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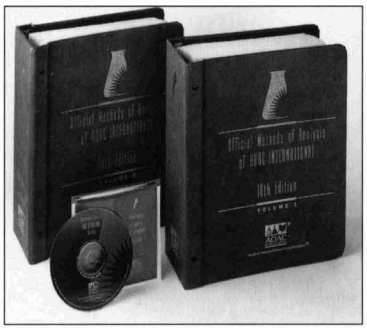

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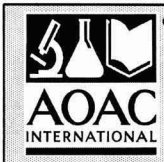

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JOURNAL OF AOAC INTERNATIONAL

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

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The *Journal of AOAC INTERNATIONAL* (ISSN 1060–3271) is published bimonthly by AOAC INTERNATIONAL, 481 North Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA. **Subscriptions** for Vol. 81 (1998) are: Members, $181.00 in North America, $232.00 outside North America; Nonmembers, $249.00 in North America, $300.00 outside North America. Periodicals postage paid at Gaithersburg, MD, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.

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TECHNICAL COMMUNICATIONS

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3. **Text** (consecutive sheets, **DOUBLE SPACED**): *Introduction*. Include information on why work was done, previous work done, use of compound, or process being studied.

Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; avoid use of brand names. (Common reagents/apparatus or those which require no special treatment need not be listed separately.) Place detailed operations in separate sections with appropriate headings (e.g., Preparation of Sample, Extraction and Cleanup). Include necessary calculations; number of significant figures must reflect accuracy of method. Use metric units for measurements of quantities wherever possible. Note hazardous and/or carcinogenic chemicals.

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JOURNAL ARTICLE REFERENCE

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

BOOK CHAPTER REFERENCE

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

BOOK REFERENCE

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, Arlington, VA, secs **29.070–29.072**

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October 22, 1998: AOAC MidAtlantic USA Section, College Park, MD, USA. Contact: Joan Pinkas, McCormick and Co., 202 Wright Ave, Hunt Valley, MD 21031, USA, +1-410-771-7811

October 22–24, 1998: AOAC Latin American and Caribbean Section, Buenos Aires, Argentina. Contact: Ricardo Sobol (50601@sicoar.com), Buenos Aires, Argentina, fax +54-1-3044141

October 26–28, 1998: AOAC Central Section, Cincinnati, OH, USA. Contact: Vernon Stubblefield, Eastern Kentucky University, Department of Chemistry, Moore Science Room No. 337, Richmond, KY 40475, USA, +1-606-622-1453

October 29–31, 1998: AOAC Central Europe Subsection, Varazdin, Croatia. Contact: Bisreka Raspor (raspor@rudjer.irb.hr), Rudjer Boskovic Institute, Center for Marine and Environmental Research, PO Box 1016, 41001, Zagreb, Croatia, +385-1-46-80-216, fax +385-1-46-80-242

August 1–5, 1999: AOAC Latin American and Caribbean Section, Santiago, Chile. Contact: Nuri Gras (ngras@gopher.cchen.cl), fax +562-3646277, or Patricia Bravo (corthon@ibm.net), fax +562-2439299

September 26–30, 1999: The 113th AOAC INTERNATIONAL Annual Meeting and Exposition, Houston, TX, USA. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, +1-301-924-7077, fax +1-301-924-7089

September 10–14, 2000: The 114th AOAC INTERNATIONAL Annual Meeting and Exposition, Philadelphia, PA, USA. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, +1-301-924-7077, fax +1-301-924-7089

AOAC INTERNATIONAL Launches Proficiency Testing Program

AOAC INTERNATIONAL has launched Phase 1 of a new AOAC® Laboratory Proficiency Testing Program aimed at helping laboratories meet global standards of quality.

AOAC® Laboratory Proficiency Testing Program

Program	Code	Matrix	Annual cost	Annual shipping	Frequency	Shipment	Analytes/Organisms
Standard microbiology	M01	Mashed potatoes	$1500	$200	Quarterly	October January April July	Qualitative: *Salmonella* species *Listeria* species *E. coli* 0157:H7 Quantitative: Coliform *E. coli* Coagulase Positive *Staphylococcus* Yeast and Mold Aerobic Plate Count
Pathogen-free microbiology	M02	Mashed potatoes	$1300	$100	Quarterly	November February May August	Quantitative: Coliform *E. coli* Yeast and Mold Aerobic Plate Count
Meat microbiology 1	M03	Ground meat	$1400	$250	Quarterly	December March June September	Qualitative: *Salmonella* species
Meat microbiology 2	M04	Ground meat	$1400	$250	Quarterly	December March June September	Qualitative: *E. coli* 0157:H7

Developed by international experts, the program seeks to promote method validation and quality measurements in the analytical sciences. It also aims to improve analytical performance by providing an independent measure of data quality.

Phase 1 involves both food safety and nutritional labeling. Proficiency testing for food safety includes standard, pathogen-free, and meat microbiology test materials. It also covers test materials for E. coli sponge testing for cattle and swine carcasses, and testing for *E. coli* and *Salmonella* in poultry rinses.

Meat chemistry and processed cheese testing for nutritional labeling also fall under Phase 1. All test materials in this phase are distributed quarterly to provide adequate time for feedback and corrective action for subscribers with analytical problems. Expert help is provided to subscribers who request assistance in determining a course of corrective action.

The AOAC® Laboratory Proficiency Testing Program focuses on compliance with international guidelines for proficiency testing. The goal is to provide a comprehensive proficiency testing program. Future phases will include proficiency testing for additional areas of analytical interest in food nutrition, pesticide residues, environmental contaminants, and

AOAC® Laboratory Proficiency Testing Program—*continued*

Program	Code	Matrix	Annual cost	Annual shipping	Frequency	Shipment	Analytes/Organisms
Meat microbiology 3	M05	Processed meat	$1400	$250	Quarterly	December March June September	Qualitative: *Listeria monocytogenes*
HACCP for cattle/swine	M06	Sponges	$1500	$100	Quarterly	November February May August	Quantitative: *E. coli*
HACCP for poultry	M07	Rinse	$1500	$200	Quarterly	November February May August	Quantitative: *E. coli* Qualitative: *Salmonella* species
Meat chemistry	C01	Meat	$1000	$100	Quarterly	December March June September	Nutritional labeling: % Moisture % Fat % Protein % Ash % Carbohydrate Cholesterol Sodium Potassium Magnesium Iron Calcium Salt Calories
Cheese chemistry	C02	Processed cheese	$1000	$100	Quarterly	December March June September	Nutritional labeling: % Moisture % Fat % Protein % Ash % Carbohydrate Cholesterol Sodium Potassium Magnesium Iron Calcium Salt Calories

water. These phases are being planned by international experts who have been members of advisory task forces.

A 21st century approach to reporting of data has been implemented to ensure accurate data transcription. The proficiency testing reporting system verifies data entry, converts results into a bar code, and reduces transcription errors.

"This program will help labs improve their credibility with national and international customers," says Ron Christensen, AOAC INTERNATIONAL executive director. "It will provide access to international experts and greatly diminish the potential for errors by reporting data through computers. It will help labs get ahead of their competition."

AOAC Publishes Revisions to OMA and BAM

AOAC INTERNATIONAL has published the 16th Edition, 4th Revision, of its Official Methods of Analysis. These new and revised methods are published in Revisions and CD-ROM updates of the *Official Methods of Analysis of AOAC INTERNATIONAL.*

The March 1998 Revision includes **16 new and 102 newly revised methods**—methods not available when the initial publication and previous revisions were produced. The new and revised methods in this revision incorporate many of the latest developments in the field. **Method additions and revisions include:**

Chapter 5: Drugs in Feeds—Added new liquid chromatographic method with post-column derivitization for determination of **monensin in all types of premixes and feeds.** Analytical concentration range is from 0.0055 mg/g to 200 g/kg, with a limit of quantitation of 0.004 mg/g.

Chapter 7: Pesticide Formulations—Added new capillary gas chromatographic method for determination of **tebuconazole in fungicide formulations.** Method uses internal standard quantitation.

Added new capillary gas chromatographic method for determination of **MGK-264, pyrethrins and BPE in technical materials, concentrates, and finished products**. Method uses internal standard quantitation.

Added new reversed-phase liquid chromatographic method for determination of imidacloprid in liquid and solid formulations.

Added new liquid chromatographic method for determination of **thiodicarb in technical products and formulations**. Method is adaptation of CIPAC method.

Chapter 9: Metals and Other Elements at Trace Levels in Foods—Added new graphite furnace atomic absorption method for determination of **lead in sugars and syrups.** Method uses external standard calibration and has a method detection limit of 3.3 ng/g sugar.

Chapter 16: Extraneous Materials—Added new method using an exposure chamber for determination of **microbial ranking of porous packaging materials**. Method is adaptation of ASTM method.

Chapter 17: Microbiological Methods—Added new method using dry rehydratable film for determination of **yeast and mold counts in foods**. The mean log counts using this method were generally greater than those found in agar plating methods.

The price of the *1998 Official Methods of Analysis of AOAC INTERNATIONAL* is $399. Add $20 for shipping the print version within the United States and Canada; add $85 for other countries. For the CD-ROM format, one user, add $5 for shipping within the United States and Canada; add $30 for other countries.

1998 Revisions to FDA *Bacteriological Analytical Manual* (BAM)

AOAC has also made revisions to the 8th Edition of the FDA *Bacteriological Analytical Manual* (BAM). You can keep your copy of the 8th Edition current by purchasing Revision A and inserting the new pages without having to buy a whole new book. The 1998 version of the 8th Edition contains more than 300 pages of revised material.

Revised sections include the following new information:

- The chapter on *Campylobacter* includes a **new** enrichment broth, simplified sample processing procedures with product-specific flowcharts, and an **improved**, blood-free isolation agar.
- **Procedures with enhanced sensitivity** have been added to the chapter on *Staphylococcal enterotoxins*, including alternative ELISA-based assays and a newly developed SDS-Polyacrylamide gel-Immunoblot assay for staphylococcal enterotoxin A (which can detect enterotoxin after heat processing and discriminate SEA from cross-reacting proteins that may be present in the food matrix).
- The chapter on *Yeasts and Molds* now uses media that retard growth of bacteria and permit more accurate enumeration of yeasts and molds.
- The *Parasitic Animals in Foods* chapter has been expanded to include the FDA's protocol for PCR identification and microscopic detection of *Cyclospora cayatenensis*, an emerging protozoan pathogen that has been involved in several recent foodborne disease outbreaks.
- Appendix 1 is a compilation of selected commercially available methods kits, which has been **updated.**
- Appendix 2, on *Most Probable Number Enumeration of Bacteria*, has been revised to accommodate **new statistical assumptions** and to **clarify this statistical treatment** for analysts without extensive background in statistics.

The price of the 1998 8th Edition of BAM is $129. The price for the **Revision A** update is only $39. Add $5 in North America and $30 elsewhere for shipping. AOAC INTERNATIONAL members subtract 10% discount.

Journal Information

The Association

The primary objective of AOAC INTERNATIONAL is to obtain, improve, develop, test, and adopt precise, accurate, and sensitive methods for analysis of foods, vitamins, food additives, pesticides, drugs, cosmetics, plants, feeds, fertilizers, hazardous substances, air, water, and any other products, substances, or phenomena affecting the public health and safety, the economic protection of the consumer, or the protection of the quality of the environment; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in the analytical sciences related to foods, drugs, agriculture, the environment, and regulatory control of commodities in these fields; and to afford opportunity for the discussion of matters of interest to scientists engaged in relevant pursuits.

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

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European Representatives. For information about AOAC INTERNATIONAL and its publications, persons outside the United States may also contact the following: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone +31-318-418725.

The Journal

The *Journal of AOAC INTERNATIONAL* (ISSN 1060-3271) is published bimonthly by AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA. Each volume (one calendar year) will contain about 1400 pages. The scope of the *Journal* encompasses the development and validation of analytical procedures pertaining to the physical and biological sciences related to foods, drugs, agriculture, and the environment. Emphases is on research and development of precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

Methods. The scientific validity of published methods is, of course, evaluated as part of the peer-review process. However, unless otherwise stated, methods published in contributed papers in the *Journal* have not been adopted by AOAC INTERNATIONAL and are not AOAC Official Methods.

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Books in Brief

HPLC in Enzymatic Analysis, Second Edition. By Edward F. Rossomando. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158-0012, USA, 1998. Price: $149.95. 451 pp. ISBN 0-471-10340-3.

The use of high performance liquid chromatography techniques in the study of enzymatic reactions has grown significantly since the publication of the first edition of this highly successful book: the role of enzymes in biological research has expanded; the application of HPLC and enzymes has extended to more disciplines; advances in separation techniques and instrumentation have increased the capability of HPLC; and the discovery of new enzymes has spawned new methods of analysis. *High Performance Liquid Chromatography in Enzymatic Analysis, Second Edition,* addresses these developments in its coverage of the refinements of HPLC methods and their use in a wide range of laboratory applications. It offers the same practical approach found in the first edition, incorporates new information into existing chapters, and adds new chapters to deal with new applications, including capillary electrophoresis, forensic chemistry, microdialysis, and the polymerase chain reaction. Easy to read and full of practical advice and hundreds of diagrams and examples, this book is an invaluable resource for students, researchers, and laboratory workers in analytical chemistry and biochemistry, molecular biology and cell biology, and for anyone interested in keeping up with this fast-growing field.

High Performance Capillary Electrophoresis: Theory, Techniques, and Applications. Edited by Morteza G. Khaledi. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158-0012, USA, 1998. Price: $150.00. 1047 pp. ISBN 0-471-14851-2.

With features and capabilities that match—and even surpass—those of conventional electrophoresis and HPLC, high performance capillary electrophoresis is the fastest developing technology for the separation and analysis of chemical compounds. Keeping pace with the rapid changes in this field and the wealth of journal articles on the subject is a difficult and time-consuming challenge for anyone needing a basic and up-to-date grasp of HPCE. This book makes it much easier to find this important information—with comprehensive one-source coverage of all of the essential aspects of HPCE theory, techniques, and applications. Featuring the contributions of well-qualified, highly regarded scientists, it is organized into sections on the theory and principles of HPCE techniques, detection systems, operation aspects and special methods in HPCE, uses in chemical analysis, and physicochemical studies. Specific topics addressed here that are not treated extensively by other books include two-dimensional separations, CE on microchips, nonaqueous CE, indirect detection, monitoring enzymatic reactions, and more. As interest in HPCE continues to grow, it is clear that this technology has much to offer researchers and others working in disciplines ranging from analytical chemistry and biochemistry to pharmaceutical chemistry and biotechnology. This book provides scientists and students with the knowledge they need to take immediate advantage of the exciting potential of HPCE.

Free Radical and Antioxidant Protocols. Edited by Donald Armstrong. Published by Humana Press, Inc., 999 Riverview Dr, Suite 208, Totowa, NJ 07512, USA, 1998. Price: $89.50 455 pp. ISBN 0-89603-472-0.

In *Free Radical and Antioxidant Protocols*, 76 leading international authorities describe cutting-edge methodolo-

gies for quantitating free radical and antioxidant analytes in tissue and body fluids using experimental models and in vitro procedures. These user-friendly and easily reproducible techniques cover the essential tasks, including radical-generating systems, direct measurement or trapping of reactive radical species and acute-phase proteins, and measurement of metabolic intermediates derived from the oxidation of lipids, proteins, and nucleic acids. There are also methods for the determination of vitamin, enzymatic, and water-soluble antioxidants, as well as of essential micronutrients and cofactors. The techniques take advantage of new instrumentation—probes, photon counting, chemiluminescence, and caged compounds, with an emphasis on HPLC—and are adaptable to a wide range of applications. This book provides state-of-the-art methodology and biotechnology in a convenient format for both academic and corporate biomedical scientists. The detailed, laboratory-tested free-radical assays, many of them presented here for the first time, will illuminate the study of both primary and secondary oxidative stress and contribute significantly to the understanding of the many disorders associated with this process.

Introduction to Bioanalytical Sensors. By Alice J. Cunningham. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158-0012, USA, 1998. Price: $64.95. 418 pp. ISBN 0-471-11861-3.

Biosensors have become virtually indispensable components in the analytical scientist's tool kit. Increasingly, researchers are called upon to design and adapt them for customized applications. Yet, surprisingly, most young scientists graduate without having acquired an integrated working knowledge of the cross-disciplinary principles underlying biosensing strategies. This book was prepared to fill that critical educational gap. *Introduction to Bioanalytical Sensors* presents to readers a broad view of scientific concepts and principles on the design and use of biosensing devices and systems. While it integrates basic information from an array of related scientific disciplines, the emphasis throughout is on applications rather than theory. Only as much basic science is covered as is necessary for an analytical scientist to use or customize biosensing devices for fulfilling experimental objectives. Major topics covered include biosensors and bioanalytical challenges, designing for performance, developing bioselective layers, fundamentals of electroanalytical sensors, optically based energy transduction, and thermal and acoustic-wave transduction. This book, written as a self-teaching guide, assumes that readers have only a bachelor's degree in chemistry or a related field using analytical and physical chemistry, and biochemistry.

UPCOMING IN THE NEXT ISSUE

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New Products

State of the Art Isolation Chamber

Schaefer Technologies, Inc. announced the Model STS-6 sterility test suite. This state of the art isolation chamber is specially designed to provide for manual or automated testing of product sterility in a Class 100 environment. The STS-6 is constructed of 316 stainless steel with clear polycarbonate windows. The suite is also designed for manual or VHP sterilization.

Contact: Clark Sayer, Schaefer Technologies, Inc., 4251 North Shadeland Ave, Indianapolis, IN 46226, telephone +800-435-7174

Labconco's Crude Fiber Apparatus Determines Crude Fiber in Food Products

Labconco Corp. offers a crude fiber apparatus for the determination of crude fiber in food samples. The apparatus is a reflux condenser designed to operate with speed and accuracy and is listed by AOAC INTERNATIONAL. The apparatus processes 6 samples at a time in its 600 mL borosilicate glass beakers, which are locked in place between the spring-loaded heaters and Type 304 stainless steel condensers with inverted cone bases. A tight seal keeps acid and alkali at constant volumes during the boiling stages. The six 350 W heater elements are operated by individual infinite-control knobs with variable heat input, and the stainless steel manifold is mounted on a sturdy anodized aluminum framework. The apparatus arrives completely factory assembled and requires connections to a water source and an electrical circuit for operation.

Contact: Ginny Pressly, Labconco Corp., 8811 Prospect Ave, Kansas City, MO 64132-2696, telephone +800-821-5525

MICROSON™ Ultrasonic Homogenizer for Processing Small Volumes

The Misonix XL2000 MICROSON™ ultrasonic homogenizer is ideal for processing small sample volumes (usually

under 50 mL) for a wide variety of applications. Portable and economical, it features a convenient thumb-activated pulsing switch for easy operation. The unit is especially useful for cell disruption, particulate dispersion, mixing and homogenizing samples, as well as focused cleaning of intricate parts. The 100 W portable unit measures 7.5" × 13" × 7" and features automatic tuning, variable amplitude control, and digitally displayed watt meter. It comes with a 1/8" × 5" titanium alloy probe; optional tips are also available in 3/32", 3/16", and 1/4" diameters.

Contact: Kim Brecher, Misonix, Inc., 1938 New Hwy, Farmingdale, NY, 11735, telephone +800-645-9846

Ultraviolet Photometric Standards

SpectroStandards™ Analytical is introducing certified NIST-traceable UV photometric reference standards. The CRM 500UV filters are used for verification of the transmittance and absorbance scales of spectrophotometers in the UV mode. Certification is provided at 5 standard UV wavelengths, with the option of certification at customized wavelengths from 200–400 nm. The CRM 500UV exhibits the highest optical neutrality throughout the spectrum, eliminating bandwidth restrictions required for potassium dichromate and other liquid photometric standards. These solid standards are permanently mounted in cuvette-style holders that require no special handling or preparation before use.

Contact: Daniel L. Campbell, SpectroStandards Analytical, 363 W. Drake Rd No. 5, PO Box 270334, Fort Collins, CO 80527-0334, telephone +970-282-3840

UNI-FILTER® 384-Well Filter Bottom Microplates for High Throughput Screening

Chemists and life scientists working in combinatorial chemistry or genomics can very easily boost their productivity by 400% with the new Whatman UNI-FILTER® 384-well filter bottom microplates for high throughput screening. These new robotics-compatible microplates have the same size footprint as the widely available standard 96-well microplate. This means that each 384-well microplate fits the majority of microplate readers that read 384 wells. These microplates can also be used with any automated liquid-handling and reagent-dispensing systems built to handle 384-well microplates. Each well has a capacity of 100 µL. The microplates are available with GF/C and GF/B glass microfibre filters (1.2 and 1.0 µm pore sizes), 0.45 µm PVDF, and WCN membrane filters.

Contact: Sharman V. Pate, Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014, telephone +888-942-8626

High-Performance Benchtop LC/MS

The Shimadzu LCMS-QP8000 liquid chromatograph/mass spectrometer puts the quantitative and qualitative resolving power of LC/MS onto the laboratory bench. Designed to be a routine LC detector for the Shimadzu VP Series HPLC, the compact, affordable LCMS-QP8000 provides sensitivity and performance features found in larger, more expensive LC/MS instruments. User-selectable electrospray and APCI interfaces enhance flexibility for use in a wide range of HPLC analyses under a variety of mobile phase conditions. The LCMS-QP8000 is rugged, easy to operate, and extremely easy to maintain. Access to the ion source can be accomplished from the front of the instrument without the need for any tools. New CLASS-8000 32-bit software for Windows® 95 provides single-point control for the entire LC/MS system.

Contact: Mary Madden, Shimadzu Scientific Instruments, Inc., 7102 Riverwood Dr, Columbia, MD 21046-2502, telephone +800-477-1227

Explosion-Proof Pressure Switch Monitors Line Pressure and Gas Usage

The Scott Specialty Gases Model 69EP Series pressure switch provides a safe and convenient way to monitor line pressure or usage of flammable gas in a cylinder manifold system. The explosion-proof switch handles an inlet pressure of up to 3000 psig and can be set to activate on either increasing or decreasing pressure. It can also be used to trigger an external alarm when a predermined pressure is reached. This single-pole, double-throw switch can be safely used with flammable gases and with oxygen. It features a hermetically sealed switching element and meets NEMA Class 1 specifications, groups A, B, C, and D. A weathertight, aluminum housing provides protection from the elements while a 316L stainless steel port and diaphragm help prevent gas contamination. Maximum power rating is 15 amps at 480 VAC.

Contact: Robert Jefferys, Scott Specialty Gases, 6141 Easton Rd, PO Box 310, Plumsteadville, PA 18949, telephone +215-766-8861

New Wall-Mounted Gas Monitor

The Guardian is a brand new family of wall-mounted, continuous gas monitors available for carbon monoxide, hydrogen sulfide, oxygen, chlorine, sulfur dioxide, hydrogen cyanide, hydrogen chloride, nitrogen dioxide, nitric oxide, hydrogen, phosphine, and others. Standard features include digital display, NEMA 4X enclosures, output relays, and multi-level audio and visual alarms. The controller is available with up to 4 remote sensors that are fast responding and unaffected by temperature and humidity changes. A system test button, local danger alarm indicator, and fault alarm assure proper operation. The unit is easy to install, operate, and maintain.

Contact: Steven R. Adelman, CEA Instruments, Inc., 16 Chestnut St, Emerson, NJ 07630, telephone +201-967-5660

Safe Containment of ^{35}S Radioactive Waste

Contamination can occur in incubators when ^{35}S-labeled amino acids, such as methionine and cysteine, are used to label proteins. The new Disposable Charcoal Cartridge from Scienceware® is designed to efficiently absorb the volatile ^{35}S radioactive components released during this metabolic labeling. The new cartridges are supplied in a 100 mm id plastic Petri dish with a highly porous filter membrane sealed over the top edge. The unit is placed in the fume hood. The filter membrane allows the free flow of air containing the contaminants. The charcoal filter absorbs and holds the radioactive waste.

Contact: Jackie Swartzberg, Scienceware by Bel-Art Products, Pequannock, NJ 07440-1992, telephone +973-694-0500

New Matrix Solid-Phase Dispersion Products for Sample Preparation

Jones Chromatography, Inc. introduces 2 matrix solid-phase dispersion (MSPD) grade ISOLUTE® sorbents developed by International Sorbent Technology. MSPD sorbents improve the extraction of drugs and other residues from complex biological and food samples. They produce a more homogenous and rapid blend of the matrix–sorbent mixture than previously used sorbents. This makes the blending process less operator dependent. Additionally, the homogenized sample provides a better packed bed for minimum analyte elution volume. MSPD sorbents are ideal for veterinary and food analysis. These sorbents are available in MSPD C_{18} and MSPD C_{18} end-capped chemistries.

Contact: Lauren Burgess, Jones Chromatography, Inc., PO Box 280329, Lakewood, CO 80228-0329, telephone +800-874-6244

Hach's Newest Technology Platform—Water Quality Test Strips

Hach's Water Quality Test Strips are designed for fast and dependable on-site testing for 8 key water quality parameters. Testing takes just seconds and costs only pennies per test. Hach's easiest testing platform yet requires no training and no handling of chemicals. For anyone performing semi-quantitative spot-checks or field tests, there is no faster way to test. The test strips use proven chemistries that are easy to read, safe, and disposable. They are available for free and total chlorine, hardness, alkalinity, iron, copper, nitrate and nitrite, pH, and chloride.

Contact: Cindee Arduino, Hach Co., PO Box 389, Loveland, CO 80539, telephone +800-227-4224

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AGRICULTURAL MATERIALS

Assay of Oxytetracycline in Animal Feeds by Liquid Chromatography with Fluorescence Detection

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LUANN WETZLER
Nebraska Department of Agriculture, 3703 South 14th St, Lincoln, NE 68502

A liquid chromatographic (LC) method is described to assay oxytetracycline (OTC) in feeds at gram-per-ton and gram-per-pound levels. Complete feeds and premixes are extracted with acid–methanol, according to AOAC Method 957.23B(i). Extracts are filtered and quantitated by LC using a C_{18} column and fluorescence detection with excitation at 390 nm and emission at 512 nm. The mobile phase (1.5 mL/min) uses a methanol gradient with an initial hold time of 1 min at 15% methanol followed by an increase to 35% methanol over 8 min. The aqueous component contains $CaCl_2$, disodium ethylenediamine tetraacetate, and sodium acetate buffered to pH 6.5. Recovery of spiked samples averaged 101.6% (coefficients of variation, 0.1–2.4%), and the limit of detection was 1 g/ton. Results correlated closely with those obtained by using AOAC microbiological Method 968.50 and with another LC method using ultraviolet detection. OTC and epi-OTC elute at approximately 7.4 and 4.8 min, respectively. No interference was observed from several drugs and other antibiotics often used in feeds in combination with OTC.

For several years our laboratory has made adjustments to a liquid chromatographic (LC) method for the assay of oxytetracycline (OTC) in feeds using ultraviolet (UV) detection (1). To improve selectivity and sensitivity, we investigated modifications of the isocratic LC method with fluorescence detection reported by Iwaki et al. for tetracyclines in fish (2). The LC and fluorescence detector conditions described here for OTC in feeds is similar to those previously reported by Houglum et al. (3) for the assay of chlortetracycline (CTC) in animal feeds. The differences are reductions in the percentage of methanol in the gradient mobile phase and in the hold time prior to beginning the gradient. Data were collected to determine accuracy, precision, sensitivity, and correlation with other assays.

Received November 4, 1997. Accepted by RN April 6, 1998.

METHOD

Safety

Use protective equipment and safe laboratory techniques. Follow appropriate precautions when handling samples and reagents to avoid direct contact or inhalation. Use HCl in a fume hood.

Apparatus

(**a**) *LC system.*—BioRad (Richmond, CA) Model 8221 HRLC gradient system equipped with Model 8221 gradient module, 2 Model 1350 Soft-Start pumps, Model AS-48 autosampler, HRLC 800 software, Rheodyne (Cotati, CA) Model 7125 fixed-loop injector (20 µL), Thermo Separation Products (San Jose, CA) on-line solvent degasser, and Model FL2000 fluorescence detector with excitation at 390 nm and emission at 512 nm.

(**b**) *LC columns.*—0.46 × 15 cm reversed-phase (5 µm, C_{18}) Prodigy cartridge column and guard column (Phenomenex, Torrance, CA), guard column and 0.46 × 15 cm reversed-phase (5 µm, C_{18}) Alltima cartridge column (Alltech Associates, Deerfield, IL).

(**c**) *Syringe filters.*—25 mm diameter, 0.45 µm nylon (Alltech Associates).

Reagents

(**a**) *Solvents.*—Reagent-grade methanol for extraction; LC-grade methanol for chromatography, filter through 0.45 µm filter under vacuum.

(**b**) *Acid–methanol extraction solution.*—Mix methanol and HCl (50 + 1).

(**c**) *Aqueous portion of gradient mobile phase.*—Prepare pH 6.5 solution of 0.1M sodium acetate, 0.055M calcium chloride and 0.020M disodium ethylenediamine tetraacetate (EDTA) as follows: add 7.44 g disodium EDTA dihydrate to ca 600 mL water and 7 mL 40% NaOH to expedite dissolution; then dissolve 6.10 g $CaCl_2$ and 8.20 g sodium acetate; adjust pH to 6.5 with concentrated HCl and bring to 1 L with water. Filter through 0.45 µm filter under vacuum. Solution may be refrigerated to prevent microbial growth.

(**d**) *OTC and CTC·HCl reference standards.*—U.S. Pharmacopeial Convention, Inc. (Rockville, MD).

(**e**) *4-Epioxytetracycline (epi-OTC).*—Pfizer, Inc. (Easton, PA).

(f) *Tetracycline·HCl.*—Sigma Chemical Co. (St. Louis, MO).

(g) *OTC standard solutions.*—Prepare stock solution (1 mg/mL) by weighing 25 mg OTC into 25 mL volumetric flask, add 3 mL methanol, dissolve, and bring to volume with 0.01N HCl. Prepare intermediate stock solution (100 μg/mL) by diluting 10 mL stock standard to 100 mL with acid–methanol solution. Prepare working standard solutions (2, 10, and 15 μg/mL) by diluting intermediate stock solution with water and/or acid–methanol solution so that the ratio of water to acid–methanol is approximately the same as in the final dilution of the sample. Intermediate stock solution may be stored at –10°C for several months.

Sample Preparation

Accurately weigh in an Erlenmeyer flask an amount of ground feed based on potency claim as follows: <50 g/ton, weigh 20 g in 250 mL flask; 50–500 g/ton, weigh 1000 ÷ X g feed in 250 mL flask where X = g/ton claim (e.g., weigh 10 g feed for OTC claim of 100 g/ton); 501–1999 g/ton weigh 2 g into 250 mL flask; ≥1 g/pound, weigh 2 g into 500 mL flask. Add 100 mL acid–methanol solution if claim is <1 g/lb and 200 mL acid–methanol solution if claim is ≥1 g/lb. Shake for 30 min and centrifuge 5 min at 2500 rpm. Dilute sample with water and/or acid–methanol solution so that final ratio of water to acid–methanol solution and the concentration of OTC are approximately the same as those in the working standard but the solutions should contain at least 50% water. For example, add 5 mL extract from 100 g/ton sample to 10 mL volumetric flask and dilute to volume with water. Use syringe filter for diluted extracts that remain cloudy.

Table 1. Effect of $CaCl_2$ and EDTA concentrations

$CaCl_2$, mM	EDTA, mM	Relative response[a]
0	25	0
40	25	71
50	0	32
50	5	81
50	15	99
50	25	100
50	35	100
60	25	108
75	25	103

[a] Values are averages of duplicate injections of OTC standard.

LC Conditions

Elute at 1.5 mL/min with a gradient containing an increasing methanol concentration. Hold initial 15/85 ratio of methanol/aqueous portion for 1 min before increasing to 35/65 over 8 min and then hold at 35/65 until no other peaks elute (usually less than 7 additional min). Before the next injection, reequilibrate the column with 15/85 for 5 min or until a steady baseline is obtained.

System suitability parameters include a linear 3-point standard curve that passes through the origin, has a correlation coefficient >0.999, and encompasses the concentration of OTC in the sample extracts; baseline separation of OTC from epi-OTC; and consistent retention times between sample and standard (± 0.5 min). If peak separation of sample is not suitable, adjust

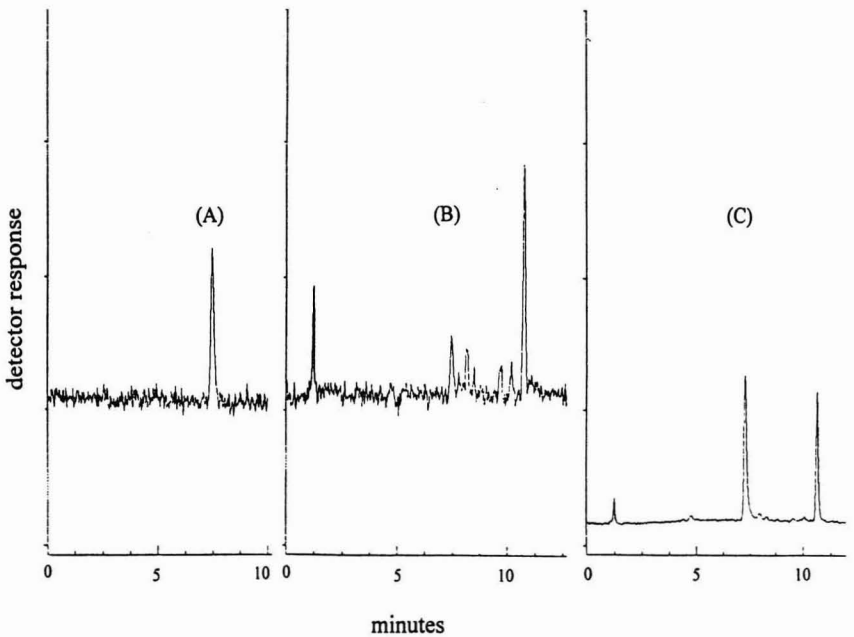

Figure 1. Chromatogram of 0.4 μg/mL OTC standard (A) and OTC in extracts from feeds containing OTC at 1.4 g/ton (B) and 130 g/ton (C). Full-scale detector response is 10× for 130 g/ton (C), compared with chromatograms A and B.

Table 2. Recovery of OTC from spiked feeds[a]

Spike level	Recovery, %	CV, %
1 g/lb	100.3	0.6
1 g/lb	101.0	1.0
4 g/lb	101.7	1.5
4 g/lb	102.0	1.0
400 g/ton	102.0	2.0
400 g/ton	98.7	2.3
400 g/ton	101.7	1.5
100 g/ton	100.7	1.9
50 g/ton	106.3	1.4
50 g/ton	102.7	2.4
50 g/ton	101.8	0.1
5 g/ton	100.0	1.0
Average	101.6	1.8

[a] Each result represents an average of 3 spikes of the same feed, except that the 100 g/ton spike level represents 5 different feeds. A total of 10 different feeds were used.

conditions of gradient slightly (e.g., alter the 8 min gradient time or the 15/85 ratio or increase initial hold time).

Determination

Repeat injections of working standard until consecutive injections provide responses within 2% of each other. Inject standard after every 3 injections of sample. Calculate as follows:

$$\text{OTC·HCl, g/ton} = (R/R') \times (C'/C) \times 454 \times 2000 \times 1.08$$

where R and R' are OTC area response of sample and average peak area of standards that bracket sample, respectively, and C' and C are concentrations (mg/mL) of OTC standard as base and sample extract, respectively. To calculate in units of grams per pound, eliminate the factor 2000 from the equation.

Table 3. Precision of LC assay

Source of variation	Average OTC found	CV, % (n = 5)
Between days[a]	3.1 g/lb	0.9
	3.5 g/lb	2.6
	572 g/ton	3.6
	273 g/ton	6.0
	141 g/ton	3.1
	86 g/ton	2.7
	58 g/ton	5.7
Within day[b]	3.1 g/lb	1.9
	3.5 g/lb	1.2
	561 g/ton	2.7
	263 g/ton	3.2
	141 g/ton	1.1
	125 g/ton	1.5
	56 g/ton	1.3

[a] Average represents different extracts.
[b] Average represents same extract.

Table 4. Comparison of results from 3 methods[a]

Sample	OTC, g/ton: LC with fluorescence	LC with UV	Microbiological assay
1	726	777	680
2	648	642	624
3	539	574	563
4	449	423	432
5	416	420	426
6	395	402	414
7	233	212	211
8	203	209	191
9	180	177	188
10	143	150	176
11	124	112	126
12	120	161	148
13	109	120	120
14	100	97	96
15[b]	86	84	103
16	85	84	90
17	80	80	83
18	76	76	82
19	74	77	74
20	72	74	80
21	72	74	80
22	68	71	60
23	66	71	59
24	57	57	64
25	7	12	8

[a] Correlation coefficient for fluorescence and UV methods = 0.997. Correlation coefficient for fluorescence and microbiological methods = 0.997. Correlation coefficient for UV and microbiological methods = 0.996.
[b] Sample contained 5 g/ton CTC as assayed by fluorescence LC. Results not included in calculation of correlation coefficients.

Comparisons with Other Methods

Results of the LC fluorescence method were compared with results from another laboratory using microbiological plate assay (AOAC Method **968.50**) and a previously reported (4) LC method using UV detection at 365 nm, a C_{18} reversed-phase column, and mobile phase components of dimethylformamide and phosphate buffer at pH 2.5. Samples were extracted with acid–methanol, and the same extracts were used for the 2 assays.

Results and Discussion

Chromatographic Response

As with the LC assay of CTC with fluorescence detection, the detector response to OTC depended on the concentration of $CaCl_2$ and EDTA in the mobile phase. Table 1 compares the detector response to OTC for various concentrations of $CaCl_2$ and EDTA in the mobile phase. To obtain near-optimum response, EDTA and $CaCl_2$ should be ≥15 and ≥50 mM, respectively. Peak width also increases slightly as EDTA decreases. EDTA at 20 mM and $CaCl_2$ at 55 mM were used for routine analysis.

Figure 1 shows chromatograms of OTC standard (0.4 μg/mL; retention time, 7.4 min) and feed extracts containing OTC at 1.4 and 130 g/ton. The chromatogram was obtained with a Prodigy ODS-3 column. As previously reported for CTC assay (1), the chromatographic responses with Alltima and Prodigy columns for OTC assay are indistinguishable without any adjustment in LC conditions. Although most of the data presented here were obtained with the Prodigy column, we have used the 2 types of columns interchangeably for more than a year and have no preference. No other columns have been tested. Dilution of extracts by at least 50% with water causes precipitation of some contaminants, improves longevity of the guard column, and prevents the increased back pressure observed prior to the use of water to dilute the samples.

A linear response was obtained for OTC standards (1–50 μg/mL), with a correlation coefficient of >0.9999. The intercept of the line was not significantly different from zero ($\alpha = 0.01$). Epi-OTC, a degradation product of OTC in feeds, and tetracycline separated from OTC with retention times of 4.8 and 16 min, respectively. CTC does not elute within the time frame of this method. On the basis of recoveries from a spiked extract, the limit of detection is estimated at 1 g/ton (2 ng/injection). As shown in Table 2, average recovery of a feed extract spiked 3 times at 5 g/ton was 100%, with a coefficient of variation (CV) of 1%.

Precision and Accuracy

Commercial feeds were extracted to obtain between-day and within-day precision (Table 3). Between-day CVs ranged from 0.9% for 3.1 g/lb to 6.0% for 273 g/ton. Within-day CVs were narrower in range (1.1–3.2%). Accuracy was determined by spiking extracts of commercial feeds (Table 2). Average recovery was 101.6% (98.7–106.3%) for 12 feeds.

The fluorescence method has better selectivity and sensitivity compared with another LC method used in our laboratory (1). The following compounds were investigated for potential interference with retention time and detector response of OTC: amprolium, arsanilic acid, decoquinate, monensin, roxarsone, sulfamethazine, sulfathiazole, penicillin, and riboflavin. Each compound was spiked at 100 μg/mL into a 10 μg/mL OTC standard. None of these compounds exhibited chromatographic interference with OTC.

Comparison with Other Methods

For comparison of the fluorescence LC method with other methods, 25 feed samples were also assayed by the official AOAC microbiological plate assay and by a previously reported LC/UV method (Table 4). Results of single analysis of these samples by each method ranged from 7 to 726 g/ton. Samples included commercial feeds for cattle, swine, and rabbit. Almost identical correlation coefficients (0.996–0.997) are obtained when comparing the data for any 2 of these 3 methods. One sample gave a notably higher result with the microbiological assay but was determined to also contain approximately 5 g CTC/ton when assayed by the fluorescence LC method (3). The ability to distinguish between these 2 tetracyclines illustrates one advantage of LC assays, because contamination of CTC in the OTC microbiological assay is known to give erroneously high OTC results. In addition, because the LC methods readily separate epi-OTC from OTC, these methods may also be useful to determine whether lower than expected results by the microbiological method are a result of significant degradation to epi-OTC.

Summary and Conclusions

This LC fluorescence method for determining OTC in animal feeds provides an accurate and reproducible alternative to the microbiological assay while using the same extraction procedure. It also provides an alternative to the previously published LC method using UV detection and allows OTC quantitation at low levels. The chromatography usually takes 16 min for each sample extract; separates OTC from epi-OTC, tetracycline, and CTC; and is not affected by other drugs typically added to OTC-containing feeds. In addition, as reported previously, a slight variation of the mobile phase conditions (i.e., higher methanol content) also allows assay of CTC. The method requires a fluorescence detector and an LC system with gradient capabilities.

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AGRICULTURAL MATERIALS

Determination of Micronutrients in Feed Products by Inductively Coupled Plasma Mass Spectrometry

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Micronutrients are widely used in feed products and are very important to the health and productivity of farm and domestic animals. It is imperative to have analytical capabilities for rapid and accurate results. By using a simple nitric acid digestion of the feed coupled with inductively coupled plasma-mass spectrometry (ICP/MS), rapid and accurate results can be obtained. ICP/MS has many advantages over classical wet chemical methods, atomic absorption methods, and other ICP methods: speed of analysis, reduced standards preparation, reduced waste stream, and qualitative scan capabilities. One gram portions of feed products were placed into 250 mL volumetric flasks and digested with 30 mL concentrated high-purity nitric acid for about 20 min. They were then brought to volume with ultra-high-purity water, shaken, and filtered. Ten milliliter portions were taken, and 0.5 mL internal standards were added. Standards were made up in concentrations to agree with the expected range of the desired element. Analysis of check samples from the Association of American Feed Control Officials shows that this method is comparable with other methods of choice.

Inductively coupled plasma mass spectrometry (ICP/MS) was first commercially introduced in 1983. A number of laboratories have used ICP/MS for environmental analysis with success (1); however, there are concerns with implementing methodology and optimizing method performance. The atomic absorption (AA) spectrophotometric method for analyzing minerals in feed products has been an AOAC INTERNATIONAL Official Method (**968.08**) since 1969 (2). With recent developments, ICP/MS has been used to analyze micronutrients in a variety of feeds (R. Beine, personal communication).

This study investigates use of ICP/MS in analyzing micronutrients in feed products to determine if the instrument would be a suitable analytical tool to improve productivity of analysis while maintaining accuracy and precision of traditional methods.

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METHOD

Apparatus

(**a**) *VG Elemental Genesis ICP/MS with Meinhard nebulizer system.*

(**b**) *Gilson Minipuls 3 with autosampler.*

(**c**) *Compaq Prolinea 4/50 computer.*

(**d**) *Epson FS-870 printer.*

(**e**) *Analytical balance.*—Sensitive to 0.1 mg.

(**f**) *Automatic pipettors.*—2.0–10.0 mL and 25 µL.

(**g**) *Thomas Wiley mill.*—Standard Model No. 3.

(**h**) *Repipetor.*—1.0 mL delivery capacity.

(**i**) *Volumetric flasks.*—With glass stoppers; 100, 250, 500, and 1000 mL; acid-washed.

(**j**) *Hot plate.*—Capable of achieving boiling temperature of acid.

(**k**) *Fume hood.*

(**l**) *Volumetric pipet.*—25 mL.

(**m**) *Graduated cylinder.*—50 mL.

(**n**) *Autosampler tubes.*—Polypropylene, round bottom, with stoppers; Falcon brand 2018 or equivalent.

(**o**) *Screen.*—1.0 mm opening.

Reagents

(**a**) *Association of American Feed Control Officials (AAFCO) check samples.*—9531 (Gilt Finisher), 9623 (Cattle Feed), 9724 (Pet Food, Cat), and 9728 (Horse Feed).

(**b**) *Nitric acid.*—Concentrated, Optima or TraceMetal grade.

(**c**) *Ultrapure water.*

(**d**) *Plasma-grade ICP/MS standards.*—1000 ppm in 2% nitric acid of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, zinc, beryllium, bismuth, cobalt, indium, lanthanum, uranium, lead, lithium-6, and yttrium.

(**e**) *Liquid argon.*—99.996% purity or better.

(**f**) *2% Nitric acid solution.*—20 mL nitric acid diluted to 1000 mL in ultrapure water.

(**g**) *Tuning solution.*—Using autopipettor, transfer 10 mL beryllium, bismuth, cobalt, indium, lanthanum, magnesium, lead, and uranium stock standards (1000 ppm) into 100 mL volumetric flask. Bring to volume with 2% nitric acid solution. Stopper and shake. Next, transfer 25 µL of this solution to another 100 mL volumetric flask and bring to volume with 2%

Table 1. Comparison of results for AAFCO check sample 9531[a]

	All methods			ICP/MS			ICP			AA		
Element	Mean[b] (grand average), %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %
Ca	0.8163	0.0569	6.97	0.7980	0.0238	2.98	0.8140	0.0554	6.81	0.8126	0.0543	6.68
Cu	0.0024	0.0003	12.50	0.0022	0.0003	13.64	0.0024	0.0003	12.50	0.0025	0.0003	12.00
Fe	0.0408	0.0060	14.71	0.0470	0.0037	7.87	0.0397	0.0057	14.36	0.0423	0.0051	12.06
Mg	0.1374	0.0082	5.97	0.1410	0.0063	4.47	0.1365	0.0093	6.81	0.1377	0.0074	5.37
Mn	0.0085	0.0008	9.41	0.0080	0.0005	6.25	0.0086	0.0006	6.98	0.0084	0.0010	11.90
P	0.5783	0.0245	4.24	0.5481	0.0296	5.40	0.5801	0.0259	4.46	—[c]	—[c]	—[c]
K	0.7419	0.0570	7.68	0.7490	0.0332	4.43	0.7423	0.0457	6.16	0.7397	0.0557	7.53
Zn	0.0307	0.0021	6.84	0.0290	0.0012	4.14	0.0305	0.0019	6.23	0.0307	0.0023	7.49

[a] All data except for ICP/MS are taken from check sample 9531 result sheets.
[b] Means are representative of results from 25 to 145 participating laboratories.
[c] Phosphorus was not analyzed by AA by any of 179 participating laboratories.

Table 2. Comparison of results for AAFCO check sample 9623[a]

	All methods			ICP/MS			ICP			AA		
Element	Mean[b] (grand average), %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %
Ca	4.46	0.2247	5.03	4.49	0.1120	2.49	4.49	0.2060	4.59	4.45	0.2122	4.77
Cu	0.0174	0.0012	6.90	0.0157	0.0009	5.73	0.0168	0.0012	7.14	0.0177	0.0012	6.78
Fe	0.0470	0.0052	11.06	0.0615	0.0024	3.90	0.0448	0.0047	10.49	0.0483	0.0039	8.07
Mg	0.5311	0.0341	6.42	0.5541	0.0060	1.08	0.5292	0.0292	5.52	0.5310	0.0359	6.76
Mn	0.0668	0.0061	9.13	0.0673	0.0077	11.44	0.0660	0.0057	8.64	0.0673	0.0055	8.17
P	1.12	0.0545	4.87	1.06	0.0129	1.22	1.12	0.0619	5.53	—[c]	—[c]	—[c]
K	1.38	0.0818	5.93	1.44	0.0206	1.43	1.37	0.0656	4.79	1.37	0.0818	5.97
Zn	0.0540	0.0041	7.59	0.0531	0.0009	1.69	0.0537	0.0042	7.82	0.0544	0.0041	7.54

[a] All data except for ICP/MS are taken from check sample 9623 result sheets.
[b] Means are representative of results from 26 to 158 participating laboratories.
[c] Phosphorus was not analyzed by AA by any of 179 participating laboratories.

Table 3. Comparison of results for AAFCO check sample 9724[a]

	All methods			ICP/MS			ICP			AA		
Element	Mean[b] (grand average), %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %
Ca	1.67	0.0962	5.76	1.69	0.0360	2.13	1.71	0.1020	5.96	1.65	0.0841	5.10
Cu	0.0020	0.0002	10.00	0.0017	0.0001	5.88	0.0020	0.0002	10.00	0.0020	0.0002	10.00
Fe	0.0337	0.0035	10.39	0.0414	0.0025	6.04	0.0322	0.0036	11.18	0.0345	0.0030	8.70
Mg	0.1004	0.0079	7.87	0.1003	0.0013	1.30	0.1025	0.0081	7.90	0.0997	0.0069	6.92
Mn	0.0082	0.0005	6.10	0.0079	0.0002	2.53	0.0082	0.0006	7.32	0.0081	0.0005	6.17
P	1.05	0.0413	3.93	1.04	0.0173	1.66	1.06	0.0465	4.39	—[c]	—[c]	—[c]
K	1.04	0.0641	6.16	1.11	0.0126	1.13	1.06	0.0610	5.75	1.04	0.0633	6.09
Zn	0.0209	0.0013	6.22	0.0212	0.0005	2.36	0.0210	0.0014	6.67	0.0209	0.0012	5.74

[a] All data except for ICP/MS are taken from check sample 9724 result sheets.
[b] Means are representative of results from 30 to 146 participating laboratories.
[c] Phosphorus was not analyzed by AA by any of 186 participating laboratories.

Table 4. Comparison of results for AAFCO check sample 9728[a]

	All methods			ICP/MS			ICP			AA		
Element	Mean[b] (grand average), %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %	Mean, %	SD	RSD, %
Ca	3.74	0.1763	4.71	3.79	0.1100	2.90	3.80	0.2022	5.32	3.71	0.1575	4.24
Cu	0.0225	0.0017	7.55	0.0204	0.0005	2.45	0.0223	0.0018	8.07	0.0227	0.0017	7.49
Fe	0.0863	0.0135	15.64	0.0888	0.0050	5.63	0.0800	0.0154	19.25	0.0922	0.0080	8.68
Mg	0.4286	0.0253	5.90	0.4489	0.0132	2.94	0.4334	0.0239	5.51	0.4249	0.0255	6.00
Mn	0.0369	0.0026	7.05	0.0359	0.0011	3.06	0.0365	0.0023	6.30	0.0373	0.0027	7.24
P	1.98	0.0787	3.97	1.84	0.0404	2.20	1.98	0.0760	3.84	—[c]	—[c]	—[c]
K	1.78	0.1101	6.18	1.94	0.0434	2.24	1.80	0.0888	4.93	1.76	0.1053	5.98
Zn	0.0498	0.0035	7.03	0.0483	0.0009	1.86	0.0503	0.0034	6.76	0.0496	0.0033	6.65

[a] All data except for ICP/MS were taken from check sample 9728 result sheets.
[b] Means are representative of results from 38 to 154 participating laboratories.
[c] Phosphorus was not analyzed by AA by any of 182 participating laboratories.

nitric acid solution. The final tuning solution contains 25 ppb of each element.

Safety Precautions

All digestions of samples should be performed in a fume hood with face velocity capability of at least 100 CFM (cubic feet per minute). Gloves, protective eyewear, and protective clothing should be worn when handling acids or metal standards. Instrument must be well ventilated to outside of building. Never look at plasma without protection from UV light.

Table 5. Parameters for ruggedness test

Parameter	Variation 1	Variation 2
Acid volume	A = 30 mL	a = 20 mL
Digestion time	B = 30 min	b = 20 min
Concentration deselection by element	C = 3 concentrations of 8 elements	c = 5 concentrations of 8 elements
Internal standard selection	D = lithium-6 and yttrium	d = scandium and rhodium
Tuning element	E = cobalt	e = magnesium
Argon pressure	F = 70 psi	f = 65 psi
Autosampler rate	G = 0.75 mL/min	g = 0.83 mL/min

Quality Assurance and Quality Control

To ensure quality, certain routine maintenance items must be addressed. Autosampler tubing and cones should be changed every 100 samples. Nebulizer needs to be cleaned every month. Quality control measures should be established to ensure that the analyst will be made aware if the method is out of control. Each instrument run should include a spiked sample or reference material, a reagent blank, and duplicates. Quality control data should be maintained through use of control charts. Data points must be posted and reviewed before any data are reported.

Procedure

(**a**) *Preparation of internal standards.*—Using 10 mL autopipettor, transfer exactly 10 mL of each 1000 ppm stock standards of lithium-6 and yttrium into a 500 mL volumetric flask. Bring to volume with 2% nitric acid solution, stopper, and shake. Place this solution into a repipet bottle set at 0.5 mL delivery.

(**b**) *Element standards.*—(*1*) *Standard 5.*—Using autopipettor, transfer 4.2 mL each of the calcium, magnesium, phospho-

Table 6. Ruggedness test: Recovery (%) of all parameters[a]

Element	s	t	u	v	w	x	y	z
Mg	96.4	104.1	109.0	103.0	108.2	72.5	99.9	92.0
P	97.0	85.6	83.0	80.3	84.2	69.2	89.0	92.0
K	97.6	98.1	106.2	103.5	103.8	73.7	97.5	99.2
Ca	98.1	99.4	107.1	106.0	101.7	79.6	98.4	100.9
Mn	91.5	93.5	98.5	93.1	104.5	78.4	90.2	92.0
Fe	126.9	108.9	114.2	117.5	128.6	111.5	109.4	125.2
Cu	74.5	99.4	90.9	92.8	88.2	88.6	73.7	103.9
Zn	90.6	82.5	87.3	85.7	97.2	78.4	84.5	93.7

[a] The letters s, t, u, v, w, x, y, z represent the results (in % recovery) of all 8 determinations.

Table 7. Ruggedness test: Recovery (%) per parameter[a]

Element	A	a	B	b	C	c	D	d	E	e	F	f	G	g
Mg	103.1	93.1	95.3	101.0	103.4	92.9	98.1	98.2	92.4	103.8	99.9	96.3	92.9	103.3
P	86.5	83.6	84.0	86.1	88.3	81.8	90.9	79.2	85.3	84.8	88.4	81.7	83.9	86.2
K	101.3	93.5	93.3	101.6	101.2	93.6	98.1	96.8	94.1	100.7	101.0	93.8	93.1	101.8
Ca	102.7	95.1	94.7	103.1	101.3	96.5	99.2	98.6	96.4	101.4	101.7	96.1	95.5	102.3
Mn	94.1	91.3	92.0	93.5	96.2	89.3	91.8	93.6	90.1	95.3	95.3	90.1	88.3	97.1
Fe	116.9	118.7	119.0	116.6	119.8	115.8	117.6	118.0	119.4	116.1	124.6	111.0	116.3	119.2
Cu	89.4	88.6	87.7	90.3	81.8	96.2	87.9	90.1	89.5	88.5	89.9	88.2	82.4	95.6
Zn	86.5	88.5	87.2	87.8	89.9	85.1	87.8	87.2	87.5	87.5	91.8	83.2	84.8	90.2

[a] A comparison of parameter A to parameter a (for example) is examined by calculating (s + t + u + v)/4 and (w + x + y + z)/4.

rus, potassium, manganese, iron, copper, and zinc stock standards (1000 ppm in 2% nitric acid) into a 100 mL volumetric flask, fill flask to volume with 2% nitric acid solution, stopper, and shake. This standard will contain 42.0 ppm of each element. Pipet 10 mL of this standard into an autosampler tube and add 0.5 mL internal standard. The final concentration will be 40 ppm. (*2*) *Standard 4.*—Using 25 mL volumetric pipet, transfer standard 5 into a 100 mL volumetric flask, fill flask to volume with 2% nitric acid solution, stopper, and shake. The concentration of each element is 10.5 ppm. Transfer 10 mL into an autosampler tube and add 0.5 mL internal standard. The final concentration will be 10 ppm. (*3*) *Standard 3.*—Using 10 mL autopipettor, transfer 10 mL standard 4 into 100 mL volumetric flask, fill flask to volume with 2% nitric acid solution, stopper, and shake. Pipet 10 mL of this standard into an autosampler tube and add 0.5 mL internal standard. The final concentration will be 1.0 ppm. (*4*) *Standard 2.*—Using 10 mL autopipettor, transfer 10 mL standard 3 into 100 mL volumetric flask, fill flask to volume with 2% nitric acid solution, stopper, and shake. Pipet 10 mL of this standard into an autosampler tube and add 0.5 mL internal standard. The final concentration will be 0.1 ppm. (*5*) *Standard 1.*—Using 10 mL autopipettor, transfer 10 mL standard 2 into 100 mL volumetric flask, fill flask to volume with 2% nitric acid solution, stopper, and shake. Pipet 10 mL of this standard into an autosampler tube and add 0.5 mL internal standard. The final concentration will be 0.01 ppm.

(**c**) *Sample preparation.*—Grind feed samples with Wiley Mill and pass through a 1 mm screen. Weigh exactly 1.0 g ground sample and place into a 250 mL volumetric flask. Using 50 mL graduated cylinder, add 30 mL TraceMetal grade nitric acid to flask. Place flask on a hot plate located under a well-ventilated fume hood and bring sample to boil. Boil sample(s) for 20 min and then remove from hot plate. *Caution*: Do not allow sample to boil to dryness. Allow the flask(s) to cool under the hood. After flask(s) are cool to touch, fill to volume with ultrapure water. Stopper flask and shake. If solid content is visible to the naked eye, filter sample through Whatman No. 5 filter paper to prevent obstruction of nebulizer. With autopipettor, transfer proper volumes into autosampler tubes and bring volume to exactly 10 mL with 2% nitric acid solution. Add to sample 0.5 mL internal standard solution. Stopper and shake. Sample(s) is now ready for ICP/MS analysis.

(**d**) *Reagent blank.*—A reagent blank is prepared by placing 30 mL of the same lot of nitric acid used in digesting samples into a 250 mL volumetric flask. The reagent blank is treated in the same manner as samples. Ten milliliters is placed into an autosampler tube and 0.5 mL internal standard is added. The reading from the reagent blank is subtracted by the instrument from all samples.

(**e**) *Standard blank.*—A standard blank is also analyzed with each batch of samples. The standard blank consists of 10 mL 2% nitric acid solution to which 0.5 mL internal standard is added. The standard blank is subtracted by the instrument from all standards.

Calculations

Calculations using fully quantitative multielement algorithms are performed by the software.

A dilution correction is made to each sample with the following formula:

$$\text{Dilution correction} = \frac{(\text{final vol. mL}) \times (\text{aliquot fraction})}{(\text{vol. or wt. of sample, mL or g})}$$

The reading from the instrument is always expressed as µg/mL or ppm. To convert ppm to percent, divide by 10 000.

Instrument Optimization

Set autosampler at delivery rate of 0.83 mL/min. Allow instrument to warm up for ca 1 h. Optimize instrument with

Table 8. Ruggedness test: Difference (%) per element

Mg	P	K	Ca	Mn	Fe	Cu	Zn
10.0A	2.9A	7.8A	7.5A	2.8A	1.8a	0.8A	2.0a
5.7b	2.1b	8.3b	8.4b	1.5b	2.7B	2.6b	0.6b
10.5C	6.5C	7.6C	4.9C	6.9C	4.0C	14.3c	4.8C
0.1d	11.7D	1.3D	0.6D	1.8d	0.4d	2.2d	0.6D
11.4e	0.5E	6.6e	5.0e	5.0e	3.3E	1.0E	0
3.6F	6.7F	7.2F	5.6F	5.1F	13.6F	1.7F	8.6F
10.4g	2.3g	8.7g	6.8g	6.8g	2.9g	13.2g	5.4g

Table 9. Ruggedness test: Ranking of parameters for elements

Magnesium		Zinc		Calcium		Potassium	
Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two
e: Tuning	Magnesium	F: Argon flow	70 psi	b: Digestion time	20 min	g: Autosampler rate	0.83 mL/min
C: Concn deselected	3 concn	g: Autosampler rate	0.83 mL/min	A: Acid volume	30 mL	b: Digestion time	20 min
g: Autosampler rate	0.83 mL/min	C: Concn deselected	3 concn	g: Autosampler rate	0.83 mL/min	A: Acid volume	20 mL
A: Acid volume	30 mL	a: Acid volume	20 mL	F: Argon flow	70 psi	C: Concn deselected	3 concn
b: Digestion time	20 min	D: Internal standards	Lithium-6 and yttrium	e: Tuning	Magnesium	F: Argon flow	70 psi
F: Argon flow	70 psi	b: Digestion time	20 min	C: Concn deselected	3 concn	e: Tuning	Magnesium
D: Internal standards	Lithium-6 and yttrium	E: Tuning	Cobalt	D: Internal standards	Lithium-6 and yttrium	D: Internal standards	Lithium-6 and yttrium
Iron		**Phosphorus**		**Manganese**		**Copper**	
Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two	Parameter ranking	Best parameter of the two
F: Argon flow	70 psi	D: Internal standards	Lithium-6 and yttrium	g: Autosampler rate	0.83 mL/min	c: Concn deselected	5 concn
C: Concn deselected	3 concn	F: Argon flow	70 psi	C: Concn deselected	3 concn	g: Autosampler rate	0.83 mL/min
E: Tuning	Cobalt	C: Concn deselected	3 concn	e: Tuning	Magnesium	b: Digestion time	20 min
g: Autosampler rate	0.83 mL/min	A: Acid volume	30 mL	F: Argon flow	70 psi	d: Internal standards	Scandium and rhodium
B: Digestion time	30 min	g: Autosampler rate	0.83 mL/min	A: Acid volume	30 mL	F: Argon flow	70 psi
a: Acid volume	30 mL	b: Digestion time	20 min	d: Internal standards	Scandium and rhodium	E: Tuning	Cobalt
d: Internal standards	Scandium and rhodium	E: Tuning	Cobalt	b: Digestion time	20 min	A: Acid volume	30 mL

Table 10. Ruggedness test: Criticalness values (CV)[a]

Parameter	1st rank = 7	2nd rank = 6	3rd rank = 5	4th rank = 4	5th rank = 3	6th rank = 2	7th rank = 1
Acid volume							
A = 30 mL	0	1	1	2	1	0	1
a = 20 mL	0	0	0	1	0	1	0
Digestion time							
B = 30 min	0	0	0	0	1	0	0
b = 20 min	1	1	1	0	1	2	1
Concentrations deselected							
C = 3 concentrations	0	3	2	1	0	1	0
c = 5 concentrations	1	0	0	0	0	0	0
Internal standards							
D = Lithium-6 and yttrium	1	0	0	0	1	0	3
d = Scandium and rhodium	0	0	0	1	0	1	1
Tuning							
E = Cobalt	0	0	1	0	0	1	2
e = Magnesium	1	0	1	0	1	1	0
Argon flow							
F = 70 psi	2	1	0	2	2	1	0
f = 65 psi	0	0	0	0	0	0	0
Autosampler rate							
G = 0.75 mL/min	0	0	0	0	0	0	0
g = 0.83 mL/min	2	2	2	1	1	0	0

[a] The number of times the parameter occurs in the individual ranking.

25 ppb 8-element tuning solution. With indium set at mass 115, tuning solution should give a detector reading of ca 250 000 counts. Next, set mass tuner to magnesium, mass 25, and finish fine tuning. Finally, have the instrument perform a mass calibration of 8 tuning analytes.

Results and Discussion

Twenty-four determinations of 4 AAFCO check samples were made. As seen in Tables 1–4, the mean grand average of all methods was used as the target value for each element. AAFCO check samples (3) were used because no reference materials were available for micronutrients in a feed matrix.

Tables 1-4 show relative standard deviation (RSD) values, which are measures of precision. RSD values for ICP/MS are comparable with or better than values for ICP, AA, or all methods. Mean values obtained with ICP/MS are comparable with those from ICP, AA, or all methods.

Instrument Stability

The stability of ICP/MS is critical to method performance (1). Over a lengthy period (2 h), the instrument drifted downward. This drift usually occurred when the reference material was first introduced into the instrument. The problem was corrected when internal standards that bracketed the mass ranges were used. Also, the internal standard chosen cannot be present in the actual samples because of corrections made by instrument algorithms.

Although the manufacturer recommends that the plasma burn for at least 20 min, our study shows that for the instrument to stabilize the plasma should burn for about 1 h before analysis begins.

Table 11. Ruggedness test: Average of differences for recovery for all elements per parameter

	A	a	B	b	C	c	D	d	E	e	F	f	G	g
	9.98	1.79	2.40	5.67	10.49	14.33	11.75	0.10	0.50	11.35	3.55			10.37
	2.85	1.95		2.07	6.52		1.28	1.83	3.32	6.56	6.66			2.33
	7.84			8.32	7.63		0.56	0.35	0.95	4.96	7.15			8.74
	7.52			8.41	4.87		0.64	2.22	0.04	5.25	5.58			6.76
	2.84			1.49	6.90						5.14			8.80
	0.82			2.65	3.99						13.57			2.93
				0.61	4.80						1.69			13.19
											8.64			5.35
Avg.	5.31	1.87	2.40	4.17	6.46	14.33	3.56	1.12	1.20	7.03	6.50	0	0	7.31

Table 12. Ruggedness test: Results of weighted criticalness evaluation

Parameter	Results		
Acid volume			
30 mL	A = 0[a] + 6 + 5 + 8 + 3 + 0 + 1 = 23[b]	23[b] × 5.3[c] =	121.9[d]
20 mL	a = 0 + 0 + 0 + 4 + 0 + 2 + 0 = 6	6 × 1.9 =	+11.4
			133.3[e]
Digestion time			
30 min	B = 0 + 0 + 0 + 0 + 3 + 0 + 0 = 3	3 × 2.4 =	7.2
20 min	b = 7 + 6 + 5 + 0 + 3 + 4 + 1 = 26	26 × 4.2 =	+109.2
			116.4
Concentration deselection			
3 concentrations	C = 0 + 18 + 10 + 4 + 0 + 2 + 0 = 34	34 × 6.5 =	221.0
5 concentrations	c = 7 + 0 + 0 + 0 + 0 + 0 + 0 = 7	7 × 14.3 =	+100.1
			321.1
Internal standard			
Lithium-6 and yttrium	D = 7 + 0 + 0 + 0 + 3 + 0 + 3 = 13	13 × 3.6 =	46.8
Scandium and rhodium	d = 0 + 0 + 0 + 4 + 0 + 2 + 1 = 7	7 × 1.1 =	+7.7
			54.5
Tuning element			
Cobalt	E = 0 + 0 + 5 + 0 + 0 + 0 + 2 = 7	7 × 1.2 =	8.4
Magnesium	e = 7 + 0 + 5 + 0 + 3 + 2 + 0 = 17	17 × 7.0 =	+119.0
			127.4
Argon flow			
70 psi	F = 14 + 6 + 0 + 8 + 6 + 2 + 0 = 36	36 × 6.5 =	234.0
65 psi	f = 0 + 0 + 0 + 0 + 0 + 0 + 0 = 0	0 × 0 =	+ 0.0
			234.0
Autosampler rate			
0.75 mL/min	G = 0 + 0 + 0 + 0 + 0 + 0 + 0 = 0	0 × 0 =	0.0
0.83 mL/min	g = 14 + 12 + 10 + 4 + 3 + 0 + 0 = 43	43 × 7.3 =	+313.9
			313.9

[a] Weighted criticalness values (WCV).
[b] Sum of weighted criticalness values.
[c] Average of difference of recovery for all elements per parameter, from Table 11.
[d] Weighted criticalness number (WCN).
[e] Sum of weighted criticalness numbers.

Interferences

Most interferences can result from sample matrix, matrix digestate, or argon fuel. In the initial stages of method development, it was noted that high results were obtained with iron. This was due to formation of argon dimers with masses that mimic those of iron.

High solids could block the nebulizer. This was prevented by filtration, if necessary, or by maintaining less than 0.5% solids in the total sample volume. This was achieved by diluting a weight of 1 g to 250 mL.

