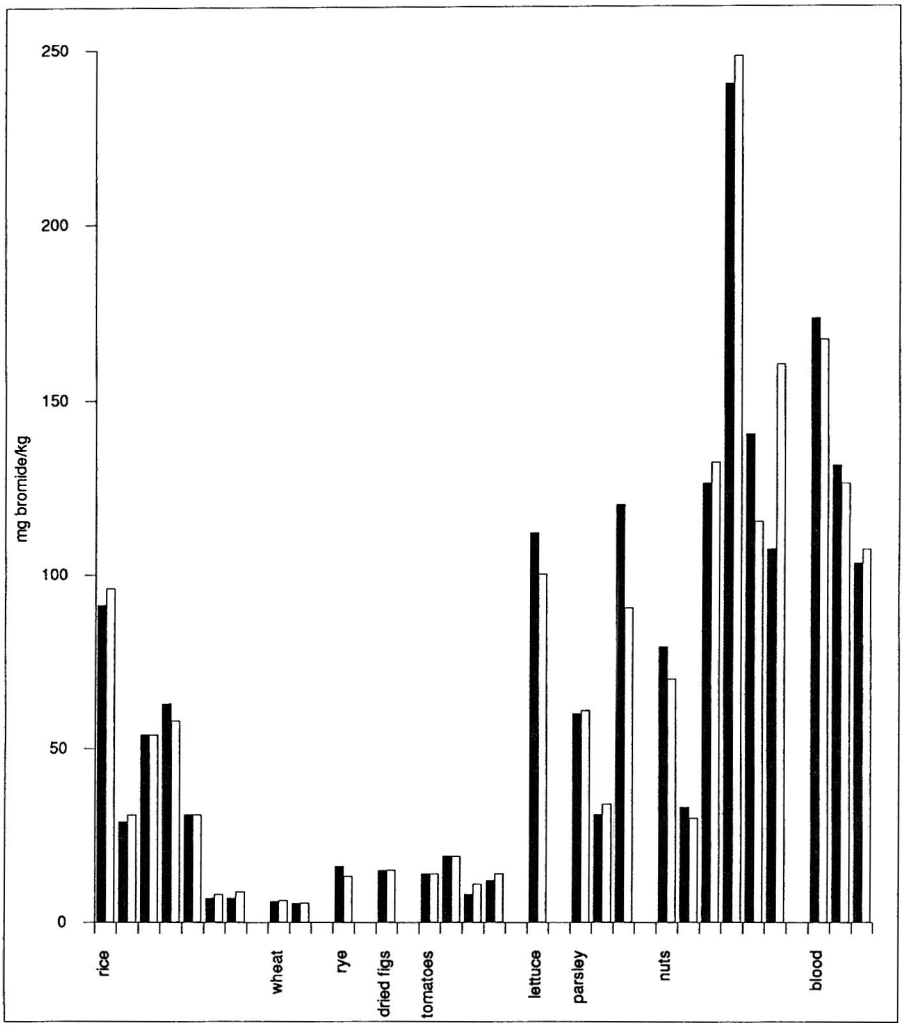

Figure 3. Results of duplicate analyses of marketed products.

treated successively with methanol and water). Discard the first few intensively colored drops.

Procedure

Transfer an aliquot of extract to a glass vial. Inject samples on chromatograph, alternating with standard solutions. Use peak heights or peak areas for calculation:

$$CN_{\text{Br smpl}} = \frac{CN_{\text{Br Std}} \times V_{\text{wtr smpl}} \times PH_{\text{Br smpl}}}{W_{\text{smpl}} \times PH_{\text{Std}}}$$

where $CN_{\text{Br smpl}}$ = bromide concentration in sample, $CN_{\text{Br Std}}$ = concentration of bromide ion in standard, $V_{\text{wtr smpl}}$ = volume of water in sample, $PH_{\text{Br smpl}}$ = peak height of sample, W_{smpl} = weight of sample, and PH_{std} = peak height of standard.

Water volume is the sum of the water in the sample and the added water. In practice, the water content of fresh fruit and vegetables is set at 0.9 mL/g sample, and that of cereals and dried fruit is set at 0.1 mL/g. For more exact figures, the water content of the sample should be measured separately.

For recovery experiments, add stock solution of potassium bromide in water to sample prior to homogenizing or mixing with water.

Results and Discussion

Chromatography

Typical retention times for chloride, bromide, and iodide were approximately 3, 4, and 9 min, respectively. Several systems have been used satisfactorily during the years, such as reversed-phase columns (e.g., Nucleosil 5 C_{18}, Spherisorb ODS2, and Sephasil C_8), as well as the Chrompack column used in this study. An ion-exchange column (Nucleosil 5SB eluted with 0.05M sodium salicylate, pH 5) was inferior to the reversed-phase columns.

Electrochemical Detection

Detection is based on the reaction between halide and silver, $X^- + Ag \rightarrow AgX + e^-$, which makes detection very selective (Figure 1). Selectivity toward bromide is adjusted by voltage applied (Figure 2). Chloride, which is naturally abundant in

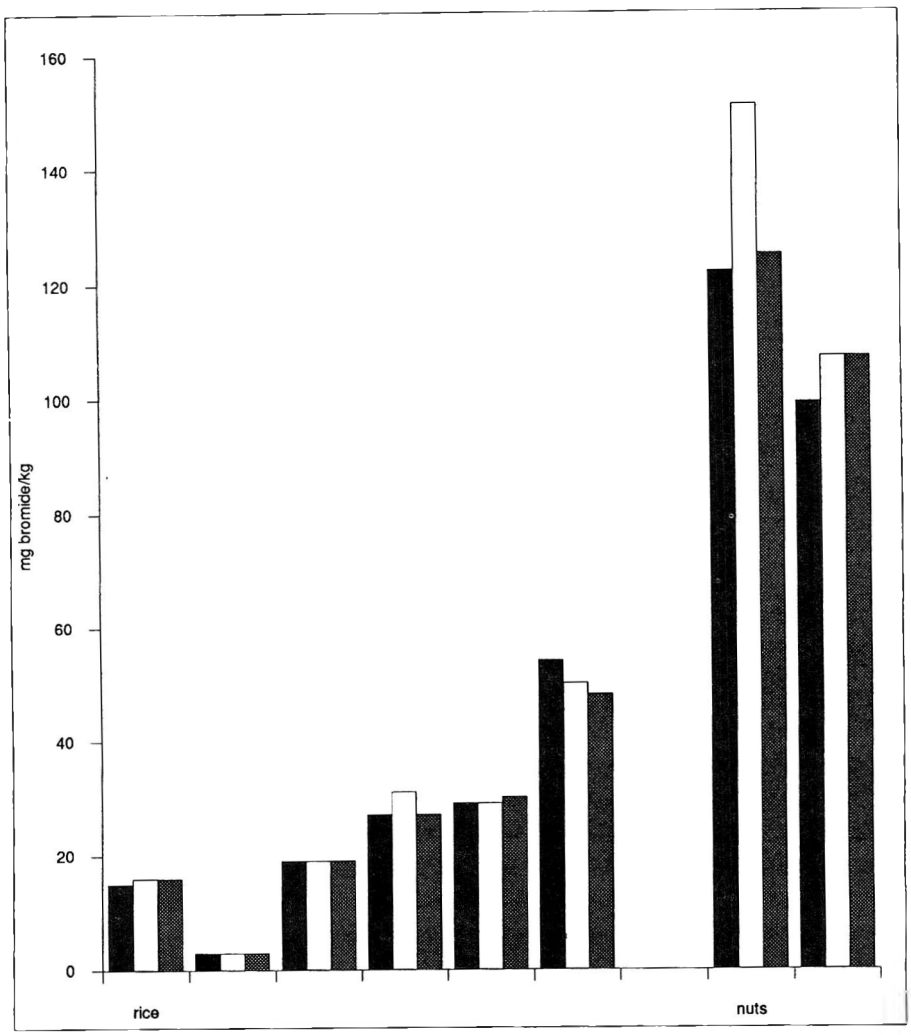

Figure 4. Results of single analyses of products sampled in triplicate.

most matrixes, rarely interferes. If it does, separation has to be increased, for example, by lengthening the column. When the method was used to determine iodide in wine, the voltage was decreased accordingly to increase iodide sensitivity. Although detection is very selective, tomatoes contain a compound that may interfere with bromide in some eluent systems.

The bromide signal is linear over 2 orders of magnitude. The detection limit of the system is 0.05–1 μg bromide ion/mL, which corresponds to 0.5–1 mg/kg crop sample. In practice, the reporting limit is 5 mg/kg, because crops naturally have a bromide content of 2–3 mg/kg (2, 9).

Preparation of Cereals

Because fumigation mainly reaches the surfaces of grains, milling of grains before extraction was supposedly unnecessary. Therefore, commercial samples of wheat and rice (both milled and unmilled) were extracted and analyzed. Repeated analyses of one wheat sample gave residues of 5.5 ± 0.2 mg/kg (n = 5) in milled and 5.4 ± 0.2 mg/kg (n = 5) in unmilled subsamples. Corresponding residues for the rice sample were 6.0 ± 0.1 mg/kg (n = 5) and 5.2 ± 0.1 mg/kg (n = 5). Further studies revealed that milling before extraction can increase the amount found. Altogether, 37 samples of wheat, barley, and rice, which had been analyzed unmilled and found to contain 1 mg/kg or less, were reanalyzed after milling. In 28 milled samples, bromide concentrations increased by up to 15 mg/kg (3.8 ± 2.6 mg/kg, n = 28). Crops naturally contain bromide at about 2–3 mg/kg (i.e., drawn from the soil) (2, 9). Therefore, it is not clear whether the additional bromide found after milling is the result of deep penetration of fumigants into the grain.

Method Validation

(**a**) *Recovery tests.*—These tests were performed with various crops: lettuce, parsley, tomatoes, sunflower seeds and oil, rice, and almonds. Bromide ion at levels between 1 and 70 mg/kg was added prior to extraction. Recoveries were consistently 85–110%. Recoveries from blood fortified with 120 μg bromide ion/mL were 100 ± 15% (n = 6).

(**b**) *Repeatability.*—Repeatability was good, as demonstrated by results from wheat and rice samples. Over periods of 4–6 months, repeated analyses of a dill sample gave 11 ± 0.6 mg/kg (n = 6), and analyses of 2 rice samples yielded 2.7 ± 0.08 (n = 9) and 12 ± 0.5 mg/kg (n = 13). Results of duplicate routine analyses are presented in Figure 3. For most commodi-

Table 1. Bromide residues[a] found by various methods

Matrix	Bromide concentration, mg/kg				
	This paper	x-ray fluorescence[b]	Gas chromatography[c]	Ion chromatography[d]	Neutron activation[e]
Lettuce	32	35			
	30	33			
	31	35			
Sunflower seeds	14	17			
	13	18			
	15	17			
Rice	54	62	58	53	62
Buckwheat	105		97		
	78		88		

[a] Single analyses.
[b] Method described in reference 10, modified.
[c] Method described in reference 4.
[d] Method described in references 8 and 13.
[e] Method described in reference 11.
[b–e] Figures kindly provided by the Finnish Customs Laboratory, Helsinki.

ties, repeatability is satisfactory, but for some nuts (shelled before analysis) and parsley samples, deviations are high. These high deviations probably are due more to the difficulty of getting homogeneous samples from these crops than to the extraction. The same is indicated in Figure 4, which shows results of analyses of samples drawn in triplicate from the same cargoes.

(c) *Comparison with other methods.*—The present method was tested in a series of intercalibrations with other methods (Table 1). The present method gives slightly lower results than the x-ray fluorescence method, which measures total bromine content and not only the concentration of the bromide ion (2, 14).

Conclusions

The method described for rapid determination of the bromide ion in commodities needs little labor and uses no noxious solvents or reagents. The method is repeatable, accurate, and suitable for routine determinations, as has been demonstrated during several years of use in food monitoring.

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RESIDUES AND TRACE ELEMENTS

Analysis of Total Free and Glucose-Conjugated Pyrethroid Acid Metabolites in Tea Infusions as Hexafluoroisopropyl Esters by Gas Chromatography with Electron Capture Detection

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A gas chromatographic method with electron capture detection (GC–ECD) has been developed to quantitate the sum of free acids of pyrethroid metabolites, their glucosides, and their other base-cleavable conjugates that are extracted from tea leaves when a tea infusion is prepared. Four representative glucoside conjugates were synthesized; hydrolytic conditions were established; and extraction, derivatization and GC conditions were developed for analysis of these pyrethroid acid metabolites at ≥0.01 ppm on dry tea. GC/mass spectrometry was used to confirm assignment of residues found in some tea samples.

The pyrethroids are a class of widely used insecticides that have relatively low mammalian toxicities and reasonably short lifetimes in the field. Their metabolism has been well studied in mammals and in plants. Hydrolysis of the ester linkage is the principal initial metabolic step in both cases (1). In plants, the acid moiety of the parent pyrethroid is subsequently conjugated with glucose and, in time, with longer sugar chains (2–4). The alcohol moiety may also be conjugated with glucose or further oxidized to the corresponding acid before conjugation (5, 6).

Work with ^{14}C-labeled permethrin on bean plants (2, 7), with ^{14}C-labeled cypermethrin applied to cabbage leaves (5), and with ^{14}C-labeled fluvalinate on lettuce and tomatoes (8) has shown that, although the residue of parent compound declines over a few weeks, an appreciable portion of the radiolabel, whether in the acid or in the alcohol moiety, is retained on the plants. The metabolites and their conjugates are apparently more persistent than the original pyrethroids. Despite this observation, little work has been done on analysis of pyrethroid metabolites and their conjugates in food products. George (9) measured permethrin and its 2 principal nonconjugated metabolites in 7 agricultural crops over a period of 7 days after the crops were sprayed with the insecticide but did not measure the conjugates. In 2 crops, the initial levels of the 2 metabolites were roughly equal to those of the parent compound, a curious finding, because little or no metabolite should have been expected immediately after the insecticide is sprayed. Fitch et al. (10) developed a gas chromatography/mass spectrometry (GC/MS) method for 2 fluvalinate metabolites and their conjugates, but only the free acids were used in recovery determinations.

Wan et al. (11) determined that less than 3% cyhalothrin, permethrin, or cypermethrin residues from tea leaves are extracted into water when a tea infusion is prepared. They attributed this result to the low water solubilities of these pyrethroids (≤0.04 ppm). One would expect, however, that the metabolite conjugates would be more readily extracted from the tea leaves because their water solubilities should be considerably higher than those of the parents. Wan et al. (11) concluded that if the water solubility of the pesticide exceeded about 170 mg/L, the pesticide would be extracted completely into the infusion. Little residue data are available for pyrethroids from tea leaves. Nakamura et al. (12) reported finding 0.9 ppm fluvalinate in a commercial green tea sample but did not measure the metabolites. Considering the relatively high levels of pyrethroids allowed on dry teas in some countries (10–30 ppm), appreciable amounts of their metabolite conjugates might be found in tea infusions and might exceed the negligible residue limit (0.1 ppm) enforced in Canada under the Foods and Drugs Act Regulations. As part of a broader project to determine these conjugates in food products, a method has been developed to quantitate these metabolites in tea infusions.

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Experimental

Reagents and Materials

(a) *Chemicals.*—The source or preparation of the free pyrethroid acids was previously reported (13). Trichloroacetonitrile, hexafluoroisopropyl alcohol, diisopropylcarbodiimide, and 10% palladium on activated carbon (Pd-C) were obtained from Aldrich (Milwaukee, WI). Tetrabenzylglucose was purchased from Pfanstiehl Labs (Waukegan, IL).

(b) *Solvents.*—Deuterated solvents were purchased from MSD Isotopes (Pointe Claire, PQ, Canada). Solvents were distilled-in-glass grade (Caledon Labs, Georgetown, ON, Canada).

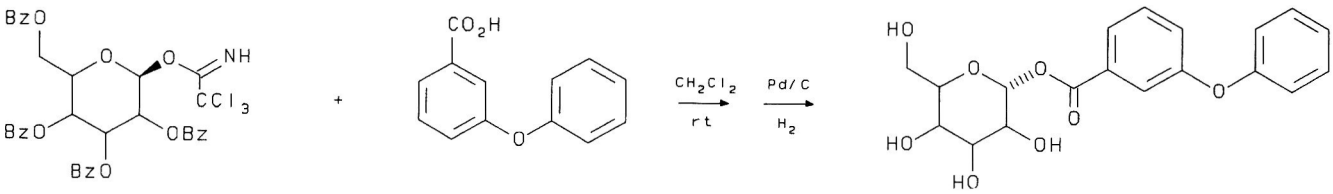

Figure 1. General synthetic scheme used to prepare glucosides of pyrethroid acid metabolites.

(c) *Materials.*—Silica gel for column chromatography was 60–200 mesh (J.T. Baker, Phillipsburg, NJ), and for flash chromatography, it was 200–425 mesh Davisil (Aldrich). Sodium hydride was 50% in oil (J.T. Baker). Thin-layer chromatography (TLC) silica plates (1 in. × 3 in., Whatman MK6F) were obtained from Chromatographic Specialties (Brockville, ON, Canada). Aminopropyl solid-phase extraction (SPE) tubes (1 mL) were purchased from Supelco (Oakville, ON, Canada). Tea samples were purchased at local stores.

Spiking Solutions

A solution of pyrethroid acids was prepared in 1.0 mL ethyl acetate for spiking studies with the free acids: cyhalothrin acid (0.176 mg), permethrin acids (0.135 mg, *cis/trans* = 56:44), fenvalerate acid (0.113 mg), deltamethrin (0.119 mg), fluvalinate acid (0.111 mg), and 3-phenoxybenzoic acid (0.122 mg). A solution of pyrethroid acid glucosides was prepared in acetone–water (9 + 1) for spiking studies with the conjugated acids: cyhalothrin acid glucoside (8.80 μg/mL), permethrin acid glucosides (8.36 μg/mL), fenvalerate acid glucoside (10.06 μg/mL), and 3-phenoxybenzoyl glucoside (8.38 μg/mL).

Apparatus

(a) *Liquid chromatography.*—LC was performed at room temperature with a Beckman 110B pump set at 1 mL/min, a Supelco LC18 reversed phase column (25 cm × 4.6 mm id, 5 μm) with a C_{18} packed guard column (J.T. Baker, 40 μm), a Waters Model 441 ultraviolet detector set at 254 nm, and a mobile phase of 30% CH_3CN in H_2O.

(b) *Gas chromatography.*—GC was done on an HP5890 with an electron capture detector (325°C), a DBWax capillary column (30 m × 0.25 mm id, 0.25 μm film; J&W Scientific, Folsom, CA), and hydrogen carrier gas set at 1.5 mL/min. An injection volume of 2 μL (equivalent to 1 mg tea) was used. Initial oven temperature (70°C) was held for 14 min and then raised to 100°C at 1°C/min and then to 145°C at 2°C/min. Data were collected with a Metrabyte Chrome1 AT data acquisition board set at 12 Hz.

(c) *Nuclear magnetic resonance (NMR) spectroscopy.*—^1H and ^{13}C NMR spectra were run on a Bruker AM400 spectrometer (9.4 Tesla) with a 5 mm probe. Chemical shifts are reported relative to tetramethylsilane.

(d) *GC/MS.*–A Fisons Instruments Autospec-Ultima mass spectrometer coupled with a Fisons 8000 series gas chromatograph was used. A 30 m × 0.25 mm id DB-5 capillary column with 0.25 μm thickness film was directly interfaced to the mass spectrometer. The head pressure of He was 103.4 kPa (15 psi), and the injector temperature was 220°C. The column temperature was held initially at 70°C for 1 min and then raised at 4°C/min to 180°C and at 40°C/min to 240°C. The mass spectrometer conditions were as follows: electron impact ionization, 70 eV; source temperature, 220°C; reentrant and capillary lines, 230°C. Data were obtained in selected ion recording (SIR) voltage-switching mode with a resolution of 2000 (10% valley definition). A dwell time of 50 ms each and a delay of 10 ms between switching were used for the following ions: *m/z* 323, 325, 345, 357, 358, 360, 362, 364, 367, 369, 445, and 447.

(e) *Fast atom bombardment mass spectrometry (FAB MS).*—Only the conventional mass spectrometer section of a VG Analytical 7070EQ hybrid tandem mass spectrometric (MS/MS) system was used. For the FAB ionization, xenon was the neutral species and glycerol was the matrix solvent. Mass spectra were obtained at a scanning speed of 10 s/decade for the mass range *m/z* 20 to 1000, with a resolution of 1000 (10% valley definition). A VG 11/250 data system was used for instrumental scanning control and data acquisition and processing.

Synthesis

(a) *Tetrabenzylglucose trichlorimidate.*—Sodium hydride dispersion (170 mg) was washed with CH_2Cl_2 (3× 2 mL) under nitrogen. A solution of tetrabenzylglucose (3 g) in CH_2Cl_2 (25 mL) was added. A clear solution was produced after initial effervescence. Trichloracetonitrile (3 mL) was added, and the reaction mixture was left at room temperature overnight (17 h). After filtration of the reaction mixture through a pad of Celite (8.5 g), the solvent was removed under reduced pressure at 40°C. The amber syrup (4.3 g) was eluted through a silica gel column (Baker, 150 g) with 15% ethyl acetate in cyclohexane and yielded 3.1 g (89%) of a pale-yellow syrup, which did not crystallize (14). ^1H NMR ($CDCl_3$) showed the β-anomeric proton as a doublet (J = 3.3 Hz) at 6.56 ppm, but there was no signal near 5.8 ppm for an α-anomeric proton. This product was used in subsequent reactions without further purification.

(b) *Cyhalothrin glucoside.*—Tetrabenzylglucose trichloroimidate (260 mg) and cyhalothrin acid, *cis*-3-(*Z*-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid (150 mg, 2× excess), were dissolved in CH_2Cl_2 (5 mL) and held at room temperature for 4 h (TLC showed that most of the imidate was consumed within 90 min). The reaction mixture was washed with 5% Na_2CO_3 (2× 5 mL) then dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent left a yellowish syrup (359 mg), which showed a major spot at Rf 0.33, an overlapping minor spot at Rf 0.41 on TLC (10% ethyl acetate in cyclohexane), and several more polar minor spots. Flash chromatography with 10% ethyl acetate in cyclo-

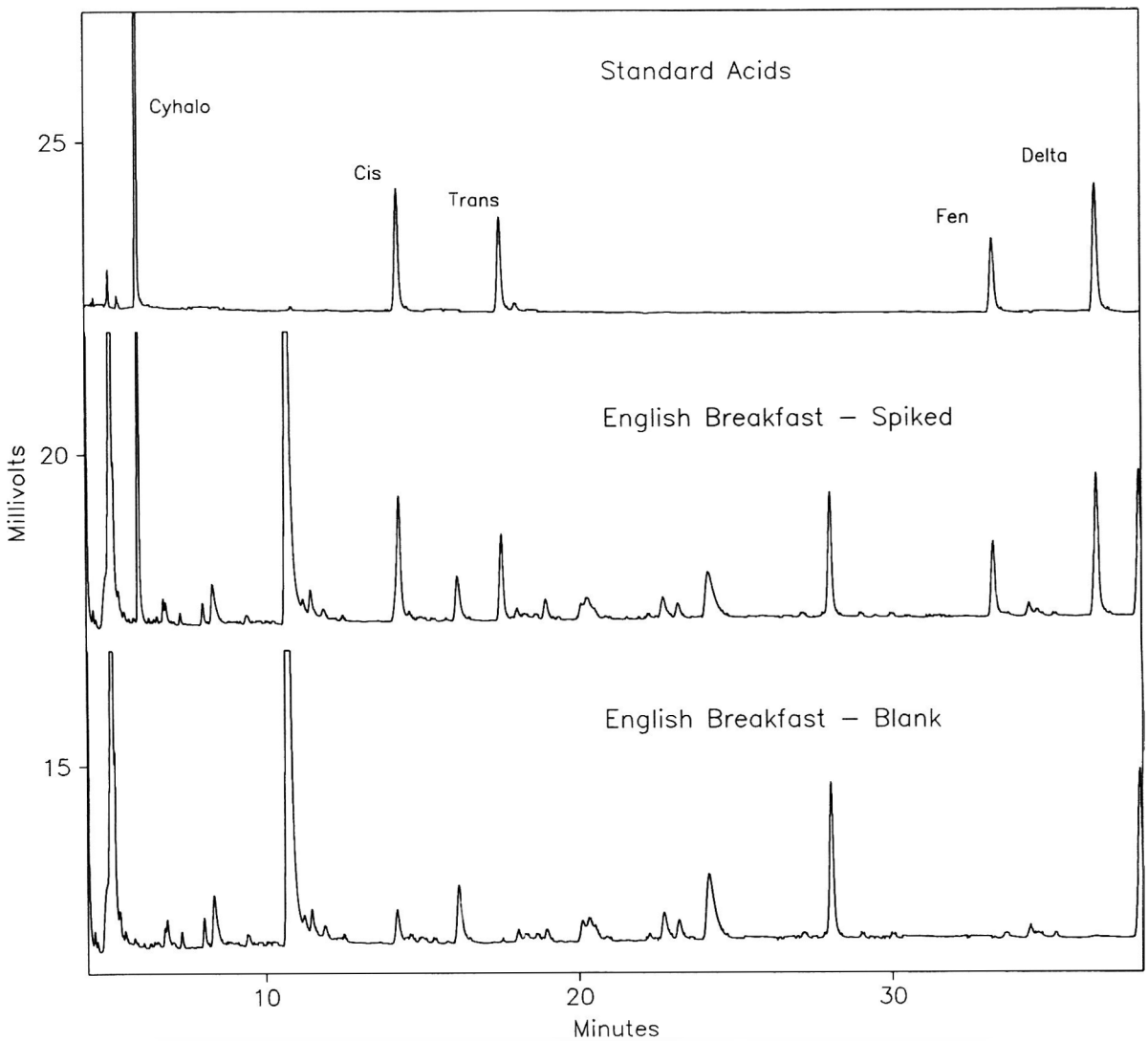

Figure 2a. Comparison of the early eluting part of the GC–ECD traces of extracts of English breakfast tea (spiked and blank) and standard pyrethroid acids (as hexafluoroisopropyl esters). Abbreviations: cyhalo, cyhalothrin acid; cis, *cis*-permethrin acid; trans, *trans*-permethrin acid; fen, fenvalerate; delta, deltamethrin.

hexane yielded a colorless oil free of minor components (253 mg, 72%). An acetone (4 mL) solution of this oil was stirred vigorously with 10% Pd-C (47 mg) under an atmosphere of hydrogen for 4 h (no further change was observed on TLC after 2 h). The reaction mixture was filtered and evaporated to yield an off-white solid (118 mg), which showed a major spot at Rf 0.45 and 2 less polar minor products on TLC (10% methanol in ethyl acetate). Flash chromatography gave a white solid (105 mg, 78%), which showed a single spot on TLC. LC analysis of this solid showed 2 peaks at 21.7 and 24.7 min in ratio of 8:92. FAB MS (in glycerol) gave *m/z* 405, 407 (9%, M + H^+); 497, 499 (10%, M + H^+ + glycerol); 225, 227 (47%, acyl group); and 163 (57%, glycosyl group), in addition to major fragments at 255 (67%), 225, 227 (45%), 145 (80%), and 127 (46%). ^{1}H NMR (acetone-d_6 + D_2O): 6.22 (dd, J = 3.8 Hz, α-anomer, 7%) and 5.62 (dd, J = 7.6 Hz, β-anomer, 93%). ^{13}C NMR (acetone-d_6): 169.34, 169.32 (C=O); 94.87, 94.83 (β-anomer, 93%); 92.78, 92.74 (α-anomer, 7%) ppm.

(c) *Fenvalerate acid glucoside.*—This glucoside was prepared as described above but with 2-(4-chlorophenyl)-3-methyl butyric acid in place of cyhalothrin acid. After chromatographic purification of the product, LC analysis of the white solid showed 2 principal peaks at 19.0 and 24.2 min (ratio, 2:98) and 2 minor impurities (≤2%). FAB MS gave *m/z* 375, 377 (8%, M + H^+); 397, 399 (5%, M + Na); 357, 359 (5%, M + H^+ – H_2O); 341, 343 (3%); 167, 169 (90%, acyl CO); 145 (70%, glycosyl – H_2O) and 125, 127 (100%, 4-Cl benzyl). ^{1}H NMR (acetone-d_6): 5.55, 5.51 (dd, J = 8.1 Hz, β-anomer, 98%) and 6.11, 5.87 (dd, J = 3.7 Hz, α-anomer, 2%) ppm. ^{13}C NMR (acetone-d_6): 173.10, 173.08 (C=O); 96.01, 95.84 (β-anomer) ppm.