Although the instrument has interference equations included in the software, the element isotopes were selected on the basis of the least amount of interferences. The following isotopes were used: ^{25}Mg, ^{31}P, ^{39}K, ^{44}Ca, ^{55}Mn, ^{56}Fe, ^{65}Cu, and ^{66}Zn.

AOAC Ruggedness Test

AAFCO sample 9531 (Gilt Finisher) was used for this test. The ruggedness test (4) was developed to evaluate reasonable variations of a single analyte in a method. Minor variations are deliberately introduced in a method. Changes in recovery are evaluated and ranked according to their critical nature. Appropriate documentation in the procedure brings to the analysts' attention the most and least rugged parameters of the method. Procedures for more than one analyte require additional statistical analysis. This paper will demonstrate and show how the ruggedness test can help identify problem areas and fine tune a method. Table 5 lists the parameters used for the test.

In Table 5, acid volume is the amount of nitric acid used in the digestion. Digestion time is the interval of time allowed for sample digestion. Concentration deselected by element refers to limiting the calibration curve to the elements and their concentrations. This allows one to include or omit a certain element concentration in order to bracket the element concentration in the sample matrix. Internal standard selection is the variation of internal standards used for the ruggedness test. Tuning element is the elemental mass incorporated for tuning the mass detector. Argon pressure is the metered pressure of argon delivered to the

instrument. Autosampler rate is the rate of standard or sample flow to the instrument.

The following steps were used in the ruggedness test and weighted criticalness evaluation (WCE): (*1*) Calculate the recoveries for all 8 elements for the 7 varied parameters shown in Table 5. This will yield a total of 64 results for recovery (Table 6). (*2*) Calculate recovery per parameter (Table 7). (*3*) Rank differences in recoveries per parameter (for example, Mg: A,b,C,d) for each of the 8 elements (Table 8). (*4*) From Table 9, calculate criticalness value (CV), which is the occurrence of each parameter by rank. An example (Table 10) using the acid volume as parameter with A = 30 mL and a = 20 mL follows. Determine the number of times 30 mL acid occurred as the first parameter. In this case, 30 mL never appears as the first parameter for the 8 elements and is assigned the value of zero. An acid volume of 20 mL also does not appear first. Therefore 20 mL is also assigned a value of zero. Continue to assign values for all ranking positions with 7 as the highest and zero as the lowest. Each weighted criticalness value (WCV) is derived by multiplying the criticalness value by the number of times it appeared in the rank under consideration (Table 10). (*5*) Multiply the average of the difference in recovery for all elements per parameter (from Table 11) by the sum of the weighted criticalness values (Table 12). This product is the weighted criticalness number (WCN). (*6*) Add the WCNs (Table 12) for each of the variants of each parameter (A + a, B + b, etc.). These sums should be ranked in numerical order from the largest to the smallest. This will indicate the relative criticalness in descending order for each parameter.

Summary of AOAC Ruggedness Test

The ranking of the ruggedness test data in descending order of criticalness with the best parameter selection is as follows: (*1*) concentration deselected by element, (*2*) 0.83 mL/min autosampler rate, (*3*) 75 psi argon pressure, (*4*) 30 mL acid volume, (*5*) magnesium used as tuning element, (*6*) 20 min digestion time, and (*7*) internal standards of lithium-6 and yttrium.

The ruggedness test shows that minor changes can result in significant improvement in method accuracy. If changes are undetected, poor quality could result.

Conclusion

The data show that ICP/MS compares well ICP, AA, and wet classical methods for analysis of micronutrients in feeds, providing an efficient, accurate, and precise method.

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AGRICULTURAL MATERIALS

Development and Implementation of an Automated Sample Preparation Procedure for Frenolicin-B in Poultry Feed

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The Zymark Tablet Processing Workstation II (TPW II) provides a versatile, cost-effective alternative to manual sample preparation for extracting drugs from medicated animal feed (Type C medicated articles). Routine manual assays require samples to be ground, extracted, filtered, diluted, and analyzed by liquid chromatography. The TPW II uses computer-controlled homogenization, a liquid management system, an internal 4-place analytical balance, and a 3-place top-loading balance to consistently provide accurate results. The TPW II is also equipped with a robotic arm articulated by 4 degrees of freedom. The relative standard deviation of the precision assays for automated versus manual extraction were 7.4 and 8.1, respectively. The workstation also provides a gravimetric audit trail for all sample weights and sample volumes transferred. Few papers have been published concerning automated extraction of drugs in feeds. We report the successful development and routine implementation of a robotically automated procedure for extracting frenolicin-B, an anticoccidial and feed efficiency enhancer, from poultry feed.

The need for analytical laboratories to meet demands of business process reengineering and to survive into the 21st century requires not doing better, which leads only to modest improvements, but doing work differently such that significant leaps in performance and productivity can be achieved. One of the clear trends of the 1990s has been the focus on productivity and speed to improve response times over a wide range of business processes including how analytical results are generated and reported to other management groups. By eliminating waste and implementing redesigned processes in a creative manner, it is possible to increase productivity and reduce sample turnaround time. For the 21st century, we see this trend continuing and even accelerating, as leading companies are projecting even greater improvements (1).

In analytical laboratories supporting the medicated-feed-additive business, sample preparation and documentation for compliance are major areas where productivity is low and slow. These areas also generate the greatest number of errors. For example, in customer support and product development laboratories, analysts can spend all day preparing samples so that they can run overnight. Such batch processing can result in instruments sitting idle for substantial amounts of time while samples are prepared. Batch processing is not necessarily the preferred process in laboratories, but it has evolved because of the constraints of fitting work in an 8-h day and the limitations of humans to keep track of too many simultaneous events (2).

In response to changing economic and regulatory environments in the pharmaceutical and chemical industries and to meet the reality of the 1990s and beyond, we have developed an automated extraction procedure for frenolicin-B (Figure 1) in Type C medicated articles (complete feeds). It was considered that automation of the feed extraction procedure would realize the greatest gain in analytical productivity, decrease sample turnaround times, and reduce the need for rework.

This paper reports on the development and implementation of a routine, robotically automated extraction procedure for frenolicin-B in poultry feeds. Frenolicin-B was discovered in 1978 by researchers at Kitasato Institute and Kitasato University, Tokyo, Japan (3). Frenolicin-B designated AM-3867 I was isolated from a fermentation broth of *Streptomyces roseofulvus* strain AM-3867, a soil isolate (3). Frenolicin-B is an important animal feed anticoccidial that is particularly effective against chick coccidiosis. It is also used as a feed efficiency enhancer in swine. *S. roseofulvus* produces a group of frenolicin-like structures (isochromanequinones) that are converted to frenolicin-B after fermentation.

Experimental

Poultry Feed (Type C Medicated Articles)

Pelleted feed samples supplemented with frenolicin-B at 60 μg/g were obtained from Hoffmann-La Roche, Animal Sci-

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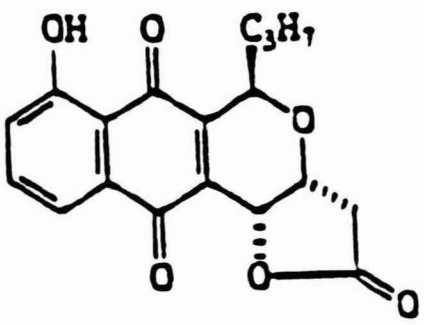

Figure 1. Structure of frenolicin-B.

ence Research Station, Wrightstown, NJ. Feed samples were pelleted in a Sproutwaldron pelleting mill (Model Junior 500B) and then ground with an S.500 Disc Mill grinder (Model 100.001, Glen Mills, Inc.) prior to analysis.

Zymark Tablet Processing Workstation II (TPW II)

Automated extraction was performed with a Zymark TPW II (Zymark Corporation, Hopkinton, MA). The TPW II is equipped with a robotic arm articulated by 4 degrees of freedom, homogenizer, homogenization vessel, capping and filtering stations, and liquid-handling station. Additionally, the system is equipped with balances to accurately weigh feed samples prior to analysis and to provide a gravimetric audit trail of un-

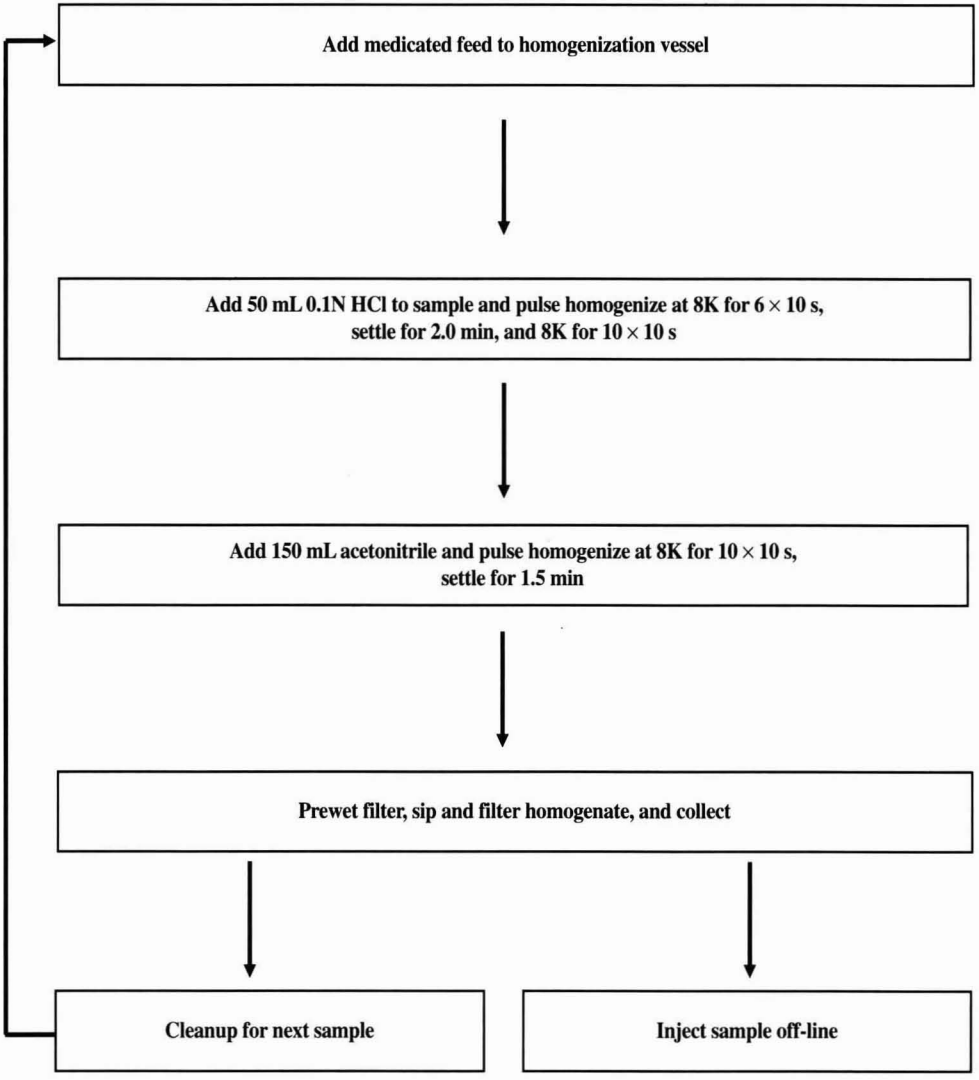

Figure 2. Steps for extracting frenolicin-B in Type C medicated articles.

attended operations. The balance also provides gravimetric monitoring of all reagent additions and liquid transfers performed on each sample. From the gravimetric data and predetermined solvent densities, the volume of solvent added to each sample is determined. The operating system supplied by the vendor codes the automated analysis method through interaction with a menu-driven method development section. This section performs the task of sequencing, compiling, and storing the proper programming codes used by the TPW II to execute sample preparation and analysis. This software also interfaces with a spreadsheet program (Excel) to provide accurate sample weights and volumes that are used to determine the final concentration of the drug in feed.

Automated Extraction

The analyst begins by weighing 10 g ground feed into the first test tube only. The remaining ground samples to be analyzed are poured into test tubes to make up 10 g with the first sample as a visual reference point. The samples are then placed in the appropriate sample tray on the bench top of the TPW II. The robotic arm uncaps the sample test tube and adds the sample into the homogenizing vessel. The empty test tube is returned to the sample tray. The sample is weighed accurately and 50 mL 0.1N HCl in water is added. After homogenization, 150 mL acetonitrile is added and the sample is further homogenized. The liquid management system removes a portion and filters the sample. The extracting vessel is washed and the next sample extracted. These unit operations are summarized in Figure 2.

Manual Extraction

Ten grams of feed is weighed in a 500 mL volumetric flask; 50 mL 0.1N HCl is added, and the mixture is sonicated for 30 min at 50°C. After the mixture has cooled, 150 mL acetonitrile is added and the flask is placed on a mechanical shaker and shaken for 1 h.

Liquid Chromatography

The liquid chromatograph consisted of a Model 510 reciprocating pump coupled to a Model 486 photometric detector and a Model 717 autosampler (Waters Chromatography, Milford, MA). Chromatography was performed on a Hypersil-BDS column (50 × 4.6 mm id, Alltech, Chicago, IL), and the effluent was monitored at 280 nm. The mobile phase was acetonitrile–water–0.1M sodium acetate/acetic acid buffer, pH 4.45 (420 + 580 + 5, v/v/v).

Results and Discussion

Method Optimization

To determine optimum extraction conditions, several trials using the basic procedure (Figure 2) were run while varying homogenization time and speed. Similar recoveries (98–101%) of frenolicin-B from poultry feed were obtained when homogenizer speed was varied between 5000 and 20 000 rpm. Excessive speeds and continuous homogenization led to considerable aggregation and heating of samples, respectively. Therefore, it was necessary to pulse homogenize the feed samples in short bursts to reduce heating and aggregation. In another set of experiments, the volume of acetonitrile was varied from 50 to 150 mL. We observed sample emulsification and blockage of the filter when 50 or 100 mL acetonitrile was used as extractant.

Table 1. Precisions of automated and manual procedures for extracting frenolicin-B

	Frenolicin-B, μg/g	
Sample No.	Automated	Manual
1	54.4	57.9
2	57.8	58.0
3	57.0	66.8
4	62.5	61.8
5	64.7	57.3
6	59.8	50.9
7	63.8	55.9
8	67.8	55.9
Mean	61.0	58.1
RSD, %	7.4	8.1

Method Validation

The precisions of automated and manual extractions are shown in Table 1. All samples were taken from a single bag containing 1 kg of thoroughly mixed feed. Results indicate that overall precisions of automated and manual procedures for extracting frenolicin-B supplemented theoretically at 60 μg/g in poultry feeds were comparable. In another experiment to determine precision of the automated procedure by a different analyst and on a different day, a coefficient of variation of 6.2% was found.

The TPW II processes samples serially. At the end of each run, the homogenizing vessel and the lines are washed and the vessel is reweighed for residual solvent. Therefore, it was important to establish the level of cross-contamination from sample to sample. During these experiments, 8 poultry feed sam-

Table 2. Within-run carryover of frenolicin-B

Run No.	Sample type	Peak heights at room temperature for frenolicin-B	Frenolicin-B, μg/g
1	Feed	3194	58.2
2	Feed	2252	40.5
3	Feed	2874	52.1
4	Blank	9	<0.1[a]
5	Feed	3166	57.2
6	Feed	3565	64.3
7	Feed	3006	54.1
8	Blank	11	<0.1[a]
9	Feed	3397	61.6
10	Feed	3399	61.3
11	Blank	15	<0.1[a]

[a] μg/mL.

ples containing frenolicin-B were extracted by the automated procedure. Interspersed between the 8 samples were reagents blanks that were also extracted by the TPW II. After extraction and analysis of reagent blanks, the level of carryover was determined to be less than 0.1 μg/mL (Table 2).

A prerequisite for successful implementation of any automated method is good correlation with previously established manual techniques. To evaluate the correlation between manual and automated procedures, samples from 8 different bags were ground and extracted by both methods.

Table 3 indicates that similar results were obtained when different samples were analyzed by the 2 methods. A statistical evaluation of these results found no significant difference between the 2 sets of values. Although the overall precision of the assay was 7.4% (Table 1), the larger coefficients of variation (Table 3) for the assay of frenolicin-B in feeds was due to the greater difficulty in obtaining representative samples from the different sample bags. This was mainly due to variation in the manufacturing process. Nevertheless, the means and coefficients of variation for both manual and automated methods were very similar.

Table 3. Comparison of manual and automated procedures for extracting frenolicin-B

	Frenolicin-B, μg/g	
Sample No.	Manual	Automated
1	75.1	58.2
2	48.3	40.4
3	53.0	52.1
4	50.2	57.2
5	51.3	64.3
6	56.0	54.1
7	57.4	61.6
8	52.3	61.3
Mean	55.5	56.2
RSD, %	15.3	13.3

Analysis of Routine Samples

We have implemented the automated extraction procedure to provide analytical support for frenolicin-B product development program. A detailed audit trail (not shown) for each sample was also obtained and filed for compliance. Such an audit trail is considerably more defensible and thorough than that one kept by an analyst.

To assess extraction variability through the entire run time, a quality control feed sample (ground pelleted feed) was extracted after every sixth sample during a run that included 30 other samples. Within-run coefficient of variation for quality control samples was about 6.0%. In a similar run comprising 40 samples, within-run precision for control feed samples (ground mash feed) was about 4.0%.

Economics of Extraction and Analytical Productivity

Automated systems allow an analyst to perform other duties concurrent with automated testing. Generally, only 20 samples could be processed manually, whereas the TPW II extracted 50 samples in the same time (24 h). Additionally, to process a sample manually an analyst cost of $18.80 was incurred (at $50.00/h). Analyst intervention in the automated procedure was reduced from 22.5 to 2.0 h per 50 samples, analyst cost per sample dropped to about $2.00. Our practical experience with the TPW II suggests that an analyst can process 150 samples in 3 days and have time to spare for other projects. This represents an increase in productivity of 150% over the manual method.

Use of TPW II has standardized our extraction operations and increased compliance by generating sample history and documentation. Additionally, rework of samples has been reduced significantly because of fewer manipulative errors and deviation from the official methods, compared with manual extraction. As a consequence, the effort expended for analytical audits is reduced considerably and the reliability of the data is increased. Dependence on a single person in the laboratory who accomplishes that particular task is also eliminated.

Automation of the frenolicin-B extraction procedure has improved the quality of the work environment by reducing exposure to toxic solvents and biohazards and by allowing time for other productive activities.

Conclusions

The work shows that it is possible to automate analytical methods for extracting drugs from complex matrixes such as poultry feed. Overall experiences with the TPW II have been enormously positive with laboratory personnel and popular with executive management for the following reasons: (*1*) Manual methods have been easy to transfer to the workstation. (*2*) The workstation offers higher productivity and faster turnaround. (*3*) Significant savings in analyst cost can be achieved. (*4*) Documentation and auditing of samples are reduced. (*5*) Regulatory compliance is increased.

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AGRICULTURAL MATERIALS

Improved Parameterization of Fertilizer Particle Size Distribution

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Particle size distribution is an important physical property of granular fertilizers that influences their bulk behavior (e.g., packing and segregation). Several parameter systems for fertilizer particle size distributions are analyzed in this paper. The most common system used by the fertilizer industry is the SGN–UI system, where SGN is the size guide number (the median particle size) and UI is the uniformity index (the 10th percentile particle size expressed as a percentage of the 95th percentile particle size). This 2-parameter system, however, has many limitations. For example, it does not give a distribution function. Furthermore, the UI parameter does not accurately reflect the spread of particle sizes. It is therefore necessary to find a better parameterization system. Three size distribution functions (the log-normal, the Rosin–Rammler and the Gaudin–Schuhmann equations) were tested on a size distribution database composed of 377 samples from 7 fertilizer materials. Each function was fitted to the data by nonlinear regression. The Rosin–Rammler function is the best parameter system on the basis of an analysis of variance of the sum of squares of error from the nonlinear fits. Comparisons between the Rosin–Rammler and the SGN–UI parameters were also made. The Rosin–Rammler system is more accurate than the SGN–UI system, possesses the ability of prediction, and provides a measure of the goodness of fit. Therefore, the Rosin–Rammler system should be used to characterize size distribution of granular fertilizer materials instead of the SGN–UI system.

Fertilizer particle size distribution is an important physical property that impacts on many fertilizer applications (1). Fine particle sizes can cause problems like dustiness and difficulties in handling, storage, and distribution. Segregation of bulk-blended granular fertilizers is another problem caused by mismatched particle size distributions. Unless certain precautions are observed, components of blends may segregate severely during handling and distribution. Such segregation not only causes difficulties in sampling and in meeting guaranteed analysis standards but also frequently results in nonuniform crop response in the field (2). Segregation is due to differences in physical properties such as size, shape, and density of the component materials. For bulk blending of fertilizers, size is the most important factor, while differences in shape and density can be neglected (3). Hoffmeister et al. (3) suggested that particle size distributions be matched to avoid segregation.

To reduce segregation and dust problems, size ranges have been specified by many fertilizer organizations. For example, the U.S. Agency for International Development (USAID), in purchasing granular fertilizers for its aid programs, generally specifies a 90% minimum in the range 1.00–3.35 mm, with 0% larger than 4.75 mm and no more than 2% finer than 0.6 mm. However, these specified ranges are not sufficiently restrictive to ensure good size matching for bulk blending (4).

The SGN–UI parameterization system is currently widely used by the fertilizer industry (5). The 2 parameters in this system are the size guide number (SGN) and the uniformity index (UI). These correspond, respectively, to the median particle size and the 10th percentile particle size expressed as a percentage of the 95th percentile particle size. This system is of limited value because it just gives 2 parameters without a distribution function. Thus, even if 2 fertilizer materials possess exactly the same parameters, it does not necessarily mean that they will have the same the size distribution. Additionally, UI is not a good parameter to describe the spread of particle sizes, as will be shown later in this paper.

Looking for a better parameterization system, we investigated 3 distribution functions that have been used previously for other granular materials: the log-normal, the Rosin–Rammler, and the Gaudin–Schuhmann equations. Similar investigations for soil particles have been conducted by Russell (6) and Buchan et al. (7).

Epstein (8) showed that the size distribution of fragments resulting from a process of repeated breakage is asymptotically log-normal. His model assumes that the probability of failure is scale-invariant. The log-normal size distribution function is given by (9):

$$P(X > x) = 50 - 50 \text{ erf } [\ln (x/\text{GMD})/\sqrt{2} \ln (\text{GSD})] \quad (1)$$

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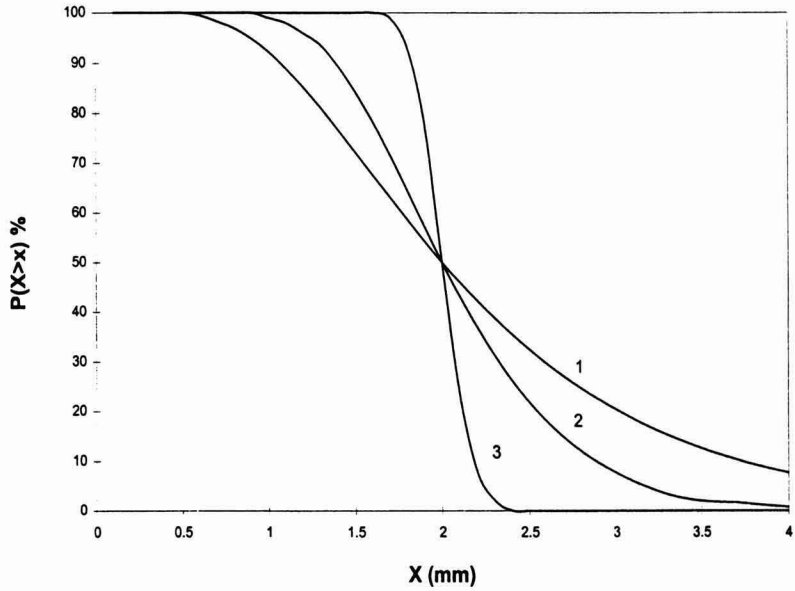

Figure 1. Log-normal distributions for a median particle size of 2 mm. Curves 1, 2, and 3 denote GSD values of 1.1, 1.5, and 2.0, respectively.

where $P(X > x)$ is the percentage by mass of particles greater than screen size x, erf is the error function, ln is the natural logarithm, GMD is the geometric mean diameter (equivalent to the median size), and GSD is the geometric standard deviation. GSD determines the shape of the distribution curve. Examples of the log-normal distribution function are given in Figure 1.

The Rosin–Rammler equation (10) was developed to describe broken coal and other comminuted earth materials. This equation also has been used to describe the size distribution of soil aggregates (11) and rock fragments (12). Bennett (13) provided a theoretical derivation of the Rosin–Rammler function. The Rosin–Rammler equation is given by (10):

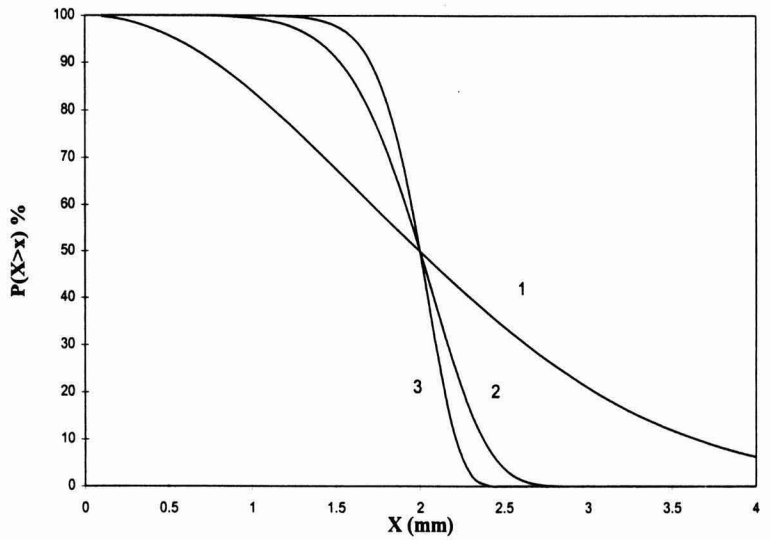

Figure 2. Rosin–Rammler distributions for a median particle size of 2 mm. Curves 1, 2, and 3 denote β values of 2.0, 7.0, and 12.0, respectively.

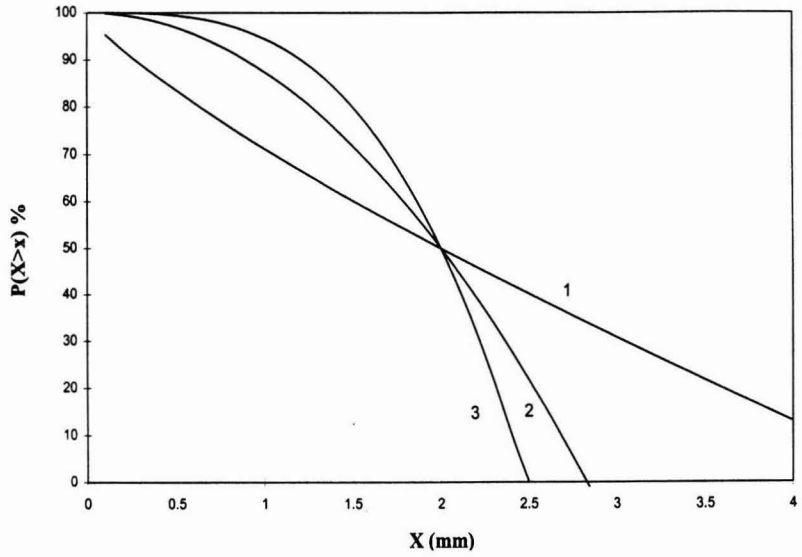

Figure 3. Gaudin–Schuhmann distributions for a median particle size of 2 mm. Curves labeled 1, 2, and 3 denote *m* values of 0.8, 2.0, and 3.2, respectively.

$$P(X > x) = 100 \exp[-(x/\alpha)^{\beta}] \quad (2)$$

where exp is the exponential function and α and β are parameters related to the characteristic size and spread of the distribution, respectively: α represents the particle size corresponding to the 36.78th percentile of the cumulative probability distribution, and β controls the shape of the distribution function. A small β denotes a wide spread of particle sizes and vice versa. Examples of the Rosin–Rammler distribution function are given in Figure 2.

When particles of a homogeneous solid are repeatedly broken by a series of single fractures, their resulting size distribution is a straight line on a log-log plot (14). This is the Gaudin–Schuhmann function, which is given by (14):

$$P(x > X) = 100[1 - (x/x_0)^{m}] \quad (3)$$

where x_0 is size of the largest particle in the particle size distribution and m is the shape parameter. The Gaudin–Schuhmann function is widely used in the mineral processing industry. It is closely related to the fractal size distribution function (15). Examples of the Gaudin–Schuhmann distribution function are given in Figure 3.

The parameters used to construct Figures 1–3 cover the range of fertilizer size distributions presented in this paper. Thus, they show the different distribution functions normalized to approximately the same median size and the same amounts of spread.

Experimental

A database of 377 particle size distributions for granular fertilizers was analyzed. These data were collected during 1994 and 1995 by the Division of Regulatory Services at the University of Kentucky, an organization responsible for regulating fertilizers in Kentucky. The database covered 7 different fertilizer materials: ammonium nitrate (AMM), diammonium phosphate (DAP), muriate of potash (MOP), sulfate of potash (SOP), sulfate–potash–magnesium (SPM), triple superphosphate (TSP), and urea (URE). These are the most common fertilizer materials in Kentucky. The guaranteed chemical analysis and number of samples of each material analyzed are shown in Table 1.

Each sample was tested individually, and its size distribution was obtained according to the SGN procedure (16). The original data for a representative sample are shown in Table 2. The first column is the sieve size in Tyler mesh. The Tyler mesh number (t) was converted into screen size in millimeters (x) by using the following relation obtained by nonlinear regression: $x = 21.60t^{-1.079}$. The R^2 for this relationship was 0.99. The third column in Table 2 is the cumulative mass percentage of material greater than the screen size of the corresponding mesh.

The SGN and UI for each sample were calculated by linear interpolation as indicated in Figure 4. SGN represents the median size corresponding to $P(X > x) = 50\%$, which means that 50% of particles by mass are greater than this size. UI, which

Table 1. Chemical analysis of materials used

Material	*N*	N, %	P_2O_5, %	K_2O, %
AMM	17	34	0.00	0.00
DAP	90	18	46	0.00
MOP	90	0.00	0.00	60
SOP	25	0.00	0.00	50
SPM	12	0.00	0.00	22
TSP	53	0.00	46	0.00
URE	90	46	0.00	0.00

Table 2. Example of an original data set[a]

Tyler sieve number *t*	Screen size, mm	P($X > x$), %[b]
5	3.80	0.1
7	2.65	18.2
10	1.80	61.8
18	0.95	94.5
30	0.55	96.7

[a] Material: SOP, SGN: 222, UI: 27.
[b] Mass percentage > sieve size, *x*.

characterizes the spread in the particle size distribution, is the ratio of the particle size corresponding to $P(X > x) = 95\%$ (x_s) to the particle size corresponding to $P(X > x) = 10\%$ (x_l) expressed as a percentage. In this paper, these parameters are referred to as observed SGN and observed UI.

The 3 size distribution functions described previously were used to fit the data. The SAS Non-Linear Regression (NLIN) procedure (17) was used for all fits. We performed nonlinear regressions and computed the sum square of error (SSE) and model parameters for individual samples. Regressions for all samples were convergent. This means that the least square of residual (error) solutions have been obtained and that the parameters for each sample were unique. SSE was used as an index for evaluating goodness of fit. The smaller this value, the better the fit.

A 2-way factorial analysis of variance was conducted on the SSE data to look for sources of variation. The dependent variable was SSE, and the sources of variation were material, equation, and the interaction between these 2 factors. The SAS General Linear Models (GLM) procedure (17) was used for this analysis. Protected *t*-tests were used to compare the equation SSE means by material and the material SSE means by parameter. Linear regression analyses between the observed SGN and UI values and the different model parameters were performed according to the SAS Linear Regression (REG) procedure (17).

Table 3. Analysis of variance for SSE from nonlinear regression analyses

Source	DF	SS[a]	*F* value	Pr > *F*
Material	6	1.91×10^7	22.8	0.0001
Equation	2	1.41×10^8	505.0	0.0001
Interaction	12	4.41×10^7	26.3	0.0001

[a] SS = sum of squares.

Results and Discussion

Analysis of SSEs showed that material, equation, and their interaction were all significant sources of variation at the 0.0001 significance level (Table 3). However, *F*-values indicated that equation contributed the most to this analysis, while material and the interaction term contributed much less (Table 3). Thus, equation was the most important factor influencing the SSE values—that is, goodness of fit depended mainly on the equation used.

To compare the 3 equations, protected *t*-tests were conducted to show the differences between averaged SSEs within each material. In Table 4, means with different superscript letters in any given row indicate that there were significant differences at the 95% confidence level between the equations in terms of their goodness of fit for that particular material. The log-normal and Rosin–Rammler equations always produced statistically smaller mean SSEs than the Gaudin–Schuhmann equation (Table 4). Although there was no statistical difference between these 2 equations, the Rosin–Rammler equation had the smallest averaged SSE for all materials except for SPM, which is not widely used and which had fewest samples of all of the materials investigated. Therefore, we can conclude that the Rosin–Rammler system is the best general equation for parameterizing the size distribution of granular fertilizers.

Although equation played a dominant role in this analysis of variance, material was still significant even though its *F*-value

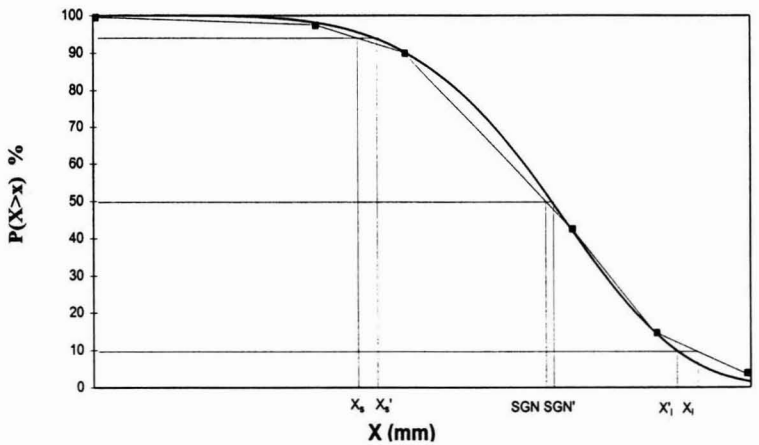

Figure 4. Calculations of SGN, SGN', UI, and UI' from a sample Rosin–Rammler function and its linear interpolation.

Table 4. Comparison of means for SSE from nonlinear regression analyses for each equation by material

Material	Equation[a] 1	2	3
AMM	93.91^{Bb}	39.96^{B}	715.55^{A}
DAP	33.85^{B}	12.57^{B}	1197.13^{A}
MOP	39.81^{B}	30.61^{B}	700.09^{A}
SOP	54.34^{B}	30.29^{B}	1045.06^{A}
SPM	36.05^{B}	72.42^{B}	563.95^{A}
TSP	32.21^{B}	7.32^{B}	1286.96^{A}
URE	29.53^{B}	4.90^{B}	1790.85^{A}

[a] 1 = log-normal equation, 2 = Rosin–Rammler equation, 3 = Gaudin–Schuhmann equation.
[b] Means with the same letters in each row are not significantly different at $p < 0.05$.

Table 5. Comparison of means for Rosin–Rammler and SGN–UI parameters for different materials

Material	α	β	SGN	UI
AMM	2.30^{Da}	4.16^{C}	226.94^{C}	31.76^{D}
DAP	2.54^{B}	5.45^{B}	248.48^{B}	43.73^{B}
MOP	2.45^{BC}	3.56^{CD}	233.28^{C}	33.72^{CD}
SOP	2.32^{CD}	3.86^{C}	224.64^{C}	36.88^{C}
SPM	2.14^{E}	2.78^{D}	198.00^{D}	27.58^{E}
TSP	2.70^{A}	5.80^{A}	266.30^{A}	45.37^{B}
URE	2.58^{AB}	7.67^{A}	258.97^{AB}	53.82^{A}

[a] Means with the same letter in each column are not significantly different at $p < 0.05$.

was relatively small (Table 3). Results of protected *t*-tests conducted between materials for the Rosin–Rammler and SGN–UI parameters are shown in Table 5. There were statistically significant differences between materials with both systems, indicating that, in general, fertilizers have their own characteristic size distribution. All 4 parameters in Table 5 were significantly higher for TSP and URE than for the other materials. This result indicates that these materials were both coarser on average and had a narrower spread of particle sizes than the other materials in the database. In another situation, this might not be the case, because particle size distribution in the result of manufacturing processes that can vary both temporally and spatially.

We will now focus on comparing the parameters of the Rosin–Rammler distribution with those of the SGN–UI system. The Rosin–Rammler equation is a distribution function. As a result, one can calculate theoretical SGN and UI values for this distribution from the α and β parameters in equation (2). However, the calculated SGN and UI values are not going to be exactly the same as the observed SGN and UI values obtained by linear interpolation. To distinguish between these different estimates, we denote the theoretical SGN and UI as SGN′ and UI′, respectively; they are also referred to as predicted SGN and predicted UI. Suppose a particle system has Rosin–Rammler parameters α and β; then $SGN' = \{x[P(X > x) = 50]\}100$ and $UI' = \{x_s[P(X > x) = 95]/x_l\,[P(X > x) = 10]\}100$. By using these definitions in equation (2), it can be shown that

$$SGN' = \alpha/\exp(-0.366/\beta) \quad (4)$$

and

$$UI' = \exp(-3.804/\beta) \times 100 \quad (5)$$

Figure 5 shows equation 4 graphically. The lines labeled 1, 2, and 3 represent β values of 12, 7, and 2, respectively. This was the range of β values from nonlinear regression analyses. The points represent observed SGN values corresponding to the α parameter. It can be seen that SGN was closely related to

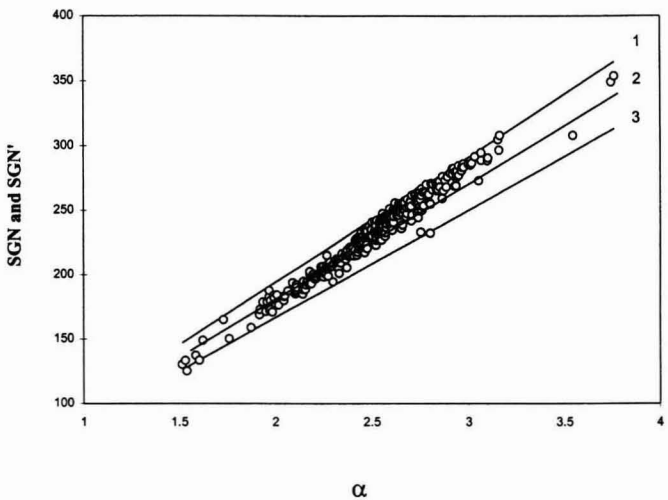

Figure 5. Experimental and theoretical relations between SGN, α, and β. Lines 1, 2, and 3 denote β values of 12, 7, and 2, respectively.

Table 6. Results of linear regressions between observed SGN and UI and predicted SGN and UI and the Rosin–Rammler parameters

Regression	Slope	Intercept	R^2
SGN′ versus SGN	0.96	–5.28	0.99
UI′ versus UI	1.13	–2.74	0.93
α versus SGN	105.98	–20.93	0.94
β versus UI	4.51	18.45	0.82

the α parameter and that β had only a small influence on the overall relationship. Corresponding regression analyses are presented in Table 6.

The curve in Figure 6 shows equation 5 graphically. The points in the figure represent observed UI values. As β increases, the deviation between the observed and theoretical relations becomes larger. Figure 4 shows the reason for this trend for a Rosin–Rammler curve with β = 12. Because $UI = x_s/x_1 100$ and $UI' = x_s'/x_1' 100$, then UI′ > UI. It is clear from Figures 4 and 6 that UI cannot be predicted accurately as β becomes large. We know that the Rosin–Rammler β parameter provides a good description of the "true" spread of particle sizes present. Therefore, the deviation of UI from UI′, which is related to β, means that the UI parameter does not reflect the "true" spread of particle sizes especially when β becomes large.

Figure 7 shows the relationship between predicted SGN from equation 4 and observed SGN. Predicted values are very close to observed values. Regression analysis indicated a very good linear relationship, with an R^2 of 0.99 (Table 6). This result indicates that SGN can be closely predicted by the α and β parameters and equation 4. In contrast, UI is less predictable (Figure 8 and Table 6) than SGN for the reasons discussed previously.

To summarize, the Rosin–Rammler parameter system is superior to the SGN–UI system. Rosin–Rammler parameters are associated with a distribution function while SGN and UI are just parameters. We do not know how well the SGN–UI parameters represent the "true" distribution, while we can evaluate the goodness of fit for the Rosin–Rammler system. The Rosin–Rammler equation has a theoretical statistical basis (13), while the SGN–UI system is empirical. With Rosin–Rammler parameters, one can calculate the corresponding SGN′ and UI′, while with SGN–UI parameters, one cannot get any additional information. Regarding the 2 parameters in the SGN–UI system, analyses show that SGN is accurate enough to represent median size, but UI is not a good descriptor for the spread of sizes. The relatively low R^2 values for the regressions between UI′ versus UI and UI versus β (Table 6) indicate that UI does not reflect the "true" distribution. This is because UI is calculated on the basis of a linear interpolation rather than from the distribution itself.

The SGN–UI system does have one advantage over the Rosin–Rammler system in its simplicity of computation. However, the Rosin–Rammler equation can be linearized by applying a double logarithmic transformation, that is:

$$\ln \{-\ln [P(X > x)/100]\} = \beta \ln (x) - \beta \ln (\alpha) \qquad (6)$$

With this transformation, α and β parameters can be easily obtained from simple linear regression, with the slope equal to β and the intercept equal to $-\beta \ln (\alpha)$. Thus, the computational costs of switching to the Rosin–Rammler equation from the SGN–UI system would be minimal.

Mixtures composed of different materials can be expected to show more complicated particle size distributions than those for individual materials. For example, with one component obeying a Rosin–Rammler distribution with α_1, β_1, and another component obeying a Rosin–Rammler distribution with α_2, β_2, the distribution function for the mixture of these 2 components will depend on all 4 parameters, as well as the relative proportions of the components. If a mixture is composed of more than 2 components, the expression will be even more complicated. One of the objectives of our future work will be to derive an index, based on the Rosin–Rammler system, to determine

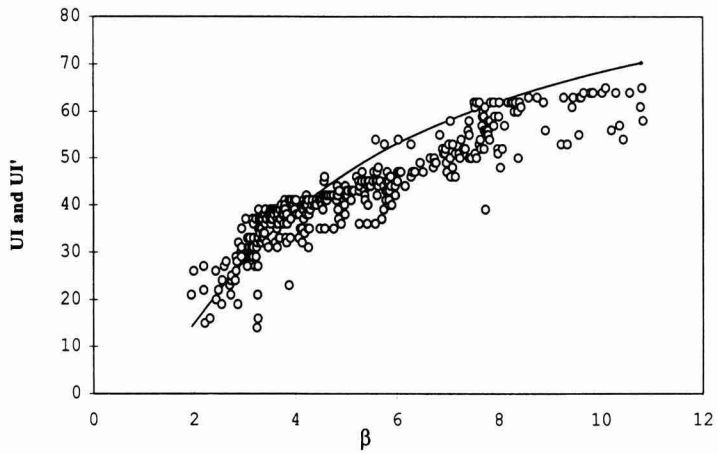

Figure 6. Experimental and theoretical relations between UI and β.

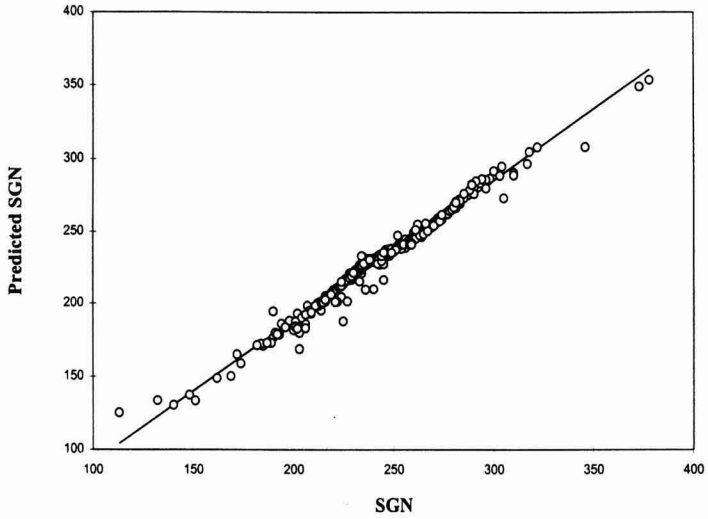

Figure 7. Predicted SGN versus observed SGN and the linear regression line.

whether 2 or more particle size distributions match (or not) within specified limits. Such an index may be useful for predicting the segregation of fertilizer materials during bulk blending.

Conclusions

The log-normal, Rosin–Rammler, and Gaudin–Schuhumann equations were compared, on the basis of goodness of fit, as potential parameter systems for characterizing size distribution of granular fertilizer materials. Although material and material by equation interaction were significant in an analysis of variance of resulting SSEs, equation was the most important source of variation. The Rosin–Rammler equation consistently gave the best fit for 6 of 7 materials investigated; the sole exception was sulfate-potash-magnesium, which is not widely used and which constituted only 3% of samples investigated. Thus, the Rosin–Rammler equation was selected

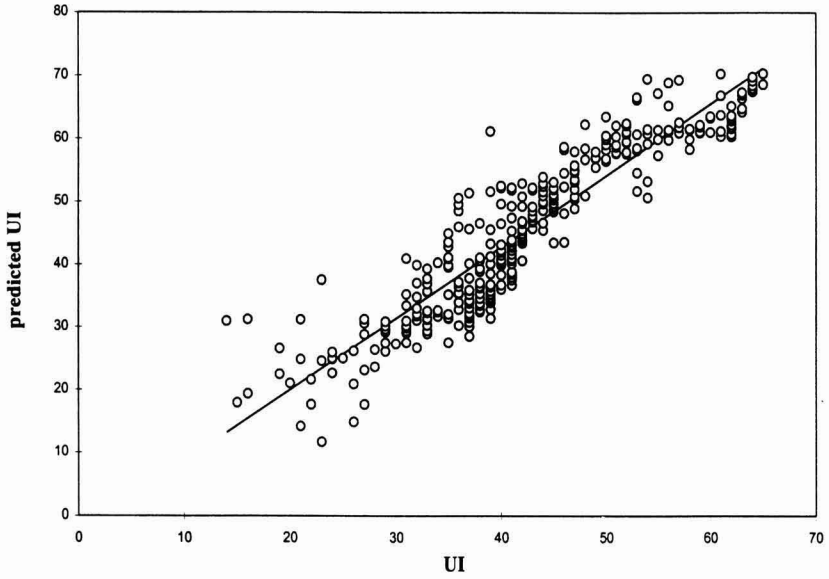

Figure 8. Predicted UI versus observed UI and the linear regression line.

as the best equation for parameterizing the size distribution of granular fertilizers.

The Rosin–Rammler system was also compared with the existing SGN–UI system and found to be superior in many aspects. The Rosin–Rammler equation has a theoretical basis, whereas the SGN–UI system does not. The Rosin–Rammler system provides a measure of goodness of fit and can be used to predict other (unmeasured) points on the particle size distribution curve. On the other hand, the UI parameter in the SGN–UI system does not provide a good estimate of the spread of the true distribution. These considerations suggest that use of the SGN–UI system should be discontinued. To obtain a better description of fertilizer particle-size distribution, the Rosin–Rammler parameter system should be used instead of the SGN–UI system. By applying a double logarithmic transformation to the Rosin–Rammler equation, it is possible to estimate the relevant parameters from the measured particle size distribution simply and conveniently by linear regression analysis.

In the database we investigated, triple superphosphate and urea had a larger median size and a narrower spread of sizes compared with other materials. Although this may not always be the case, care should be taken when materials with different size distributions are used in a bulk-blended fertilizer product because segregation can occur. Further research is needed to predict the particle size distribution of mixtures and their tendency to segregate from knowledge of the Rosin–Rammler parameters for each material.

Acknowledgments

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Diethanolamine and *N*-Nitrosodiethanolamine in Fatty Acid Diethanolamides

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Diethanolamine (DEA) is a precursor of *N*-nitrosodiethanolamine (NDELA), an animal carcinogen. A gas chromatographic (GC) method was developed for determining DEA in fatty acid diethanolamides that are commonly used in cosmetic products. Methanolic solutions of the amides were analyzed by GC with flame ionization detection on either a wide-bore methyl silicone (Rt_x-1) or 95% dimethyl–5% diphenyl polysiloxane (SPB-5) capillary column. Recovery of DEA from fatty acid dialkanolamides at fortification levels of 0.50, 1.00, and 5.00% ranged from 94 to 100%. In a survey of commercial fatty acid diethanolamides, DEA was found at levels ranging from 1.1 to 14.0%, and most were in good agreement with manufacturer's DEA specifications. Fatty acid diethanolamides also were analyzed for NDELA by liquid chromatography interfaced to a thermal energy analyzer. Recovery of NDELA from fatty acid diethanolamides at fortification levels of 50, 100, and 200 ppb averaged 95%. No NDELA was found in any of the fatty acid diethanolamide samples analyzed.

Alkanolamines such as diethanolamine (DEA) and triethanolamine (TEA), and fatty acid diethanolamides such as cocamide DEA and lauramide DEA, are used commonly in cosmetic products. Fatty acid diethanolamides provide cosmetic products with such properties as thickening, cleansing, foam boosting, and foam stabilizing. Although most commonly found in detergent formulations (i.e., shampoos, bubble baths, and liquid body cleaners), fatty acid diethanolamides also are useful in products such as creams and lotions.

Fatty acid diethanolamides usually contain residual DEA; the amounts depend on how they are synthesized (1, 2). DEA is also found as an impurity in TEA. The high reactivity of secondary amines such as DEA with nitrosating agents such as nitrite, can result in the formation of *N*-nitrosodiethanolamine (NDELA; 3), a potent animal carcinogen (4). NDELA has been found in cosmetic products formulated with TEA or fatty acid diethanolamides (5). DEA is the major amine precursor of NDELA in cosmetics, although TEA itself can be nitrosated (6). Although NDELA has been found in some cosmetic raw materials, studies suggest that NDELA is predominantly formed in cosmetic products from precursors after the product is formulated (5).

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Since 1977, the U.S. Food and Drug Administration has been actively involved in the identification of *N*-nitrosamines in cosmetic products (7). The Cosmetic, Toiletry, and Fragrance Association (CTFA) also initiated a comprehensive program to minimize *N*-nitrosamine contamination of cosmetic products. The CTFA Nitrosamine Task Force studied the effect of inhibitors on nitrosation in emulsion systems (8, 9).

In a 1986 survey of cosmetic products purchased on the German market, 40% of the products were found to be contaminated with NDELA (10). The German Federal Health Office issued an official recommendation to the cosmetic industry to stop using secondary amines in cosmetic products (11). This recommendation was adopted by the German Manufactures' Association. Further recommendations were that (*1*) fatty acid diethanolamides should contain as low as achievable residual DEA, and (*2*) TEA used in cosmetics should have a minimum purity of 99% and contain less than 1.0% DEA, less than 0.5% monoethanolamine, and less than 50 μg/kg (ppb) NDELA. Cosmetic products from the German market analyzed 6 to 18 months after the recommendation was issued showed that only 15% were contaminated with NDELA (10). The results of the study demonstrated a strong downward trend in both levels and frequency of NDELA contamination and proved that *N*-nitrosamine contamination in cosmetics could be minimized by instituting simple preventive measures.

The European cosmetic industry has adopted similar measures to minimize nitrosamine contamination of cosmetic products. These measures resulted in the 15th Adaptation in 1992 to the European Economic Community (EC) Cosmetics Directive, which states that fatty acid diethanolamides (raw materials) should contain less than 5% DEA and less than 50 ppb NDELA and that cosmetic products should contain less than 0.5% dialkanolamine (12). The directive also warns that fatty acid dialkanolamines should be stored in nitrite-free containers and should not be used in cosmetic products together with nitrosating agents.

Although considerable information on the presence of NDELA in cosmetic products exists, little data on levels of DEA and NDELA in fatty acid diethanolamides are available. This information would be valuable in determining the poten-

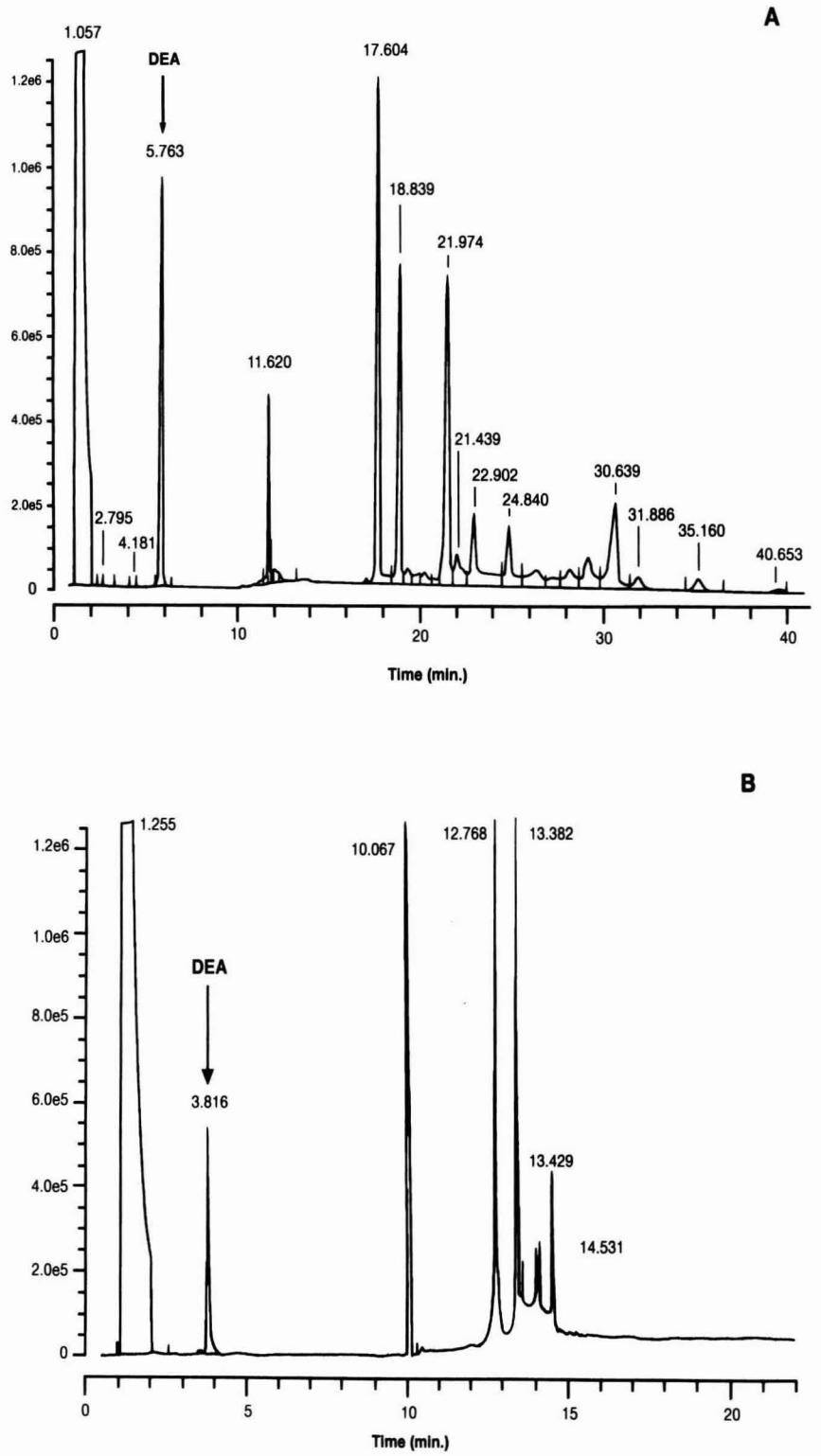

Figure 1. Typical gas chromatograms of a solution of lauramide DEA on (A) Rt_x-1 column and (B) SPB-5 column.

Table 1. Recovery of diethanolamine from cocamide DEA

GC column	Diethanolamine, %		
	Added	Found	Recovery
Rt_x-1	0.5	0.46	92
		0.45	90
		0.44	88
	1.0	0.95	95
		0.94	94
		1.00	100
	5.0	5.00	100
		4.80	96
		5.00	100
SPB-5	0.5	0.45	90
		0.44	88
		0.43	86
	1.0	0.95	95
		0.94	94
		0.91	91
	5.0	5.00	100
		5.10	102
		5.00	100

tial for *N*-nitrosamine formation in these raw materials and in evaluating the quality of U.S. cosmetic raw materials against the recommended German and EC standards.

Previously published methods for determining alkanolamines in aqueous solution include gas chromatography (GC; 13) and liquid chromatography (LC) and ion chromatography coupled with electrochemical or conductivity detection (14, 15). However, methods for determining DEA in fatty acid diethanolamides have not been reported. This paper describes a capillary GC method for determining DEA in fatty acid diethanolamides. The method uses splitless injection of analyte in methanol into a wide-bore capillary column with flame ionization detection.

Analytical methods for detecting NDELA have been reported; however, analysis of fatty acid diethanolamides was not part of the study (16). Results of analysis of fatty acid diethanolamides for NDELA by LC interfaced with a thermal energy analyzer (TEA) is also described in this report.

METHOD

Caution: N-Nitrosodiethanolamine is a suspected human carcinogen. Extreme care should be exercised when handling it.

Apparatus

(a) *Gas chromatograph.*—Model 5890 Series II (Hewlett-Packard, Palo Alto, CA) equipped with flame ionization detector. General operating conditions: carrier gas, helium at 48.4 cm/s; split/splitless injector temperature, 250°C; detector temperature, 280°C; temperature program for Rt_x-1 column: 120° to 170°C at 6°C/min, 170° to 250°C at 30°C/min (hold for

Table 2. Diethanolamine in fatty acid dialkanolamides determined by using 2 different capillary GC columns

Sample No.	Fatty acid alkanolamide	Diethanolamine, %		
		Manufacturer's specification	GC column[a]	
			Rt_x-1	SPB-5
1	Lauramide DEA	6.5	7.3	8.0
2	Lauramide DEA	6.0	5.5	5.1
3	Cocamide DEA	7.0	14.0	13.6
4	Cocamide DEA	7.0	7.2	6.9
5	Cocamide DEA	6.5	8.0	7.8
6	Lauramide DEA	6.5	10.5	9.1
7	Cocamide DEA	4–6	6.8	6.2
8	Linoleamide DEA	≤7.0	5.0	4.3
9	Lauramide DEA	≤8.5	1.4	1.2
10	Cocamide DEA	≤7.0	5.0	5.8
11	Lauramide DEA	<10.0	5.7	6.5
12	Cocamide DEA	6.0	5.3	4.7
13	Cocamide DEA	<5.0	3.9	4.7
14	Lauramide DEA	10.0	11.6	12.4
15	Lauramide DEA	7.0	6.6	5.5
16	Cocamide DEA	<5.0	3.5	3.7
17	Cocamide DEA	5.0	3.4	3.2
18	Lauramide DEA	5.0	4.0	3.6
19	Lauramide DEA	None	3.4	3.1

[a] Values are averages of 2 determinations.

Table 3. Recovery of *N*-nitrosodiethanolamine from cocamide DEA and lauramide DEA

Alkanolamide	*N*-Nitrosodiethanolamine, ppb		
	Added	Found	Recovery, %
Cocamide DEA	50	50	100
		40	80
		50	100
	100	90	90
		90	90
		100	100
	200	190	95
		180	90
		200	100
Lauramide DEA	50	50	100
		50	100
		40	80
	100	90	90
		90	90
		100	100
	200	190	95
		190	95
		180	90

30 min); temperature program for SPB-5 column: 120° to 170°C at 6°C/min, 170° to 280°C at 30°C/min (hold for 30 min); injection mode, splitless, purge valve initial: off, on: 0.8 min, off: 3.0 min.

(b) *GC column.*—Rt_x-1 capillary (Restek Corp., Bellefonte, PA) 30 m x 0.53 mm with 5 µm film and SPB-5 capillary (Supelco, Inc., Bellefonte, PA) 30 m × 0.53 mm with 1.5 µm film.

(c) *Data collection.*—All GC data were collected on an HP 3365 Series II ChemStation (Hewlett-Packard, Wilmington, DE).

(d) *LC/TEA system.*—LC solvent delivery system Model 6000A (Waters Associates, Milford, MA) equipped with Model 7120 Rheodyne injector with 50 µL loop (Rheodyne, Inc., Cotati, CA), and TEA Model 502 (Thermedics Detection, Inc., Chelmsford, MA), Model 3392A recording integrator (Hewlett-Packard), LC column (25 cm × 4.6 mm id, Zorbax Sil; Du Pont, Wilmington, DE). TEA operating conditions: pyrolyzer temperature, 550°C; total TEA reaction chamber pressure, 0.5 torr, with argon carrier gas; 2 glass cold traps in dry ice/acetone bath (–70°C).

Reagents

(a) *Solvents.*—Hexane, acetone, methylene chloride, isooctane, and methanol; UV grade (Burdick and Jackson, Muskegon, WI).

(b) *DEA.*—Eastman Organic Chemicals, Rochester, NY.

(c) *LC/TEA mobile phase.*—Hexane–acetone (70 + 30); flow rate, 1.5 mL/min.

(d) *4-Hexyloxyaniline.*—99% pure (Aldrich Chemical Co., Milwaukee, WI). Prepare 20 mg/mL solution in ethyl acetate.

(e) *Cocamide DEA, lauramide DEA, and linoleamide DEA.*—Gifts from Croda, Inc. (Parsippany, NJ), Henkel Corp. (Cincinnati, OH), Chemron Corp. (El Paso de Robles, CA), Stepan Company (Northfield, IL), Albright & Wilson Americas (Richmond, VA), and Lonza, Inc. (Fairlawn, NJ).

Preparation of Standard Curve

Accurately weigh ca 250 mg DEA into a 50 mL volumetric flask, dilute to volume with methanol, and mix. Prepare by serial dilution a series of 5 DEA standard solutions in methanol with concentrations ranging from 0.25 to 4 µg/µL. Inject 2 µL of each standard solution into GC system in duplicate. Using average peak areas, calculate regression equation $Y = A + BX$, where Y = peak area of DEA in the test solution, A = intercept of the standard curve, B = slope of standard curve, and X = weight of DEA (µg).

Raw Material Analysis

(a) *Raw material preparation for DEA determination.*—Accurately dissolve ca 10 mg test sample in 10 mL volumetric flask, dilute to volume with methanol, and mix. Inject 2 µL solution into GC system.

(b) *Calculation of percentage of DEA in fatty acid diethanolamides.*—Calculate quantity of DEA by using the equation for the DEA regression line:

$$\text{DEA, \%} = \frac{(Y - A) \times 500 \times V}{B \times W}$$

where Y = peak area of DEA in test sample solution, A = intercept of standard curve, V = total volume of test sample solution (mL), B = slope of standard curve, and W = total weight of test portion (mg).

(c) *Raw material preparation for NDELA determination.*—Thoroughly mix 5 g fatty acid diethanolamide with 10 mL ethyl acetate and 250 µL 4-hexyloxyaniline solution. Analyze 50 µL test solution by LC/TEA.

Results and Discussion

Determination of DEA in Fatty Acid Diethanolamides

Wide-bore capillary GC columns were selected to separate DEA because they have higher efficiency than packed columns. Two nonpolar GC columns, Rt_x-1 and SPB-5, were evaluated and found to be suitable for separating DEA from other raw material components (Figure 1). GC peak area for DEA was linear from 0.50 to 5.00 µg/µL. Recovery studies were conducted with 2 different fatty acid alkanloamides, cocamide DEA and lauramide DEA. The alkanolamides were fortified with DEA at levels ranging from 0.50 to 5.00%. Results for cocamide DEA are shown in Table 1. Recoveries of added DEA averaged 95 ± 4.28% with the Rt_x-1 column and 94 ± 5.72% with the SPB-5 column. The limit of detection was approximately 0.05 µg/g sample for both columns. Recovery of DEA from lauramide DEA was similar to that obtained for cocamide DEA.

Fatty acid diethanolamides are prepared by the condensation of DEA with fatty acids, fatty acid methyl esters, or fatty triglycerides. Amount of unreacted DEA in final product depends on the concentrations of the reactants. DEA can range

from 3 to 5% when 1 mol DEA is reacted with 1 mol fatty acid methyl ester (1:1 type), less than 7% when 3 moles DEA is reacted with 1 mol triglyceride (1:1 type), and 20 to 27% when 2 moles DEA is reacted with 1 mol fatty acid derivatives (2:1 type; 1, 2). According to manufacturers, the fatty acid diethanolamides surveyed in this study were produced via a 1:1 reaction.

Nineteen samples of lauramide DEA, cocamide DEA, and linoleamide DEA obtained from several manufacturers were analyzed for DEA by GC on 2 different GC columns to increase the possibility of detecting other components coeluting with DEA. Results of analyses are shown in Table 2. Manufacturers' DEA specifications for the raw materials analyzed also are shown. The presence of DEA in each raw material was confirmed by mass spectrometry. The data obtained with the 2 columns were in close agreement. Eight of 19 raw materials contained less than the 5% DEA recommended by the EC Cosmetics Directive. For 3 raw materials (samples 3, 6, and 19), the data differed significantly from manufacturers' stated DEA specification, perhaps because of differences in methodology used to determine DEA. Manufacturers typically use titration to determine DEA in cosmetic raw materials (17). The presence of basic compounds may affect results obtained by titration. The GC method is more selective than titration.

Determination of NDELA in Fatty Acid Diethanolamides

A sensitive, specific analytical technique was desired to quantitate NDELA at part-per-billion levels in fatty acid alkanolamides. Direct dilution of these raw materials with ethyl acetate followed by LC/TEA analysis provided simple and selective technique. Because fatty acid diethanolamides contain unreacted DEA, the antioxidant 4-hexyloxyaniline was added to the test sample to avoid artifact formation of NDELA during raw material preparation. Recovery studies with 2 different alkanolamides (cocamide DEA and lauramide DEA) were conducted to validate the method. Cocamide DEA and lauramide DEA were fortified with NDELA at levels ranging from 50 to 200 ppb. Results are shown in Table 3. Recoveries of added NDELA averaged 94% (standard deviation [SD], 7.0) for cocamide DEA and 93% (SD, 6.6) for lauramide DEA. The detection limit of NDELA was 0.6 ng, corresponding to 25 ppb in the test sample.

The 19 samples of cocamide DEA, lauramide DEA, and linoleamide DEA previously analyzed for DEA also were analyzed for NDELA. No NDELA was found in any sample, even though some contained more than 10% DEA, perhaps because of absence of nitrite or other nitrosating agents in these raw materials.

The use of fatty acid diethanolamide raw materials containing significant levels of free DEA can result in considerable levels of DEA in cosmetic products. Products that also contain nitrosating agents are likely candidates for in situ formation of NDELA. Cosmetic manufacturers have the responsibility to ensure that their products are free of *N*-nitrosamines.

Cosmetic manufacturers can minimize NDELA levels in cosmetic finished products by reducing the level of DEA in cosmetic raw materials such as fatty acid diethanolamides, avoiding use of nitrite-releasing preservatives in cosmetic formulations, avoiding contamination of finished products and raw materials with nitrite and oxides of nitrogen, and using nitrosation inhibitors (9, 12). The method described could provide raw material manufacturers a means of obtaining rapid and reliable measurements of DEA levels in fatty acid alkanolamides.

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Detection of Thiazide-Based Diuretics in Equine Urine by Liquid Chromatography/Mass Spectrometry

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Thiazide-based diuretics are included in the list of banned drugs in the horse-racing industry. One effect of their misuse is increased urine flow, contributing to dilution of other doping agents. Their determination is essential in ensuring compliance to horse-racing regulation. This study evaluates the feasibility of using liquid chromatography/mass spectrometry (LC/MS) with electrospray and atmospheric pressure chemical ionization interfaces to analyze thiazidic diuretics in equine urine samples. Existing LC and gas chromatography/MS methods are limited in their applicability to thiazide analysis. Sample preparation, analyte extraction, chromatographic separation, ion-source collision induced dissociation, solvent composition, ionization mode, and ion polarity are discussed. The practicality of LC/MS for this analysis is demonstrated with actual equine administration samples collected at specified time intervals. Detection limits were 270 ng/mL for chlorothiazide, 131 ng/mL for hydrochlorothiazide, and 384 ng/mL for trichlormethiazide.

Thiazidic diuretics are used in the equine sport industry to mask ingestion of other doping agents by diluting their concentration in urine. These diuretics are fused heteroatomic ring systems with at least one chlorine and 2 benzosulfonamide moieties, as shown in the insets to Figures 1 and 2. Detection of these compounds is crucial to preserving the integrity of the equine sport industry.

For forensic screening, multiresidue methods are preferred because they can provide simultaneous information in a single run. Liquid chromatography with ultraviolet absorption detection (LC/UV) has been used to analyze thiazidic compounds in human urine (1–5). LC/UV methods, however, do not provide the necessary thiazide confirmation accomplished with mass spectroscopic (MS) detection. Hyphenated techniques such as chromatography combined with MS are particularly powerful for these applications. Capillary gas chromatography (CGC)/MS is the standard method for analysis of thiazidic diuretics. However, CGC/MS is limited by the need for a derivatization step to improve chromatographic separation, eliminating the possibility of direct confirmation of thiazidic compounds. The most common derivatization procedure involves some type of methylation (4–6).

One successful alternative to CGC/MS is LC/MS. LC/MS allows direct separation of parent compounds with concurrent structural information without chemical derivatization. Therefore, LC/MS is a more efficient means of confirming the presence of thiazides in equine urine.

Currently, few methods that confirm thiazides in urine matrixes use LC/MS. Ventura et al. (7) developed an LC/MS method using thermospray and plasma spray interfaces to determine residual hydrochlorothiazide and other thiazidic diuretics in human urine. Using the plasma spray interface, they obtained a detection limit of 150 ng in full-scan mode for hydrochlorothiazide. Garcia et al. (8) developed an LC/MS/MS method using an electrospray ionization (ESI) interface to determine residual hydrochlorothiazide and trichlormethiazide in equine urine. They obtained a detection limit of 1 ng/mL in selective reaction monitoring mode for hydrochlorothiazide. The method described in this paper simultaneously separates and confirms (in terms of molecular and fragment marker ions) chlorothiazide, hydrochlorothiazide, and trichlormethiazide residues in equine urine through LC/MS with an atmospheric pressure chemical interface (APCI).

This study evaluates use of LC/MS with ESI and APCI interfaces for analysis of thiazidic diuretics in equine urine samples. Chlorothiazide, hydrochlorothiazide, and trichlormethiazide were examined because they are the most commonly used thiazide species in equine veterinary practice. Common oral or intravenous dosages range from 0.1 mg/kg body weight for trichlormethiazide to 10 mg/kg body weight for chlorothiazide (1). Sample preparation, analyte extraction, chromatographic separation, ion-source collision induced dissociation, solvent composition, ionization mode, and ion polar-

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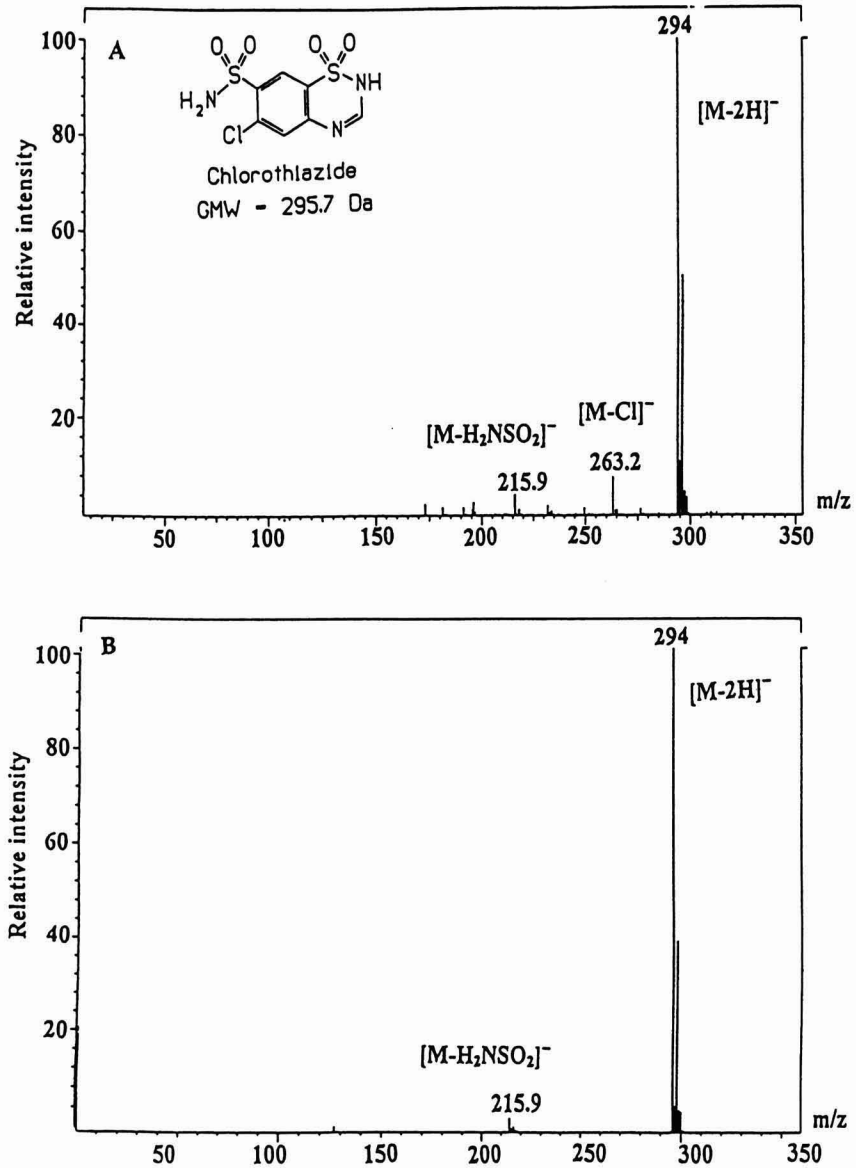

Figure 1. (A) Negative-ion APCI mass spectrum of chlorothiazide generated with an sCID offset potential of 3 eV. (B) Negative-ion ESI mass spectrum of chlorothiazide generated with an sCID offset potential of 15 eV.

ity are discussed. The practicality of LC/MS for this analysis is demonstrated with actual equine administration samples collected at specified time intervals.

METHOD

Reagents

(**a**) *Solvents.*—LC grade methanol and ethyl acetate (Fisher Scientific, Fair Lawn, NJ).

(**b**) *Glacial acetic acid.*—Reagent grade (J.T. Baker, Phillipsburg, NJ).

(**c**) *Water.*—LC grade (Fisher Scientific).

(**d**) *N,N-Dimethylformamide (DMF).*—99.5%, UV spectrophotometric grade (Adrich Chemicals, Milwaukee, WI).

(**e**) *LC mobile phase.*—3% glacial acetic acid in water (pH 2.6)–methanol, with linear elution gradient from 95 + 5 at initial time to 5 + 95 in 15 min, hold at this composition until 25 min; flow rate, 100 µL/min.

(**f**) *100 mM buffer solution at pH 6.0.*—Dissolve 1.70 g Na_2HPO_4 (Adrich Chemicals) and 12.14 g $NaH_2PO_4 \cdot H_2O$ (Adrich Chemicals) in 800 mL deionized water. Bring to 1 L

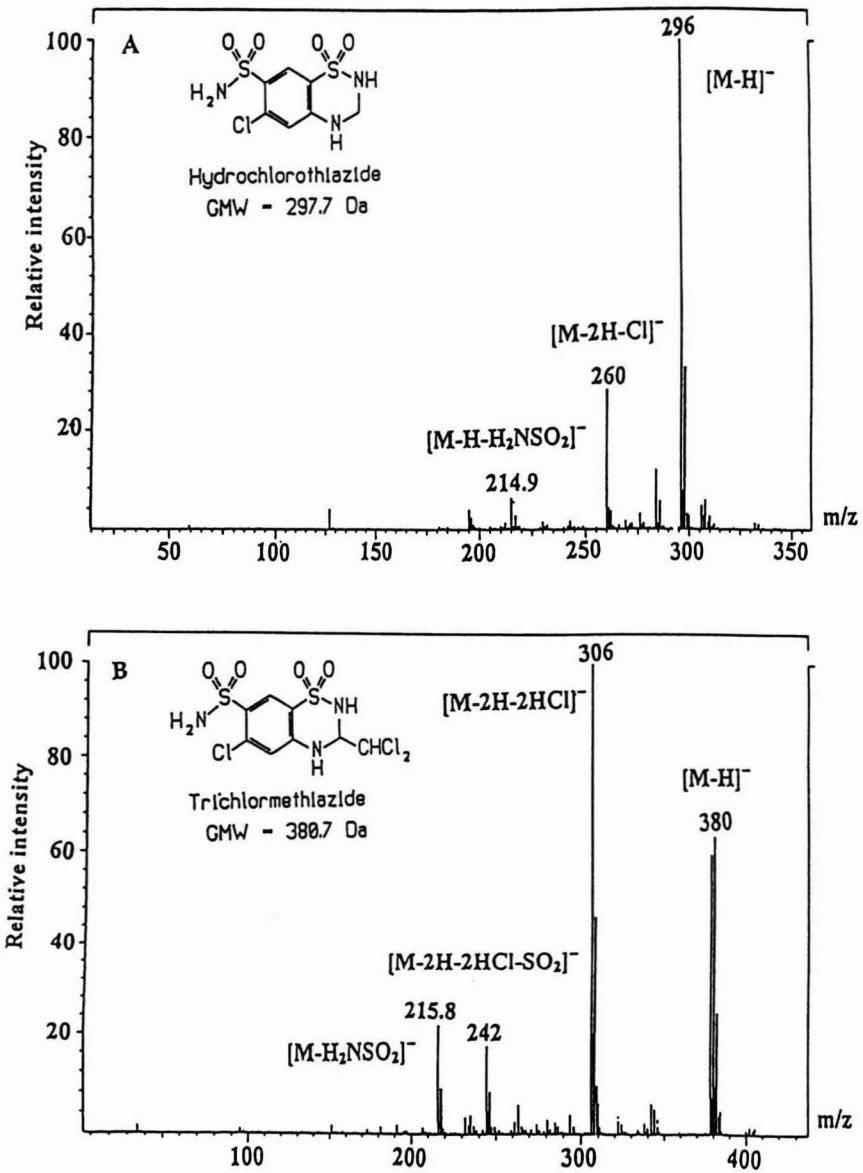

Figure 2. Negative-ion APCI mass spectra of (A) hydrochlorothiazide and (B) trichlormethiazide generated with an sCID offset potential of 3 eV.

volume with deionized water. Adjust pH to 6.0 ± 0.1 with $NaH_2PO_4 \cdot H_2O$ to lower the pH or with Na_2HPO_4 to raise the pH.

Materials and Equipment

(**a**) *Chromatographic column.*—Phenomenex Luna C_{18} (series 2; Torrance, CA) completely end-capped, 3 μm particle size, 150 × 3.2 mm id, 100 Å void space, protected by Luna C_{18} (series 2) guard column, 3 μm particle size, 30 × 3.2 mm id, 100 Å void space.

(**b**) *Liquid chromatograph.*—Thermo Separation Products (TSP) Model P4000 quaternary pump (TSP, Fremont, CA) equipped with TSP Model AS3000 autosampler with 100 μL sample loop, TSP in-line membrane degasser, and TSP spectrophotometer Model 5000 photodiode array detector.

(**c**) *Mass spectrometer.*—Finnigan MAT Model SSQ 7000 equipped with a single quadrupole mass analyzer with 2000 daltons upper mass limit and ESI and APCI ion source modules (Palo Alto, CA).

Standards

(**a**) *Reference standards.*—Chlorothiazide, hydrochlorothiazide, and trichlormethiazide (Aldrich Chemicals).

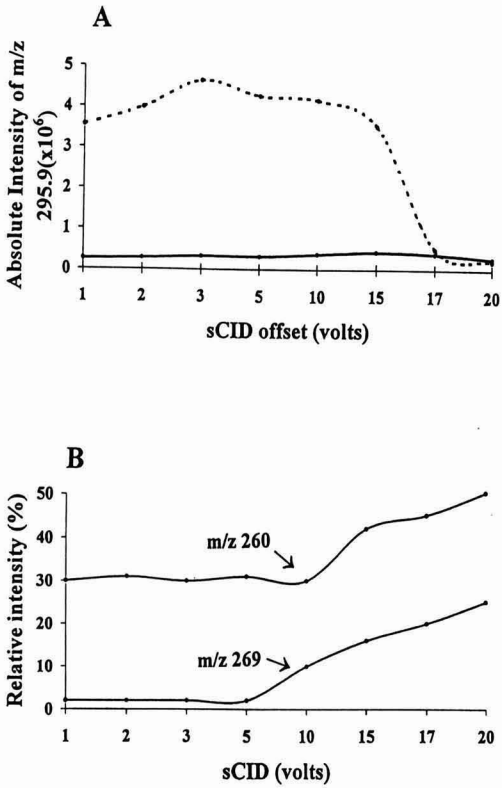

Figure 3. (A) sCID signal responses of hydrochlorothiazide with APCI (broken line) and ESI (solid line) interfaces. (B) Effect of sCID offset on specific marker ions *m/z* 260 and *m/z* 269 relative intensities for hydrochlorothiazide with negative-ion APCI interface.

(**b**) *Macrolide stock solutions.*—Dissolve each standard in methanol to obtain a 2 μg/μL solution and store at 4°C. Chlorothiazide will require an initial dissolution with 5 μL DMF.

(**c**) *Working composite standard (100 ng/μL each).*—Dilute 50 μL of each macrolide stock solution in 850 μL methanol and store at 4°C.

(**d**) *Composite spiked urine standards.*—To determine recoveries and linearity of response of thiazides in equine urine samples, 5, 15, 30, 45, and 60 μL of the 100 ng/μL working composite standard were introduced to 3 mL volumes of mobile phase and negative control urine. These yielded final concentrations of 0.17, 0.5, 1.0, 1.5, and 2.0 μg/mL. For higher concentrations, 15 and 30 μL were introduced to 3 mL portions each of mobile phase and negative control urine to yield final concentrations of 10.0 and 20 μg/mL. For determination of same-day variation, 3 replicate spiked urine extracts at the same concentration level with one absolute standard were analyzed in a single LC/MS session. For determination of day-to-day variation, urine samples spiked at different concentrations with corresponding absolute standards were analyzed on 5 different days over 2 months.

Thiazide Administration Scheme

Chlorothiazide (3.5 g; Diuril; MSD, Willmington, DE) was administered orally to a 12-year-old thoroughbred mare weighing 480 kg. Urine samples were collected prior to administration and at 0–1, 1–2, 4–6, and 53 h postadministration.

Hydrochlorothiazide (500 mg; Hydrodiuril; Endo, Willmington, DE) was administered orally to a 21-year-old standard bred mare weighing 410 kg. Urine samples were collected prior to administration and at 0–1, 1–2, 4–6, and 29 h postadministration.

Trichlormethiazide (100 mg; Naqua; Schering, Kenilworth, NJ) was administered orally to an 8-year-old standard bred mare weighing 460 kg. Urine samples were collected prior to administration and at 0–1, 1–2, 4–6, and 27 h postadministration.

One milliliter volumes of each administration sample were collected at 0, 0–1, 1–2, 4–6, and >24 h intervals and pooled prior to liquid–liquid extraction. This scheme was used to evaluate the LC/MS technique in detecting a multiresidue sample from a more generalized equine urine matrix.

Sample Extraction

Adjust 3 mL volumes of negative control urine, composite spiked urine, and composite administration samples to pH 6.0 with glacial acetic acid. Add 3 mL 100 mM phosphate buffer, mix, and then introduce 5 mL ethyl acetate. Mix with a rotating stirrer for 15 min at ca 50 rotations per minute (rpm). Centrifuge at 3000 rpm for 10 min to separate phases. Transfer organic layer to drying tube and evaporate to dryness under a stream of dry nitrogen gas in a 40°C water bath. Reconstitute residue by first mixing with 50 μL DMF and then mixing with 50 μL methanol. Finally, mix with 400 μL mobile phase (3% acetic acid in water–methanol, 95 + 5). Pass solution through a syringe filter (Nylon 66, 0.2 μm pore size × 13 mm id; Whatman, Clifton, NJ) and into autosampler vial for analysis.

Mass Spectrometer Conditions

(**a**) *Infusion analysis.*—To evaluate detector response of each thiazide, macrolide stock solutions were introduced at a flow rate of 3 μL/min with a syringe pump (Model 980-532; Harvard Apparatus, South Natick, MA) and sprayed from a fused-silica capillary tubing (0.025 id × 370 mm length) connected to a stainless steel syringe (Unimetrics Corp., Shorewood, IL).

Prior to a given day's analysis, the mass spectrometer was tuned with a syringe infusion of the chlorothiazide macrolide stock standard, optimizing on the most abundant marker ion at *m/z* 293.9. Tube lens, capillary, and lens stack assembly potentials were optimized at *m/z* 293.9 with the Instrument Control Language software package. All other parameters were determined iteratively on the basis of signal abundance response.

(**b**) *ESI interface.*—To eliminate electrical discharge problems, silicon hexaflouride (SF_6) gas was used as the carrier gas with a sheath gas pressure of 10 psig and an auxiliary gas flow of 10 mL/min. Negative-ion polarity was used. Sample solutions were sprayed at a spray potential of 6.5 keV, resulting in a discharge current of 2.5–10.5 μA throughout the chroma-

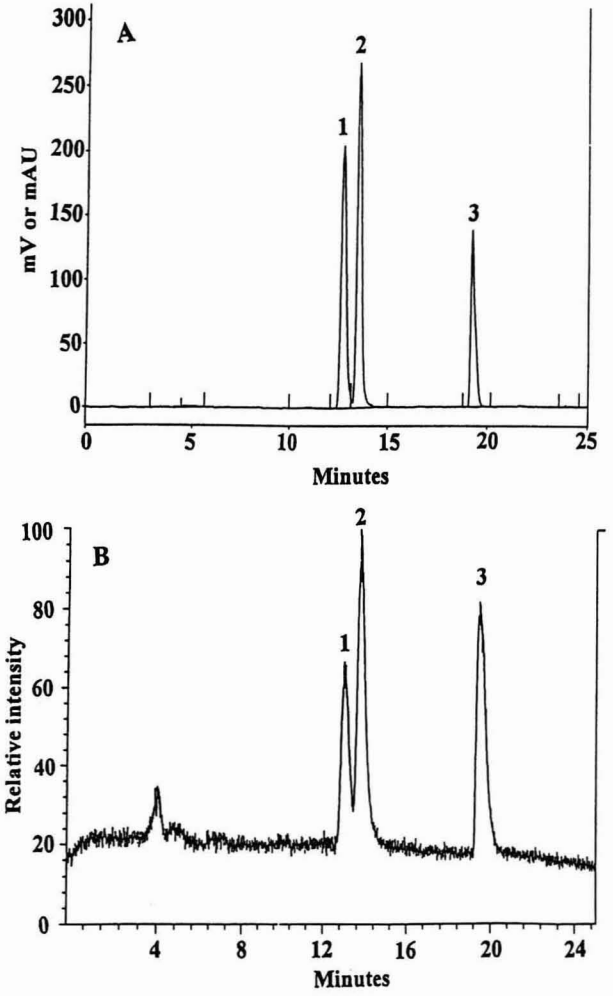

Figure 4. (A) Liquid chromatogram of working composite standard at detection wavelength of 270 nm. (B) Corresponding reconstructed ion chromatogram with APCI/MS interface at 3 eV sCID. Peaks: 1 = chlorothiazide, 2 = hydrochlorothiazide, 3 = trichlormethiazide.

tographic gradient run. Capillary temperature was 275°C at a potential of –12.5 eV. Tube lens potential was –40.7 eV. Spray needle was positioned on axis at ca 2 mm from the capillary orifice.

(c) *APCI interface.*—Negative-ion polarity was used. Corona potential was 2.2–2.9 keV throughout the gradient run at a corona current of 5.0 μA. Vaporizer temperature was 500°C, and capillary temperature was 275°C. Carrier gas was dry nitrogen with a sheath gas pressure of 82 psig and an auxiliary flow of 10 mL/min. Capillary potential was –12.5 eV, and tube lens potential was –38.5 eV.

(d) *MS acquisition parameters.*—The detector was operated under full-scan mode with a mass range of *m/z* 20–650 at a scan rate of 500 amu/s. A band pass filter of 690 Hz was used.

Results and Discussion

Optimization of MS Detection of Thiazidic Compounds

Experiments were performed to determine which interface, ESI or APCI, gave the strongest mass spectral signals and sufficient fragmentation to allow positive confirmation. Fragmentation was accomplished by skimmer collision-induced dissociation (sCID). This technique generates characteristic fragment ions by collision of unseparated ions with carrier gas molecules in the expansion region of the ion source between the skimmer baffle and the octapole ion guide (9). During syringe infusion of macrolide stock solutions, the magnitude of the sCID signal was varied by changing the offset potential between the skimmer element and the octapole ion guide. All

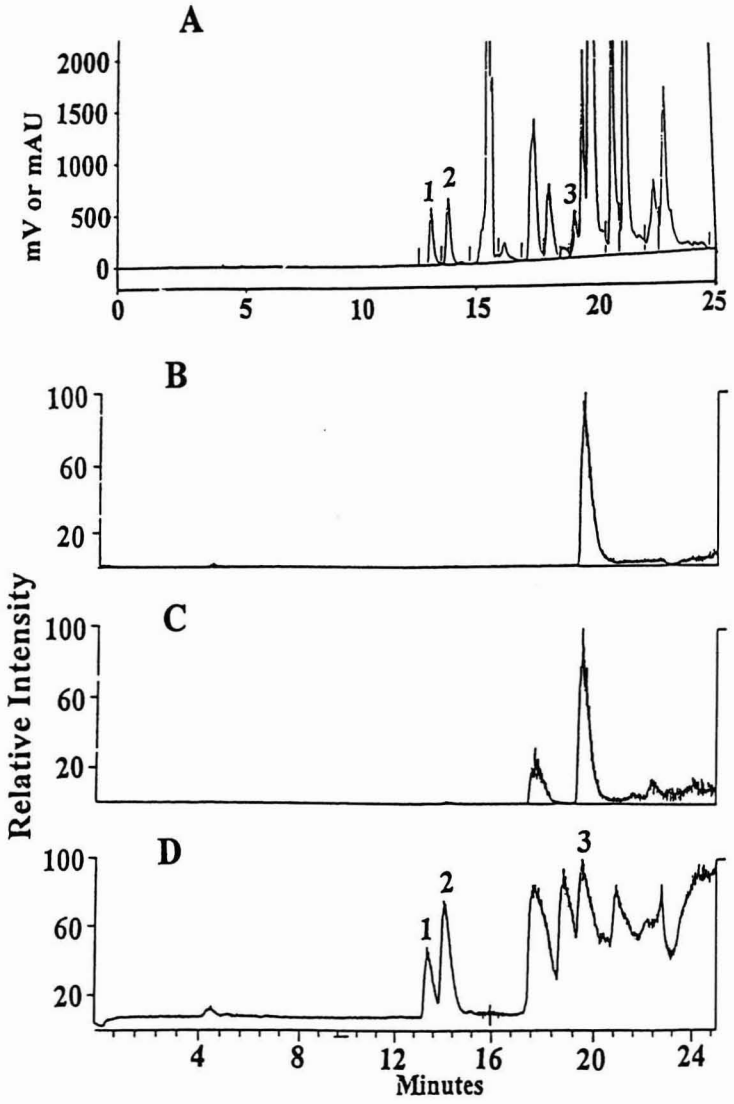

Figure 5. (A) Liquid chromatogram of 10 μg/mL composite spiked urine extract at detection wavelength of 270 nm, recordings of (B) *m/z* 306 and (C) *m/z* 380 for trichlormethiazide, and (D) corresponding reconstructed ion chromatogram with APCI/MS interface at 3 eV sCID. Peaks: 1 = chlorothiazide, 2 = hydrochlorothiazide, 3 = trichlormethiazide.

other MS conditions remained unchanged from those established during chlorothiazide tuning.

APCI mass spectra of chlorothiazide were measured across an sCID offset range of 1–20 eV. Figure 1A shows a mass spectrum at an sCID potential of 3 eV. The most abundant marker ion was the deprotonated anion $[M–2H]^-$ at *m/z* 294.0. Also observed but with substantially less abundance were marker anions $[M–HCl]^-$ at *m/z* 263.2 and $[M–H_2NSO_2]^-$ at *m/z* 215.9. No significant changes to the ratios of these ions were observed across the entire 1–20 eV range of sCID offset values. The largest absolute signal response was at an sCID offset potential of 3 eV (a unitless value of 1.81×10^6 was given by the instrumental software; all subsequent absolute signal intensities are referenced to this value).

Figure 1B shows the mass spectrum of chlorothiazide generated with ESI at an sCID potential of 15 eV. For an sCID offset range of 1–20 eV, the most abundant marker ion was the deprotonated anion $[M–2H]^-$ at *m/z* 294.0, the same primary ion observed with APCI. The only other marker ion observed with ESI, albeit at much less abundance, was the $[M–H_2NSO_2]^-$ at *m/z* 215.9. No significant changes to the ratios of these ions were observed for this range of sCID offset values.

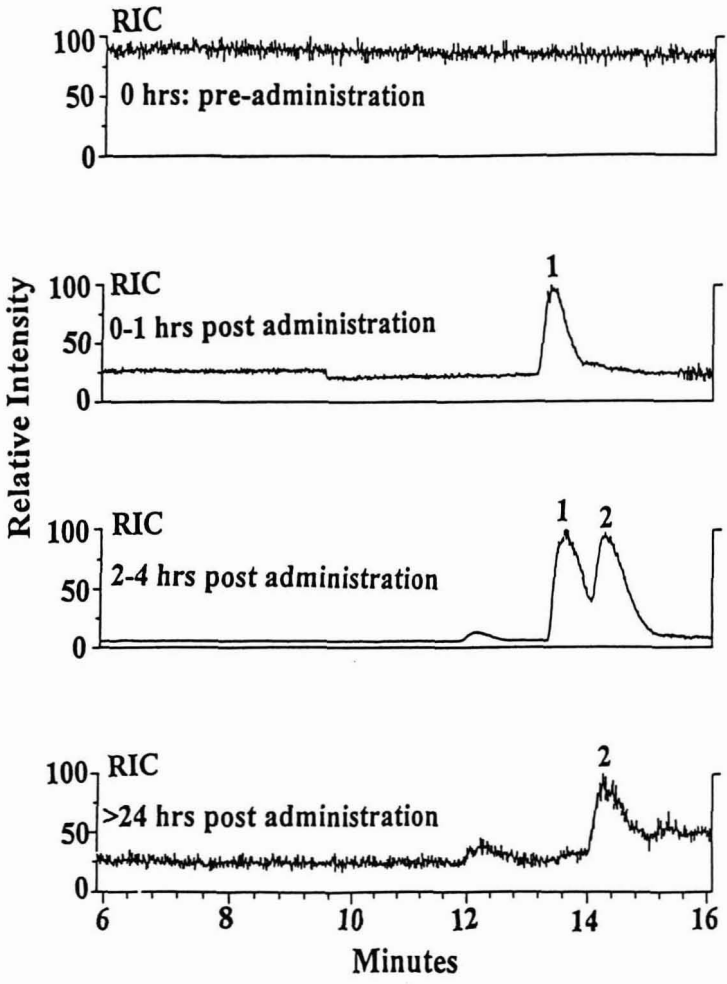

Figure 6. Reconstructed ion chromatogram traces with APCI/MS interface at 3 eV sCID resulting from analysis of composite administration urine extracts at various sampling time intervals. Peaks: 1 = chlorothiazide, 2 = hydrochlorothiazide.

This difference in fragmentation character signifies that the APCI interface provides greater fragmentation of chlorothiazide. The strongest absolute signal response was observed at an sCID offset potential of 15 eV, but this was only 0.1× the absolute signal observed with APCI at an sCID offset of 3 eV.

Mass spectra of hydrochlorthiazide were also recorded with APCI with an sCID offset range of 1–20 eV. Figure 2A shows a mass spectrum of hydrochlorothiazide at a 3 eV sCID offset with 3× higher absolute signal strength than observed for chlorothiazide (Figure 1A). The most abundant marker ion was the deprotonated anion $[M–H]^-$ at *m/z* 296.0. Other marker ions were $[M–2H–Cl]^-$ at *m/z* 260.0 and $[M–H–H_2NSO_2]^-$ at *m/z* 214.9. Even though hydrochlorothiazide differs from chlorothiazide by only an additional proton on one of the heterocyclic amide groups, it fragmented much more extensively than did chlorothiazide. This result is consistent with the less aromatic character of hydrochlorothiazide compared with chlorothiazide, increasing the former's probability of fragmentation during ionization. Overall, the 3 thiazides showed parent and fragment ions similar to those observed by Garcia et al. (8).

Mass spectra of trichlormethiazide were also measured with APCI across the sCID offset range of 1–20 eV. The mass spectrum of trichlormethiazide (Figure 2B) generated with the APCI interface with an sCID offset at 3 eV has signals 0.6× those of chlorothiazide (Figure 1A). The most abundant marker ion for this range was $[M–2H–2HCl]^-$ at *m/z* 306.0. Other abundant marker ions were $[M–H]^-$ at *m/z* 380.0, $[M–2H–2HCl–SO_2]^-$ at *m/z* 242.0, and $[M–H_2NSO_2]^-$ at *m/z* 215.8. Mass spectra were also obtained with ESI interface at an sCID offset range of 1–20 eV (data not shown). The strongest absolute signal intensity was observed at 15 eV, with the most abundant marker anion being $[M–H]^-$ at *m/z* 380.0. However, the

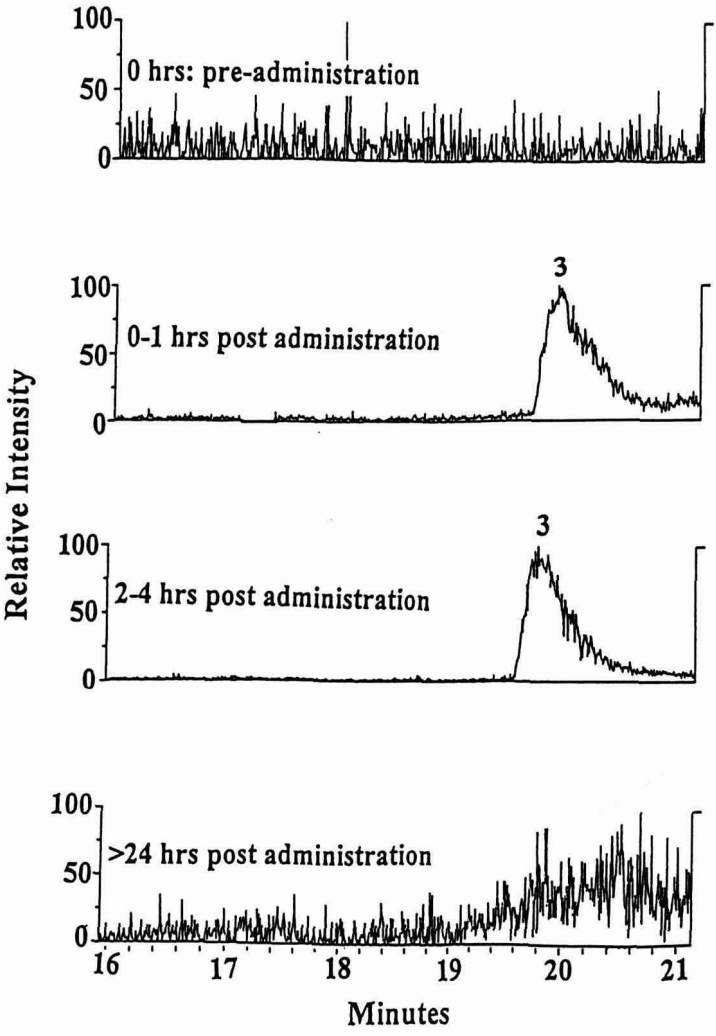

Figure 7. Recordings of *m/z* 306 for trichlormethiazide resulting from analysis of composite administration urine extracts at various sampling time intervals.

absolute signal strength with ESI at 15 eV sCID offset was only 0.1× that observed with APCI at 3 eV sCID offset. Also, the marker compound $[M–H_2NSO_2]^-$ at *m/z* 215.8 was never observed with ESI. These differences indicate that ESI with SF_6 buffer gas provides a softer ionization compared with APCI. This softer ionization was evident for molecules that are more prone to fragmentation, such as trichlormethiazide. Trichlormethiazide is less conjugated and has more branching than the other 2 thiazidic compounds. In this study, the need for maximum sensitivity with structural confirmation for trichlormethiazide points to APCI as the better interface.

Figure 3A shows the total MS signal for hydrochlorothiazide recorded with APCI (broken line) and ESI (solid line) as a function of sCID offset voltage. Up to 15 eV, the APCI signal was about 10× higher than the ESI signal. The most abundant marker ion for all sCID offset voltages and for both ESI and APCI is the deprotonated anion $[M–H]^-$ at *m/z* 296.0. However, hydrochlorothiazide was much more resistant to fragmentation with ESI, resulting in much lower abundance of other marker ions. Furthermore, fragmentation patterns for ESI and APCI were different. Figure 3B shows the primary fragment marker anions for ESI and APCI of hydrochlorothiazide. The intensity of both anions increased dramatically above 5 eV sCID voltages. The other marker anions observed with ESI were $[M–HCN]^-$ at *m/z* 269.0 and $[M–HCN–SO_2]^-$ at *m/z* 204.9. These fragment anions were also observed by Garcia et al. (8), using ESI and sCID. The marker anion $[MHCN]^-$ at *m/z* 269.0 was also observed with APCI at sCID offset values of >5 eV. However, the marker anion $[M–HCN–SO_2]^-$ at *m/z* 204.9 was never observed with APCI. Similarly, the marker anion $[M–2H–Cl]^-$ at *m/z* 260.0 was always seen with APCI but not with ESI.

MS responses observed for all 3 thiazides were based on dissociative electron capture negative-ion production, as described by Christophoru et al. (10). The 10× higher signal intensity for APCI compared with ESI (i.e., Figure 3A) was observed for all 3 thiazides. SF_6 was used as the carrier gas here to suppress electrical discharges in negative-ion ESI, but it also reduced overall signal intensity, as indicated by Wampler et al. (11). Given that optimum MS signal responses will lead to lower limit of quantitation and better sensitivity and that strong relative abundance in marker anions contributes to compound confirmation, APCI was chosen as the ionization source for the LC/MS detection of all 3 thiazidic compounds. All mass spectra discussed from here on were recorded with APCI.

Optimization of the LC Separation

Efficient LC separation is essential for reproducibile and selective MS detection from syringe infusion experiments. This is especially true for single quadrupole MS detectors with sCID where nonselective collisions occur when multicomponent samples are introduced. The combination of efficient LC separation and sCID can provide the desired specific and selective MS detection normally attributed to tandem MS detection systems.

To optimize LC separation, a gradient phase was implemented to maximize resolution of the 3 thiazidic compounds in the working composite standard. Next, 3% glacial acetic acid in water (pH 2.6) was used for the mobile phase to increase peak intensity and to provide sharper peaks for all 3 compounds. This improvement allowed baseline separation of adjacent chromatographic peaks of chlorothiazide and hydrochlorothiazide. Apparently, the lower pH allowed full ionization of all thiazides in the mobile phase, which in turn contributed to narrower peak widths. Such an effect was confirmed when additives other than acetic acid were used, including ammonium acetate and trifluoroacetic acid at various concentrations and pH values. However, these other mobile phase systems resulted in lower peak intensities with broader half-height peak widths.

Another important factor was choice of column. Several column types were evaluated for resolution, chromatographic run time, peak shape, and peak intensity. The smaller 3 µm particle size with the midbore geometry (3.2 mm id) of the Luna reversed-phase column provided maximum analyte peak resolution and intensity at lower flow rates and reasonable chromatographic run times.

Figures 4A and 4B display typical chromatographic responses of the working composite standard with UV/visible detection at 270 nm and APCI/MS detection in reconstructed (or total) ion chromatogram (RIC) mode, respectively. Both detection schemes demonstrate reasonable analyte peak sharpness and good chromatographic separation. Spiked composite urine extracts were run to test the efficiency of the LC/APCI/MS method in separating and detecting the 3 thiazides in the presence of endogenous matrix components. The sample was an extracted 3 mL aliquot of a negative control urine sample spiked with each thiazide at 10 µg/mL. Figures 5A and 5D show typical LC responses with UV/visible and RIC detection, respectively. Figures 5B and 5C show selected ion chromatographs of the marker anions $[M–2H–2HCl]^-$ at *m/z* 306.0 and $[M–H]^-$ at *m/z* 380.0 for trichlormethiazide. Chlorothiazide and hydrochlorothiazide (peaks 1 and 2) remained well resolved from each other and from other endogenous urine extract components for both UV/visible and MS detections. It appears that the negative-ion MS mode contributes to masking of many endogenous positive urine components normally observed in the positive-ion mode. Unfortunately, the trichlormethiazide peak (peak 3) was still not fully resolved from endogenous urine extract components, as determined from peak purity assessments with the photodiode array mode UV/visible detector. However, full discrimination of the trichlormethiazide peak from all other matrix components was possible by its extensive fragmentation behavior. Incomplete LC separation of the trichlormethiazide peak was remedied by confirming its presence by its increased fragmentation behavior. As a supplementary technique for confirming all thiazides, with emphasis on the partly resolved trichlormethiazide, the composite spiked and administration urine samples were run at 15 eV sCID offset (data not shown). These runs demonstrated that changes in *m/z* anion ratios were consistent with those obtained from syringe infusion experiments. Also, the larger sCID offset potential provided an even greater reduction in the endogenous urine extract components in the chromatographic response. However, the large sCID offset potential resulted in a decline in absolute signal, as expected.

Method Validation

(**a**) *Linearity.*—Data for a full set of LC/MS runs were pooled from 5 different analysis sessions recorded on different days spanning 2 months. Each session involved analysis of extracted composite spiked urine standards in the mobile phase. Analyte concentrations ranged from 0.17 to 20.0 µg/mL. Linear regression analyses gave a standard calibration curve for chlorothiazide of $Y = 370\,X + 184$, where Y is the RIC signal abundance and X is the chlorothiazide concentration (µg/mL; $R^2 = 0.9798$). The standard calibration curve for hydrochlorothiazide was $Y = 670\,X - 191$ ($R^2 = 0.9859$) and that for trichlormethiazide gave $Y = 239\,X + 175$ ($R^2 = 0.9859$).

(**b**) *Recovery.*—This was evaluated for each thiazide by comparing the regression response of extracted spiked urine samples to the regression of absolute (unextracted) standards. Recoveries were 68 ± 7.3% for chlorothiazide, 69 ± 6.4% for hydrochlorothiazide, and 48 ± 8.2% for trichlormethiazide.

(**c**) *Precision.*—This was evaluated by conducting replicate analyses of each thiazide component in the composite spiked urine extract at various concentrations. Relative standard deviation (RSD) values were 9.2 to 5.2% from the lowest to the highest concentration for chlorothiazide, 10.3 to 6.7% for hydrochlorothiazide, and 17.6 to 8.4% for trichlormethiazide. The high values for trichlormethiazide are due to matrix interference.

(**d**) *Detection limit.*—This was determined on the basis of 3 standard deviations of the average of 5 assay values for each of the 3 lower concentrations of each thiazide in the composite spiked urine extracts. Detection limits were chlorothiazide, 270 ng/mL; hydrochlorothiazide, 131 ng/mL; and trichlormethiazide, 384 ng/mL.

(e) *Day-to-day variation.*—This was determined from the uncertainty obtained by comparing the average regression output of each analysis session taking place on the same day for 5 different days. RSD values ranged from 11.7 to 6.8% from the lowest to the highest concentration for chlorothiazide, 12.4 to 7.3% for hydrochlorothiazide, and 19.1 to 9.6% for trichlormethiazide.

LC/APCI/MS Analysis of Composite Administration Samples

Figure 6 shows results of LC/APCI/MS analysis of composite administration samples for pre- and several postadministration time intervals. This figure displays the RIC region where chlorothiazide (peak 1) and hydrochlorothiazide (peak 2) were detected, demonstrating the feasibility of monitoring the concentration of these 2 analytes simultaneously. Figure 6 shows that the resolution between analytes is reasonable for all administration sample LC/APCI/MS chromatograms. Chlorothiazide appeared within 1 h of administration and was fully excreted after 4 h. Hydrochlorothiazide appeared within 2–4 h of administration and remained after 24 h.

Trichloromethiazide could not be distinguished solely by monitoring RIC. As discussed above, the increased fragmentation associated with trichlormethiazide allowed for confirmation in the presence of partially coeluting matrix components. Figure 7 shows the recordings of the specific marker ion at *m/z* 306 for trichlormethiazide at the appropriate RIC region. Trichloromethiazide (peak 3) appeared within 1 h of administration and remained beyond 4 h. These results also illustrate the ability to monitor trends in the concentration of trichlormethiazide during the respective time intervals. Because the MS instrument software allows construction of RIC and selected ion chromatographs from a single run, all 3 thiazides could be detected simultaneously.

No excretion curve (to support pharmacokinetic and pharmacodynamic studies) could be determined definitively on the basis of the results. Such a curve would require more administration samples at more time intervals than was done for this study. Furthermore, multiple horses (i.e., 5–7) would need to be administered and the samples pooled. However, results from the administration samples indicate that the LC/APCI/MS method is sensitive enough to construct more comprehensive excretion curves. Also, the sCID technique can be used effectively in structural confirmations of the analytes and, when applicable, their metabolites.

To assist in screening large numbers of urine samples, the photodiode array detector of the LC instrument was used to provide characteristic UV spectra from 190–360 nm for compounds eluting at the retention times observed for the 3 thiazidic compounds. When positive UV spectral matches were obtained at these retention times, the APCI/MS detection system was used for confirmation. This protocol avoided exposure of dirty urine extracts to the MS detector, therefore reducing labor-intensive and costly maintenance.

Conclusion

LC/APCI/MS allowed simultaneous determination of chlorothiazide, hydrochlorothiazide, and trichlormethiazide in equine urine. The method has good selectivity in terms of both fragmentation pattern and molecular weight. The dynamic combination of LC and negative-ion sCID-APCI/MS with a conventional quadrupole mass analyzer yielded a reliable, relatively rapid, and informative method for characterizing thiazides in complex matrixes. Negative-ion sCID-ESI/MS with SF_6 as the carrier gas also detected the 3 thiazides. However, ESI was not chosen for analysis because it has less sensitivity to marker ions.

Acknowledgments

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Determination of Pseudoephedrine Hydrochloride and Carbinoxamine Maleate in Combination Drug Formulation by Liquid Chromatography

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An isocratic, reversed-phase liquid chromatographic (LC) method was developed for simultaneous determination of pseudoephedrine hydrochloride (I) and carbinoxamine maleate (II) in a pharmaceutical dosage form. Analysis was conducted on a CN column (10 μm), with a mobile phase consisting of acetonitrile–methanol–phosphate buffer (pH 5.3)–water (140 + 170 + 40 + 100) and at a detection wavelength of 262 nm. The method was validated for linearity, precision, system reproducibility, and accuracy. Recoveries at 80–120% of labeled claim ranged from 97.4 to 100.7% and from 98.5 to 100.2% for I and II, respectively. Results were linear (correlation coefficient, r > 0.9995 for I in the range 250–750 μg/mL and r > 0.9999 for II in the range 20–60 μg/mL).

Pseudoephedrine and its salts are taken orally for relief of nasal congestion. They are also commonly combined with other ingredients in preparations intended for relief of cough and cold symptoms. Pseudoephedrine hydrochloride or sulfate are generally given in doses of 60 mg 3–4 times daily by mouth. Suggested oral doses for children 2–5 years are 15 mg 3 times daily; 6–12 years, 30 mg 3 times daily (1).

Carbinoxamine maleate, an ethanolamine derivative, is an antihistamine with antimuscarinic, central sedative, and serotonin antagonist effects. It is used for relief of hypersensitivity disorders such as rhinitis and is a common ingredient of preparations for symptomatic treatment of coughs and the common cold. The dose of carbinoxamine maleate by mouth, used alone or in combination preparations, is usually 2–4 mg 3–4 times daily (2).

No procedure has been reported for analysis of pseudoephedrine hydrochloride and carbinoxamine maleate in combination. Araujo et al. (3) determined carbinoxamine maleate but in a mixture with phenylpropanolamine hydrochloride. Anktar et al. (4) used liquid chromatography (LC) to determine pseudoephedrine hydrochloride but in a mixture with paracetamol and triprolidine hydrochloride. Fong et al. (5) separated pseudoephedrine hydrochloride by LC but from a mixture with dextromethorphan hydrochloride and doxylamine succinate.

No compendial method exists for such a combination of drugs. The U.S. Pharmacopoeia contains official methods for carbinoxamine maleate (alone) in tablet dosage form and for pseudoephedrine hydrochloride (alone) in tablet and syrup dosage forms. Methods also exist for combinations of pseudoephedrine and diphenhydramine in capsule dosage form, combinations of pseudoephedrine hydrochloride with acetaminophen in tablet dosage form, and combinations of pseudoephedrine and triprolidine hydrochloride in syrup and tablet dosage forms (6).

METHOD

Apparatus

(**a**) *LC system.*—Two Shimadzu LC-10A pumps, PSD-10A UV visible detector, SCL-10A system controller, DGU-4A degasser unit, CTO-10A column oven, fixed 20 μL loop manual injector, C-R7A plus Chromatopac (Shimadzu Corp., Kyoto, Japan). LC conditions: flow rate, 0.75 mL/min; detector sensitivity, 0.01 absorbance unit full scale (AUFS); attenuation, 6; detector wavelength, 262 nm; chart speed, 3 mm/min; column temperature, 25°C.

(**b**) *Chromatographic column.*—Bondclone CN, 150 × 3.9 mm, 10 μm particle size (Phenomenex, Torrance, CA).

(**c**) *Filters.*—Millipore; filter type, HA; pore size, 0.45 μm (Millipore Corp., Bedford, MA).

Reagents

(**a**) *Acetonitrile.*—LC grade (Hi Per Solv, BDH Laboratory Supplies, Pool, UK).

(**b**) *Methanol.*—LC grade (Honil Limited, London, UK).

(**c**) *Distilled water.*

(**d**) *Triethanolamine.*—Aldrich Chemical Company Ltd., Gillingham, UK.

(**e**) *Acetic acid.*—Glacial (Riedel-de-Haen AG, Seelze, Germany).

(**f**) *Phosphate buffer, pH 5.3.*—Dissolve 0.71 g dibasic sodium phosphate in 500 mL distilled water. Add 5.97 g triethanolamine and dilute to 1000 mL with distilled water. Ad-

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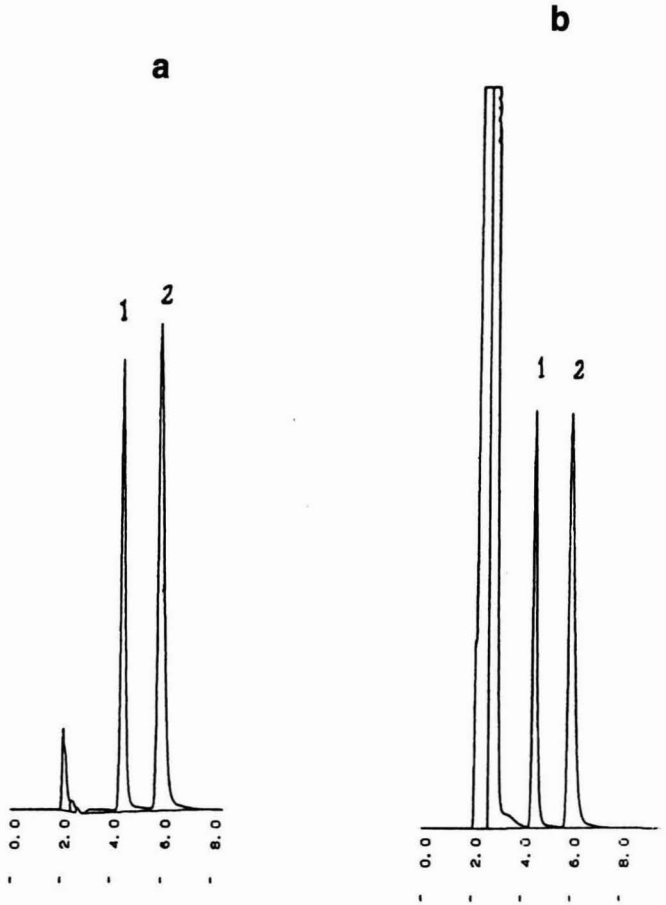

Figure 1. Chromatogram of (a) standard working solution and (b) commercial oral drops formulation: 1, pseudoephedrine hydrochloride; 2, carbinoxamine maleate.

just pH of solution to 5.3 with glacial acetic acid and filter through 0.45 μm Millipore filter.

(**g**) *Dibasic sodium phosphate.*—Anhydrous, analytical grade, Prolabo (El-Nasr Chemical and Pharmaceutical Co., Abu-Zabal, Egypt).

(**h**) *Potassium dihydrogen orthophosphate.*—Analytical grade, Prolabo (El-Nasr Chemical and Pharmaceutical Co.).

(**i**) *Mobile phase.*—Acetonitrile–methanol–phosphate buffer (pH 5.3)–water (140 + 170 + 40 + 100, v/v/v/v).

Standards

(**a**) *Pseudoephedrine hydrochloride.*—Selectchemie AG, Zurich, Switzerland; batch No. B950128; assay, 100.46%.

(**b**) *Carbinoxamine maleate.*—Rovensberg GmbH Chemische Fabrik, Konstanz, Germany; batch No. 6563; assay, 99.92%.

Samples

(**a**) *Experimental formulated sample.*—Prepared in author's laboratory so that each 1 mL contains pseudoephedrine hydrochloride, 25.0 mg; carbinoxamine maleate, 2.0 mg; methylparaben, 1.8 mg; propylparaben, 0.2 mg; sorbitol solution 70% (crystalline), 400.0 mg; propylene glycol, 20.0 mg; saccharine sodium, 1.0 mg; tutti-frutti flavor, 0.3 mg; and purified water, to 1.0 mL.

(**b**) *Commercial oral drops.*—Rhinostop oral drops, manufactured by Medical Union Pharmaceutical Co., Abu-Sultan, Ismailia, Egypt. Each 1 mL contains pseudoephedrine hydrochloride, 25.0 mg, and carbinoxamine maleate, 2.0 mg.

Mixed Standards

(**a**) *Carbinoxamine maleate stock solution.*—Accurately weigh 25 mg carbinoxamine maleate standard and transfer to

Table 1. Recoveries of pseudoephedrine hydrochloride and carbinoxamine maleate at 80, 100, and 120% of labeled claim

Ingredient	Added, mg	Recovered, mg	Recovery, %
Pseudoephedrine hydrochloride	0.399296	0.388928	97.4
	0.499120	0.502652	100.7
	0.598944	0.596581	99.6
Carbinoxamine maleate	0.033024	0.032526	98.5
	0.041280	0.041343	100.2
	0.049536	0.048937	98.8

Table 2. Assay of pseudoephedrine hydrochloride and carbinoxamine maleate in a commercial preparation (Rhinostop oral drops; $n = 5$)

Ingredient	Strength, mg	Average found, mg	% of declared	RSD
Pseudoephedrine hydrochloride	25	25.3	101.2	0.3
Carbinoxamine maleate	2	2.05	102.5	0.2

25 mL volumetric flask. Dissolve in and dilute to volume with methanol.

(**b**) *Pseudoephedrine hydrochloride stock solution.*—Accurately weigh 250 mg pseudoephedrine hydrochloride standard and transfer to 50 mL volumetric flask. Dissolve in and dilute to volume with methanol.

(**c**) *Mixed standard preparation.*—Transfer 2.0 mL from (**a**) and 5.0 mL from (**b**) into 50 mL volumetric flask. Dilute to volume with methanol and mix well.

Sample Preparation

Transfer quantitatively 2.0 mL oral drops solution into 100 mL volumetric flask. Dilute to volume with methanol and mix well.

Procedure

Inject 20 µL of mixed standard into chromatograph and record peak areas. The relative standard deviation (RSD) for 10 replicate injections should not exceed 2.0% for each analyte.

Separately inject duplicate 20 µL portions of the standard and sample preparations into chromatograph and record the peak areas. Pseudoephedrine hydrochloride will elute first, after ca 4.5 min, and carbinoxamine maleate will follow after ca 6.0 min. The number of theoretical plates of the column (N) should not be less than 4000 for both pseudoephedrine hydrochloride and carbinoxamine maleate, and the resolution factor (R_s) should not be less than 1.5. The capacity factors (K') were 1.13 and 1.82 for pseudoephedrine hydrochloride and carbinoxamine maleate, respectively. The alpha factor (α) was 1.6, and R_s was >2.0.

Calculations

Calculate quantity of pseudoephedrine hydrochloride as follows:

$$\text{Pseudoephedrine hydrochloride, mg/mL} = \frac{T \times m_s \times a \times 5 \times 100}{S \times 50 \times 100 \times 50 \times 2}$$

where T is the peak area corresponding to pseudoephedrine hydrochloride in sample chromatogram, S is the peak area corresponding to pseudoephedrine hydrochloride in standard chromatogram, m_s is weight of standard pseudoephedrine hydrochloride (mg), and a is potency of standard pseudoephedrine hydrochloride (%).

Calculate quantity of carbinoxamine maleate as follows:

$$\text{Carbinoxamine maleate, mg/mL} = \frac{T \times m_s \times a \times 2 \times 100}{S \times 25 \times 100 \times 50 \times 2}$$

where T is the peak area corresponding to carbinoxamine maleate in sample chromatogram, S is the peak area corresponding to carbinoxamine maleate in standard chromatogram, m_s is weight of standard carbinoxamine maleate (mg), and a is potency of standard carbinoxamine maleate (%).

Results and Discussion

Chromatograms of a standard solution and a commercial oral drops solution (Rhinostop oral drops) are presented in Figure 1.

Table 3. Assay of pseudoephedrine hydrochloride and carbinoxamine maleate in 3 commercial batches of Rhinostop oral drops

Batch No.	Ingredient	Strength, mg	Average found, mg		Sample SD
			Laboratory 1	Laboratory 2	
970993	Pseudoephedrine hydrochloride	25	24.53	25.07	0.38
	Carbinoxamine maleate	2	1.99	1.96	0.02
970994	Pseudoephedrine hydrochloride	25	24.28	25.89	1.14
	Carbinoxamine maleate	2	1.97	2.04	0.05
970996	Pseudoephedrine hydrochloride	25	24.62	25.44	0.58
	Carbinoxamine maleate	2	1.97	1.97	0.00

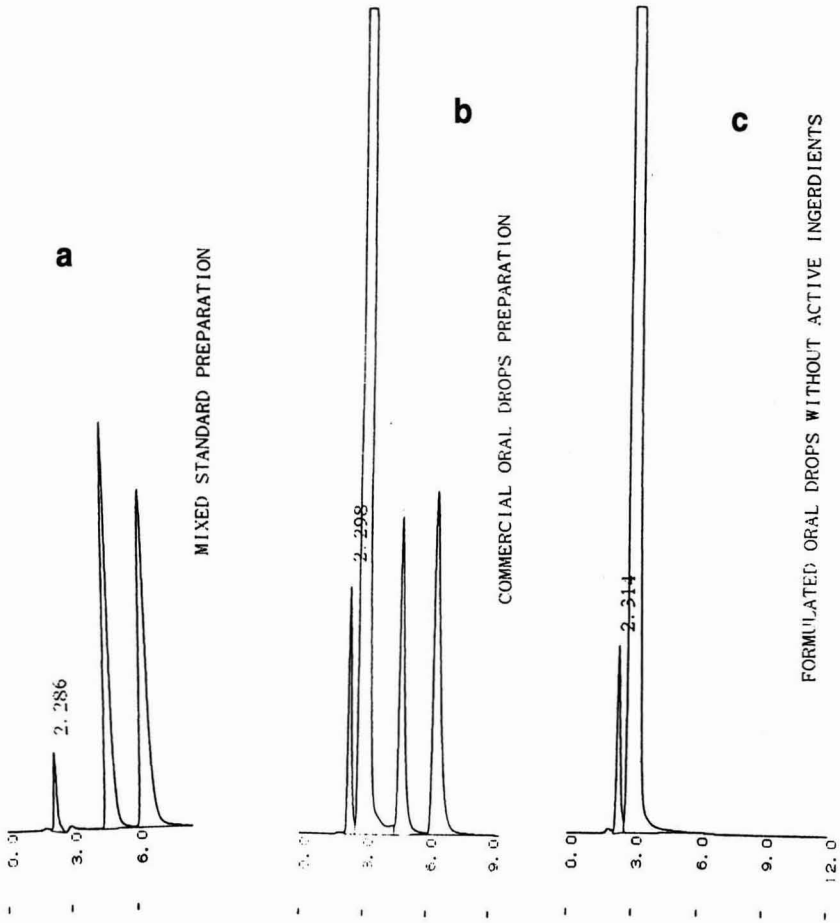

Figure 2. Chromatograms of (a) mixed standard preparation, (b) commercial oral drops preparation, and (c) formulated oral drops without active ingredients. The last chromatogram shows no peaks at retention times of pseudoephedrine hydrochloride and carbinoxamine maleate.

Method accuracy (as recovery), precision, linearity, and reproducibility were validated. Method accuracy was tested by analyzing previously assayed portions of product spiked with equivalent amounts of pseudoephedrine hydrochloride and carbinoxamine maleate standards.

Studies were performed at 80, 100, and 120% of the theoretical sample preparation concentrations for oral drops. Results are given in Table 1.

Precision was determined by analyzing 5 separate containers of a homogeneous experimental batch of oral drops prepared by the author. The precision test was repeated with 5 separate containers from a production batch of oral drops. Results are presented in Table 2.

Precision was also determined by analysis of samples from 3 different batches of the commercial preparation in 2 different laboratories by 2 different analysts using 2 different LC instruments. The other LC system consisted of a Waters 610 fluid unit, Waters 486 detector, Waters 600 controller, Waters 717_{plus} autosampler, and Waters 746 data module (Waters Corporation, Milford, MA). Two samples were taken from each batch for assay, and 2 tests were done for each assay. The sample standard deviation was always less than 2.0. Results are presented in Table 3.

Mixed standards prepared at 25% intervals from 50 to 150% of the mixed working standard preparation concentrations were injected to test method linearity. A plot of peak area versus concentration produced a straight line passing through the origin and with correlation coefficients of 0.9995 for pseudoephedrine hydrochloride and 0.9999 for carbinoxamine maleate. Reproducibility of detector response was tested and recorded through method development. RSD was always <2.0%.

As an additional test, 10 consecutive injections of the mixed working standard were injected; RSDs for pseudoephedrine hydrochloride and carbinoxamine maleate were 0.62 and 0.55, respectively.

The method is selective, as it allows determination of the amount of each active ingredient without any interference from other ingredients such as methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, saccharine sodium, sorbitol, and tutti-frutti flavor. This selectivity was determined by injection of a formulated sample containing neither pseudoephedrine hydrochloride nor carbinoxamine maleate under the same chromatographic conditions. No peaks eluted at the retention times of either pseudoephedrine hydrochloride or carbinoxamine maleate, as shown in Figure 2.

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DRUGS, COSMETICS, FORENSIC SCIENCES

A Screening Method for β-Lactams in Tissues Hydrolyzed with Penicillinase I and Lactamase II

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Antibiotic residues above tolerance levels are not allowed in foods derived from farm animals. Microbial inhibition assays are used to screen antibiotics in U.S. regulatory laboratories. We developed a screening approach to classify β-lactams through selective hydrolysis of the β-lactam ring with Penase™ or lactamase II, thereby inactivating the β-lactam activity. Optimum conditions for hydrolysis of β-lactams with Penase and lactamase II were determined. β-Lactams were detected by a microbial inhibition assay and with enzyme-linked immunosorbent assays before and after hydrolysis. β-Lactams (10–100 ppb) were spiked in kidney extracts and hydrolyzed. Results indicate a pattern that tentatively classified the β-lactams into 3 subgroups. Desfuroyl-ceftiofur-cysteine, a major metabolite of ceftiofur, was clearly detected. Penicillin G, ampicillin, amoxicillin, and cloxacillin were distinguishable from cephapirin, ceftiofur metabolite, and high levels of hetacillin. Liver and kidney tissue samples were analyzed with the combined enzyme hydrolysis and screening assays, which tentatively identified the residues. This approach can speed up screening analysis of β-lactam residues prior to identification and quantitation by chromatographic analysis, thus enhancing positive identification of residues to provide a safer food supply.

Antibiotic residues above tolerance levels are not allowed in foods derived from farm animals. β-lactams are used commonly as antimicrobial drugs to treat or prevent infectious diseases. Their tolerance levels in tissues and fluids are shown in Table 1 (1). Improved rapid screening methods are needed to identify tentatively the specific β-lactam or class of compounds prior to identification and quantitation by chromatographic methods.

Various bioassay and screening techniques and on-site tests used by the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) were reviewed by MacNeil and Ellis (2) and Moats (3). Kidney, liver, and muscle tissues of farm animals are screened on-site in the slaughter house with STOP (Swab Test on Premises; 4), CAST (Calf Antibiotic and Sulfa Test; 5), and more recently with FAST (Fast Antimicrobial Screen Test; 6). LAST (Live Animal Swab Test; 7) detects antibiotic residues in urine of live farm animals.

Tissues of animals found positive by screening tests are analyzed further with a 7-plate assay (8) to determine the class of compounds or specific compounds present. The 7-plate assay is based on the susceptibility or resistance of 4 microorganisms against various drugs, including 3 strains of *Micrococcus* (formerly *Sarcina*) *lutea*. *M. lutea* (ATCC 9341a) is susceptible to penicillin and erythromycin but resistant to streptomycin. *M. lutea* ATCC 9341 and ATCC 15957 are resistant to neomycin and streptomycin, respectively. *Staphylococcus epidermidis* is susceptible to neomycin but resistant to tetracycline. *Bacillus subtilis* is susceptible to streptomycin, *B. cereus* var. *mycoides* is susceptible to tetracycline. These drugs show characteristic patterns of results with these assays.

The presence of β-lactam antibiotics is confirmed by digestion with Penase (EC 3.5.2.6), a type I penicillinase, or with a β-lactamase (9). Penicillins are assayed with *M. lutea* plate assay containing penase incorporated in the agar gel. Other antimicrobials that cannot be identified by the 7-plate bioassay are classified as unidentified microbial inhibitors (UMI).

β-Lactamases catalyze hydrolysis of the β-lactam ring of the 6-aminopenicillanic acid moiety of penicillin compounds or the 7-aminocephalosporanic acid moiety of cephalosporin compounds (10, 11). These β-lactam structures are shown in Figure 1. Penicillinase is used commonly for β-lactamase preparations that predominantly catalyze hydrolysis of penicillins, and cephalosporinase is an enzyme active against cephalosporin compounds (10). β-Lactamases are classified by the Enzyme Commission (EC) as 3.5.2.6 denoting their class as

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Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1. Maximum residue tolerance levels of β-lactams[a]

β-Lactam	Residue level	Species	Tissues or fluids
		5-Membered thiazolidine ring	
Amoxicillin	10 ppb	Cattle	Milk, edible tissues[b]
Ampicillin	10 ppb	Swine, cattle	Milk, edible tissues
Cloxacillin	10 ppb	Cattle	Milk, edible tissues
Hetacillin	No tolerance		
Penicillin	50 ppb	Cattle	Edible tissues
	10 ppb	Turkey	Edible tissues
	0	Chicken, pheasants, quail, swine, sheep	Edible tissues, eggs, milk, dairy processed foods
		6-Membered dihydrothiazine ring	
Cephapirin	20 ppb	Cattle, dairy cattle	Milk
	100 ppb		Edible tissues of dairy
Ceftiofur	Tolerance not required for parent[c]		
	50 ppb[d]	Cattle	Milk
	12 ppm[d]	Cattle	Edible tissues

[a] From reference 1.
[b] Uncooked edible tissues.
[c] Ceftiofur is not detectable as a parent drug.
[d] Action levels for ceftiofur in edible tissues determined by FDA include "ceftiofur equivalent" present as metabolites.

hydrolases [3] catalyzing the cleavage of C–N bonds other than peptide bonds [5], cleaving cyclic amides [2], and hydrolyzing β-lactam substrates [6]. The designation EC 3.5.2.8 originally was assigned to cephalosporinase (12) but has been deleted, and this enzyme is now included in EC 3.5.2.6. As more enzymes were isolated and characterized, these enzymes were further classified as β-lactamase type I, II, or III or as β-lactamase A, B, C, or D. These classifications are based on characteristics such as isoelectric pH, temperature stability, localization, molecular weight and structure, substrate specificity, and metal ion requirement (13, 14).

Type I penicillinases from various sources also vary in hydrolytic rates. The bacterial source of Penase from Difco (Detroit, MI) is not known because of proprietary restriction. Assays utilizing Penase or penicillinase confirm the presence of penicillins except cloxacillin and methicillin, which are resistant to penicillinase (8, 15–18). In addition, Gilbertson et al. (18) reported that the cephalosporin compounds (ceftiofur and cephapirin) also are resistant to type I penicillinase.

Cephalosporinase, a type II penicillinase or lactamase (from *B. cereus* 569/H), rapidly hydrolyzes cephalosporin C, suggesting its selectivity for this subclass of β-lactams (13, 19). Kuwabara and Abraham (19, 20) reported that the V_{max} values (a measure of hydrolytic property obtained from Lineweaver–Burk plots) of lactamase II (from *B. cereus* 569/H) relative to benzylpenicillin (100) are 80, 64, and 89 for cephalosporin C, ampicillin, and cloxacillin, respectively. Thatcher (14) found that the relative hydrolysis rates of lactamase II (from *B. cereus* 569/H) for ampicillin and cephalosporin C are 47 and 14%, respectively, when measured against benzylpencillin (100%). Hydrolytic activities against other veterinary β-lactams were not determined. Recently, Gilbertson and coworkers (18) analyzed the hydrolytic effects of Difco Penase and cephalosporinase (from *Enterobacter cloacae*) on β-lactam antibiotics in milk, using the *B. stearothermophilus* Disk Assay (BSDA). Their findings showed that hydrolytic activities depend on concentration and source of enzyme. Ceftiofur was distinguishable from ampicillin, amoxicillin, penicillin, cephapirin, and cloxacillin.

We studied the hydrolytic selectivities of lactamase II from *B. cereus* 569/H and of penase to determine if the enzyme combination can be used to distinguish cephalosporins from penicillins in a UMI sample. In this study, β-lactam drugs were identified tentatively by rapid screening assays such as the *B. stearothermophilus* diffusion assay (Delvotest [DT]) and the enzyme-linked immunosorbent assays (ELISAs), LacTek-BL (LBL) and LacTek-Cef (LTC) before and after hydrolysis with Penase and lactamase II. Portions of aqueous extracts of tissues were treated separately with these enzymes, and results were compared with those from untreated samples. The presence of β-lactams was shown by loss of inhibitory activity in the diffusion assay (DT), and the absence of β-lactams was shown by negative responses to the ELISAs. If inhibitory activities persisted after Penase and lactamase II treatments, the presence of antibiotics other than β-lactams was suspected, and samples did not have to be analyzed for β-lactams. Also, if extracts retained inhibitory activity after Penase treatment but lost the activity after lactamase II treatment, the presence of a cephalosporin compound (ceftiofur or cephapirin) was suspected. The efficacy of Penase and lactamase II to selectively hydrolyze β-lactams was evaluated by analyzing tissues containing incurred antibiotic residues and tissue samples obtained from the market.