(d) *3-Phenoxybenzoyl glucoside.*—This glucoside was prepared as described above but with 3-phenoxybenzoic acid in place of cyhalothrin acid. After chromatographic purification of the product, LC analysis of the white solid showed 2 peaks at 13.7 and 15.7 min (ratio, 10:90). FAB MS gave *m/z* 377 (7%, M + H^+), 469 (4%, M + H^+ + glycerol), 185 (65%), 149 (35%),

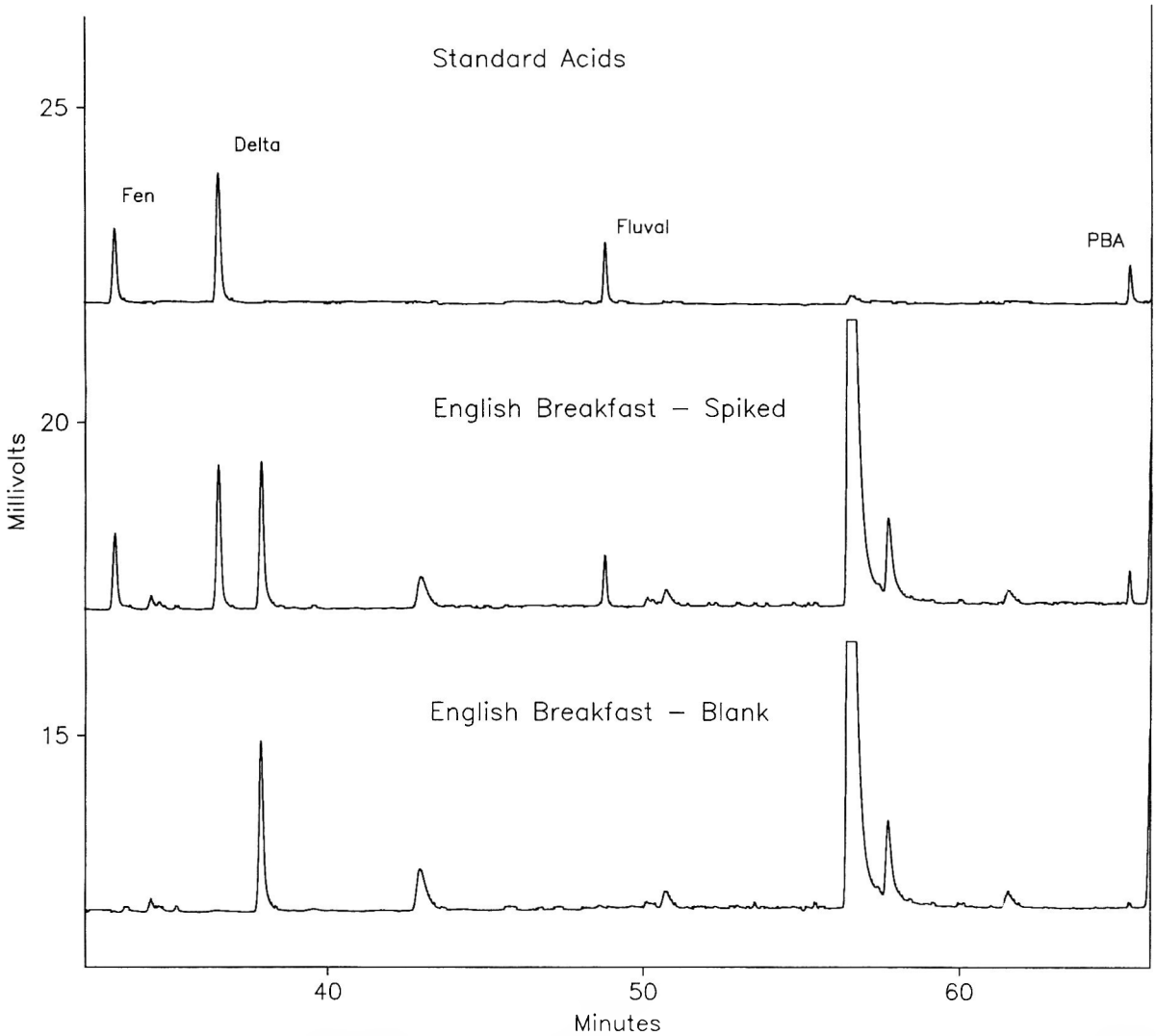

Figure 2b. Comparison of the late eluting part of the GC–ECD traces of extracts of English breakfast tea (spiked and blank) and standard pyrethroid acids (as hexafluoroisopropyl esters). Abbreviations are as defined in the caption to Figure 2a. Fluval, fluvalinate acid; PBA, 3-phenoxybenzoic acid.

and 75 (100%). ^{1}H NMR (acetone-d_6): 6.37 (d, J = 3.7 Hz, α-anomer, 9%) and 5.75 (d, J = 7.8 Hz, β-anomer, 91%). ^{13}C NMR (acetone-d_6): 165.41 (C=O), 96.44 (β-anomer, 91%), 94.37 (α-anomer, 9%) ppm.

(e) *cis- and trans-permethrin acid glucoside.*—This mixture was prepared as described above by substituting a mixture of *cis-* and *trans*-permethrin acids (ratio, 36:64) for cyhalothrin acid. After chromatographic purification of the product, LC analysis of the white solid showed 2 peaks at 12.4 and 15.0 min (ratio, 7:93). FAB MS gave *m/z* 371, 373 (20%, M + H^+); 463, 465 (3%, M + H^+ + glycerol); 191, 193 (60%, acyl ion); 145 (100%); 127 (90%). ^{1}H NMR (acetone-d_6): 6.12 (m, α-anomers, 6%), 5.52 (m, β-anomers, 94%), 6.35 (m, *cis*-vinyl proton, 34%), 5.95 (m, *trans*-vinyl proton, 66%) ppm. ^{13}C NMR (acetone-d_6): 170.71, 170.25 (C=O, *trans/cis* = 64:36); 95.80, 95.52 (β-anomers, *trans/cis* = 64:36).

Sample Preparation

(a) *Tea extracts.*—Tea leaves (5 g) were added to boiling Milli-Q water (250 mL), and the infusion was removed from the hot plate. The brew was left for 5 min after stirring once to ensure complete immersion of tea leaves. The hot tea infusion was then filtered through a prewashed (water) absorbent cotton plug in a filter funnel. The tea leaves were rinsed twice with Milli-Q water (25 mL), which was also filtered through the cotton plug. The infusion was cooled to room temperature, NaOH (2 g) was added, and the alkaline solution was left for 15 min at room temperature. The sample was then acidified with 10N H_2SO_4 to pH 3.5 and extracted with hexane (4× 40 mL). The combined hexane layers were dried over anhydrous Na_2SO_4, filtered, and evaporated just to dryness. The residue was taken up in 5.0 mL CH_2Cl_2.

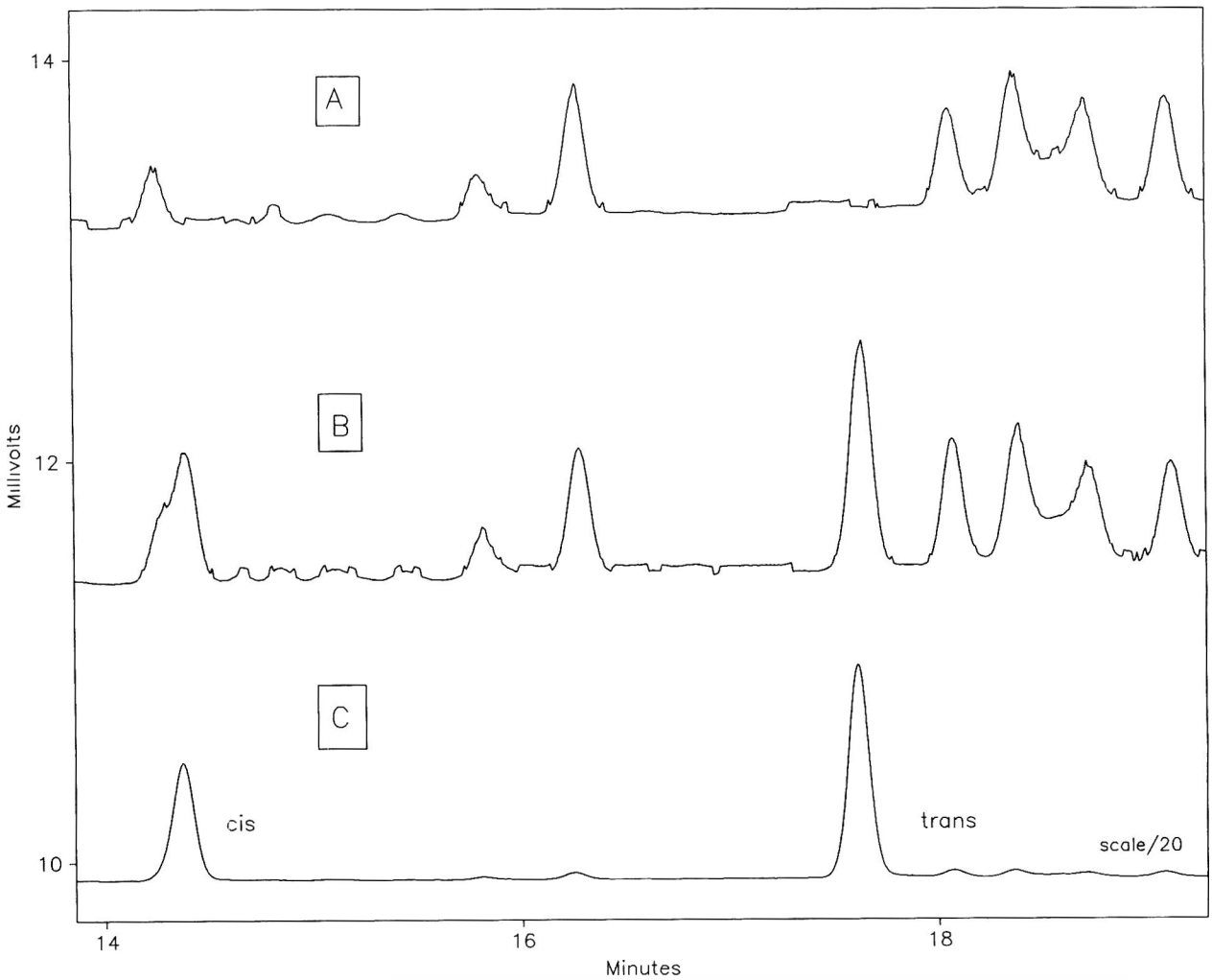

Figure 3. Comparison of a section of the GC–ECD chromatograms of extracts of orange pekoe: (A) blank, (B) spiked with permethrin glucosides at 0.05 ppm, and (C) spiked with permethrin glucosides at 1 ppm. Samples were hydrolyzed and derivatized prior to analysis.

(b) *Cleanup and derivatization.*—An aliquot of the CH_2Cl_2 tea extract (1 mL) was applied to an aminopropyl column. The column was washed first with 1% methanol in CH_2Cl_2 (1.5 mL) and then with 1% formic acid in CH_2Cl_2 (1.5 mL). The latter fraction was collected in a 3 mL vial and evaporated under N_2 on a warm hot plate to ca 0.1 mL. Hexane (2 mL) was added, and the solution reevaporated under N_2 to roughly 0.1 mL. The volume was adjusted with hexane to about 1 mL, hexafluoroisopropyl alcohol (15 µL) was added, and the mixture was shaken briefly on a Vortex mixer to disperse the reagent. Then diisopropylcarbodiimide (20 µL) was added (13). The sample was shaken on a Vortex mixer and then left at room temperature for 1 h. The derivatized sample was transferred to a 10 mL volumetric flask and made up to volume with hexane. An aliquot (1.0 mL) of this solution was transferred to a silica column (120 mg, Davisil) that had been prewashed with hexane. Eluant collection was begun when the sample was added. The column was washed with 1% ethyl acetate in hexane (1.0 mL). The volume of the combined eluants was adjusted to 2.0 mL where necessary for GC analysis. The samples showed no changes in the pyrethroid esters within 48 h at room temperature, although changes due to reagents were observed.

Results and Discussion

Although methods for synthesizing 1-*O*-acyl glucosides are numerous, only a few give high percentages of the naturally occurring β-anomer. We chose Schmidt's approach (15) because it uses a commercially available starting material (tetrabenzyl glucose) whose protecting benzyl groups are easily removed without cleavage of the acyl moiety and because it usually gives high β-selectivity (typically 92–95%) (16). The reaction sequence is illustrated in Figure 1 for 3-phenoxybenzoic acid; it involves reaction of the preformed α-trichloroimidate of tetrabenzylglucose with the free acid at room temperature followed by hydrogenolysis of benzyl groups. The trichloroimidate is easily produced and is used typically without extensive purification. One complication we observed was regeneration of tetrabenzylglucose from the trichloroimidate during cleanup on the Davisil silica (200–400 mesh). For some reason, this did not happen on the larger mesh, Baker grade silica.

Four representative glucosides of pyrethroid acid metabolites were synthesized, purified by column chromatography, and analyzed by LC, FAB MS, and ^1H and ^{13}C NMR. The β/α

Table 1. Measured values of pyrethroid acids in teas (ppm)

Tea	Cyhalothrin	*trans*-Permethrin[a]	Fenvalerate	Deltamethrin	Fluvalinate	3-Phenoxy benzoate
Lapsang	0.002	0.024[b]	0.012[b]	0.006	0.006	0.042[b]
Kenya	≤0.001	≤0.001	≤0.001	≤0.001	0.002	0.002
Darjeeling	≤0.001	≤0.001	0.002	≤0.001	0.002	≤0.001
Orange pekoe	≤0.001	0.002	0.005	0.003	0.005	0.005
Jasmine	≤0.001	≤0.001	0.012[b]	0.004	0.002	0.017[b]
Oolong	≤0.001	0.004	0.015	0.003	0.004	0.017
Japan green	0.008	≤0.001	0.004	≤0.001	0.002	0.020
Gunpowder	0.003	0.002	0.030[b]	≤0.001	0.003	0.053[b]
Earl Grey	≤0.001	0.003	0.008	≤0.001	0.005	0.011
E. breakfast	≤0.001	0.003	0.005	≤0.001	0.004	0.011

[a] For *cis*-permethrin, shoulder peak makes quantitation at ≤0.01 ppm imprecise.
[b] Confirmed by GC/MS.

ratio of anomers was ≥90:10, and purity was ≥95% in all cases. ^{13}C NMR spectra for fenvalerate acid glucoside and 3-phenoxybenzoyl glucoside were identical to those reported (17) for material isolated from pyrethroid-treated cabbage (17) except that we were able to see many doubled peaks in the fenvalerate acid glucoside spectrum. The doubled peaks, which differ by only a few Hz arise from the 2 diastereomers and are evident in our spectra because of the more powerful 9.4-Tesla magnetic field used. The NMR signals were consistent with the structures proposed and with reported NMR data for 1-*O*-acyl glucosides (18, 19) and were readily assigned except those for the permethrin acid glucosides. In the latter case, we were dealing with a mixture of *cis*/*trans* isomers, α/β anomers, and diastereomers. As a result, some signals were complex multiplets of overlapping peaks. The signals showed some solvent dependencies, but these were not fully explored. Adding a drop of D_2O to the acetone-d_6 solution, for example, caused the α-anomer proton signals to separate nicely into 4 doublets with J = 3.7 Hz (2 geometrical isomers, 2 diastereomers). Unfortunately, the β-anomer proton signals remained overlapped. Integration of the multiplets, however, revealed that the *cis*/*trans* ratio remained 34:66 as in the original acids.

The selection of acid metabolites is not exhaustive but represents one or both of the principal acid metabolites of most pyrethroids in use. For example, the structural moiety 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (permethrin acid) is found in permethrin, cypermethrin, and cyfluthrin; *cis*-3-(*Z*-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid (cyhalothrin acid) is found in cyhalothrin, bifenthrin, and tefluthrin; and 3-phenoxybenzoic acid is formed from oxidation of the alcohol moiety in at least 9 different pyrethroids. The 3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid, 2-(4-chlorophenyl)-3-methylbutyric acid, and 2-(2-chloro-4-trifluoromethylanilino)-3-methylbutyric acid are specific to deltamethrin, fenvalerate, and fluvalinate, respectively.

Table 2. Mass values used to monitor pyrethroid acids[a]

Acid (molecular weight)	Ion, *m/z* (intensity, %)
Cyhalothrin (392.5)	357 (75)
Permethrins (358.5)	358 (5), 360 (4), 323 (100), 325 (25)
Fenvalerate (362.5)	362 (30), 364 (6)
Deltamethrin (447)	367 (100), 369 (95)
Fluvalinate (445.5)	445 (20), 447 (5)
3-Phenoxybenzoic (364)	364 (100), 345 (12)

[a] As hexafluoroisopropyl esters.

Teas can be divided into 3 categories by how they are processed: green teas, partially fermented teas, and black teas. To develop an analytical method that is representative of the 3 groups, we examined tea infusions prepared from Japan sencha green, China gunpowder green, and jasmine (green teas); orange pekoe (2 brands), Kenya, English breakfast, lapsang souchong, darjeeling, and earl grey (black teas); and oolong (partially fermented).

Tea infusions were prepared as described by Wan et al. (11) except only one brew was done. The only problem encountered in preparation of the tea infusion extract was emulsion formation in the organic phase during extraction. Although the hexane layer rapidly settled from the bulk of the aqueous phase after the mixture was shaken, it always contained a frothy 'slime' whether the aqueous layer was acidic or basic. Keeping the pH at no lower than 3.5 in the acidified infusion minimized precipitation of tannins but did not prevent emulsion formation. Even in relatively clear, acidified infusions, emulsion formation was observed. Adding methanol (10%) or salt to the infusion did not help. Although the emulsion was readily broken by centrifugation (2500 rpm for 5 min), we found it more convenient to stand the combined hexane layers on a warm hot plate for several minutes. The heat degassed the emulsion and separated the layers. The use of other solvents did not avoid the emulsion problem. In fact, the quality of the chromatograms worsened because the quantity of coextractives increased in the order hexane <CH_2Cl_2 <ether <ethyl acetate.

We anticipated that it would be more convenient to work with the free pyrethroid acids than with the glucosides, so the first reaction we studied was hydrolysis of the conjugate. More

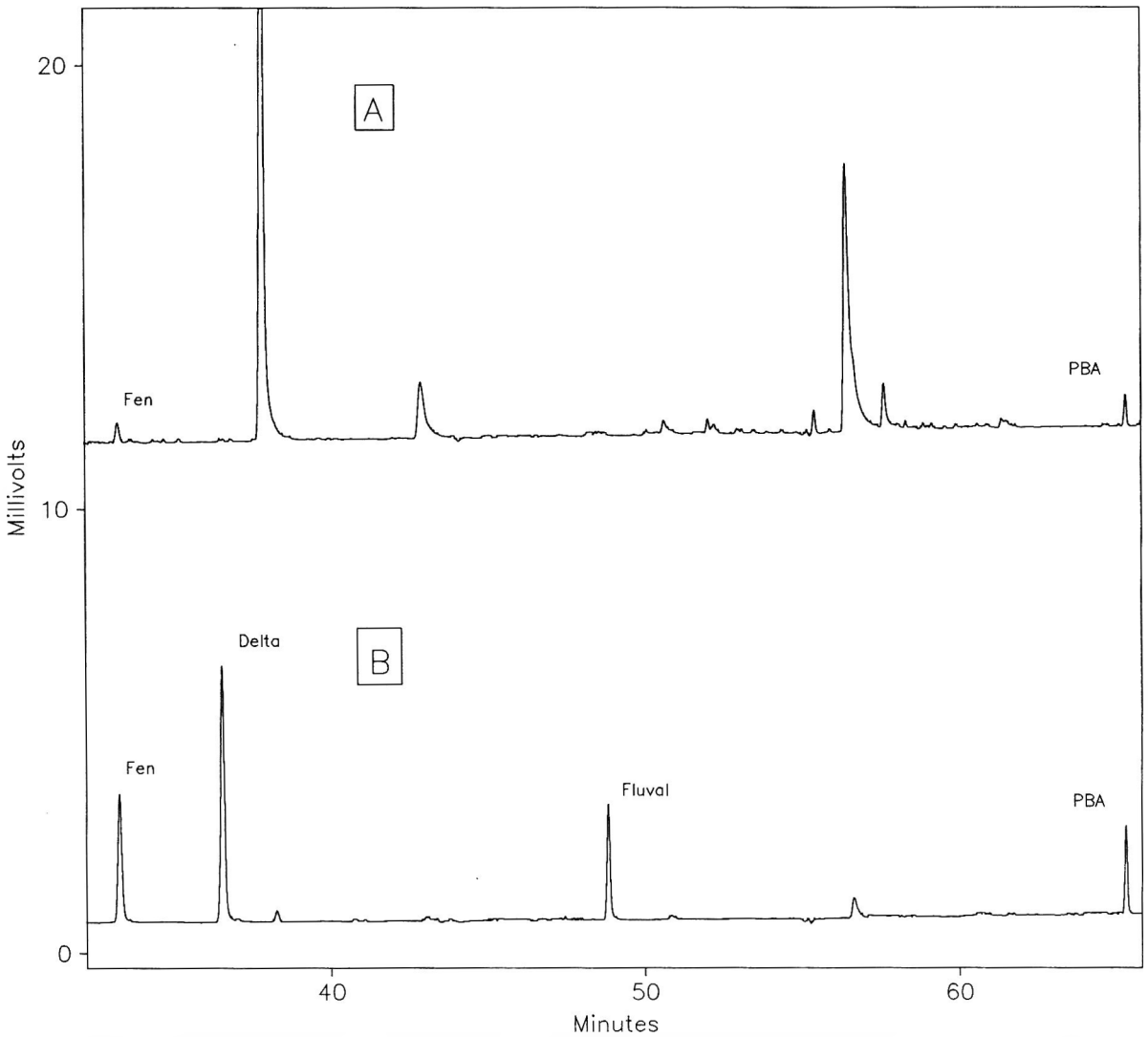

Figure 4. Comparison of a section of the GC–ECD chromatograms of (A) gunpowder green tea and (B) standard pyrethroid acids (as hexafluoroisopropyi esters) showing a residue of fenvalerate acid (Fen) and 3-phenoxybenzoic acid (PBA) in the tea.

et al. (3) used 1N HCl at 60°C for 24 h to cleave 3-phenoxybenzoyl glucoside. Quistad et al. (8) used 0.1M KOH at 38°C for 16 h, and Fitch et al. (10) refluxed their extracts in 0.1N KOH for 1 h to cleave the glucoside of fluvalinate acid. In retrospect, these conditions are unnecessarily severe. We observed that the half-lives for hydrolysis of 3-phenoxybenzoyl glucoside in 1N HCl at 75°C and in 0.1N NaOH at room temperature were 27 min and ≤20 s, respectively. The speed of basic hydrolysis is consistent with the character of 1-*O*-acyl glucosides as 'activated' esters; their reactivity with nucleophiles is well documented (20, 21). We did not examine the hydrolysis of other glucosides but arbitrarily set 10–15 min at room temperature as a convenient period for hydrolysis. Coresidues of the parent pyrethroids are not expected to hydrolyze under these conditions of infusion and isolation because they are not 'activated' esters. A tea sample prepared from an infusion of orange pekoe spiked with pure permethrin (0.5 ppm) showed no pyrethroid acids in the GC–ECD chromatogram even though 1% conversion would have been detectable. The other pyrethroids have no functionality that would make them behave differently.

Because of the emulsions observed during extraction of tea infusions, neutral and acidic constituents were separated on a small, commercial aminopropyl column rather than by extracting the tea infusion at basic and then at acidic pH. The aminopropyl column not only easily and effectively separated the neutral coextractives from the tea acids but also removed the bulk of the color. The acid fraction was only faintly colored. Evaporation of the concentrated acid fraction with hexane removed excess formic acid (which forms an azeotrope with hexane) and residual methylene chloride.

Derivatization with hexafluoroisopropyl alcohol–diisopropylcarbodiimide easily provided the acids with an ECD-detectable group (13). Previous derivatizations of pyrethroid acid metabolites were either more cumbersome (22) or the products were not amenable to EC detection (10). Final cleanup on silica gel removed polar coextractives and the remaining color. Although a commercial silica column (1 mL) was convenient for

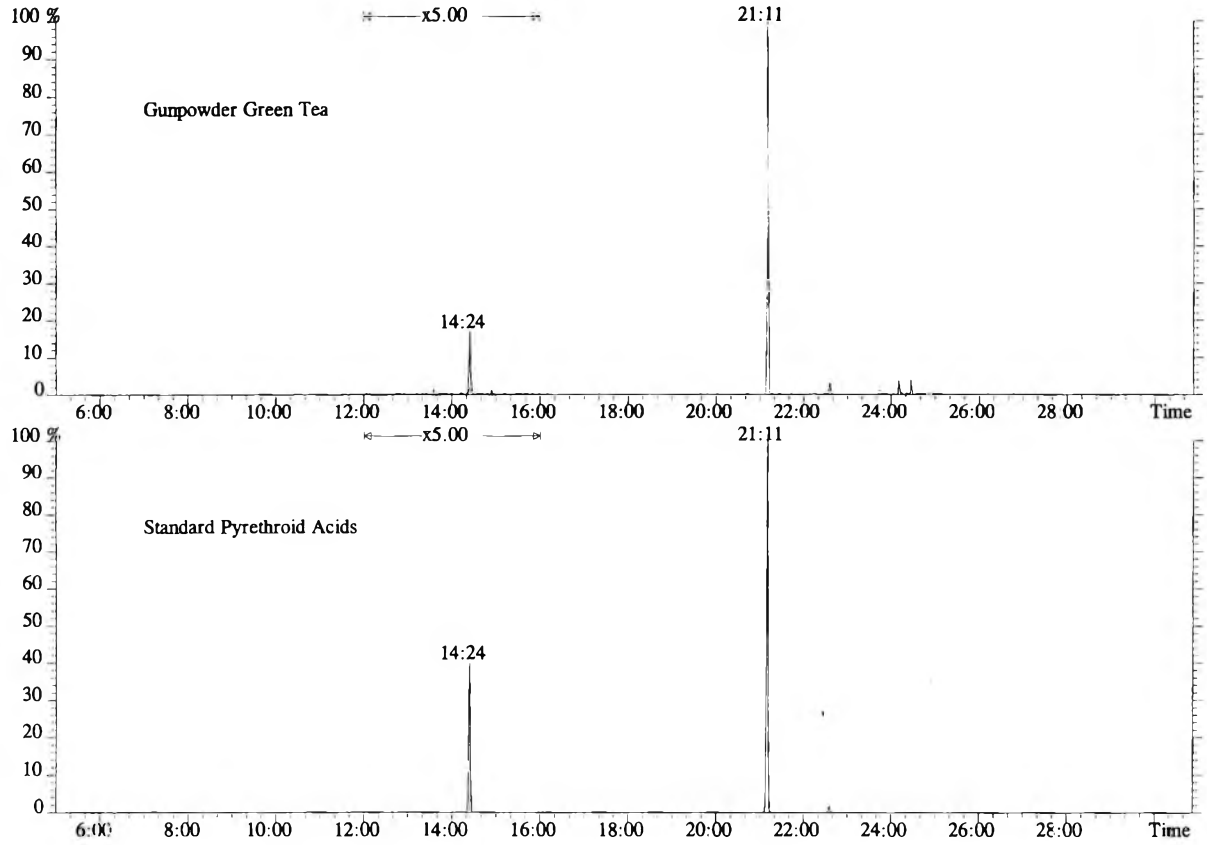

Figure 5. Comparison of GC/MS traces at *m/z* 364 of an extract of gunpowder green tea and standard hexafluoroisopropyl esters of pyrethroid acids that confirm the presence of fenvalerate (14.25 min) and 3-phenoxybenzoic acid (21.11 min) in the tea (each trace is individually normalized to its largest peak).

this step, we observed that a number of small interferences leached from the column and complicated the chromatograms. These interferences were avoided by using an equivalent amount of glass-bottled silica in a small pipette.