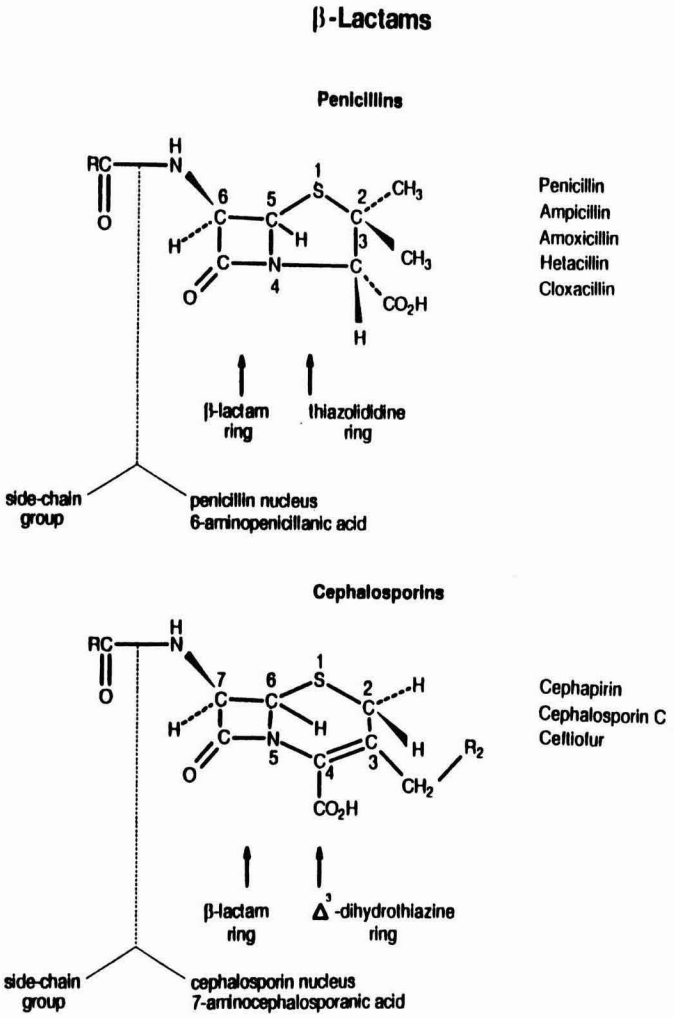

Figure 1. Structure of β-lactams. Penicillins possess a 5-membered thiazolidone ring fused with β-lactam. Cephalosporin compounds consist of a 6-membered d^3-dihydrothiazine ring fused with β-lactam ring (11).

Experimental

Equipment

(**a**) *Seward stomacher.*—Model 80 (Tekmar-Dohrman, Cincinnati, OH).

(**b**) *Tomy high-speed microrefrigerated centrifuge.*—MTX-150 (Peninsula Laboratories, Belmont, CA).

(**c**) *Enzyme-linked immunosorbent assay (ELISA) reader.*—EL312 (Biotek, Winooski, VT).

(**d**) *Photometer.*—For 12 × 75 cm tubes (Idexx Laboratories, Westbrook, ME).

(**e**) *Multiblock heater.*—Lab-Line, Melrose Park, CA.

(**f**) *Incubator.*—Model 1525 (VWR Scientific, Philadelphia, PA).

(**g**) *Extraction/digestion tubes.*—Polypropylene tubes with caps, 12 × 72 cm (5 mL) and 16 × 92 cm (10 mL), and conical tubes, 17 × 110 cm (12 mL) and 25 × 107 cm (30 mL).

Reagents

(**a**) *Extraction/dilution buffer.*—73 mM phosphate buffer, pH 6, contained 8.71 g KH_2PO_4 and 2.04 g $K_2HPO_4 \cdot 3H_2O$ diluted in 1 L. (This is equivalent to 1% KH_2PO_4 described for the 7-plate assay [8]). NaOH (1M) was used to adjust extraction buffer pH to 6.5 and 7.

(**b**) *β-Lactams.*—Sodium salts of ampicillin (A9518), amoxicillin (A8523), cephapirin (C8270), cloxacillin (C9393), and penicillin G (Pen-Na), and hetacillin, potassium salt (H0509) were purchased from Sigma Chemical Company (St. Louis, MO). Desfuroylceftiofur cysteine (DFCC) metabolite

was a gift from M. Beconi-Barker, Pharmacia & Upjohn Company (Kalamazoo, MI).

(c) *Stock solutions of β-lactams.*—Dilutions were prepared for each β-lactam. Stock A contained a specific β-lactam at 1 mg/mL in deionized water. Stock B (10 μg/mL) was prepared by diluting 0.1 mL stock A with 9.9 mL phosphate buffer. Stock C (1 μg/mL or 1 ppm) was prepared by taking 0.5 mL stock B and diluting with 4.5 mL phosphate buffer.

(d) *Lactamases.*—β-lactamase II (EC 3.5.2.6; molecular weight [MW], 23 000) produced from *B. cereus* 569/H, K_m of 1100 μM and specific activity of 2000 units [U]/mg vs cephalosporin C, Cat. No. 191493) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). One unit is the amount of enzyme that will inactivate 1 μmol cephalosporin C/min at 30°C, pH 7. One micromole cephalosporin C (formula weight [FW], 415.4; free acid) is equivalent to 0.415 μg/mL. A stock solution of 1000 U/mL (stock 1) was prepared, divided in 100 μL portions, and stored in a –80°C freezer. A working dilution of 100 U/mL (stock 2) was prepared by diluting stock 1 (1:10) with phosphate buffer, pH 6.

BactoPenase (EC 3.5.2.6; 20 000 Levy unit [LU]/mL/min) was purchased from Difco. One LU inactivates 59.3 IU (international units) of sodium penicillin G/h at 25°C, pH 7. With the Sigma sodium penicillin G (FW, 354.4), which has an activity of 1650 U/mg, 1 LU inactivates 0.5995 μg/min. Penase was used at 20, 2, and 0.2 LU/mL. Penase was diluted with 73 mM phosphate buffer, pH 6.

(e) *Screening assays.*—(*1*) *β-Lactams.*—LacTek-BL (Idexx Laboratories, Inc.). (2) *Ceftiofur.*—LacTek-CEF (Idexx). (*3*) *Delvotest P Mini.*—Manufactured by Gist-Brocades (Delft, The Netherlands) and purchased from Eastern Crown, Inc. (Vernon, NY).

Methods

(a) *Sample extraction.*—Conditions for enzyme hydrolysis (e.g., time, temperature, pH of extraction buffer, and enzyme concentration) were optimized. The tissue extraction method of the USDA FSIS laboratory was adopted with slight modifications (4, 8). Tissues were weighed (5 or 10 g), cut into small pieces (ca <5 mm^2), and transferred quantitatively into Tekmar stomacher bags (9 × 16 cm). Extraction buffer was added at 4 mL/g tissue (8), and the mixture was blended for 60 s. Blended samples were kept at 4°C for 1 h to allow residues to diffuse into the extraction buffer and to allow solid materials to settle. Extracts were transferred to polypropylene tubes and centrifuged at 12 000 rpm (11 850 × *g*) for 10 min. (If a high-speed centrifuge is not available, samples may be centrifuged at 3000 × *g* for 15 min.) Supernatants or clarified extracts were analyzed as described in the following paragraphs.

(b) *Optimum enzyme hydrolysis conditions.*—Portions (5 mL) of supernatants or clarified extracts were transferred to polypropylene tubes and spiked with 50 and 100 μL of stock C β-lactam solution (1 μg/mL) to final β-lactam concentrations of 10 and 20 ppb, respectively. Penase concentrate (50 μL) and lactamase II (50 μL lactamase, stock 2) were added to spiked extracts. Samples were incubated for 30, 45, or 60 min at 37°C, pH 6. Effects of pH on enzyme hydrolysis and screening assays were studied at pH 6, 6.5, and 7. Effects of temperature on enzyme activity in pH 6 buffer were studied at 37°C and at room temperature (ca 25°C).

(c) *Hydrolytic activity of lactamase II in spiked tissue extracts.*—A 10 g kidney tissue was extracted with 40 mL buffer (pH 6) by the procedure described in the preceding section. Clarified extracts were spiked with β-lactams (ampicillin, amoxicillin, cephapirin, cloxacillin, hetacillin, penicillin G, and DFCC) at 0, 10, 50, and 100 ppb (ng/mL). To 2 mL spiked extracts, 20 μL lactamase II stock 1 or 2, containing 10 or 100 U/mL, was added to give a final enzyme concentration of 0.1 or 1 U/mL, respectively. Extracts without β-lactams were used as negative control. Spiked samples without enzymes also were tested. Samples were hydrolyzed for 45 min at 37°C and stored in an ice bath or at 4°C until assayed with DT, LTB, and LTC, as described in later sections.

(d) *Hydrolytic activity of Penase in spiked tissue extracts.*—A 10 g kidney tissue was extracted with 40 mL buffer (pH 6) by the procedure described previously. Clarified extracts were spiked with β-lactams (ampicillin, amoxicillin, cephapirin, cloxacillin, hetacillin, penicillin G, and DFCC) at 0, 10, 50, and 100 ppb (ng/mL). To 2 mL spiked extracts, 20 μL Penase at 0.2, 2, or 20 LU/mL was added to give a final enzyme concentration of 2, 20, or 200 LU/mL extract, respectively. Samples were hydrolyzed for 45 min at 37°C and stored in an ice bath or at 4°C until assayed with DT, LTB, and LTC.

(e) *Analysis of β-lactams in market tissue samples.*—Twenty kidney and muscle samples were purchased from local markets. Five gram portions were extracted with 20 mL buffer (pH 6) as described previously. To 2 mL clarified extracts of unknown samples, 20 μL Penase concentrate and 20 μL stock 2 lactamase II containing 100 U/mL were added separately. Samples were hydrolyzed for 45 min at 37°C. Extracts of the unknown samples that were not treated with enzymes also were tested. Untreated tissue extracts ("blank") served as negative controls, and tissue extracts (2 mL) spiked with 20 μL of 1 ppm (1 μg/mL) penicillin G and cephapirin (resulting in final β-lactam concentration of 10 ng/mL) served as positive controls. Control, untreated, and enzyme-treated extracts were tested with DT, LTB, and LTC.

(f) *Analysis of β-lactams in incurred tissues.*—Incurred residues or incurred tissues is a term used by U.S. regulatory agencies to refer to residues or tissues from animals that had been treated with veterinary drugs. Eleven samples of beef kidney and liver tissues from 7 animals that tested positive for UMI were analyzed. These samples were obtained from FSIS Eastern and Midwestern Laboratories. A 5 g liver or kidney tissue was extracted with 20 mL buffer. To 2 mL clarified extract, Penase concentrate (20 μL) was added, and to another 2 mL extract, lactamase II (20 μL of 100 U/mL) was added. Extracts were hydrolyzed for 45 min. After hydrolysis, samples were kept at 4°C or in an ice bath to inhibit further enzymatic hydrolysis at room temperature. Treated, negative control, and positive control samples were assayed by DT, LTB, and LTC.

Beef kidney extract was used for negative control. Penicillin G and cephapirin (10 ppb) spiked in clarified kidney extracts were used as positive reference standards. Control sam-

Table 2. Detection of β-lactams in spiked tissue extracts after hydrolysis with Penase and lactamase II[a]

β-Lactam, ppb	DT			LTB			LTC		
	No enzyme	+Penase	+L II	No enzyme	+Penase	+L II	No enzyme	+Penase	+L II
Penicillin G									
0	–	–	–	–	–	–	–	–	–
10	+	–	–	+	–	–	–	–	–
50	+	–	–	+	–	–	–	–	–
100	+	–	–	+	–	–	–	–	–
Ampicillin									
0	–	–	–	–	–	–	–	–	–
10	+	–	–	+	–	–	–	–	–
50	+	–	–	+	–	–	–	–	–
100	+	–	–	+	–	–	–	–	–
Amoxicillin									
0	–	–	–	–	–	–	–	–	–
10	+	–	–	+	–	–	–	–	–
50	+	–	–	+	–	–	–	–	–
100	+	–	–	+	–	–	–	–	–
Cloxacillin									
0	–	–	–	–	–	–	–	–	–
10	–	–	–	+	–	–	–	–	–
50	+	–	–	+	– (Trace)	–	–	–	–
100	+	–	–	+	– (Trace)	–	–	–	–
Hetacillin									
0	–	–	–	–	–	–	–	–	–
10	+	–	–	+	–	–	–	–	–
50	+	–	–	+	–	–	–	–	–
100	+	+	+	+	–	–	–	–	–
Cephapirin									
0	–	–	–	–	–	–	–	–	–
10	+	+/–	–	+	–	–	–	–	–
50	+	+	–	+	–	–	–	–	–
100	+	+	–	+	–	–	–	–	–
Ceftiofur (DFCC)[b]									
0	–	–	–	–	–	–	–	–	–
10	+	–	–	–	–	–	+	NH[c]	–
50	+	+	–	–	–	–	+	NH	–
100	+	+	–	–	–	–	+	NH	–

[a] β-Lactams were added to kidney tissue extracts and treated with Penase at 200 LU/mL extract and hydrolyzed for 45 min at 37°C. DT = Delvotest, LTB = LacTek-BL, LTC = LacTek-CEF. No enzyme = unhydrolyzed samples, +Penase = Penase addded, +L II = lactamase II added. Positive (+) results were indicated by >30% decrease in optical density of spiked sample vs zero (blank) control. Negative (–) results were indicated when >30% increase in optical density was observed after enzyme hydrolysis. – (Trace) indicates <30% increase in optical density after enzyme digestion compared with undigested samples. +/– indicates borderline positive/negative.

[b] Desfuroylceftiofur cysteine.

[c] NH = not hydrolyzed. Indicates no change in OD after penase treatment.

ples and unknown samples were tested for β-lactams with DT, LTB, and LTC before and after digestion with Penase and lactamase II. One sample that retained inhibitory activity after Penase treatment but lost its activity after lactamase II hydrolysis was suspected to contain ceftiofur on the basis of screening tests. This sample was further analyzed by LC (21, 22) to determine the presence of ceftiofur metabolite. Twenty additional beef kidney and liver samples with incurred β-lactams (21) that had been tested with the 7-plate microbial inhibition assay also were analyzed with DT and LTB. A total of 31 incurred tissue samples were analyzed.

Rapid Screening Tests

Assay protocols described by manufacturers were followed. Samples were analyzed within 20 min or stored at 4°C or in an ice bath for later analysis. Hydrolyzed samples were kept in the freezer (–20°C) for storage overnight or a few days.

(a) *Delvotest (DT).*—A multiblock heater with precise temperature control was used. Sample tube holders of the heating block were filled with water about one-third full and equilibrated at 65°C. Hydrolysates (0.1 mL) were transferred to vials, and a nutrient tablet was added to each vial. The sample and nutrient tablet were mixed gently and incubated for 2.5 h at 65°C in the block heater.

(b) *ELISA tests: LacTek-BL (LBL) and LacTek-CEF (LTC).*—Portions (0.25 mL) of hydrolysate were transferred to antibody-coated tubes. Tracer reagent (enzyme-labeled β-lactam) was added, and the mixture was incubated for 3 min at room temperature (RT). Contents of ELISA tubes were discarded, and the tubes were washed 3 times with LacTek wash buffer. Color developer was added, and the tubes were incubated for another 3 min at RT. Stopping reagent was added, and the absorbance was measured at 405 nm with an Idexx photometer.

Results and Discussion

Optimization of Penase and Lactamase II Hydrolysis

For the microbial inhibition test DT, pH 6 was optimum for extraction. A higher pH yielded false-positive results when β-lactams were hydrolyzed with Penase or lactamase II.

A pH near 7 was ideal for the ELISA assay LTB, suggesting optimal antibody–antigen interactions. Extraction buffer at pH 6 was selected for subsequent assays because pH 6 phosphate buffer is being used in regulatory methods (4, 8). However, up to pH 6.5 may be used in DT and LTB assays. Optimum hydrolysis time of 45 min was sufficient to hydrolyze β-lactams at levels ranging from 10 ppb (ng/mL) to 1 ppm (μg/mL). A 30 min hydrolysis was not sufficient to hydrolyze completely higher levels of β-lactams (100 ppb and above).

With this time (45 min) and temperature (37°C), optimum lactamase II concentration was 1 U/mL tissue extract. Lactamase II at less than 1 U/mL was not sufficient to hydrolyze the β-lactams. Lactamase II concentrations of 2, 10, 50, and 100 U/mL hydrolyzed both types of β-lactams and did not show selectivity. The ceftiofur compound was evaluated only with 0.1 and 1 U lactamase II/mL extract. Theoretically, 1 U lactamase II hydrolyzes approximately 1 μM cephalosporin C (415 ppb)/min at 30°C. The concentration of Penase that effectively hydrolyzed β-lactams was 200 LU/mL extract. Lower concentrations, 2 and 20 LU/mL extract, were not sufficient to hydrolyze higher β-lactam concentrations at 37°C for 45 min. Hydrolysis proceeded with longer exposures of the drugs to the enzymes at either 37°C or room temperature.

Rates of hydrolysis by Penase (200 LU/mL) and lactamase II (1 U/mL) at room temperature and at 37°C were equal when tested with β-lactam concentrations below 1 ppm (1 μg/mL) in buffer. β-Lactam concentrations greater than 1 ppm may require higher enzyme concentration or longer hydrolysis time. Time, temperature, and enzyme concentration must be controlled to obtain described results.

Penase and lactamase concentrations influenced ELISA results. The tracer reagent, a β-lactam labeled with an enzyme, can be altered with high concentrations of Penase and lactamase II, which would open the lactam ring. An altered β-lactam does not bind with the antibody, resulting in lower optical density (OD) because of reduced binding of the tracer with the antibody coated on the tube. Samples containing borderline amounts of β-lactams may then be classified as positives. Samples hydrolyzed with a combination of Penase and lactamase II also gave lower absorbances compared with samples hydrolyzed with a single enzyme. This result again indicates partial digestion of the tracer. Thermal inactivation of the enzyme also may degrade β-lactams. Chemical inactivation would interfere with ELISAs and microbial inhibition assays.

β-Lactam Hydrolytic Activities of Lactamase II and Penase

The hydrolytic activity of lactamase II from *B. cereus* has not been evaluated recently. Kuwabara and Abraham (19) reported its hydrolytic activity against benzylpenicillin, ampicillin, cloxacillin, and cephalosporin C. Our findings show that the hydrolytic activity of lactamase II depends on the concentrations of enzyme and drug (substrate): Less than 1 U/mL did not digest completely the β-lactams in the prescribed time and temperature, but 1 U/mL was sufficient to digest β-lactams up to 100 ppb. These results are summarized in Table 2.

In this study, Penase also was evaluated systematically for its hydrolytic activity against the veterinary β-lactams, Penase at 200 LU/mL extract was sufficient to hydrolyze β-lactams up to 100 ppb, except ceftiofur metabolites. In addition, cephapirin, hetacillin, and cloxacillin were only partially hydrolyzed. Data resulting from separate Penase or lactamase hydrolysis of β-lactams spiked in tissue extracts are not shown, but results from simultaneous enzyme hydrolysis of similarly spiked extracts are shown in Table 2.

Penicillin G (10, 50, and 100 ppb) was completely hydrolyzed by 1 U lactamase II/mL extract but only partially digested by 0.1 U/mL. Penicillin G was completely hydrolyzed by Penase. Intact drugs were detected by DT and LTB but not by LTC. The digested drugs indicated negative results. DT indicated presence of penicillin G, other β-lactams, and antimicrobials by generating a blue color. Negative results—that is, absence of antimicrobial activity due to absence of antibiotics or hydrolysis of β-lactam rings—were indicated by a yellow

Table 3. Detection of β-lactams in market tissues before and after hydrolysis with Penase or lactamase II[a]

Sample No. and identification	Delvotest P Mini			LacTek-BL			LacTek-CEF		
	No enzyme	Penase	Lactamase II	No enzyme	Penase	Lactamase II	No enzyme	Penase	Lactamase II
Kidney extract blank	–	–	–	–	–	–	–	–	–
+ 10 ppb Pen G	+	–	–	+	–	–	–	–	–
+10 ppb cephapirin	+	–	–	+	–	–	–	–	–
1 Calf liver 1	+	+	+	–	–	–	–	–	–
2–7 Calf liver 2–7	–	–	–	–	–	–	–	–	–
8 Calf liver 8	+	+	+	–	–	–	–	–	–
9 Beef kidney 1	+	–	–	–	–	–	+	Trace	–
10–14 Beef kidney 2–6	–	–	–	–	–	–	–	–	–
15 Beef muscle	–	–	–	–	–	–	–		–
16–18 Pork kidney 1–3	+	+	+	–	–	–	–	–	–
19 Pork kidney 4	+/–(Trace)	+/–(Trace)	+/–(Trace)	–	–	–	–	–	–
20 Pork liver 1	–	–	–	–	–	–	–	–	–

[a] Extracts (2 mL) were hydrolyzed with 20 μL Penase concentrate and 20 μL lactamase II (Lactamase II). Positive (+) results with Delvotest were indicated by a purplish blue color; negative (–) results were indicated by yellow color, and trace (+/–) was indicated by yellowish-purple color. Positive antimicrobial activity not due to β-lactams was indicated by (+) in undigested (No Enzyme) and digested samples. Trace results by LacTek assays indicated detectable amounts of β-lactams, such as a decrease in optical density compared with blank control and an increase in optical density after enzyme hydrolysis. Trace results (<10 ppb) were indicated by 20–30% change in optical density before and after enzyme hydrolysis. Extracts of samples 1, 8, and 16–18 were resistant to either Penase or lactamase II, as indicated by antimicrobial activity not due to β-lactams and as confirmed by LacTek assays. Presence of UMI compounds is indicated in samples 1, 8, and 16–19. Ceftiofur metabolites are indicated in sample 9.

color. Positive results in the LTB or LTC assay were indicated by >30% lower OD compared with a blank sample extract (without drug) and a concomitant increase in OD (>30%) after enzyme hydrolysis. Changes in OD between 20 and 30% were reported as trace amounts, because such amounts were below action or tolerance levels.

Cloxacillin (native or hydrolyzed) was detected at 50 and 100 ppb by DT. No antimicrobial activity was detected after lactamase II hydrolysis. Cloxacillin at 10 ppb was not detected by DT. LTB detected 10–100 ppb cloxacillin (hydrolyzed and unhydrolyzed) but indicated partial hydrolysis of cloxacillin with Penase. LTC did not detect cloxacillin at any concentrations.

Intact hetacillin (10, 50, and 100 ppb) was detected by DT and LTB. Lower levels (10 and 50 ppb) were completely hydrolyzed by lactamase II at 1 U/mL and by Penase at 200 LU/mL, as shown by absence of microbial inhibitory activity with DT. However, 100 ppb hetacillin was resistant to Penase and lactamase II, as indicated by bluish-purple positive results with DT. Hetacillin at 10–100 ppb was detected by LTB, giving a significant decrease in OD after Penase and lactamase II digestion. LTC did not detect native or hydrolyzed hetacillin. Hetacillin is a precursor drug of ampicillin and does not exist as a parent compound in residues.

Cephapirin (10, 20, and 100 ppb) was digested by lactamase II at 1 U/mL and by Penase at 200 LU/mL and was detected by LTB. Lactamase II digested cephapirin, and no microbial activity was detected by DT. However, Penase did not completely inactivate cephapirin, resulting in a positive/negative biological activity at 10 ppb. However, positive antimicrobial activities were detected at 50 and 100 ppb. The intact and enzyme-treated drugs were not detected by LTC.

The biologically active ceftiofur metabolite DFCC was added to kidney extracts (pH 6) at 10, 50, and 100 ppb, digested by lactamase II, and detected by DT and LTC. DFCC was not detected by LTB. Samples hydrolyzed by 1 U lactamase II gave a >200% increase in OD, as tested with LTC. However, the hydrolysate from digestion with lactamase II at 0.1 U/mL showed only 20–25% increase in OD when DFCC was present at 10 and 50 ppb, although a 205% increase in OD was observed with hydrolysate when DFCC was present at 100 ppb. DFCC was resistant to Penase hydrolysis, as indicated by no change in OD of digested drug compared with undigested drug. The resistance of DFCC (50 and 100 ppb) to Penase digestion was detected by DT. A low level (10 ppb) of DFCC was digested by Penase, and DT did not show any microbial activity. The combination of these assays and enzyme digestion indicate a unique response of DFCC.

Ampicillin (10, 50, and 100 ppb) was digested by lactamase II at 1 U/mL and Penase at 200 LU/mL. DT and LTB assays detected the native and enzyme-inactivated drug in extracts containing the drug at 10–100 ppb. Digests by lactamase II at 0.1 U/mL were not detectable by DT but were detectable with LTB. LTC assays did not detect intact or hydrolyzed ampicillin.

Amoxicillin (10, 50, and 100 ppb) was detected by DT and LTB. The drug was hydrolyzed by lactamase II at 1 U/mL and by Penase at 200 LU/mL. No β-lactam activity was detected by DT and LTB after hydrolysis. Digestion with lactamase II at 0.1 U/mL was not complete, and DT and LTB indicated positive results because of incomplete digestion of the β-lactams. Unhydrolyzed and hydrolyzed amoxicillin were not detected by LTC in all concentrations.

Table 4. Detection of β-lactams in UMI tissue hydrolyzed with Penase or lactamase II[a]

Sample No. and tissue (No. of analysis)	Delvotest P Mini			LacTek-BL		LacTek-CEF	
	Penase (Pens)	Lactamase II (Cephs)	UMI (Others)	Penase (Pens)	Lactamase II (Cephs)	Penase (Pens)	Lactamase II (Cephs)
Kidney extract blank	–	–	–	–	–	–	–
+ 10 ppb Pen G	+	–	–	+	+	–	–
+ 10 ppb cephapirin	–	+	–	Trace	+	–	–
1 Kidney (1)	–	–	+	–	–	–	–
1 Liver (1)	–	–	+	–	–	–	–
2 Kidney (1)	–	–	+	Trace	Trace	–	–
3 Kidney (3)	–	–	+	Trace	Trace	–	–
3 Liver (1)	–	–	+	Trace	Trace	–	–
4 Kidney (3)	–	+	–	–	+	–	+
4 Liver (2)	–	+	–	–	+	–	+
5 Kidney (1)	–	–	–	–	–	–	–
6 Kidney (1)	+	–	–	+	+	–	–
6 Liver (2)	+	–	–	+	+	–	–
7 Liver (2)	–	–	–	–	–	–	–

[a] Two milliliter extracts hydrolyzed with 20 μL penase concentrate and 20 μL lactamase II. Pens = penicillins. Cephs = cephalosporins. Positive (+) results with Delvotest were indicated by disappearance of β-lactams after enzyme hydrolysis. Others = results indicating positive antimicrobial activity not digested by either Penase or lactamase II. Positive results by LacTek assays were indicated by >30% decrease in optical density compared with blank control and >30% increase in optical density after digestion. Trace results were indicated by 20–30% change in optical density before and after enzyme hydrolysis but were below the 10 ppb positive control standard. Samples 1–3 contained UMI antibiotics. Samples 2 and 3 also had trace levels of β-lactam. Results for sample 4 indicate presence of ceftiofur compound. Other β-lactams were detected in sample 6.

In summary, penicillin G, ampicillin, amoxicillin, hetacillin (*10, 50,* and *100 ppb*), and cloxacillin (*50* and *100 ppb*) were detected by DT and LTB. Cloxacillin at 10 ppb was not detectable by DT. Only DFCC was detected by LTC, indicating the high specificity of the ceftiofur antibody. Lactamase II hydrolyzed all β-lactams except hetacillin. At 100 ppb, hetacillin was only partially hydrolyzed, retaining microbial activity as shown by DT. Penase hydrolyzed low levels of DFCC and cephapirin (10 ppb), inactivating microbial activity, but it did not inactivate DFCC and cephapirin at 50 and 100 ppb, as indicated by DT. Resistance of ceftiofur and cephapirin to Penase also was reported by Gilbertson and coworkers (18). Higher levels of cloxacillin also were only partially digested by Penase, as detected by LTB (<30% in OD after hydrolysis). Previous reports (8, 15–18) also indicated cloxacillin resistance to Penase.

DFCC was detected by DT and LTC at 10, 50, and 100 ppb. Although resistant to Penase, DFCC was not detectable with LTB, thus displaying a unique pattern of responses. High levels of cephapirin (50 and 100 ppb) also showed a unique pattern: negative response to LTB, positive response to LTC, but resistant to penase as shown by DT. Cloxacillin may be classified with the penicillin G group. However, its Penase resistance was detectable only with LTB as trace amounts. Penicillin G, ampicillin, and amoxicillin had similar patterns of being detected by DT and LTB but not by LTC. By using the combination of enzyme digestion and these 3 screening tests, the results of these assays can tentatively classify the β-lactams into 3 subgroups: (*1*) DFCC, (*2*) cephapirin, and (*3*) penicillin G, ampicillin, amoxicillin, and cloxacillin.

Detection of β-Lactams in Market Tissue Samples

A positive control containing 10 μg penicillin G/mL extract was used. This concentration is equivalent to 50 μg/g tissue, on the basis of a dilution factor of 5, wherein samples were extracted with 4 mL buffer/g tissue (8). In addition, a cephapirin standard (10 ppb) was used at 10 μg/mL extract. In future studies, however, a cephapirin standard of 20 μg/mL extract must be used to conform with the minimum residue levels (MRL) tolerance. Results (Table 3) indicate positive antimicrobial activity of calf livers (samples 1 and 8) and pork kidney (samples 16–18) that were resistant to Penase and lactamase II hydrolysis. No β-lactam activity was detected in these samples, indicating a non-β-lactam microbial inhibitor. Another beef kidney sample (sample 9) was positive for a ceftiofur compound (DT and LTC), but this sample was not analyzed by LC to confirm the presence of a ceftiofur compound. Samples showing antimicrobial activity with DT after enzyme digestion were classified as non-β-lactam positive or UMI. Samples 1 and 9–16 were analyzed at least twice, and the others were analyzed once with combined enzyme hydrolysis. Samples 9–14 were tested further with DT, LTB, and LTC (3, 7, 27, 4, 16, and 12 times, respectively) and market samples 10–14 were used as negative control. Some of these samples contained trace amounts of β-lactams, but the amounts were technically negative because levels below 50 ppb penicillin G are nonviolative (1). Tolerance levels (Table 1) are zero for chickens, swine, and sheep and 10 ppb for turkeys.

Detection of β-Lactams in Incurred Tissues

Kidney and liver tissues that tested positive for UMI (21) were extracted, treated with Penase and lactamase II, and tested with DT, LTC, and LTB. As shown in Table 4, tissues from 3 animals showed antimicrobial activity that was not due to β-lactams. Kidney and liver tissue samples from one animal (sample 6) gave a response pattern indicating the presence of cloxacillin. Tissues from 2 animals (samples 5 and 7) did not contain detectable antimicrobial compound. Kidney and liver tissues from another animal (sample 4) was positive for the presence of a ceftiofur compound. The residue was resistant to Penase but was digested by lactamase II and was positively detected by LTC (an ELISA test for ceftiofur) but was not detected by LTB (an ELISA test for β-lactams). These samples were analyzed further by a multiresidue LC method (22, 23), and the presence of DFCC was confirmed (data not shown). DFCC is the major biologically active metabolite that can be detected after administration of ceftiofur, which is not detectable after 2 h (24).

Additional 20 samples that tested positive for incurred β-lactam residues by the FSIS 7-plate assay (21) were screened for β-lactams by using this modified enzyme hydrolysis method. Results indicated that 4 samples did not contain detectable antimicrobial activity or β-lactams. These samples had been stored at –20°C for at least 3 months prior to analysis and trace levels of β-lactams may have been lost. With our screening procedure, 16 of these 20 samples were positive for β-lactams, and 2 contained mixtures of β-lactams and other antimicrobials. In later studies (25), additional incurred samples that tested positive for UMI by the 7-plate assay were evaluated with this screening procedure. Positive samples were identified by LC (22, 25, 26).

Conclusions

Systematic analysis of hydrolyzed and unhydrolyzed β-lactams indicated that detection of these drugs before and after hydrolysis depends on the type of β-lactam drug and the concentration of enzyme used. Lactamase II at 1 U/mL and Penase at 200 LU/mL were sufficient to hydrolyze β-lactams up to 100 ppb under specified conditions. The drugs can be identified through the pattern of results of enzyme-treated samples (compared with untreated samples) followed by simultaneous detection of nonspecific and specific β-lactams with ELISA.

Use of both Penase and lactamase II hydrolysis and screening assays can tentatively classify the β-lactams into 3 subgroups prior to chromatographic analysis: ceftiofur metabolite represented by DFCC; cephapirin; and penicillin G, ampicillin, amoxicillin, and cloxacillin. This approach eliminates a large number of negative samples for chromatographic analysis, which typically has a lower throughput than the bioassays, and therefore increases the efficiency of the assay. Further analysis of tissue samples by this screening procedure will be evaluated with LC methods.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Cephapirin and Ceftiofur Residues in Bovine Milk by Liquid Chromatography with Ultraviolet Detection

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A method capable of quantitating cephapirin at a level of 20 ng/mL and ceftiofur at a level of 50 ng/mL was developed for raw bovine milk. Raw bovine milk is deproteinated with acetonitrile. The supernatant is collected and then acetonitrile is removed under reduced pressure while warming in a water bath at 40°–50°C. The extract is mixed with water and loaded onto a conditioned C_{18} solid-phase extraction column. Analytes are eluted with acetonitrile, which is removed completely under a stream of nitrogen gas. Analytes are separated from coextractives by gradient elution with an ion-pair mobile phase on a reversed-phase column and are detected by ultraviolet absorbance at 290 nm. Mean recoveries from fortified milk samples ranged from 79 to 87% for cephapirin and from 76 to 86% for ceftiofur, with intralaboratory coefficients of variation ranging of 6–10% and 7–14%, respectively.

Cephapirin (Figure 1) and ceftiofur (Figure 2) are β-lactam antibiotics commonly used in veterinary medicine. In the United States, ceftiofur is approved for use in treatment of respiratory diseases of beef and dairy cattle (1). The safe level for ceftiofur (parent drug) in milk is 50 ppb (2). Cephapirin is approved for use in lactating dairy cattle. The tolerance in milk, set by the U.S. Food and Drug Administration (FDA), is 20 ppb (3). Since 1992, under the Appendix N of the Pasteurized Milk Ordinance (PMO), all bulk milk transported by truck tankers must be monitored for β-lactam residues prior to entering the food supply (4). Commercially available test kits accepted by FDA and the Association of Official Analytical Chemists Research Institute (FDA-AOACRI) are commonly used for this purpose. Although test kits give rapid results, they are generally nonspecific, do not distinguish between β-lactams, and do not provide a quantitative measure of residues detected. Chemical assays capable of identifying and quantitating β-lactams in milk are needed to verify test kit results and to provide the basis for regulatory action.

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Several liquid chromatographic (LC) methods are available for determination of cephapirin alone (5–7), ceftiofur alone (8), or both (9). MacIntosh (6) reported a procedure for determining cephapirin in milk using a methylene chloride wash on a C_{18} solid-phase extraction (SPE) column followed by LC with ultraviolet (UV) detection. McNeilly et al. (8) developed a procedure for determining ceftiofur in milk using a C_{18} SPE cleanup followed by LC with UV detection. Tyczkowska et al. (7) developed a procedure for determining cephapirin in milk and serum using LC with UV detection and confirmation by thermospray mass spectrometry at higher levels than the tolerance set by FDA. The MacIntosh, Tyczkowska et al., and McNeilly et al. procedures are for a single analyte only. A multiresidue procedure for determining 6 β-lactams, including cephapirin and ceftiofur, was developed by Moats and Harik-Kahn (9). In this procedure, milk was deproteinated with acetonitrile and then cleaned up by LC with fraction collection. LC fractions corresponding to retention times of each analyte of interest were collected and evaporated to dryness. Each residue was reconstituted in appropriate solvent and assayed on a second LC system with UV detection. The method is versatile in that it can determine all 6 β-lactams commonly found in milk. However, this method is lengthy, involves use of 2 LC systems, and requires significant expertise to produce good results. In this paper, we describe a method that will determine both ceftiofur and cephapirin in a sample of milk.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Perkin-Elmer Series 410 pump, Perkin-Elmer LC-95 UV/visible spectrophotometer detector (Norwalk, CT) interfaced to a PE-Nelson Turbochrom version 4.1 data system, and a Perkin-Elmer ISS-100 autosampler equipped with a 200 µL loop, or equivalent LC system.

(b) *LC column.*—Supelcosil LC-18 (Cat. No. 5-8230), 5 µm particle size, 150 × 4.6 mm column equipped with a Supelcosil LC-18, 2 cm, guard column (Bellefonte, PA), no substitutions. Both analytical and guard columns are placed in a column heater set at 40.0° ± 1.0°C.

(c) *Filters.*—LC mobile phase solvent filtration apparatus with 0.2 µm Nylon-66 (N-66) filters to fit. Sample filtration with 13 mm LC certified 0.2 µm Whatman polyvinylidene fluoride (PVDF) filter, no substitution, (Cat. No. 6779-1302; Whatman Laboratory Div., Clinton, NJ).

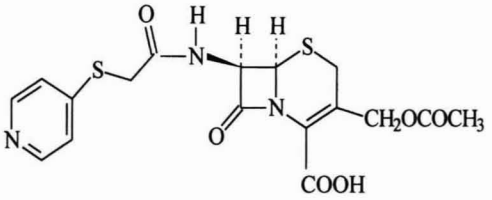

Figure 1. Structure of cephapirin.

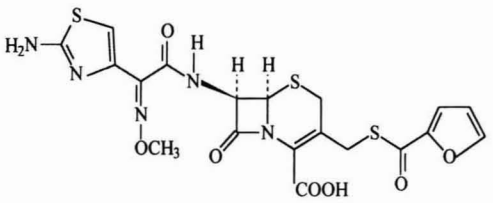

Figure 2. Structure of ceftiofur.

(**d**) *SPE columns.*—Sep-Pak, Vac 6 cc (1 g) C_{18} (Cat. No. WAT036905; Millipore Corp., Waters Chromatography, Marlborough, MA), no substitution.

(**e**) *Rotary evaporator.*—Buchi Rotavapor Model EL131, Buchi Model 461 water bath (Buchi/Brinkmann, Westbury, NY), Trivac "A" dual-stage vacuum pump (Leybold-Heraeus Vacuum Products, Inc., Export, PA), multitrap (FTS Systems, Inc., Stone Ridge, NY), or equivalent.

Reagents

(**a**) *LC grade water.*—Distilled, deionized water prepared in-house with Milli-Q Plus water system was used in preparing all solutions.

(**b**) *Solvents.*—LC grade acetonitrile (ACN) and methanol (MeOH).

(**c**) *Chemicals.*—Sodium dodecyl sulfate (SDS), Ultrapure Bioreagent; *o*-phosphoric acid (H_3PO_4), 85%, LC grade.

(**d**) *Analytical standards.*—Ceftiofur, sodium salt, bulk drug, Pharmacia & Upjohn Company, Kalamazoo, MI. Cephapirin, sodium salt, Cat. No. 10250-0, reference standard, U.S. Pharmacopeial Convention, Rockville, MD.

(**e**) *33 mM H_3PO_4, 9 mM SDS ion-pair solution for mobile phase.*—Weigh 5.20 g SDS into a 2 L volumetric flask. Fill half the volume with distilled, deionized water. Add 4.5 mL H_3PO_4. Bring to mark with distilled, deionized water.

(**f**) *Mobile phase B.*—33 mM H_3PO_4, 9 mM SDS–ACN (90 + 10). Measure 900 mL of 33 mM H_3PO_4, 9 mM SDS ion-pair solution into a 1000 mL graduated cylinder. Add 100 mL ACN into same 1000 mL graduated cylinder. Thoroughly mix. Filter through N-66, 0.2 µm filter. SDS is a detergent and foams during mixing and degassing. Degas mobile phase very carefully. This solution may be stored for up to 3 months in the refrigerator.

Standard Solutions

(**a**) *Stock solutions.*—100 µg/mL. On the basis of the listed potency or purity of each standard (e.g., 10.6 mg of 94.3% cephapirin), calculate amount of cephapirin and ceftiofur needed to prepare 100 mL of a 100 µg/mL solution. Weigh each standard to the nearest ± 0.1 mg, into separate 100 mL volumetric flasks. Bring to the mark with distilled, deionized water. These solutions may be stored refrigerated for 2 weeks.

(**b**) *Fortification solution.*—2 µg cephapirin/mL and 5 µg ceftiofur/mL. Pipet 2 mL stock standard cephapirin (100 µg/mL) solution into a 100 mL volumetric flask. Pipet 5 mL stock standard ceftiofur (100 µg/mL) solution into same 100 mL volumetric flask. Bring to the mark with distilled, deionized water. This solution may be stored refrigerated for 2 weeks.

(**c**) *Calibration standards.*—Prepare from fortification solution (2 µg cephapirin/mL and 5 µg ceftiofur/mL). Dilute fortification solution with water to prepare at least a 4-point standard curve of the following concentrations: (*1*) 40 ng cephapirin/mL and 100 ng ceftiofur/mL, (*2*) 80 ng cephapirin/mL and 200 ng ceftiofur/mL, (*3*) 200 ng cephapirin/mL and 500 ng ceftiofur/mL, and (*4*) 400 ng cephapirin/mL and 1000 ng ceftiofur/mL. These solutions may be stored refrigerated for 2 weeks.

Samples

The control milk used for development work was grade A milk obtained from the bulk tank at the U.S. Department of Agriculture's Agricultural Research Center in Beltsville, MD. This milk was commingled from a herd of ca 100 animals. Incurred milk was obtained from 2 cows, one treated by intramammary infusion with cephapirin and the other cow treated similarly with ceftiofur. Fresh raw control milk was stored in the refrigerator at <7°C for up to 3 days. If analyses were not performed within 3 days, the milk was subdivided and stored at ≤–60°C for up to a year. Frozen milk was slowly thawed in warm water and mixed gently before sampling.

Procedure

(**a**) *Extraction/deproteination.*—Ten milliliters milk was measured into a disposable 50 mL polypropylene centrifuge tube. For recovery measurements, 100 µL fortification solution was added at this step. This is equivalent to 20 ng cephapirin/mL and 50 ng ceftiofur/mL in milk. The tube was gently mixed with a Vortex mixer. ACN (30 mL) was added to milk in the centrifuge tube. The centrifuge tube was capped and mixed with Vortex mixer at high speed for 20 s. The extract was centrifuged for 20 min at 2500 × *g* and –4°C to effect phase separation. While the sample was still cold (<5°C), ca 30 mL of the clear supernatant was transferred with a 10 mL disposable glass pipet into a 100 mL pear-shaped flask, and the flask was stoppered. ACN (20 mL) was added to the precipitate in the centrifuge tube. The tube was capped, mixed on a Vortex mixer at high speed for 20 s, and centrifuged for 15 min at 2500 × *g* and –4°C. The supernatant was decanted carefully into the same pear-shaped flask; thus the clear supernatants were combined. The solid protein in the disposable centrifuge tube was discarded. Water (2 mL) was added to the pear-shaped flask to prevent extract from drying during evaporation of ACN. The extract was evaporated under reduced pressure while warming in a bath at 40°–50°C to a final volume of 1–1.5 mL. Complete

Table 1. Gradient for LC analysis

Step	Time, min	A (ACN), %	B (H_3PO_4, SDS–ACN), %	C (MeOH), %
1	2	25	75	0.00
2	5	20	60	20
3	5	20	60	20
4	7	45	35	20
5	11	45	35	20

removal of ACN is critical because the amount of ACN present in the extract affects retention and recovery of both analytes on the SPE column. Water (10 mL) was added to each evaporated extract. The extract was shaken on a Vortex mixer at high speed for at least 10 s. Extracts were stable at room temperature for up to 2 h. If analysis could not be done in 2 h, extracts were refrigerated overnight, before SPE cleanup.

(**b**) *SPE cleanup.*—C_{18} SPE columns were conditioned with 5 mL MeOH followed by 2× 5 mL water. Care was taken that columns did not go to dryness. A slight vacuum (<3 in. Hg) was applied. The extract was loaded onto the conditioned SPE column. The flow rate was adjusted to 1–2 drops/s. The pear-shaped flask was rinsed with 5 mL water, stoppered, and shaken on a Vortex mixer. Once the loaded extract drained to the surface of the sorbent bed, the rinse was added to the SPE column. The flow rate was maintained at 1–2 drops/s. Once the water rinse drained to the surface of the sorbent bed, a vacuum of 5 in. Hg was applied for 2 min to completely drain and dry the SPE column. The waste was discarded. Analytes were eluted off the SPE column with 3 mL ACN at a rate of 1–2 drops/s. The eluate was collected in a 15 mL glass graduated centrifuge tube. Water (1 mL) was added to the graduated centrifuge tube. The tube was gently shaken on a Vortex mixer. The extract was evaporated under a slow stream of nitrogen in a water bath set at 40°–50°C to a volume of <1 mL. (Do not evaporate to a volume less than 0.5 mL.) The final volume was adjusted to 1 mL by adding water (this represents a 10-fold concentration of analytes). The extract was gently shaken on a Vortex mixer. The contents were transferred into a 3 cc syringe fitted with a 13 mm, 0.2 μm pore size, disposable PVDF filter. The extract was filtered into a glass autosampler vial, and 100 μL was analyzed on the LC system.

(**c**) *Chromatographic conditions.*—Milk extracts are analyzed for cephapirin and ceftiofur with the following LC conditions. Gradient elution, *see* Table 1. Mobile phase A, ACN; mobile phase B, (33 mM H_3PO_4, 9 mM SDS)–ACN (9 + 1); and mobile phase C, MeOH. Equilibration time, 10 min. Flow rate, 1.0 mL/min. Wavelength, 290 nm. Run time, 30 min. Sample extracts are stable for 48 h prior to injection if stored in the freezer ≤–20°C. Inject a water blank to equilibrate LC system to gradient conditions. Inject 100 μL of each standard so-

Table 2. Concentration and mean percent recovery of cephapirin and ceftiofur measured in fortified milk samples

	Measured concentration of cephapirin, ng/mL, in samples fortified at indicated levels		
	10 ng/mL	20 ng/mL	40 ng/mL
	6.5	17	31
	8.0	18	31
	8.3	17	37
	8.6	19	34
	8.1	17	32
			36
Average[a]	7.9	17	34
Mean recovery, %[a, b]	79	87	84
CV, %[a]	10	6	8
	Measured concentration of ceftiofur, ng/mL, in samples fortified at indicated levels		
	25 ng/mL	50 ng/mL	100 ng/mL
	17	44	86
	21	46	75
	22	44	90
	17	41	86
	18	38	71
			63
Average[a]	19	43	78
Mean recovery, %[a, b]	76	86	78
CV, %[a]	12	7	14

[a] Calculated from data prior to rounding of individual values.

[b] Mean recovery, % = (average amount found/amount fortified) × 100.

Table 3. Concentration of cephapirin and ceftiofur measured in milk samples from treated cows

	Cephapirin, ng/mL	Ceftiofur, ng/mL
	29	69
	30	74
	30	77
	31	79
	31	63
Average[a]	30	72
CV, %[a]	3	9

[a] Calculated from data prior to rounding of individual values.

lution prior to injecting a sample set. Quantitation is performed by using peak height and linear regression.

Because the mobile phase is highly acidic, the analytical column and guard column should be flushed with at least 120 mL water after a sample set (e.g., 8–12 samples). In addition to water flush, 30 mL MeOH should flow through columns to wash off matrix buildup. Final short-term storage (overnight) should be water–ACN (65 + 35). With proper care, the system is capable of running ca 100–150 samples without changing guard column and 600–800 samples on the analytical column without degradation.

Results and Discussion

LC conditions for cephapirin described by Moats and Harik-Kahn (5, 9) initially were used for our analyses. The mobile phase was (33 mM H_3PO_4, 9 mM SDS)–ACN (66 + 34). However, in this mobile phase, the retention times of cephapirin in standard solution and in sample extracts did not coincide. This effect was traced to use of tetraethylammonium chloride during sample preparation. Although addition of the ion-pair reagent tetraethylammonium chloride to milk prior to deproteination with ACN improved recoveries of cephalosporins to nearly 100% (6), the high concentration of tetraethylammonium chloride altered the ionic strength of milk extracts, leading to competition between tetraethylammonium chloride and the ion-pair reagent in the mobile phase (SDS) and resulting in the shift of the retention time of cephapirin. Tetraethylammonium chloride was, therefore, eliminated from the deproteination step. Instead, analytes were extracted from milk with ACN and the extract was centrifuged to precipitate proteins. By extracting milk twice, analyte recovery was markedly improved.

For LC, gradient analysis was used to elute cephapirin and ceftiofur into chromatographic regions free from endogenous peaks. Chromatography was optimized by using a ternary gradient consisting of MeOH, ACN, and SDS. Gradient elution decreased the retention time for ceftiofur, thus improving the sensitivity of its detection.

The method was validated by using control milk fortified with cephapirin at 10, 20, and 40 ng/mL cephapirin and with ceftiofur at 25, 50, and 100 ng/mL. Results are presented in Table 2.

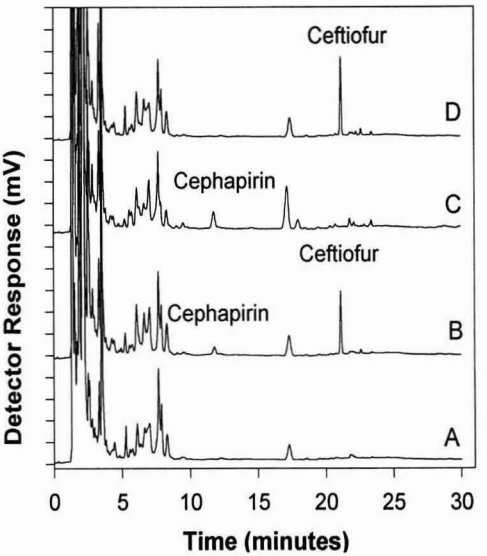

Figure 3. Chromatograms of (A) control milk, (B) control milk fortified with 20 ng cephapirin/mL and 50 ng ceftiofur/mL, (C) incurred milk containing cephapirin, and (D) incurred milk containing ceftiofur.

The method was used to assay milk from cows treated with cephapirin and ceftiofur. Milk was collected twice daily and analyzed until levels needed to validate the method were reached. These data were collected to demonstrate the suitability of the method in analyzing samples from treated animals. This was not meant to be a depletion study or a pharmacokinetics exercise. Multiple replicates (n = 5) of incurred samples were analyzed at time points where levels were within the range of the tolerance or safe level for each drug (Table 3). Incurred samples were found to contain 30 ng cephapirin/mL (intralaboratory coefficient of variation [CV], 3%) and 72 ng ceftiofur/mL (intralaboratory CV, 9%). No problems were encountered with the analyses. Typical chromatograms of control milk, milk fortified with cephapirin and ceftiofur, and incurred milk samples containing cephapirin and ceftiofur are shown in Figure 3. To properly characterize low-level performance, the limit of detection (LOD) and limit of quantitation (LOQ) for each drug were calculated as defined in the U.S. Pharmacopeia–National Formulary (10). Average background signal responses were measured for 5 control milk extracts in the retention time windows of both cephapirin and ceftiofur. LOD (average + 3 times standard deviation) and LOQ (average + 10 times standard deviation) were estimated to be 1 and 2.5 ng/mL, respectively, for cephapirin and 2.5 and 4 ng/mL, respectively, for ceftiofur.

To demonstrate method selectivity, other approved veterinary drugs, including tetracyclines, amphoteric β-lactams, sulfonamides, aminoglycosides, macrolide antibiotics, corticosteroids, and diuretics, were chromatographed. The drugs tested did not interfere with the analysis. Milk obtained from other geographic areas was evaluated to eliminate the possibility of

interference from endogenous peaks due to different animal husbandry practices. No peaks were observed in the chromatographic windows of interest from these regional milk samples.

This method is simple, rapid, and direct. In a normal working day, a single analyst can prepare 8–10 samples. The method is highly practical for use in a regulatory environment.

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Delvotest SP for Detection of Cloxacillin and Sulfamethoxazole in Milk: IDF Interlaboratory Study

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Under the auspices of the International Dairy Federation's Group E503, a collaborative study of the Delvotest SP multiplate microbial inhibitor test was performed to gain information about the detection limits of 2 antimicrobial agents (a β-lactam and a sulfa compound), the variation of test results between users and 2 batches of the test, and the reasons for deviating results. Lyophilized milk samples spiked with various concentrations of cloxacillin or sulfamethoxazole were analyzed. Each substance/concentration combination was tested with each of 2 test batches 14 or 15 times per participating laboratory. Test results were to be read by more than one person and reported on separate forms. Results were obtained from 29 laboratories, which included 5 with no experience and 11 with limited experience with this test. Detection limits for cloxacillin (22.5 or 30 μg/kg, depending on batch) and sulfamethoxazole (45 μg/kg) were established from dose–response curves. A small difference in cloxacillin detection levels between the 2 test batches was observed. Analyses of samples gave almost unanimous results (≥95%). Of deviating results, defined as anomalous results (1.4% of readings), half could be attributed to human errors and half to procedural errors.

Demands on tests to detect antibacterial compounds in milk have changed markedly during recent decades (1). Milk factories began testing for antimicrobial compounds to prevent contaminated milk from entering the factory. In the early 1960s, attention was drawn to toxic and allergenic residues of antimicrobial drugs. Today, maximum residue limits (MRLs), established by the Codex Committee on Residues of Veterinary Drugs in Food or according to European Union Regulation 2377/90 (2) or safe/tolerance levels (as in the United States and Canada), are fixed for numerous antimicrobials. Because the incidence of antimicrobial residues in milk in most areas is less than 1%, there is a need for easy, inexpensive tests to detect a broad spectrum of antimicrobials for screening purposes.

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Microbial inhibitor tests play an important role as screening methods. Within the integrated detection system of the International Dairy Federation (IDF), milk samples are screened for example by microbial inhibitor tests, and positive samples in relation to subsequent requirements—such as quality payment at the farm level, self control in the dairies according to article 14 of European Union Directive 92/46 (3), and food inspection—are analyzed further by more sophisticated methods for identification and quantitation of contaminant(s) (4).

Under the pressure of an increasing number of antimicrobials with fixed MRLs or comparable limits, numerous microbiological inhibitor tests with sufficient detection sensitivities of as many antimicrobials as possible are or will be developed or modified. To make it possible to compare different methods with respect to their suitability for certain field(s) of application, IDF Group E503 "Antibiotics" prepared a guidance document for evaluation of microbiological inhibitor tests, which forms the basis for an expert's opinion on applicability for intended use (5). IDF Group E503 "Antibiotics" tested the guidance with emphasis on item collaborative study. Included in this study were one microbial inhibitor test (Delvotest SP multiplate; Gist-Brocades, Delft, The Netherlands) and 2 antimicrobials (cloxacillin and sulfamethoxazole).

Earlier intercomparison tests organized by that group, whose aim was to obtain insight into the actual "state of proficiency" of routinely applied tests and/or laboratories, led to the conclusion that results obtained by various participants with the same method differed considerably (6). The trial was performed with a strict protocol to gain information about the detection limits of 2 antimicrobials by one test kit, the variation of test results between participating laboratories and/or between 2 batches of the test kit, and the reasons for deviating or anomalous results.

Experimental Design

Detection of cloxacillin and sulfamethoxazole in spiked milk samples by 2 different batches of Delvotest SP (batch H and J, 8 and 16 weeks old at the time of shipment) was investigated. Test samples were prepared in one laboratory and pre-

served by lyophilization. The study included a pretest to check incubation conditions and the collaborative study with spiked milk samples. To obtain a sufficient basis for analysis of potential problems with the test, participants were asked to have test results read by different persons and to report individual results. For evaluation of the collaborative study, only one randomly chosen report from each laboratory was included.

Participants

Thirty laboratories from 17 countries participated.

Time Schedule

Shipment of insulated parcels with ice bags by courier took 1–2 days in most cases. Participants were advised to store test samples, control samples, and test kits in a refrigerator after arrival and until use. Analyses had to be performed within 3 weeks of dispatch.

Content of Parcels

Each parcel contained 24 test samples (*see Test Samples*); 2 control samples (negative and positive with 4 μg penicillin G/kg); Delvotest SP kit with 2 test plates each of 2 batches (J and H), nutrient tablets, and a tablet dispenser (in addition, one extra plate of batch J was included for the pretest); questionnaire; protocols for performing the Delvotest SP multiplate test, the pretest, and the collaborative study; and schemes for test results and control samples.

Test Samples

Test samples were coded from 1 to 24 at random (Table 1). Control samples were prepared according to Section 3.1 of IDF guidelines (5). Commingled milk from 7 cows of the experimental herd of Bundesanstalt fur Milchforschung (Federal Dairy Research Center), Kiel, Germany, was spiked with aqueous solutions with different concentrations of cloxacillin (Sigma Cat. No. C-9393; Deisenhof, Germany) and sulfamethoxazole (Sigma Cat. No. S-7507). Test and control samples had to be reconstituted by adding exactly 2.0 mL distilled water, thoroughly mixing, and warming in a water bath (5 min/50°C) if necessary. Reconstitution had to be performed on the day of analysis, and storage time of reconstituted samples in the refrigerator had to be ≤4 h before analysis.

Principle of Delvotest SP

Samples to be examined and nutrient tablets are added to wells of test plate. Each well contains the test microorganism *Bacillus stearothermophilus* and a pH indicator (bromcresol purple) in agar. During incubation, microbial metabolism will result in a change in color of the indicator from purple to yellow. If substances are present in the sample that are inhibitory to the test microorganism, the color remains purple.

METHOD

Materials and Apparatus

In addition to test materials contained in the kit, the following devices are required:

Table 1. Codes and concentrations of test samples

Substance	Concentration, μg/kg	Sample No.
Cloxacillin	0	14/19
	1	6/20
	22.5	3/23
	30	1/15
	37.5	10/11
	45	18/24
Sulfamethoxazole	0	9/13
	12.5	8/21
	25	7/17
	50	5/16
	75	4/22
	100	2/12

(**a**) *Forceps.*

(**b**) *Pipets or syringes.*—Suitable for dispensing 100 μL milk samples.

(**c**) *Water bath or dry incubator.*—Thermostatically controlled at 64° ± 0.5°C. The dry incubator should contain a perforated shelf or a grille to ensure constant heat circulation underneath the whole plate and plates should not be placed on top of each other.

(**d**) *Pipets.*—Suitable for dispensing 2.0 mL.

(**e**) *Water.*—Distilled, for reconstitution of lyophilized samples.

(**f**) *Milk.*—About 200 mL inhibitor-free milk obtained locally for pretest (*see Pretest*).

Precautions: The test is very sensitive to antibiotics, sulfa drugs, and other antimicrobial substances, such as disinfectants and sanitizers. Any contamination with such substances must be avoided during the whole test procedure. Participants were advised to clean working tables and hands thoroughly before starting the test.

Preparatory Steps

Instructions were given to draw off slowly the aluminum foil in which the test tablets are sealed, open the bottle of nutrient tablets after it had adjusted to room temperature and to remove the white silica gel capsule and the foam cushion by means of forceps, load the dispenser included in the test kit with test tablets, add one tablet to each well of the test plate, confirm whether each well contained one tablet, return tablets not required to the bottle, put in the foam cushion, add the silica gel capsule, and close the bottle.

Addition of Milk Samples

To avoid cross-reactions, a clean pipet (tip) was required for each test and control sample. During the pretest, because the same milk sample was used to inoculate all wells of one test plate, only one pipet (tip) was necessary. A 100 μL portion of each sample was added to each well of the test plate according to procedures described in *Pretest* and *Collaborative Study with Spiked Test Samples.*

Incubation

Inoculated test plates were sealed with the adhesive foil included in the test kit. Plates were incubated at 64° ± 0.5°C either floating in a waterbath or standing in a dry incubator. Incubation was continued until the color of the negative control sample changed to yellow-greenish (ca 2.5 h).

Reading of Test Results

Plates were removed from the incubation device. Results were read from underneath immediately after incubation as follows: negative (–), yellow-greenish like the negative control sample or even more decoloration (N); positive (+), completely purple or a slightly muddy purple color (P); and questionable (±), a mixture of greenish-yellow and slightly muddy purple color (Q).

Pretest

The pretest was performed to ensure that the incubation device was working appropriately and to obtain an indication of the required incubation time. Each well of the test plate was inoculated with 100 µL of the negative milk sample obtained locally according to *Reading of Test Results*. The time for the color to change to yellow-greenish (optimal incubation time), which should not exceed 2 h and 45 min, was recorded. When individual wells of the plate gave marked variations in color, the incubator was considered unsuitable.

Collaborative Study with Spiked Test Samples

Instructions described under *Principle of Delvotest SP* were followed. A 100 µL portion of test or control sample was pipetted according to a defined scheme avoiding cross-contamination. Results were recorded as negative (–), questionable (±), or positive (+). If possible, test results were read independently by different persons. The individuals performing the test and reading the results were identified by an internal laboratory code (e.g., by letters A, B; *see Experimental Design*).

Results

Participants

All participants except one returned test results ($n = 29$; 97% of nominated laboratories). One laboratory (laboratory 16) made a mistake when pipetting samples according to the scheme (*see Collaborative Study with Spiked Test Samples*); therefore, fewer data were available from this laboratory for both batches. Because of different combinations of test-performing and test-reading persons, participating laboratories reported 1–5 sets of data (data not presented in detail).

Five laboratories had no experience and 8 had limited experience with Delvotest SP. Of those who used the test regularly, 3 used Delvotest SP ampoules and thus also had no or limited experience with Delvotest multiplates as applied in this interlaboratory study. Participating laboratories used the test mainly for screening purposes.

Pretest

Ten laboratories used a dry incubator. The average incubation time with a dry incubator was 20 min longer than with a waterbath (2 h and 50 min compared with 2 h and 30 min). Within each group, incubation times varied; dry incubator, from 2 h and 15 min to 3 h and 25 min; water bath, from 2 h and 10 min to 2 h and 45 min. Temperature variations were within specified limits.

A number of laboratories reported variations in color across the plate; most of these laboratories used a dry incubator. Although each laboratory provided its own negative control milk to check test conditions, in each case, the same sample was used for the whole plate. Thus, the color should have been uniform. Of 2784 negative control tests (29 laboratories × 96 tests), 2 (0.07%) gave positive results (one each from laboratories 5 and 28).

Interlaboratory Study

In the evaluation of the collaborative study, one set of results from each laboratory was analyzed. The set was chosen at random for those laboratories reporting several readings (Tables 2–5). Problems with sample reconstitution were not reported.

Control Samples

Positive control samples ($n = 398$) gave positive results in every case. One laboratory (laboratory 10) interpreted the results of the negative control sample ($n = 398$) as questionable in 2 cases (0.5%) and as positive in 2 cases (0.5%).

Test Samples

Of 1646 negative milk samples, 22 (1.3%) were reported as questionable and 19 (1.2%) as positive. Results are reported in Tables 2–5 and summarized in Table 6. Dose–response curves for cloxacillin and sulfamethoxazole are shown in Figures 1 and 2, respectively. Detection limits derived from these curves are given in Table 7.

MRLs valid in the European Union were detected in the following percentage of results when positive test results were considered: cloxacillin: batch H, 96.0%; batch J, 100%; total 98.0%; sulfamethoxazole: batch H, 99.8%, batch J, 99.7%, total 99.8%.

Anomalous Results

In this evaluation, all results due to different combinations of persons who performed the tests and sent the test results were included (data are not presented in detail). The large volume of results made it possible to determine the reasons for the limited number of cases where results different from the majority were obtained.

For a number of samples, almost unanimous results were obtained (Table 6). If in ≥95% of readings the same result was reported (defined as the common result), deviating results for the same sample were defined as anomalous.

When the common result of a certain sample was negative, results reported as questionable or positive were considered anomalous. In the same way, when the common result was positive, results reported as negative or questionable were con-

Table 2. Analysis of cloxacillin in test samples by Delvotest SP batch H

	No. of test samples giving indicated results[a] for sample No.[b]																	
	14/19			6/20			3/23			1/15			10/11			18/24		
Lab code	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P
100	14	0	0	14	1	0	0	14	1	0	0	15	0	0	15	0	0	14
200	14	0	0	14	0	0	0	13	2	0	0	15	0	0	15	0	0	14
300	14	0	0	14	1	0	6	8	1	0	0	15	0	0	15	0	0	14
400	14	0	0	15	0	0	0	15	0	0	0	15	0	0	15	0	0	14
500	14	0	0	15	0	0	0	11	4	0	0	15	0	0	15	0	0	14
600	14	0	0	15	0	0	0	9	6	0	0	15	0	0	15	0	0	14
700	14	0	0	15	0	0	0	5	10	0	0	15	0	0	15	0	0	14
800	13	1[c]	0	4	7	4	0	0	15	0	0	15	0	0	15	0	0	14
1000	10	3[c]	1[c]	5	5	5	0	2	13	0	0	15	0	0	15	0	0	14
1100	14	0	0	14	1	0	0	7	8	0	0	15	0	0	15	0	0	14
1200	14	0	0	15	0	0	0	14	1	0	0	15	0	0	15	0	0	14
1300	14	0	0	15	0	0	0	1	14	0	0	15	0	0	15	0	0	14
1400	14	0	0	13	1	1	0	0	15	0	0	15	0	0	15	0	0	14
1500	14	0	0	14	1	0	0	15	0	0	3[c]	12	0	0	15	0	0	14
1600	7	0	0	0	0	7	0	0	7	0	0	8	0	0	8	0	0	7
1700	13	1[c]	0	15	0	0	0	12	3	0	0	13	0	0	15	0	0	14
1800	14	0	0	15	0	0	0	14	1	0	0	15	0	0	15	0	0	14
1900	14	0	0	15	0	0	0	15	0	0	0	15	0	0	15	0	0	14
2000	14	0	0	2	13	0	0	0	15	0	0	15	0	0	15	0	0	14
2100	14	0	0	0	8	7	0	0	15	0	0	15	0	0	15	0	0	14
2200	14	0	0	15	0	0	6	9	0	0	12[c]	3	0	0	15	0	1[c]	13
2300	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2400	14	0	0	1	13	1	0	0	15	0	0	15	0	0	15	0	0	14
2500	14	0	0	15	0	0	0	14	1	0	0	15	0	0	15	0	0	14
2600	13	0	1[c]	14	0	1	6	0	9	1[c]	0	14	0	0	15	0	0	14
2700	12	0	0	14	0	0	2	9	3	0	0	14	0	0	15	0	0	12
2800	13	1[c]	0	9	5	1	0	9	6	0	1[c]	14	0	0	15	0	0	14
2900	14	0	0	13	2	0	1	2	12	0	0	15	0	0	15	0	0	14
3000	14	0	0	2	7	6	0	0	15	0	0	15	0	0	15	0	0	14
Total	389	6[c]	2[c]	312	65	48	21	198	207	1[c]	16[c]	408	0	0	428	0	1[c]	396
%	98	1.5[c]	0.5[c]	73	15	11	5	46	49	0.2[c]	3.8[c]	96	0	0	100	0	0.3[c]	100

[a] N = negative, Q = questionable, P = positive.

[b] Concentrations (μg/kg) of cloxacillin in test samples are 14/19, 0; 6/20, 15; 3/23, 22.5; 1/15, 30; 10/11, 37.5; and 18/24, 45.

[c] Values are anomalous results.

Table 3. Analysis of cloxacillin in test samples by Delvotest SP batch J

	No. of test samples giving indicated results[a] for sample No.[b]																	
	14/19			6/20			3/23			1/15			10/11			18/24		
Lab code	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P
100	14	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	14
200	14	0	0	0	14	1	0	0	15	0	0	15	0	0	15	0	0	14
300	14	0	0	6	6	3	0	0	15	0	0	15	0	0	15	0	0	14
400	14	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	14
500	14	0	0	0	4	11	0	0	15	0	0	15	0	0	15	0	0	14
600	14	0	0	0	9	6	0	0	15	0	0	15	0	0	15	0	0	14
700	12	1[c]	1[c]	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
800	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
1000	7	2[c]	5[c]	1	1	13	0	0	15	0	0	15	0	0	15	0	0	14
1100	14	0	0	0	11	4	0	0	15	0	0	15	0	0	15	0	0	14
1200	14	0	0	0	13	2	0	0	15	0	0	15	0	0	15	0	0	14
1300	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
1400	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
1500	14	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	14
1600	5	0	0	0	0	5	0	0	5	0	0	6	0	0	6	0	0	5
1700	13	1[c]	0	0	12	3	0	0	15	0	0	12	0	0	15	0	0	14
1800	14	0	0	0	14	1	0	0	15	0	0	15	0	0	15	0	0	14
1900	14	0	0	2	13	0	0	0	15	0	0	15	0	0	15	0	0	14
2000	12	2[c]	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2100	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2200	14	0	0	8	7	0	0	0	15	0	0	15	0	0	15	0	0	14
2300	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2400	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2500	14	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	14
2600	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2700	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
2800	14	0	0	10	5	0	0	3[c]	12	0	0	15	0	0	15	0	0	14
2900	14	0	0	0	4	11	0	0	15	0	0	15	0	0	15	0	0	14
3000	13	1[c]	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	14
Total	384	7[c]	6[c]	27	173	225	0	3[c]	422	0	0	423	0	0	426	0	0	397
%	97	1.8[c]	1.5[c]	6	41	53	0	1[c]	99	0	0	100	0	0	100	0	0	100

[a] N = negative, Q = questionable, P = positive.
[b] Concentrations (μg/kg) of cloxacillin in test samples are 14/19, 0; 6/20, 15; 3/23, 22.5; 1/15, 30; 10/11, 37.5; and 18/24, 45.
[c] Values are anomalous results.

Table 4. Analysis of sulfamethoxazole in test samples by Delvotest SP batch H

	No. of test samples giving indicated results[a] for sample No.[b]																	
	9/13			8/21			7/17			5/16			4/22			2/12		
Lab code	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P
100	15	0	0	1	14	0	0	0	15	0	0	15	0	0	15	0	0	15
200	15	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	15
300	15	0	0	4	11	0	0	0	15	0	0	15	0	0	15	0	0	15
400	15	0	0	0	15	0	0	1	14	0	0	15	0	0	15	0	0	15
500	15	0	0	0	11	4	0	1	14	0	0	15	0	0	15	0	0	15
600	15	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	15
700	15	0	0	0	13	2	0	1	14	0	0	15	0	0	15	0	0	15
800	15	0	0	0	7	8	0	0	15	0	0	15	0	0	15	0	0	15
1000	13	2[c]	0	3	1	11	0	0	15	0	0	15	0	0	15	0	1[c]	14
1100	15	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	15
1200	15	0	0	0	15	0	0	0	15	0	0	15	0	0	14	0	0	15
1300	15	0	0	0	11	4	0	0	15	0	0	15	0	0	15	0	0	15
1400	15	0	0	0	1	14	0	0	15	0	0	15	0	0	15	0	0	15
1500	15	0	0	7	8	0	0	15	0	0	1	14	0	0	15	0	0	15
1600	8	0	0	0	7	0	0	1	7	0	0	8	0	0	7	0	0	8
1700	14	1[c]	0	9	6	0	1	9	5	0	0	15	0	0	15	0	0	15
1800	12	0	3[c]	0	15	0	0	0	15	0	0	15	0	1[c]	14	0	0	15
1900	14	1[c]	0	13	2	0	0	15	0	0	0	15	0	0	15	0	0	15
2000	15	0	0	0	13	2	0	6	9	0	0	15	0	0	15	0	0	15
2100	15	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
2200	15	0	0	15	0	0	6	9	0	0	6[c]	9	0	0	15	0	0	15
2300	15	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
2400	15	0	0	0	2	13	0	0	15	0	0	15	0	0	15	0	0	15
2500	15	0	0	0	15	0	0	2	13	0	0	15	0	0	15	0	0	15
2600	15	0	0	4	0	11	0	0	15	0	0	15	0	0	15	0	0	15
2700	14	0	0	11	3	0	4	8	2	0	0	14	0	0	14	0	0	15
2800	14	1[c]	0	2	12	1	0	6	9	0	1[c]	14	0	0	15	0	0	15
2900	15	0	0	14	1	0	0	5	10	0	0	15	0	0	15	0	0	15
3000	14	1[c]	0	0	1	14	0	0	15	0	0	15	0	0	15	0	0	15
Total	418	6[c]	3[c]	83	229	114	11	79	337	0	8[c]	419	0	1[c]	424	0	1[c]	427
%	98	1.4[c]	0.7[c]	19	54	27	3	19	79	0	2[c]	98	0	0.2[c]	100	0	0.2[c]	100

[a] N = negative, Q = questionable, P = positive.
[b] Concentrations (μg/kg) of sulfamethoxazole in test samples are: 9/13, 0; 8/21, 12.5; 7/17, 25; 5/16, 50; 4/22, 75; and 2/12, 100.
[c] Values are anomalous results.

Table 5. Analysis of sulfamethoxazole in test samples by Delvotest SP batch J

	No. of test samples giving indicated results[a] for sample No.[b]																	
	9/13			8/21			7/17			5/16			4/22			2/12		
Lab code	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P	N	Q	P
100	15	0	0	0	15	0	0	2	13	0	0	15	0	0	15	0	0	15
200	15	0	0	0	15	0	0	4	11	0	0	15	0	0	15	0	0	15
300	15	0	0	7	7	1	0	0	15	0	0	15	0	0	15	0	0	15
400	15	0	0	0	15	0	0	5	10	0	0	15	0	0	15	0	0	15
500	15	0	0	0	2	13	0	0	15	0	0	15	0	0	15	0	0	15
600	15	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	15
700	13	1[c]	1[c]	0	5	10	0	0	15	0	0	15	0	0	15	0	0	15
800	15	0	0	0	2	13	0	0	15	0	0	15	0	0	15	0	0	15
1000	8	2[c]	5[c]	1	2	12	0	0	15	0	0	15	0	0	15	0	0	15
1100	15	0	0	0	13	2	0	1	14	0	0	15	0	0	15	0	0	15
1200	15	0	0	0	15	0	0	0	15	0	0	15	0	0	15	0	0	15
1300	15	0	0	0	4	11	0	0	15	0	0	15	0	0	15	0	0	15
1400	15	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
1500	15	0	0	8	7	0	0	14	1	0	5[c]	10	0	0	15	0	0	15
1600	6	0	0	0	4	1	0	1	5	0	0	6	0	0	5	0	0	6
1700	15	0	0	3	12	0	0	7	8	0	0	15	0	0	15	0	0	15
1800	14	0	1[c]	0	15	0	0	1	14	0	0	15	0	0	15	0	0	15
1900	15	0	0	2	13	0	0	14	1	0	0	15	0	0	15	0	0	15
2000	15	0	0	0	5	10	0	0	15	0	0	15	0	0	15	0	0	15
2100	15	0	0	0	7	8	0	8	7	0	0	15	0	0	15	0	0	15
2200	15	0	0	5	10	0	1	12	2	0	0	15	0	0	15	0	0	15
2300	15	0	0	1	0	14	0	0	15	0	0	15	0	0	15	0	0	15
2400	15	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
2500	15	0	0	0	15	0	0	3	12	0	0	15	0	0	15	0	0	15
2600	14	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
2700	14	0	1[c]	2	7	6	0	0	15	0	0	15	0	0	15	0	0	15
2800	15	0	0	9	6	0	0	14	1	0	1[c]	14	0	1[c]	14	0	1[c]	14
2900	15	0	0	0	5	10	0	0	15	0	0	15	0	0	15	0	0	15
3000	15	0	0	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15
Total	414	3[c]	8[c]	38	216	171	1	86	339	0	6[c]	420	0	1[c]	424	0	1[c]	425
%	97	0.7[c]	1.9[c]	9	51	40	0	20	80	0	1[c]	99	0	0.2[c]	100	0	0.2[c]	100

[a] N = negative, Q = questionable, P = positive.

[b] Concentrations (μg/kg) of sulfamethoxazole in test samples are: 9/13, 0; 8/21, 12.5; 7/17, 25; 5/16, 50; 4/22, 75; and 2/12, 100.

[c] Values are anomalous results.

Table 6. Summary of results

			Samples giving indicated result with			
			Batch H		Batch J	
No. of samples tested[a]	Concentration, µg/kg	Result[b]	*n*	%	*n*	%
			Cloxacillin			
397/397	0	N	389	98.0	384	96.7
		Q	6	1.5	7	1.8
		P	2	0.5	6	1.5
425/425	15.0	N	312	73.4	27	6.4
		Q	65	15.3	173	40.7
		P	48	11.3	225	52.9
426/425	22.5	N	21	4.9	0	0
		Q	198	46.5	3	0.7
		P	207	48.6	422	99.3
425/423	30.0	N	1	0.2	0	0
		Q	16	3.8	0	0
		P	408	96.0	423	100
428/426	37.5	N	0	0	0	0
		Q	0	0	0	0
		P	428	100	426	100
397/397	45.0	N	0	0	0	0
		Q	1	0.3	0	0
		P	396	99.8	397	100
			Sulfamethoxazole			
427/425	0	N	448	97.9	414	97.4
		Q	6	1.4	3	0.7
		P	3	0.7	8	1.9
426/425	12.5	N	83	19.5	38	8.9
		Q	229	53.8	216	50.8
		P	114	26.81	71	40.2
427/426	25.0	N	11	2.6	1	0.2
		Q	79	18.5	86	20.2
		P	337	78.9	339	79.6
427/426	50.0	N	0	0	0	0
		Q	8	1.9	6	1.4
		P	419	98.1	420	98.6
425/425	75.0	N	0	0	0	0
		Q	1	0.2	1	0.2
		P	424	99.8	424	99.8
428/426	100.0	N	0	0	0	0
		Q	1	0.2	1	0.2
		P	427	99.8	425	99.8

[a] Values are for batch H/batch J.
[b] N = negative, Q = questionable, P = positive.

sidered anomalous. Analysis of anomalous results is summarized in Table 8.

Reading of the test result by more than one person in 21 participating laboratories made it possible to distinguish between human and procedural errors: If a certain test was read differently by only one person in a laboratory only, it may be considered a human error (reading or recording results). If the interpretations of the test by several persons in a laboratory were the same, there was reason to suspect a procedural error (mistakes in sample inoculation, differences between tests within batch, or differences in temperature due to unequal heating).

Among 20 999 results, 245 (1.2%) were anomalous. They can be ascribed in almost equal proportions to procedural and human errors (51.4 and 48.6%, respectively).

Anomalous results with the negative control sample (4 positive and 6 questionable) came from 2 laboratories (laboratories 10 and 20). Of these, all positive and 3 of 6 questionable results were considered as procedural errors. No

Table 7. Detection limits of cloxacillin and sulfamethoxazole based on dose–response curves for 2 batches of Delvotest SP

Substance	Detection limit, µg/kg	
	Batch H	Batch J
95% Positive results		
Cloxacillin	30.0	22.5
Sulfamethoxazole	45.0	45.0
95% Positive + questionable results		
Cloxacillin	22.5	17.5
Sulfamethoxazole	22.5	17.5

negative results were reported with the control sample (4 µg penicillin G/kg).

Table 9 presents the anomalous results reported by 21 of 29 laboratories. Remarkably, the 4 laboratories with the highest percentage of anomalous results used dry incubators.

Comparison of anomalous results with the incubation device and the Delvotest experiences of the laboratories are summarized in Tables 10 and 11, respectively.

No obvious relationship was found between the percentage of anomalous results and the positions on the test plates or the laboratory's experience with Delvotest SP multiplates.

Discussion and Conclusions

Earlier intercomparison tests were organized by the IDG Group E503 "Antibiotics" to obtain insight into the actual

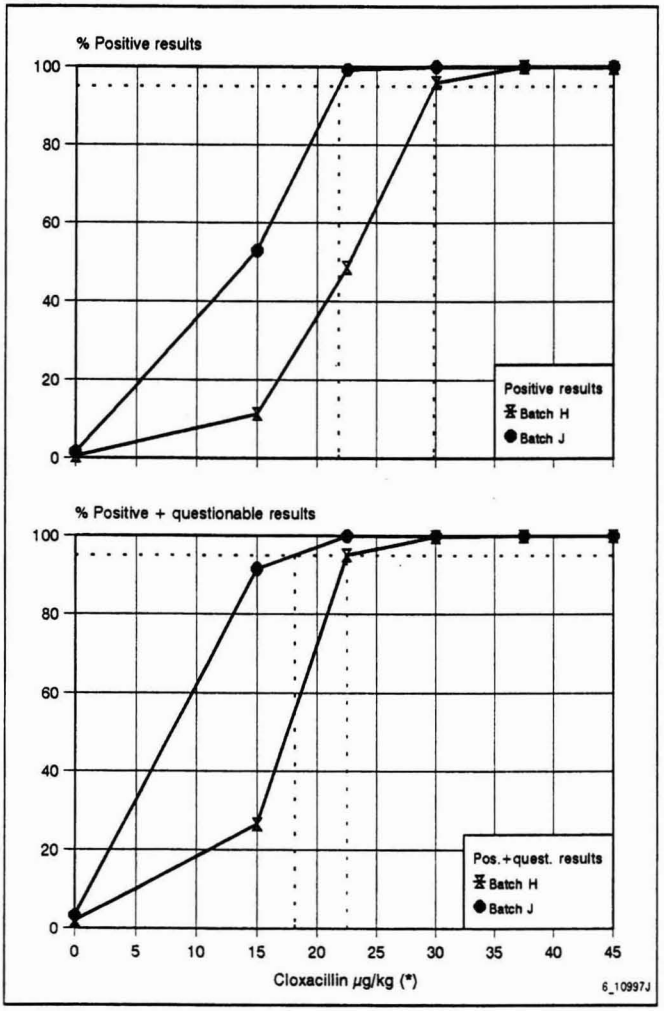

Figure 1. Dose–response curves for determination of cloxacillin detection limits by Delvotest SP batches H and J. (*) n = 397–428 per concentration and batch.

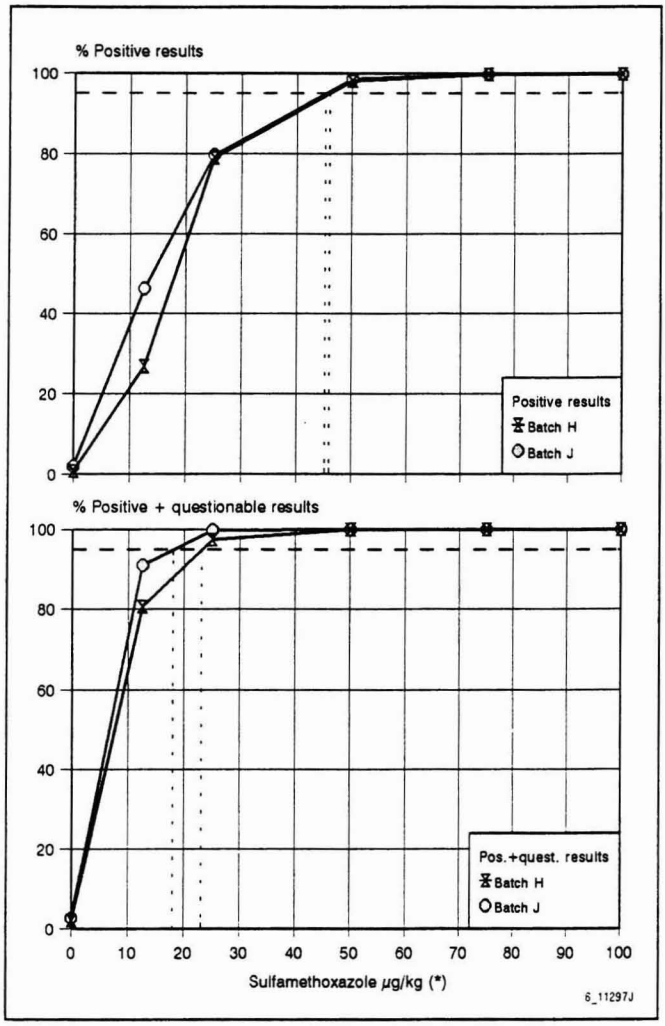

Figure 2. Dose–response curves for determination of sulfamethoxazole detection limits by Delvotest SP batches H and J. (*) n = 425–428 per concentration and batch.

"state of proficiency" of routinely applied tests and/or laboratories. Those earlier studies suggest that results obtained with the same method differed considerably (8). The present trial was performed with a strict protocol to gain information about the detection limits of 2 antimicrobial agents by one test kit, the variation of test results between participating laboratories and/or between 2 batches of the test kit, and the reasons for deviating results.

Results were obtained from 29 of 30 participants. Five laboratories had no experience with Delvotest plates, 11 had limited experience, and 13 were regular users. Ten laboratories used a dry incubator, and 19 used a water bath. On average, use of a dry incubator required 20 min longer incubation time compared with use of a water bath, because of slower warming of the plates in a dry incubator than in a water bath.

In the pretest, the whole test plate was inoculated with one locally obtained negative milk sample. Some laboratories reported variations in color for the pretest; most of those laboratories used a dry incubator. The nonuniform color of the test plate could be due to temperature fluctuations across the plate during incubation. Two of 2784 (0.07%) pretest results were positive.

Among 1646 negative milk samples, 1.3% were reported as false questionable and 1.2% as false positive. About half of these deviating results were reported by one participating laboratory (laboratory 10). Because of the interpretation of the results of the control samples, problems with the test procedure cannot be excluded. The percentage of false positives in this study was much lower than that reported by Zomer and Lieu (6), who found 7% false positives with Delvotest SP in a collaborative study with 5 participating laboratories.

Table 8. Analysis of anomalous results of samples with ≥95% identical test results (common result) (control samples, n = 901; test samples, n = 1802–1984)

Sample	Concn, µg/kg	Samples giving common results, %	Samples giving anomalous results: Negative n	Negative %	Questionable n	Questionable %	Positive n	Positive %
Common result: negative								
Negative control		98.9			6	0.7	4	0.4
Cloxacillin	0	96.8			36	2.0	21	1.2
Sulfamethoxazole	0	91.1			29	1.5	26	1.3
Common result: positive								
Positive control		100	0	0	0	0		
Cloxacillin	22.5	95.7[a]	0	0	41	2.1		
	30	98.4	5	0.3	25	1.3		
	37.5	99.9	2	0.1	0	0		
	45	99.7	3	0.2	2	0.1		
Sulfamethoxazole	50	98.4	0	0	30	1.5		
	75	99.5	1	<0.1	8	0.4		
	100	99.8	1	<0.1	3	0.2		

[a] Batch J only.

Detection limits for cloxacillin and sulfamethoxazole were established from dose–response curves. These were prepared on the basis of 397–428 results per substance/concentration combination. The experimental design made it possible to detect a small difference in the detection limits of the 2 test kit batches. At least 95% positive results were indicated at the following substance/concentration combinations: cloxacillin, 22.5–30.0 µg/kg, depending on the batch; sulfamethoxazole, 45 µg/kg.

Detection of cloxacillin at the European Union MRL agrees with the finding of McGrane et al. (7). Zomer and Lieu (6)

Table 9. Analysis of anomalous results within laboratories

Laboratory	Incubator type[a]	N	Anomalous results n	Anomalous results %	Human error	Procedural error
10	D	558	47	8.4	13	34
22	D	279	19	6.8	0	19
28	D	1395	57	4.1	25	32
15	D	279	9	3.2	0	9
18	W	558	15	2.7	15	0
26	D	833	19	2.3	12	7
7	W	1116	17	1.5	1	16
24	D	1395	18	1.3	10	8
20	W	837	9	1.1	9	0
19	W	837	9	1.1	9	0
2	W	1116	6	0.5	6	0
17	W	548	3	0.5	3	0
30	D	558	3	0.5	1	2
11	W	558	2	0.4	2	0
8	W	279	1	0.4	1	0
27	D	271	1	0.4	1	0
12	W	684	2	0.3	2	0
3	W	1116	3	0.3	3	0
5	W	558	1	0.2	1	0
13	W	558	1	0.2	1	0
25	D	558	1	0.2	1	0

[a] W, water bath; D, dry incubator; N, total number of results reported by the laboratory.

Table 10. Analysis of anomalous results with respect to incubator type

Incubator	N[a]	Anomalous results		Procedural error		Human error	
		n	%[b]	*n*	%[b]	*n*	%[b]
Dry incubator	6684	174	2.6	111	63.8	63	36.2
Water bath	11135	69	0.6	16	23.2	53	76.8
Total	17819	243	1.4	127	52.2	116	47.8

[a] N = total number of results reported for the indicated incubation device.
[b] Percent of total results.

reported only 25% positive results for a collaborative study with 5 participating laboratories.

It may be concluded that (*1*) the trial design made it possible to ascertain small differences in detection limits, (*2*) batch J is more sensitive than batch H for detecting cloxacillin when only positive results are considered, (*3*) the difference in cloxacillin detection limits between the 2 test kit batches is less pronounced when positive and questionable results are considered, and (*4*) there is no difference between batches in sulfamethoxazole detection limits when only positive results are considered, but a small difference exists when questionable results also are included.

For most samples, almost unanimous results (≥95%) were reported. Results deviating from these common results were regarded as anomalous. From the data of 21 participating laboratories reporting more than one set of results, the following conclusions concerning the reasons for nonconformity could be drawn: 48.6% of anomalous results could be attributed to human errors, such as errors in reading or recording the results, and 51.4% to procedural errors, such as mistakes in sample inoculation, differences between wells within a batch, or differences in temperature during incubation. Most (87%) procedural errors were from laboratories using a dry incubator, whereas human errors came in nearly equal proportions from laboratories using a dry incubator and a water bath (54 and 46%, respectively). There was no obvious relationship between anomalous results and the position of the test plates or with the laboratory's experience in using Delvotest SP multiplates.

The procedure described in the IDF's *Guidance for the Standardized Description of Microbial Inhibitor Tests* (5) provides a sound basis for evaluating the performance of a microbial inhibitor test by an interlaboratory study.

Table 11. Analysis of anomalous results with respect to the laboratory's experience

Experience	No. of laboratories, *n*	Anomalous results, %	
		Range	Mean
First-time user	5	0.4–2.3	1.0
Limited experience	10	0–8.4	1.6
Regular user	14	0–6.8	1.3

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FOOD CHEMICAL CONTAMINANTS

Liquid Chromatographic Determination of Histamine in Fish, Sauerkraut, and Wine: Interlaboratory Study

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An interlaboratory study of the liquid chromatographic (LC) determination of histamine in fish, sauerkraut, and wine was conducted. Diminuted and homogenized samples were suspended in water followed by clarification of extracts with perchloric acid, filtration, and dilution with water. After LC separation on a reversed-phase C_{18} column with phosphate buffer (pH 3.0)–acetonitrile (875 + 125, v/v) as mobile phase, histamine was measured fluorometrically (excitation, 340 nm; emission, 455 nm) in samples and standards after postcolumn derivatization with *o*-phthaldialdehyde (OPA). Fourteen samples (including 6 blind duplicates and 1 split level) containing histamine at about 10–400 mg/kg or mg/L were analyzed singly according to the proposed procedure by 11 laboratories. Results from one participant were excluded from statistical analysis. For all samples analyzed, repeatability relative standard deviations varied from 2.1 to 5.6%, and reproducibility relative standard deviations ranged from 2.2 to 7.1%. Average recoveries of histamine for this concentration range varied from 94 to 100%.

Biogenic amines originate from decarboxylation of specific free amino acids in foods and food products. This decarboxylation may be related to fermentation processes involved in food production or to deterioration and spoilage by microorganisms. Histamine, a biogenic amine, is formed by decarboxylation of the amino acid histidine. Histamine is found in high levels in protein-rich or fermented foods, marine products like scombroid fish, meat and meat products, wines, alcoholic beverages, sauerkraut, cheese, etc. Among biogenic amines, histamine is considered as the principal toxic agent causing histamine poisoning syndrome, although other biogenic amines possibly potentiate histamine toxicity. The possible toxic effects on individuals of excessive levels of histamine in foods have been reviewed extensively.

Food legislators, consumers, and manufacturers have a common interest in monitoring levels of histamine in foods. For this reason the European Union (EU) has set maximum tolerance levels for histamine contents of *Scombridae*, *Clupeidae*, *Engraulidae*, and *Coryhaenidae* at 400 mg/kg (1–8). According to an EU directive, the mean histamine content in a group of 9 samples of fresh fish or canned fish should not exceed 100 mg/kg. Two of 9 samples may have levels between 100 and 200 mg/kg, but no sample may contain more than 200 mg histamine/kg. For fermented fish, these tolerance levels are doubled to 200 and 400 mg/kg.

By now, all EU member states have implemented this directive in their national food regulations. Various analytical methods have been published to determine histamine in food and food products, including capillary electrophoresis (4, 5), continuous flow analysis (6), enzyme-linked immunosorbent assay (7, 8), enzymatic analysis (9), spectrofluorometric (10–14), and liquid chromatographic (LC) methods (15–24). The laboratories of the Inspectorate for Health Protection, Food Inspection Services in The Netherlands use LC methods to monitor various foods for histamine.

It was decided to validate a recommended LC method for histamine through an interlaboratory study. The method and the validation results of this study are presented in this paper.

Interlaboratory Study

Study Design

The Project Group of Collaborative Studies (PCS), who organized this study, decided to test the method with 14 samples consisting of white wine, sauerkraut, mackerel, and anchovy having levels of interest ranging from about 10 to 400 mg his-

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Table 1. Recovery of histamine from spiked samples

Sample	Histamine, mg/kg[a] or mg/L[b] Blank	Amount added	Amount found	Recovery, %
Wine	0	10.3	10.3	100.0
Wine	0	25.8	25.0	96.9
Sauerkraut	50.9	0		
Sauerkraut	50.9	5.2	55.8	94.2
Mackerel	4.0	100.2	102.5	98.3
Mackerel	4.0	200.4	203.5	99.6
Anchovy	4.0	199.9	198.0	97.0
Anchovy	4.0	401.0	402.0	99.3

[a] For fish.
[b] For wine and sauerkraut juice.

tamine/kg or L: 2 pairs of blind duplicates of white wine (2 levels; range, about 10–25 mg/L), 2 pairs of blind duplicates of mackerel (2 levels; range, about 100–200 mg/kg), 2 pairs of blind duplicates of anchovy (2 levels; range, about 200–400 mg/kg), and 1 pair split level of sauerkraut (1 level; about 50 mg histamine/L).

Preliminary Study

Method instructions were sent to participating laboratories in advance to allow the person conducting the test to become familiar with the procedure. A few months before the study, participants received 2 training samples consisting of a standard solution and mackerel. The samples had histamine contents of about 100 and 200 mg/kg, respectively. The training samples were analyzed to establish if the method is suitable for interlaboratory testing.

Participants were requested to analyze each sample 2 times in succession starting from different test portions and to send results and comments on the method to the study organizer.

Preparation of Samples

White wine, sauerkraut, and fish samples were collected from routine survey. Fish samples (mackerel fillets and fresh anchovies) were chopped with a food cutter and homogenized in a cooled commercial-size mixer. The sauerkraut sample was pressed, and the juice was collected and mixed. The wine sample was obtained by mixing several bottles of white wine. Immediately after being homogenized, each sample was divided into 2 batches. After blank values were determined, the 8 sample batches were spiked with histamine·2HCl as specified in Table 1 and homogenized. Then, portions (about 16 g for fish and 50 mL for wine and sauerkraut juice) from each batch were transferred into plastic air-tight containers, identified by code numbers, and stored at <–18°C. Before shipment of samples, their homogeneities were verified by taking 10 test samples at

Table 2. Collaborative results for histamine (mg/kg) in 2 training samples

Collaborator	Mackerel 1	Mackerel 2	Mackerel Mean	Standard solution 1	Standard solution 2	Standard solution Mean
1	208	226	217.0	101.8	100.9	101.4
3	212	215	213.5	100.7	102.2	101.4
4	226	227	226.5	114.0[a]	103.8[a]	108.9
5	219	212	215.5	97.2	95.9	96.6
6	213	222	217.5	96.9	98.6	97.8
7	214	206	210.0	100.9	100.1	100.5
8	224	230	227.0	105.5	104.8	105.2
9	202	208	205.0	98.6	96.6	97.6
10	201	197	199.0	102.1	101.3	101.7
11	188	209	198.5	110.9	108.6	109.8
12	223	230	226.5	98.0	93.9	96.0

Statistical analysis

Parameter	Value for mackerel	Value for standard solution
No. of laboratories	11	11
No. of results	22	22
No. of accepted results	22	20
Mean, mg/kg	214	100.8
s_r, mg/kg	7.1	1.3
RSD_r, %	3.3	1.3
s_R, mg/kg	11	4.3
RSD_R, %	5.4	4.3
r, mg/kg	19.9	3.7
R, mg/kg	32	12.2

[a] Laboratory marked as outlier by the Cochran outlier test ($P = 99\%$).

Table 3. Collaborative results for histamine (mg/L) in wine and sauerkraut

	Wine level 1		Wine level 2		Sauerkraut level 3	
Collaborator	F	I	G	H	J	E
1	10.0	10.6	24.9	24.7	57.8	52.0
2	9.7	8.9	22.4	21.8	50.6	49.2
3	10.4	10.5	25.5	28.3	55.9	53.6
4	10.9	10.2	26.4	26.1	58.6	53.5
5	3.9[a]	3.8[a]	14.7[a]	15.4[a]	45.5[a]	40.9[a]
6	10.8	10.8	25.6	25.5	58.1	54.1
7	9.7	11.2	24.6	25.4	57.1	52.3
8	10.6	11.2	27.0	28.5	56.7	53.5
9	10.7	9.1	24.6	24.4	54.2	52.8
10	10.0	10.0	23.3	24.0	53.7	47.5
11	10.2	10.2	22.9	24.9	55.6	51.1

[a] Values are outliers by the Grubb's outlier test ($P = 99\%$).

random from each batch and analyzing them for histamine according to the prescribed procedure. The 14 samples (6 pairs of blind duplicates and 1 split level) were mailed by express delivery to each of 11 participating laboratories. Special care was taken to keep samples frozen. Simultaneously, each laboratory received the method under investigation, an instruction form for participants, a sample delivery form, a reporting sheet, and an agreement-to-participate form. Laboratories were instructed to store samples in the freezer at <–18°C and to perform analyses within 8 weeks of receipt of samples. Each laboratory was asked to perform single analysis on each sample and to analyze a reagent blank.

Statistical Calculations

Statistical evaluation of the interlaboratory study data was performed according to the International Union of Pure and Applied Chemistry (IUPAC)/International Organization for Standardization (ISO)/AOAC protocol (25) for a uniform-level and split-level study, as described by Pocklington (26).

METHOD

Principle

Homogenized solid samples are suspended in water. Both sample suspensions and liquid samples are purified by treatment with perchloric acid. After filtration and dilution with water, extracts are analyzed by LC on a reversed-phase C_{18} column with phosphate buffer (pH 3.0)–acetonitrile (875 + 125, v/v) as mobile phase. Histamine (and other primary amines) are quantitated by postcolumn derivatization with *o*-phthaldialdehyde (OPA). Separated histamine peaks are measured fluorometrically (excitation, 340 nm; emission, 455 nm).

Apparatus

Trade names and sources are for user information only.

Table 4. Collaborative results for histamine (mg/kg) in mackerel and anchovy

	Mackerel level 4		Mackerel level 5		Anchovy level 6		Anchovy level 7	
Collaborator	A	N	B	M	C	L	D	K
1	110	106	203	198	209	204	410	418
2	104	106	208	206	210	206	426	412
3	104	102	205	204	205	207	431	423
4	101	109	201	209	198	218	381	412
5	98[a]	99[a]	172[b]	183[b]	180[a]	179[a]	392[a]	411[a]
6	106	105	205	207	214[c]	152[c]	404	400
7	102	108	210	203	192	206	410	421
8	96	100	197	203	189	199	379	408
9	94	96	211	200	171	200	388	408
10	106	106	204	204	185	193	375	380
11	94	97	200	193	196	181	390	372

[a] Values removed by hand because being 4 times an outlier of the 7 levels.
[b] Values are outliers by the Grubb's outlier test ($P = 99\%$).
[c] Values are strangglers by the Cochran outlier test ($P = 95\%$); rejected because of analytical problems.

Table 5. Statistical analysis of collaborative results for histamine (in mg/L or mg/kg) in food samples

	Wine[b]		Sauerkraut[b]	Mackerel[c]		Anchovy[c]	
Parameter[a]	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
No. of collaborators	11	11	11	11	11	11	11
No. of results	22	22	22	22	22	22	22
No. of accepted results	20	20	20	20	20	18	20
Mean	10.29	25.0	53.9	102.6	203.6	198	402
s_r	0.58	0.89	1.2	2.8	4.2	10	12
RSD_r, %	5.6	3.5	2.3	2.7	2.1	5.1	3.0
s_R	0.62	1.8	2.3	5.1	4.6	12	18
RSD_R, %	6.0	7.1	4.3	4.9	2.2	5.9	4.6
Acceptable RSD_R, %	11.3	9.9	8.7	8.0	7.2	7.2	6.5
r	1.62	2.49	3.4	7.8	11.7	29	34
R	1.74	5.0	6.4	14.2	12.8	35	52

[a] s_r and s_R are repeatability and reproducibility standard deviations, respectively; RSD_r and RSD_R are repeatability and reproducibility relative standard deviations, respectively; r and R are repeatability ($s_r \times 2.8$) and reproducibility ($s_R \times 2.8$), respectively.
[b] Values expressed in mg/L.
[c] Values expressed in mg/kg.

(**a**) *pH meter.*—Calibrated; graduated in units of 0.1 pH or less and equipped with combined glass/calomel electrode.

(**b**) *Centrifuge.*—Capable of centrifuging mixtures at 2500 × *g* for 20 min.

(**c**) *Ultra Turrax.*—For grinding solid foods like fish, marine products, meat and meat products, and fermented products.

(**d**) *LC system.*—Consisting of 2 high-pressure pumps capable of delivering 1.0 mL/min, injection system calibrated to deliver 25 µL, column oven installed at 30°C, postcolumn reaction equipment (mixing chamber or capillary mixing T), fluorescence detector capable of wavelength selections for excitation at 340 nm and emission at 455 nm, recorder, and data acquisition system.

Keep column and postcolumn reaction equipment under temperature-controlled conditions, preferably at 30°C, to obtain reproducible and accurate results.

(**e**) *LC analytical column.*—Reversed-phase C_{18}, 250 × 4.6 mm, 5 µm particle size (e.g., Inertsil ODS-2, available from GL Science or equivalent). Equilibrate column by flushing overnight with mobile phase. When column is not in use (less than one week), store column filled with eluant.

Reagents

All reagents should be of analytical quality unless otherwise stated. The water used should be distilled water or water of at least equivalent purity.

(*Caution*: Consult safety data sheets or labels for additional information on safe handling, toxicity, flammability, and explosivity of chemicals used, especially for potassium hydroxide, perchloric acid, acetonitrile, 2-mercaptoethanol, and methanol. Soak all laboratory glassware and plasticware in detergent before washing or disposal. Dispose of waste solvents in appropriate manner compatible with applicable environmental rules and regulations.)

Trade names and sources are for user information only.

(**a**) *Solvents and reagents.*—Acetonitrile (LC grade) and methanol; boric acid, histamine ($C_5H_9N_3 \cdot 2HCl$), 2-mercaptoethanol, perchloric acid (70–72% m/v), phosphoric acid (25% m/v), OPA, potassium dihydrogen phosphate, potassium hydroxide (all available from, for example, E. Merck, Darmstadt, Germany); cadaverine ($C_5H_{14}N_2 \cdot 2HCl$), and tyramine ($C_8H_{11}NO \cdot HCl$) (available from, for example, Sigma, St. Louis, MO); sodium-1-heptane sulfonate ($C_7H_{15}O_3SNa \cdot H_2O$) (e.g., Fluka Chemie AG, Buchs, Switzerland).

(**b**) *Perchloric acid solution.*—6% m/v. Dilute 85 mL perchloric acid with 915 mL water and mix.

(**c**) *Potassium hydroxide solution.*—8 mol/L. Dissolve 44.8 g KOH in ca 50 mL water. Cool to room temperature and dilute to 100 mL with water in a volumetric flask.

(**d**) *Borate buffer, pH 10.0.*—Dissolve 12.3 g boric acid in 800 mL water. Adjust to pH 10.0 with KOH solution (**c**), using pH meter. Dilute to 1000 mL with water and mix. Buffer is stable for 1 year at 4°C.

(**e**) *Postcolumn derivatizing reagent.*—Dissolve 400 mg OPA in 10 mL methanol. Add this solution to 1000 mL borate buffer (**d**). Mix and degas. Add 2 mL 2-mercaptoethanol and mix. This solution is stable for 48 h at 4°C. Store in capped container when in use.

(**f**) *Stock solution.*—100 mg histamine/L, 100 mg cadaverine/L, and 100 mg tyramine/L. Dissolve a mixture of 165.7 mg histamine, 171.0 mg cadaverine, and 126.5 mg tyramine in water. Dilute to 1000 mL in volumetric flask and mix. This solution is stable for 2 months at 4°C.

(**g**) *Reference standard solution.*—Containing histamine, cadaverine, and tyramine, each at 1.0 mg/L. Pipet 10 mL stock solution (**f**) into a 1000 mL volumetric flask and add 25 mL perchloric acid solution (**b**). Dilute to volume with water and mix. Prepare on day of use.

(**h**) *Histamine standard solutions.*—0.5, 1.0, and 1.5 mg histamine/L. Dissolve 165.7 mg histamine in water, dilute to

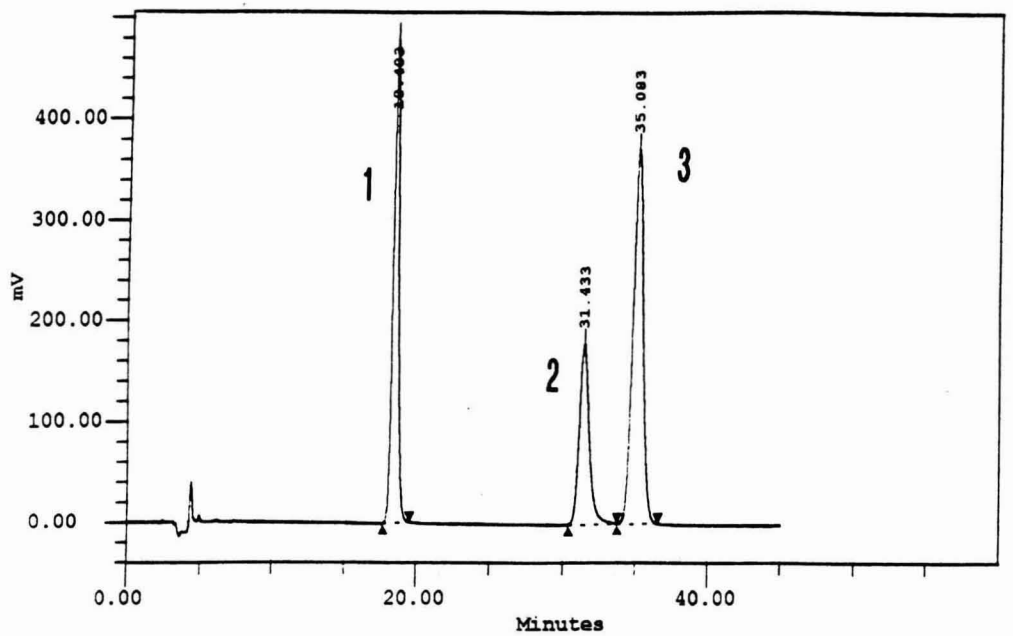

Figure 1. LC separation of tyramine (1; 18.5 min), cadaverine (2; 31.4 min), and histamine (3; 35.1 min) in reference standard solution containing 1 mg of each amine/L.

1000 mL in volumetric flask, and mix. Pipet 5, 10, and 15 mL of this solution into separate 1000 mL volumetric flasks, add 25 mL perchloric acid solution (**b**), and dilute to volume with water. Prepare on day of use.

(**i**) *Phosphate buffer, pH 3.0.*—Dissolve 2.9 g sodium-1-heptane sulfonate and 1.9 g potassium dihydrogen phosphate in 900 mL water. Adjust to pH 3.0 with phosphoric acid, using pH meter. Dilute to 1000 mL with water and mix. Buffer is stable for 2 months at 4°C.

(**j**) *LC mobile phase.*—Dilute 875 mL phosphate buffer (**i**) with 125 mL acetonitrile. Mix and degas solution. Store in capped container when in use. This solution is stable for 2 months at 4°C.

Sample Preparation

Store laboratory samples at <–18°C. Homogenize defrozen samples in suitable mixer. If necessary, keep homogenized samples at ≤–18°C just before analysis.

Procedure

(**a**) *Liquids (wines and alcoholic beverages).*—Pipet 5 mL perchloric acid solution (**b**) in 50 mL volumetric flask. Dilute to mark with sample solution (dilution factor perchloric acid, F_P = 10/9) and mix. Pipet 10 mL of this solution into a 100 mL volumetric flask and dilute to volume with water (dilution factor = F) and mix. Dilute sample solution with water when histamine concentration exceeds 1.25 mg/L (dilution factor = f). Proceed as described in *LC Analysis*, (**b**).

(**b**) *Semiliquids (sauerkraut juice and suspensions).*—Pipet 5 mL perchloric acid solution (**b**) and 5 mL sample solution in a tube, mix, and centrifuge (dilution factor perchloric acid, F_P = 10/5). Pipet 4 mL supernatant into a 100 mL volumetric flask, dilute to volume with water (dilution factor = F), and mix. Dilute sample solution with water when histamine concentration exceeds 1.25mg/L (dilution factor = f). Proceed as described in *LC Analysis*, (**b**).

(**c**) *Solids (marine products, meat and meat products, cheese, and fermented products).*—Accurately weigh ca 10.00 g sample in a 250 mL beaker, add 45 mL water, and homogenize for 1 min. Add 50 mL perchloric acid solution (**b**), homogenize again for 1 min, and filter through fluted filter paper. Pipet 10 mL of this solution into a 100 mL volumetric flask, dilute with water (dilution factor = F), and mix. Dilute sample solution with water when histamine concentration exceeds 1.25mg/L (dilution factor = f). Proceed as described in *LC Analysis*, (**b**).

LC Analysis

(**a**) *System suitability test.*—(*1*) *Retention.*—Run entire LC system to stabilize it. Inject 25 µL reference standard solution (**g**) and chromatograph with mobile phase (**j**) at 1.0 mL/min for at least 40 min. Adjust sensitivity to yield ca 90% FSD (full scale deflection) for histamine peak. Retention times should be ca 19 min for tyramine, 31 min for cadaverine, and 34 min for histamine, depending on brand, age, and condition of column. (*2*) *Resolution.*—After system equilibration, check resolution (R) between cadaverine and histamine by us-

ing reference standard solution. R, calculated as shown below, should not be less than 1.50.

$$R = \frac{2(t_2 - t_1)}{(W_1 + W_2)}$$

where t = elution time of relative maximum of separated peak (mm) and W = width of separated peak at baseline, measured between points of intersection between tangent and baseline (mm). If necessary, adjust acetonitrile concentration of mobile phase or select a more appropriate column. (*3*) *Linearity.*—Check linearity of LC system for concentration range between 0.5 and 1.5 mg histamine/L by injecting 25 µL individual histamine standard solutions (**h**).

(**b**) *Samples and standards.*—Inject 25 µL sample extracts or reference standard solution (**g**) onto analytical LC system. Chromatograph with mobile phase (**j**) at 1.0 mL/min for at least 40 min. Record separated peaks of derivatized histamine from samples and standards and quantitate peak areas by electronic integration.

Calculation

(**a**) *Solids.*—Calculate histamine content of solid samples as follows:

$$\text{Histamine, mg/kg} = \frac{A_{SA}}{A_{ST}} \times C_{ST} \times \frac{100}{m} \times F \times f \times \frac{95 + (W \times \frac{m}{100})}{100}$$

(**b**) *Liquids and semiliquids.*—Calculate histamine content of liquid and semiliquid samples as follows:

$$\text{Histamine, mg/L} = \frac{A_{SA}}{A_{ST}} \times C_{ST} \times F_P \times F \times f$$

For either equation, A_{SA} = peak area of histamine in sample solution, A_{ST} = peak area of histamine in reference standard solution, C_{ST} = concentration of histamine in reference standard solution (mg/L), m = mass of test portion (g), F_P = dilution factor for perchloric acid addition, F = dilution factor, f = additional dilution factor, and W = moisture content of sample (m/m, %). Report results to nearest 1 mg/kg product for solids and to 1 decimal place for liquids and semiliquids.

Notes on Procedure

Tyramine, cadaverine, and histamine are well separated from each other. Putrescine and cadaverine are not separated, and the 2 peaks will coincide. Check linearity of the LC system for samples with high (≥200 mg/kg or mg/L) histamine concentrations. Do not refresh mobile phase or OPA reagent during LC analysis. If necessary, recondition LC system and control linearity and retention times by using standards.

Results and Discussion

Results received from 11 participants for the 2 training samples are listed in Table 2. Participants were asked to analyze these samples in duplicate and to send comments and chromatograms to the study organizer. As a result of this study, it was decided to proceed with the interlaboratory study for histamine.

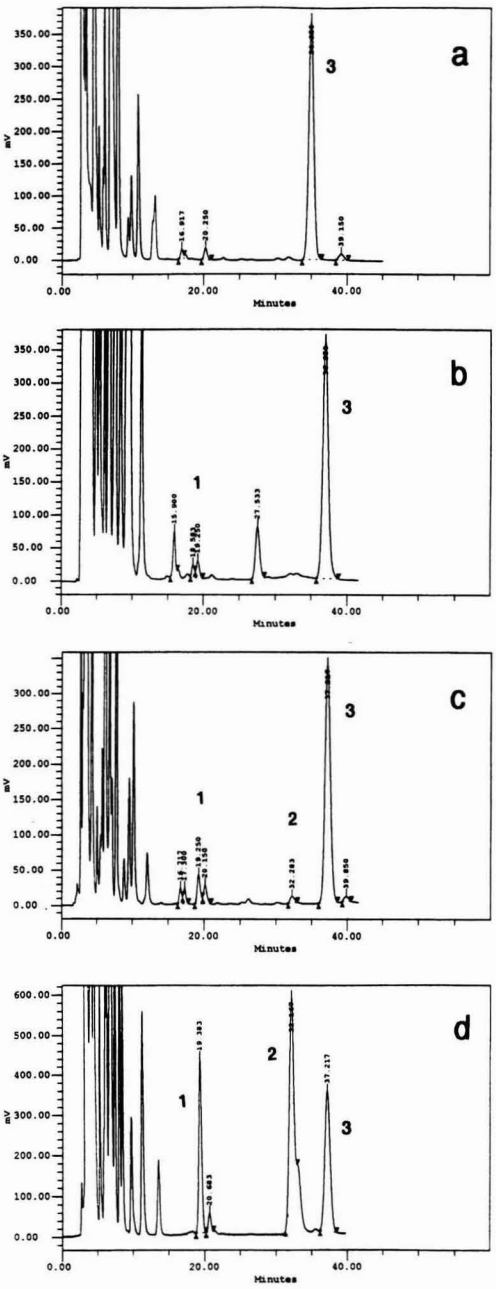

Figure 2. Liquid chromatograms of histamine-containing samples: (a) mackerel (106 mg/kg), (b) anchovy (418 mg/kg), (c) wine (10.5 mg/L), and (d) sauerkraut (50.9 mg/L). Peaks: 1 = tyramine, 2 = cadaverine, and 3 = histamine.

Data collected from participants for collaborative samples are presented in Tables 3 and 4. Results of duplicate samples for each concentration level ($N = 6$ levels) and the split level ($N = 1$ level) were paired for each laboratory and examined for outliers by the Cochran and Grubb's tests at the 1% level of significance. Results of participant 5 were identified as Grubb's outliers for levels 1, 2, 3, and 5. Because of analytical problems, results of participant 6 were Cochran stragglers ($P = 95\%$) for level 6. Data submitted by participant 5 were considered outlying results and were discarded from the study.

All reported data (except those from participant 5) were used for statistical evaluation (Table 5). Average histamine concentrations of all levels ranged from 10.29 mg/L to 402 mg/kg. Overall repeatability relative standard deviation (RSD_r) values ranged from 2.1 to 5.6%. They are considered to be in agreement with this concentration range. Reproducibility relative standard deviation (RSD_R) values of all levels, reflecting the variation between the laboratories, ranged from 2.2 to 7.1%. These values are acceptable according to the 1987 harmonized IUPAC protocol (27, 28) and the Horwitz equation (29, 30) dealing with acceptability of interlaboratory studies representing a wide range of analytes, matrixes, and measurement techniques. According to these publications, predicted RSD_R values for analyte concentrations between 10.29 mg/L and 402 mg/kg should vary from 6.5 to 11.3%. The 1989 IUPAC harmonized protocols for collaborative studies (31) considers values within 0.5 to 2 times the predicted RSD_R value as acceptable. For all samples analyzed, repeatability (r) values varied from 1.62 mg/L to 34 mg/kg, and reproducibility (R) values ranged from 1.74 mg/L to 52 mg/kg.

Results and statistical parameters obtained with training samples summarized in Table 2 do not significantly differ from those found in the interlaboratory study and confirm the latter's results. Recoveries were calculated from spiking levels and mean histamine concentrations for 7 levels calculated from results reported by participants. Recoveries ranged from 94 to 100% (Table 1). Figure 1 shows a typical chromatogram of a standard solution, and Figure 2 shows separation patterns for mackerel, anchovy, sauerkraut juice, and wine.

The findings of this study were compared with those of other interlaboratory investigations. Rogers and Staruszkiewicz (32) reported results of the study for fluorometric assay of histamine in canned tuna and raw frozen mahi-mahi (dolphin fish). Mean histamine values ranged from 5.63 to 158.4 mg/kg. RSD_r values ranged from 3.6 to 21.4%, and RSD_R values ranged from 8.9 to 23.0%. Recoveries of 85–125% were obtained from tuna spiked with 57 mg histamine/kg. As a result of this study, modification of the AOAC INTERNATIONAL Official Method of Analysis for histamine (14) was recommended.

A German interlaboratory study (11) yielded repeatability values of 11 and 73 mg/kg for fluorometric histamine determinations in fish at levels of 104 and 834 mg histamine, respectively. Reproducibily values for these levels were 32 and 205 mg/kg, respectively.

During the past 10 years, proficiency studies for histamine determinations in fish and processed cheese (spiking range, 100–500 mg/kg) have been organized in The Netherlands (33–35). Precision parameters of these studies (with RSD_r and RSD_R values ranging from 2 to 5% and from 7 to 12%, respectively) are comparable with those found in this interlaboratory study.

Participant's Comments

In general, most participants commented favorably on the method. The only minor point experienced by nearly all participants was the relatively long retention times for the biogenic amines of interest. No significant difficulties were encountered with the procedure. One participant recommended use of *N,N*-dimethyl-2-mercaptoethylammonium chloride instead of the rather toxic mercaptoethanol in the postcolumn derivatizing reagent.

Some participants mentioned minor changes on the method, which has been amended accordingly.

Conclusions

Validation results from this interlaboratory study reveal that the proposed LC method is reliable and reproducible for determining histamine in various foods at levels up to 400 mg/kg or mg/L. The LC method has demonstrated its usefulness for laboratories of the Food Inspection Services, The Netherlands.

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FOOD CHEMICAL CONTAMINANTS

High-Performance Liquid Chromatographic Method for the Determination of Moniliformin in Corn [1]

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A high-performance liquid chromatographic method using UV absorption was developed for determining moniliformin in corn. The toxin was extracted with water containing 1% tetrabutylammonium hydrogen sulfate (w/v). Paired moniliformin was partitioned into dichloromethane, which was evaporated to dryness at 50°C. The residue was dissolved in water and applied to a disposable strong-anion exchange solid-phase extraction tube. Adsorbed moniliformin was eluted from the tube with 0.05M sodium dihydrogen phosphate monohydrate (pH 5). It was determined by ion-pair reversed-phase chromatography and UV measurement at 229 nm. The minimum detectable amount of pure moniliformin was 0.25 ng/injection (signal-to-noise ratio = 3:1). The detector response was linear from 0.25 to at least 20 ng. The limit of determination was 0.025 μg/g corn. Recoveries of moniliformin from corn spiked at 0.025, 0.05, 0.25, and 1.0 μg/g averaged 96.5, 96.2, 97.2, and 97.8% respectively.

Moniliformin is a fungal metabolite structurally characterized as 3-hydroxycyclobut-3-ene-1,2-dione (Figure 1). It was first isolated in 1973 from corn culture that had been inoculated with *Fusarium proliferatum* but that had been misidentified as *F. moniliforme*, thus the name moniliformin (1). The metabolite is produced by at least 15 other *Fusarium* species (2–7). Of these, several species are particularly important pathogens of cereal grains throughout the world, *F. proliferatum* and *F. subglutinans* being the most important in corn. Moniliformin has been reported to occur naturally in corn, wheat, rye, triticale, oats, and rice from different parts of the world (8–15).

Moniliformin is a highly toxic metabolite. It is acutely toxic to many experimental animals including chickens, ducklings, and rats (1, 2, 16–18). The predominant mechanism of its acute toxicity is believed to be inhibition of pyruvate dehydrogenase. The toxin binds to pyruvate dehydrogenase, preventing entrance of pyruvate into the tricarboxylic acid cycle and therefore decreasing mitochondrial respiration (19–21). Dietary exposure indicated that moniliformin is more toxic to chickens than fumonisin B_1, another mycotoxin produced by *F. proliferatum* and *F. subglutinans* (22). It is also more cytotoxic than fumonisin B_1 on cultured chicken cells and other cultured mammalian cell lines (23, 24). Moniliformin is a potent cardiotoxic mycotoxin (1, 25–27). Although the acute and long-term toxicity of moniliformin for humans is not yet known, some Chinese scientists suggest that moniliformin is involved in the heart disease known as Keshan disease occurring in Chinese regions where inhabitants eat home-grown corn infected by *F. subglutinans* and contaminated with moniliformin (28). The lack of mutagenicity to *Salmonella typhimurium* (29) suggests that moniliformin is probably not carcinogenic.

Only a few analytical methods have been published for determination of moniliformin in agricultural products. These methods include thin-layer chromatography (TLC), gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC). Most of the published methods are not entirely satisfactory for routine determination of moniliformin. They are either not sensitive and selective or not practical for routine analysis of moniliformin in a large number of samples. TLC methods allow detection limits usually in the range 0.1–1.0 μg/g sample depending on sample cleanup (2, 17, 30, 31). A GC/MS method with a detection limit of 5 pg of derivatized standard moniliformin was developed (32) but not applied to corn or other cereal grains. HPLC is generally preferred over TLC because of its improved sensitivity and resolution. Thiel et al. (8) used ion-pair reversed-phase and ion-exchange LC for determining moniliformin in corn. Recovery rates of the procedure were not given and were stated to be low and to vary considerably. The procedure included a 4 h sample cleanup. Shepherd and Gilbert (33) described an LC procedure that uses ion pairing for extraction and separation of moniliformin. The procedure was relatively sensitive (minimum detectable amount of standard moniliformin, 10 ng; determination limit, 0.1 μg/g corn), with recovery rates in the range 60–80% at spiking concentrations of 0.1–1.6 μg/g. However, the procedure suffered from coeluting interfering peaks that made interpretation of chromatograms difficult. The procedure, which required a 3-step cleanup, also was not practical for routine determination of moniliformin. Scott and Lawrence (10) developed an LC method with recovery rates of

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74–83% at spiking concentrations of 0.05–1.0 μg/g corn and a detection limit of about 0.01 μg/g. However, chromatographic separations were very poor. Thiel (34) described 2 LC procedures using ion-exchange and ion-pair reversed-phase separation. The detection limit of standard moniliformin was 20 ng, and overall recovery rates were about 70%. The procedures depended upon a lengthy sample cleanup (4 h) that did not eliminate major interfering compounds and a lyophilization step. Sharman et al. (11) described a sensitive LC method for determining moniliformin in corn, wheat, rye, and triticale. Samples were extracted with 95% acetonitrile in water. Extracts were concentrated by evaporation at 40°C and cleaned up on a combination of reversed-phase and strong-anion-exchange (SAX) disposable cartridge columns. Extracts were analyzed by ion-pair reversed-phase HPLC with UV detection. Recoveries ranged from 81 to 96% for samples spiked at 0.25 and 0.5 μg/g and the limit of detection was 0.05 μg/g. Efforts to use this method in our laboratory were not successful. Recoveries of moniliformin extracted with mixtures of acetonitrile and water were very low when extracts were concentrated by evaporation. Recently, Filek and Lindner (35) reported a very sensitive and selective LC method. The procedure detected moniliformin at 0.02 μg/g, with overall recoveries of about 70% at spiking concentrations of 0.02–0.25 μg/g. The smallest detectable amount of moniliformin derivative was 0.5 ng. Unfortunately, the method required a time-consuming (2 h) fluorescence derivatization step.

We found the ion-pairing phenomenon very useful in a redesigned analytical procedure for determining moniliformin in corn. Compared with other published LC methods using UV detection, the primary advantages of this procedure are simple and efficient sample extraction and cleanup resulting in improved recoveries, chromatographic separation, and sensitivity.

METHOD

Moniliformin is a toxic substance and should be handled with caution. All apparatus and reagents may be replaced by equivalent substitutions.

Apparatus

(a) *Liquid chromatograph.*—Model 510 HPLC pump operated at 1 mL/min, Model 486 tunable absorbance detector set at 229 nm and 0.003 absorbance unit full scale (AUFS; Waters, Milford, MA), Valco EC6W injector valve (Vici Valco Instruments Co., Inc., Houston, TX) with a 20 μL injection loop, and Model HP3395 integrator with chart speed of 0.8 cm/min (Hewlett-Packard, Avondale, PA).

(b) *Analytical and guard columns.*—Ultremex C_{18} reversed-phase column (150 × 4.6 mm id, 5 μm) and Partisil 10 SAX guard column (30 × 4.6 mm id, 10 μm; Phenomenex, Torrance, CA).

(c) *Shaker.*—Wrist-action shaker (Burrell Corp., Pittsburgh, PA).

(d) *Solid-phase extraction (SPE) column.*—Disposable LC SAX tubes, 1 mL capacity containing 100 mg sorbent (Supelco, Inc., Bellefonte, PA).

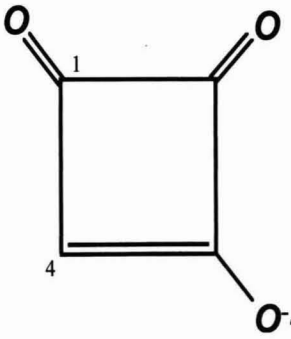

Figure 1. Structural formula of moniliformin (sodium salt).

Reagents

(a) *Solvents.*—Acetonitrile, dichloromethane, methanol, and water (all LC grade).

(b) *Chemicals.*—Tetrabutylammonium hydrogen sulfate (TBAHS) (98%; VWR Scientific products, Chicago, IL), potassium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate (all LC grade), and *o*-phosphoric acid (85%).

(c) *Solution of ion-pair modifiers.*—Fifty milliliters of 40% tetrabutylammonium dihydrogen sulfate mixed with 100 mL 1.1M potassium dihydrogen phosphate (both solutions were prepared in LC grade water and filtered through 0.20 μm nylon membrane).

(d) *LC mobile phase.*—Prepare by diluting 10 mL ion-pair modifiers with acetonitrile–water (8 + 92) to a final volume of 1 L. Adjust pH of mobile phase to 6.5 with 5N KOH and filter through a 47 mm × 0.45 μm nylon membrane. Degas before use. Run and allow the HPLC system to equilibrate for ca 1 h prior to use.

(e) *Moniliformin standard solution.*—Pure sodium salt of moniliformin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05M sodium dihydrogen phosphate monohydrate, pH 5.0 (200 μg/mL) and stored at 4°C.

(f) *Working standards.*—Prepare an intermediate solution of moniliformin standard (10 μg/mL) in 0.05M sodium dihydrogen phosphate monohydrate (pH 5). Use portions of the solution to prepare different concentrations of moniliformin in the range 0.01–1.0 μg/mL. Store solutions at 4°C. They are stable for at least 6 months.

Sample Extraction

Grind corn to pass U.S. No. 20 sieve. Place 10 g ground corn sample into 125 mL polyethylene sample bottle. Add 50 mL 1% tetrabutylammonium hydrogen sulfate (TBAHS) prepared in LC grade water and shake for 30 min at maximum speed on a wrist-action shaker. Filter extract by gravity through Whatman No. 4 filter paper, taking care to retain most solids in the sample bottle. Add 50 mL 1% TBAHS to solids in sample bottle and shake for additional 30 min. Filter extract through the same filter paper and combine the 2 extracts. Transfer 25 mL extract into a separatory funnel or 125 mL sample bottle and add 25 mL dichloromethane. Mix gently and avoid vigorous shaking. Let phases separate and drain lower phase into a 100 mL container. If an emulsion is formed, centrifuge at

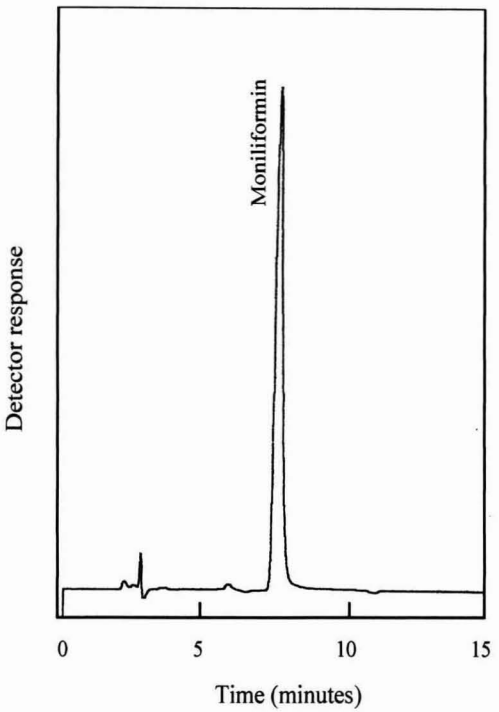

Figure 2. Chromatogram of standard moniliformin (20 ng injection).

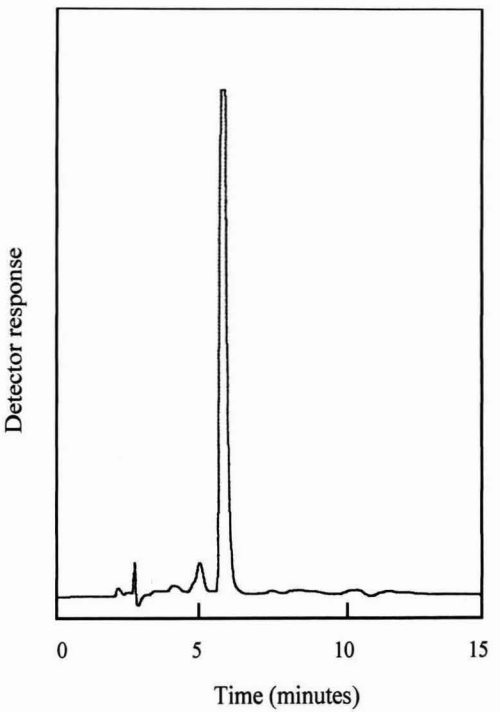

Figure 3. Chromatogram of moniliformin-free corn sample; 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

3000 rpm for 5 min to allow good phase separation. Repeat the partition with additional 25 mL dichloromethane and combine dichloromethane extracts. Evaporate dichloromethane to 5–10 mL at 50°C in a water bath under a stream of blowing air. Transfer the reduced volume of dichloromethane into a small vial and evaporate to dryness.

Extract Cleanup

Fit disposable SAX SPE tube on the end of a 10 mL syringe or port of vacuum manifold. Condition tube by washing successively with 1 mL methanol, 1 mL water, and 1 mL 0.1M *o*-phosphoric acid. Do not allow tube to dry. Dissolve extract residue into 1 mL LC-grade water and load onto the SPE tube. When all extract has passed through tube, wash tube with 1 mL water and force air through tube to expell all the wash solution. Elute adsorbed moniliformin with 1 mL 0.05M sodium dihydrogen phosphate monohydrate (pH 5.0). Filter eluate through a 0.2 μm nylon membrane and save eluate at 4°C before LC analysis.

Liquid Chromatography

Prepare a standard curve by injecting 20 μL moniliformin working standards. The retention time of moniliformin is ca 7.5 min. There is no need to prepare the standard curve daily, but injection of a moniliformin standard solution is required for each analysis. Inject 20 μL test solution. Identify peak and determine the quantity of moniliformin by comparing retention time and area with those of reference standard.

Spiking of Samples and Recovery

Prepare moniliformin standard solutions in LC grade water (2.5, 5.0, 25, and 100 μg/mL) and spike ground sample at 0.025, 0.05, 0.25, and 1.0 μg/g with a spiking volume of 0.1 mL for 10 g sample. Analyze samples according to the procedure described above and calculate percentage recoveries. Use 3 replicates of spiked samples at each concentration for each run and repeat analysis to determine recoveries and daily variation of the analytical procedure.

Results and Discussion

Extraction of Moniliformin

Water appeared to be the ideal solvent for extracting moniliformin because of the polarity and high solubility of the toxin in water. Thiel (34) used water (40 mL) to extract moniliformin from spiked ground corn (3 g) and reported a 95% recovery rate for the extraction step.The extraction procedure described here is based on the extraction procedure developed by Shepherd and Gilbert (33). However, conditions were profoundly modified to improve removal of interferences and recoveries. These investigators used water (150 mL) containing ion-pairing reagent (tetra-*n*-butylammonium hydroxide) to extract moniliformin in ground corn (30 g). The extraction was followed by

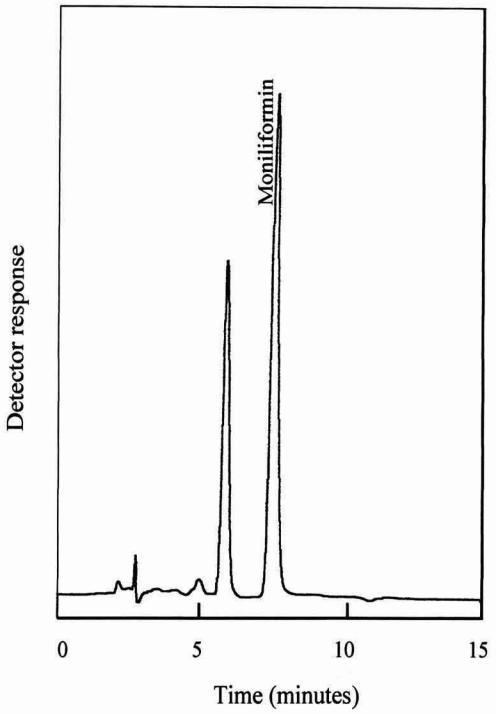

Figure 4. Chromatogram of corn sample spiked with moniliformin (1 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

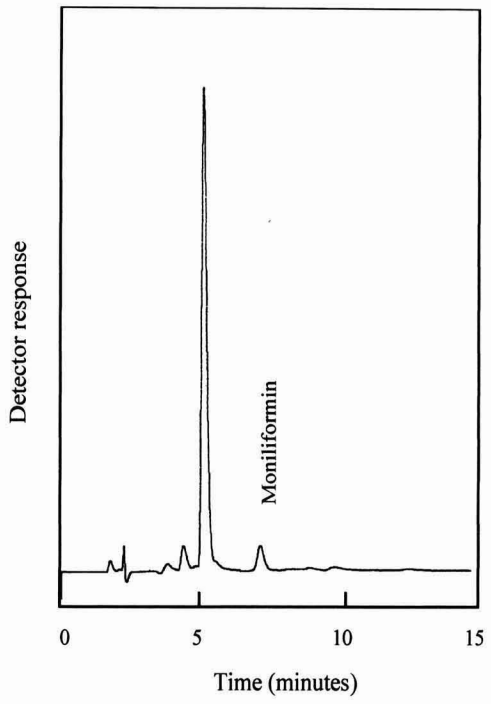

Figure 5. Chromatogram of corn sample spiked with moniliformin (0.025 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

cleanup on Amberlite IRC-50 resin and C_{18} Sep-Pak cartridge before another ion-pairing and final cleanup on "Chem Tube." Compared with extraction, ion-pairing, and cleanup procedures described by these investigators, conditions in our procedure were simplified very much, therefore making the procedure easier and more practical and reducing handling time. Extraction and ion-pairing of moniliformin were accomplished in a single step by using 1% TBAHS in water. No pre-cleanup on Amberlite IRC-50 resin or C_{18} Sep-Pak cartridge was required for successful ion pairing and subsequent partition of moniliformin. Pre-cleanup on C_{18} Sep-Pak cartridge was particularly omitted in our procedure because C_{18} Sep-Pak cartridges or columns bound up to 35% of free moniliformin and 100% of paired moniliformin in water solution. Two 50 mL volumes of 1% TBAHS were used to extract moniliformin. In general, the first volume extracted 70–75% of recovered moniliformin.