Column type (DB-5, DB-1701, DB210, and DBWax), carrier gas (He, H_2), temperature profile, and flow rate were examined to provide retention windows for the 7 pyrethroid metabolites in the teas examined. The best conditions are outlined in the experimental section, and Figures 2a and 2b show the retention windows available for the 7 pyrethroid hexafluoroisopropyl esters in a sample of English breakfast tea. Using H_2 rather than He allowed a lower maximum temperature (145°C rather than 175°C) and gave an excellent retention window for the 3-phenoxybenzoic ester. This was not possible with He as the carrier gas. The teas still had a trace coextractive with a retention time very close to that of the *cis*-permethrin ester (14.23 ± 0.01 and 14.37 ± 0.01 min, respectively). As a result, the 2 peaks overlapped. Fortunately, the *trans*-permethrin ester had a clear retention window, and because the *cis* is not found without the *trans*, doubts about the presence of the *cis* are minimized by reference to the *trans*. Figure 3 shows a comparison of blank orange pekoe extract with extracts of the same tea spiked with glucosides at 0.05 and 1.0 ppm (acid equivalent). The ester of *cis*-permethrin acid (0.02 ppm) is clearly shifted from the coextractive but difficult to quantitate. At 1 ppm, the interference is swallowed up in the *cis*-isomer peak. This particular coextractive was found in each tea but varied significantly in concentration: gunpowder green, 0.004 ppm; orange pekoe, 0.009 ppm; Japan sencha green, 0.011 ppm; lapsang souchong, 0.024 ppm; and jasmine, 0.031 ppm. The GC samples were relatively free from nonvolatiles, judging by the maintenance of peak symmetry and sharpness over a period of 4 to 6 weeks with the same retention gap.

Calibration curves of the standard mixture of acids as their hexafluoroisopropyl esters were generated in the range of interest, 0.02 to 2.0 ppm. Linearity was excellent in all cases, with R values of ≥ 0.999. The esters of the *cis*- and *trans*-permethrin acids gave essentially identical slopes indicating equal responses on the EC detector.

Limits of detection (at a signal-to-noise ratio of 3) for the standard acids are ≤ 0.005 ppm. Cyhalothrin acid is the most sensitive (0.0014 ppm), in part because it elutes rapidly as a very narrow peak, and 3-phenoxy benzoic acid is the least sensitive (0.004 ppm). In actual tea samples, however, the limits of detection will depend on the coextractive interferences. Table 1 lists the acid equivalent values measured at each acid retention time for the teas. Values of ≤ 1–2 ppb represent baseline noise, while higher values are observable peaks. Although some variation in coextractives was expected with the tea varieties, in fact, the chromatograms were quite similar for most of the teas, differing more in the relative intensities of the peaks than in the

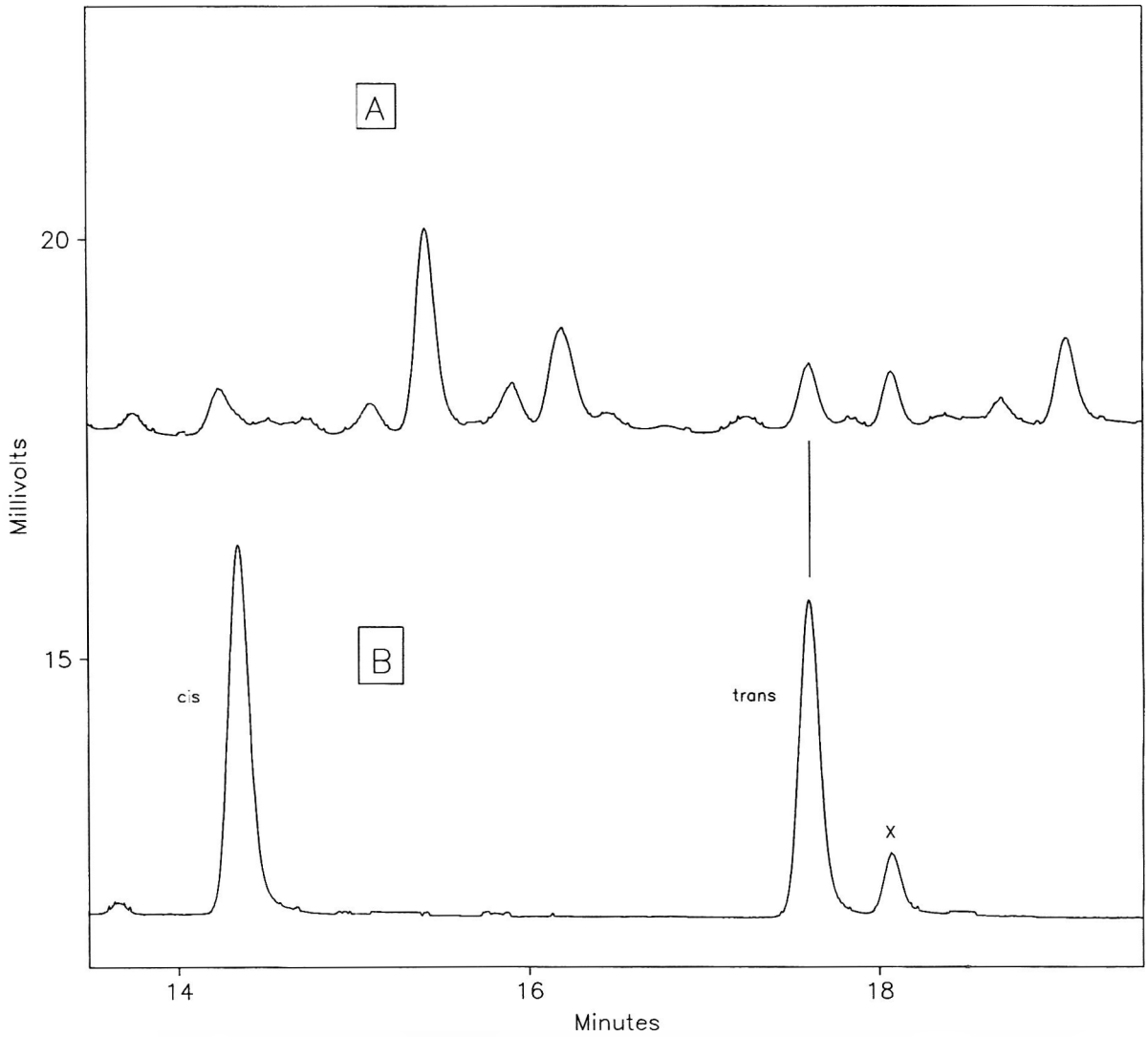

Figure 6. Comparison of a section of the GC–ECD chromatograms of (A) an extract of lapsang souchong tea and (B) standard *cis*- and *trans*-permethrin acids (as hexafluoroisopropyl esters) that show a residue of these acids in the tea (X is a reagent blank contaminant).

number of peaks. Lapsang souchong, which is flavored with natural smoke, was the most complex.

GC/MS conditions were established, but not optimized at this point, for the tea extracts. Although it is preferable to use at least 2 ions for selected-ion monitoring, cyhalothrin showed a very weak parent ion in its electron impact (EI) mode, so only the fragment at *m/z* 357 (75%) was monitored. The next highest fragment was *m/z* 197 (100%). We considered that this ion was too distant from the upper masses (445, 447) being scanned to be used efficiently. The ions used for the GC/MS monitoring are listed in Table 2. GC/MS analyses on a few tea samples have shown that some of the apparent interferences are not co-extractives but actual pyrethroid metabolite residues. By using the listed ions, we were able to show that the China gunpowder green tea contained fenvalerate acid and 3-phenoxybenzoic acid (Figures 4 and 5); the lapsang souchong tea contained fenvalerate, permethrin, and phenoxybenzoic acids (Figure 6); and the jasmine tea had residues of fenvalerate and phenoxybenzoic acids. Japan sencha green had an appreciably higher cyhalothrin peak than the other teas but it has not yet been examined by GC/MS. The second ion for phenoxybenzoic acid at *m/z* 345 is difficult to see in some cases, because it is only 10% of the parent ion's intensity. Nevertheless, this ion was discernible in the GC/MS trace of an extract of orange pekoe tea spiked with glucoside at the 0.05 ppm acid equivalent level.

Both the free pyrethroid acid metabolites and their conjugates are well recovered by our analytical procedure. Extraction of acids from either a 0.2N NaOH solution (after acidification) or an infusion of English breakfast tea (5 g) that had been spiked with 5 µL of the acid stock solutions gave 78–109% recoveries of the acids. Recoveries of glucoside spikes were equally good. For example, duplicate samples of orange pekoe (5 g) were spiked with the glucoside stock solution at 2 levels equivalent to 0.05 and 1.0 ppm free acid (57 µL and 1.14 mL). At the lower level, recoveries were 99, 108, 79, and 105% for cyhalothrin, permethrin, fenvalerate, and phenoxybenzoic acids, respectively. At the higher level, recoveries were 107, 77, 101, and 95%. The range of the duplicates was ≤9%.

In summary, we have developed an analytical method using GC–ECD that can reliably determine residues of pyrethroid acid metabolites and their base-cleavable conjugates that are leached from dry tea when an infusion is prepared. The limit of detection is typically ≤0.01 ppm, and the presence of suspected pyrethroid metabolites may be confirmed by GC/MS.

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Multiresidue Method for the Chromatographic Determination of Triazine Herbicides and Their Metabolites in Raw Agricultural Products

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A method is described for the determination of 19 triazine herbicides and 4 metabolites in 6 agricultural products that represent diverse matrixes. In addition, a modification of this method to determine the more water-soluble metabolite, desethyldesisopropylatrazine, is described. In both these procedures, residues are extracted with methanol, and the product coextractives are removed using solvent partition and cation-exchange solid-phase extraction chromatography. A nitrogen phosphorus detector and DB-17 megabore column are used for the temperature-programmed chromatographic determination of samples fortified at 0.02–1.0 ppm levels. Average recoveries ranged from 81.1 to 106.2% for the parent herbicides and from 59.6 to 87.5% for the metabolites on all crops. An average recovery of 101.1% was obtained for desethyldesisopropylatrazine.

As a group, the triazines are one of the most familiar and widely used classes of herbicides. In 1987, the U.S. Environmental Protection Agency estimated that atrazine, a member of this group of compounds, was the most heavily used pesticide in the United States (1).

As their name implies, the triazines are 6-membered ring compounds containing 3 nitrogen atoms within the ring. The general structure for the symmetrical triazines is illustrated in Figure 1 (2). In this figure, R_1 usually designates –Cl (as in atrazine, simazine, and propazine), $-OCH_3$ (as in atratone, prometone, and simetone), or $-SCH_3$ (as in ametryne, dipropetryne, and simetryne). R_2 and R_3 are usually of the form $-NHC_nH_{2n+1}$, and the alkyl group is generally ethyl, propyl, or butyl. R_2 and R_3 may be the same or different. The triazine metabolites that were studied were those that lost C_nH_{2n+1} from either the R_2 or R_3 positions or from both positions. These compounds are also the metabolites included in the tolerances for atrazine and simazine (3).

Several multiresidue methods have been developed for the analysis of triazines in various commodities. The majority of these procedures analyze triazines in either water or soil and involve some type of adsorptive chromatographic cleanup step.

Multiresidue procedures for determination of these herbicides from water use either solvent extraction followed by purification on an aluminum oxide column or solid-phase extraction (SPE) (4, 5). The SPE techniques often involve C_8 or C_{18} bonded silica (6–8) with gas chromatography (GC) and nitrogen selective detection, and have become the more widely used of the 2 multiresidue procedures. Cation-exchange resin has also been used as SPE media to isolate triazines from water (9). This procedure uses liquid chromatography (LC) with UV detection to analyze the concentrated triazines.

Studies (10, 11) of surface and groundwater that included metabolites in their determinations found the desalkyl metabolites of atrazine present as residues. These metabolites were also analyzed by methods developed for determining residues of these herbicides in soil (12–15). Recovery studies (12) conducted for 3 triazine pesticides and 4 metabolites (desethylatrazine, desisopropylatrazine, desethyterbutylazine, and hydroxyterbutylazine) used aqueous acetone extraction and a micro on-line method for determination of the residues by reversed-phase LC (12).

Other studies (13–17) for analyzing triazine herbicides and atrazine metabolites in soil included both LC and GC. These methods extract the residues with an organic solvent followed by cleanup on a combination of columns and Sep-Paks made from alumina, silica, florisil, strong cation-exchange (SCX), or C_{18}.

Work by Ramsteiner et al. (18) represented the first serious attempt to develop a broad spectrum, multiresidue method for determining triazine residues in food crops. They conducted recovery studies with 12 triazine herbicides in pome fruits, vegetables, cereals, alfalfa, grass, potato tubers, and straw. However, recovery studies of the metabolites of any of the herbicides were not conducted. Other weaknesses are the complexity of the chromatographic systems that used packed columns of 3% Carbowax and the 4 different element-specific detectors (electrolytic conductivity, alkali flame ionization, flame photometric, and microcoulometric) needed.

A later study conducted by Roseboom et al. (19) determined 13 triazine herbicides in onions, leeks, peas, rye. and cabbage. This detection system was simpler than that of Ramsteiner et al. (18) but still used packed and capillary columns and 2 de-

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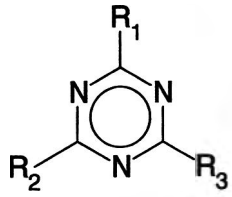

Figure 1. General structure of symmetrical triazine.

tection systems. As with the earlier work (18), Roseboom et al. (19) did not include recoveries of any triazine metabolites.

A more recently developed method (20) used tandem cartridges of Carbopack and SCX to concentrate and purify the extracted triazines. The carbon black cartridge was used to concentrate the spiked herbicides from aqueous vegetable extracts. The compounds were then eluted onto a cation ion-exchange column and desorbed with aqueous KCl–methanol before their determination by LC.

In the present study, a method was sought to determine parent triazines and their metabolites in a variety of agricultural products. The methods described in this study recovered 4 metabolites and 19 triazines from corn, celery, apples, silage, wheat, and milk. The methods used methanolic extraction followed by liquid–liquid partition and purification on a cation-exchange cartridge. The purified extract can then be analyzed for both the chlorotriazines and the nonhalogenated triazines using a single GC system with nitrogen–phosphorus detection (NPD).

METHOD

Reagents

(a) *Solvents.*—Methanol, ethyl acetate, methylene chloride, and acetone (Baxter, Burdick and Jackson Div., Muskegon, MI).

(b) *Filter aids.*—Celite 545 and Hyflow Super Cel (Fisher Scientific, Fair Lawn, NJ).

(c) *Phosphate buffer.*—pH 6.5.

(d) *Elution solution.*—Aqueous 1N NH_4OH–methanol (1 + 3).

(e) *Reference standard solutions.*—Dissolve standards in acetone. Prepare mixed standard solutions by serial dilution of combined aliquots of individual stock solutions to a concentration of 5.0 µg/mL for spiking solutions and 0.25 µg/mL and 0.05 µg/mL for GC solutions. (Reference standard materials were obtained from U.S. Environmental Protection Agency, Pesticides and Industrial Chemicals Repository, Research Triangle Park, NC; Ultra Scientific, North Kingstown, RI; and Crescent Chemical Co., Hauppauge, NY.)

Apparatus

(a) *Blender, high speed.*—One quart (Waring Products Div., New Hartford, NJ).

(b) *Filter paper.*—Shark skin analytical paper, 11 cm (Schleicher & Schuell, Kene, OH).

(c) *Vacuum rotary evaporator with refrigerated, circulating condenser.*—Buchi Rotavapor EL 130 (Brinkmann Instruments, Inc., Westbury, NY) and Neslab TRE 210 (Neslab Instruments, Inc., Portsmouth, NH).

(d) *SPE tubes.*—Supelclean LC-SCX, 3 mL (Supelco, Inc., Bellefonte, PA).

(e) *Gas chromatograph.*—HP 5890 Series II with 7673 automatic injector, 19234B/C nitrogen–phosphorus detector, and HP 3396 series II Integrator (Hewlett-Packard Co., Wilmington, DE).

(f) *Column.*—DB-17 column with 1 µm film thickness and DB-1 with 1.5 µm film thickness, 30 m × 0.53 mm with 5 m fused-silica retention gap for both (J&W Scientific, Folsom, CA).

GC Conditions

Temperatures (°C) were as follows: inlet, 220; detector, 220; DB-17 column, programmed from 150 to 230 at a rate of 4°C/min and held at 230 for 2 min (hold for 12 min at 230°C to determine hexazinone); DB-1 column, programmed from 150° to 210° at a rate of 3°C/min (no hold time). Gas flows (mL/min) were as follows: carrier helium, 15; auxiliary helium, 35; hydrogen, 3.5; air, 90; sensitivity, 1×10^9 amp full scale. Adjust integrator attenuation and power source so that 1 ng atrazine gives ca 50% full-scale deflection.

Sample Preparation

Remove husks from ears of corn. Homogenize corn (kernels plus cob), apples, silage, celery, and milk in food chopper. Grind wheat to pass through 20 mesh sieve.

Extraction

(a) *Corn, apples, and celery.*—Weigh 100 g composite into high-speed blender jar and blend for 2 min with 200 mL methanol. (Add ca 15 g Celite 545 to corn as filter aid.) Filter with suction through 12 cm Buchner funnel fitted with shark skin filter paper and into 500 mL flask. Transfer portion of filtrate equivalent to 50 g sample to 500 mL separatory funnel. Reserve remaining filtrate for determination of water-soluble metabolite, desethyldesisopropylatrazine.

Calculate the volume to transfer with the following formula:

$$\text{Volume} = (\text{percent water in sample} \times 0.5) + 100 - 5$$

(b) *Silage and wheat.*—Weigh 25 g composite into high-speed blender jar and blend for 2 min with 250 mL 10% water in methanol. Proceed as in (a) except take portion equivalent to 10 g sample.

$$\text{Volume} = (\text{percent water in sample} \times 0.1) + 100$$

(c) *Milk.*—Weigh 100 g composite into high-speed blender jar and blend for 2 min with 200 mL methanol and ca 20 g Hyflow Super Cell. Filter with suction through shark skin filter paper overlaid with ca 1/2 in. Hyflow Super Cell into 500 mL flask. Proceed as in (a).

Partitions and SPE Cleanup

(a) *Parent herbicides and majority of metabolites.*—Add 10 mL saturated sodium chloride solution, 100 mL water, and 100 mL methylene chloride to sample extract in 500 mL separatory funnel. Shake funnel vigorously for 30 s and let layers separate. (*Note*: An emulsion will probably form with milk extracts. In such cases, drain lower, organic layer with any emulsion into 500 mL centrifuge bottle and centrifuge at ca 1500 rpm for 5 min. Slowly pour contents of bottle back into separatory funnel, being careful not to reform emulsion.) Drain lower methylene chloride layer through a 1 in. layer of anhydrous sodium sulfate into a 1 L round-bottom flask. Reextract aqueous layer with an additional 100 mL methylene chloride. Evaporate combined extracts to dryness using vacuum rotary evaporator with water bath (<35°C). Dissolve residue in 10.0 mL hexane delivered from a pipet. Decant solution through a tightly packed pledget of glass wool in a small funnel into a glass tube with Teflon-lined screw cap.

Prewash a Supelclean LC/SCX SPE tube with 2 mL acetone and then with 2 mL methylene chloride. Let these and subsequent washes drip through tube by gravity. Pipet 1.0 mL sample solution in hexane to LC/SCX tube. When solution has eluted into tube, wash with 2 mL methylene chloride and two 2 mL portions of acetone. Let each portion pass into the tube before adding the next wash. Expel excess acetone from tube by applying slight positive pressure to top of tube or vacuum to bottom. (*Note*: Commercially available vacuum manifolds are convenient for holding and applying vacuum to the tubes.)

Add 10 mL pH 6.5 phosphate buffer and 10 mL methylene chloride to 60 mL separatory funnel. Place LC/SCX tube so that it will drip into the separatory funnel. Elute tube with 2.5 mL 1N NH_4OH–methanol (1 + 3). At this point, slight positive pressure may need to be applied to top of tube to maintain flow. After all NH_4OH–methanol solution has eluted, shake separatory funnel vigorously for 1 min. Open stopcock often to relieve pressure, which builds up at start of shaking. After layers have separated, drain lower layer into 500 mL round-bottom flask. Reextract aqueous layer with a second 10 mL portion of methylene chloride and combine into same round-bottom flask.

Evaporate contents of flask just to dryness on a rotary evaporator with <35°C water bath. Dissolve residue into 2.0 mL acetone by holding bottom of pipet ca 1 in. from bottom of flask and then swirling. Transfer immediately to glass tube with Teflon-lined screw cap or directly into automatic injection vials for GC analysis.

(b) *Desethyldesisopropylatrazine.*—This method is used only for those samples on which atrazine, simazine, or propazine use is suspected. An indicator would be finding the parent compound, desethylatrazine, or desisopropylatrazine by using the previously described method.

Measure a portion of the reserved filtered methanolic extract equivalent to 25.0 g for corn, celery, apples, or milk, and 5.0 g for silage or wheat. Calculate the appropriate volume for transfer with the following formulas:

$$\text{Volume for 25 g sample} = (\text{percent water in sample} \times 0.25) + 50 - 2.5 \text{ mL}$$

$$\text{Volume for 5.0 g sample} = (\text{percent water in sample} \times 0.05) + 50 \text{ mL}$$

Add the appropriate volume to 250 mL separatory funnel containing 10 mL saturated NaCl solution, extract with two 25 mL portions of methylene chloride, and discard the methylene chloride layers. (*Note*: Add 15 mL distilled water to silage and wheat extracts before partitioning with methylene chloride.) Transfer methanolic layer to 1 L round-bottom flask and evaporate to ca 10 mL on a vacuum rotary evaporator.

Decant through a pledget of glass wool contained in small funnel into original 250 mL separatory funnel containing 50 mL ethyl acetate. Wash round-bottom flask and funnel with two 5 mL portions of water.

Shake separatory funnel for 1 min and let layers separate. Transfer lower aqueous layer to second 250 mL separatory funnel containing 50 mL ethyl acetate and shake for 1 min. Drain acetate layer from first separatory funnel into a 500 mL round-bottom flask through Na_2SO_4. Shake second separatory funnel for 1 min, let layers separate, and drain lower aqueous layer back into first separatory funnel, to which a third portion of 50 mL ethyl acetate was added. Drain solution remaining in second separator through same funnel into same round-bottom flask. Repeat with third extraction and discard aqueous layer. Evaporate contents of flask to dryness on rotary evaporator and dissolve residue in 5.0 mL acetone. Transfer solution into a glass tube with Teflon-lined screw cap.

Prewash a Supelclean LC/SCX tube with 2 mL acetone. Pipet 1.0 mL sample solution into LC/SCX tube and wash tube with two 2 mL portions of acetone. Expel excess acetone and place tube so that it will drip into 125 mL separatory funnel containing 10 mL pH 6.5 phosphate buffer and 50 mL ethyl acetate. Elute tube with 2.5 mL 1N NH_4OH–methanol (1 + 3). Extract buffer layer with three 50 mL portions of ethyl acetate as before, and collect extracts in a 500 mL round-bottom flask.

Evaporate contents of flask just to dryness on a rotary evaporator with <35°C water bath. Dissolve residue in 2.0 mL acetone by holding bottom of pipet ca 1 in. from bottom of flask and then swirling. Transfer immediately to glass tube with Teflon-lined screw cap or directly into automatic injection vials for GC analysis.

GC Determination

Inject 3.0–5.0 µL sample solutions onto GC system using conditions described in *GC Conditions*. (*Note*: For analysis of milk at 0.02 ppm, double sensitivity of instrument and inject 5.0 µL.) Identify residues by comparing retention times from sample injections to those of standard solutions, and calculate amounts by comparing peak responses (either areas or heights) of samples to standards.

Results and Discussion

The method was evaluated by recovery studies of added standards of 6 different crops. The products were chosen to

give a wide range of sample types and consisted of corn, apples, celery, wheat, silage, and milk. All products have established tolerances for at least some of the herbicides on which the fortified studies were conducted. Fortifications were added to composites that were prepared on the whole, edible portion except for corn and apples. Because all triazine tolerances (3) are established for corn kernels plus cob with husks removed, this product was prepared in this manner. Apples were prepared by grinding the entire fruit including core.

Recovery results for the spiked samples are shown in Table 1. Wheat and silage were fortified at 1.0 ppm for each of the 19 triazine herbicides and 4 metabolites. Corn, apples, celery, and milk were all fortified at 0.1 ppm for each of the same 23 compounds. Milk was also fortified at 0.02 ppm to reflect the lower tolerances that this product has for many of the herbicides of interest.

Except for silage, all recoveries of the parent herbicides were 75% or better. The reason for the sporadic low recoveries (36% for hexazinon) for silage is not known.

Because of the poor response on the GC system used in this study, a fortified recovery was not obtained for hexazinone in milk at the 0.02 ppm level. Hexazinone has the lowest response of any of the parent compounds recovered through the method and is only one-tenth as sensitive as atrazine.

Average recoveries for each of the 23 compounds tested were calculated from individual recoveries on each of the 7 commodities or spike levels. These recovery results and the standard deviations and coefficients of variation are listed in Table 2. Except for desisopropylatrazine, all recoveries averaged greater than 80%.

Early attempts to recovery desethyldesisopropylatrazine through the initial method yielded very low recoveries. The cause of these low results was suspected to be the increased solubility in water of the metabolites over the parent. This difference was shown by the recovery rates: the desisopropyl compound was lower than the desethyl, which was lower than atrazine. Because of this solubility problem, the initial method was modified by reducing the amount of methanol–water solution from which the desethyldesisopropylatrazine was extracted. Also, ethyl acetate was substituted for methylene chloride as the extraction solvent. These modifications and other minor changes described in the method resulted in good recoveries for this metabolite.

The modified method used to successfully analyze desethyldesisopropylatrazine was also tried with the desisopropyl metabolite. Recoveries for desisopropylatrazine using the second method were on the average 10% lower than with the original procedure. For this reason, recoveries reported for this compound are those obtained using the initial procedure.

Hydroxyatrazine is a metabolite of atrazine (21) but would not separate on the GC system used in this study. Hydroxy metabolites were analyzed by LC using UV, photodiode-array, and mass spectrometric detection systems (12, 22, 23).

Table 1. Recovery (%) of triazine compounds through entire method from fortified crops[a]

Compound	Corn (0.1 ppm)	Apples (0.1 ppm)	Celery (0.1 ppm)	Wheat (1.0 ppm)	Silage (1.0 ppm)	Milk (0.1 ppm)	Milk (0.02 ppm)
Ametryn	96.2	95.0	98.4	99.7	94.1	96.5	106.3
Atraton	108.4	96.8	101.3	106.2	87.2	105.2	115.7
Atrazine	94.9	96.7	98.1	101.5	102.4	92.3	107.0
Cyprazine	98.5	99.9	92.9	99.9	101.6	97.1	103.7
Desethylatrazine	94.1	56.3	80.0	86.6	85.5	85.4	95.7
Desethylterbuthylazine	90.1	88.0	87.3	99.9	83.0	86.0	78.2
Desisopropylatrazine	56.1	41.9	55.8	59.5	48.8	57.3	97.5
Desmetryn	102.5	89.4	114.4	79.7	71.9	98.1	77.7
Dimethametryn	106.8	87.2	90.8	107.9	99.2	96.1	105.2
Dipropetryn	79.3	83.4	99.3	97.7	86.7	103.5	108.4
Hexazinone	93.7	77.0	87.1	90.1	36.3	106.8	—
Methoprotryne	124.6	83.7	85.8	114.4	104.6	98.9	131.6
Prometryn	92.8	96.1	100.1	101.8	97.1	98.9	107.5
Propazine	91.3	93.0	97.8	99.7	101.7	93.2	106.0
Secbumeton	99.3	97.8	116.5	85.0	70.8	107.9	88.6
Simazine	96.3	89.5	95.3	98.4	99.2	92.4	107.0
Simeton	89.9	76.8	95.2	93.0	68.4	110.3	100.1
Simetryn	91.3	81.7	81.4	99.7	75.5	93.5	88.6
Terbumeton	109.6	126.6	98.6	91.4	67.7	98.4	120.3
Terbutryn	93.3	99.2	101.7	105.3	95.9	101.6	108.6
Terbuthylazine	90.4	88.0	84.4	99.0	90.2	83.7	88.9
Trietazine	92.4	11.4	112.7	84.6	91.9	91.9	74.7
Desethyldesisopropylatrazine[b]	102.0	102.0	111.8	96.1	88.2	97.1	110.8

[a] Value in parenthesis is compound spike.
[b] Desethyldesisopropylatrazine determined using modification of method specific for this compound.

Table 2. Statistical summary of recoveries through entire method of crops fortified at 1.0, 0.1, and 0.2 ppm

Compound	Average, %	SD, %	CV, %	Compound	Average, %	SD, %	CV, %
Ametryn	98.0	4.1	4.2	Prometryn	99.2	4.7	4.7
Atraton	103.0	9.1	8.8	Propazine	97.5	5.4	5.5
Atrazine	99.0	5.0	5.0	Secbumeton	95.1	15.2	16.0
Cyprazine	99.1	3.5	3.5	Simazine	96.9	5.6	5.8
Desethylatrazine	83.4	13.1	15.7	Simeton	90.5	14.1	15.5
Desethylterbuthylazine	87.5	6.7	7.7	Simetryn	87.4	8.3	9.5
Desisopropylatrazine	59.6	17.8	29.8	Terbumeton	101.8	19.6	19.3
Desmetryn	90.5	15.3	16.9	Terbutryn	100.8	5.2	5.2
Dimethametryn	99.0	8.1	8.2	Terbuthylazine	89.2	5.1	5.7
Dipropetryn	94.0	11.0	11.7	Trietazine	94.2	13.7	14.5
Hexazinone	81.8	24.3	29.7	Desethyldesisopropylatrazine[b]	101.1	8.3	8.2
Methoprotryne	106.2	18.4	17.3				

[a] SD, standard deviation; CV, coefficient of variation.
[b] Desethyldesisopropylatrazine determined using method specific for this compound.

Methanol was chosen as the extraction solvent for both methods because it seemed to extract fewer plant coextractives then acetone. The extraction efficiency of methanol was already demonstrated (18). One of these experiments (18) used radiolabeled triazines that resulted in methanol extracting between 85 and 99% of the total radioactivity. A second experiment compared methanolic extraction and exhaustive Soxhlet extraction. This experiment demonstrated that methanol extracted 84.2 to 93.6% of those residues extracted by the exhaustive Soxhlet procedure.

Because recoveries were conducted at 0.02 ppm, the limit of detection for the methods would be at least this low for milk, celery, corn, and apples. Fortifications were not conducted at this level for celery, corn, and apples, but the 0.1 ppm chromatograms of these commodities appeared as free of interferences as that of the 0.1 ppm spike for milk. Sample cleanup, which results in a good chromatogram, appears to be the major factor in obtaining the lower analytical limit. The sensitivity would probably not be quite as low for silage and wheat as for the other commodities because of sample cleanup. More intensely

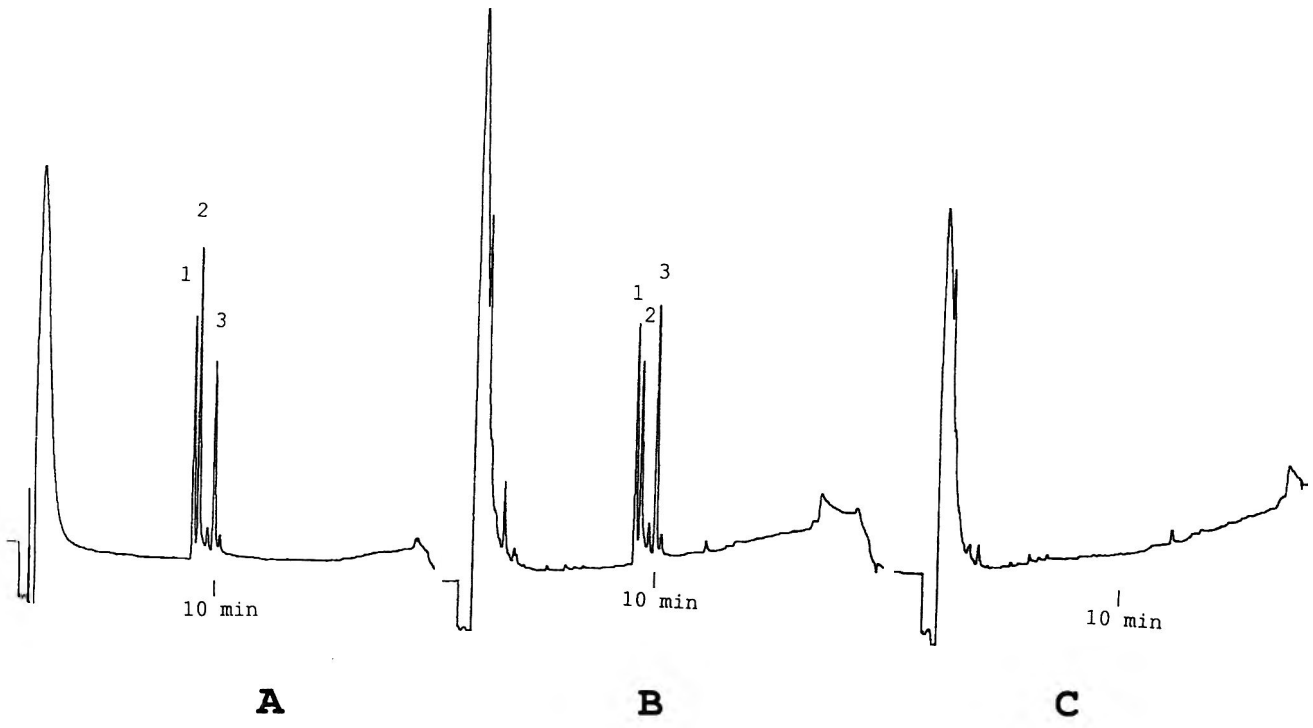

Figure 2. Chromatogram of A, a mixed standard of 1.0 ng each: 1, desethylatrazine; 2, desisopropylatrazine; and 3, atrazine. Chromatogram of B, 10 mg injection of corn extract spiked with 0.10 ppm each of the triazine compounds. Chromatogram of C, 10 mg injection of unspiked corn sample.

colored extracts and larger extraneous peaks in the GC chromatograms indicated more crop coextractives were obtained for these 2 commodities than for the other products.

Several different manufacturers' cation-exchange tubes were tried. We specified the only SCX tube that would work using the eluting solutions listed in the method. The triazine compounds were not eluted from the other tubes by using ammonium–methanol solutions, even when the concentrations of ammonia were increased. This irreversible adsorption indicates a considerable difference in the tube characteristics between manufacturers. Five different lots of Supelco's tubes were tried, and all gave comparable results.

The capacity of the SCX tubes can be exceeded easily. This phenomenon occurred when acetone was used as extraction solvent or when more than 1 mL hexane solution obtained from evaporation of the methylene chloride extract was added to the extraction tubes. The addition of 3 mL (equivalent to 1.5 g sample) of the hexane extract from corn spiked with triazines at 0.1 ppm resulted in the chlorotriazines being desorbed with the acetone and methylene chloride washes. To avoid overloading the cation-extraction tubes, the method should be strictly followed.

The triazine compounds cyromazine, melamine, metribuzin, cyanazine, and procyazine are not recovered by this method. Apparently, cyromazine and melamine are too water soluble to be extracted with either methylene chloride or ethyl acetate. A small amount of cyromazine, approximately 10%, was recovered using the alternate method for desethyldesisopropylatrazine. However, none of the cyromazine metabolite, melamine, was recovered.

Unlike cyromazine and melamine, which are eluted from the SCX tube, the other 3 triazines are not eluted with the conditions used in the method. The reason for their strong attraction for the cation-exchange is not known. Metribuzin differs from the other compounds tested in that it is an asymmetrical triazine, whereas cyanazine and procyazine differ in that each has a nitrile group in one of their alkyl substitution chains. Apparently, these molecular modifications affect the basicity of the compounds to such an extent that methanolic ammonia solutions will no longer remove the compounds from the cation-exchange resin, at least at the concentrations tried.

Figure 2 represents chromatograms of a standard mixture of desethylatrazine, desisopropylatrazine, and atrazine; the extract from a corn sample spiked with the 3 compounds; and an unspiked corn sample extract. The chromatogram obtained from the unspiked corn sample is typical of those obtained from the other 5 commodities tested.

All recovery studies were conducted using the DB-17 column. Several temperature programs were tried with this column, and a rate of 4°C/min from 150° to 230°C gave the best separation in a reasonable amount of time. At least a 2 min final hold time at 230°C was necessary to remove a small extraneous crop peak that would sometimes elute at about 21 min. To determine residues of hexazinone, the hold time needed to be increased to 12 min.

The DB-17 chromatographic system would not completely separate all of the compounds tested. Table 3 lists the relative retention times and response factors of the compounds tested. A DB-1 column differentiated between the 4 pairs of triazines that had identical retention times on the DB-17 column. With the DB-1 column, temperature programming from 150° to 210°C at the rate of 3°C/min gave the most desirable separation for these compounds. By using the DB-1 column as a confirmation to residue findings on the DB-17 column, the identity of any of the 23 compounds could be determined.

Conclusions

Because many different plant materials contain nitrogen compounds that can be determined by GC–NPD, a strong cleanup procedure was needed. This procedure was not obtained using alumina, silica, florisil, or C_{18} columns, which rely primarily on their adsorptive properties to separate the residues of interest from coextractives. The cation-exchange tube

Table 3. Relative retention times for triazine herbicides and their metabolites on DB-17 and DB-1 columns (0.53 mm) and response factors

Compound	Relative retention time[a]		Response factor, ng for 50% FSD[b]
	DB-18	DB-1	
Melamine	0.76	0.46	22.7
Desethyldesisopropylatrazine	0.80	0.52	14.3
Desethylterbuthylazine	0.89	0.82	1.4
Desethylatrazine	0.89	0.76	2.0
Desisopropylatrazine	0.92	0.72	3.3
Prometon	0.95	1.00	1.2
Atraton	0.96	0.95	1.3
Propazine	0.96	1.04	1.0
Simeton	0.98	0.90	1.4
Terbumeton	1.00	1.06	1.4
Atrazine	1.00	1.00	1.0
Trietazine	1.01	1.11	1.2
Terbuthylazin	1.02	1.10	1.2
Simazine	1.03	0.96	1.1
Secbumeton	1.14	1.22	1.8
Cyromazine	1.26	1.00	16.5
Cyprazine	1.30	1.39	1.4
Desmetryn	1.33	1.37	1.6
Prometryn	1.35	1.57	1.3
Ametryn	1.38	1.52	1.4
Metribuzin	1.41	1.37	2.7
Simetryn	1.41	1.47	1.3
Terbutryn	1.42	1.65	1.4
Dipropetryn	1.44	1.75	2.1
Dimethametryn	1.68	2.07	1.4
Cyanazine	1.71	1.72	2.7
Procyazine	1.99	2.19	1.5
Methoproteryne	2.07	2.64	2.3
Hexazinone	2.94	3.15	10.0

[a] Retention times are relative to atrazine, which elutes in 10 min on the DB-17 column and in 6.3 min on the DB-1.

[b] Response factors were determined on a DB-17 column. FSD, full-scale deflection.

cleanup procedure yielded a chromatogram nearly free of interfering responses. The methods described are suitable for the simultaneous determination of a wide range of triazine herbicides and their metabolites.

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RESIDUES AND TRACE ELEMENTS

Determination of Hydramethylnon Residues in Grass by Liquid Chromatography with Confirmation by Liquid Chromatography/Mass Spectrometry

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An improved method for determination of hydramethylnon residues in pasture grass is described. The method uses (*1*) the hydrochloride salt of hydramethylnon to improve its water solubility and (*2*) an acid–methanol precipitation to remove chlorophylls while leaving the analyte in solution. The liquid chromatographic method has a validated sensitivity of 0.05 ppm with controls showing <0.004 ppm. The overall average recovery from 0.05 to 0.50 ppm was 98%, with a standard deviation of 11%. Samples showing a positive response (0.05 ppm or higher) and requiring mass spectrometric confirmation are directly amenable to liquid chromatography/mass spectrometry without additional sample preparation.

Hydramethylnon (Figure 1), the active ingredient in Amdro (American Cyanamid) fire ant insecticide, is formulated as a 0.88% bait on pregel corn grits containing 30% w/w soybean oil as an attractant. Hydramethylnon is selectively toxic to insects whose mode of feeding results in in-

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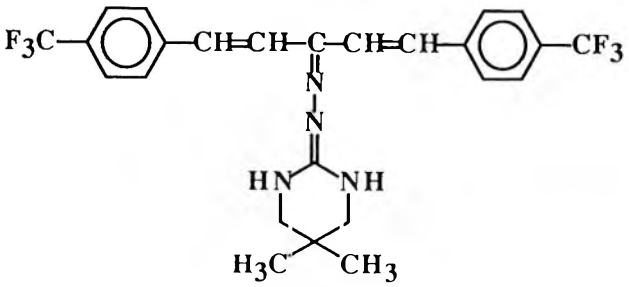

Figure 1. Chemical structure of hydramethylnon.

gestion of the toxicant and shows little or no toxicity to insects with piercing sucking mouth parts or to insects whose exposure is limited to cuticular contact. Hydramethylnon is a slow-acting stomach poison and is very active against the imported fire ant (*Solenopsis invicta*) (1–3). The oral LD_{50} of hydramethylnon for the male rat is 1131 mg/kg of body weight; thus, the compound is considered safe for mammals.

Vander Meer et al. (4) reported rapid photodegradation of hydramethylnon during daylight hours under ambient summer climatic conditions but no decomposition during evening hours. Because no thermal decomposition was detected in the absence of light, decomposition was attributed to photolysis. Mallipudi et al. (5) reported that hydramethylnon rapidly photodegrades in distilled water ($T_{1/2}$ = 42 min) and identified the major photodegradation products. Because hydramethylnon rapidly photodegrades and neither it nor its metabolites are taken up by plants, the only mechanism that allows for the occurrence of residues is lodging of the bait on the commodity (6).

We previously reported a method for determining hydramethylnon residues in pasture grass and crops (6). That method was quite involved and required careful avoidance of light to prevent photodegradation, use of low-actinic glassware throughout, and preparation and use of an XAD-2 column. The first determinative procedure used gas chromatography with electron capture detection (GC–ECD) for final analysis after final cleanup by liquid chromatography (LC). A subsequent shortened procedure accomplished the determination with LC alone with a reversed-phase paired-ion chromatographic system and a variable wavelength UV detector. As a third alternative, GC/negative ion chemical ionization (GC/NICI) mass spectrometry (MS) could be used to simplify sample cleanup. Both GC–ECD and GC/NICI required conditioning of the GC column with several injections of 1% lard solution.

In this paper, we report on advances in the method for determination of hydramethylnon residues in pasture grass. We also have incorporated LC/MS as a confirmatory procedure in such a way as to use the same final extract analyzed by the LC determinative procedure.

METHOD

Rinse all clean glassware thoroughly with methanol and dry before using. Solvents should be distilled-in-glass and suitable for pesticide analyses (Burdick & Jackson Laboratories, Inc., or equivalent). Water should be purified by a Milli-Q water purification system (Millipore Corp.), or equivalent. Samples (typically 4 per set) should be run completely through the method in one day. Unless otherwise specified, allow solvents and sample mixtures to flow only to the top of the cartridge sorbent bed. Do not allow the cartridge to dry.

Reagents and Apparatus

(**a**) *Hydramethylnon standard solutions.*—From a stock solution of hydramethylnon in acetonitrile (100 μg/mL), prepare standard solutions of 10, 5, and 0.5 μg/mL in acetonitrile. When stored in amber bottles in a refrigerator, the stock solution is stable for 1 month; the 10 and 5 μg/mL dilutions are stable for 1 week. The 0.5 μg/mL solution is prepared daily. Hydramethylnon is available from American Cyanamid (Princeton, NJ).

(**b**) *LC standard solutions.*—Pipet 2.0 and 1.0 mL of the 10 μg/mL standard solution and 10 and 5 mL of the 0.5 μg/mL standard solution into separate 100 mL volumetric flasks. Dilute each flask to the mark with the LC dilution solvent to give LC standard solutions containing 0.20, 0.10, 0.05, and 0.025 g/mL. These solutions are prepared daily.

(**c**) *LC dilution solvent.*—Dilute 200 mL deionized water to 1 L with acetonitrile.

(**d**) *LC mobile phase.*—Mix 5 mL triethylamine with 150 mL deionized water in a 1000 mL graduated mixing cylinder. Dilute to 1 L with acetonitrile and shake to mix. Filter through a Rainin Nylon-66 (0.45 μm) filter, or equivalent.

(**e**) *Pre-elution solvent.*—Add 0.5 mL 0.05N HCl to a 100 mL volumetric flask and dilute to the mark with acetone.

(**f**) *Elution solvent.*—Add 10.0 mL 0.05N HCl to a 200 mL volumetric flask and dilute to the mark with acetone.

(**g**) *Extraction solvent.*—Add 40 mL concentrated HCl to 200 mL water and dilute to 4 L with acetone. Mix well.

(**h**) *Vac-Elut processing station.*—Varian, Cat. No. A16000.

(**i**) *Solid-phase extraction cartridge.*—Isolute, MF C_{18} cartridge, 1000 mg/6 mL capacity tube, Cat. No. 240-0100-C, International Sorbent Technology, distributed by Jones Chromatography, Lakewood, CO. Condition prior to use with 5 mL methanol followed by 5 mL water using the Vac-Elut processing station.

(**j**) *Liquid chromatograph.*—An ABI Spectroflow Model 400 pump equipped with an ABI Spectroflow Model 783 UV absorbance detector, a Spectra-Physics Model 4290 recording integrator, and a Rheodyne Model 7125 injector fitted with a 200 μL loop. Use a Rexchrom S5-100-ODS (25 cm × 4.6 mm id) column fitted with a 0.5 μm in-line frit filter (Supelco, Inc.) just before the column. Do not use a guard column. Replace the frit when the mobile phase pressure becomes excessive because of plugging of the frit by sample matrix. LC operating conditions: column temperature, ambient; mobile phase flow rate, 0.85 mL/min; detector wavelength, 400 nm; volume injected, 200 μL; retention time for hydramethylnon, ca 7.0 min.

(**k**) *Liquid chromatograph/mass spectrometer.*—Same basic LC system as described earlier except a Whatman Partisil 5

ODS-3 (25 cm × 4.6 mm id) column is used with a 100 µL loop at a flow rate of 1.5 mL/min. (Retention time of hydramethylnon, ca 3.5 min.) For mass spectrometry, a Finnigan-MAT TSQ70 triple-stage quadrupole equipped with a Finnigan-MAT thermospray LC/MS accessory. Operate in the negative ion detection mode with discharge ionization (discharge voltage, 1500 V; discharge current, 100 µA). Thermospray interface parameters: vaporizer temperature, 80°C; aerosol temperature, 225°C; repeller voltage, −5 V. Mass spectrometric parameters: scan mode, Q3MS; conversion dynode voltage, +20 kV; electron multiplier voltage, −1250 V; preamplifier gain, 10^{-9} A/V; ion monitored, *m/z* 494; dwell time, 1.0 s.

Procedure

Freeze a quantity of commodity sufficient to provide representative sampling (typically 1 to 2 lb from a field study), mix with powdered dry ice, and chop with prechilled food chopper. Mix chopped sample well and let stand in freezer until dry ice has completely dissipated.

Accurately weigh 20 g pasture grass into a 1 quart Mason jar. Add 250 mL extraction solvent and extract at medium-high speed on an Omni-Mixer for 10 min. Filter ca 60–100 mL slurried extract through a glass microfiber filter paper set in a 600 mL sintered glass funnel (medium porosity) under vacuum. Pipet a 25 mL aliquot of the filtrate into a 250 mL evaporation flask and evaporate to ca 2 mL on a rotary evaporator. After complete evaporation of the acetone, add 25 mL methanol to the aqueous mixture and dissolve the residue by sonication.

With a pipet, add 1 mL 2N HCl and 12 mL water to the sample. Swirl the mixture gently and allow to stand for 1–2 min. Then pour the mixture into a 75 mL disposable fritted reservoir fitted on the top of the C_{18} cartridge. Using vacuum, pull the precipitation mixture through the cartridge at the rate of ca 1 drop/s and discard the eluate. Rinse the evaporation flask with 5 mL water, add rinse to the reservoir, and pull the rinse through the cartridge. Stop the vacuum when the wash reaches the top of the sorbent bed. Discard the eluate and remove the 75 mL reservoir. Rinse the cartridge with an additional 5 mL water to ensure complete removal of acid and discard the wash. In succession, rinse the cartridge with the following solvents at a rate of ca 1 drop/s: 5 mL 10% water in methanol, 5 mL methanol, 5 mL acetone, 8 mL ethyl acetate. Finally, rinse the cartridge with 3 mL (measured by pipet) of the pre-elution solvent. Carefully elute to the top of the bed only, discard the wash, and remove the C_{18} cartridge from the Vac-Elut processing station.

Attach a 10 mL plastic syringe barrel to the top of the C_{18} cartridge and add 3 mL elution solvent to the syringe. Carefully insert the plunger and elute at a rate of ca 1 drop/s. Push all the elution solvent through the cartridge bed into a 100 mL pear-shaped flask. Add ca 5–15 mL methanol to the eluate and evaporate to apparent dryness on a rotary evaporator. Add 2 mL LC dilution solvent to dissolve the residue and swirl to mix. If the final sample appears turbid, use a disposable syringe to filter through a 0.5 µm filter. Inject 200 µL aliquots onto the LC system. Dilute samples containing high residues with LC dilution solvent to fit on the standard curve.

Quantitate samples and standards by peak height. Check the linearity of the standard curve by using the LC standard solutions (0.025–0.20 µg/mL). The 0.10 µg/mL standard is used as the working standard for each set of samples analyzed. Calculate the residue with the following formula:

$$\text{Residue (ppm)} = 10 \times R(samp) \times V \times C(std) \times \frac{DF}{R(std) \times W}$$

where 10 is the ratio of the total extraction solvent volume to the volume of extract taken for analysis (250 mL/25 mL), R(sample) is the response of the sample, R(std) is the response of the standard, V is the volume (mL) of the final solution for LC (usually 2 mL), DF is the dilution of the sample if V is too concentrated (DF = 1 if no dilution is made), C(std) is the concentration (µg/mL) of the standard solution (usually 0.10 µg/mL), and W is the amount of sample (g) used in the analysis (usually 20 g).

For extracts requiring LC/MS confirmation at the 0.05 ppm level, inject 100 µL aliquots of the 0.05 µg/mL standard solution and the same extracts analyzed previously by LC. Measure the retention time and response of the analyte peak in the *m/z* 494 mass chromatogram. The extract contains hydramethylnon at >0.05 ppm when the response of the analyte peak in the extract exceeds that of the 0.05 µg/mL LC standard solution. Alternatively, calculate the residue (in ppm) by using the equation given above.

Results and Discussion

The first key to improvement of this residue methodology over that reported previously (6) was the recognition that the solubility of hydramethylnon in water could be increased dramatically by working in an acidic medium. Hydramethylnon itself is essentially insoluble (5–7 ppb) in water (5). By working with aqueous acid, a hydrochloride salt is formed, which greatly enhances the water solubility of the parent molecule. Not only did this modification improve extractability into acetone–water, it also prevented the analyte from binding onto glassware when hydramethylnon was in an aqueous solution. Although hydramethylnon is still photosensitive and must be handled with some care, many losses encountered previously were found to arise not from photodecomposition but from binding to glassware.

The increased water solubility of hydramethylnon with aqueous acid was also crucial in effecting the second key to successful implementation of the improved methodology, acid–methanol precipitation. As discussed in our previous report (6), removing chlorophylls, carotenes, and other plant matrix materials from the initial extract of green foliage without also removing the analyte was a difficult challenge. With the hydramethylnon dissolved in aqueous acidic methanol after initial extraction, further addition of water was found to precipitate the chlorophylls but leave the analyte in solution. The amount of water added was optimized to maximize the removal

Table 1. Recovery of hydramethylnon from pasture grass[a]

		Recovery (%) at indicated fortification level in ppm		
Commodity	Control, ppm	0.05	0.10	0.50
Forage	<0.004	88.4	97.2	99.2
Hay	<0.004	101.0	98.8	102.8

[a] Average of duplicate samples processed through the procedure.

of chlorophylls without occluding the analyte in the precipitate. The precipitate was conveniently removed with the frit of the disposable reservoir during loading of the extract on the C_{18} cartridge.

The selection of the C_{18} cartridge, a monofunctional non-end-capped C_{18}, was critical. Without residual silanol groups for hydrogen bonding, the acid salt of hydramethylnon would not be retained on the cartridge during loading. After loading, a series of organic solvent washes was used for a fractional cleanup of the extract. Bands of various colors representing different plant matrix materials were visibly observed to elute from the cartridge. The final wash with the pre-elution solvent (1/10 the acid concentration of the elution solvent) was required to eliminate 6 major late eluting LC peaks. These peaks, which eluted over the span of an hour in the LC analysis, caused carryover problems in subsequent analyses if the stronger elution solvent was used immediately after the organic solvent washes.

The higher wavelength used for UV detection in this study (400 nm versus 300 nm previously) also imparted additional specificity to the UV detection of the method. The 300 nm absorbance maximum reported previously (6) for hydramethylnon was determined with an LC mobile phase not containing triethylamine. With triethylamine in the mobile phase, the 300 nm absorbance maximum was still present but a second UV peak of approximately the same absorbance was generated at 400 nm.

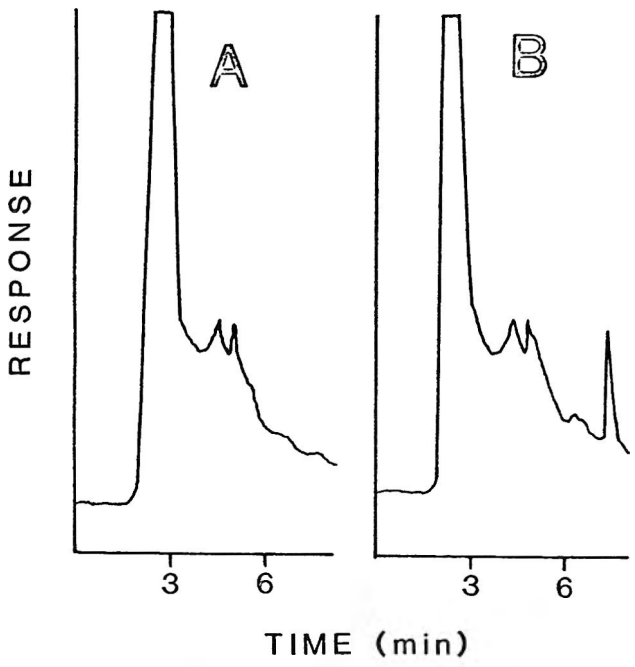

Figure 2. LC/UV chromatograms of (A) control grass hay and (B) grass hay fortified at 0.05 ppm.

To validate the LC determinative method, untreated grass samples (forage and hay) were spiked with the appropriate hydramethylnon standard solution and immediately carried through the assay. Recoveries were run in the fortification range of 0.05 to 0.50 ppm, and results are given in Table 1. Overall, the recoveries expressed as the average ± 1 standard deviation were 95 ± 14% for forage and 101 ± 6% for hay. Control tissues showed apparent hydramethylnon residues of <0.004 ppm, the limit of detection for the method. Figure 2 shows typical LC/UV chromatograms for hay fortified at 0.05 ppm and control hay processed through the procedure.

Although the previous mass spectrometric procedure was relatively short (6), it still necessitated separately processing another sample for analysis. To eliminate additional sample preparation or the workup of an entirely different extract, the optimal mass spectrometric confirmatory method would simply tack on the end of the determinative method and use the same extract and, preferably, the same chromatographic technique as the determinative procedure. Essentially, one would like to replace the detector of the determinative method with a mass spectrometer. For confirmation of hydramethylnon residues in grass commodities, we found that LC/MS could be directly applied to the same extract analyzed by LC/UV without additional sample preparation. Further, the same mobile phase and essentially the same LC column were also directly usable for LC/MS confirmation.

Because the high electron capture cross-section of hydramethylnon previously led to successful negative ion detection with GC (6), discharge ionization coupled with negative ion detection was used in the LC/MS procedure. Under these mass spectrometric conditions, almost solely the M^- of hydramethylnon was generated at *m/z* 494. This is the same ion generated and monitored in the GC/NICI method (6). The NICI mass spectrum of hydramethylnon has been previously published (5). An alternative source of thermal electrons for negative ion generation could be obtained from the filament rather than from discharge ionization. As expected, this mode of ionization gave essentially identical results. An attempt at conventional thermospray LC/MS with buffer ionization with ammonium acetate in the mobile phase rather than triethylamine gave an *m/z* 493 ion, $[M - H]^-$, resulting from proton abstraction from hydramethylnon by acetate. However, the response with buffer ionization was only 1% of that obtained with discharge ionization. Consequently, buffer ionization was abandoned. Thermospray LC/MS in the positive ion mode was not investigated because compounds showing elevated negative ion response also exhibit depressed positive ion response (7).