Moniliformin is frequently extracted with mixtures of water and organic solvents (10–12, 35). Initial efforts in our laboratory to extract moniliformin with various ratios of acetonitrile–water or methanol–water and concentrate extracts by evaporation gave overall recoveries not exceeding 40% and sometimes as low as 10% depending on the spiking level. Low or zero recoveries of moniliformin from water or mixed organic-aqueous extracts taken to dryness or near dryness have been reported (33). To prevent low recoveries of moniliformin, Rottinghaus recommended use of siliconized glassware and not overdrying when evaporating the extract of moniliformin (personal communication). However, this procedure did not improve recoveries of moniliformin from solutions taken to near dryness.

Moniliformin spiked into water or an aqueous extract of corn (0.1 μg/mL) could not be partitioned into dichloromethane, chloroform, or ethyl acetate without prior pairing of the toxin with tetrabutylammonium counter ion. When a moniliformin-free corn sample was extracted with 1% TBAHS and the extract spiked with moniliformin, about 100% of moniliformin was partitioned into dichloromethane, 53% into ethyl acetate, and less than 5% into chloroform. Paired moniliformin in aqueous extract was partitioned into 2 volumes of dichloromethane. Approximately 85% of paired moniliformin partitioned in the first volume and 15% in the second volume. Compared with previously published methods, pairing moniliformin with TBAHS followed directly by partition into dichloromethane before cleanup was a new step in moniliformin analysis. The described pairing and partition procedure enhanced overall recoveries of moniliformin from spiked corn.

Shepherd and Gilbert (33) indicated that exposure of moniliformin to low pH leads to loss of the toxin. Therefore the stability of moniliformin in 1% TBAHS aqueous extract of corn (pH of about 2.2) was studied by holding extracts at room temperature for up to 24 h before cleanup and analysis. Results indicated that paired moniliformin was very stable in 1% TBAHS extracts with no loss at all. Additional studies also in-

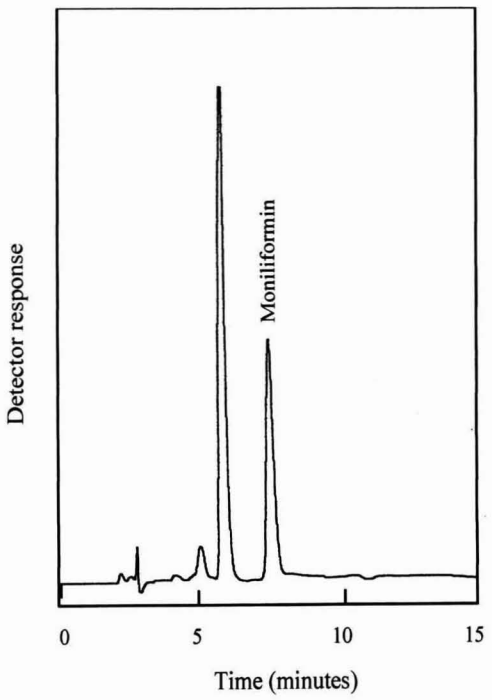

Figure 6. Chromatogram of corn sample naturally contaminated with moniliformin (0.2 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

dicated that the toxin was very stable in dichloromethane after partition and in water after evaporation of dichloromethane (data not shown). This high stability of moniliformin is a real advantage of the proposed analytical procedure.

Cleanup Procedure

Cleanup on disposable SAX SPE tubes was adapted from procedures described by other investigators (11, 35). However, the C_{18} SPE column used in combination with SAX SPE column (11) was omitted in our procedure because of adverse effects of the C_{18} cleanup column on recovery. A 1 mL SAX tube was preferred over the 3 mL tube used by other investigators. It required lower amounts of solvents and appeared to be more efficient than a 3 mL tube at retaining interference compounds. Sodium phosphate buffer (pH 5) was preferred over solvents used by Sharman et al. (11) or Filek and Lindner (35) for eluting moniliformin from the SAX tube. The buffered water was more efficient at eluting adsorbed moniliformin, and it eluted fewer interferences than other solvents.

HPLC Separation and Determination

The optimized extraction–cleanup procedure gave excellent chromatograms free of coextractive interferences. Typical chromatograms of standard moniliformin (20 ng injection), moniliformin-free corn, spiked corn samples (0.025 and 1 μg/g), and naturally contaminated corn (0.2 μg/g) are shown in Figures 2–6. In all cases, moniliformin eluted as a very sharp peak without tailing and well separated from other constituents. Reproducibilities of retention time, peak area, and width determined over many days were all excellent (Table 1). The limit of detection of pure moniliformin was 0.25 ng (signal-to-noise ratio = 3:1), which is lower than the 1 ng reported by Thiel (34) or 0.5 ng moniliformin-1,2-diamino-4,5-dichlorobenzene fluorescent derivative reported by Filek and Lindner (35). The chromatographic response was linear ($R^2 = 1.00$) between 0.25 and at least 20 ng moniliformin injected onto the column.

Different reversed-phase columns and guard columns were compared for chromatographic separation of moniliformin from other compounds in corn extracts. The combination of a Partisil 10 SAX guard column and Ultremex C_{18} column gave the best separation of moniliformin from interfering compounds, which could not be obtained by using either a Partisil 10 SAX column in combination with a similar guard column or an Ultremex C_{18} column and a similar guard column. The limit of determination of moniliformin spiked into ground corn was 0.025 μg/g corn, which is lower than the 0.1 μg/g corn reported by Shepherd and Gilbert (33) and comparable with the 0.01 and 0.02 μg/g corn reported by Scott and Lawrence (10) and Filek and Lindner (35), respectively. Chromatograms were better than any previously published. The peak corresponding to moniliformin was sharp and free of interfering compounds. Average recovery rates of moniliformin spiked into ground corn at 0.025–1 μg/g varied from 96 to 98% (Table 2), which are higher than the 70–80% recovery rates reported by other investigators (10, 33–35).

Extraction and recovery rates were compared when moniliformin spiked in ground yellow corn at 1 μg/g was extracted with water alone followed by ion pairing with TBAHS, 1% aqueous solution of TBAHS, and aqueous solutions of tetrabutylammonium hydroxide (TBAH) or tetrabutylammonium hydrogen phosphate (TBAHP) (Sigma Chemical Co.,

Table 1. Reproducibility of moniliformin determination by ion-pairing reversed-phase chromatography[a]

Parameter	Retention time, min	Peak area	Peak width
Mean (n = 15)	7.50	1839536	0.244
Standard deviation	0.01	15815	0.002
Coefficient of variation, %	0.16	0.9	1.0

[a] Column: Ultremex C_{18} reversed-phase column (150 × 4.6 mm; 5 μm) with Partisil 10 SAX guard column (30 × 4.6 mm, 10 μm). Mobile phase: 10 mL of modifiers (50 mL 40% TBAHS + 100 mL 1.1M potassium dihydrogen phosphate) diluted with acetonitrile–water (8 + 92); final volume, 1 L; pH, 6.5. Flow rate, 1 mL/min; detection wavelength, 229 nm (0.003 AUFS); injection, 20 ng standard moniliformin.

Table 2. Recoveries of moniliformin added to ground corn

Moniliformin added, µg/g	Recovery		
	Average, %	Range, %	CV, %
0.025	96.5	86.3–109.9	8.1 (n = 9)
0.050	96.2	83.0–109.1	9.7 (n = 12)
0.250	97.2	88.3–102.2	4.4 (n = 12)
1.0	97.8	95.4–105.7	2.8 (n = 15)

St. Louis, MO) with molar concentration equal to that of 1% TBAHS solution. Extraction with 1% TBAHS was the prefered procedure; extracts were very clear and filtered faster than others. TBAHP solution extracted more solids than TBAHS or water alone, thus making filtration by gravity very difficult. Extracts formed a thick emulsion upon partition with dichloromethane. TBAH solution extracted yellow pigments and a large amount of solids. It is not possible to filter the extracts by simple gravity. Similar average recovery rates, about 98%, were obtained with TBAHS solution, water alone followed by pairing with TBAHS, and TBAP solution. Chromatograms were excellent in all 3 cases. Moniliformin was not detected in spiked samples extracted with TBAH solution.

Compared with previously published HPLC methods for determining moniliformin in corn, this analytical procedure is excellent in terms of efficient and easy sample extraction and cleanup, resulting in improved recovery rates and chromatographic separation. Handling time was reduced. The time required for complete analysis of 15 spiked samples was about 8–9 h. The method was reproducible, and its sensitivity was at least comparable with that reported for previous methods.

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FOOD COMPOSITION AND ADDITIVES

Validation of Sample Preparation Procedures for Botanical Analysis

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A 2-part method was developed to validate the extraction efficiency (accuracy) of specific marker compounds from crude herb samples. This method has several advantages over the standard addition method of validating efficiency. In part 1, recovery optimization, recovery of marker compound is optimized by determining the maximum weight of sample that may be extracted in a specific volume of solvent before affecting recovery. The choice of extraction solvent was based on physicochemical parameters of the specific marker compound. In part 2, extraction efficiency testing and validation, the extraction solvent and optimal sample weight are used to test and validate extraction efficiency by extracting and then re-extracting 10 replicate samples of each botanical. The average recoveries of marker compound from extraction and re-extraction are compared by analysis of variance at the 95% confidence level. Extraction is considered 100% efficient if there is no significant difference between these averages. The solvent and/or conditions used for extraction are modified and the efficiency is retested if the average recoveries are significantly different. Extraction precision and ruggedness are evaluated from coefficients of variance and by testing the efficiency of several chemically different marker compounds. Levels of marker compounds are determined by liquid chromatography and by comparison with peak areas produced by reference standard solutions of known concentration. The method is extremely precise and rugged, making it a valuable tool for evaluating and validating the efficiency of extracting marker compounds from crude herb samples.

Development of accurate quantitative methods of analysis for specific marker compounds in herbal manufacturing is critical for determining the quality and consistency of raw materials and finished products. The accuracy of these methods, however, depends on the ability to completely and reproducibly extract the analyte of interest. A crucial prerequisite for accurate measurement is an efficient extraction (1).

Standard addition and matrix fortification (spiking) are the primary methods used to determine the efficiency of extractions (2). Standard addition requires that the true concentration of analyte in reference materials be known (2). Because concentrations of marker compounds in raw herb samples may be quite variable, the utility of standard addition for crude herbs can be limited. Matrix fortification (spiking) requires use of a matrix blank that does not contain the analyte of interest (3). This criterion cannot be met for crude herbal materials, because they naturally contain at least some level of the marker compounds. In addition, both methods require use of pure, well-characterized reference materials with known stability (2, 4). Unfortunately, many herbal marker compounds are either commercially unavailable or too costly to be acquired as pure, well-characterized standards. Use of either method in these cases would require substantial investment for isolation and characterization.

This report presents a 2-part procedure that may be used to evaluate and statistically validate the efficiency of extracting specific marker compounds from crude herb samples. The method does not require use of a pure reference standard. It is extremely precise and rugged, making it a valuable tool for evaluating and validating the efficiency of extracting marker compounds from crude herbal material. Also, the procedure rapidly processes many samples simultaneously, making it very useful for quality control.

Experimental

Sample Preparation

Dried, voucher samples (50 g each) of green tea leaves (*Camellia sinensis*), guarana seed (*Paullinia cupana*), ginseng root (*Panax ginseng*), *Echinacea* root (*Echinacea angustifolia*), and goldenseal root (*Hydrastis canadensis*) were ground in a laboratory-size grinder (Waring, New Hartford, CT). They were then milled to 60 mesh in a laboratory-size mill (Glen Mills, Clifton, NJ), stored separately in air-tight containers at room temperature, and shielded from light until use. Water contents of all samples were determined with a Karl Fischer titrator (Photovolt, Indianapolis, IN) and subtracted from the sample weights prior to recording.

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Reference Standards for Marker Compound

Caffeine (Sigma Chemical Co., St. Louis, MO) was used as a reference standard for green tea leaves and guarana seed. Hydrastine hydrochloride (Sigma) was used as a reference standard for goldenseal root. Pure ginsenosides Rb1, Rc, Re, Rg1, Rb2, and Rd (Indofine, Somerville, NJ) were used as reference standards for ginseng root. An *Echinacea angustifolia* standardized extract (Indena, Seattle, WA) was used as the standard for the for *Echinacea* root. Reference standard solutions of known concentration were prepared by weighing or transferring a known amount into a clean, dry volumetric flask; dissolving; and diluting to volume with an appropriate extraction solvent.

Solvents

Solvents for extractions and analyses were LC grade (EM Science, Gibbstown, NJ). Concentrated acetic and ortho phosphoric acids were reagent grade (VWR, Piscataway, NJ).

Extraction Solvents

Extraction solvents for recovery optimization (part 1) were chosen on the basis of the physicochemical properties and solubility characteristics of the marker compounds. Water was used to extract caffeine from green tea leaves and guarana seed. Methanol (100%) was used to extract ginsenosides from ginseng root, hydrastine from goldenseal root, and echinacoside from *Echinacea* root. Extractions were performed at room temperature.

In part 2 (extraction efficiency testing and validation), water at 25° and 100°C was used to extract caffeine from green tea leaves and guarana seed powder. Methanol (100%) and methanol–water were used to extract ginsenosides from ginseng root. Methanol (100%) and water–methanol–glacial acetic acid were used to extract echinacoside from *Echinacea* root. Methanol (100%) and a methanol–water acidified with concentrated ortho-phosphoric acid were used to extract hydrastine from goldenseal root.

Liquid Chromatographic Assay Protocols

Portions of each extract (10–15 µL) were analyzed with a modular liquid chromatographic (LC) system (Waters Associates, Milford, MA) consisting of a Model 717 WISP autosampler, a Model 996 photodiode array detector, and a Model 600 solvent delivery system. Marker compounds were eluted with various combinations of acetonitrile and water or methanol and water at 1.0 mL/min. Caffeine was chromatographed on a 150 × 4.6 mm, 5 µm, C_{18} Hypersil BDS column (Supelco, Belefonte, PA). Ginsenosides and echinacoside were chromatographed on a 150 × 4.6 mm, 5 µm, C_{18} Nucleosil column (Supelco). Hydrastine was chromatographed on a 150 × 4.6 mm, 5 µm, C_{18} ABZ Plus column (Supelco). Data were collected and integrated with Millenium software (Waters). The linear dynamic range and the limit of detection for each marker compound were determined prior to collection of experimental data. Experimental results were quantitated by using peak area counts vs a reference standard of a concentration within the linear dynamic range of the assay. Experimental results below the limit of quantitation were not used. Results greater than the linear range of the assay were diluted to fall within the linear range and reassayed. Standards and samples were assayed in duplicate.

Part 1: Recovery Optimization

Extractions for recovery optimization were performed by accurately weighing incremental amounts (0.1–4.0 g) of each ground plant sample on an analytical balance (A & D Engineering, Milpitas, CA). Each sample was transferred to separate clean and dry 100 mL volumetric flasks. About 60 mL extraction solvent was added. Each flask was then placed in an ultrasonic bath (Branson, Danbury, CT) for 15 min and then agitated for 30 min with a wrist action shaker (Lab-Line, Melrose Park, IL). Each flask was filled to volume with extraction solvent; the contents were mixed well and then left to stand for a few minutes to allow visible solids to settle. A 5 mL portion from each flask was withdrawn and centrifuged for 10 min at 2500 rpm in a bench top centrifuge (Fisher Scientific, Pittsburgh, PA) to remove particulate material. A volume of supernatant was transferred to individual borosilicate vials (Waters) for LC analysis. Portions of appropriate reference standards of known concentration were prepared simultaneously for LC analysis.

Recovery of each marker compound was determined by injecting equal volumes of extract and reference standard of known concentration and comparing peak areas. The amount of marker compound was then converted to amount per milligram sample weighed and finally to percent of sample weighed. The sample weight of each plant sample tested yielding the highest percentage recovery of marker compound, as well as extraction solvents and conditions, were used in part 2 (*Extraction Efficiency Testing and Validation*) to test and validate the efficiency of each extraction.

Part 2: Extraction Efficiency Testing and Validation

Ten replicates of each sample, each having approximately the same weight, were weighed accurately on an analytical balance. Each sample was then transferred to a separate clean and dry 100 mL volumetric flask, and ca 60 mL extraction solvent was added. Each sample was extracted as described in the previous section, and a volume of the supernatant from each of the 10 flasks was analyzed by LC. An additional 45 mL portion (50 mL total) was then removed from each flask and discarded. Approximately 30 mL fresh extraction solvent was added to each flask; samples were re-extracted and prepared for LC analysis as described above. Portions of appropriate reference standards of known concentration were prepared simultaneously for LC analysis.

Recovery of each marker compound was determined by injecting equal volumes of extract, re-extract, and reference standard of known concentration and comparing peak areas. The concentration of the analyte was then converted to amount of marker compound per milligram sample weighed and finally to percent of sample weighed. The average percentage recovery of marker compound was calculated from each set of 10 extractions and corresponding re-extractions. The average value calculated from re-extractions was doubled to compensate for the 50 mL extract that was discarded and replaced with fresh extraction solvent. These 2 averages were compared statistically

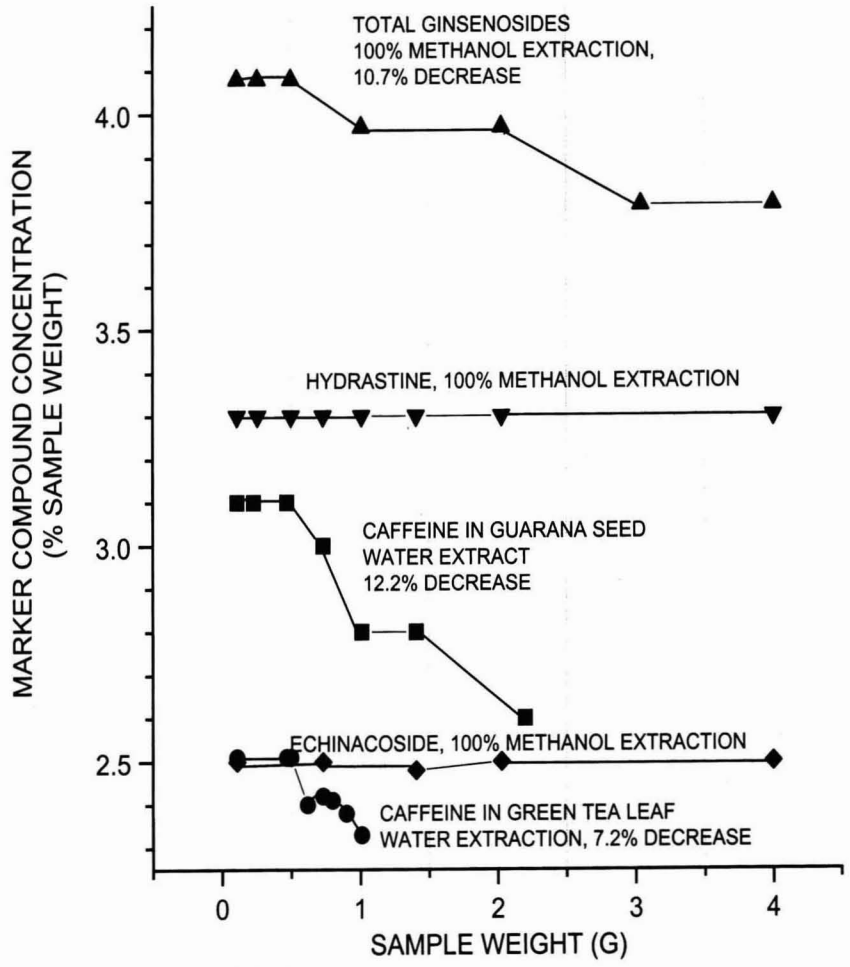

Figure 1. Results for recovery optimization.

to determine if they were significantly different. If the average value determined from 10 extracts was not significantly different from the average value determined from the corresponding 10 re-extracts, the extraction efficiency was considered validated at 100%. If the averages were significantly different, the extraction was considered not to be 100% efficient. Extraction parameters (solvent composition, temperature, and pH) were then modified and the efficiency retested.

Statistical Analysis

One-way analysis of variance (ANOVA) and coefficients of variance were calculated with the Origin graphic and statistical software package (Microcal, Northampton, MA). Significance of differences between mean values was assessed at the 95% confidence level.

Results and Discussion

Part 1: Recovery Optimization

This part was done to establish solvent capacity parameters for ultimately validating the efficiency of extracting specific marker compounds from various samples. Solvent volume was held constant to evaluate the effect of increasing sample weight and ultimately analyte concentration on recovery of marker compound. Recovery of marker compound (as a percentage of sample weight) versus sample weight is graphed in Figure 1. Results show that recovery of marker compound from some samples may be affected adversely by increasing the amount of sample extracted, as illustrated by a 12.2% decrease in recovery of caffeine from guarana seed powder, a 10.7% decrease in recovery of ginsenosides from ginseng root powder, and a 7.2% decrease in recovery of caffeine from green tea when the weight of sample extracted is more than 0.5 g/100 mL. These

results indicate that the solvents chosen (water at 25°C for caffeine and 100% methanol for all others) have the appropriate selectivity to solubilize and extract the respective marker compound. They also indicate, however, that the extraction capacity of 100 mL solvent is saturated when more than 0.5 g sample is extracted under these experimental conditions. To optimize recovery of marker compound from these samples under these conditions, therefore, a maximum of 0.5 g sample should be extracted with 100 mL solvent.

The results in Figure 1 also show that for other samples, recovery of marker compound is not affected by increasing the amount of sample extracted, as illustrated by no apparent decrease in recoveries of hydrastine from goldenseal powdered root and of echinacoside from *Echinacea* powdered root for sample weights of 0.1–4.0 g extracted with 100 mL solvent. The results indicate that the solvents chosen to extract these samples have the appropriate selectivity to solubilize and extract the respective marker compounds. They also indicate that the extraction capacity of each solvent used is not saturated when more than 0.5 g of these samples are extracted in 100 mL under these experimental conditions. Therefore, to optimize recovery for these samples under these conditions, up to 4.0 g sample may be extracted with 100 mL solvent. As with guarana, ginseng, and green tea, these established experimental parameters imply a very highly efficient extraction. Unfortunately, they are inadequate to determine if all of the marker compound is recovered from the sample. The efficiency of extraction or accuracy of the assay, therefore, cannot be determined. For accurate quantitative determination of marker compound in a crude herb sample, however, it is critical that the efficiency of extraction be validated.

Part 2: Extraction Efficiency Testing and Validation

A set of 2 average values for each marker compound recovered from each sample is listed in Tables 1 and 2. The first average was calculated by determining the percentage, by sample weight, of marker compound recovered from the extraction of 10 replicate samples of each botanical. The second average was calculated by determining the percentage by weight, of marker compound recovered from re-extraction of the same samples. Average values from all reextractions were multiplied by 2, to compensate for the dilution caused by adding 50 mL fresh extraction solvent. Results were compared by 1-way ANOVA at the 95% confidence level (Tables 1 and 2). Extraction was considered validated at 100% efficiency when there was no significant difference between a respective set of averages. A significant difference, required the extraction parameters to be modified and the efficiency retested until 100% efficiency was achieved. Ten sample replicates were used for each sample tested, because at least 6 replicates are recommended for adequate statistical analysis, and a larger number provides better estimates of mean values and acceptance range (2). A confidence level of 95% was used to compensate for variations that may occur in sample preparation, chromatography, and peak integration.

Each of the samples listed in Table 1 were extracted with identical extraction solvents and conditions used in part 1. A sample weight of about 0.5 g per 100 mL solvent was used for all extractions of caffeine and ginsenosides to test the validity of the optimized sample weights and conditions determined in part 1. For consistency, the same sample weight and solvent volume were used to extract echinacoside and hydrastine. The results in Table 1 show a statistically significant difference between each set of respective averages when tested for 100% efficiency. This indicates that even though extraction conditions were thought to be optimized in part 1, they were still inadequate to produce a 100% efficient extraction of the respective marker compounds. Extraction parameters were then modified to increase extraction efficiency.

Some parameters that can affect extraction efficiency include particle size, temperature, pH, solvent, solvent movement around particles, and diffusion of solvent into sample, which can affect dissolution of marker compound (5). Samples were finely ground to 60 mesh and subjected to 15 min in an ultrasonic bath followed by 30 min of agitation. This treatment would seem adequate to avoid inefficient extraction due to large particle size or insufficient movement of solvent around particles. Temperature, pH, extraction solvent capacity, and selectivity remained as possible variables that could be manipulated to increase efficiency.

The solubility of caffeine in water increased greatly with increased temperature (6). Increased temperature also increases the rate of extraction (5). The efficiency of extracting caffeine

Table 1. Statistical comparison of recoveries of marker compounds from 10 replicate extractions and re-extractions for validation of extraction efficiency with various solvents

Marker compound	Extraction solvent[a]	Average recovery from extraction[b] ± CV[c], %	Average recovery from re-extraction[b] ± CV[c], %	Significant difference between averages at 0.05[d]
Caffeine from green tea	25°C water	2.19 ± 10.70	2.63 ± 2.21	Yes
Caffeine from guarana	25°C water	3.11 ± 1.51	4.16 ± 1.37	Yes
Ginsenosides from ginseng root	100% methanol	4.39 ± 5.36	4.66 ± 4.21	Yes
Echinacoside from *Echinacea*	100% methanol	1.91 ± 3.29	1.99 ± 2.07	Yes
Hydrastine from goldenseal root powder	100% methanol	3.80 ± 1.09	3.94 ± 1.62	Yes

[a] Solvent volume for all extractions was 100 mL.
[b] Average of 10 replicate samples.
[c] Coefficient of variance for average.
[d] One way ANOVA at 0.05 (95%) level of significance.

Table 2. Statistical comparison of recoveries of marker compounds from 10 replicate extractions and re-extractions using various solvents and conditions

Marker compound	Extraction solvent[a]	Average recovery from extraction[b] ± CV[c], %	Average recovery from re-extraction[b] ± CV[c], %	Significant difference between averages at 0.05[d]
Caffeine from green tea	100°C water	2.51 ± 2.36	2.57 ± 3.49	No
Caffeine from guarana	100°C water	3.01 ± 3.67	3.04 ± 3.37	No
Ginsenosides from ginseng root	Methanol–water	3.88 ± 1.02	3.88 ± 1.78	No
Echinacoside from *Echinacea*	Water–methanol–ACOH[e]	1.92 ± 2.92	1.88 ± 2.37	No
Hydrastine from goldenseal root powder	Water–methanol–OPA[f]	3.78 ± 3.06	3.78 ± 2.91	No

[a] Solvent volume for all extractions was 100 mL.
[b] Average of 10 replicate samples.
[c] Coefficient of variance for average.
[d] One way ANOVA at 0.05 (95%) level of significance.
[e] Glacial acetic acid.
[f] Concentrated ortho-phosphoric acid.

from guarana and green tea was, therefore, re-evaluated with water at 100°C as the extraction solvent. Ginsenosides, hydrastine, and echinacoside are very soluble in methanol, making this solvent an obvious choice for extraction. Use of 100% methanol, however, may inhibit adequate rehydration (swelling) of the dried sample, which is necessary for complete diffusion of solvent. Incomplete diffusion of solvent may cause incomplete dissolution of marker compound and incomplete equilibration with solvent. Complete diffusion of solvent and a high capacity to dissolve marker compound are both essential for extraction of a substance from a crude herb sample. In this way, an equilibrium of extractable substances will be established between sample and solvent, completing the extraction (5). The efficiency of extracting ginsenosides, hydrastine, and echinacoside was therefore re-evaluated with mixtures of alcohol and water. The ratio of water to alcohol was determined experimentally to maximize extraction capacity and selectivity for marker compounds while minimizing recovery of unwanted substances.

Adjusting the pH of the extraction solvent may increase extraction efficiency by making the marker compound more soluble. It may also increase recovery by stabilizing the molecule in solution. Lowering the pH of the extraction solvent increased the recovery of hydrastine and the precision of the extraction, most likely because of ionization of the tertiary amine group, leading to increased water solubility. Lowering the pH of the extraction solvent had no significant effect on recovery of echinacoside but it increased extraction precision. The increased precision is most likely due to decreased hydrolysis of the ester when extraction is done at a low pH.

The effects of temperature, pH, extraction solvent capacity, and selectivity on extraction efficiency are shown in Table 2. In every example, there is no significant difference between the average recovery of marker compound from extraction and re-extraction of the same sample. This result validates the efficiency of the extraction at 100%, because the recovery marker compound is not significantly different from the recovery after re-extraction. A 100% efficiency also validates the accuracy of the extraction, which is defined as the closeness of agreement between the value found by the method and the value accepted either as a conventional true value or a reference value (2). For the examples in Table 2, the average value obtained from extraction may be considered to be the value found by the method. It may be accepted as the conventional or true value if it is identical to the value obtained from re-extraction.

The ability to validate efficiency and accuracy supports the use of this method as a tool for validating extractions. For a validation to be complete, however, the method must also be precise and rugged. Method precision was evaluated by calculating the coefficient of variation (CV) for each average recovery. The results show that the CV for each recovery except one in Table 1 is below 10%, which has been suggested as the acceptance criterion for precision of an analytical method (2). This result indicates that overall this method is very precise and has a very high degree of reproducibility. The results also indicate that when CV is greater than 10% there may be a significant difference between the recoveries of marker compound from extraction and re-extraction. The ruggedness of this procedure was demonstrated by its ability to discern differences in recovery with a variety of chemically different marker compounds. Similar results, however, must be produced by different individuals and different laboratories to confirm ruggedness.

This method for validating efficiency was found to have several advantages over the standard addition method for recovery of caffeine from ground green tea leaves (7). Ten replicate samples of approximately identical weight were used in both methods. This procedure produced a 100% recovery with CV values of 2.4% for extraction and 3.5% for re-extraction. Standard addition produced a 112.8% recovery, with a CV of 4.6%. A recovery greater than 100% indicates a possible problem with integration, extraction conditions, or both. More importantly, the precise amount of marker compound naturally present in the tested material must be known to accurately calculate efficiency. Because concentrations of marker compounds in raw herbs can be quite variable, it is not clear whether a recovery of 112.8% truly represents more or less than a 100% recovery. When using the standard addition method, it must be assumed that the reference material added reaches equilibrium with the solid sample and is extracted with the same ease or

difficulty as the naturally occurring marker compound. Lastly, the standard addition method requires a marker compound standard of known purity. This may not be economically feasible in some cases or may require its isolation and characterization before the validation can be done. The re-extraction method presented here provides a reproducible, statistical procedure with a level of certainty that 100% efficiency is obtained. Also it does not require addition or even the use of a reference standard for validation. Instead, the average peak area or height of the compound extracted and re-extracted may be statistically compared for testing efficiency without needing to calculate actual percent recovered.

Conclusions

Quantitative analysis of marker compounds in crude herbs requires a validated extraction procedure of known efficiency to evaluate quality and consistency. Presented here is a simple 2-part method by which this can be done with confidence. *Part 1: Recovery Optimization*, provides a procedure for optimizing recovery by adjusting the amount of sample extracted in a given volume of solvent. *Part 2: Extraction Efficiency Testing and Validation*, provides a procedure to statistically validate the efficiency and precision of a particular extraction method without need of a pure reference standard. This method has several advantages over the standard addition method and may be used to validate recovery of marker compounds from extracts, tablets, and other finished products containing excipients that could affect extraction efficiency.

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FOOD COMPOSITION AND ADDITIVES

Rapid Determination of Volatile Bases in Fish by Using an Ammonia Ion-Selective Electrode

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A simple and rapid method using an ammonia ion-selective electrode (ISE) to measure volatile bases in fish is proposed. Accuracy and precision were determined with 5, 10, 20, and 30 ppm NH_3 standard solutions. Ammonia values obtained with the method correlate strongly with total volatile basic nitrogen ($r^2 = 0.88$). Recoveries of added ammonia to homogenized fish samples ranged from 83.7 to 96.0%. Responses of the probe to trimethylamine (TMA), calculated as NH_3 (mg/100 mL), ranged from 74.9 to 91.7%. These findings indicate that the probe measured TMA as well as ammonia. Storage trials on 8 fish species illustrated that the results obtained with the ISE method reflected nitrogen concentrations based on total volatile base (TVB) analysis. This procedure may be used in lieu of the traditional TVB method for on-site rapid screening of fish.

Volumes of research have addressed the quality and spoilage profiles of finfish. The effects of time and temperature are clear, and the types of compounds that reflect freshness and decomposition have been elucidated. Procedures have been developed to analyze seafood for many of these compounds. Many are complex, require expensive equipment and extensive training, require use of dangerous chemicals, or are laborious and time consuming (1).

Traditionally, sensory evaluation of fish quality has been the method for routine assessment of fish freshness and overall quality profiles. While the importance of sensory assessment cannot be ignored, many individuals making the assessments can be subjective (1–4). If evaluators are not properly trained, their responses can vary depending on individual likes and dislikes, fatigue, prejudices, and ability to communicate accurately sensory sensations (2, 3). At the same time, highly trained, calibrated sensory analysts are highly paid, are too few in numbers (5, 6), and are not the individuals who routinely make determinations as to the condition of the product. Sensory assessment—size, appearance, raw odor—may be carried out in the processing plant by an experienced person without formal sensory training or by individuals who are not well trained or calibrated to assess properly the signs of quality decline and decomposition. As the final determinant in situations that may result in legal disputes or regulatory conflicts, sensory assessment of quality and decomposition, as it is currently being practiced, is insufficient (2). Although most trade is based on sensory assessments, these measurements are not always regarded as objective (4). In many instances, an objective scientific test, whether chemical, physical, or microbial, would be performed.

Various chemical indicators have been used to assess fish freshness and the overall spoilage profile. Test selection often depends upon available equipment and the species under study. Trimethylamine (TMA), formed by spoilage bacteria from the precursor trimethylamine oxide (TMAO); dimethylamine (DMA), formed by autolytic enzymes during frozen storage; and ammonia (NH_3), produced via protein (amino acid) degradation and enzymatic breakdown of nucleotides, make up the major components of what has been known as the total volatile bases (TVBs) (7). TVB nitrogen (TVB-N) has been used routinely as an indicator of fish quality and decomposition (2, 7–9).

Currently, methods available for TVB and TMA analyses (9–11) can be performed only in a laboratory. These methods can be time consuming, requiring skilled analysts while also posing potential safety hazards. Consumer demand for high-quality commodities (12, 13) and regulations of the U.S. Food and Drug Administration to guard against safety hazards and decomposition make a simple seafood quality screening procedure desirable for both industry and government. Ward et al. (14) described the use of a specific-ion electrode (ammonia) for determining the quality of raw, iced shrimp. This paper describes the use of an ammonia ion-selective electrode (ISE) to determine ammonia and TMA and to correlate ammonia and TMA results to TVB-N. The ISE measures NH_3 and TMA contents of a water-extracted fish sample without requiring protein precipitation or filtration. Aside from being simple, rapid, and accurate, the procedure offers on-site portability and application without need for extensive training requirements or full-scale laboratory or expensive equipment.

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Table 1. Precision and accuracy of ammonia electrode[a]

Standard concentration, ppm	Ammonia concentration recorded, ppm			
	Trial 1	Trial 2	Trial 3	Trial 4
5	5.5 ± 0.5	5.2 ± 0.1	4.7 ± 0.2	5.4 ± 0.6
	n = 9	n = 8	n = 9	n = 8
10	9.6 ± 0.4	8.4 ± 0.5	9.9 ± 0.5	10.0 ± 0.4
	n = 10	n = 7	n = 7	n = 8
20	19.5 ± 1.0	18.8 ± 0.4	19.7 ± 0.3	19.0 ± 1.1
	n = 34	n = 9	n = 10	n = 10
30	—	—	29.3 ± 0.3	28.9 ± 0.6
	—	—	n = 8	n = 7

[a] Slope repeatability of Orion ISE meter with ammonia probe, −54.9 ± 1.9 on 34 separate determinations; n = number of samples.

METHOD

Apparatus and Reagents

(a) *pH meter.*—Orion Model 290A portable pH/ISE meter (Fisher No. 13-641-248; Pittsburg, PA) with BNC connector.

(b) *Ammonia electrode.*—Orion Model 95-12 ammonia gas-sensing electrode (Fisher No. 13-641-922) with ammonia sensor membranes (Fisher No. 13-641-929).

(c) *Ammonia standard solutions.—(1) 1000 ppm stock solution.*—Weigh 0.315 g ammonium chloride (Fisher No. A661-500) into 100 mL volumetric flask and dilute to volume with distilled water. Alternatively, pipet 58.8 mL commercially prepared 1700 ppm NH_3 stock solution—0.1M NH_4Cl (Fisher No. 13-641-923)—into a 100 mL volumetric flask. Bring to volume with distilled water. This solution can be refrigerated for a week. Keep stock standard solutions refrigerated when not in use. *(2) Calibration solutions.—(a) 50 ppm solution.*—Pipet 5 mL 1000 ppm NH_3 solution into a 100 mL volumetric flask and bring to volume with distilled water. *(b) 20 ppm solution.*—Pipet 2 mL 1000 ppm NH_3 solution into a 100 mL volumetric flask and bring to volume with distilled water. *(c) 5 ppm solution.*—Pipet 0.5 mL 1000 ppm NH_3 solution into a 100 mL volumetric flask and bring to volume with distilled water.

(d) *Ionic strength adjuster (ISA).*—5M NaOH, 0.05M disodium ethylenediaminetetraacetate (EDTA) and 10% methanol in distilled water (Fisher No. 13-641-883). Alternatively, to prepare 200 mL ISA solution, weigh 3.72 g diso-

Table 2. Recovery of known additions of ammonia as part of tuna tissue homogenate

Statistic	Recovery of ammonia, mg/100 g, from tissue homogenate spiked with indicated amount of ammonia[a]			
	10 mg/100 g	20 mg/100 g	40 mg/100 g	60 mg/100 g
	13.6	17.6	39.2	49.2
	8.90	14.4	35.2	49.7
	13.3	13.4	36.6	55.9
	9.30	12.7	36.8	54.1
	10.2	19.8	35.4	52.9
	8.90	17.8	37.8	51.9
	10.9	15.8	33.2	52.1
	9.30	17.1		
	8.60	15.9		
	10.7	14.9		
	7.90	20.4		
	8.10	17.3		
	8.70	17.7		
	10.3	18.6		
	9.20	15.7		
Mean	9.86 ± 1.70	16.6 ± 2.21	36.3 ± 1.94	52.2 ± 2.35
Coefficient of variation, %	17.2	13.3	5.34	4.49
Percent recovery[b]	98.6	83.0	90.8	87.2

[a] Ammonia added as ammonia chloride. Amounts are final known concentration of added standard in tissue homogenate. Amounts recovered are background corrected depending on background determined on the day of analysis.

[b] Calculated from mean divided by standard addition multiplied by 100.

Table 3. Calculated response of ammonia electrode to trimethylamine (TMA) standards

TMA standard concentration, mg/100 mL[a]	No. of samples	TMA calculated from electrode determinations, mg/100 mL[a]	Relative recovery, %
5	5	3.7 ± 0.1	74.9 ± 1.9
10	5	8.5 ± 0.4	85.3 ± 4.1
20	10	15.9 ± 1.5	79.6 ± 7.3
30	10	27.5 ± 3.7	91.7 ± 12.4

[a] TMA concentrations calculated as relative ammonia because the ISE was standardized as ammonia.

dium EDTA into a 200 mL volumetric flask and add 20 mL methanol. Weigh 40 g NaOH in a 500 mL beaker and dissolve in 100 mL distilled water. Quantitatively transfer to 200 mL volumetric flask containing EDTA and methanol solution and bring to volume with distilled water.

(e) *Electrode filling solution.*—0.1M NH_4Cl (Fisher No. 13-641-928).

Table 4. Calculated response of the ammonia electrode with known additions trimethylamine (TMA) to tuna and cod tissue matrixes

TMA added, mg/100 g[a]	No. of samples	TMA found, mg/100 g fish[b]	Increase, %
		Tuna	
0	2	31.5[c]	—
10	4	41.8 ± 2.3	133
20	4	46.2 ± 1.6	147
30	4	72.0 ± 12.3	229
		Cod	
0	5	15.8 ±1.6[c]	—
10	7	18.4 ± 1.2	116
20	7	25.7 ± 1.2	163
30	7	34.3 ± 2.7	217

[a] Final known concentration of standard added to tissue homogenate.
[b] Calculated as ammonia.
[c] Background ammonia concentration.

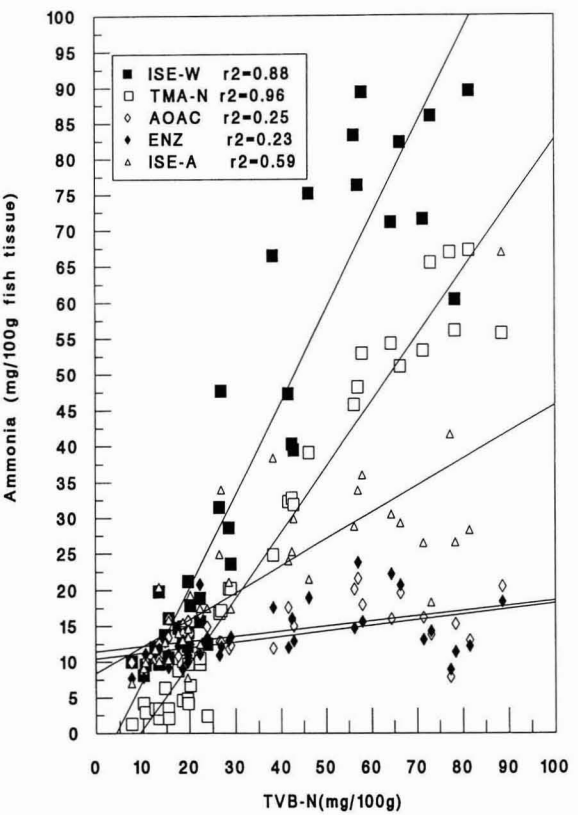

Figure 1. Correlation of total volatile base (TVB-N) with trimethylamine (TMA-N) and 4 methods of determining ammonia for 8 fish species (n = 39): ammonia ion-selective electrode and water-extracted sample (ISE-W); ammonia ion-selective electrode with acid-extracted sample (ISE-A); colorimetric (AOAC) and UV/enzymatic (ENZ).

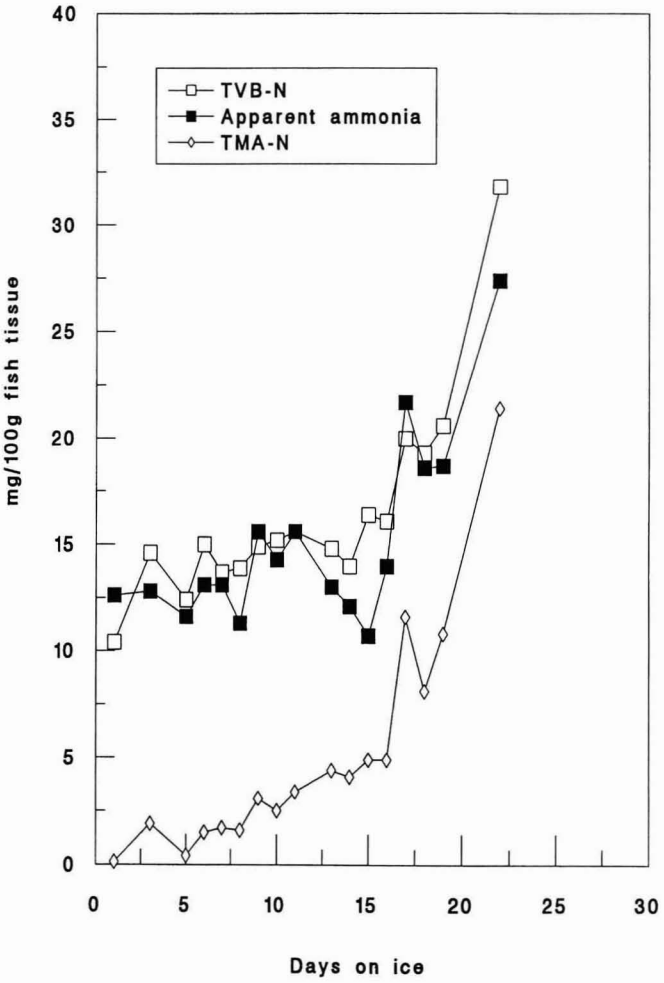

Figure 2. Pattern of volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of whole summer flounder (fluke, *Paralichthys dentatus*) held on ice at refrigeration temperature (3°–4°C).

Sample Collection

(a) *Random collections.*—The first sampling protocol involved random collections of fish from supermarkets by Rhode Island Department of Health sanitarians or purchase by investigators. The 39 samples included 8 fish species—cod, 17; haddock, 7; perch, 4; pollack, 3; hake, 2; salmon, 2; sole, 2; and flounder, 2—as identified by market labels.

(b) *Controlled aging.*—The second sampling scheme involved controlled aging of fish samples. Fish were obtained from local Rhode Island seafood processors, immediately transported on ice to the Rhode Island Department of Health, Food Chemistry Laboratory, for storage trials. These species included summer flounder (fluke, *Paralichthys dentatus*), skate (*Raja* sp.), whiting (silver hake, *Merluccius bilinearis*), cod (*Gadus morhua*), dogfish (*Squalus acanthias*), mackerel (*Scombers scombrus*), monkfish (*Lophius americanus*), and squid (*Loligo pealeii*). The fish were stored whole (flounder, whiting, mackerel, squid) or in other common market forms (cod fillets, skate wings, headed/gutted skin-off dogfish, or monkfish tails) on ice at refrigeration temperature (3°–4°C). Samples were held up to 3 weeks to achieve a full quality profile. Fish were sampled for chemical analysis initially and every 2–3 days thereafter. Samples were filleted when necessary and either analyzed immediately or frozen at –20°C until analyses could be completed. Frozen samples were thawed with cold running tap water prior to analysis.

Analysis of Ammonia

(a) *Calibration of ISE meter.*—*(1)* Prepare electrode according to the manual. Place ammonia membrane over outer tip of electrode casing similar to placing Parafilm over a test tube opening. Avoid touching the membrane that will come in contact with the inner reference solution and the external sample and standard solutions. A pair of tweezers may help. Screw on the external protector tip that holds the membrane in place.

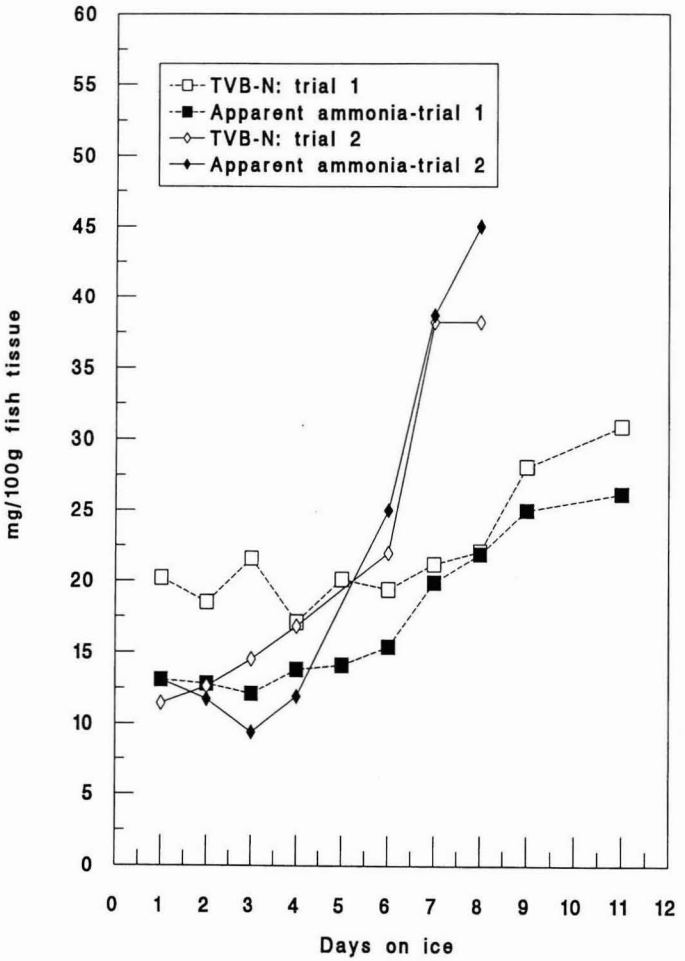

Figure 3. Pattern of volatile base (TVB-N) and apparent ammonia development during 2 storage trials of skate wings (*Raja* sp.) held on ice at refrigeration temperatures (3°–4°C).

With a Pasteur pipet, add NH_3 electrode filling solution (e) to inner casing of electrode. Insert reference electrode into casing with membrane on the tip and screw electrode into casing. Some filling solution will overflow but this ensures enough solution has been added. Allow membrane to equilibrate overnight in 100 ppm NH_3 solution. (*2*) Pour 100 mL 5.0 ppm NH_3 solution into a 250 mL beaker. Place electrode in beaker and add 2 mL ISA. The ISA will liberate NH_3 so measurement must be run immediately. While holding the electrode between index and middle finger, grab and rotate beaker with the same hand to thoroughly mix contents. This initial mixing is necessary to alkalinize the solution. Further mixing during the measurement may disrupt the equilibrium process and is not necessary as long as the ISE is thoroughly dispersed in the sample solution. Immediately go to the next step. (*3*) Turn ISE meter on. Wait for "P" to appear at the bottom of the liquid-crystal display (LCD). Press "mode" to select "conc." (*4*) Press "2nd" key then "cal" key. The LCD will show "P1." Wait for "ready" prompt. The word "Ready" will be flashing on the LCD once equilibration is reached. (*5*) To set the decimal point, press a scroll key "∧ or ∨" to bring dot to the right. When there is only one digit to the right of the dot, press "yes." (*6*) To enter "005.0" ppm, press "yes" to enter first digit "0." The second digit will be flashing. Using the "∧ or ∨" key, change the number to "0" and press "yes." The third digit will be flashing. Enter "5" using the "∧ or ∨" key and press "yes." The last digit will be flashing. Change the number to "0" and press "yes." "P2" will appear at the bottom of the LCD. (*7*) Rinse electrode with deionized water and place in a 250 mL beaker with 100 mL 50 ppm NH_3 solution. Add 2 mL ISA and mix as directed above. (*8*) Proceed as in step 6 to enter "050.0" ppm. When this step is completed, "P3" will be displayed. Press "measure" key to get the slope. While the slope should read (–)57 ± 3 according to the Orion manual, actual use on standards has shown that a slope of –55 ± 3 is acceptable. Rinse and place the electrode in a 250 mL beaker containing 100 mL 20 ppm NH_3 so-

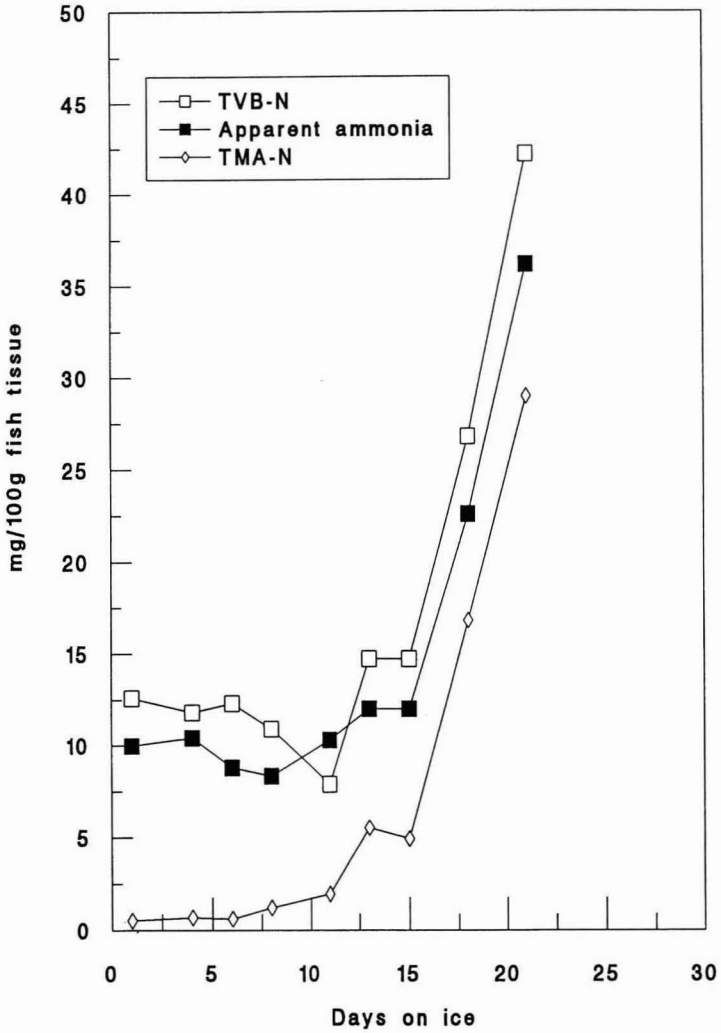

Figure 4. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of whole whiting (silver hake, *Merluccius bilinearis*) held on ice at refrigeration (3°–4°C) temperature.

lution (control). Add 2 mL ISA and wait for "ready" prompt to record meter reading. The 95% confidence interval for 34 replicate analyses of the 20 ppm control was 19.1–19.8 ppm. (*9*) Between measurements, keep electrode in a standard NH_3 solution with ISA (the 20 ppm control solution will do for this purpose). When not in use, store electrode in a 100 ppm NH_3 solution without ISA. Do not allow electrode to sit in a sample solution if analysis is interrupted to avoid clogging the electrode with fish tissue. Try to complete the current sample and place the electrode in a standard NH_3 solution as described above if further testing is postponed.

(b) *Determination.*—Weigh 5.0 ± 0.1 g comminuted fish in 250 mL blender. Record exact weight to 0.1 g. Add 95 mL distilled water. Blend at high speed for 2 min. Transfer mixture into 250 mL beaker and cover beaker with aluminum foil or Parafilm until measurement. Prepare 4–5 samples at a time for analysis. Run a fresh 20 ppm control after every fifth sample. If the reading is 18–22 ppm, more samples can be analyzed without recalibration. If the reading is outside these values, recalibrate with freshly prepared 5 and 50 ppm standards and verify with fresh 20 ppm control. Add ISA one sample at a time before placing electrode in sample solution. Do not add ISA to all the blended samples prior to batch analysis, otherwise NH_3 will be given off prematurely and lower readings will result on subsequent samples.

For determination of accuracy, weigh 5.0 ± 0.1 g comminuted fish in a 250 mL blender jar with 94 mL deionized water and then volumetrically pipet 1 mL 1000 ppm NH_3 standard solution to represent a 20 mg/100 g spike. Blend for 2 min at high speed and follow the same procedure as above.

(c) *Calculations.*—Use the following equation to determine amount of ammonia if sample is 5 g comminuted fish tissue:

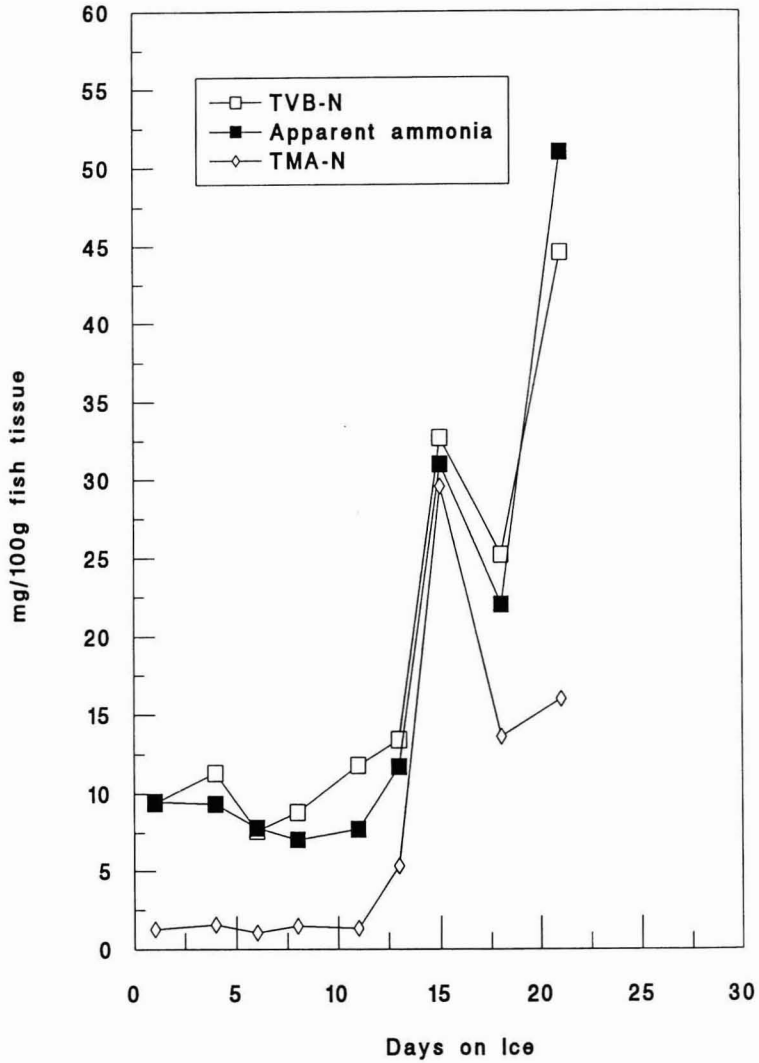

Figure 5. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of skin-off cod fillets (*Gadus morhua*) held on ice at refrigeration (3°–4°C) temperature.

$$NH_3, \text{ mg } \% = (100/Wt)(ppm)(1 \text{ mg}/1000 \text{ ppm})(100) = (2)(\text{ppm reading})$$

where Wt = weight of sample (5.0 g), ppm = direct ISE meter reading, 1000 = conversion of ppm to mg, 100 = conversion to 100 g sample.

$$\text{Recovery, } \% = (\text{mg } \% \ NH_3 \text{ spike} - \text{mg } \% \ NH_3 \text{ background})/20 \times 100$$

Analysis of TVB (8, 10)

(a) *Determination.*—Weigh 50 g comminuted fish in a 1000 mL blender jar, add 100 mL 7.5% (w/v) trichloroacetic acid solution (TCA) and blend at high speed for 2 min. Filter homogenate through Whatman No. 1 or equivalent filter paper. Pipet 25 mL TCA extract into a distilling flask (250 mL Tecator digestion tube, Fisher No. TC1000-0155 or equivalent) and add 10 mL 10% NaOH. Steam distill (Tecator Model 1002 precision distiller, Fisher No. TC1002-001 or equivalent) 75 mL liquid into 125 mL Erlenmeyer flask containing 10 mL Kjeldahl indicator solution (4 g boric acid in distilled water containing 0.7 mL 0.1% alcoholic solution of methyl red and 1.0 mL 0.1% alcoholic solution of bromocresol green diluted to 100 mL in distilled water) as found in AOAC Method **981.10**, reagent **(b)** (15). Titrate green alkaline distillate with 0.025N sulfuric acid to original red color.

(b) *Calculations.*—Use the following equation to determine TVB:

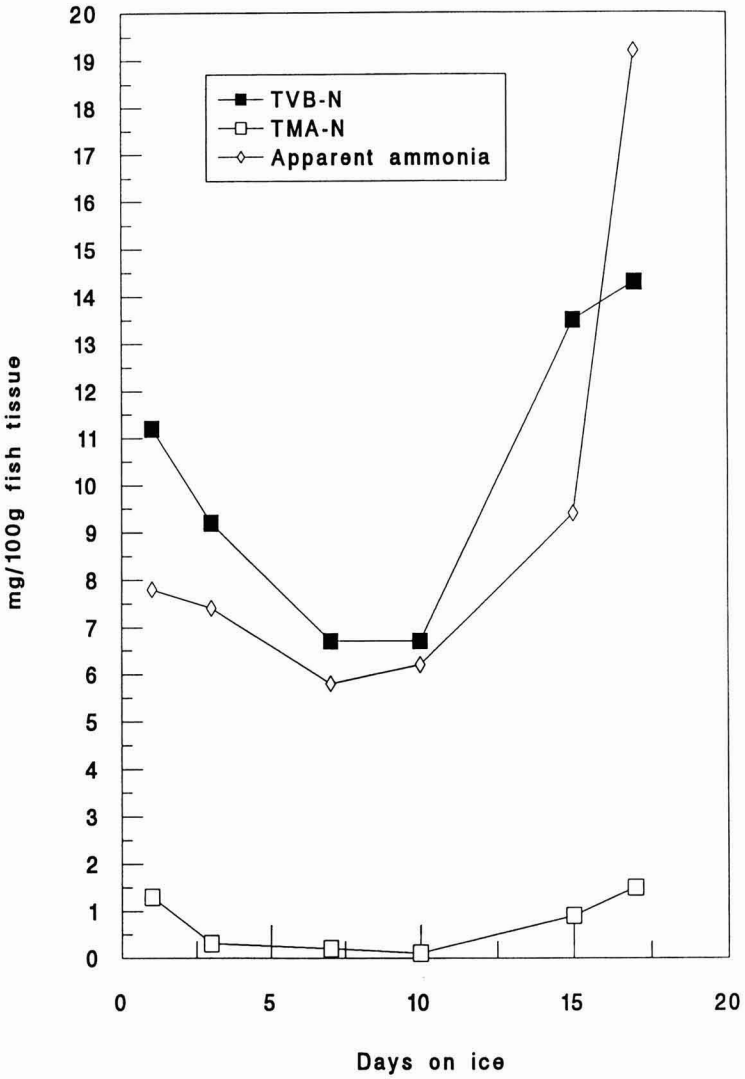

Figure 6. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of headed/gutted, skin-off dogfish (*Squalus acanthias*) held on ice at refrigeration (3°–4°C) temperature.

$$\text{TVB, mg N/100 g} = (\text{mL titrated}) \times 4.2$$

The factor 4.2 factor is derived as follows: (*1*) 50 g fish/[50 g fish + 100 mL TCA] = 0.33 g fish/mL blended. (*2*) 25 mL filtrate × 0.33 g fish/mL = 8.33 g fish equivalent in distillation tube. (*3*) Converting to 100 g fish, multiply by 100/8.33 = 12.00. (*4*) mg N to 1.0 mL acid = (N acid)(mL acid) (meq wt nitrogen) = (0.025)(1.0)(14) = 0.35. (*5*) mg TVB N/100 g = (0.35)(12.00) = 4.2.

Analysis of TMA

Use extract from TVB procedure and follow AOAC Method **971.14,** *Trimethylamine Nitrogen in Seafood* (11), beginning with step *D, Determination.*

Analysis of Ammonia by Other Methods

(a) *Colorimetric.*—Follow AOAC Method **973.25,** *Ammonia in Crabmeat, Colorimetric Method* (16).

(b) *Enzymatic.*—Ammonia was determined on prepared fish extracts by using a commercial enzyme test kit combination (available through Gene Trax, Hopkinton, MA; 17). The enzyme test was run on samples according to a modified version of a method developed by McCarthy et al. (18).

Ten grams minced fish tissue was blended with 50 mL 0.6N perchloric acid. The entire homogenate was transferred quantitatively with distilled water (30–40 mL) through Whatman No. 1 filter paper into a 100 mL beaker. The filtrate was adjusted to pH 7.0–7.5 with 20% KOH (3.0–3.5 mL) by

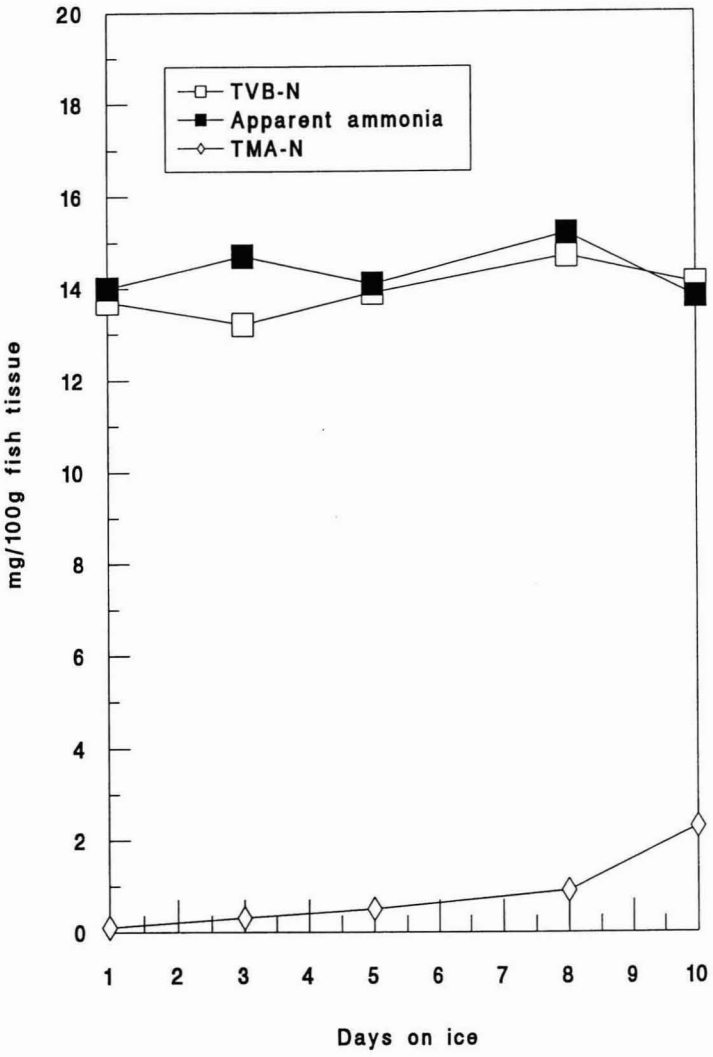

Figure 7. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of Atlantic mackerel (*Scomber scombrus*) held on ice at refrigeration (3°–4°C) temperature.

means of a pH meter. If pH 7.5 was overshot, 5N HCl was added to bring the pH to within the specified range. The pH-adjusted filtrate was then brought to a final volume with water in a 100 mL volumetric flask. The dilution factor was 10. Results were expressed in g/L (mg/mL), as determined with the enzymatic kit calculations. The g/L values were multiplied by the dilution factor and 100 to express results as mg/100 g fish tissue.

Results and Discussion

Tables 1 and 2 document the precision, accuracy, and reproducibility of the ISE method. Table 1 shows the method's good reproducibility, with calculated ammonia in standards ranging from 84 to 110% in standards. The percent recovery reflects the mean value of the individual trial divided by the standard concentration that was assayed. Table 2 shows good recoveries from a homogenized tuna matrix—83.0–98.6%—at the 4 ammonia addition levels tested.

Tables 3 and 4 show data that support a hypothesis that TMA has an impact on the probe response. Standard concentrations of TMA, as measured by the probe, produced a relative high response (calculated as ammonia) and were reasonably consistent and predictable. This probe response pattern is further documented by the increase in the calculated response of the ammonia electrode when known amounts of TMA were added to tuna and cod tissue matrixes. Therefore, these results

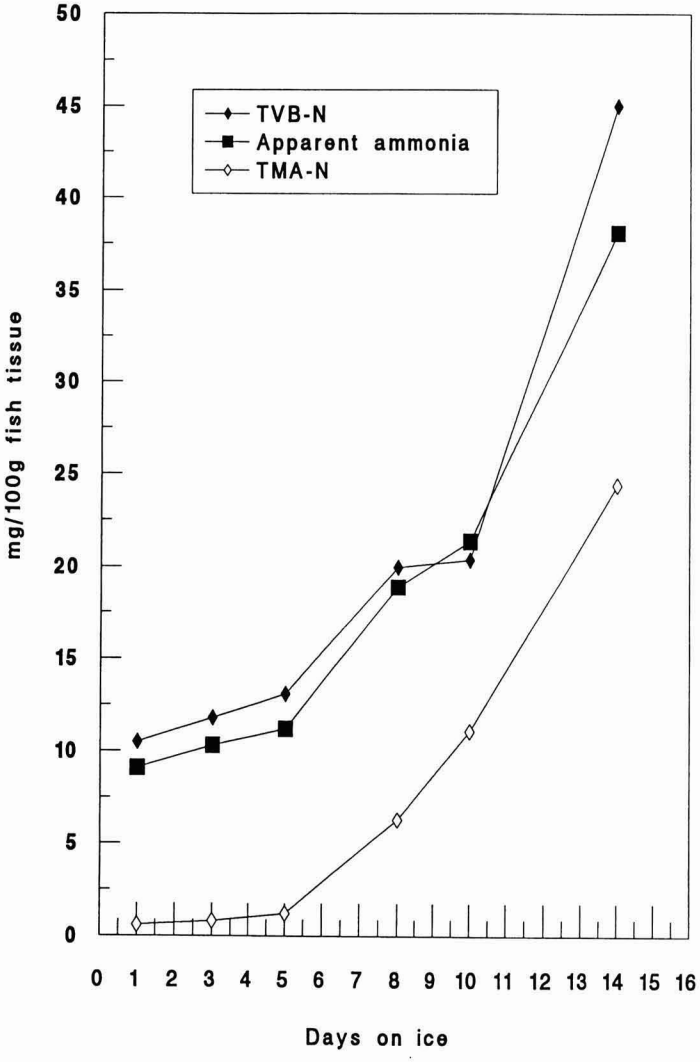

Figure 8. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of monkfish (*Lophius americanus*) tails held on ice at refrigeration (3°–4°C) temperature.

give evidence to the ability of the ISE to act more as a TVB probe. The lack of selectivity to the small-molecular-weight volatile bases and its apparent ability to act as a TVB probe increase the value and usefulness of this method in terms of evaluating the magnitude of fish quality changes from fresh to marginal to unacceptable. Therefore, "apparent" or "relative" ammonia concentrations will be used to further describe results.

Thirty-nine random fish samples, mostly lean, were collected from stores within the state of Rhode Island and analyzed for quality by the various methods outlined in the methods section of this paper. Figure 1 shows the relationship between TVB-N analysis and a variety of ammonia determination procedures including the ISE-W method, and TMA measurements. The figure illustrates that only the ISE-W and the TMA methods are strongly correlated with the TVB-N measurements thereby clearly reflecting the changes that were occurring due to spoilage.

Figures 2–9 illustrate the patterns of TVB, TMA, and apparent ammonia development during storage of the 8 species tested. Many of these—skate, dogfish, mackerel, monkfish, and squid—are highly exportable species in the Northeast region and are subject to judgment by inspectors at a foreign port of entry. For the species evaluated, apparent ammonia, TMA, and TVB analyses clearly show the same trends over the storage periods. More significantly, however, the ISE method mirrors the TVB concentrations, as determined by TCA extraction and Tecator steam injection distillation methodology. The consistency of this trend among the species tested indicate the

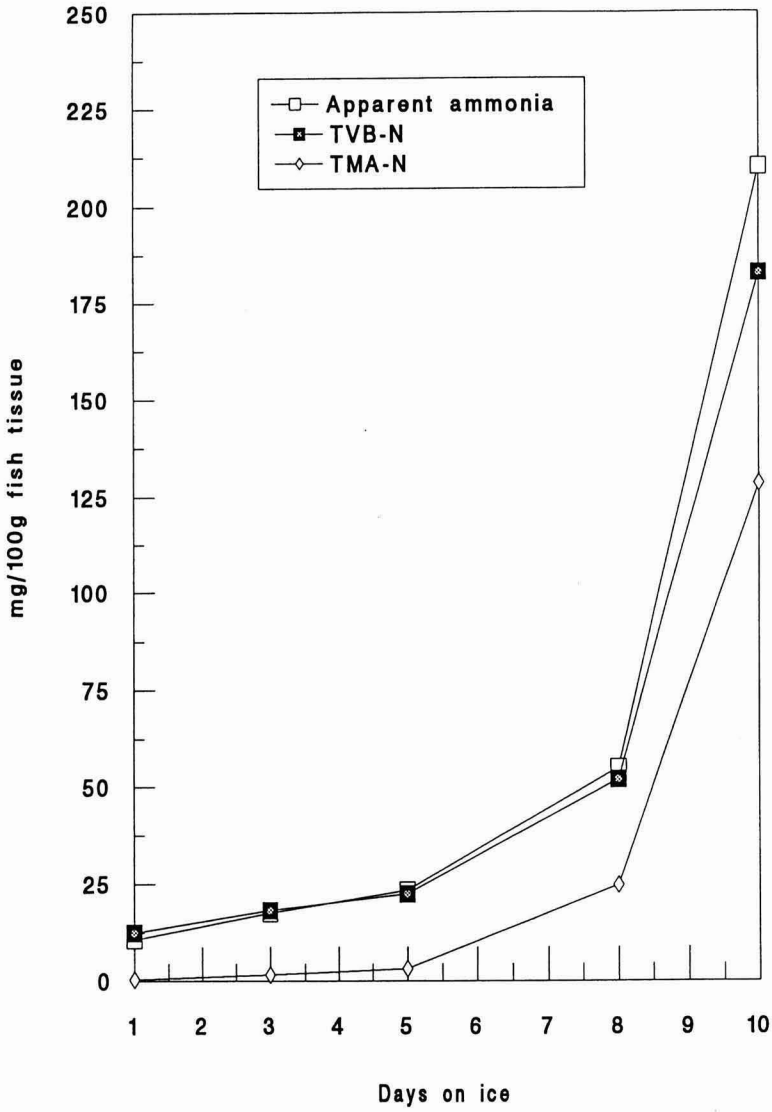

Figure 9. Pattern of total volatile base (TVB-N), trimethylamine (TMA-N), and apparent ammonia development during storage of whole squid (*Loligo pealeii*) held on ice at refrigeration (3°–4°C) temperature.

enormous potential of the ISE method in reflecting seafood quality and decomposition. However, identification of distillation method is critical to the quantitative agreement between TVB-N and ISE methodologies on fish. As previously reported by Botta et al. (19) on Atlantic cod and reconfirmed during method development in this laboratory (results not shown), the distillation method chosen had an impact on the empirical TVB-N values obtained but not on the overall quality profile with storage.

Preliminary sensory analysis was conducted on the fish, prior to freezing, by 10–12 untrained panelists using a 5-point hedonic scale (20) based primarily on the acceptance or rejection of odor and appearance. There appeared to be a relationship between apparent ammonia levels, as measured by the probe, and quality decline. This association is currently being addressed by ongoing research in our laboratories involving carefully designed chemical and sensory experimental protocol.

The ammonia ISE method for determining volatile bases in fish has enormous potential as a simple, rapid method for screening fish quality and decomposition. This procedure could be applied in a processing plant for routine on-site quality control monitoring or by regulatory agencies for rapid screening for decomposition of suspect fish.

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Determination of Malathion Residues in Some Medicinal Plants by Liquid Chromatography with Gas Chromatographic/Mass Spectrometric Confirmation

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A method for determining malathion residues by reversed-phase liquid chromatography (LC) using methanol only as mobile phase is described. Malathion [diethyl(dimethoxyphosphinothiol)succinate] was applied on marjoram, mint, and chamomile. Residues were detected in fresh and dry crops by LC and confirmed by gas–LC/mass spectrometry. Average recovery of malathion was 85%. Residues detected in fresh marjoram, mint, and chamomile were 0.18, 0.23, and 0.083 mg/kg, respectively. Residues detected in dry marjoram and mint were 0.024 and 0.050 mg/kg, respectively. No malathion residues were detected in dry chamomile. The minimum detectable concentration with this method is 0.013 mg/kg. The study suggests it is safe to use malathion up to 2 sprays per season provided the crop is harvested not less than 3 weeks from the last spray.

The demand for herbal plants is currently on the increase. People who object to use of synthetic drugs because of the side effects they may elicit often revert to herbal medicines. Moreover, extracts of herbal plants are widely used in the cosmetics and pharmaceutical industries.

Some herbal plants are attractive prey to insect pests and plant pathogens. Use of pest control chemical agents is often imperative to keep pests in check and to fetch a sound yield. International codes of pesticides residue levels could restrict sales of crops that contain pesticide residues.

Several reports have identified residues of commonly used pesticides in major vegetable crops (1–4). Pesticide residues in medicinal and aromatic plants also have been identified. Mikolajewisz et al. (5) studied the rate of disappearance of the organophosphate fenitrothion in some medicinal plants. Molto et al. (6) used gas chromatography/mass spectrometry (GC/MS) to detect residues of some organochlorine pesticides in mint, vervain, chamomile, and tea. Pluta (7) studied residues of some organochlorine insecticides in some medicinal plants. Belanger (8) investigated residues of azinophos-methyl, cypermethrin, benomyl, and chlorothalonil in peppermint and monarda oil. Azinophos-methyl applied at 1.1 kg/ha showed no residues at harvest or 28 days after application. Cypermethrin applied at 0.2 kg/ha resulted in residue levels of 0.07 ppm in peppermint oil and 0.8 ppm in monarda oil at harvest. Chlorothalonil applied at 2.5 L/ha resulted in 0.44 and 0.07 ppm residues in peppermint oil and monarda oil, respectively.

In the present study, 3 major herbal plants, marjoram (*Majorana hortensis* L.), mint (*Mentha* sp.), and chamomile (*Matricaria chamomilla* L.), were treated with malathion [diethyl(dimethoxyphosphinothioyl)succinate] at a rate of 87 g active ingredient/100 L water. Residues were detected in fresh and dry crops. A reversed-phase liquid chromatographic (LC) method based on use of methanol as mobile phase was developed as an easy alternative to the previously reported normal-phase method (9) or GC method (10).

Experimental

Field Applications

Malathion formulated at 57% emulsifiable concentrate was applied on marjoram, mint, and chamomile at the manufacturer recommended rate, 87 g active ingredient/100 L water. A knapsack sprayer fitted with a single-nozzle boom applied the malathion solution at 300 L/acre.

Malathion was applied on marjoram and mint twice, with a 21-day interval. Chamomile was treated only once. Control plots were treated with similar volumes of water with no insecticide. Malathion and control treatments were applied according to a completely randomized block design. The recommended 2-week security period of malathion was observed.

Sampling

Ripe marjoram, mint, and chamomile (1 kg) were collected from treated and control plots 44 days after treatment. In addition, portions of ripe plants were dried under room conditions for 1 month. At ca 75 days after treatment, samples of each dried plant were extracted to monitor malathion residues. Representative samples of each crop were chopped and mixed thoroughly. Three representative of fresh (50 g) and dry (10 g) subsamples of each crop were taken for analysis.

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Table 1. Recovery from fortified samples

Sample and wt, g	Malathion added, μg	Malathion recovered, μg			Recovery, %
Fresh, 50	0	0	0	0	0
	10	8.2	7.8	8.6	82
	25	21	23	21	86.6
	50	44	43	47	89.3
Dry, 10	0	0	0	0	0
	2	1.8	1.5	1.6	81.6
	5	4.1	4.2	4.3	84
	10	9	8.5	8.5	86.6

Extraction

Extraction of malathion residues was performed according to the procedure of Luke (11, 12).

Cleanup

Solid-phase extraction Florisil cartridges (Sep Pak 0.9 g; Waters Associates, Milford, MA, part No. 51960) were used for cleanup. Each cartridge was activated by passing 0.5 mL hexane–acetone (9 + 1, v/v) with a Luered tip glass syringe. Sample extract (1 mL) was poured into the syringe barrel and passed through the cartridge. Malathion was eluted with 9 mL hexane–acetone (9 + 1, v/v) at 1 mL/min. Extracts were evaporated to near dryness with a gentle stream of nitrogen, and residues were made up to 1 mL with acetone and kept in air-tight glass vials ready for analysis.

Validation Study

Fresh and dry control mint samples were fortified with a standard solution of malathion in acetone (200 mg/L) at 3 levels. To 50 g fresh sample, 50, 125, or 250 μL standard solution was added. To 10 g dry sample, 10, 25, or 50 μL standard solution was added. Final concentrations of malathion in fresh and dry samples were equivalent to 0.2, 0.5, and 1 mg/L. At each fortification level, 3 replicates were made.

Liquid Chromatography

Reversed-phase LC was performed with a Beckman 432 liquid chromatograph (Beckman, Arlington Heights, IL) equipped with 2 pumps (Model 112), a solvent programmer (Model 340), an injector (Model 210), and a fixed-wavelength ultraviolet detector (Model 160). An Ultrasphere (Beckman) C_{18} octadecyltrichlorosilane (ODS) analytical column (25 cm × 4.6 mm id) was used. Chromatography-grade methanol (Merck, Darmstadt, Germany) was filtered through membrane filters (Millipore, Waters Associates; pore size, 0.45 μm) and degassed in an ultrasonic bath (Cole Parmer, Niles, IL, part No. G-08851-02) before use. The mobile phase was methanol at a flow rate of 0.7 mL/min. Malathion was detected and quantitated by monitoring UV absorbance of column eluates at 254 nm. Peak areas were measured with a Spectra Physics Data Jet computing integrator. Under these conditions, the retention time of malathion was ca 3.8 min. A standard curve for malathion was constructed by plotting peak area against concentrations (external standard method). Good linearity was observed over a 200-fold range (0.5–100 ng).

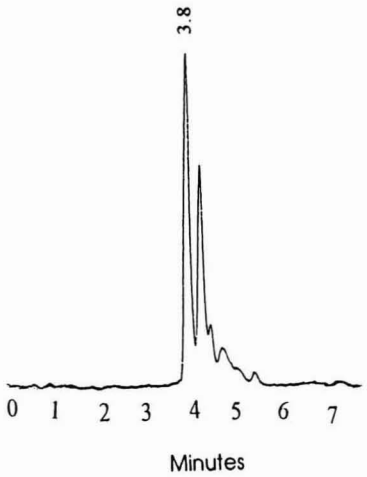

Figure 1. Typical chromatogram of malathion residues in marjoram. Malathion retention time is 3.8 min.

Gas Chromatography/Mass Spectrometry

A Hewlett-Packard gas chromatograph 5830 Series II equipped with Model 5972 quadrupole mass spectrometer and with HP pesticides library data system was used to identify and confirm malathion. An HP-5 MS capillary column (cross-linked, 5% phenyl methyl silicone; 30 m × 0.25 mm id) with 0.25 μm film thickness was used. GC operating conditions were as follows: splitless injections; injector temperature, 225°C; detector temperature, 280°C; helium carrier gas flow rate, 1 mL/min. The temperature program was 150°C for 0 min, 20°C/min to 270°C for 15 min.

A standard solution of malathion was analyzed with a scan range from *m/z* 50 to 550 under full-scale conditions. The criteria for malathion identification were coelution of all characteristic ions within ± 0.02 min, and agreement of retention time windows and relative abundances of selected masses within 20%. Determination was performed by comparing the peak area of a single primary ion obtained by external standardization.

Results and Discussion

The efficiency and reproducibility of the method were determined with samples fortified at 3 levels (Table 1). Average recovery of malathion was 85% (standard error of the mean, 5.2). No significant differences in recoveries from fresh and dry samples were observed. Results were not corrected according to recovery rate. The minimum detectable concentration that gave a signal 2 times higher than the blank signal at the malathion retention time when 25 μL blank extract was injected was 0.013 mg/kg.

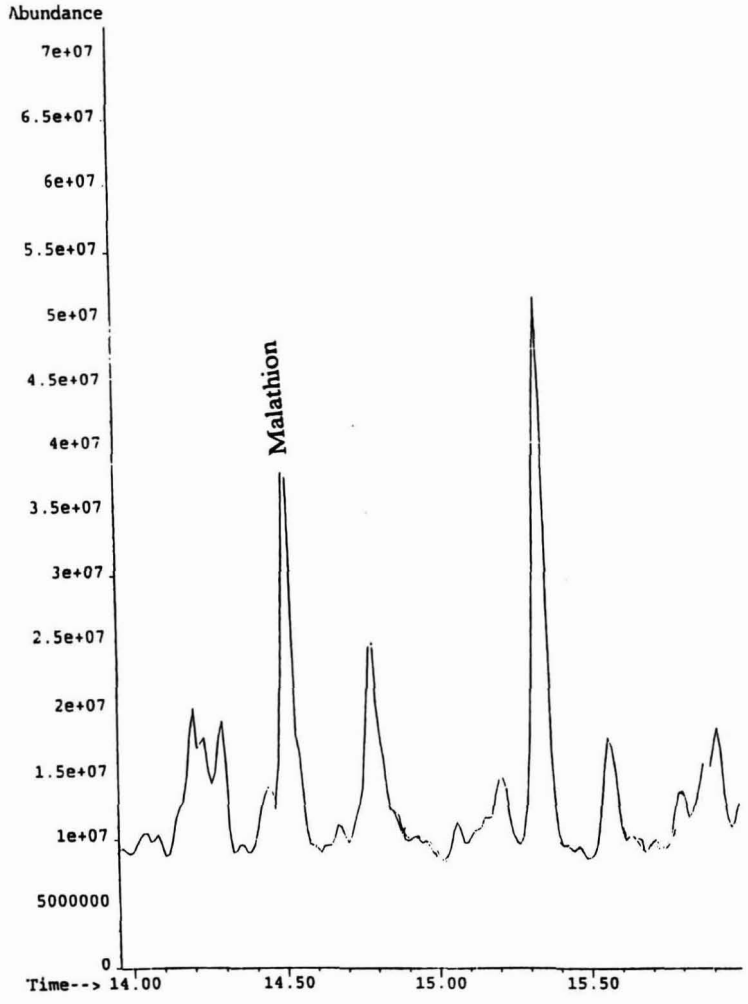

Figure 2. GC/MS chromatogram of malathion residues in mint extract.

The reversed-phase chromatography reported here offers an easy alternative to normal-phase chromatography (9), which is a cumbersome method with fairly erratic baseline. A representative reversed-phase liquid chromatogram of malathion residues in marjoram is shown in Figure 1.

Results were further confirmed by GC/MS. A GC/MS chromatogram of malathion residues in mint extracts is shown in Figure 2. The mass spectrum of malathion separated from chamomile is shown in Figure 3.

Fresh and dry samples of mint and marjoram from crops that were sprayed twice with malathion had detectable concentrations of malathion (Table 2). Variations in residue levels in crops treated twice are probably due to variations in plant morphology, such as posture and leaf texture. Various studies have confirmed the influence of plant shape and texture on the content of pesticide residues (3, 13). Chamomile, which was sprayed only once, had detectable concentrations of malathion in fresh samples but not in dry samples. Results also indicate a major decrease in malathion residues in dried crops, due to degradation of malathion.

The maximum residue limit (MRL) set by the Codex Alimentarius Committee for Pesticides Residues (14) for malathion residues in crops vary to a large extent. For example, MRLs for nuts, citrus, lentils, and lettuce are 8, 4, 8, and 8 mg/kg, respectively. On the other hand, the MRL for many vegetables, including eggplant, peas, pepper, and root vegetables, is 0.5 mg/kg. The committee has not set an MRL for pesticides in herbal plants.

In view of the present results and considering the MRL set by the Codex Aliminarius Committee for vegetable crops, it is probably safe to recommend use of malathion on herbal plants up to 2 applications per season, provided the crop is harvested not less than 3 weeks after the last spray.

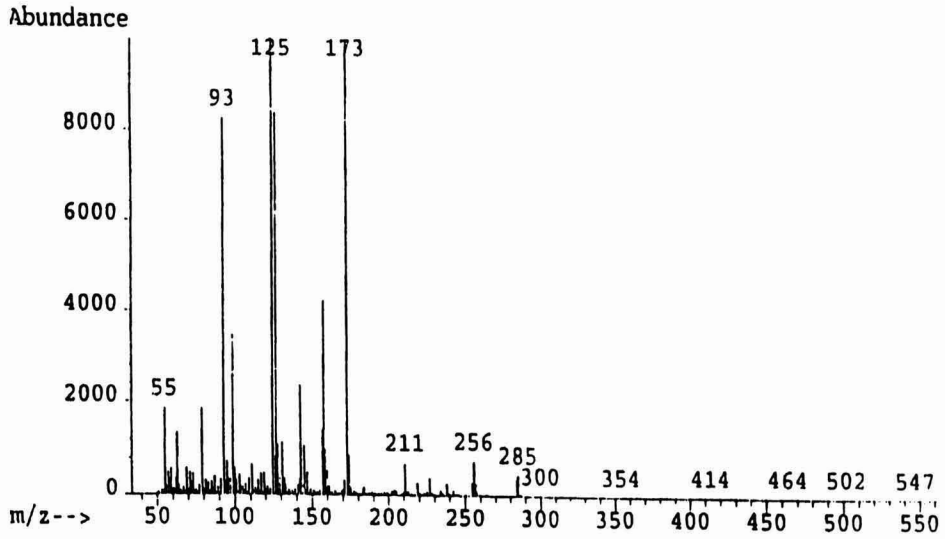

Figure 3. Mass spectrum of malathion separated from chamomile extract.

Table 2. Malathion residues on fresh and dry margoram, mint, and chamomile[a]

Crop	Condition	Residue detected, mg/kg
Marjoram	Fresh	0.18 ± 0.056
	Dry	0.024 ± 0.014
Mint	Fresh	0.23 ± 0.0078
	Dry	0.050 ± 0.023
Chamomile	Fresh	0.083 ± 0.045
	Dry	ND

[a] Results are expressed as means ± standard deviations for 3 determinations. ND = not detected.

Acknowledgment

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RESIDUES AND TRACE ELEMENTS

Evaluation of Passive Samplers for Analysis of Chlorinated Solvents

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Chlorinated solvents like tetrachloroethylene, chloroform, and trichloroethylene were trapped on commercially available diffusive sampling devices and then desorbed with carbon disulfide (1.5 mL) containing surrogate standards. The extract was analyzed by gas chromatography with electron capture detection (ECD). To overcome the incompatibility of carbon disulfide with the electron capture detector, an electronically driven, high-temperature, 6-port valve was attached to the oven of a the gas chromatograph. An RTX 502 (105 m × 0.53 mm) capillary column was connected between the injector and the detector through the 6-port valve. The solvent peak was vented by rotating the valve before the peak reached the detector, and the valve was rotated to its original position before the peak due to chloroform eluted. The sampling time of 8–24 h and the use of ECD have led to detection limits of 0.5 μg/m^3 for many chlorinated solvents.

Analysis of volatile organic compounds (VOCs) in air can be performed by pulling air with a pump through adsorbents like Porapak N (1), charcoal (2), Tenax, and other adsorbents (3) or by collecting air samples in evacuated deactivated canisters (4). Before analysis, the adsorbed samples can be extracted with a solvent (Porapak N and charcoal) or thermally desorbed (Tenax). Samples collected in canisters can be cryogenically concentrated. Then, samples may be analyzed by gas chromatography (GC) with flame ionization, mass spectrometric, electron capture, or photoionization detection.

Active (pump-based) sampling methods work very well but require special equipment, which can be cumbersome. For large-scale sampling, active methods can become very expensive. Passive samplers that collect VOCs by molecular diffusion are relatively inexpensive, are easy to use, and require no additional special equipment. They provide another approach for collecting VOCs.

Lewis et al. (5, 6) developed a passive sampler with Tenax TA as adsorbent that could be thermally desorbed, thus achieving low detection limits for most compounds. Halmans et al. (7) described a tube-type passive sampler that is easy to desorb thermally with standard commercially available desorbers. However, thermal desorbers do not have the flexibility provided by automatic liquid injectors.

Commercially available passive samplers with carbon-based sorbents have been used extensively by the National Institute for Occupational Safety and Health (8) to monitor exposure of workers to various solvents in industrial work settings. Hammond et al. (9) and Weschler et al. (10) have performed validation experiments as well as analyses of various VOCs using commercially available passive samplers. They exposed these samplers up to 3 weeks and were able to achieve low detection limits (1 μg/m^3), which are required to monitor VOC levels in ambient air. Coutant (11) tried to desorb various VOCs from charcoal-based passive samplers using 5% carbon disulfide in methanol and analyzed the compounds by GC with electron capture and photoionization detection in series, but overall desorption yields of various compounds were low.

Most commercially available passive samplers are based on charcoal and require carbon disulfide to completely desorb most of the adsorbed organic compounds. The carbon disulfide eluate is then analyzed by GC with flame ionization detection. This detector has good sensitivity for hydrocarbons, aromatics, etc., but is not good for chlorinated solvents like tetrachloroethylene (TCE). To achieve good sensitivity and reduce sampling time for chlorinated solvents, electron capture detection (ECD) is preferred, but unfortunately, carbon disulfide is not compatible with ECD. We overcame this difficulty by venting the solvent before it reaches the detector. With a 24 h sampling time, the approach has enabled us to achieve low detection limits (0.5 μg/m^3) for a number of chlorinated solvents.

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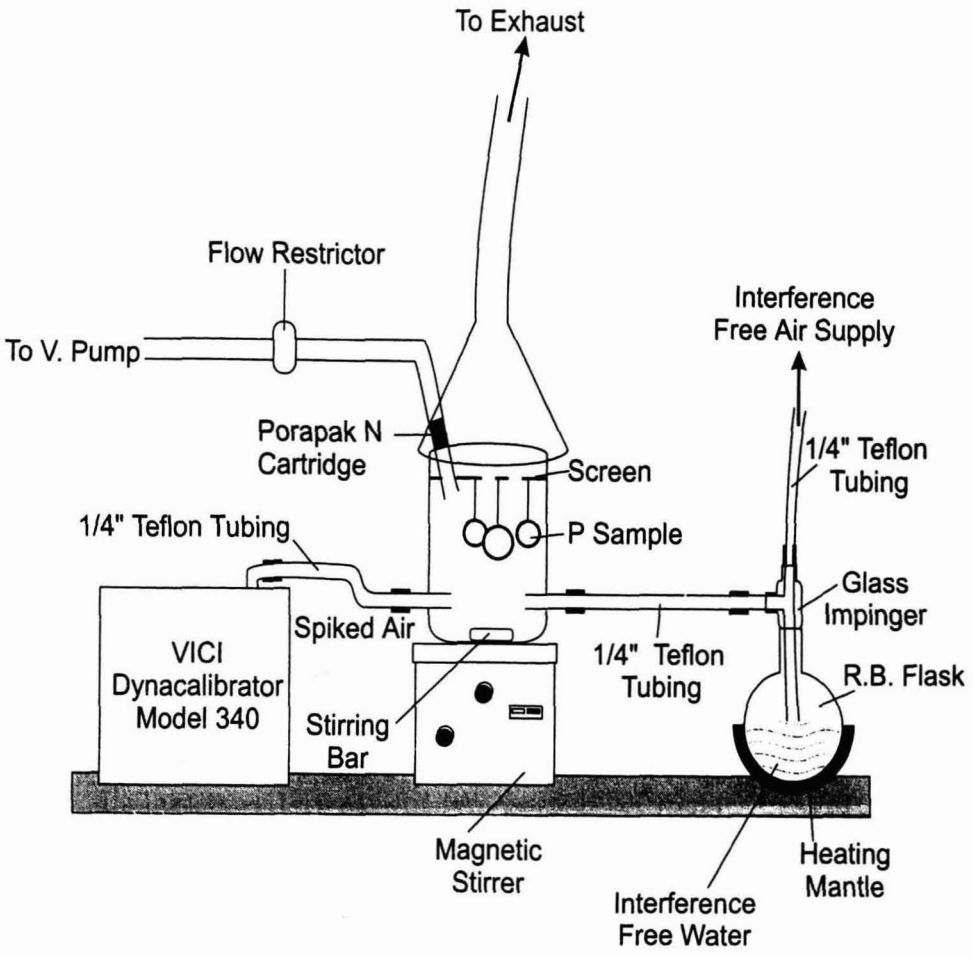

Figure 1. Vapor generator and collection system. All glass to teflon connections made using 1/4" Swaselok fittings.

Experimental

The Dynacalibrator Model 340 and the permeation tubes containing various solvents were purchased from Vici Metronics, Santa Clara, CA. Passive samplers used were 3500 series samplers (3M, St. Paul, MN) 575 series samplers, and charcoal (20–40 mesh; SKC, Inc., Eighty Four, PA). RH-30 humidity/temperature indicator was purchased from Omega Engineering, Stamford, CT; mass flow controllers were from Teledyne Hastings, Hampton, VA; electronically driven 6-port valve was from Valco Instruments Co., Inc., Houston, TX; flow restrictors, Model SC423SXFIHT, were from Veri Flow Corp., Richmond, CA; RTX-502.2 capillary column (105 m, 0.53 mm id) was from Restex Corp., Bellefonte, PA; liquid chromatography grade carbon disulfide was from Aldrich, Milwaukee, WI; methanol (high purity) was from Burdick and Jackson, Muskegon, MI. Porapak N was purchased from Alltech Assoc., Inc., Deerfield, IL. All the reagents used to prepare analytical standards were of high purity (99.99%).

Active Cartridges with Porapak N

These cartridges were prepared according to a published procedure (1). Briefly, Porapak N, 80–100 mesh (Analabs, Inc., Milford, MA), is conditioned overnight in wide-bore glass columns (1.2 × 20 cm) at 180°C under a flow of carrier gas (He or N_2, 20 mL/min). The conditioned Porapak N (400 ± 10 mg) is sandwiched between 2 glass-wool plugs in a Pyrex tube (25 cm × 6 mm). These tubes are washed under methanol (30 mL) with gentle suction and reconditioned at 160°–170°C under He or N_2 flow (20 mL/min). After being reconditioned, the cartridges are allowed to cool in a solvent-free atmosphere and then plugged with polyethylene caps until use. Used cartridges are reconditioned by washing with methanol (30 mL) under gentle suction and heating at 160°–170°C for 30 min under He or N_2 flow (20 mL/min).

Active Cartridges with Porapak N/Charcoal

To the Porapak N cartridge prepared above was added charcoal (20–40 mesh; 120 ± 10 mg) and the end was plugged with

Table 1. Solvent effect on response of internal standards

Source of standards	Solvent	*n*	1,2-Dibromoethane		Chlorofluorobenzene	
			Average area	RSD, %	Average area	RSD, %
3M Passive Sampling Devices extract	CS_2	15	62 500	3.2	58 000	4.1
SKC Passive Sampling Devices extract	CS_2	4	59 600	1.5	51 300	2.4
TCE standard	CS_2	16	67 500	8	63 300	6.4
TCE standard	Methanol	12	29 100	4	37 500	5

glass wool. The cartridge was reconditioned at 150°–170°C with nitrogen (ca 20 mL/min) flowing through it, the direction of flow being through charcoal first followed by Porapak N.

Calibration of Permeation Tubes

Permeation tubes were weighed and placed in the heater of the Dynacalibrator. The temperature of the heater was maintained at 50°C. These tubes were reweighed after 3 weeks, and the loss per minute of various analytes was calculated.

Gas Chromatography

A Hewlett Packard 5840 gas chromatograph was fitted with a high-temperature, electronically-driven, 6-port valve. The column end was attached to the detector through this valve in such a way that, during venting, the detector makeup gas flow (ca 45 mL/min) was not interrupted. A wide-bore capillary column (RTX-502.2, 105 m, 0.53 mm id) was used. General conditions were: detector temperature, 300°C; injector temperature, 200°C; column oven was kept initially at 50°C for 5 min and then programmed to 200°C at 4°C/min and kept at the final temperature for 20 min. The venting valve was opened 2 min after injection of sample and closed at 14.2 min into the analytical separation.

Exposure Chamber

The chamber (Figure 1) consists of a round glass jar (15 × 25 cm) with 2 inlets near the bottom. One inlet was used to introduce the air spiked with volatile haloorganics, and the second, to add dry or humidified air for dilution. The ratios of these gases were adjusted to achieve proper concentration and humidity. The air used for dilution, as well as that for the calibrator, was purified by passing through traps containing molecular sieves, silica gel, and charcoal. The flow of purified air was controlled with mass flow controllers. A magnetic bar (10 cm long) was placed in the jar. The jar was placed on a magnetic stirrer, and the contents were stirred continuously to create an upward draft during sampling. Purity of the air used was monitored by sampling the air in the jar with Porapak N tubes (1).

Sample Exposure

Spiked air was generated by placing calibrated permeation tubes in the heater of the Dynacalibrator. Sampling rate through Porapak N tubes was adjusted (28–32 mL/min) with the help of restrictors to match the diffusion rate of different compounds through the 3M passive samplers. The passive samplers, as well as the active tubes containing Porapak N or Porapak N/charcoal, were placed in the middle of the jar, as shown in Figure 1. These were removed after the required exposure time had elapsed. The passive samplers were sealed according to the manufacturer's directions, and the ends of the active sampling tubes were sealed with Teflon caps (1).

Sampling at Above-Normal Levels of Relative Humidity

Interference-free air used for dilution was passed through an impinger filled with interference-free water. The flow of the air and the temperature of the water in the impinger were adjusted to achieve a relative humidity of 40–85% at 25°C. The humidity was maintained at the lower level (ca 40%) for 1 h before the passive samplers, as well as the active sampling tubes, were

Table 2. Recovery of direct spikes for 3M passive monitors (*n* = 6)

Analyte	Spiking level, µg	Recovery ± RSD, %	Amount in blanks (µg)[a] ± RSD
$CHCl_3$	0.15	85 ± 20	0.006 ± 0.003
CH_3CCl_3	0.15	100 ± 11	0.042 ± 0.004
CCl_4	0.15	98 ± 12	0.017 ± 0.001
$C_2H_3Cl_3$	0.15	90 ± 7	0.011 ± 0.003
C_2Cl_4	0.15	103 ± 5	0.009 ± 0.002
C_2Cl_4	5.0[b]	103 ± 4	
C_2Cl_4	10.0[b]	83 ± 9	
C_2Cl_4	50.0[b]	94 ± 5	
C_2Cl_4	100.0[b]	91 ± 2	

[a] *n* = 4.
[b] *n* = 3.

Table 3. Collection of TCE with different samplers

Sampler	*n*	Sampling time, min	Sampling volume, L	Experimental air concn ($\mu g/m^3$) ± RSD (%)	Calculated amount,[a] $\mu g/m^3$
3M3500	6	0.5	0.849	200 ± 10	122
SKC575	3	0.5	0.387	130 ± 20	122
Porapak N	6	0.5	3.46	150 ± 10	122
3M3500/3520 front	6	18.25	30.989	150 ± 10	122
3M3520 back[b]	3	18.25	30.989	<0.5	122
3M3500	3	64.16	108.9	520 ± 20	430
SKC575	3	1	0.77	500 ± 0	430
Porapak N	3	1	1.68–1.95	530 ± 40	430
3M3500	3	20	33.96	1.7 ± 0.6	1.2
Porapak N	3	20	30.96	1.2 ± 0.8	1.2

[a] Amount calculated based on permeation rate and dilution factor.
[b] Back portion of sampler (3M3520) to determine breakthrough.

Table 4. Analysis at different concentration levels and effect of humidity[a]

			Passive samplers[b]		Active samplers[c]		
Analyte	Sampling time, min	Relative humidity, %	Amount found, $\mu g/m^3$	RSD, %	Amount found, $\mu g/m^3$	RSD, %	Calculated value, $\mu g/m^3$
$CHCl_3$	16.0	25 ± 5	INT[d]	—	2.7	5	2.9
CH_3CCl_3	16.0	25 ± 5	5.8	10	3.9	4	2.7
CCl_4	16.0	25 ± 5	2.9	5	2.5	11	2.7
C_2HCl_3	16.0	25 ± 5	2.3	6	INT	—	2.4
C_2Cl_4	16.0	25 ± 5	3	2	2.5	3	2.6
$CHCl_3$	16.0	25 ± 5	4.6	6	2.9	3	6.7
CH_3CCl_3	16.0	25 ± 5	7.1	8	5.0	5	8.2
CCl_4	16.0	25 ± 5	5.0	5	4.6	6	7.9
C_2HCl_3	16.0	25 ± 5	2.9	4	2.2	5	6.1
C_2Cl_4	16.0	25 ± 5	3.9	4	3.6	4	6.8
$CHCl_3$	16.0	25 ± 5	47.3	4	58.6	3	41.5
CH_3CCl_3	16.0	25 ± 5	54.9	4	42.8	3	51.0
CCl_4	16.0	25 ± 5	53.6	4	41.9	5	49.1
C_2HCl_3	16.0	25 ± 5	23.9	3	22.7	5	38.0
C_2Cl_4	16.0	25 ± 5	47.8	3	37.0	6	42.2
$CHCl_3$	18.67	40–85	6.4	6	7.6	2	7.5
CH_3CCl_3	18.67	40–85	13	8	9.5	1	10.2
CCl_4	18.67	40–85	9.1	8	9.0	0	9.7
C_2HCl_3	18.67	40–85	6.9	7	6.1	2	7.5
C_2Cl_4	18.67	40–85	8.5	8	8.5	3	8.4
$CHCl_3$	19.5	40–85	0.9	11	0.9	7	1.0
CH_3CCl_3	19.5	40–85	2.4	20	1.7	2	1.3
CCl_4	19.5	40–85	1.1	2	1.1	3	1.2
C_2HCl_3	19.5	40–85	0.7	3	0.9	7	1.0
C_2Cl_4	19.5	40–85	1.7	2	1.7	2	1.1

[a] All the calculations are done after subtracting the blanks.
[b] 3M3500 series samplers, $n = 3$.
[c] Porapak N cartridges, $n = 4$.
[d] INT = because of interference in chromatography, amounts could not be calculated.

Table 5. Results of holding-time study[a]

	CH_3CCl_3		CCl_4		C_2Cl_4	
Holding time, days	Amount, μg	RSD, %	Amount, μg	RSD, %	Amount, μg	RSD, %
1	0.150	12	0.060	12	0.076	10
24	0.120	6	0.050	5	0.061	5
44	0.140	6	0.050	9	0.068	6

[a] $n = 3$; exposure time, 19 h.

placed in the chamber. The relative humidity was monitored throughout the experiment with a portable probe.

Desorption

Passive samplers were extracted by injecting into the container 1.5 mL carbon disulfide (desorbing solvent) containing known amounts of internal (surrogate) standards; bromochloromethane, 40 ng/1.5 mL; 1,2-dibromoethane, 85 ng/1.5 mL; and chlorofluorobenzene, 450 ng/1.5 mL. The sampler–solvent system was allowed to stand for 30 min with occasional shaking. After 30 min, the solvent was removed from the container with a disposable pipet and placed into a vial, which was sealed immediately with a septum-sealed aluminum cap. The Porapak N tubes were eluted with methanol through a previously described method (1).

Results

Carbon disulfide is very volatile. Loss of solvent during desorption and transfer could lead to erroneous results. To overcome this problem, the carbon disulfide was spiked with known amounts of 3 internal standards. Table 1 shows peak areas obtained for 2 of the 3 internal standards from the solvent recovered after desorption of the passive sampling devices. It also shows peak areas of internal standards obtained from different gas chromatographic runs of standards during the same period. Loss of solvent during the whole process was within experimental errors. The response of various halogenated VOCs in carbon disulfide was greater than their response in methanol. This result suggests there might be some synergistic effect in the presence of carbon disulfide. In view of this observation, it is essential that standards used for quantitation be prepared in carbon disulfide. It was also observed that switching the venting valve on and off caused the detector signal to drift and that the surrogate standard bromochloromethane did not separate very well from the drift. Thus, this surrogate standard could not be used to perform quantitative experiments.

Table 2 shows recoveries of various analytes spiked directly onto the surface of the passive samplers. Also shown are background levels of some analytes present on the surface of samplers.

Initially, 2 types of commercially available passive samplers were evaluated for analysis of TCE: SKC 575 and 3M3500 or 3M3520. 3M3520 has a backup section of the adsorbent, which is useful in determining whether breakthrough of analytes is taking place. These samplers, along with an active sampling tube, were placed in the chamber and exposed to air containing known amounts of TCE for different lengths of time; results are shown in Table 3. There is good agreement among the analyses with different passive samplers and the dynamic method as well as with the projected amounts of analyte calculated from the permeation rate of TCE. Results in Table 3 also show that no breakthrough of TCE occurs up to at least 20 h of sampling.

To determine whether these samplers could be used for analysis of halogenated VOCs in air other than TCE at low ($\mu g/m^3$) levels, the 3M samplers were exposed in the chamber described above. Sampling cartridges filled with Porapak N/charcoal also were placed alongside the passive samplers, and spiked air was pulled through them at a known flow rate so as not to exceed the safe sampling volume of the respective analytes. 3M samplers were preferred over the SKC samplers

Table 6. Comparison of samples collected by passive and active samplers with Porapak N

Passive sampler[a]		Active sampler (Porapak N)[b]		
Amount, $\mu g/m^3$	RSD, %	Amount, $\mu g/m^3$	RSD, %	Difference, %
275	6	255	0	7
4920	6	5665	5	–15
1000	6	1103	2	–10
157	6	175	9	–12.6
68	4	73	3	–7
265	5	268	1	–1
3607	2	4060	4	–12.5

[a] Passive samplers, $n = 3$.
[b] Active (Porapak N) samplers, $n = 2$.

mostly because of the larger sampling area available, thus increasing the diffusion rates of various analytes. For example, the diffusion rate of TCE through 3M samplers is 28.3 mL/min but only 12.7 mL/min through SKG 575 samplers. The faster diffusion rate in turn, helps reduce sampling time to achieve low detection limits. Table 4 shows results for passive samplers and active sampling tubes exposed to spiked air at normal humidity levels (25 ± 5%) at room temperature (22° ± 1°C). VOC levels varied from 2.4 to 5.1 μg/m^3 (values calculated from permeation rates of VOCs). There is good agreement between the 2 methods and the calculated values. In a few instances where experimental values differed (>40%) from calculated values, experimental values obtained by the 2 methods agreed well with each other.

With our setup, it was difficult to control the relative humidity in the sampling jar over an extended period. So we decided to run experiments at above-normal levels of humidity. The relative humidity was at least 40% but varied up to 85% over the length of the experiments at room temperature (22° ± 1°C). Under these conditions, passive samplers and active sampling tubes were exposed to different VOC levels (1–10.2 μg/m^3), based on the calculated values. These results are also shown in Table 4. They show good agreement between the 2 methods and the calculated values, showing that high levels of humidity, at least 2–3 times the normal levels, has no effect on VOC adsorption by passive samplers.

These samplers were designed for analysis of VOCs at mg/m^3 levels. At these levels, interfering impurities in the sampler are usually of no consequence. But as detection limits are lowered, these impurities become more significant. To overcome this problem, a number of blanks (at least 3 but preferably 5) should be analyzed from every batch and the average amounts of these impurities should be subtracted from the samples being analyzed.

The effect of holding time on analytes once adsorbed on the sampler was studied by exposing 12 samplers to room air for 19 h. These samplers were stored at room temperature and analyzed 3 at a time over a period of 44 days. Results are shown in the Table 5. The data suggest there was little loss (or gain) in the concentration of the analytes collected. The data also show that containers supplied by the manufacturer, when used properly, are air-tight and no more diffusion takes place.

The method, along with dynamic sampling method, was used to collect indoor samples in residences near dry-cleaning establishments in various parts of New York. Samples were collected in the same rooms and at the same time. Results (Table 6) show reasonable agreement between data obtained by the 2 methods.

The detection limit of halogenated VOCs depends on the sensitivity of the compound as well as the sampling time. The longer the sampling time, the lower the detection limit. Typically, for most halogenated compounds like chloroform under normal conditions, the detection limit (with ECD) is 1–5 pg injected on the column. The diffusion rates of these compounds through the 3M 3500 passive samplers are close to 30 mL/min. On this basis, 24 h sampling will yield enough quantity to achieve 0.5 μg/m^3 detection limits for most of these compounds.

Conclusions

Commercially available passive samplers can be used to monitor indoor air levels of chlorinated solvents with boiling points greater than that of chloroform. This technique can be beneficial in a large-scale monitoring program because it significantly cuts down the cost of sampling while providing analyses that are comparable with those obtained by dynamic methods.

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RESIDUES AND TRACE ELEMENTS

Gel Permeation and Florisil Chromatographic Cleanup and Gas Chromatographic Determination of Organochlorine Pesticides in Eggs

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Cleanup of residual organochlorine pesticides (OCPs; α-, β-, γ-, and δ-BHC; aldrin; dieldrin; *p,p′*-DDE; *o,p′*-DDT; *p,p′*-DDD; and *p,p′*-DDT) in eggs by gel permeation chromatography (GPC) and Florisil minicolumn chromatography is described. Ten OCPs in purified extract are determined by gas chromatography with electron capture detection. The lipids extracted from whole egg are cleaned up first by GPC with an Envirogel column and an ethyl acetate–cychlohexane (1 + 1, v/v) mobile phase and then by Florisil minicolumn chromatography with 15% (v/v) diethyl ether–hexane eluant. Cleanup is highly efficient. Average recoveries of 10 spiked OCPs (0.0025–0.0125 ppm) ranged from 81 to 101%, with coefficients of variation between 1 and 14%. The detection limit was 0.001 ppm for the 10 OCPs.

Analytical methods for organochlorine pesticide (OCP) residues in foods should be accurate, simple, harmless to the environment, and economical in time and cost. To determine OCP residues in high-lipid-content samples, such as foods of animal origin, the starting point will be extraction of lipid from sample (1–4).

Agencies for food safety routinely analyze table eggs for OCP residues by extracting OCPs from eggs with organic solvents and removing coextractants by multiple liquid–liquid partition and Florisil macrochromatography. Requiring many stages and large volumes of extracting solvents, this approach is time consuming and troublesome, gives low recoveries for some target pesticides, and is harmful to the environment. The Japanese official method (4) and methods described in AOAC's *Official Methods of Analysis* (2) and the U.S. Food and Drug Administration's *Pesticide Analytical Manual* (3) are all in this category.

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A relatively simple cleanup procedure using gel permeation chromatography (GPC) is effective for cleaning up OCPs in various crops (5–9) and bovine tissues (10). However, a better method for cleanup of OCPs in eggs is lacking so far. Because they are highly nutritious, cheap, and readily available, eggs are a very important food. Monitoring of eggs for OCP residues must be rigorous and economical, hence the need for better analytical techniques.

This paper describes optimization of a GPC/minicolumn chromatographic method for cleanup of 10 OCPs—α-BHC, β-BHC, γ-BHC, δ-BHC, aldrin, dieldrin, *p,p′*-DDE, *o,p′*-DDT, *p,p′*-DDD, and *p,p′*-DDT—from eggs. The method involves quantitative gas chromatography (GC) with electron capture detection (ECD).

Experimental

Apparatus

(**a**) *GPC system.*—Waters Model 600E equipped as follows: 300 × 19 mm Envirogel GPC column (Waters Corp., Milford, MA); 150 × 19 mm Envirogel GPC guard column; column temperature, 30°C; elution solvent, ethyl acetate–cyclohexane (1 + 1, v/v); flow rate, 5 mL/min; fractions, 8 min dumped (40 mL), 1 collect/min from 41 to 110 mL.

(**b**) *GC system.*—HP-5890 Series II (Hewlett Packard Corp., Palo Alto, CA) equipped as follows: ^{63}Ni electron capture detectors; 30 m × 0.25 mm × 0.25 μm SPB1 (Supelco, Inc., Bellefonte, PA) capillary column; He carrier gas flow, 20 cm/s; detector makeup gas, N_2 at 10 cm/s; column temperature program: 140°C for 2 min, 10°C/min to 200°C, 2.5°C/min to 260°C; splitless injection; injector temperature, 200°C; detector temperature, 300°C; injection volume, 2 μL by HP 7796 autoinjector.

(**c**) *Homogenizer.*—Model BM-1 (Nippon Seiki Co., Ltd., Tokyo).

(d) *Centrifuge.*—Sorvall RC2-B.

(e) *Vacuum rotary evaporator.*—EYELA N-1M (Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

(f) *Funnel.*—Form with fritted disk (Bucher type), RYREX 11G1 (Iwaki Glass, Chiba, Japan).

Reagents and Materials

(a) *Solvents.*—Acetone, acetonitrile, cyclohexane, dichloromethane, diethyl ether, ethyl acetate, *n*-hexane, and petroleum ether suitable for residue analysis (Wako Pure Chem. Ltd., Osaka, Japan).

(b) *Sodium sulfate.*—Anhygrous, pesticide residue grade (Wako).

(c) *Florisil.*—Florisil PR, 60–100 mesh (Wako). Heated at 130°C for 15 h, cooled in a desiccator, and stored in a sealed bottle.

(d) *Florisil cleanup minicolumn.*—A 2 g portion Florisil was placed with *n*-hexane into a glass column (130 × 10 mm id, with Teflon stopcock) and topped with 1 g anhydrous sodium sulfate. The minicolumn was sequentially washed with 30 mL *n*-hexane.

(e) *Organochlorine pesticide standards.*—α-BHC, β-BHC, γ-BHC, δ-BHC, aldrin, dieldrin, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDT were obtained from Wako.

(f) *Working standard solutions.*—Mixed solutions of pesticides at concentrations ranging from 0.005 to 0.025 μg/mL were prepared in *n*-hexane.

Lipid Extraction and Cleanup

Lipid in sample was extracted by a procedure described previously (4). An accurately weighted 50 g amount of whole egg was homogenized in 150 mL acetonitrile by using a homogenizer. After the mixture was centrifuged at 3000 rpm for 10 min, the supernatant was poured into a separating funnel. Extraction was repeated twice, and the combined supernatant was shaken vigorously with 150 mL petroleum ether. The petroleum ether layer was collected, washed with 1 L 2% (w/v) NaCl solution in a separatory funnel, dried with anhydrous sodium sulfate, and filtered. After evaporating the filtrate to dryness, the egg lipid was obtained.

Table 1. Recoveries of organochlorine pesticides from gel permeation chromatography (GPC) with 2 mobile phases[a]

Pesticide	Average recovery, %	
	A	B
α-BHC	72.3	92.4
β-BHC	75.0	104.3
γ-BHC	72.9	93.2
δ-BHC	90.1	99.4
Aldrin	79.4	97.5
Dieldrin	78.7	91.0
p,p'-DDE	84.8	93.5
o,p'-DDT	89.7	90.0
p,p'-DDD	87.0	91.1
p,p'-DDT	90.0	89.6

[a] A 2 mL volume of a mixed standard solution (0.005–0.025 ppm) was injected into the GPC system. Data are means of triplicate analyses when the elution volume from GPC was 110 mL. Mobile phases: A, dichloromethane–cyclohexane (1 + 1, v/v); B, ethyl acetate–cyclohexane (1 + 1, v/v).

The lipid extract was dissolved in 5 mL ethyl acetate–cyclohexane (1 + 1, v/v). A 2 mL volume was injected into the GPC system. The OCP fraction (70–110 mL) was evaporated to dryness. The residue was dissolved in 2 mL *n*-hexane and applied to a Florisil minicolumn. OCPs were eluted with 30 mL 15% (v/v) diethyl ether–hexane (flow rate, <3 mL/min). The eluate was evaporated to dryness, and the residue was dissolved in 2 mL *n*-hexane. A 2 μL portion was injected into the GC system.

Table 2. Elution patterns of egg lipids and organochlorine pesticides from a gel permeation chromatography (GPC) system[a]

Analyte	Fractionate volume, mL							Elution volume, mL
	40–50	50–60	60–70	70–80	80–90	90–100	100–110	
Egg lipid	20.8	41.7	21.0	12.0	4.5	0	0	40–90
Organochlorine pesticides								
α-BHC	0	0	0	0	33.0	56.0	11.00	80–110
β-BHC	0	0	0	13.0	78.0	9.0	0	70–100
γ-BHC	0	0	0	0	18.0	66.0	16.0	80–110
δ-BHC	0	0	0	5.0	81.0	12.0	2.0	70–110
Aldrin	0	0	0	0	13.2	68.1	18.8	80–110
Dieldrin	0	0	0	0	3.4	61.1	35.5	80–110
p,p'-DDE	0	0	0	0	8.4	76.1	15.5	80–110
o,p'-DDT	0	0	0	3.0	62.0	33.0	2.0	70–110
p,p'-DDD	0	0	0	0	9.0	72.0	19.0	80–110
p,p'-DDT	0	0	0	0	28.0	65.0	7.0	80–110

[a] Data are recoveries (%). An egg lipid was dissolved in GPC mobile phase (250 mg/mL) and a 2 mL volume was applied to the GPC system.

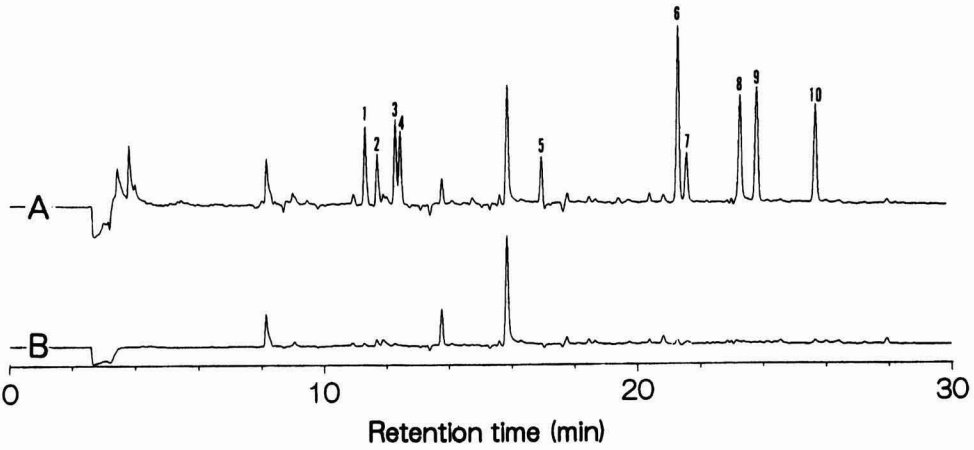

Figure 1. Typical gas chromatograms of spiked (A) (0.0025–0.0125 ppm, *see* Table 3) and blank (B) egg samples. For GC conditions *see* text. Peaks: 1, α-BHC; 2, β-BHC; 3, γ-BHC; 4, δ-BHC; 5, aldrin; 6, *p,p'*-DDE; 7, dieldrin; 8, *o,p'*-DDT; 9, *p,p'*-DDD; 10, *p,p'*-DDT.

Recovery Studies

Samples were fortified with working standard solutions of 10 OCPs and mixed. Recovery studies were done in triplicate.

Results and Discussion

The focus of this study is effective cleanup of OCPs in eggs to minimize lipid content. Therefore, samples were doubly cleaned up by GPC and Florisil minicolumn chromatography.

The First Step of Cleanup (GPC)

Two mobile phases were compared: dichloromethane–cyclohexane (1 + 1, v/v) and ethyl acetate–cyclohexane (1 + 1, v/v). Recoveries of 10 OCPs with dichloromethane–cyclohexane (72–90%) were worse than those with ethyl acetate–cyclohexane (90–104%) when the elution volume was 110 mL (Table 1). Therefore, the latter mixture was used as mobile phase.

Table 2 presents the elution patterns of egg lipids and OCPs from the GPC system. Lipids and other unknown large molecules eluted in the first 90 mL, and OCPs eluted between 70 and 110 mL. Although the 70–90 mL fraction still contained some lipids (16.5%, w/w), the 70–110 mL fraction was collected and cleaned up further by minicolumn chromatography.

The Second Step of Cleanup (Florisil Minicolumn)

Residual lipids in sample after GPC were purified further to minimize lipid content. Earlier methods have shown that Florisil (packed 20 g) macrocolumn chromatography was suitable for cleanup of OCP residues in various foods of animal origin. Therefore, in this study, Florisil was used as the packing material for minicolumn chromatography. Target compounds were eluted from the Florisil (packed 2 g) minicolumn with 30 mL 15% (v/v) diethyl ether–hexane solution. Good results were obtained; average recoveries (n = 3) of all OCPs varied from 88.7 to 133.2% (detailed data not shown).

Use of a commercial cartridge column to extract and purify OCP residues in honey (11), macadamia nuts (12), beef (13), and vegetable oils (14) has been reported. Schenck et al. (15) have used 2 cartridge columns to extract and cleanup OCPs from a small amount of egg sample (5 g). However, our preliminary experiments showed that OCP-related compounds in the lipids obtained from a large amount of the sample could not be determined simultaneously after cleanup with the cartridges because of interfering coextractants. Moreover, packing materials such as Florisil are cheaper than commercial cartridges. Although use of 2 cleanup steps, GPC and minicolumn chromatography, as described here may be considered more work, the steps involved and the volumes of organic solvents used are minimal.

Table 3. Recoveries of organochlorine pesticides from eggs[a]

Pesticide	Spike, ppm	Recovery, %	
		Present method	Official method[b]
α-BHC	0.005	81 (3)	64 (13)
β-BHC	0.005	95 (7)	49 (25)
γ-BHC	0.005	82 (9)	51 (20)
δ-BHC	0.005	94 (1)	47 (24)
Aldrin	0.0025	96 (8)	40 (46)
Dieldrin	0.0025	92 (11)	50 (20)
p,p'-DDE	0.0125	98 (14)	72 (13)
o,p'-DDT	0.0125	101 (8)	81 (19)
p,p'-DDD	0.0125	99 (8)	83 (18)
p,p'-DDT	0.0125	98 (9)	74 (19)

[a] Data are averages of triplicate analyses. Values in parentheses are coefficients of variation (%).
[b] Japanese official method (4).

Cleanup Efficiency

Figure 1 shows GC chromatograms of spiked (0.005–0.025 ppm, 10 OCPs) and blank egg samples. The extracts gave a clean chromatogram with a minimum of ECD-sensitive compounds that did not interfere with the pesticides. The clean extracts should extend the lifetime of the capillary column. Target compounds could be successfully separated within 22 min. These findings indicate that the double cleanup and the GC conditions worked well.

With the proposed cleanup procedure, cleaner extracts, shorter analysis time, and use of less organic solvents were achieved. Analysis time and solvent consumption were reduced by >80% and >70%, respectively, compared with the current procedures (2–4).

Recoveries

Recoveries of 10 OCPs (0.0025–0.0125 ppm) spiked into an egg sample are summarized in Table 3. Earlier methods (2–4) for screening of residues in eggs were used as reference methods. Satisfactory results were obtained with the present method. Average recoveries of 10 OCPs ranged from 81 to 101%, with coefficients of variation (CV) between 1 and 14% ($n = 3$). These results are much better than those for the Japanese official method. The detection limit (signal-to-noise ratio, 10) was 0.001 ppm for all compounds. The findings indicate that this method has good precision and may be accurate.

The method presented here is a technical improvement over earlier procedures (2–4). The proposed procedure gives higher efficiency of cleanup and shorter analysis time, requires less use of organic solvents, and is highly precise and economical. Therefore, this procedure may be useful for monitoring residual OCPs in table eggs.

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RESIDUES AND TRACE ELEMENTS

Liquid Chromatographic Determination of Five Benzoylurea Insecticides in Fruit and Vegetables

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A liquid chromatographic (LC) method was developed to determine 5 benzoylureas—diflubenzuron, hexaflumuron, teflubenzuron, flufenozuron, and lufenuron—in peppers, tomatoes, eggplants, cucumbers, and oranges. Preparation of samples involve extraction with acetone and partitioning into dichloromethane–petroleum ether. A portion of this extract is cleaned up with a solid-phase extraction aminopropyl disposable column. With LC analysis using an RP-8-DB microbore column, acetonitrile–water (70 + 30, v/v) as mobile phase, and photodiode array detection at 254 nm, recovery and repeatability data were collected for the 5 benzoylureas on 4 vegetables and citrus in the range 0.04–2.0 mg/kg. Validated limits of detection and quantitation were 0.01 and 0.04 mg/kg, respectively. The method is reliable for routine analysis of vegetables and fruits.

Benzoylureas are a group of insecticides that inhibit chitin synthesis and thus interfere with formation of the cuticle and further growth of the insect. The compounds lack plant systemic action and thus do not penetrate plant tissue.

Gas chromatography (GC) with electron capture or mass spectrometric (MS) detection has been used to analyze benzoylureas after derivatization with heptafluorobutyric anhydride to avoid thermal degradation (1–4). Reversed-phase liquid chromatograph (LC) with UV detection is also widely used for analysis of plant material (5–7), animal tissues (8, 9), and forestry substracts (10) that are thermally unstable. LC/MS has been used to analyze these compounds in crops (11, 12).

We report a rapid and sensitive LC method for determining 5 benzoylureas—diflubenzuron, hexaflumuron, teflubenzuron, flufenoxuron, and lufenuron—in plant material.

The most common cleanup technique for extracts containing benzoylureas residues are liquid–liquid partitioning, adsorption chromatography, and gel permeation chromatography. However, solid-phase extraction (SPE) can reduce analysis time and costs.

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Our goal was to perform a simple, rapid, and sensitive routine LC method with a portion of an extract obtained for multiresidue and GC methods (13) and for analysis of *N*-methylcarbamates (14) and benzimidazoles (15).

Experimental

Chemicals

(**a**) *Standard materials.*—All standard materials were of the highest purity available. Diflubenzuron was 99.5% pure from Solvay Duphar (Weesp, The Netherlands), hexaflumuron was 99.6% pure from Dow-Elanco (Middlesex, UK), teflubenzuron was 99.5% pure from Cyanamid (Princeton, NJ), lufenuron was 99.7% pure from Ciba Geigy (Basel, Switzerland), and flufenoxuron was 96.1% pure from Shell (Sittingbourne, Kent, UK). For each standard, a stock solution of 1 mg/mL in acetonitrile was prepared and stored at 6°C. Working standards solutions (0.1–1.0 mg/L) were prepared in the mobile phase.

(**b**) *Spike standard stock solution.*—One hundred milligrams of each benzoylurea standard was dissolved in 100 mL dichloromethane to obtain a 1 mg/mL stock solution. Dilute standard mixture was prepared by transferring 100 µL of each standard in a volumetric flash and diluting to 100 mL with dichloromethane to obtain 1 µg/mL standard mixture of 5 benzoylureas.

(**c**) *Solvents.*—Acetone, dichloromethane, *n*-hexane, methanol, petroleum ether (b.p., 4°–60°C) were all pesticide residue quality from Scharlau (Barcelona, Spain). Acetonitrile and dioxane were LC grade from Scharlau, and water was LC grade, obtained from a Nanopure II system (Warnstead, Dubuque, IA). MTBE (methyl *tert*-ether) was from Merck (Darmstad, Germany).

(**d**) *SPE cartridges.*—6 cc/1 g, Mega Bond Elut, aminopropyl bonded-phase columns (Varian/Analytichem, Code 0319; Harbor City, CA).

(**e**) *Filters.*—Millex LCR13 LC certified 0.5 µm, No. SLCR013NS (Millipore, Bedford, MA).

Instruments

(**a**) *Homogenizer.*—Heidolph Diax 600 (Schawabach, Germany).

(**b**) *Centrifuge.*—Hereaus Sepatech Model Labofuge GL (Hanau, Germany).

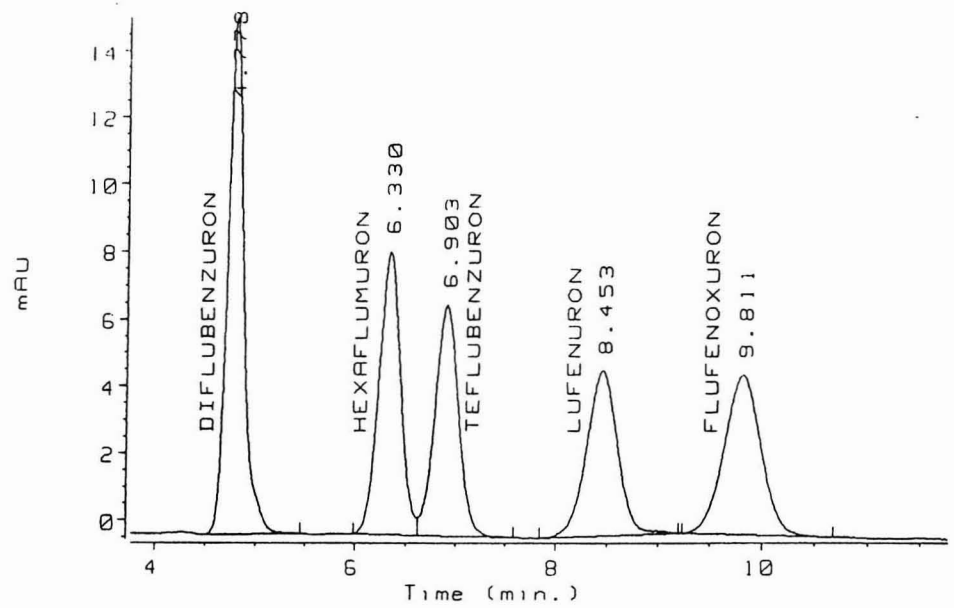

Figure 1. Chromatogram of standard mixture containing 15 ng of each benzoylurea on-column. Mobile phase, CH_3CN–H_2O (70 + 30, v/v); flow rate, 0.25 mL/min; photodiode array detection at 254 nm.

(**c**) *Food chopper.*—Dito-Sama K-55 (Aubusson, France).

(**d**) *LC system.*—Model 1090 Series II liquid chromatograph (Hewlett-Packard, Palo Alto, CA) with a ternary gradient feature, an autoinjection system, and a photodiode array detector. The instrument was controlled with a Model 9000 Series ChemStation using LC-Pascal software (Rev. 5.22). Benzoylureas were separated with a Supercosil LC-8-DB column (5 µm, 25 cm × 2.1 mm id) from Supelco (Bellefonte, PA) and a guard column cartridge ODS Hypersyl (5 µm, 20 × 2.1 mm id) from Hewlett-Packard. Data were acquired at 254 nm with a 20 nm bandwidth. The reference signal was at 450 nm with a 100 nm bandwidth. Detector sensitivity was set at 10 milliabsorbance units full scale. Separation was performed under isocratic conditions at room temperature. The injection volume was 15 µL, and the flow rate was 0.25 mL/min (Figure 1). The mobile phase was CH_3CN–H_2O (70 + 30, v/v) for all benzoylureas in every matrix tested, except for diflubenzuron in oranges, in which case CH_3CN–H_2O–dioxane (45 + 45 + 10, v/v/v) was used.

Analytical Procedure

(**a**) *Extraction.*—A representative sample of whole fruit or vegetable (15 g) was weighed into a 250 mL Teflon centrifuge bottle and then homogenized with 30 mL acetone for 30 s. Sixty milliliters dichloromethane–petroleum ether (50 + 50) was added, and the mixture was homogenized for 1 min. After centrifugation of homogenate for 5 min at 4000 rpm, the upper layer (organic phase) was decanted into a graduated flask, and the volume of extract was measured (usually ca 85 mL). Twenty-five milliliters of the extract was concentrated to dryness in a rotary evaporator with a bath water at 35°C. The residue was dissolved in 2 mL *n*-hexane.

(**b**) *SPE cleanup.*—A Mega Bond Elut cartridge was washed with 15 mL *n*-hexane. The sample, dissolved in 2 mL *n*-hexane, was applied to the cartridge, and then the cartridge was rinsed with 9 mL *n*-hexane and with 8 mL MTBE–*n*-hexane (20 + 80, v/v). The eluates were discarded. Analytes were eluted with 5 mL acetone, and the eluate was evaporated to dryness in a rotary evaporator. The dry residue was dissolved in 0.5 mL acetonitrile, and 0.5 mL water was added. This extract was filtered over a 0.5 µm filter, and the filtrate was collected in a 2 mL autosampler vial.