Figure 3 compares the LC/MS analyses of a 5 ng standard (equivalent to 0.05 ppm hydramethylnon in the commodity), an extract of control grass hay, and an extract of grass hay fortified at 0.05 ppm. LC/MS analyses of grass forage are essen-

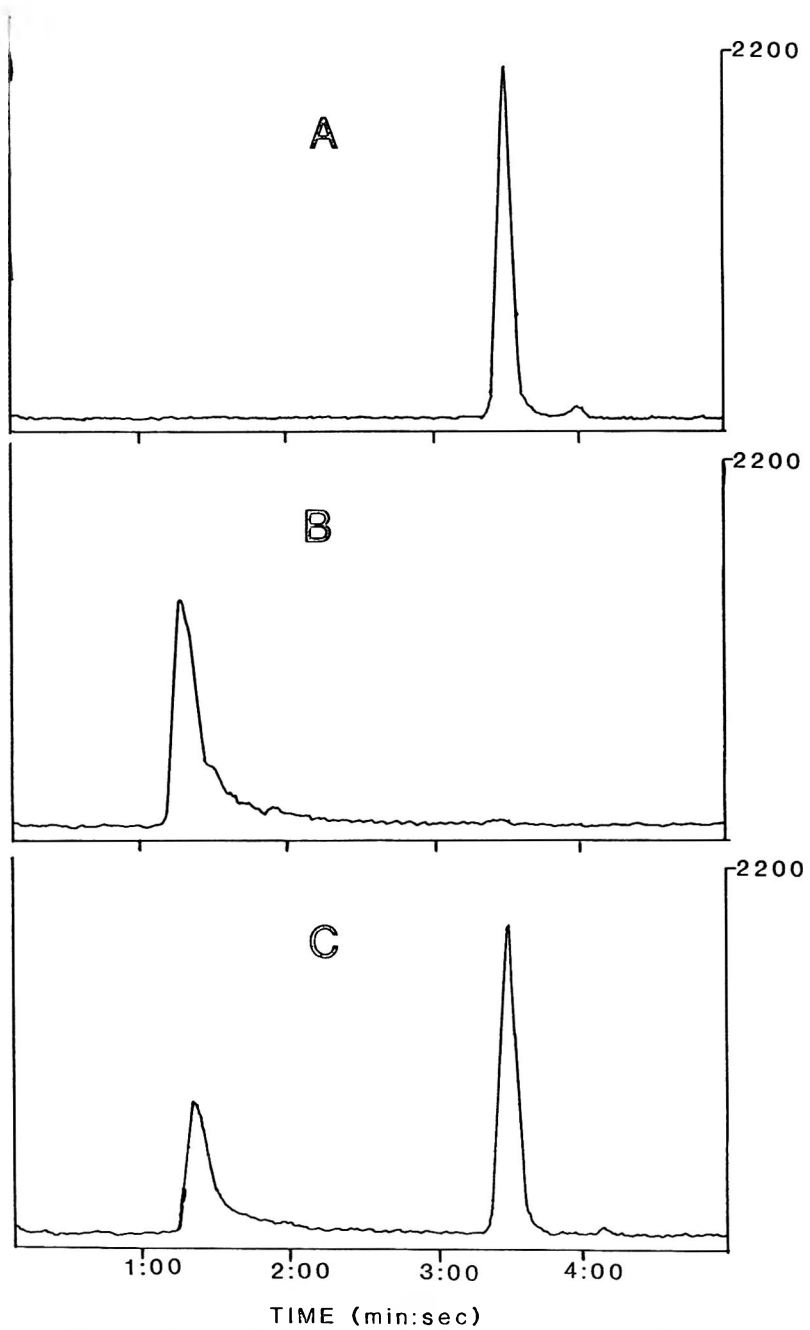

Figure 3. Chromatograms from LC/MS of (A) 5 ng hydramethylnon standard, (B) control grass hay, and (C) grass hay fortified at 0.05 ppm.

tially identical. Although quantitative data are not as important to a confirmatory procedure as the qualitative information from the mass spectrometer, recoveries at the 0.05 ppm level ranged from 56 to 109%. Controls showed apparent residues of <0.005 ppm.

In conclusion, an improved method for determination of hydramethylnon in pasture grass has been developed. By working with the analyte in acidic media, its solubility in water is dramatically improved, thus minimizing loses from binding of the analyte to glassware. Increased water solubility also facilitates a novel acid–methanol precipitation to remove the bulk of the chlorophylls from the initial extract without also removing hydramethylnon. A subsequent fractional cleanup on a C_{18} cartridge was all that was required to purify the extract sufficiently for LC determination. With the improved method, the time to process a set of 4 samples was reduced from 15 h to only 3 h. Samples showing a positive (0.05 ppm or higher) response and requiring mass spectrometric confirmation were directly amenable to LC/MS without additional sample preparation.

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RESIDUES AND TRACE ELEMENTS

Extraction of Methamidophos Residues from Vegetables with Supercritical Fluid Carbon Dioxide

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A simple supercritical fluid extraction (SFE) method has been developed to efficiently extract incurred residues of methamidophos (a very polar pesticide) from fresh vegetable samples by using commercial SFE equipment and moderate SFE conditions. Vegetable samples were mixed with anhydrous magnesium sulfate and extracted with supercritical carbon dioxide at 300 atm and 50°C with methanol as static modifier and a few milliliters of ethyl acetate as trapping system. Methamidophos recoveries were >70% from 33 pepper, cucumber, and tomato samples at incurred levels ranging from 0.1 to 2.2 mg/kg. Triplicate analysis from 2 replicates at an incurred level of 0.53 mg/kg gave a coefficient of variation of 14%. Results for incurred residues of chlorpyrifos, endosulfan, and procymidone in some samples show that the SFE technique could be used as a multiresidue method.

The unique physicochemical properties of supercritical fluids (1, 2) and the increasing concerns over hazards, environmental pollution, and disposal costs associated with organic solvents (3) have led to the growing interest in supercritical fluid extraction (SFE) as a sample preparation method in analytical chemistry (4). In the past few years, a number of papers have described extraction of pesticides from a wide variety of matrixes using supercritical CO_2 (5–9), but the majority of these reports focused on extraction of nonpolar or intermediate-polar pesticides from soils and solid matrixes with low water content. To date, only a few applications of SFE for pesticide residue analysis in vegetables have been reported. Hopper and King (10) described SFE of pesticides from foods using pelletized diatomaceous earth.

This paper describes a method for analysis of methamidophos residues in fresh vegetable samples in which extraction is carried out with supercritical CO_2 after the sample is mixed with anhydrous magnesium sulfate.

This work is focused on methamidophos for several reasons. First, methamidophos is widely used all over the world, and its residues are one of the most commonly found by regulatory agencies in residue monitorings (11, 12) and total diet studies (12, 13). In Almería (a Spanish province where 500 000 metric tons of vegetables are produced annually for export) during the past 3 years, methamidophos has been the most commonly found pesticide in residue monitoring carried out by COEXPHAL (an association of producers and exporters of fruits and vegetables in Almería). The widespread use of this pesticide means that vegetable samples containing incurred residues of methamidophos are readily available, which is a very important aspect in developing an SFE method, because different results can be obtained from the same matrix depending on whether spiked or incurred samples are used in recovery studies (14). Second, methamidophos is a very polar compound, with a water solubility close to 2 kg/kg; therefore, its extraction from a vegetable matrix with a high water content presents a special challenge for development of an SFE method. Third, results for methamidophos may indicate whether the preposed method

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may be used as the prevailing multiresidue method. In fact, the excellent methamidophos recoveries obtained with an ethyl acetate–sodium sulfate-based extraction method, developed in 1971 by Leary (15) to determine methamidophos residues in crops, led some analysts to test it as a multiresidue method (16, 17). At present, the method of Leary, with few modifications, is used by both the Swedish National Food Administration (NFA) and the COEXPHAL monitoring programs of pesticide residues (16, 18).

Experimental

Apparatus and Reagents

(a) *SFE System.*—Isco 1200 (Lincoln, NE) equipped with a Model 260 D syringe pump and controller (maximum pressure, 510 atm), an SFX 2-10 dual-chamber extractor (temperature control, ambient to 150° ± 1°C), a restrictor heater (control temperature, ambient to 100°C), and 2.5 mL (7.6 mm id) and 10 mL (15.1 mm id) stainless steel extraction cartridges with removable 2 μm frits in both ends of cartrige. Uncoated, deactivated, fused silica capillary column (30 cm × 50 μm id) was attached to the outlet of the extractor as a restrictor. Graduated test tubes (10 mL) with Teflon caps, immersed in a 15°–20°C water bath, were used as collection vessels. The restrictor was passed through a hypodermic needle inserted through the cap and immersed in the collection solvent until ca 2 mm above the test tube bottom. In all cases, the volume and flow rate of carbon dioxide were read at the pump and corresponded to the fluid state.

(b) *Gas chromatograph.*—Perkin-Elmer Model 8600 (Beaconsfield, UK) equipped with a 30 m × 0.53 mm SPB-1701, 0.5 μm film thickness fused silica column (Supelco, Bellefonte, PA) attached to a flame photometric detector (FPD) in phosphorus mode. Chromatographic conditions: injector temperature, 220°C; detector temperature, 300°C; oven temperature program, 1 min at 110°C, 30°C/min to 226°C, 4°C/min to 260°C; helium flow rate, 10 mL/min.

(c) *Gas chromatograph.*—Hewlett-Packard Model HP-5890 (Palo Alto, CA) equipped with a 30 m × 0.25 mm HP-5, 0.25 μm film thickness fused silica column attached to a ^{63}Ni electron capture detector (ECD). Chromatographic conditions: injector temperature, 280°C; detector temperature, 300°C; oven temperature program, 1 min at 140°C, 5°C/min to 280°C, and hold for 5 min; helium flow rates, 1 mL/min (carrier), 10 mL/min (split), and 30 mL/min (makeup); splitless time, 0.75 min.

(d) *Carbon dioxide.*—99.995% purity, supplied by SEO (Madrid, Spain).

(e) *Solvents.*—Acetone, ethyl acetate, cyclohexane, and methanol; pesticide residue grade; Panreac (Barcelona, Spain).

(f) *Anhydrous magnesium sulfate.*—>99% purity; Fluka (Buchs, Switzerland).

(g) *Glass wool.*—Pure and washed (Panreac).

(h) *Pesticide standards.*—Pestanal 99% purity; obtained from Riedel de Haën (Seelze, Germany). Pesticide standard solutions of 500 mg/L (stock standard solutions) and methamidophos standard solution of 50 mg/L (spiking solution) were prepared in acetone. Pesticide standard solutions for chromatographic analysis were prepared by suitable dilution of the stock standard solutions with cyclohexane and ethyl acetate.

CO_2 Bubbling Experiments

Experiments were carried out to select the solvent and solvent volume for the trapping system. In these experiments, 15 or 30 mL of compressed CO_2 (300 and 500 atm), after passing through an empty extraction cartridge (kept at 50°C), was decompressed in the collection vessel, bubbling into 3 mL (15 mL of CO_2) or 5 mL (30 mL CO_2) of cyclohexane–ethyl acetate (4 + 1), cyclohexane–ethyl acetate (1 + 1), and ethyl acetate spiked with 2.5 μg methamidophos. Solvents were spiked by adding 50 μL methamidophos spiking solution to the solvent volume in the collection vessel. After the bubbling process, the remaining solvent volume was adjusted to 1 mL by addition of ethyl acetate or by evaporation with nitrogen stream and then to 3 mL with cyclohexane. These extracts were analyzed by GC–FPD, and methamidophos losses during the bubbling process were evaluated. Methamidophos analytical standards were prepared in graduated test tubes by diluting 50 μL methamidophos spiking solution to 3 mL with cyclohexane–ethyl acetate (2 + 1).

Preliminary SFE Experiments

Preliminary SFE experiments were done to evaluate the effectiveness of different conditions to extract methamidophos from spiked glass wool and anhydrous magnesium sulfate. Extractions were carried out in 10 mL extraction cartridges filled with 2.4 g glass wool, 11.2 g anhydrous $MgSO_4$, or 11.2 g anhydrous $MgSO_4$–water (2 + 1) pounded mixture. Materials were weighed into the extraction cartridge and then spiked with 2.5 μg methamidophos by direct addition of 50 μL methamidophos spiking solution. Acetone was allowed to evaporate at ambient temperature until the cartridge gave a constant weight.

Extractions were performed in dynamic mode (after a static equilibrium period of 1 min) at 50°C, at a pressure of 300 or 500 atm, and with 15 or 30 mL compressed CO_2. Additional extraction conditions (40°–90°C temperatures and 5–50 mL flow volumes) were assessed on spiked glass wool. Ethyl acetate (3 mL for experiments using 15 mL or less of CO_2 or 5 mL for experiments using more than 15 mL CO_2) was used as collection solvent. Analytical extracts were obtained as described for the CO_2 bubbling experiments. Analytical extracts from the $MgSO_4$–water mixture were filtered through anhydrous sodium sulfate before GC analysis, because these samples separated into 2 phases when cyclohexane was added to the collection vessel (these samples also partially plugged the capillary restrictor during the elution step). Methamidophos recoveries were determined by GC–FPD analysis. The analytical standard was prepared by diluting 50 μL methamidophos spiking solution to 3 mL with cyclohexane–ethyl acetate (2 + 1).

Spiked and Incurred Vegetable Samples

Peppers, tomatos, and cucumbers to be used as spiked vegetable samples were grown in a greenhouse (Campos de Nijar

Table 1. Conditions and results of CO_2 bubbling experiments

CO_2 conditions			Collection volume, mL		Methamidophos recovery[a], %		
Volume, mL	Pressure, atm	Flow rate, mL/min	Initial	Final	C–EA (4 + 1)	C–EA (1 + 1)	EA
15	300	1.2–1.4	3	1.1–1.3	75	89	95
15	500	1.7–1.9	3	1.6–1.8	66	83	103
30	300	1.2–1.4	5	0.8–1.1	73	81	94
30	500	1.7–1.9	5	0.9–1.3	80	77	98

[a] Three trapping solvents were tested; C, cyclohexane; EA, ethyl acetate.

S.A., Almería, Spain). Spiked vegetable samples were prepared from a 1 kg sample of blended vegetables that were determined not to contain any detectable pesticide residue by a conventional ethyl acetate–sodium sulfate-based extraction and GC/FPD–ECD analysis (18, 19). The pepper sample, however, was determined to contain chlorpyrifos residues at 1.32 mg/kg. Pepper, tomato, and cucumber were spiked with methamidophos at 0.5 and 2.5 mg/kg by adding 50 and 250 µL, respectively, methamidophos stock solution to 50 g aliquots of blended vegetable sample and then mixed and homogenized for 5 min. Spiked vegetable samples were prepared for SFE as described below.

Thirty-one vegetable samples (pepper, tomato, and cucumber) from the 1993 COEXPHAL monitoring program previously determined to contain methamidophos were used to evaluate the effectiveness of the SFE technique in extracting methamidophos residues from incurred vegetable samples. Check samples, determined not to contain any detectable pesticide residue, were also selected. Incurred methamidophos levels in the selected samples ranged from 0.10 to 2.26 mg/kg. Sample 1, a pepper sample with a methamidophos level of 0.53 mg/kg, also contained an endosulfan residue level of 0.09 mg/kg (sum of endosulfan I, endosulfan II, and endosulfan sulfate). After conventional analysis, ca 100 g of blended vegetable sample was kept frozen for ca 6 months until SFE sample preparation.

Table 2. Methamidophos recoveries from spiked glass wool, anhydrous $MgSO_4$, and anhydrous $MgSO_4$–water (2 + 1) by SFE[a]

Pressure, atm	CO_2 volume, mL	Recovery[b], %		
		Glass wool[c]	$MgSO_4$	$MgSO_4$–H_2O (2 + 1)
300	15	100	0	50
300	30	97	0	78
500	15	107	0	43
500	30	102	0	61

[a] Dynamic extraction (1 min static equilibrium time) at 50°C.
[b] Single determination.
[c] Mean of 3 determinations.

Preparation of Vegetable Samples for SFE

Supercritical fluid carbon dioxide extractions of spiked and incurred vegetable samples were carried out on blended sample previously mixed with anhydrous $MgSO_4$ at a vegetable/$MgSO_4$ ratio of 5/7. Specifically, vegetable samples for SFE were prepared as follows: 20 g blended vegetable sample was thoroughly mixed with 28 g anhydrous $MgSO_4$ in a 200 mL glass beaker immersed in an ice water bath. After 5 min, the mixture was thoroughly pounded in a porcelain mortar until a dry and homogeneous powdered mixture was obtained.

Extraction and Analysis of Vegetable Samples

Vegetable–$MgSO_4$ mixtures (SFE vegetable samples) were extracted in 2.5 or 10 mL extraction cartridges. The smaller (2.5 mL) extraction cartridges were filled with ca 0.25 g anhydrous $MgSO_4$ (at the bottom) and 2 g SFE vegetable sample, whereas the 10 mL extraction cartridges were filled with ca 1 g anhydrous $MgSO_4$ (at the bottom) and 8 g SFE vegetable sample. Extractions were performed at 50°C in dynamic mode after a static equilibrium period of 1 min with 15 mL CO_2 and 3 mL ethyl acetate as collection solvent.

Three pressure–modifier conditions were assessed: 300 atm, no modifier; 500 atm, no modifier; and 300 atm, 200 µL methanol (static modifier). Modifier was added directly to the extraction cartridge just before the start of extraction. Samples spiked at 0.5 mg/kg were extracted in 10 mL extraction cartridges, whereas samples spiked at 2.5 mg/kg were extracted in 2.5 mL extraction cartridges.

SFE samples prepared from incurred vegetable samples were extracted in duplicate at 300 atm, in 10 mL extraction cartridges, and with 200 µL methanol as static modifier. Incurred vegetable sample 1 was used to prepare 2 more SFE samples, which were also extracted in duplicate under the same conditions but at pressures of 300 and 500 atm. Method reproducibility was evaluated from results obtained for this sample.

Final SFE extracts were adjusted to 1 mL by addition of ethyl acetate or by evaporation under a nitrogen stream and then diluted to 3 mL with cyclohexane. These solutions contained 0.28 g vegetable sample per mL for extractions in 2.5 mL cartridges and 1.11 g vegetable sample per mL for extractions in 10 mL cartridges. Methamidophos in these solutions (and chlorpyrifos in extracts from the spiked pepper samples) was quantitated with external standards by FPD.

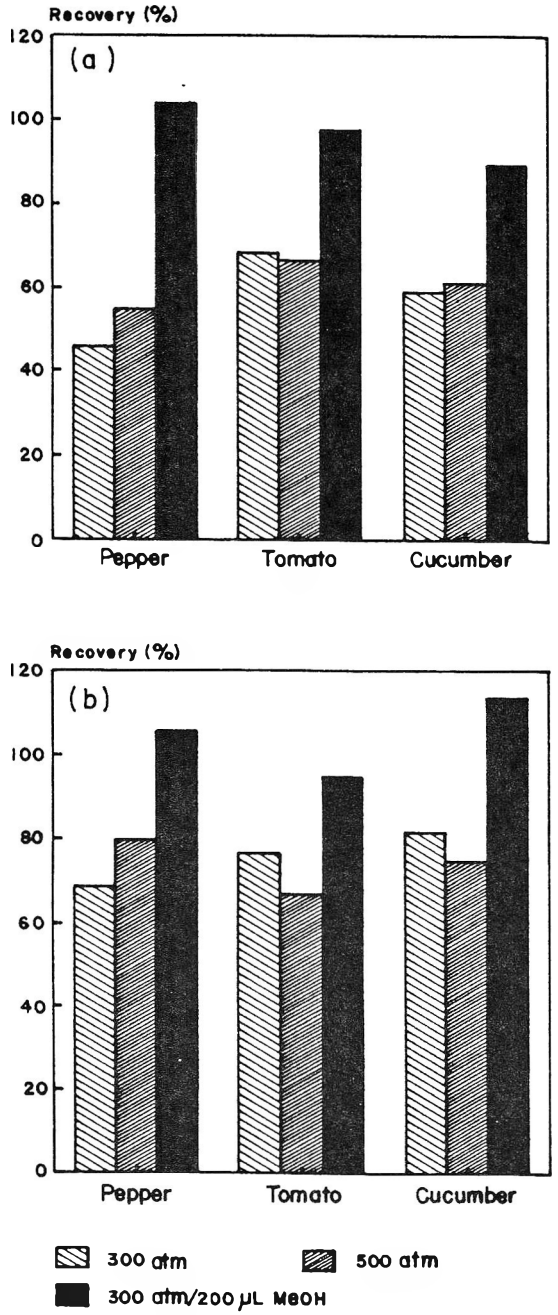

Figure 1. Methamidophos recoveries from spiked vegetable samples by SFE (dynamic mode after a 1 min static equilibrium with 15 mL of compressed CO_2 at 50°C and 3 pressure–modifier conditions): (a) 2.5 mg/kg spiking level and 2.5 mL extraction cartridge; (b) 0.5 mg/kg spiking level and 10 mL extraction cartridge.

Solutions obtained from incurred pepper sample 1 were also analyzed by GC–ECD after diluting the 2.5 mL FPD analytical extracts to 5 mL with cyclohexane. These ECD analytical extracts contained 0.55 g of incurred vegetable sample per mL.

Table 3. Methamidophos and endosulfan levels determined by SFE[a] in sample 1 from the COEXPHAL Monitoring Program[b]

SFE sample	Extraction	Methamidophos, mg/kg 300 atm	Methamidophos, mg/kg 500 atm	Endosulfan, mg/kg 300 atm	Endosulfan, mg/kg 500 atm
A	A_1	0.66	0.63	0.16	0.18
	A_2	0.79	0.66	0.15	0.14
B	B_1	0.73	0.56	0.09	0.14
	B_2	0.81	0.92	0.15	0.13
C	C_1	0.56		0.08	
	C_2	0.80		0.10	
Mean		0.73	0.69	0.12	0.15
CV, %		14	23	29	15

[a] Dynamic extraction (1 min static equilibrium time) at the indicated pressure and at 50°C with 15 mL compressed CO_2 and 200 µL methanol as static modifier.

[b] This sample was determined to contain methamidophos at 0.53 mg/kg and endosulfan 0.09 mg/kg in the COEXPHAL control analysis.

Complementary SFE Studies of Vegetable Samples from the NFA

Additional SFE experiments were carried out in the Chemistry Division 1 of the Swedish National Food Administration (NFA) on 2 pepper samples from the NFA Surveillance Sampling Programme (1992–1993), sampled from 2 different pepper lots imported by Sweden from Almería (Spain). These samples (labeled in the NFA Chemistry Division 1 as K1/93-136 and K1/93-138 pepper samples) were previously analyzed by the Swedish Monitoring of Pesticide Residues in Fruits and Vegetables with the NFA ethyl acetate–GC multiresidue method (16) and determined to contain methamidophos residues at 0.54 mg/kg (K1/93-136) and 0.10 mg/kg (K1/93-138). This last sample was also determined to contain procimidone at 0.78 mg/kg and endosulfan at 0.27 mg/kg. After conventional analysis, a portion of each blended sample was deep-frozen for ca 3 months until SFE was performed. Samples for SFE were prepared by the proposed method but at a vegetable/$MgSO_4$ ratio of 1/1 and with 2.5 mL extraction cartridges.

SFE experiments were performed with an ISCO SFE System 2200, which was similar to that used in our laboratory but which includes 2 Model 260 pump modules for modifier dynamic addition. Extraction conditions (pressure, temperature, and flow volume) were identical to those used in our laboratory for incurred vegetable samples (300 atm, 50°C and 15 mL of compressed fluid), but 3 modifier conditions were assessed: no modifier, 200 µL methanol as static modifier, and 3% (v/v) methanol as dynamic modifier. Ethyl acetate (10 mL) was used as collection solvent. Analytical extracts were prepared as described earlier and analyzed by NFA capillary GC–FPD and GC–ECD methodology (16).

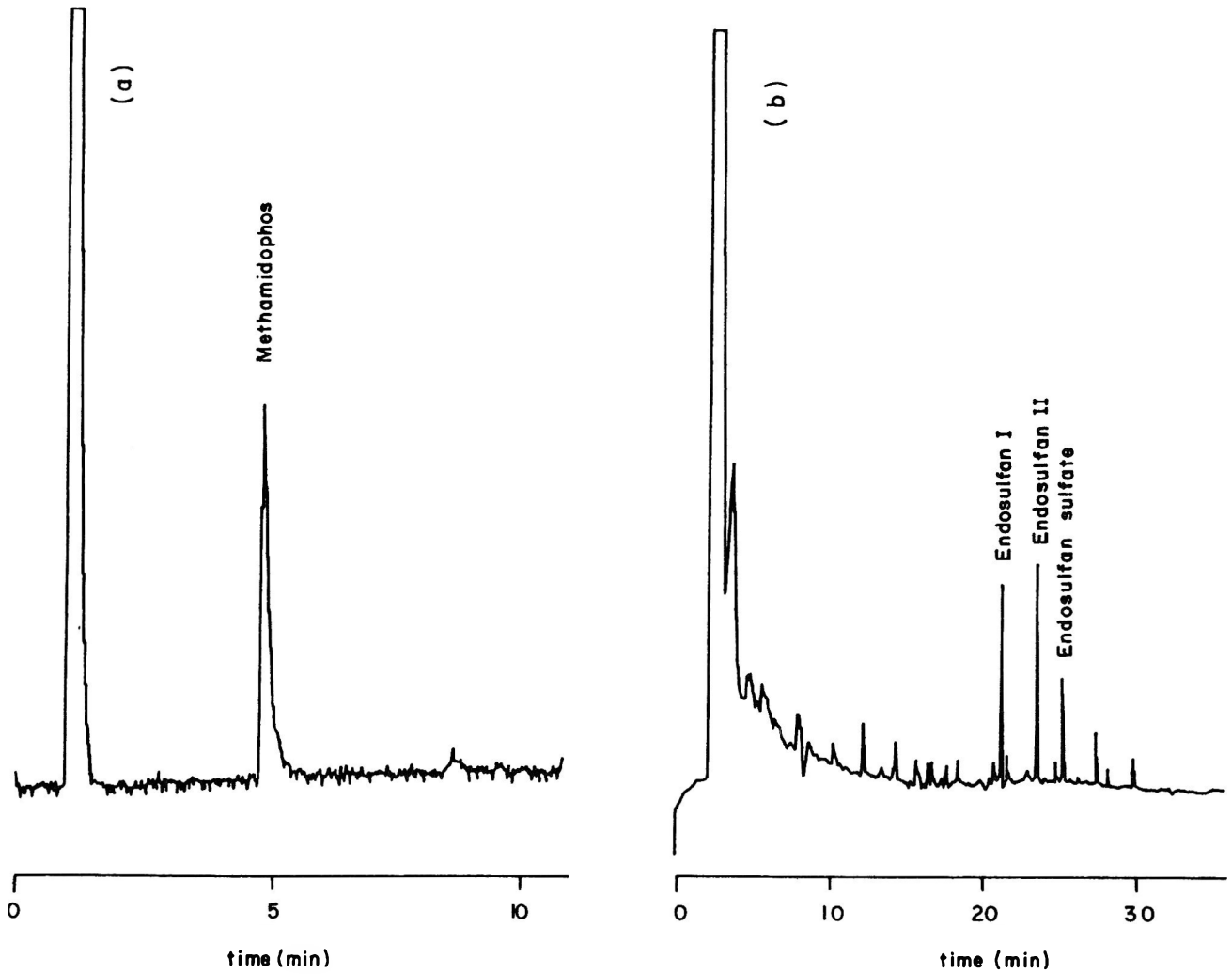

Figure 2. FPD (a) and ECD (b) chromatograms corresponding to extracts obtained for COEXPHAL sample 1 in extraction B_1 at 300 atm (*see* Table 3 for extraction conditions and determined pesticide levels).

Results and Discussion

Table 1 summarizes results of the CO_2 bubbling experiments. Also indicated are the CO_2 flow rate ranges during the bubbling process and the remaining volume of the methamidophos solutions at the end of the experiments. Results of the preliminary experiments with spiked glass wool showed that methamidophos is quantitatively recovered with only 10 mL of compressed CO_2 at pressures of 300 and 500 atm and variations in temperature (40°–90°C) have no effect on recovery. These results demonstrate that methamidophos, like many other organophosphorus pesticides (20), despite its high polarity, is soluble in pure supercritical CO_2 and confirm that ethyl acetate is an efficient trapping solvent for methamidophos.

In Table 2, methamidophos recoveries obtained from spiked anhydrous $MgSO_4$ and anhydrous $MgSO_4$–water (2 + 1) mixtures are compared with those obtained from spiked glass wool. Table 2 reveals that supercritical CO_2 cannot extract methamidophos from spiked anhydrous $MgSO_4$, but dramatic increases in recoveries are obtained when spiked $MgSO_4$–water mixtures are extracted. This result agrees with those reported by others (5, 8, 21) on the effect of moisture on recoveries of polar pesticides from solid matrixes with supercritical CO_2.

Results for spiked vegetable samples are showed in Figure 1. Extractions performed without modifier yielded methamidophos recoveries ranging from 45 to 82%. For all samples, recoveries were higher (90–114%) when methanol was used as modifier, as expected from earlier studies (2, 5). For the spiked pepper sample, GC–FPD analysis showed incurred chlorpyrifos levels ranging from 1.04 to 1.41 mg/kg (average, 1.18 mg/kg; coefficient of variation [CV], 12%). This value represents a recovery of 89% in relation to the level determined by conventional analysis and reveals that the proposed SFE method also may be suitable for determination of chlorpyrifos residues in vegetables.

Table 3 shows methamidophos and endosulfan levels determined by SFE in incurred pepper sample 1 from the COEXPHAL monitoring program. In this table, each of 3 SFE samples prepared from sample 1 are designated by A, B, and C, and each of 2 separate extractions under identical conditions of the same SFE sample is identified with the subscript 1 or 2. No significant differences were observed between extractions at

Table 4. Methamidophos levels determined by SFE[a] in incurred vegetable samples from the COEXPHAL Monitoring Program

COEXPHAL sample No.	Methamidophos, mg/kg: COEXPHAL analysis	Methamidophos, mg/kg: SFE analysis[b]	Relative recovery[c], %	COEXPHAL sample No.	Methamidophos, mg/kg: COEXPHAL analysis	Methamidophos, mg/kg: SFE analysis[b]	Relative recovery[c], %
2 (Pepper)	0.11	0.14	127	17 (Pepper)	0.18	0.19	106
3 (Pepper)	0.15	0.17	113	18 (Pepper)	0.52	0.43	83
4 (Pepper)	0.18	0.22	122	19 (Pepper)	0.21	0.29	138
5 (Pepper)	0.44	0.37	84	20 (Pepper)	0.86	0.66	77
6 (Pepper)	0.38	0.30	79	21 (Pepper)	1.07	1.04	97
7 (Pepper)	0.56	ND[d]	—	22 (Pepper)	0.50	0.75	150
8 (Cucumber)	1.25	1.29	103	23 (Pepper)	0.17	0.15	88
9 (Tomato)	0.47	0.60	128	24 (Tomato)	0.10	ND	—
10 (Pepper)	0.14	0.18	129	25 (Pepper)	0.31	0.43	139
11 (Pepper)	0.11	0.08	73	26 (Pepper)	0.13	0.10	77
12 (Pepper)	0.49	0.61	124	27 (Cucumber)	0.10	0.18	180
13 (Pepper)	2.26	1.59	70	28 (Cucumber)	0.22	0.27	123
14 (Pepper)	0.19	0.18	95	29 (Pepper)	0.17	0.29	171
15 (Cucumber)	0.45	0.53	118	30 (Pepper)	0.10	ND	—
16 (Cucumber)	0.43	0.52	121	31 (Pepper)	0.15	0.21	140

[a] Dynamic extraction (1 min equilibrium static period) at 50°C and 300 atm, with 15 mL compressed CO_2 and 200 µL methanol as static modifier.
[b] Mean of 2 determinations.
[c] SFE level × 100/COEXPHAL level.
[d] ND, not detected.

300 and 500 atm, but ECD chromatograms at 300 atm were cleaner. At 300 atm, the average value for incurred methamidophos in sample 1 was 0.73 mg/kg, which represents a recovery of 138% relative to the level determined in the COEXPHAL control analysis of this sample. This result is excellent, because the extraction method used by COEXPHAL gives recoveries of methamidophos in pepper samples close to 70% (18). Table 3 also reveals that the proposed SFE technique also may be suitable for analysis of incurred residues of endosulfan in vegetables. Figure 2 shows the FPD and ECD chromatograms corresponding to extraction B_1 at 300 atm.

Methamidophos levels, determined by the proposed method, in incurred vegetable samples 2–31 from the COEXPHAL monitoring program are listed in Table 4. Except for pepper samples 7 and 30 and tomato sample 24, all the samples contained detectable levels of methamidophos; relative recoveries (SFE level × 100/COEXPHAL level) ranged from 70 to 180%. The mean relative recovery (n = 27; SFE level range, 1.59–0.08 mg/kg) was 113%, with a CV of 26%. Results in Table 4 for samples 7, 24, and 30 could be justified either by a false positive in the COEXPHAL control analysis or a false negative in the SFE analysis or by degradation problems during sample storage. The 3 check samples analyzed by SFE were determined not to contain any pesticide residue.

Finally, SFE results for incurred pepper samples K1/93-136 and K1/93-138 from NFA are presented in Table 5. Methamidophos levels determined by the proposed method for both samples were in excellent agreement with levels determined by

Table 5. Pesticide levels determined by SFE[a] in pepper samples from the NFA Surveillance Sampling Program (1992–1993)

NFA sample code	Pesticide	NFA control analysis, mg/kg	SFE analysis[b], mg/kg: No modifier	SFE analysis[b], mg/kg: Static modifier	SFE analysis[b], mg/kg: Dynamic modifier
K1/93-136	Methamidophos	0.54	0.46	0.57	0.57
K1/93-138	Methamidophos	0.10	0.07 (14)[c]	0.08	0.10
	Procimidone	0.78	0.54 (7)[c]	0.51	0.50
	Endosulfan	0.27	0.18 (19)[c]	0.12	0.17

[a] Dynamic extraction at 300 atm and 50°C, with 15 mL compressed CO_2 (without modifier, with 200 µL methanol as static modifier, or with 3% [v/v] methanol as dynamic modifier).
[b] Single determination.
[c] Mean of 3 determinations (coefficient of variation).

conventional analysis when methanol was used as static or dynamic modifier. Results for sample K1/93-138 confirm that incurred residues of other pesticides (in this case, procimidone and endosulfan) also may be extracted from vegetables with supercritical carbon dioxide, when samples are prepared in the manner described here.

The SFE sample preparation method, SFE conditions, and collection system proposed are inexpensive and easy to perform, and they yield final SFE extracts that are ready for GC analysis without additional cleanup. The method gives good recoveries for incurred residues of methamidophos (and other pesticides) in vegetables.

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RESIDUES AND TRACE ELEMENTS

Microwave Digestion–Inductively Coupled Plasma Atomic Emission Spectrometric Determination of Boron in Raw Noodles

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Samples of raw noodles in a mixture of nitric acid and hydrogen peroxide were microwave digested in sealed tetrafluoromethaxil (PTFE TFM) vessels. The digests were diluted to volume with distilled water and quantitated by inductively coupled plasma atomic emission spectroscopy (ICP–AES). The recoveries of boron for spikes of 2.5, 250, and 500 μg were 88, 101, and 102%, respectively. The quantitation limit was estimated to be 1.5 mg B/kg. The method is rapid and ideally suited to large numbers of samples.

Boric acid and borax are sometimes added to food to disguise and mask incipient putrefaction (1). In view of their cumulative toxicity, these compounds have been declared unsafe for use as food additives by a Food and Agriculture Organization–World Health Organization expert committee (2). Although these substances are not permitted as food additives in New South Wales, the deliberate addition of these compounds is indicated by the high boron content of some Asian-style food products. Since 1989, boron levels up to 1500 mg/kg have been found by this laboratory in some Asian-style food products such as pickled fruit, meat, noodle, beef, pork, chicken, and seafood. That many similar products tested concurrently with these samples yielded little or no boron (less than 50 mg/kg) further substantiates the deliberate addition of these illegal food additives.

The toxicity of boron, its occurrence in various foods, and its distribution in human tissues is discussed in reference 3.

Boric acid has been determined by various techniques including a titrimetric method using mannitol (4) and colorimetric procedures based on the reactions with carminic acid (5) or curcumin (4). Boric acid also has been analyzed by flame atomic absorption spectroscopy (FAAS) after chelation and solvent extraction (6). This paper describes a rapid microwave digestion–inductively coupled plasma atomic emission spectrometric (ICP AES) method for boron in noodles. The main advantages of this procedure compared with earlier methods are its relative simplicity and rapidity. At least 30 samples can be processed in a day. A limiting factor for increased sample output is the number of digestion vessels and rotors available for the microwave unit.

Other advantages are good sensitivity, freedom from interferences, and minimal contamination because of closed-vessel digestion in tetrafluoromethaxil (PTFE TFM) vessels. Details of microwave digestions of a variety of difficult-to-digest sample types with a system similar to ours can be found in reference 7.

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METHOD

Use only analytical grade reagents and boron-free distilled or deionized water unless otherwise specified. Prepare all standards (except the stock standard) with plastic volumetric flasks and plastic pippetors. Digestions and subsequent dilutions are performed in plasticware. Plasticware should be soaked in 10% nitric acid for at least 1 h and then thoroughly rinsed with boron-free water before use. Detergents containing boron should not be used for cleaning any vessels.

Reagents

(**a**) *Concentrated HCl (32%).*—Analytical grade (Ajax Chemicals, Sydney, Australia).

(**b**) *Concentrated HNO_3 (70%, w/w).*—Analytical grade (Ajax).

(**c**) *Hydrogen peroxide (30%, w/w).*—Analytical grade (Rhône-Poulenc Chemicals Pty, Ltd, Clayton South, Australia).

(**d**) *Boron standard solutions.*—(*1*) *Stock solution.*—1000 μg/mL. Dissolve 5.714 g H_3BO_3 (>99.5% pure, BDH Chemicals (Australia) Pty, Ltd, Port Fairy, Australia) in 100 mL concentrated HCl. Dilute to 1 L with distilled water. (*2*) *Working solution A.*—Pipet 0, 0.5, 1.0, and 2.0 mL stock solution into separate 100 mL polypropylene volumetric flasks, add 16 mL concentrated HNO_3 (**b**) and dilute to volume with distilled water to give boron concentrations of 0, 5.0, 10, and 20 μg/mL, respectively. (*3*) *Working solutions B.*—Pipet 1.0, 5.0, and 10.0 mL of a 10 μg/mL solution into separate 100 mL polypropylene volumetric flasks, add 16 mL concentrated HNO_3 (**b**), and dilute to volume with distilled water to give boron concentrations of 0.1, 0.5, and 1.0 μg/mL, respectively.

Apparatus

(**a**) *Inductively coupled plasma atomic emission spectrometer.*—Spectroflame Model FMV05 (Spectro, Germany) equipped with concentric nebulizer.

(**b**) *High-performance microwave digestion unit.*—Model mls 1200 mega (Milestone, Italy) equipped with 10-place rotor MDR 300/10 and 10 PTFE TFM digestion vessels. Digestion vessels are 100 mL capacity and have a maximum pressure rating of 30 bar. The microwave unit has a 1200 W rated magnetron with 1000 W delivered power. Microprocessor control of power emission is from 10 to 1000 W, in 10 W increments. Programming of power and time steps is via keyboard entry from a control panel.

(**c**) *Waring blender.*—Model 32 BLBO (Waring Products Division, Dynamics Corp., New Hartford, CT).

(**d**) *Polypropylene measuring cylinder.*—25 mL (Azlon, UK).

(**e**) *Polypropylene volumetric flask.*—100 mL (Azlon).

Validation of Standards

The purity of our boric acid source (which was used direct from the bottle without drying) was tested against a multielement quality control standard containing boron at 100 μg/mL in 5% nitric acid (QC standard APS-1031, Alpha Resources, Inc., Stevensville, MI). The standard was diluted to contain boron at 10 μg/mL in 16% nitric acid. No difference could be detected between our 10 μg/mL working standard and the QC standard.

Preparation of samples

Homogenize ca 100 g noodle sample in a Waring blender for 1 min or finely chop sample with a knife and mix thoroughly. Samples are analyzed as purchased and are not dried or treated in any way. Clean the microwave digestion vessels by carrying out the digestion program in Table 1 up to step 3; use the normal digestion mixture of 4 mL HNO_3 (**b**) and 2 mL hydrogen peroxide (**c**) without sample present. Allow vessels to cool for 30 min in a water bath kept at ca 20°C and then rinse them at least 3 times with distilled water. Accurately weigh 1.0 g sample into digestion vessel. Add 4 mL HNO_3 (**b**) and 2 mL hydrogen peroxide (**c**), mix, and then place the sealed vessels into the microwave unit. Start digestion program with the parameters described in Table 1 (other brands of microwave digestion units require different parameters). Once the program is complete, allow vessels to cool for 30 min in a water bath kept at ca 20°C. Transfer digest to a 25 mL polypropylene measuring cylinder. Rinse digestion vessel with 2–3 ca 5 mL portions of distilled water and combine rinses in the measuring cylinder. Dilute to 25 mL with distilled water and mix. Filter diluted digest through Whatman No. 542 paper into clean dry plastic tube for quantitation by ICP AES.

Determination

Set up the spectrometer according to the manufacturer's recommendations. For our instrument, the operating parameters and other technical specifications are given in Table 2. Select appropriate background correction positions after scanning an actual sample matrix at the chosen analytical line. Check for possible interfering elements, such as iron, by reference to interference libraries (or tables) and then analyze for level of interferent in the actual sample. Calibrate the instrument to read out concentration of boron at the 249.773 nm line. Prepare a first-order linear regression calibration curve by using working standard solutions (**d**).

Table 1. Microwave digestion parameters

Step	Power, W	Time, s
1	0	5
2	250	30
3	0	120
4	250	300
5	400	180
6	0	120

Calculations

Calculate amount of boron in sample with the following formula:

$$\text{Boron in sample } (\mu g/g) = \mu g/mL \times 25/m$$

Table 2. Instrument conditions for boron determination by ICP AES

Parameter	Setting
Rf (radio frequency) generator frequency	27.120 MHz (free running)
Nitrogen purge spectrometer	
Grating	
Radius of curvature	750 mm
Number of grooves	2400/mm
Wavelength range	165–460 nm
Reciprocal linear dispersion	0.55 nm/mm
Entrance slit width	10 μm
Exit slit width	25 μm
Plasma output power	1.1 kW
Argon flow rate	
Coolant	14 L/min
Plasma	1.0 L/min
Auxiliary	1.0 L/min
Purge (nitrogen)	0.5 m^3/h
Observation height in plasma	15 mm
Integration time	3 s
Boron analytical line	249.773 nm
Background correction	0.021 nm (set to left and right side of peak)
Sample aspiration rate	2 mL/min
Rinse time between samples	60 s
Sample preflush time before measurement	60 s
Number of readings per sample	3

where 25 is the analyte solution volume (mL) and m is the sample weight in grams.

Results and Discussion

The method was used to analyze 3 samples that were suspected to contain an added boron preservative. One sample had only trace levels of about 1 μg/g, but the other 2 samples had boron levels of 66 and 202 μg/g. The previous method for determination of boron in food was a chelation–solvent extraction procedure followed by FAAS (6), which was lengthy and not ideally suited to large sample numbers. The main disadvantage, however, was sensitivity. Quantitation of samples with boron levels less than about 50 μg/g was a problem. The method described in this paper, however, does not have these difficulties.

Sensitivity is good, and a limit of quantitation (LOQ) of 1.5 μg/g in actual samples can be achieved. The LOQ was defined as that concentration in the sample that produced a signal 10 s above the reagent blank signal (where s is the standard deviation of 9 determinations of the reagent blank taken through the entire analysis). Method repeatability was tested at different levels by performing replicate analyses of samples before and after spiking. A minimum of 5 determinations were performed in each case. Relative standard deviations (RSDs) were generally better than 4% for boron levels above 60 μg/g and around 10% for a low-level spike of 2.5 μg/g. These data are summarized in Table 3.

Boric acid is steam volatile, and open-vessel digestion may cause concern. The closed-vessel microwave procedure in our method eliminates these concerns. Recoveries at 3 spike levels were 88% for the low-level 2.5 μg/g spike and 101 and 102% for the 250 and 500 μg/g spikes, respectively. The lower recovery (88%) for the low-level spikes is likely due to poorer method precision at that low level, rather than actual loss of boron. The recovery data are summarized in Table 3. Spikes were carried through the entire digestion process. Boric acid standard was added to samples contained in their digestion vessels prior to digestion. No difference was observed when borax was used in place of boric acid for spiking.

NIST (National Institute of Standards and Technology) reference material Tomato leaves 1573 with a boron content of 30 μg/g (non-certified value) was analyzed. Samples (0.5 g) of the reference material were soaked with 2.0 mL distilled water in the digestion vessels for ca 15 min prior to digestion. Three replicate analyses gave results of 32.7, 32.8, and 33.4 μg/g.

Reagent blank levels are always a concern when analyzing low levels. Blank levels could be minimized by performing operations in plasticware. The mean of 9 blank determinations was 0.02 μg/mL; the values ranged from 0 to 0.032 μg/mL, with a standard deviation of 0.0062. If glass volumetric ware was used for dilutions, the blanks could increase by up to a factor of 10.

The goal of our analyses was to detect illegal addition of boron at levels above about 20 μg/g. Our method readily can do this. For accurate determinations at lower levels of boron (1–5 μg/g), it may be desirable to decrease the final sample volume of 25 mL and/or increase the sample weight. Our digestion parameters are based on "as-received or -purchased" noodle products, some with relatively low water content. For determinations of high water content samples such as some fruit and vegetables, higher sample weights can be used. We found no problem in using 2.0 g samples for materials with high water content. Decreasing the final sample solution volume may require a corresponding increase in the acid strength of the calibration standards, and this must be investigated for each particular case.

The method should be applicable to other food matrixes after investigation of suitable operating parameters. Care must be taken, however, when other sample matrixes are first attempted. Samples containing high percentages of animal fat and gas-producing samples can overpressurize microwave digestion vessels. Manufacturer recommendations as to maximum sample weights for different matrixes and various digestion vessel pressure ratings should always be considered carefully. Domestic microwave units are not recommended because of safety considerations. Care must be exercised when selecting digestion mixtures; there can be a serious explosion hazard with perchloric acid and organic matrixes.

The possibility of interference by other elements was investigated. The only likely interferent in our samples is iron. We found that an iron standard of 1000 μg/mL gave an equivalent boron reading of 0.4 μg/mL at the wavelength used for boron (249.773 nm). This problem was not encountered with our samples, which contained iron at about 1 μg/mL in the actual solutions being read for boron (ca 25 μg/g in the actual sample). We also found no effect on the boron response when the nitric acid concentration is changed. A 10 μg/mL boron standard prepared in 10, 15, and 20% nitric acid showed no difference in the boron response.

Table 3. Repeatability and recovery data for boron in noodles

Sample	Boron found, μg/g	RSD, %	Spike level, μg/g	Recovery, %	RSD, %
Hokkien noodles	0.98	16	2.5	88	10.3
			250	102	1.2
			500	101	1.1
Hokkien noodles	66	5.6			
Egg pastry[a]	202	3.7			

[a] Nine replicate analyses; all other RSDs are 5 replicates.

The method is a rapid, simple, sensitive, and interference-free procedure for determination of boron in noodles.

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TECHNICAL COMMUNICATIONS

Improved Determination of Chlorite and Chlorate in Rinse Water from Carrots and Green Beans by Liquid Chromatography and Amperometric and Conductivity Detection

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A method is presented for determining chlorite and chlorate in the presence of interfering organic compounds in rinse water from vegetables. Rinse water from cut raw carrots and green beans was fortified separately with chlorite and chlorate, filtered (0.45 μm), and analyzed by liquid chromatography with amperometric and conductivity detection. Detection limits for chlorite and chlorate in carrot rinse water were 17 and 50 ppb, respectively. Average recoveries from rinse water were 95% for chlorite in a 0.084–1.00 ppm range and 90% for chlorate in a 0.078–1.00 ppm range.

Chlorite and chlorate can be quantitated by amperometric titration (1–3), *N,N*-diethyl-*p*-phenylenediamine colorimetry (2), and liquid chromatography (LC) (4–7). Most of these procedures were developed for analysis of drinking water and have not been tested on rinse water from produce (1–4, 6). One LC method quantitates chlorite in candied foods, but the detection limit is only 5 ppm (5). To evaluate the safety of chlorine dioxide-treated produce, a method was needed to determine chlorite at a detection limit of <0.1 ppm in vegetable rinse water without interference from other anions. With rinse water from produce, one LC method (6) eluted foreign compounds after the water peak and prevented the conductivity detection of chlorite. Interfering chemicals were not removed adequately from samples by C_{18} filtration or 5000 Dalton ultrafiltration. Filtering acidified extracts through C_{18} or cation-exchange filters still did not solve the problem. Changing the concentration, the flow rate, and even the composition and ratios of components of the mobile phase also were unsuccessful. However, by adding an amperometric detector to the LC system, chlorite was detectable. This paper describes a sensitive method using an anion-exchange column and both amperometric and conductivity detectors for quantitating chlorite and chlorate in the presence of interfering compounds in rinse water from cut raw carrots and green beans.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Dual-piston pump, 50 μL injection loop, AMM-II membrane suppressor, and conductivity detector. Operating conditions: 0.5 mL/min, 2000 psi maximum limit, and local settings on gradient pump; 5 mL/min flow rate for regenerating solution; 80 psi injection valve N_2 pressure; temperature compensation of 1.7, 30 μsiemens (S) output range, and <50 nS stable conductivity settings (series 4500i, Dionex, Sunnyvale, CA).

(b) *Chromatographic column.*—IonPac AS9-SC (250 × 4 mm, 13 μm) with an IonPac AG9-SC guard column (50 × 4 mm) (Dionex).

(c) *Amperometric detector.*—Operating conditions: 700 mV ouput, (+) potential, 0–1000 nA offset, 500 nA range, and 0.1 Hz filter settings (Model LC-4B/17A, Bioanalytical Systems, Inc., West Lafayette, IN). Connect the outlet port for the flow cell of the conductivity detector to the inlet port of the amperometric detector.

(d) *Glassy carbon electrode.*—Bioanalytical Systems.

Reagents and Standards

All chemicals are ACS grade except where noted. Use deionized, carbon-filtered water to prepare all solutions (Super-Q Plus Water System, Millipore, Bedford, MA).

(a) *Mobile phase.*—4.0 mM sodium carbonate–1.50 mM sodium bicarbonate. Dissolve 10.600 g sodium carbonate (Curtin Matheson Scientific, Houston, TX) and 3.150 g sodium bicarbonate 100.2%, Curtin Matheson) in deionized water in a 500 mL volumetric flask, bring up to volume, and mix. Pipette 20.0 mL of this solution into a 1 L volumetric flask, dilute to volume with deionized water, and mix. Suction filter in a vacuum-filtering apparatus attached to a 2 L flask by using a house vacuum through a 0.45 μm filter. Do not sparge oxygen from the solution before use.

(b) *AMM-II membrane suppressor regenerate solution.*—25 mM sulfuric acid. Dilute 2.8 mL concentrated sulfuric acid (EM Science, Norwood, OH) in a 2 L volumetric flask containing deionized water, bring to volume, and mix. Suction filter as described for the mobile phase.

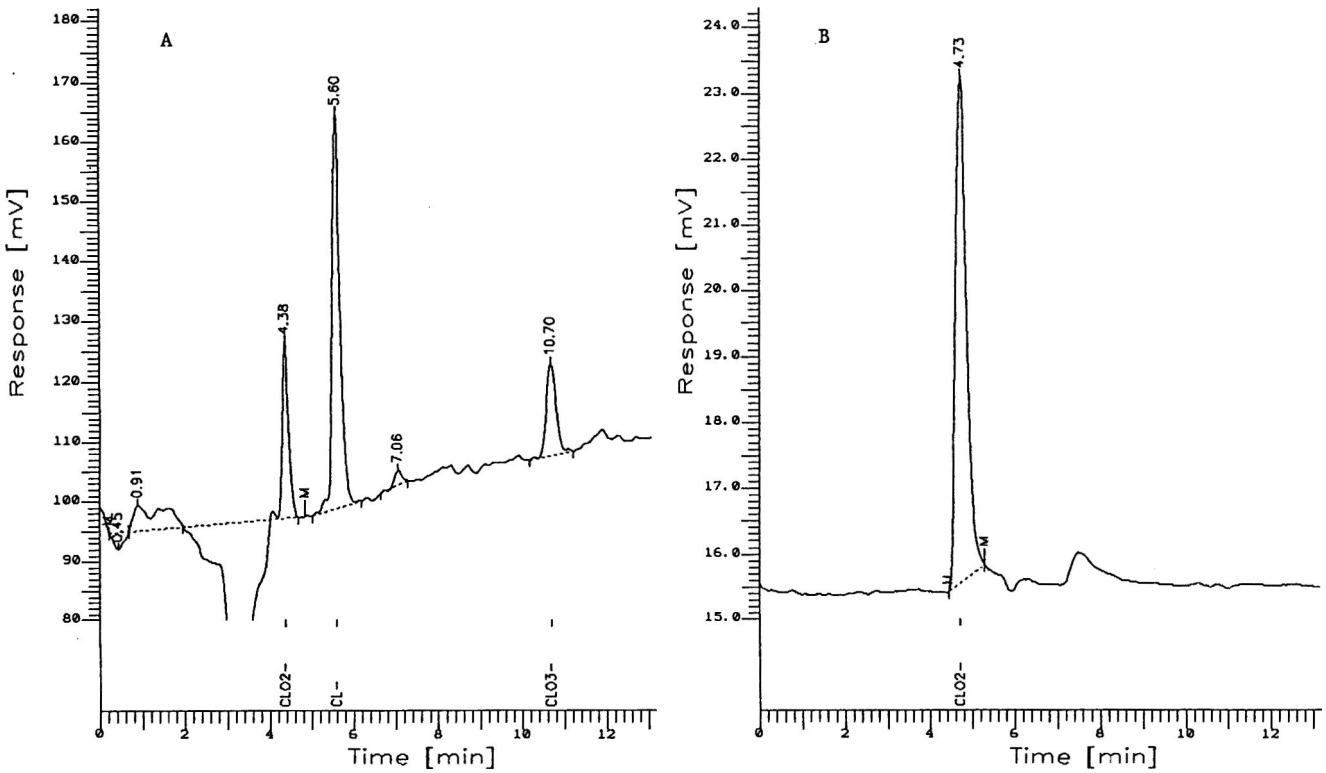

Figure 1. Chromatograms of representative 0.281 ppm chlorite, chloride, and chlorate standard solutions. (A) Conductivity detector. (B) Amperometric detector. Response scales are dissimilar.

(c) *Sodium chlorite, sodium chloride, and sodium chlorate standard solution (900 ppm each anion).*—Prepare daily. Dissolve 0.3018 g sodium chlorite (80%; EM Science, Gibbstown, NJ), 0.2968 g sodium chloride (VWR Scientific, San Francisco, CA), and 0.2318 g sodium chlorate (99.0%; J.T. Baker, Phillipsburg, NJ) in 200 mL deionized water in a volumetric flask. Serially dilute standard solution with deionized water to obtain working standards (containing 1.13, 0.563, 0.281, and 0.141 ppm for each anion). Chloride was included in the standard to verify that chlorite was detected electrochemically, on the basis of comparisons of retention times.

(d) *Chlorite and chlorate fortification solution (900 ppm each anion).*—Prepare daily. Weigh and dissolve each anion as described for the working standard. Serially dilute the solution with deionized water to form fortification solutions (22.5 and 2.25 ppm each anion). Analyze appropriately diluted samples with the working standard to determine the actual concentrations of anions.

Determination

(a) *Sample preparation.*—Rinse 31–101 g raw carrots and 100 g raw green beans under running deionized water to remove soil. Cut carrots into ca 0.25 in. slices and green beans into 1–1.25 in. sections. Separately soak the produce in deionized water (1 + 1, w/v) in 100–125 mL beakers at 25°–27°C. Drain and discard the water, repeat the soaking procedure, and collect the rinse water.

(b) *Rinse water fortification.*—Fortify rinse water samples on the day of analysis. Place 5.00 mL rinse water into 15 mL test tubes and 0.222 mL of each fortification solution into separate tubes, seal tubes, and mix thoroughly. Prepare 3–4 replicates for each type of rinse water and each fortification level. Prepare unspiked rinse water samples to serve as controls. Hold tubes at 25°–26°C until filtration.

(c) *Sample filtration.*—Separately filter the fortified rinse water samples through a Nylon-66 filter (0.45 μm, 25 mm diameter; Alltech Assoc., Deerfield, IL) attached to a 5 mL syringe into a 6 mL test tube immediately before analysis.

Analysis

Inject at least 10 injection loop volumes of standards and filtered samples (a requirement of the Dionex LC system) every 25–30 min. Analyze the working standards before and after injecting 9–11 samples.

Calculations

Construct linear regression curves of analyte concentration vs peak area for the standard solutions:

$$\text{Concentration of anion in fortified sample (ppm)} = (C)(F) - C_u$$

where C is anion concentration of fortified sample determined from linear regression curve, F is sample + fortification solution volumes/ sample volume, and C_u is anion concentration of unfortified sample determined from linear regression curve.

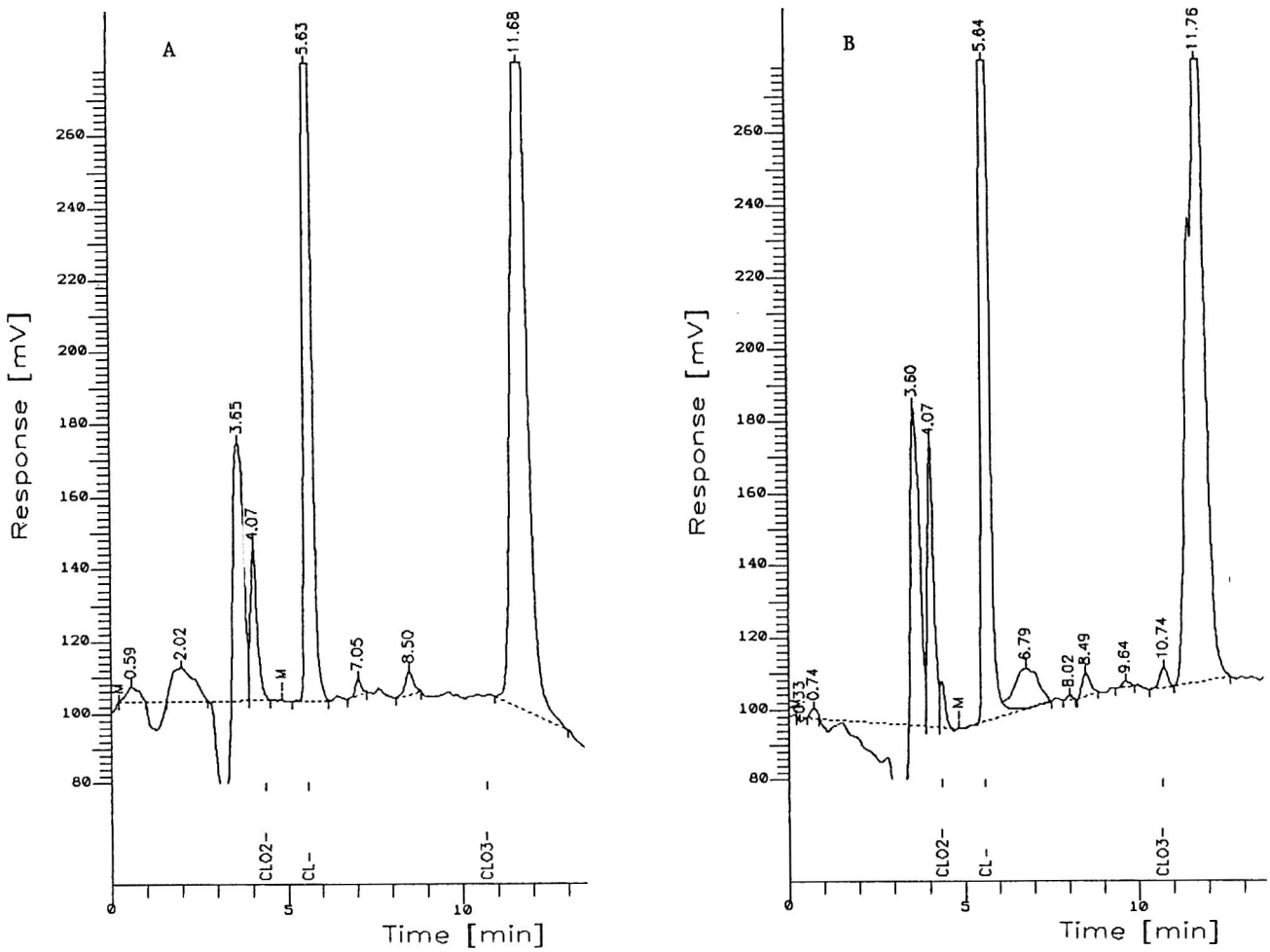

Figure 2. Chromatograms of representative rinse water samples from cut, raw green beans before (A) and after (B) fortification with 0.100 ppm chlorite and 0.086 ppm chlorate. Conductivity detection was used.

Results and Discussion

Figure 1 illustrates the conductivity detection of chlorite, chloride, and chlorate along with the amperometric detection of chlorite in a representative standard solution containing each anion at 0.281 ppm. The 3 ions are well resolved in the chromatogram from conductivity detection. The amperometric detector was installed after the conductivity detector in the LC system. The peak retention times indicate that chlorite was first detected by conductivity and then was oxidized by the amperometric detector before chloride and chlorate eluted. The flow rate of the mobile phase was reduced from 2 mL/min (6) to 0.5 mL/min to improve resolution of chloride and chlorate from interfering compounds in rinse water samples. The analysis of drinking water is generally conducted with a mobile phase flow rate of 1–2 mL/min (4, 7).

Organic compounds in rinse water from produce can create problems when a conductivity detector is used to determine chlorite (Figure 2). Figure 2A shows that the rinse water from cut, raw green beans contained substances that eluted between 3.5–4.5 min. After the rinse water was spiked with chlorite at 0.100 ppm, chlorite was detected in the tail of the second peak shown in Figure 2B. This type of interference was also observed with the rinse water from the carrots (not shown). Therefore, conductivity detection of low concentrations of chlorite in rinse water from vegetables can produce inconclusive results.

In contrast, improved detection of chlorite in rinse water samples was observed with amperometric detection (Figure 3). Figures 3A and 3B illustrate magnified chromatograms of rinse water from green beans before and after fortification, respectively, with chlorite and chlorate. Figures 3C and 3D show chromatograms of rinse water from carrots before and after fortification, respectively. Chlorite was not present initially in water from both vegetables but was detected at 4.74 min after fortification. A small peak was observed after 5 min, but it did not shift during analyses to interfere with quantitation of chlorite at all levels of fortification.

To determine the detection limit and reproducibility of the LC system, rinse water from carrots was fortified at 2 concentrations of chlorite and chlorate and analyzed 6 times (Table 1). To calculate the detection limit, the U.S. Environmental Protection Agency equation (8) was used:

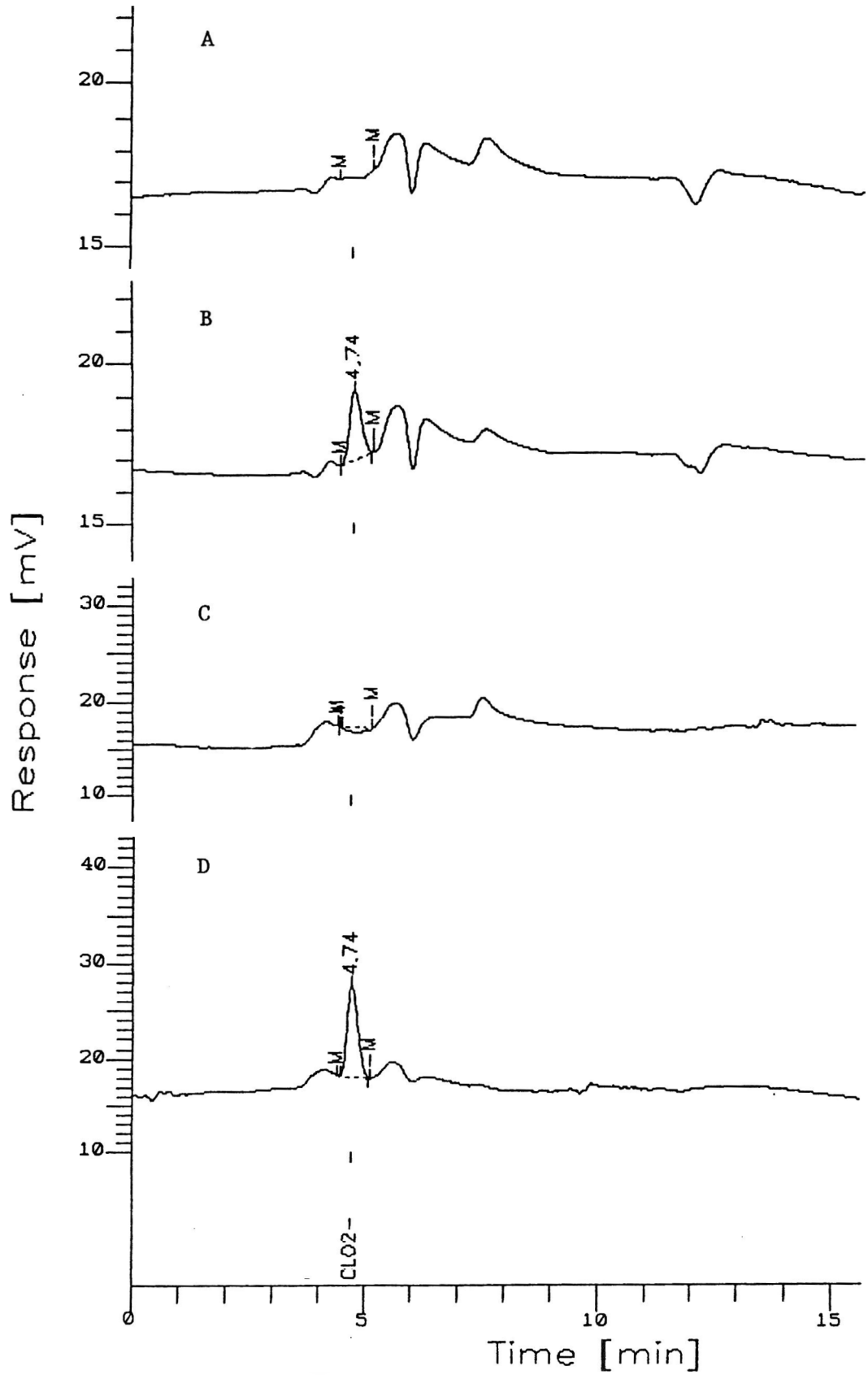

Figure 3. Chromatograms of representative rinse water samples from cut, raw green beans before (A) and after (B) fortification with 0.100 ppm chlorite and 0.086 ppm chlorate and sliced, raw carrots before (C) and after (D) fortification with 0.084 ppm chlorite and 0.078 ppm chlorate. Amperometric detection was used. Response scales are dissimilar.

Table 1. Variability of 6 multiple injections of 2 carrot rinse water samples fortified[a] with chlorite and chlorate

Analyte	Sample	Amount added, ppm	Amount recovered, ppm[b]	CV, %
Chlorite	1	0.100	0.103 ± 0.005	5
	2	1.00	0.975 ± 0.016	2
Chlorate	1	0.090	0.067 ± 0.015	22
	2	0.901	0.911 ± 0.071	8

[a] Fortification solutions contained both chlorite and chlorate.
[b] Values are means ± standard deviations.

$$\text{Method detection limit} = t_{(n-1,\ 1-\text{alpha}\ =\ 0.99)} \times \text{SD}$$

where t is Student's t value for 99% probability for $(n - 1)$, and SD is the standard deviation of replicate analyses.

The detection limits for chlorite and chlorate were calculated as 17 and 50 ppb, respectively, from analysis of sample 1. Therefore, this method was sensitive for determining chlorite and chlorate in carrot rinse water. Low coefficients of variation (CVs) were noted for both concentrations of chlorite tested as well as for chlorate at 0.901 ppm; therefore, the LC system yielded reproducible results. The highest CV was noted for the sample fortified with chlorate at 0.090 ppm. This concentration is near the detection limit of this anion, which may explain the high variability.

Amperometric detection of chlorite in rinse water from carrots and green beans performed well (Table 2). All fortified rinse water samples had CVs of <11%, a result that suggests the method was reproducible for both types of water and at all concentrations of chlorite tested. Greater than 87% of the fortified chlorite was detected in all rinse water samples, representing an average recovery of 95%. These results indicate that amperometric detection of chlorite is accurate.

Conductivity detection of chlorate in vegetable rinse water performed better at higher fortification concentrations than at lower levels (Table 2). Samples spiked with chlorate at 0.895–1.00 ppm had CVs of <1–13%, whereas rinse water fortified with chlorate at <0.13 ppm yielded CVs of >18%. This trend suggests that as the concentration of chlorate approaches its detection limit of 50 ppb, the variability of the method increases. This tendency is seen in results for experiments 5 and 6, where low and high recoveries for chlorate, respectively, were noted for samples fortified at only 1.7–2.5 times the detection limit of chlorate. Overall, an average of 90% of the chlorate added to the samples was detected.

This method reduces interferences from organic compounds to produce a reliable and sensitive analysis of chlorite and chlorate in rinse water from cut, raw carrots and green beans.

Acknowledgments

We appreciate the technical assistance and advice of Andy Adsetts, Don Gates, and Richard Higby (Rio Linda Chemical Co.).

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Table 2. Recovery of anions in carrot and green bean rinse water samples fortified[a] with chlorite and chlorate

Analyte	Rinse water	Experiment	Amount added, ppm	Number of replicates	Amount recovered, ppm[b]	CV, %	Recovery, %[b]
Chlorite	Carrot	1	0.084	3	0.082 ± 0.002	2	97 ± 2
		2	0.100	4	0.092 ± 0.008	9	92 ± 8
		3	0.855	3	0.751 ± 0.039	5	88 ± 5
		4	1.00	4	0.93 ± 0.05	5	93 ± 5
	Green beans	5	0.100	4	0.097 ± 0.010	10	97 ± 10
		6	0.100	3	0.100 ± 0.004	4	100 ± 4
		7	0.903	3	0.806 ± 0.011	1	89 ± 1
		8	1.00	4	1.02 ± 0.03	3	103 ± 3
Chlorate	Carrot	1	0.078	3	0.066 ± 0.016	24	85 ± 20
		2	0.100	4	0.089 ± 0.032	36	89 ± 32
		3	0.895	3	0.910 ± 0.058	6	102 ± 6
		4	1.00	4	0.97 ± 0.06	6	97 ± 6
	Green beans	5	0.126	4	0.054 ± 0.016	30	43 ± 13
		6	0.086	3	0.091 ± 0.017	19	105 ± 19
		7	1.00	3	0.98 ± <0.01	<1	98 ± <1
		8	1.00	4	1.01 ± 0.13	13	101 ± 13

[a] Fortification solutions contained both chlorite and chlorate.
[b] Values are means ± standard deviations.

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TECHNICAL COMMUNICATIONS

Homogeneous Sample Preparation of Raw Shrimp Using Dry Ice

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Sample homogeneity is critical to accurate and reproducible analysis of trace residues in foods. A method of uniform sample preparation using dry ice is described for shrimp. Other sample preparation techniques for raw shrimp produce nonhomogeneous samples. Sample homogeneity was determined through analysis of chloramphenicol added to intact tiger or white shrimp prior to sample preparation. Simulated chloramphenicol residue levels were 50, 15, 10, and 5 ppb. No significant differences were noted when analyses of shrimp inoculated with chlor-amphenicol prior to sample preparation with dry ice were compared with analyses of shrimp spiked after grinding with dry ice. Grinding shrimp with dry ice produced samples with homogeneous chloramphenicol residues. This technique should be applicable to other tissues and vegetable products.

Without a homogeneous sample, it is not possible to accurately evaluate the performance of an analytical method. The common assumption that the analyte is uniformly distributed throughout the sample may not be valid, particularly for tissue samples. In fact, Love (1) believed that sample homogenization is essential for determinations of pesticides in fish, where the fat is heterogeneously distributed. Sample preparation is especially critical in analyses for drugs, pesticides, or residues of environmental contaminants at the part-per-million or part-per-billion levels. If the ground sample does not have a uniform physical appearance, it is unlikely that any analytical residue will be uniformly distributed in that sample. The Food and Drug Administration's *Pesticide Analytical Manual* (2) is a good reference with detailed descriptions of sample preparation procedures for a wide variety of food commodities.

In aquaculture, shrimp in one pond may be treated with drugs for disease while others at the same or nearby sites may be untreated. When harvested shrimp reach the processing plant, shrimp contaminated with drug or other residues may be commingled with clean animals. Therefore, a sample representative of the processed lot may include only a small percentage of residue-contaminated shrimp. If sample preparation does not yield a homogeneous composite, high residue pockets will exist in the ground shrimp.

In this laboratory, chloramphenicol was added to samples with incurred residues taken from different sections of a shrimp composite thought to be homogeneous. The chloramphenicol analytical results for these samples differed significantly from the relative standard deviation, 12% (10 ppb), previously established by Munns et al (3). The disparity of these analyses raised serious questions about the homogeneity of the ground shrimp sample and prompted investigation into sample preparation techniques for trace residue analyses.

Raw tiger and white shrimp form a viscid mass when ground at room temperature. Typically, samples are prepared by passing an entire 10–12 lb sample through a meat grinder 3 times, mixing in between each pass through the grinder. As a preliminary test of sample homogeneity, 0.4 ppm of a water-insoluble red dye was added as a visible marker to 2.2 lb shrimp, which was then passed through a meat grinder 3 times. Close examination of the resultant pink ground shrimp revealed streaks of red in the shrimp, thereby confirming that homogeneous mixing was not being achieved with a meat grinder.

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Clearly, if only part of the shrimp sample contained high residue levels, this residue would not be distributed uniformly in the ground mass. Close examination of the ground shrimp also revealed that it contained numerous strings of fibrous material and did not appear to have a uniform particle size.

Shrimp also may be ground in a blender. With this technique, whole shrimp can fall beneath the mixer blades and remain intact. Ground shrimp forms a ball, which rolls on top of the spinning mixer blades. This ball, as well as tissue on the walls of the blender jar, have to be scraped into the remaining mass to attempt to form a uniform composite. Grinding small portions in a blender and then combining the separate batches is very time and labor intensive. This technique does not produce a sample with uniform size particles.

Previously, this laboratory had used dry ice to prepare citrus samples for analysis of the volatile fumigants ethylene dibromide and 1,2-dibromo-3-chloropropane (4, 5). In this case, the dry ice was used to prevent dissipation of the fumigant residue in the fruit during sample preparation. The ground citrus sample was a finely divided dry powder. The California Department of Food and Agriculture reports the routine use of dry ice to prepare food products that do not have a homogeneous composition, for example, a mixture of fluid and pulp, or that form a sticky aggregate (Terry Jackson, 1994, personal communication). The use of liquid nitrogen to uniformly distribute a viscous material, coal tar, in feed for animal toxicity studies has been reported. With liquid nitrogen, the tar can be hardened and finely fractured, and then the resulting powder can be distributed uniformly throughout the feed (Harold Thompson, 1993, personal communication). For analyses of pesticide residues Benville and Tindle (6) favored the use of dry ice and a blender to prepare fish homogenates over hand grinding with anhydrous sodium sulfate in a mortar.

Liquid nitrogen and dry ice were each investigated as a means of producing homogeneous shrimp samples. Liquid nitrogen was evaluated by grinding shrimp in a gallon-size, metal blender jar. Although a finely divided powder was obtained, liquid nitrogen was found to be unsuitable in our laboratory for the following reasons: (*1*) The rubber gaskets in the blender crystallized and were incorporated into the composite. (*2*) Liquid nitrogen escaped from around the seals in the blender blade assembly and had to be continuously replaced. About 3.5 L of liquid nitrogen were required to prepare 1000 g shrimp. (3) Shrimp powder was expelled and lost whenever additional liquid nitrogen was added. (4) This technique required a heavy Dewar flask and protective gloves for the analyst, as liquid nitrogen can be a safety hazard. Since dry ice (mp, −56.6°C) is more readily available and safer to handle than liquid nitrogen (mp, −195°C), it was used as the compositing aid for shrimp.

When shrimp were ground with dry ice, a uniform, fine powder was produced. Long strings of fibrous material were not present. Analysis of 4 shrimp samples spiked with chloramphenicol at different residue (ppb) levels prior to grinding with dry ice found the chloramphenicol to be uniformly distributed throughout the entire sample.

METHOD

Apparatus

(**a**) *Gas chromatograph.*—Model 7673 automated liquid sampler and 3396 Series II integrator interfaced to a Model 5980 Series II gas chromatograph equipped with electronic pressure control and an electron capture detector (Hewlett Packard, Palo Alto, CA), a Merlin Microseal™ Septum (Merlin Instrument Co., Half Moon Bay, CA), and a HP-5 (5% diphenyl 95% dimethylpolysiloxane) column, 25 m × 0.2 mm id, 0.33 μm film thickness (Hewlett Packard). GC conditions: helium carrier gas with an initial pressure of 30 psi for 2 min, 99 psi/min ramp, final pressure 20 psi; initial oven temperature 160°C for 2.5 min, 15°C/min ramp, and final temperature held at 270°C for 5.5 min. Splitless injection, 3 μL, at 250°C with injector purge at 2 min. Detector temperature, 350°.

(**b**) *Vertical-cutter-mixer (VCM).*—25 quart, Model VCM-25 (Stephan Machinery, Manhasset, NY), or equivalent.

(**c**) *Homogenizer.*—Polytron Model PT 10/35 with Model PTA 20S generator (Brinkmann Instruments, Westbury, NY), or equivalent.

(**d**) *Centrifuge.*—Model UV (International Equipment, Needham Heights, MA), or equivalent.

(**e**) *Pipettors.*—100 μL adjustable volume and 1 mL fixed volume (Gilson, Worthington, OH), or equivalent.

(**f**) *Mixer.*—Vortex, or equivalent.

(**g**) *Kuderna Danish concentrator.*—Three-chamber micro distillation column 24/25; 250 mL flask 24/40 Top, 19/22 Bottom; 10 mL concentrator tub 19/22; polyacetal clamps (Kontes, Vineland, NJ).

(**i**) *Solvent evaporator.*—N-Evap Model 111 (Organomation Associates, South Berlin, MA), or equivalent at 55°C.

(**j**) *Dry bath.*—Temp-Blok Module Heater with 8-hole block (20 mm) (Scientific Products, McGaw Park, IL), or equivalent at 70°C. Add sand to block cavities to promote efficient heat transfer.

(**k**) *Gas-tight syringe.*—1 mL.

(**l**) *Polypropylene tubes.*—Graduated 50 mL centrifuge tubes with screw caps (Becton Dickinson, Franklin Lakes, NJ), or equivalent.

Reagents and Solutions

(**a**) *Dry ice.*—Commercial 5–10 lb slabs or nuggets.

(**b**) *Residue analysis grade solvents.*—Ethyl acetate, hexane, methanol, and toluene.

(**c**) *NaCl solution, 4%.*—Dissolve 40 g reagent grade NaCl in deionized water and dilute to 1 L.

(**d**) *Derivatizing reagent.*—Bis(trimethylsilyl) trifluoroacetamide–trimethylchlorosilane (99 + 1) (BSTFA-TMCS) Sylon BFT (Supelco, Bellefonte, PA), or equivalent.

Standards and Standard Solutions

Store working and stock standard solutions in low-actinic glassware or protected from light.

(a) *Chloramphenicol (CAP).*—Analytical grade (Sigma Chemical, St. Louis, MO).

(b) *m-Nitrochloramphenicol (mCAP).*—Synthesized at the University of Georgia, School of Chemical Science, Department of Chemistry, Athens, GA.

(c) *Chloramphenicol standard solutions.*—(*1*) *Stock solution.*—100 µg/mL. Dissolve 10 mg CAP in acetone in a 100 mL volumetric flask. (*2*) *Intermediate solution.*—10 µg/mL. Pipet 10 mL stock solution into a 100 mL volumetric flask and dilute to volume with acetone. (*3*) *Working solution.*–1000 ng/mL. Pipet 10 mL intermediate solution into a 100 mL volumetric flask and dilute to volume with acetone.

(d) *m-Nitrochloramphenicol internal standard solutions.*—(*1*) *Stock solution.*—10 µg/mL. Dissolve 1 mg mCAP in acetone in a 100 mL volumetric flask. (*2*) *Working solution.*—1000 ng/mL. Pipet 10 mL stock solution into a 100 mL volumetric flask and dilute to volume with acetone.

(e) *Analytical standards.*—Pipet 100 µL each CAP and mCAP working solutions into a 10 mL glass-stoppered test tube. Evaporate just to dryness with a jet of N_2. Prepare silyl derivative of mixed standard solution concurrently with sample series.

Shrimp Samples

Randomly select ca 12 lb shrimp (individually quick frozen or frozen block) to ensure a representative sample from the lot. Thaw if shrimp are covered with ice or to remove chitin shell and tail. When frozen, headless shrimp may be ground with carapace and tail in place. Apply an appropriate correction factor in the calculation formula to compensate for the weight of the chitin shell and tail if incorporated in the composite.

Procedure

(a) *Sample preparation.*—Use 150 to 200% by weight of dry ice to shrimp (15–20 lb). Break dry ice blocks into large pieces with a hammer. Grind about half of the dry ice (10 lb) to a fine powder in the Stephan VCM. Add remaining dry ice chunks to VCM and grind briefly until dry ice is reduced to a uniform, fine powder. Add ca one-half of shrimp (6 lb) to powdered dry ice. Grind until shrimp have been reduced to small pieces. Add remaining shrimp and grind until a uniform powder is produced. If shrimp pieces greater than ¼ in. square remain, continue grinding until no large pieces remain.

Transfer ground shrimp to plastic quart containers and place in a freezer. Cover loosely with a lid until CO_2 has sublimed. (*Caution:* Exercise caution when entering a walk-in freezer that may contain sublimed CO_2 gas. Humans loose consciousness when air contains more than 10% CO_2 [7]). Alternatively, place the dry ice–shrimp mixture in a metal pan and allow the CO_2 to sublime at room temperature. Stir occasionally to reduce the time required to remove the CO_2.

Clean the VCM food chopper immediately with a spray of warm water; otherwise the gelatinous shrimp adheres to the metal surface and is difficult to remove.

(b) *Sample analysis.*—Weigh 10 g ground shrimp into a 50 mL plastic, screw-cap centrifuge tube. Add 100 µL mCAP working solution and 20 mL ethyl acetate to test tube. Homogenize for 10–15 s at medium speed. Centrifuge at 1000–2000 rpm for 1–2 min. Decant solvent into Kuderna Danish concentrator (KD). Homogenize with an additional 20 mL ethyl acetate, centrifuge, and combine extracts in KD. Place KD, supported by a cork ring, into an active steam bath. Evaporate until about 2 mL remains in KD evaporator. Rinse KD flask with about 2 mL ethyl acetate and transfer concentrated extract to a 30 mL screw-cap test tube. Rinse KD tube with three 2 mL portions of ethyl acetate and combine rinses with extract. Place test tube in N-Evap and evaporate combined extracts to dryness under a jet of N_2.

Add 15 mL heptane, 2 mL methanol, and 25 mL 4% NaCl to the dried residue in the 30 mL test tube. Shake capped tube vigorously for about 1 min. Centrifuge if necessary to break the emulsion. Remove heptane layer with aspirator or Pasteur pipet and discard. Repeat extraction with heptane twice. Centrifuge after last extraction to ensure complete expression of heptane. Add 15 mL ethyl acetate to saline sample solution and shake vigorously for about 30 s. Centrifuge if necessary to break the emulsion. Transfer the ethyl acetate extract to a KD with a Pasteur pipet. Extract again and centrifuge. Combine the ethyl acetate extracts in the KD and evaporate to a volume of 2–4 mL in a steam bath. Rinse the KD flask with about 2 mL of ethyl acetate. Place the KD tubes in the N-Evap and evaporate the ethyl acetate concentrate just to dryness under a jet of N_2. (*Note*: Heating the dried residue under a flow of N_2 for an extended period will lead to loss of analyte.)

(c) *Preparation of trimethylsilyl chloramphenicol derivative.*—Add 100 µL BSTFA from a gas-tight syringe to sample and standard preparations. Tightly stopper. Mix briefly on Vortex mixer. Place tubes in the Dry-Blok and heat for 15 min at 70°C. Remove excess BSTFA with a gentle flow of N_2. When test tube is dry, immediately remove from N_2 jet because the CAP silyl derivatives are volatile. Add 1 mL toluene and mix on Vortex mixer. Transfer toluene solution to an autosampler vial.

(d) *Calculations.*—Calculate the concentration of CAP on the basis of the area or peak height ratios (PR) of CAP/mCAP peak responses as follows:

$$\text{CAP, ppb} = \frac{PR_{smpl}}{PR_{std}} \times \frac{C}{W}$$

where PR_{smpl} and PR_{std} are the peak ratios for the sample and standard, respectively; C is the CAP standard concentration (ng); and W is the weight of shrimp (g).

Determination of Uniformity of Distribution

Use 10 to 12 lb shrimp. With a gas-tight hypodermic syringe, inject shrimp (10% by weight) with sufficient CAP solution to procuce the desired residue level. Prepare as described in *Sample Preparation*. Allow CO_2 to sublime at room temperature. Transfer ground shrimp to plastic quart containers. Weigh at least six 10 g samples into 50 mL centrifuge tubes. Store shrimp composite at –35°C. Analyze samples as described in *Sample Analysis* beginning at "Add 100 µL mCAP . . . ." If in-

Table 1. Analysis of spiked shrimp samples ground with dry ice

Target spike level, ppb	Amount analyzed, ppb						Average, ppb	% of target	RSD, %
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6			
50	52.5	51.7	52.2	52.4	53.0	52.1	52.3	104.6	0.83
15	13.5	15.1	14.0	16.6	16.0	15.0	15.0	100.0	7.8
10	11.4	10.5	11.0	10.7	9.9	10.7	10.7	107.0	4.7
5[a]	4.0	3.9	4.0	4.1	3.8	3.9	4.0	80.0	2.6
5[a]	3.8	3.8	4.1	3.8	4.0	4.6[b]	3.9	78.0	3.6

[a] Single composite divided into 6 quart containers; samples from containers 2 and 5 were analyzed on consecutive days.
[b] Data point fails the Dean and Dixon test and was discarded as an outlier (8).

ternal standard has been incorporated into the spiking solution, begin at "20 mL ethyl acetate ... ."

Results and Discussion

When dry ice sublimes in a freezer, a friable shrimp powder remains. As this powder thaws, it changes form and becomes stiff and viscid. When dry ice sublimes at room or refrigerated temperatures, the composited shrimp becomes a stiff, viscous mass. Ground shrimp samples were stored at –35°C or lower to retard degradation of the CAP residue.

Four shrimp samples were spiked at part-per-billion levels and analyzed to demonstrate the effectiveness of the dry ice compositing procedure. Each analytical series consisted of 6 portions from the spiked samples. Table 1 shows the results of the analyses for the 4 simulated residue levels.

Both CAP and mCAP were injected into the shrimp used to prepare the 50 ppb level. Because a 50 ppb residue exceeds the linear range of 50–250 ng established for the analytical method, the method was modified. The final ethyl acetate extract was collected in a 25 mL volumetric flask. An aliquot equivalent to about 100 ng was evaporated to dryness and analyzed as described in *Sample Analysis* starting at "Add 15 mL heptane... ." Because the internal standard was included in the sample preparation step, this sample had the lowest relative standard deviation (RSD), 0.83%, of the samples tested.

The 16.3 ppb sample had the highest RSD, 7.78%. This sample was not ground at the highest speed of the Stephan VCM and contained some small chunks of shrimp tissue. However, the RSD was well below the desired limit of 20% RSD for residues at this level.

The sample spiked at the 5 ppb level was prepared from 12 lb of thawed, peeled white shrimp and was divided into 6 quart containers after the CO_2 sublimed. Six replicate portions were analyzed from each of 2 separate quarts (containers 2 and 5). The overall average of 11 analyses was 4.0 ppb with an RSD of 3.0%. The average of the 6 portions from container 2 was 4.0 ppb (3.95 ppb) with an RSD of 2.6% and that from container 5 was 3.90 ppb with an RSD of 3.6%. These data confirm that when CAP is present in about 10% of the lot, it is uniformly distributed throughout the sample when composited with dry ice. As the amount of CAP added to the shrimp decreases, the RSD does not increase. The data in Table 1 indicate that factors other than the level of CAP residue affect the reproducibility of analytical results when the sample is homogeneous.

The data in Table 2 were generated by adding sufficient CAP to 10 g shrimp to emulate 5 and 10 ppb residues. This shrimp had been ground with the aid of dry ice. This technique tests the analytical method rather than the uniformity of sample preparation. The RSDs for these analyses are comparable with those shown in Table 1.

Fresh shrimp samples that are composited with either a blender or a meat grinder typically exhibit numerous chromatographic peaks prior to elution of mCAP and CAP peaks. When dry ice is used as a compositing aid, these extraneous peaks either disappear or are almost eliminated.

Table 3 presents data compiled by weighing headless shrimp before and after peeling. The percent shell includes the tail and chitin shell. These data can be incorporated into the calculation formula to correct the analytical result (ppb CAP) for the weight of the shell in the sample. The presence of shell in the composited shrimp does not interfere with CAP analysis.

Table 2. Chloramphenicol standard addition to shrimp ground with dry ice

Amount added, ppb	Amount analyzed, ppb						Average, ppb	Recovery, %	RSD, %
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6			
10	11.0	10.6	11.2	10.8	12.6[a]	10.7	10.8	108.0	2.2
5	4.5	4.7	4.1[a]	4.7	4.6	4.7	4.6	92.0	1.9
5	5.6[a]	5.1	5.0	—[b]	5.0	4.8	5.0	100.0	2.5

[a] Data point fails Dean and Dixon test and was discarded as an outlier (8).
[b] Internal standard was not added to this sample.

Table 3. Amount of shell in shrimp

Type of shrimp	Shell, %
Freshwater prawn	20
White shrimp	19
White shrimp	21
Tiger shrimp	20
Average	20

Inclusion of shell in the sample does not interfere in the analysis for oxytetracycline residues in shrimp either (Steve Hadley, 1994, personal communication). Compositing unpeeled shrimp not only reduces sample preparation time but also allows using frozen samples.

Conclusions

Use of dry ice as an aid in sample preparation produces a homogeneous composite. Although dry ice has been evaluated only with shrimp tissue, it should be applicable to other shellfish and finfish, as well as other tissue and vegetable products.

Acknowledgments

We thank the following for their helpful comments and technical assistance: Terry Jackson (CDFA) and Jim Seiber (UNR) for their remarks on the use of dry ice in sample preparation, Harold Thompson, Jr (FDA) for his suggestion on the use of liquid nitrogen, Steve Hadley (FDA) for oxytetracycline analysis. We also thank Bob Munns (FDA) for providing *m*-nitrochloramphenicol.

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TECHNICAL COMMUNICATIONS

Gas Chromatographic/Mass Spectrometric Analyses of Unknown Analytical Response in Imported *Fava* Beans: 4-Chloro-6-methoxyindole

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A halogenated unidentified analytical response (UAR) was encountered in a number of imported *Fava* bean samples during the Food and Drug Administration's routine pesticide-monitoring program. Gas chromatographic/mass spectrometric (GC/MS) analyses identified the halogenated component as 4-chloro-6-methoxyindole, a naturally occurring promutagen in *Fava* beans that has been linked to incidents of gastric cancer. Data from electron impact, positive and negative chemical ionization, collision-induced dissociation, and deuteration studies of this compound are presented, along with GC retention time data.

The continuing growth of a diversified ethnic population in this country along with changes in trade regulations has resulted in a large increase in the quantity and diversity of food commodities being imported. The port of New York receives a large volume of imported foods, and as a direct result, our pesticide monitoring program consists of a large percentage of these imported foods. Many so called unidentified analytical responses (UARs) are encountered in the Food and Drug Administration's (FDA) import pesticide-monitoring program. A UAR is a compound that produces a gas chromatographic (GC) response on certain specialized GC detectors, such as the electron capture (EC), flame photometric (nitrogen–sulfur), or Hall (halogen-specific) detector, but that cannot be identified with FDA's relative retention time database of pesticides and industrial chemicals (1). A halogenated UAR has been repeatedly found by a number of FDA laboratories during routine pesticide screening of *Fava* beans (canned, dried, raw, and split) imported from UK, Portugal, Canada, and the Netherlands. The UAR was extracted from the products with the official multiresidue procedure for nonfatty foods (2) and eluted from a Florisil column (3) in the 15% ethyl ether–petroleum ether fraction. The UAR produced a Hall response with GC retention times, relative to chlorpyrifos (RRTc) (1), of 0.54, 0.66, and 3.5 on packed columns of OV-101, OV-17, and DEGS, respectively. RRTc on a DB-5 megabore was 0.58. All GC analyses were isothermal at 200°C. Gas chromatography/mass spectrometry (GC/MS) was used to identify the compound.

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Experimental

Instrumentation

(a) *Low resolution GC/MS.*—Finnigan 9611 gas chromatograph directly interfaced to a Finnigan TSQ-45 mass spectometer. Data were acquired with a SuperIncos data system (Rev 6.6). The gas chromatograph was equipped with a 30 m × 0.32 mm id, 0.25 μm DB-5MS fused-silica capillary column; the temperature was programmed from 50°C (held 3 min) to 270°C at a rate of 20°C/min. The injector and transfer lines were heated at 220°C. The injector was operated in the splitless mode with a helium flow rate of 1 cc/min.

Electron impact (EI) analyses were performed at 70 eV ionization energy, 500 μA emission current, and 150°C source temperature. Scan range was 35–650 daltons in 0.45 s. Positive ion chemical ionization (PICI) analyses were performed with methane as the reagent gas at 0.2 torr pressure. Scan range was 90 to 300 daltons in 0.45 s. Negative ion chemical ionization (NICI) was carried out under the same conditions, except that the scan range was 15 to 300 daltons in 0.45 s. EI collision-induced dissociation (CID) analyses were performed under the same EI conditions described earlier with the addition of argon collision gas at 1 mtorr.

(b) *High resolution GC/MS.*—High resolution data were obtained with a Hewlett Packard 5890 series II gas chromatograph interfaced to a VG Autospec Q mass spectrometer. The gas chromatograph was equipped with a SPB-5, 30 m × 0.25 mm id, 0.25 μm film thickness. The oven was programmed from 60°C (2 min) to 270°C at a rate of 20°C/min and held at 270°C for 10 min. The split–splitless injector was operated in the splitless mode at 220°C. The temperature of the transfer lines and ion source was 250°C. Helium head pressure was 10 psi. The mass spectrometer was tuned to 10 000 resolution in the EI mode, and *m/z* 138, 166, and 181 of the UAR

were examined in 3 separate GC/MS high-resolution voltage scan analyses. Scan time was 0.5 s, and the scan windows were 168–194, 154–182, and 130–144. These windows included perfluorokerosene reference ions as well as UAR fragment ions.

Reagents

(a) *Standards.*—4-Chloro-6-methoxyindole (93490-31-4), 92 ng/μL in methanol, and 6-chloro-4-methoxyindole (117970-23-7), 32 ng/μL in methanol (Toronto Research Chemicals, ON, Canada).

(b) *Deuterium oxide.*—99.8 atom % D (Aldrich Chemical, Milwaukee, WI).

Results and Discussion

Initial EI analyses indicated a tentative molecular weight of 181 for the compound. The 3:1 ratio of the isotope cluster at *m/z* 181/183 indicated one chlorine atom on the molecule, and the odd molecular weight required an odd number of nitrogen atoms on the molecule. The major fragment ions at *m/z* 166, $[M - 15]^+$, and *m/z* 138, $[M - 43]^+$, both ions exhibiting a 1-chlorine-atom isotope cluster, suggested losses of CH_3· and $[CH_3$· + CO], respectively. A search of the National Institute of Standards and Technology mass spectral library failed to identify the compound, but the observed losses of 15 and 43 were consistent with spectra of methoxyindoles in the library. A search of *Mass Spectral Compilation of Pesticides and Industrial Chemicals* (4) also failed to identify the compound, but again, the losses of 15 and 43 were consistent with spectra of chloromethoxybenzenes (anisoles) in the library. These correlations suggested that the UAR might be a chloromethoxyindole-type compound.

PICI confirmed the molecular weight of 181 by exhibiting the characteristic protonated molecular ion cluster at *m/z* 182 and the corresponding adduct ions at *m/z* 210, $[M + 29]^+$, and *m/z* 222, $[M + 41]^+$, representing $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$, respectively. The NICI spectrum, which was quite weak, showed no evidence of resonance electron capture (no molecular ion isotope cluster at *m/z* 181/183).

To determine the number of exchangeable protons on the molecule, the sample extract was evaporated to dryness, 2 drops of deuterium oxide were added, and the extract was mixed and reconstituted with acetone. The deuteration study indicated that the UAR contained one exchangeable proton. This was evident by the shift of the molecular ion cluster from *m/z* 181 to *m/z* 182. The fragment ions at *m/z* 166 and 138 were also shifted 1 mass unit to *m/z* 167 and 139, respectively, indicating that these fragments also retained the exchangeable proton. All these ions exhibited a 1-chlorine isotope cluster. The nonchlorinated fragment ions at *m/z* 102, 103 were also shifted one mass unit to *m/z* 103, 104, respectively.

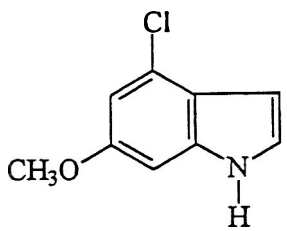
High-resolution GC/MS data confirmed the elemental composition of the UAR as C_9H_8NOCl and the exact mass as 181.029190 (+1.4 ppm). The fragment ions were determined as being *m/z* 166.006330 (−2.2 ppm) and *m/z* 138.011360 (−2.2 ppm), representing C_8H_5NOCl, $[M - CH_3]^+$, and C_7H_5NCl, $[M - (CH_3 + CO)]^+$, respectively.

The mass spectral data, EI, PICI, NICI, CID, and the deuteration study supported the proposed identity of the UAR as being a chloromethoxyindole with an elemental composition of C_9H_8NOCl. A literature search revealed that Yang et al. (5) isolated a naturally occurring chloromethoxyindole promutagen from *Fava* beans and unambiguously identified it using ultraviolet, infrared, and nuclear magnetic resonance spectroscopy, and high-resolution MS as 4-chloro-6-methoxyindole.

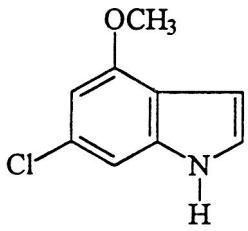
Reference standards of 4-chloro-6-methoxyindole (Structure 1) and 6-chloro-4-methoxyindole (Structure 2) were obtained for direct comparison. The 2 isomers were resolved completely on the DB-5MS capillary column (Figure 1). The GC retention time and mass spectral data agreed with that of the 4-chloro-6-methoxy isomer. On the basis of GC/MS data and the previous identification of this isomer as naturally occurring in *Fava* beans (5), the UAR is probably the 4-chloro-6-methoxy isomer.

The EI spectra of the isomers (Figure 2) were quite similar. The only observable difference was the relative intensity of the ion at *m/z* 165, $[M - CH_3]^+$. The relative abundance of this ion for the 4-chloro isomer was consistently slightly higher than for the 6-chloro isomer but was not sufficiently different to warrant identification based on the spectrum alone. This is consistent with the fragmentation of methoxyindoles, in which the $[M - CH_3]^+$ ion is more intense for the 6-methoxy isomer than for the 4-methoxy isomer (6).

CID analyses of *m/z* 181 produced daughter ions at *m/z* 166, $[M - CH_3]^+$; 138, $[M - (CH_3 + CO)]^+$; and 102, $[M - (CH_3 + CO + HCl)]^+$. The CID parent ion spectrum of *m/z* 103 indicated that this ion was primarily derived from 2 ions: *m/z* 166 (100%), by loss of [CO + Cl·], and *m/z* 138 (20%), by loss of [Cl·]. These data indicate that the *m/z* 103 ion is not the characteristic ion, $[M - (H + HCN)]^+$, observed in the EI spectrum of indole (7) but instead represents $[M - (CH_3 + CO + Cl)]^+$. The



Structure 1. 4-Chloro-6-methoxyindole.



Structure 2. 6-Chloro-4-methoxyindole.

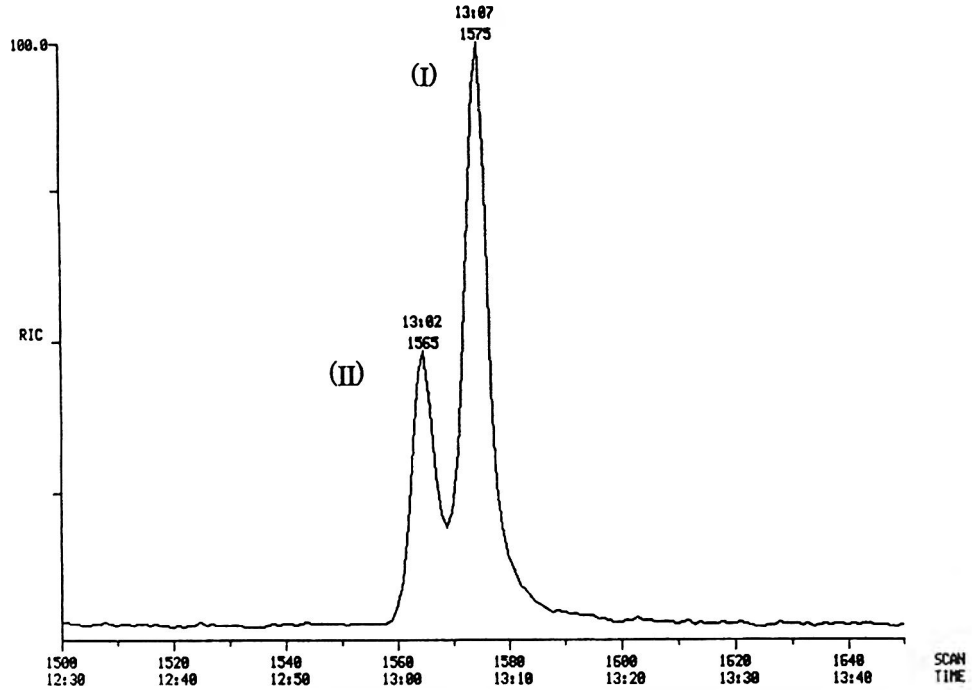


Figure 1. DB-5MS capillary column resolution of 4-chloro-6-methoxyindole (I) and 6-chloro-4-methoxyindole (II).

fragmentation of methoxyindoles (6) was shown to be directed by the methoxy moiety, resulting in loss of [CH_3·] and [CH_3· + CO], rather than by the indole moiety, which would have produced the characteristic $[M - H]^+$ and $[M - HCN]^+$ ions.

The PICI spectra of the 2 isomers (Figure 3) show a significant difference in the relative abundances of the *m/z* 146, $[M - HCl]^+$, ion. The relative abundance of *m/z* 146 for the 4-chloro isomer was approximately 15–20%, whereas for the 6-chloro isomer, it was 100%. Both isomers produced a protonated molecular ion chlorine isotope cluster $[M + H]^+$ at *m/z* 182. The chlorine isotope cluster at *m/z* 181 (relative abundance, approximately 40%) is the result of a PICI process rather than the product of a high-energy EI process. If the *m/z* 181 cluster resulted from an EI process, the PICI spectrum would also contain the other major EI fragment ions, *m/z* 166, 138, and 103, in relative abundances greater than 90%. The *m/z* 181 isotope cluster is probably formed by either of 2 processes: loss of a proton from the protonated molecular ion or a low-energy PICI

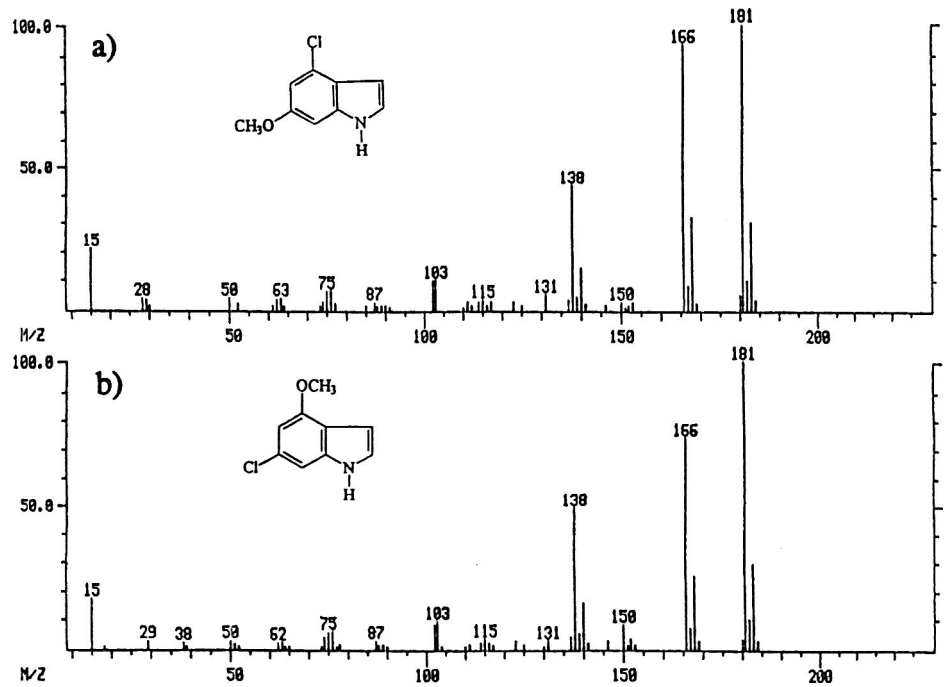


Figure 2. EI mass spectra of 4-chloro-6-methoxyindole (a) and 6-chloro-4-methoxyindole (b).

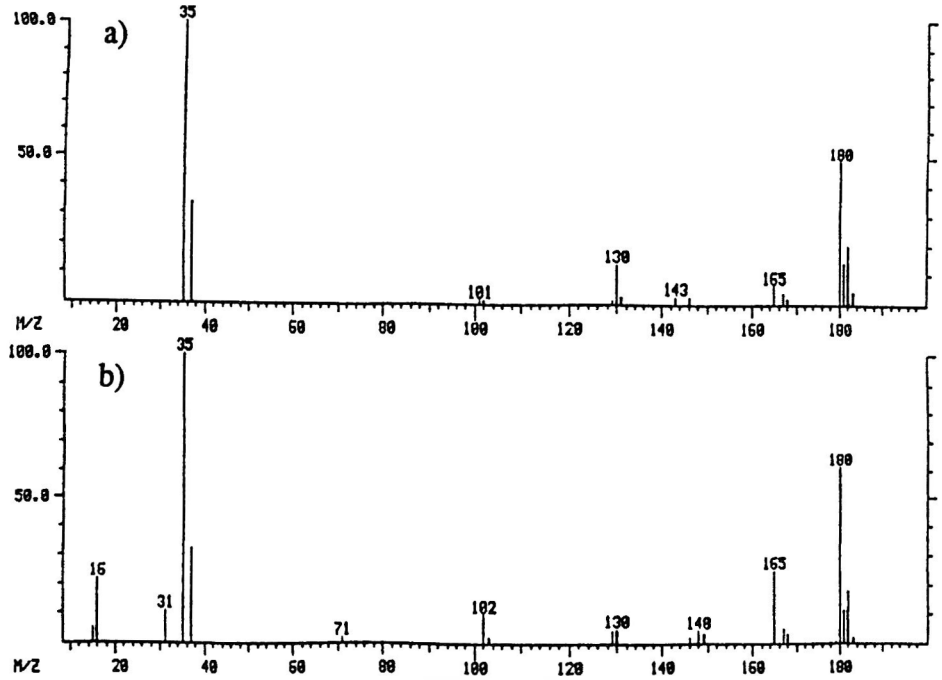


Figure 3. PICI mass spectra of 4-chloro-6-methoxyindole (a) and 6-chloro-4-methoxyindole (b).

charge-exchange reaction (11). Charge exchange would produce an odd-electron molecular ion as in EI, and therefore the fragment ions produced would be the same as those in EI. The energy, however, of the $[M]^{+\cdot}$ produced by charge exchange is limited to the exothermic reaction of its formation, and therefore the abundance of these ions would be less than those observed in the EI spectra (Figures 2 and 3).

PICI spectra of both isomers were also obtained with 2 additional instruments, a Finnigan TSQ-700 and a Fissons (VG) Autospec. The same characteristic PICI spectra were produced on these instruments. The ratio of the *m/z* 181 to *m/z* 182 cluster remained approximately 40%. Characteristic PICI spectra were obtained for a mixture of pesticide standards on all 3 instruments. These data support the hypothesis that the *m/z* 181 cluster is the result of a low-energy PICI process specific to these indole compounds.

The NICI spectra of both isomers (Figure 4) were quite weak compared with both EI and PICI spectra. There was no significant difference in the NICI spectra of the 2 isomers. The lack of a molecular ion $[M]^-$ at *m/z* 181 indicated that no resonance electron capture was taking place. It is believed the $[M - H]^-$ is formed by dissociative electron capture from the molecular ion $[M^-]^*$ (12, 13). It is also possible, however, to form an $[M - H]^-$ ion by proton abstraction from the molecular ion by O^- or OH^- ions. The presence of O^- or OH^- ions could result from traces of oxygen or water in the system. This proton abstraction would occur if the proton affinity of the compound is less than the proton affinity of O^- or OH^- (12–14). The major fragment ion in the spectrum was *m/z* 35, $[Cl]^-$.

The lack of resonance electron capture observed in the NICI spectrum of 4-chloro-6-methoxyindole agrees with the results of routine GC screening of the samples. An EC detector response comparable to that produced by 1.5 ng chlorpyrifos (50% scale deflection) was not possible with 500 ng of the compound. However, only 2.3 ng of the 4-chloro isomer was required to produce such a a response with the Hall GC detector. Therefore, this component was only detected when samples were screened with the Hall detector; it was not detected when an EC detector was used. The estimated amount of 4-chloro-6-methoxyindole in the *Fava* beans was approximately 0.1 ppm. Recovery studies were not performed, because this component is a naturally occurring compound and not a pesticide.

Yang et al. (5), in addition to isolating and identifying 4-chloro-6-methoxyindole as the naturally occurring promutagen in *Fava* beans, also showed that nitrosation of this compound produces 4-chloro-6-methoxy-2-hydroxy-N^1-nitrosoindolin-3-one oxime, a highly mutagenic compound (5). Similar nitrosation under simulated gastric conditions forms the same direct-acting mutagen (8). Hypotheses for the development of gastric cancer suggesting exposure in the stomach to activated *N*-nitroso compounds may be involved (9). A nutritional survey conducted in Columbia revealed a positive correlation between the incidence of stomach cancer and the consumption of *Fava* beans (10).

Conclusions

On the basis of GC, EI, EI high resolution, PICI, NICI, CID, and deuteration data and literature information, the halogenated UAR encountered in the pesticide residue analyses of imported *Fava* beans was identified by GC/MS as being 4-chloro-6-methoxyindole, a naturally occurring promutagen. This component will be detected by a Hall detector but not by an EC detector at the levels present in the samples. RRTc values of this compound on commonly used GC columns is given to facilitate identification.

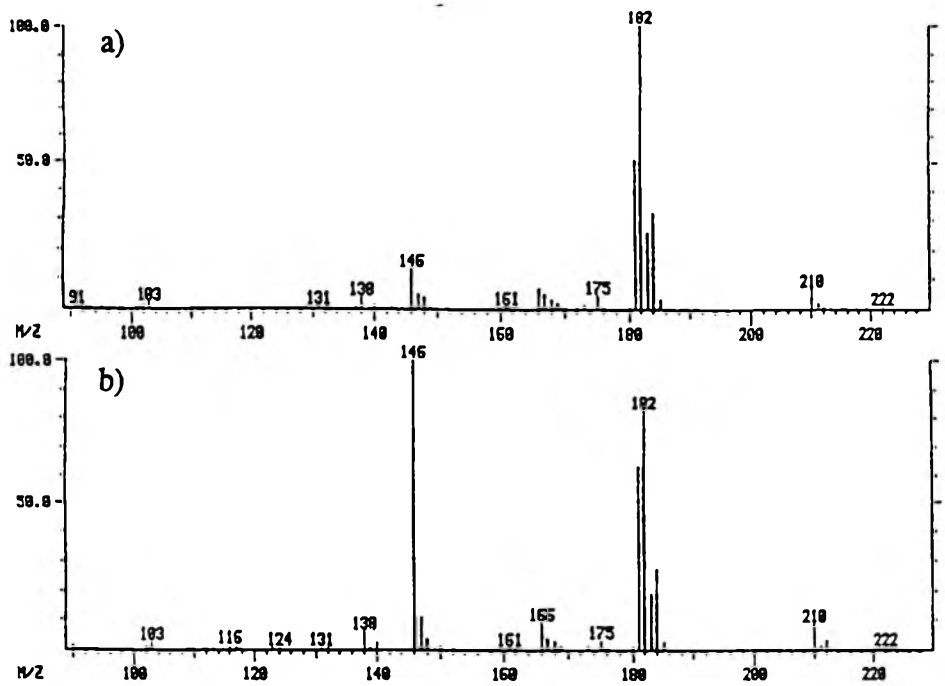


Figure 4. NICI mass spectra of 4-chloro-6-methoxyindole (a) and 6-chloro-4-methoxyindole (b).

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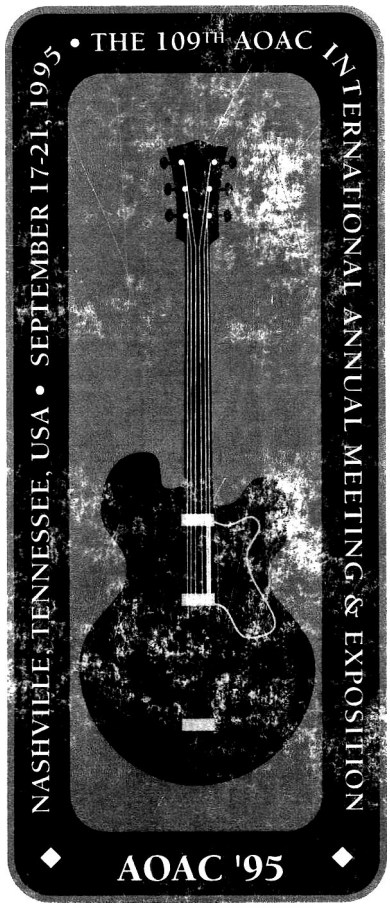

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