Results and Discussion

This method for benzoylureas is fast and inexpensive, and it can be used for routine analysis of vegetables and citrus fruits. It also allows use of extract prepared for multiresidue methods (16–18) or for analysis of other analytes, such as benzimidazoles and *N*-methylcarbamates. The procedure does not involve partitioning and laborious cleanup. SPE aminopropyl disposable columns are used to eliminate interferences.

The method gives good recoveries. Matrix interferences in vegetables are reduced to a minimum and are well separated from the analytes through the optimized isocratic run with UV detection at 254 nm (Figures 2 and 3).

Citrus samples included peel and pulp and interferences such as essential oils and waxes from the peel interfered with

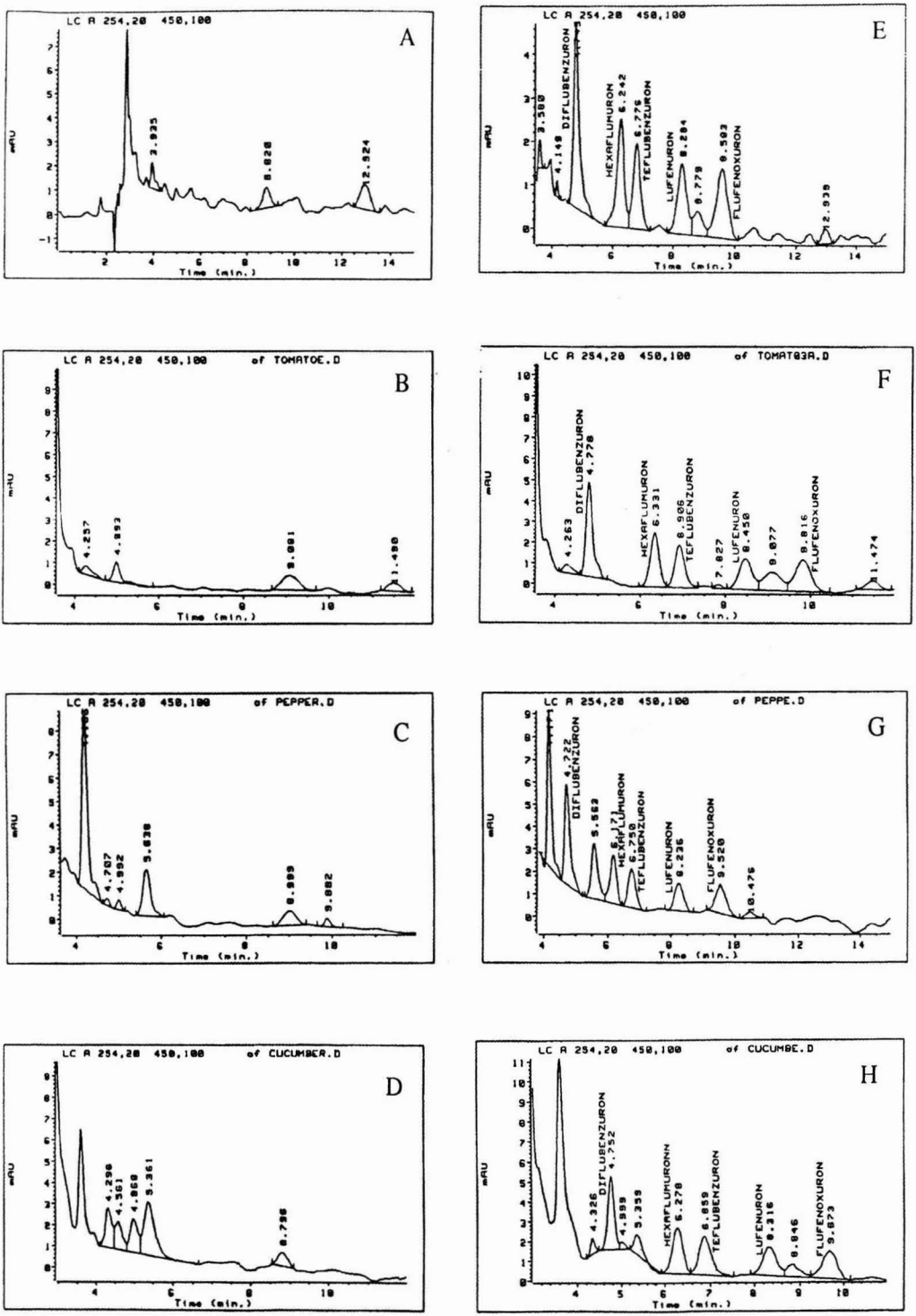

Figure 2. Chromatograms of untreated extract—(A) eggplants, (B) tomatoes, (C) peppers, and (D) cucumbers—and samples spiked with benzoylureas at 0.13 mg/kg—(E) eggplants, (F) tomatoes, (G) peppers, and (H) cucumbers. Same conditions as described in Figure 1.

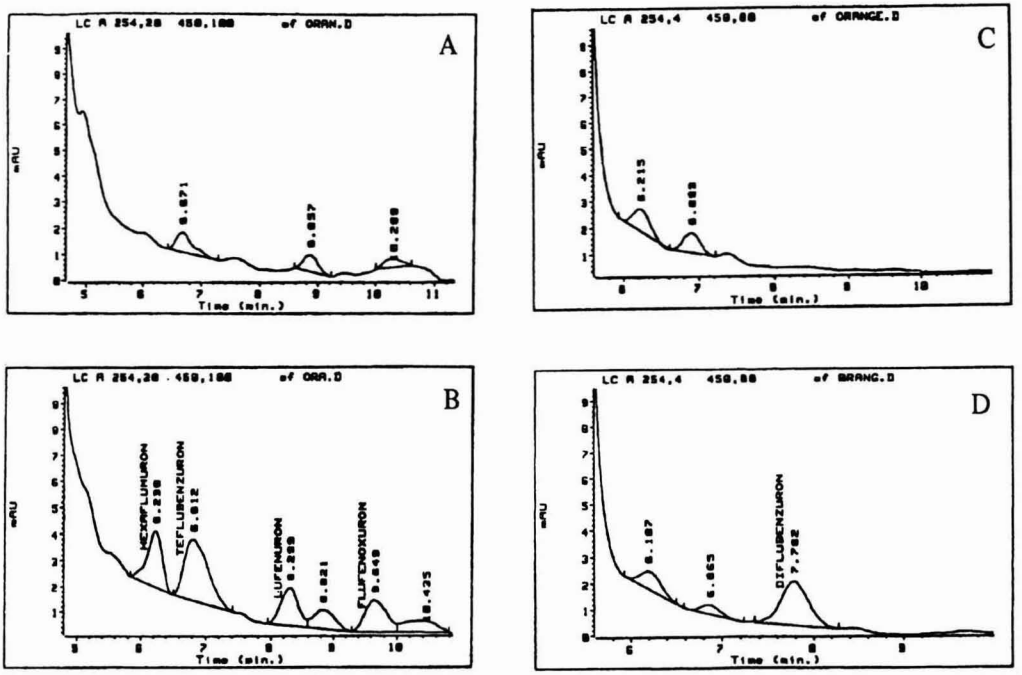

Figure 3. Chromatograms of (A) untreated oranges and (B) oranges spiked with benzoylureas at 0.13 mg/kg. Same conditions as described in Figure 1.

Figure 5. Chromatograms of (C) orange extract after cleanup and (D) sample spiked with diflubenzuron at 0.13 mg/kg. Same conditions as described in Figure 4.

determination of diflubenzuron. This problem was solved by modifying the mobile phase with dioxane while using the same analytical conditions (Figures 4 and 5).

Recoveries from homogenized untreated peppers, tomatoes, cucumbers, eggplants, and oranges spiked with each analyte at 0.04, 0.13, and 2.0 mg/kg were good (Table 1). Mean recoveries were between 58 and 118%, with relative standard deviation (RSD) values between 2 and 22%. These are considered acceptable for these types of matrix.

In some cases, higher concentrations (e.g., 2.0 mg/kg) of analytes overloaded the solid-phase column, giving lower recoveries compared with other levels. For example, mean recov-

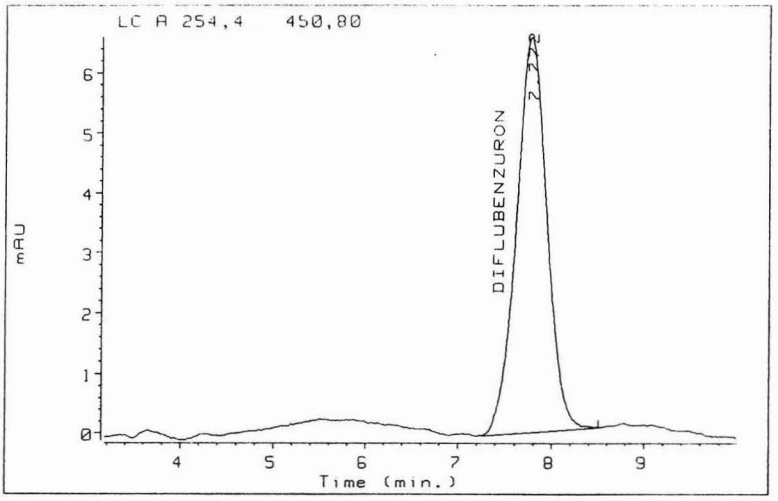

Figure 4. Chromatogram of diflubenzuron standard containing 15 ng diflubenzuron on-column. Mobile phase, CH_3CN–H_2O–dioxane (45 + 45 + 10, v/v/v); flow rate, 0.25 mL/min; photodiode array detection at 254 nm.

Table 1. Recovery of 5 benzoylureas from spiked peppers, tomatoes, cucumbers, eggplants, and oranges

		Diflubenzuron				Hexaflumuron				Teflubenzuron				Lufenuron				Flufenoxuron			
Matrix	Spike, mg/kg	Range, %	*n*	Mean, %	RSD, %	Range, %	*n*	Mean, %	RSD, %	Range, %	*n*	Mean, %	RSD, %	Range, %	*n*	Mean, %	RSD, %	Range, %	*n*	Mean, %	RSD, %
Peppers	2.0	67–86	6	72.8	9.5	59–74	6	61.9	9.7	56–71	6	60.5	8.7	55–69	6	59.6	8.2	54–66	6	57.8	7.6
	0.13	81–94	7	84.4	4.7	77–90	7	79.3	6.2	74–87	6	79.6	5.2	65–103	7	82	13.8	57–100	7	85.6	14.5
	0.04	84–133	6	110	14.2	66–80	6	72.9	6.6	64–73	6	70.1	5.6	55–79	6	68.5	13.1	81–124	6	110	15.4
Tomatoes	2.0	67–97	6	81.5	14.8	65–95	6	79.4	14.8	64–95	6	78.8	15.2	63–94	6	77.5	15.4	62–93	6	76.8	15.3
	0.13	73–102	7	88.7	9.8	88–100	7	94.8	4.4	95–100	6	98.1	2.1	98–117	7	95.8	9.7	87–110	7	95.8	9.4
	0.04	76–89	6	83.1	6.7	82–93	6	87.2	5.2	66–87	6	77.5	9.9	83–98	6	90.2	7.3	91–123	6	108	9.9
Cucumbers	2.0	75–84	6	79.7	4.4	72–82	6	77.4	4.9	72–85	6	79.2	6.2	69–85	6	79.5	8.3	63–79	6	72.2	7.5
	0.13	57–74	7	65.1	6.8	70–106	7	88.5	13.8	80–102	6	90.3	9.9	67–97	7	82.2	10.9	63–94	7	83.2	10.9
	0.04	75–87	6	80.4	6.5	73–97	6	85.2	9.8	93–117	6	105	9.6	78–100	6	89.6	9.9	91–108	6	98.8	6.7
Eggplants	2.0	64–98	6	83.5	16.5	59–91	6	76.5	16.8	47–92	6	74.7	22.7	58–95	6	77.2	18.6	58–90	6	74.8	16.6
	0.13	64–105	7	87.4	15.6	77–109	7	94.4	11.5	70–117	6	99.8	16	77–130	7	99	22.1	89–106	7	96.1	6.1
	0.04	80–112	6	101	11.4	54–74	6	67.1	10.4	96–138	6	118	12.2	—[a]	—	—	—	77–94	6	84.3	10.5
Oranges	2.0	67–84	6	74.9	9.1	54–72	6	63.4	9.6	53–71	6	63.2	9.9	65–86	6	77.1	10.4	56–76	6	67.2	10.2
	0.13	56–80	7	69.2	7.5	67–90	7	78.4	8.8	75–94	6	83.1	7.9	84–109	7	93.5	11.1	81–97	7	86.7	5.9
	0.04	61–100	6	73.5	19	90–108	6	98.4	8.2	73–99	6	86.8	11.6	64–82	6	71.9	8.8	87–104	6	97.5	6.4

[a] —, matrix interferences appear at retention time of lufenuron (0.04 mg/kg).

ery of teflubenzuron in oranges spiked at 2.0 mg/kg was 63%, compared with 83 and 87% for oranges spiked at 0.13 and 0.04 mg/kg, respectively.

The estimated limit of detection for benzoylureas in the tested matrixes was 0.01 mg/kg, and the limit of determination was 0.04 mg/kg, except for lufenuron in eggplants (0.04 and 0.10 mg/kg, respectively) because of matrix interferences.

Conclusions

A rapid and simple procedure for determining benzoylureas in crops and fruits was developed. Recovery and repeatability values are good for these kind of matrixes. For samples with high essential oil and wax contents, such as whole citrus fruit, a slight modification of the mobile phase composition is required to determine diflubenzuron. An additional advantage of this method is that an extract such as the one commonly obtained in the Luke procedure can be used, facilitating application of the method for routine purposes.

Acknowledgment

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RESIDUES AND TRACE ELEMENTS

Determination of Chromium in Wine and Other Alcoholic Beverages Consumed in Spain by Electrothermal Atomic Absorption Spectrometry

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A reliable and rapid method is described for the determination of chromium in alcoholic beverages by electrothermal atomic absorption spectrometry (ETAAS) after optimization of the temperature–time program for the graphite furnace. Chromium in wines was determined directly without sample pretreatment. Other samples were first mineralized with HNO_3 and V_2O_5. The method was validated, and its analytical characteristics were checked. The detection limit was 1 pg (for a sample volume of 10 μL). Accuracy was 99.20 ± 3.80% for wines and 98.90 ± 2.00% for other beverages. Precision ranged from 3.50 to 4.50%. The proposed method was applied to 124 samples of alcoholic beverages frequently consumed in Spain. Mean values obtained (μg/L) were 20.28 for wines, 8.75 for beer, 7.85 for cider, 14.60 for brandy, 7.50 for rum, 10.25 for whisky, 8.80 for gin, 9.25 for vodka, 24.45 for anisette, and 10.08 for liquors. The advantages of the proposed method (rapidity, sensitivity) and versatility) make it useful for routine analyses and for estimations of dietary intake of chromium.

Chromium is an essential element for carbohydrate, cholesterol, and protein metabolism (1–4). Cr toxicity depends on its chemical form. Cr(VI) compounds show toxic, mutagenic, and even carcinogenic character (5–7). Cr(III), which is the most frequent form found in foods and beverages, has low toxicity (5). Although humans can absorb chromium compounds through inhalation or dermal contact, intake through the diet is the most important route of Cr entry into humans (2, 8).

Several studies have analyzed Cr in fish (9, 10), vegetables (11, 12), oils (13–15), and dairy products (16), but not in alcoholic beverages. In 1989, the U.S. National Research Council indicated that the lack of reliable analytical methods for Cr determination made it difficult to determine Cr requirements for humans. The council established the recommended dietary intake of this element at 50–200 μg/day for adults (17).

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Problems in Cr determination are due mainly to contamination of samples and analyte losses through volatization (18, 19). Electrothermal atomic absorption spectrometry (ETAAS) has numerous advantages, including selectivity, precision, accuracy, and versatility over most techniques used for Cr quantitation (11–13, 15, 19).

In this study, a reliable and rapid method is described for determining Cr in wine and other alcoholic beverages widely consumed in Spain (beer, cider, brandy, rum, whisky, gin, vodka, anisette, and liquors). Cr in wines was determined directly; other samples were mineralized with acid before Cr analysis. For each sample, instrumental and analytical conditions were optimized, and precision, accuracy, and selectivity of the proposed method were evaluated. A total of 100 samples were analyzed to determine the contribution of alcoholic beverages to dietary intake of Cr.

Experimental

Apparatus

(a) *Spectrophotometer.*—Model 1100B double-beam atomic absorption spectrophotometer equipped with a deuterium arc background corrector and a hollow cathode lamp of Cr (Perkin-Elmer, Norwalk, CT); D-5000 chart recorder (Bausch & Lomb, Rochester, NY).

(b) *Graphite furnace.*—HGA-700 (Perkin-Elmer); pyrolytically coated graphite tubes; pyrolytically coated L'Vov platforms fixed in grooves (Perkin-Elmer).

(c) *Digestion block.*—Selecta (Rotaterm, Barcelona, Spain) and 25 mL Pyrex tubes.

(d) *Microwave acid digestion bomb.*—Model 4782 (Parr Instrument, Moline, IL).

(e) *Microwave oven.*—Model FM-460 with 15–100% full power (600 W) capability in 25% increments (Moulinex).

(f) *Ultrasonic bath.*—Selecta.

(g) *Milli-Q purifier system.*—Model R015 (Millipore, Gif-sur-Yvette, France).

(h) *Micropipette.*—Universal pipettor of 10 μL Sealpette (Jencons, UK); Micropipette Microtransferpettor, Type Digital de Brand of 250–1000 μL (Brand, Wertheim, Germany).

Reagents

(a) *Chromium standard solution.*—Titrisol solution (1000 mg/L; E. Merck, Darmstadt, Germany).

(b) *Magnesium nitrate.*—Analytical reagent grade (E. Merck).

(c) *Nitric acid.*—Suprapure, 65% (E. Merck).

(d) *Vanadium pentoxide.*—Analytical reagent grade (E. Merck).

(e) *Ammonium molybdate.*—Analytical reagent grade (E. Merck).

(f) *Water.*—All solutions were prepared with deionized water with specific resistivity of 18 MΩ·cm.

Materials

To eliminate contamination due to detergents and samples, glassware and polyethylene sample containers were washed with tap water after each use, soaked in 6M HNO_3 (at least overnight), and rinsed several times with ultrapure water.

Samples

A total of 124 samples including wine (red, white, sherry, oloroso, sherry, and cava wines) and other alcoholic beverages (beer, cider, brandy, rum, whisky, gin, vodka, anisette, and liquors) were analyzed. Different brand names of each product, representing the most widely accepted and most frequently consumed beverages in Spain, were selected for testing. All wine and beer samples and most of the other alcoholic beverages were produced in Spain. To optimize sample amount, preliminary assays were done to ensure that samples were homogeneous and representative of the product investigated.

Sample Preparation

All samples except wine were mineralized in a digestion block. Cava, beer, and cider samples were degassed in an ultrasonic bath prior to analysis to increase the precision of the determinations. A 5.0 mL portion of sample was treated with 1 mL 65% HNO_3 and 35 mg V_2O_5 (as a catalyst) in Pyrex tubes placed in the digestion block and heated at 120°C for 90 min. Solutions were left to cool to room temperature, transferred to a calibrated flask, and diluted to a final volume of 10.0 mL with deionized water. Chromium in the resulting solutions was determined by ETAAS. Three portions of each sample were analyzed.

Method Validation

To validate the proposed method, a microwave digestion bomb was used for acid mineralization of samples, as described Mena et al. (20).

Sample Analysis

Chromium in wine samples and in the mineralized solutions of the other alcoholic beverages was determined directly by ETAAS under the optimized conditions shown in Table 1. The same procedure was followed for blanks, and all determinations were done in triplicate. Samples were injected manually with a micropipet. The chemical modifier was injected after the sample. Argon of 99.999% purity at 300 mL/min flow was used as internal gas. To avoid formation of refractory carbides, the graphite tubes and the L'Vov platforms were pretreated with a saturated ammonium molybdate solution and heated several times in accordance with the temperature program. This pretreatment was effective for ca 100–150 atomizations. Appropriate calibration graphs were prepared for blanks. To evaluate method selectivity, standard addition graphs were prepared for blanks and samples and used to calculate blank-to-sample slope ratios for the different samples (21).

Results and Discussion

Sample Treatment

The sample mineralization procedure proposed for all samples except wine and the direct method for wine samples were validated by comparison with the microwave acid digestion as the reference method (Table 2). Results for both procedures showed good agreement for all samples considered. Comparison of the results by the *F*-test revealed no significant differences at a 95% confidence level.

Optimization of Graphite Furnace Program

Furnace conditions were optimized on the basis of time–temperature assays. For determination of Cr in wine samples, the matrix was destroyed completely after heating at 1000°C. An atomization temperature of 2600°C yielded maximum signals with an integration time of 6 s. Atomization from wall graphite tube gave more reproducible results. Among several matrix modifiers tested, the best was 10 µL 0.5% (v/v) HNO_3. For determination of Cr in other samples of alcoholic beverages, the optimum ashing temperature was 1650°C, and an atomization temperature of 2500°C yielded maximum signals with an integration time of 6 s. Use of a L'Vov platform gave more reproducible results. In accordance with other publications (22, 23), several matrix modifiers were also tested, and 10 µL 0.5% (w/v) $Mg(NO_3)_2$ gave the best results. Argon flow was stopped during atomization to increase sensitivity; this did not alter the usable life of the tube. Analyses were done in peak

Table 1. Analytical conditions for Cr determination in alcoholic beverages by ETAAS

Parameter	Value or setting	
	Wine[a]	Other alcoholic beverages[b]
Wavelength, nm	357.9	357.9
Slit width, nm	0.7	0.7
Atomization system	Wall tube	L'Vov platform
Dry temperature, °C	120	150
Ashing temperature, °C	1000	1650
Atomization temperature, °C	2600	2500
Sample volume, µL	10	10
Matrix modifier	0.5% HNO_3	0.5% $Mg(NO_3)_2$
Modifier volume, µL	10	10

[a] Direct determination.
[b] Determination of mineralized samples.

Table 2. Validation of method to determine Cr in alcoholic beverages by ETAAS

Sample	Chromium, μg/L[a]	
	Proposed method[b]	Reference method[c]
White wine	5.52 ± 0.04	5.55 ± 0.06
Red wine	5.92 ± 0.05	5.92 ± 0.04
Beer	8.42 ± 0.05	8.40 ± 0.07
Cider	7.27 ± 0.06	7.25 ± 0.04
Anisette	14.73 ± 0.10	14.75 ± 0.05
Gin	8.95 ± 0.06	8.90 ± 0.08
Whisky	10.78 ± 0.09	10.80 ± 0.10
Rum	6.50 ± 0.02	6.55 ± 0.04
Brandy	14.50 ± 0.08	14.58 ± 0.07

[a] Values are means of 3 replicate analyses each of 5 samples ± standard deviation at 95% confidence level.
[b] Direct determination of Cr in wine samples and determination of Cr in mineralized samples of other beverages.
[c] Mineralization with a microwave acid digestion bomb.

area mode (integrated absorbance). The furnace was cleaned by heating to 2650°C, and the graphite tube was cooled to 20°C between determinations. Optimized assay conditions obviate most matrix interferences and other sources of unspecific absorption.

Analytical Characteristics

The detection limit was calculated according to rules of the International Union of Pure & Applied Chemistry (24). Sensitivity and selectivity of analytical conditions were evaluated. Precision was checked with 10 determinations of 5 samples. Precision (as interday reproducibility) was tested by analysis of 5 samples in 6 days. Accuracy was checked by recovery of known amounts of analyte added to 5 samples chosen at random. Results are summarized in Table 3. A known amount of Cr was added to a household reference material in wine samples. This material showed the mean composition of a normal wine (25): absolute alcohol (10 mL), glucose (0.15 g), glycerol (0.50 g), Ca (100 mg/L), Mg (100 mg/L), potassium sodium tartrate (0.72 g), citric acid (0.02 g), tartaric acid (0.20 g), ammonium phosphate monohydrogen (0.035 g), and water (up to 100 mL); pH was 3.5. Recovery of Cr was 99.20 ± 3.80%. This outcome demonstrates the accuracy of the method and indicates that most compounds in wine do not interfere.

The detection limit and the sensitivity were suitable for the range of Cr concentrations encountered and are compatible with earlier estimates (26). Moreover, the analytical precision and the accuracy were acceptable (27–29). In the application of the addition method for all samples, slope ratio values were close to 1 (Table 3), indicating that the standard addition method was unnecessary and consequently simplifying the analysis greatly.

Analysis of Samples

The proposed method was applied to 124 samples of 10 alcoholic beverages frequently consumed in Spain. Results are given in Table 4. The Cr levels in wines varied widely. Results indicate differences in the Cr contents of red and white wines: 32.50 and 19.49 μg/L, respectively. Cabrera-Vique et al. (30) reported Cr levels ranging from 6.60 to 90.00 μg/L in French red wines (mean, 22.60 μg/L), from 6.60 to 43.90 μg/L in French white wines (mean, 21.30 μg/L), and from 10.50 to 36.00 μg/L in champagne (mean, 25.10 μg/L). On the basis of Cr analysis of different vintage wines from the same vineyard and winery, these authors suggested that Cr concentrations significantly increase with wine age.

Etievant et al. (31) found that Cr content of grapes could be modified by the processes of winemaking, especially maceration, yeast fermentation, and aging. Extended maceration of husks extracts Cr, and for this reason, red wines contain more Cr than white wines. Darret et al. (32) reported significant differences in Cr levels depending on the type of fermentation, aging time, and packing quality. Cr levels can increase from 2 to 10 times in relation to Cr concentrations in musts.

Among beer samples, significant differences also were observed, with Cr levels ranging from 3.94 to 30.10 μg/L. Canned and draught beers gave the highest values; lower concentrations were found in bottled beers. These data agree with earlier reports. Darret et al. (32) found variations from 70 to 200 times in Cr levels of commercial beers and even in levels of the same sample. Cr content can increase from 24 to 30% as influenced by the package.

Among other alcoholic beverages, mean concentrations of Cr ranged from 7.50 μg/L in rum to 24.45 μg/L in anisette. The

Table 3. Analytical characteristics of method to determine Cr in alcoholic beverages by ETAAS

Sample	Detection limit, pg[a]	Characteristic mass, pg[b]	Recovery, %[c]	Precision RSD, %	Blank-to-sample slope ratio
Wine	1.0	3.0	99.10 ± 3.60	3.50[d] 4.00[e]	0.96–1.00
Other alcoholic beverages	1.0	3.0	98.90 ± 2.00	4.50[d] 4.95[e]	0.95–1.05

[a] Detection limit is analyte concentration corresponding to 3 times the standard deviation of the blank for an injection volume of 10 μL.
[b] Characteristic mass in pg/0.0044 A·s.
[c] Results obtained from recovery assays of 5 samples.
[d] Relative standard deviation for 10 replicate determinations each of 5 samples.
[e] Relative standard deviation for 6 replicate determinations (interdays) each of 5 samples.

Table 4. Chromium in alcoholic beverages consumed in Spain

Sample	*n*	Chromium, μg/L Mean	Chromium, μg/L Range
Wine			
Red wine	36	32.50	6.84–80.00
White wine	11	19.49	3.94–39.50
Sherry	5	14.60	10.30–15.80
Oloroso sherry	7	29.21	20.20–35.10
Cava	5	5.61	2.90–8.94
Other alcoholic beverages			
Beer	23	8.75	3.94–30.10
Cider	9	7.85	3.85–15.30
Brandy	4	14.60	3.50–26.05
Rum	5	7.50	6.45–9.00
Whisky	5	10.25	4.50–15.70
Gin	4	8.80	8.75–10.80
Vodka	2	9.25	9.00–9.50
Anisette	6	24.45	3.29–55.28
Liquor	2	10.08	3.02–17.65

highest values were obtained for beverages with sugar. Darret et al. (32) analyzed Cr in various beverages and found 3 μg/L in water, 13 μg/L in alcoholic beverages (wine, beer, cider, and liquors), 16 μg/L in soft drinks and fruit juices, and 23 μg/kg in tea and coffee.

Several authors (8, 32) have reported that consumption of wine and other alcoholic beverages could contribute to total Cr intake. In France, Darret et al. (32) determined that Cr intake from beverages (alcoholic beverages, water, juices, infusions, etc.) was 130 μg/day, or 11% of the total dietary intake of Cr. Alcoholic beverages, especially wine and beer, contributed 35% of this amount. Cabrera-Vique et al. (30) estimated that Cr intake from wine for the French population is 4.06 μg/day on the basis of the official wine consumption rate of 66 L/year per adult.

Buchet et al. (33) estimated a dietary Cr intake in Belgium of 10–500 μg/day. Anderson et al. (34) evaluated the dietary Cr intake in a large group of the U.S. population, obtaining values of 23.1 ± 2.90 μg/day for women and 38.8 ± 6.50 μg/day for men. Barbera et al. (35) determined a dietary Cr intake in Spain of 120 μg/day, emphasizing the effects of environmental conditions, dietary habits, and food processing and technology, as well as the bioavailability of diet constituents.

Taking into account data about Spanish consumption of alcoholic beverages in 1995 (36) and considering the mean values of Cr found in this study, we estimated the contribution of alcoholic beverages to the total dietary Cr intake. Results (Table 5) show a Cr intake of 4 μg/day. These data indicate that wine and other widely consumed alcoholic beverages can contribute significantly to the dietary intake of Cr.

Table 5. Estimation of Cr intake through consumption of alcoholic beverages in Spain

Beverage	Mean consumption, L/person/year[a]	Cr level, μg/L[b]	Cr intake, μg/day
Wine	32.5	20.28	1.80
Beer	71.5	8.75	1.71
Others	8.5	11.60	0.27

[a] From reference 36.
[b] Mean value in analyzed samples.

Conclusions

The proposed method is suitable for determining Cr in alcoholic beverages. The direct quantitation of Cr in wines considerably simplifies the analysis. Acid mineralization of other beverage types (beer, cider, brandy, rum, whisky, gin, vodka, anisette, and liquors) completely mineralizes samples without loss of analyte or additional contamination. The method is useful for routine control analyses because of its rapidity, sensitivity, and versatility. We have considered it appropriate to sensitivity to perform periodic determinations of Cr in all foods and beverages making up the usual diet to establish Cr requirements in accordance with the current dietary habits of certain groups of the population and to detect potential risks due to Cr deficiencies or excesses.

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RESIDUES AND TRACE ELEMENTS

Determination of Phenylurea Herbicide Residues in Vegetables by Liquid Chromatography after Gel Permeation Chromatography and Florisil Cartridge Cleanup

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A liquid chromatographic method for quantitative determination of 9 phenylurea herbicide residues (metoxuron, metobromuron, monolinuron, chlortoluron, isoproturon, diuron, linuron, chloroxuron, and neburon) in potatoes, carrots, and mixed vegetables is described. Samples are extracted with acetone, partitioned with ethyl acetate–cyclohexane (50 + 50, v/v) and cleaned up by gel permeation chromatography with ethyl acetate–cyclohexane (50 + 50, v/v) as eluant. A small column (1 cm id) packed with Biobeads SX3 resin is used to reduce solvent consumption and analytical time. After solid-phase extraction on a Florisil cartridge, herbicide residues are successfully separated on a C_{18} column by gradient elution and determined by UV detection at 242 nm. Average recoveries of 9 compounds from different samples range from 70 to 98% at 0.010 and 0.100 mg/kg fortification levels. Quantitation limits are 0.010 mg/kg.

Phenylurea herbicides are used extensively to protect a large number of crops (vegetables, fruits, and cereals) against weeds. Monitoring of their residues in foods, soil, and water is of importance to public health.

Most published methods for separation, detection, and quantitation of phenylurea herbicides in foods and water are based on gas chromatography (GC) and liquid chromatography (LC). However, GC determination without prior derivatization is difficult because of thermal instability of phenylureas (1–4). LC methods allow direct analysis and have been used to determine phenylurea residues in various matrixes (5–12). UV detection used in many methods is sensitive enough in most cases but lacks selectivity, especially when trace levels in complex matrixes need to be assayed. This shortcoming led Luchtefeld (13) to develop an LC method with selective postcolumn reaction followed by fluorescence detection. On the other hand, effective cleanup procedures before LC/UV determination could be sufficient to isolate phenylureas from matrix interferences well enough to allow determination at low levels.

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This paper describes an LC method for determining 9 phenylurea herbicides in carrots, potatoes, and mixed vegetables. The multiresidue extraction procedure used by Specht (14) for a wide range of pesticides (organophosphorus, organochloride, pyrethroid, organonitrogen, and triazine compounds) was modified by miniaturizing the whole extraction/partition step. The extract was cleaned up by gel permeation chromatography (GPC) using a narrow column to reduce substantially solvent consumption and analytical time. A further purification on Florisil cartridge was necessary to obtain an extract completely free from interferences when detected by UV at 242 nm.

This paper presents a multiresidue method for phenylurea herbicides in vegetable matrixes using GPC cleanup with small, 1 cm id, column and nonchlorinated solvents.

Experimental

Reagents

(**a**) *Solvents.*—Methanol and acetonitrile were LC grade (Prolabo, Fontanay, France). Acetone, cyclohexane, ethyl acetate, and *n*-hexane were for residue analysis (Merck, Darmstadt, Germany).

(**b**) *LC water.*—Obtained with a Waters purification system (Smeg, Parma, Italy).

(**c**) *Phenylurea standards.*—99.9% purity, obtained from S.I. Ehrenstorfer, Ausburg, Germany.

(**d**) *Standard solutions.*—100 μg/mL stock solutions were prepared by dissolving 0.010 g pure pesticide standards in 100 mL acetone, except for isoproturon, which was dissolved in methanol. Portions of stock solutions (2 mL) were diluted to 20 mL with acetone to make an intermediate mixed standard solution (10 μg/mL). Standard solutions for LC determinations were prepared as follows: a volume of mixed intermediate solution was evaporated to dryness under a stream of nitrogen, and the residue was taken up in methanol–acetonitrile–water (85 + 15 + 100).

(**e**) *Sodium sulfate.*—Anhydrous ACS reagent grade (Carlo Erba, Milan, Italy). The material was heated at 550°C for at least 4 h, cooled in a dessicator, and stored in a sealed bottle.

(**f**) *Sodium chloride.*—ACS reagent grade (Carlo Erba).

Apparatus

(a) *GPC system.*—Dedicated sample cleanup system Model 18L MLS (Lab Service Analytica S.r.l., Bologna, Italy) with 1 mL sample loop, equipped with a 1 cm id column packed with 9 g Biobeads SX3 resin (200–400 mesh, compressed to bed length of ca 30 cm). Flow rate, 1 mL/min; 16 min dump cycle; 7 min collect cycle; 12 min wash cycle; elution solvent, ethyl acetate–cyclohexane (50 + 50, v/v).

(b) *Vacuum rotary evaporator.*—Buchi Rotavapor Model R110 (Buchi, Flawil, Switzerland).

(c) *Homogenizer.*—Ultrax-Turrax T25 (Janke & Kunkel, Staufen, Germany).

(d) *Glass microfiber filter.*—Whatman GF/A (Whatman International, Ltd., Maidstone, UK).

(e) *Membrane filters.*—For filtering LC mobile phase, Millipore GV 0.22 µm (Millipore Corp., Milford, MA). For filtering samples prior to LC analysis, hydrophilic Durapore-PVDF, 4 mm Millex-HV, 0.45 µm pore size (Millipore Corp.).

(f) *Centrifuge.*—ALC 4233R refrigerated centrifuge (ALC, Milan, Italy).

(g) *Florisil cartridge.*—Sep-Pak (Waters Associates, Milford, MA) containing 0.900 g Florisil.

LC System

(a) *Mobile phase delivery system.*—Model 600E (Waters).

(b) *Injector.*—Wisp 715 automatic sample injection module (Waters Chromatography Div., Milford, MA).

(c) *UV detector.*—Waters 490E programmable multiwavelength detector.

(d) *Software for analysis and quantitation.*—Waters 820 Maxima Chromatography.

(e) *LC column.*—Lichrospher R18 5 µm end-capped 25 cm × 4.6 mm id (Hichrom, Berkshire, UK).

(f) *Mobile phase.*—The following elution program was used: at the start 50% organic modifier (methanol–acetonitrile, 85 + 15), 50% water; the percentage of organic solvent was linearly increased to 55% in 15 min and to 75% in 30 min; ramped to original composition in 10 min; and then equilibrated for 30 min.

Samples

Carrots and potatoes were untreated fresh products. The mixed vegetable sample was a frozen product purchased from a supermarket in Parma.

GPC Calibration

Before samples could be processed with GPC, 1 mL 0.100 µg/mL phenylurea standard was injected to accurately determine the dump and herbicide collect volumes.

Extraction

Both carrot and potato samples were washed to remove adhering soil and then dried with paper towels. Unpeeled samples were chopped into small pieces and then homogenized with a food mixer. Frozen mixed vegetables were homogenized directly.

A 20.0 g portion of sample was weighed into a 250 mL Teflon centrifuge bottle. Acetone (40 mL) and sodium chloride (7 g) were added, and the mixture was blended for ca 2 min with the Ultra-Turrax mixer at 5000 rev/min. Ethyl acetate–cyclohexane (1 + 1, v/v) (20 mL) was added, and the sample was blended again for 1 min at 9000 rev/min. The homogenate was centrifuged for 15 min at 5000 rpm. The volume of organic phase, measured out in a graduated cylinder, was 55 mL. A 50 mL portion (18.2 g) was filtered through a glass fiber filter previously washed with 15 mL ethyl acetate–cyclohexane (50 + 50, v/v) and containing a bed of anhydrous sodium sulfate (20 g). The filter was rinsed with 20 mL ethyl acetate–cyclohexane (50 + 50, v/v). Effluents were collected in a 100 mL tared boiling flask, evaporated under vacuum to a small volume (ca 0.5 mL) at a bath temperature of 30°C and quantitatively transferred to a 25 mL graduated cylinder with GPC eluant diluting to a volume of 9 mL (2.02 g sample/mL).

GPC Cleanup

A portion of extract (ca 5 mL) was loaded into one of 18 calibrated, 1 mL GPC loops with a 10 mL glass syringe. Then, 1 mL extract was injected (2.02 g sample). The eluate was collected in a 10 mL culture tube and evaporated to dryness with a gentle stream of nitrogen at 30°C. The residue was taken up with 2 mL ethyl acetate–hexane (20 + 80, v/v).

Table 1. Recoveries of phenylurea herbicides from spiked vegetable products

Sample	Spiking level, mg/kg	Recovery, % (CV, %)[a]								
		Metoxuron	Monolinuron	Chlorotoluron	Metobromuron	Isoproturon	Diuron	Linuron	Chloroxuron	Neburon
Carrot	0.01	92.3 (7.4)	100.0 (0.0)	83.3 (5.7)	97.0 (3.1)	89.0 (9.3)	91.7 (5.5)	85.7 (7.6)	92.7 (9.0)	68.3 (2.4)
	0.10	89.7 (9.2)	89.3 (8.9)	94.3 (8.8)	91.7 (9.5)	92.0 (9.1)	87.0 (13.5)	86.3 (6.7)	92.7 (9.3)	68.0 (3.7)
Potato	0.01	81.3 (7.3)	90.0 (4.5)	101.5 (4.4)	88.5 (4.7)	108.5 (1.6)	88.3 (10.5)	85.0 (6.5)	87.5 (9.2)	69.3 (5.2)
	0.10	78.0 (9.6)	84.8 (7.2)	95.8 (7.9)	87.3 (8.4)	91.5 (5.9)	89.3 (8.3)	82.3 (7.1)	88.8 (6.2)	69.0 (4.7)
Mixed vegetables	0.01	88.3 (8.5)	88.5 (8.6)	108.5 (4.4)	82.8 (6.5)	102.3 (7.2)	107.3 (9.7)	78.5 (7.4)	105.3 (6.8)	75.3 (6.1)
	0.10	93.3 (9.8)	90.5 (7.8)	105.0 (3.0)	83.4 (5.4)	99.8 (6.4)	98.9 (7.9)	81.4 (4.9)	109.0 (8.6)	70.8 (4.1)
Mean,%		87.1	90.5	98.1	88.4	97.2	93.7	83.2	96.0	70.1
CV, %		7.1	5.6	9.2	6.0	7.8	8.4	3.6	9.3	3.9

[a] Each value is the mean of 4 determinations.

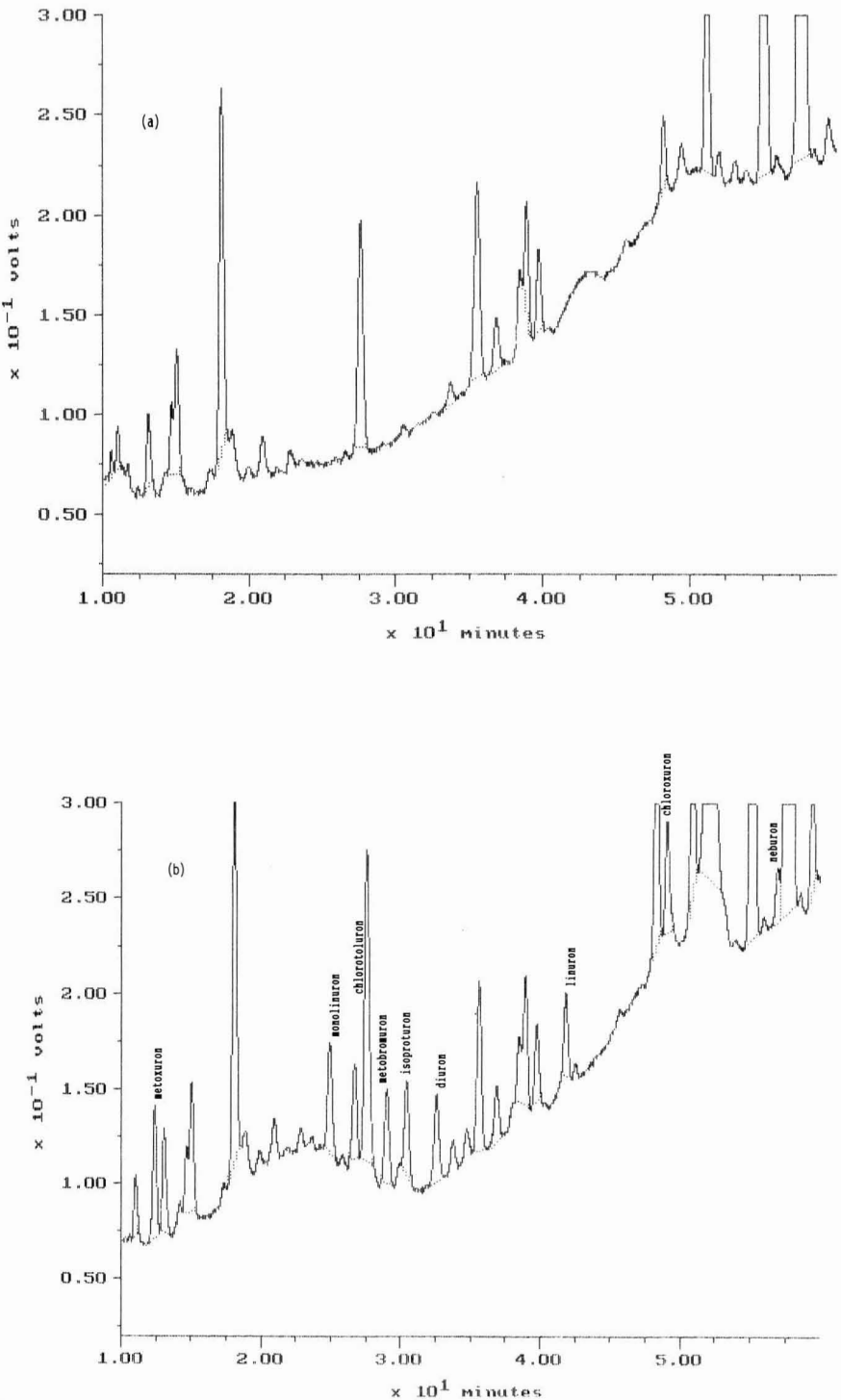

Figure 1. LC/UV chromatograms of (a) unspiked carrot extract and (b) the same sample spiked with herbicides at 0.010 mg/kg. Injected amount, 40 μL of 4 g/mL purified samples. Lichrospher R18 5 μm end-capped 25 cm × 4.6 mm id column. Gradient program: at the start 50% organic modifier (methanol–acetonitrile, 85 + 15), 50% water; the percentage of organic solvent was linearly increased to 55% in 15 min and to 75% in 30 min.

Florisil Cleanup

Before use, the Florisil cartridge was washed sequentially with 5 mL acetone–*n*-hexane (50 + 50, v/v), 5 mL acetone–*n*-hexane (15 + 85, v/v), and 5 mL ethyl acetate–*n*-hexane (20 + 80, v/v).

The cartridge was eluted by gravity flow.

A portion (1 mL) of extract from GPC cleanup (1.01 g sample) was passed through the cartridge, and the effluent was discarded. Ethyl acetate–*n*-hexane (3 mL, 20 + 80, v/v) was added, and the eluate was discarded. Phenylurea herbicides were eluted with 1 mL ethyl acetate–*n*-hexane (20 + 80, v/v) (fraction 1), 5 mL acetone–*n*-hexane (15 + 85, v/v) (fraction 2), and 4 mL acetone–*n*-hexane (50 + 50, v/v) (fraction 3). For carrot and potato samples, fractions 1–3 were collected together in a 10 mL culture tube and evaporated to dryness under a stream of nitrogen at ca 30°C. The residue was dissolved in 250 µL methanol–acetonitrile–water (85 + 15 + 100, v/v/v) (4.04 g sample/mL). For mixed vegetable samples, fractions 1 + 2 and 3 were collected separately.

LC Analysis

The system was equilibrated with 50% organic modifier (methanol–acetonitrile, 85 + 15) in water at flow rate of 0.7 mL/min for at least 30 min. The wavelength of the UV detector was set at 242 nm (absorption unit fill scale = 0.01).

A portion (40 µL) of filtered sample or standard solution was injected. Gradient elution was performed as described earlier. Quantitation was done by comparison of peak areas using external standardization.

Recovery Study

For recovery experiments, homogenized carrot, potato, and mixed vegetable samples were spiked at 0.010 and 0.100 mg/kg. Then, 2 different volumes (20 or 200 µL) of phenylurea intermediate solution (10 µg/mL) were added to 20 g of each matrix in a centrifuge bottle. Resulting samples were mixed and allowed to stand for 15 min before extraction. Four replicates at each fortification level were prepared.

Results and Discussion

Crops analyzed in this study (carrots, potatoes, and mixed vegetables) have established tolerances for at least some of the herbicides on which fortification tests were conducted (15, 16).

Recoveries of 9 phenylurea herbicides spiked at 0.010 and 0.100 mg/kg are presented in Table 1. Extraction with acetone and partition with ethyl acetate–cyclohexane (50 + 50, v/v) combined with GPC and SPE cleanup resulted in good precision and accuracy for all samples. Average recoveries ranged from 70 to 98%, and coefficients of variation (CVs) were between 5.6 and 9.3%.

Figure 1 shows the separation of 9 compounds on a C_{18} LC column obtained by injecting a carrot extract fortified at 0.010 mg/kg. The chromatogram of the unfortified sample is free from peaks interfering with active compounds.

Extraction

The extraction and partition steps used in this study were based on a procedure described by Specht (14) for determining pesticide residues with a wide range of polarity in various vegetables. Sample preparation involved extraction with acetone after the watery phase was saturated with sodium chloride. Active compounds were then partitioned with ethyl acetate–cyclohexane (50 + 50, v/v). We modified this method by miniaturizing the whole extraction and partition procedures and separating phases (watery and organic) through centrifugation. These changes reduced solvent consumption and analytical time.

A major advantage of the method described here is use of single extraction to produce an extract that could also be used for multiresidue analysis of various pesticide classes in vegetable samples. It is planned, in fact, to validate this method for organophosphorus insecticides, organonitrogen fungicides, and pyrethroids in various processed fruits and vegetables.

GPC Purification

To lower the threshold of detectability, it is necessary to concentrate the extract considerably. Concentration increases the amount of coextracts. An initial partial purification was obtained by GPC. Ethyl acetate–cyclohexane (50 + 50, v/v) was used for solvent partitioning, and Biobeads SX3 resin was used for column packing. Except for a few studies that used a miniaturized GPC column (17, 18), most published methods used large-capacity columns to separate pesticides from coextractives (14, 19).

The combination of narrow chromatographic column (1 cm id instead of classic column of 2.5 cm id), reduced loop volume (1 mL instead of 5 mL), and low flow rate (1 mL/min instead of 5 mL/min) allowed herbicides to be eluted in a narrow band (7 mL) while consuming only 35 mL solvent for each sample. However, GPC purification was insufficient as it did not eliminate interferences in LC separation. An additional purification step was therefore necessary.

SPE Purification

A Sep-Pak cartridge containing 0.900 g Florisil was chosen as SPE stationary phase. The procedure was based on the

Table 2. Distribution of 9 phenylurea herbicides in fractions eluting from SPE with Florisil[a]

Compound	Fraction 1	Fraction 2	Fraction 3
Metoxuron			****
Monolinuron		***	
Chlorotoluron			***
Metobromuron		***	
Isoproturon			****
Diuron			***
Linuron	*	***	
Chloroxuron			****
Neburon		****	

[a] Fractions are specified in *Experimental* under *Florisil Cleanup*. *, ca 10–30%; ***, ca 70–90%; ****, >90%.

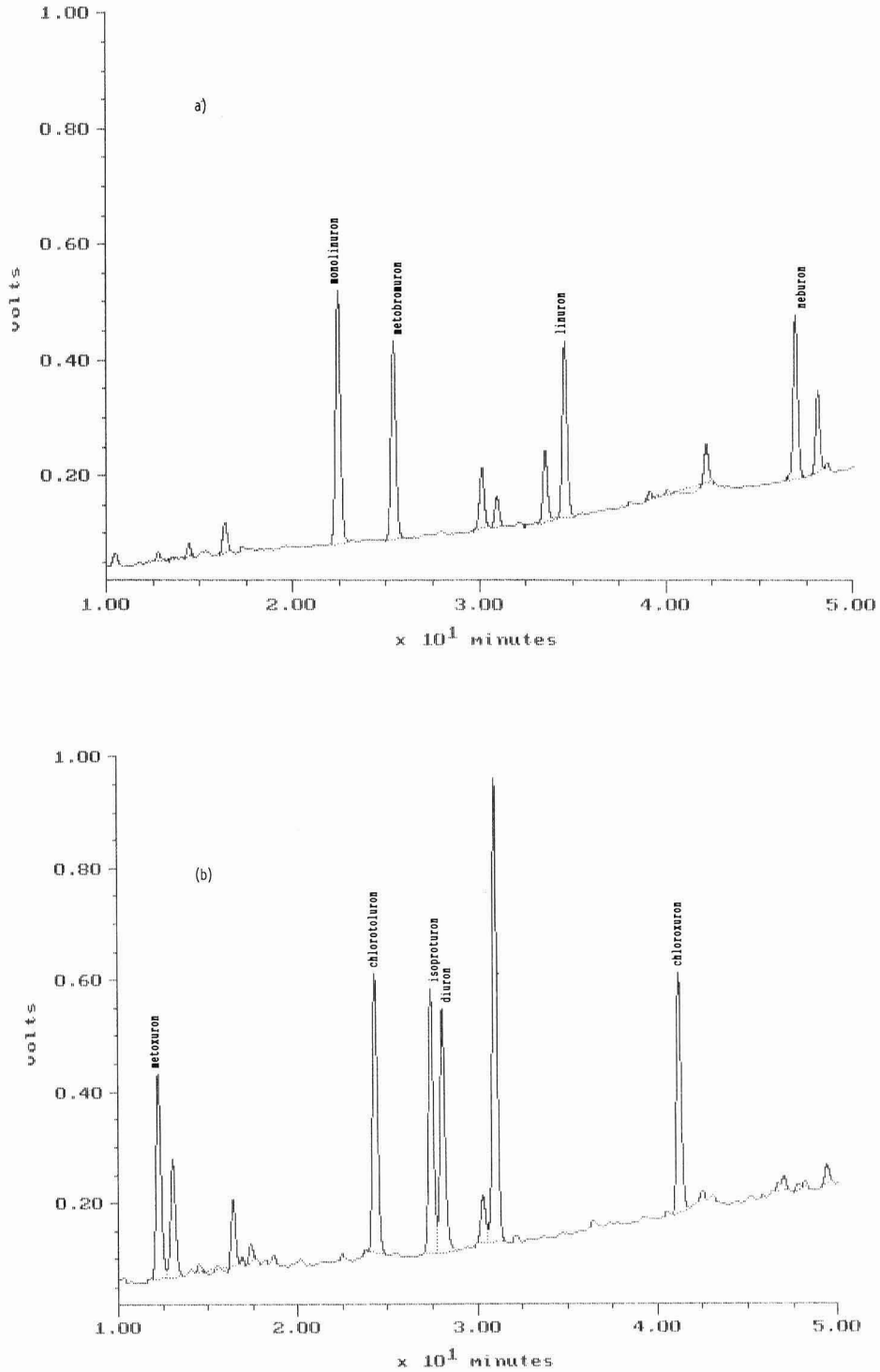

Figure 2. LC/UV chromatograms of mixed vegetables spiked with herbicides at 0.100 mg/kg: (a) fraction 1 + 2 and (b) fraction 3 eluted from Florisil cartridge. Injected amount, 40 μL of 4 μg/mL purified samples. Conditions as described in caption of Figure 1.

method described by Lawrence (5), using a 1.5 cm id column packed with 20 g Florisil. Volumes of eluant were consequentially reduced, and dichloromethane (30% in *n*-hexane) was replaced with the less hazardous ethyl acetate (20% in *n*-hexane). Carrot and potato extracts were cleaned up, and 3 fractions were collected, as described earlier. Preliminary recovery tests were performed with a mixed standard solution. Results demonstrated that the 9 phenylurea herbicides are distributed among the different fractions as shown in Table 2.

The sample of mixed vegetables analyzed was a frozen product for vegetable soup preparation. It contained a variety of vegetables (carrot, potato, bean, pea, tomato, pumpkin, zucchini, chard, string bean, cabbage, parsley, basil, garlic, onion, and celery). Determination of 9 active compounds in this complex matrix was obtained with the lowest number of coextractive peaks in the liquid chromatogram by analyzing fractions 1 + 2 and 3 separately. Monolinuron, metobromuron, linuron, and neburon eluted in fraction 1 + 2, while metoxuron, chlorotoluron, isoproturon, diuron, and chloroxuron eluted in fraction 3 (Figure 2). In all cases, analysis of separate fractions can be used as a method of identifying compounds.

LC Determination

Several elution programs using various concentrations of methanol/water and methanol/acetonitrile/water were tried with the C_{18} column. Gradient elution gave the best separation. Moreover, it caused late-eluting compounds to elute sooner, thus giving narrower bands, increased peak heights, and a lower limit of detection.

All 9 herbicides gave linear responses over the concentration range 1.6–20 ng, with correlation coefficients of about 0.999 (number of data points, 10; 2 replicates for each data point).

A fortification level of 0.010 mg/kg represents the quantitation limit for the 9 phenylurea compounds in potatoes and carrots, on the basis of a 40 µL injection of the final extract. This level is equivalent to 160 mg sample (signal-to-noise ratio, >10). For the mixed vegetable sample, the quantitation limit of chlorotoluron was 0.030 mg/kg because of an interfering peak at the retention time of chlorotoluron.

Conclusions

The method allows simultaneous determination of 9 phenylurea herbicides in carrots, potatoes, and mixed vegetables. The method involves extraction and cleanup by GPC and SPE on a Florisil cartridge. Active compounds are determinated by LC with UV detection. The method yields 70–98% recoveries at 2 fortification levels. Limits of quantitation are 0.010 mg/kg.

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RESIDUES AND TRACE ELEMENTS

Microwave-Assisted Extraction Coupled with Gas Chromatography with Nitrogen–Phosphorus Detection or Electron Capture Negative Chemical Ionization Mass Spectrometry for Determination of Dimethomorph Residues in Soil

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Dimethomorph, a cinnamic acid derivative, is a crop protection product for the control of fungi in grapes, tomatoes, potatoes, and tobacco. Of the extraction techniques evaluated, microwave-assisted extraction (MAE) gave the best extractability of the analyte at trace levels from soil. After an appropriate cleanup, dimethomorph residues could be determined by gas chromatography (GC) with either nitrogen–phosphorus detection (NPD) or mass spectrometry with electron capture negative chemical ionization (ECNCI). The sensitivity and specificity of the latter detection technique made it possible to use a greatly simplified cleanup procedure. The limit of quantitation of both procedures was 10 ppb. Soils fortified over a range of 10 to 500 ppb gave an average recovery of 88%, with a standard deviation of 11% by GC–NPD and an average recovery of 99%, with a standard deviation of 8.8% by GC/ECNCI. Control soils generally showed apparent residues of less than 1 ppb.

Dimethomorph, (*E,Z*)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)acryloyl]morpholine (Figure 1), is a new systemic oomycete fungicide showing specific activity against members of the family Peronosporaceae and the genus *Phytophthora* (1). Important pests controlled are *Plasmopara viticola* on vines (1–3), *Phytophthora infestans* on potatoes (4–7) and tomatoes (7), and *Peronospora tabacina* in tobacco (8). Dimethomorph has a biological mode of action that differs from other fungicides and shows no cross-resistance to such products. It can be used to control fungal strains that have developed resistance to other fungicides. Dimethomorph causes no phytotoxicity or crop effects at dose rates well in excess of those required for effective disease control (1).

To determine a compound's persistence in the environment, analytical methods must be developed for monitoring the compound at low part-per-billion levels from soil rate-of-dissipation (ROD) studies. Analytical methods have been reported for the determination of dimethomorph residues in potatoes and tomatoes by thin-layer chromatography (TLC; 9), in grapes by liquid chromatography (LC; 10), in barley and flue-cured tobacco by gas chromatography (GC) and LC (11), and as part of a multiresidue screen from soil by supercritical fluid extraction coupled with high-performance TLC (12). Reversed-phase LC for dimethomorph in technical and formulations has received provisional Collaborative International Pesticides Analytical Council (CIPAC) method adoption status (13) and has been recommended for adoption as an AOAC–CIPAC method (14).

The purpose of this study was to develop appropriate extraction, cleanup, and instrumental techniques for determining dimethomorph in soil at 10 ppb. The effectiveness of different extraction techniques was evaluated, as was the impact of the final determinative technique on the requirements for sample cleanup prior to analysis.

METHOD

Special Notes

Rinse all clean glassware thoroughly with methanol and dry before using. All solvents should be distilled-in-glass and suitable for pesticide analysis (Burdick & Jackson Laboratories, Inc., Muskegon, MI, or equivalent). Water should be purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA), or equivalent. All samples should be run completely through the method in 1 day. When using the solid-phase extraction (SPE) cartridge, allow each liquid to drain just below the top of the frit above the sorbent bed. Do not allow the cartridge to go to dryness. Appropriate care should be taken when handling CH_2Cl_2, a possible carcinogen.

Reagents and Apparatus

(**a**) *Dimethomorph standard solutions.*—Prepare fortification standard solutions of 10, 5.0, and 1.0 μg/mL in ethyl acetate. For GC with nitrogen–phosphorus detection (GC–NPD), prepare analytical standard solutions of 0.40, 0.20, 0.10, and 0.05 μg/mL from the 10 μg/mL standard solution. For GC with detection by mass spectrometry (MS) using electron capture negative chemical ionization (GC/ECNCI), prepare analytical standard solutions of 0.05, 0.01, and 0.005 μg/mL from the

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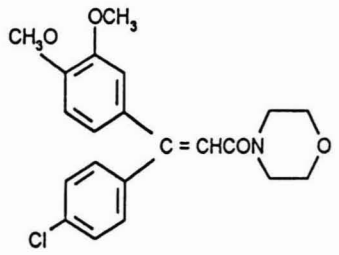

Figure 1. Chemical structure of dimethomorph (50:50 *E:Z*).

1.0 µg/mL standard solution. When stored in amber bottles in a refrigerator, standard solutions are stable for at least 1 month. Dimethomorph is available from American Cyanamid Company, Agricultural Products Research Division, Princeton, NJ.

(**b**) *Extraction solvent.*—Prepare 10% water in acetonitrile by adding 100 mL water to 900 mL acetonitrile and mix well.

(**c**) *SPE cartridge.*—For GC–NPD, Isolute silica gel (2 g/6 mL; International Sorbent Technology, Mid Glamorgan, UK) and a Supelclean LC-alumina-neutral (1 g/3 mL; Supelco, Inc., Bellefonte, PA). For GC/ECNCI, Varian Bond Elut C_{18} (200 mg/3 mL; Varian, Harbor City, CA).

(**d**) *Elution solvents for SPE cartridges.*—Prepare 10% CH_3CN in CH_2Cl_2 by adding 10 mL CH_3CN to 90 mL CH_2Cl_2 and mix well. Prepare 30% CH_3CN in CH_2Cl_2 by adding 30 mL CH_3CN to 70 mL CH_2Cl_2 and mix well. Prepare 25% CH_2Cl_2 in ethyl acetate by adding 25 mL CH_2Cl_2 to 75 mL ethyl acetate and mix well.

(**e**) *Vacuum processing station.*—VacMaster equipped with a polytetrafluoroethylene stopcock/needle assembly (International Sorbent Technology).

(**f**) *Soils.*—Tippecanoe (IN) silt loam, Beardon (ND) clay loam, Sharkey (AR) clay loam, and Sassafras (NJ) sandy loam. *See* reference 15 for additional details. Aged field-treated samples were taken from a soil ROD study 2 months after application of radiolabeled dimethomorph and were a North Carolina sandy clay loam (61% sand, 19% silt, 20% clay). Fortified samples were provided by spiking a 20 g soil subsample weighed into a microwave extraction tube with the appropriate analyte solution and allowing the solvent to evaporate. Unspiked soils were used as controls.

(**g**) *Microwave extractor.*—Model No. MES 1000 (CEM Corporation, Matthews, NC).

(**h**) *Disposable reservoirs (for GC–NPD).*—25 mL (International Sorbent Technology).

(**i**) *Centrifuge (for GC/ECNCI).*—Adams Compact II (Becton-Dickinson, Cockeysville, MD).

(**j**) *Disposable centrifuge tubes (for GC/ECNCI).*—10 mL (Kimble Glass, Inc., Vineland, NJ).

(**k**) *Disposable scintillation vials (for GC/ECNCI).*—20 mL (Fisher Scientific, Pittsburgh, PA).

(**l**) *GC–NPD system.*—Hewlett Packard Model 5890 Series II equipped with an NPD and a 15 m × 0.53 mm id (1.0 µm film), Rtx-1 fused silica capillary column (Restek Corp., Bellefonte, PA), and a Hewlett Packard 3396A integrator. Operating conditions: He carrier gas 6 mL/min; He makeup gas 40 mL/min; 3 µL splitless injection; injector, 250°C; detector, 280°C; column, 270°C; retention time, ca 4 min.

(**m**) *GC/ECNCI system.*—Finnigan-MAT SSQ710 operated in the negative-ion mode and equipped with a Finnigan-MAT Model A200S autoinjector. GC operating conditions: column, 5 m × 0.25 mm id (0.25 µm film) DB-5MS (J&W Scientific, Folsom, CA); column oven temperature, 60° to 280°C at 20°C/min and hold at 280°C for 2 min; injector temperature, 280°C; transfer line temperature, 250°C; H_2 carrier gas at 5 psi; 1 µL splitless injection (split open at 0.5 min); retention time, ca 9.5 min. MS operating conditions: ions monitored, *m/z* 387 and 389; scan time, 0.5 s; CI reagent gas, CH_4 at 9000 mT (indicated); source temperature, 150°C; electron energy, 70 eV; filament emission current, 400 µA; conversion dynode voltage, +15 kV; electron multiplier voltage, 1350 V; preamplifier range, 10^{-8} A/V.

Procedure for GC–NPD

Weigh 20 g soil into a microwave extraction vessel, add 20 mL extraction solvent, and stir with a spatula. Place vessel in the microwave and extract for 3 min at 125°C. After vessel has cooled, filter extract into a 250 mL filtration flask through 7 cm glass-fiber filter paper placed on a 7 cm Buchner funnel. Wash extraction vessel and filter cake 2 times with 40 mL fresh extraction solvent. Pour combined filtrates into a 100 mL graduated mixing cylinder, dilute to 100 mL with extraction solvent, mix, and pour 50 mL (10 g-equiv) into 250 mL separatory funnel. Add 50 mL water to separatory funnel followed

Table 1. Recovery of dimethomorph from soil by GC–NPD

Soil	Dimethomorph control, ppb	Recovery of added dimethomorph, %			
		10 ppb	20 ppb	100 ppb	500 ppb
IN	<1.0	—[a]	97 (3)[b]	88 (1)	—
MS	<1.0	98 (2)	—	89 (2)	87 (2)
AR	<1.0	84 (3)	96 (1)	—	80 (1)
NJ	<1.0	—	88 (2)	87 (1)	—
FL	<1.0	—	—	71 (2)	—

[a] Recovery experiment not performed at this fortification level.
[b] Number of replicate analyses indicated in parentheses.

by 50 mL hexane and shake vigorously for ca 15 s. Draw and save lower aqueous acetonitrile layer in a 250 mL beaker and discard upper hexane layer. Pour aqueous acetonitrile back into the separatory funnel, add 50 mL CH_2Cl_2, and partition vigorously for ca 15 s. Collect lower CH_2Cl_2 layer in a 250 mL round-bottom flask and evaporate just to dryness on a rotary evaporator (ca 30°C). Dissolve residue in 10 mL CH_2Cl_2.

Prepare a silica gel cartridge (2 g/6 mL) by conditioning the cartridge with 10 mL CH_2Cl_2. Do not allow cartridge to run dry. Using an adaptor, connect a 25 mL disposable reservoir onto the top of the silica gel cartridge. Add sample to reservoir. Rinse flask with 2 mL CH_2Cl_2 and add to reservoir. Pass sample through column under gravity at ca 2–3 drops/s. Wash cartridge with 10 mL CH_2Cl_2 followed by 10 mL 10% CH_3CN in CH_2Cl_2 at a flow rate of 2–3 drops/s. Pass 25 mL 30% CH_3CN in CH_2Cl_2 through column at 2–3 drops/s and collect in a 100 mL pear-shaped flask. Using a rotary evaporator, evaporate sample to dryness and dissolve residue in 5 mL CH_2Cl_2.

Prepare a Supelclean alumina-neutral cartridge by conditioning the cartridge with 5 mL CH_2Cl_2. Do not allow cartridge to run dry. Connect a 25 mL disposable reservoir onto top of cartridge. Add sample to reservoir. Rinse flask with 2 mL CH_2Cl_2 and add to reservoir. Pass sample through column under gravity at ca 1–2 drops/s. Pass through 15 mL 25% CH_2Cl_2 in ethyl acetate at 2–3 drops/s and collect in 100 mL pear-shaped flask. Evaporate sample just to dryness, add 2 mL methanol to flask, and reevaporate just to dryness. Dissolve residue in 1 mL ethyl acetate for GC–NPD analysis.

Quantitate analyte in samples and standards by summing peak heights (or areas) of the 2 peaks. Check linearity of standard curve by using the analytical standard solutions (50–400 ng/mL). The 200 ng/mL standard is used as the working standard for each set of samples analyzed. Calculate the amount of residue as follows:

$$\text{Residue, ppb} = 2 \times R(\text{samp}) \times V \times C(\text{std}) \times \text{DF}/[R(\text{std}) \times W]$$

where 2 = ratio of the total extraction solvent volume to the volume of extract taken for analysis (100 mL/50 mL), R(samp) = response of sample, R(std) = response of standard, V = volume (mL) of final solution for analysis (usually 1 mL), DF = dilution of sample if V is too concentrated (DF = 1 if no dilution made), C(std) = concentration (ng/mL) of standard solution (usually 200 ng/mL), and W = amount of sample used (g) in analysis (usually 20 g).

Procedure for GC/ECNCI

Weigh 20 g soil into a microwave extraction vessel, add 20 mL extraction solvent by pipet, and stir with a spatula. Place vessel in the microwave and extract for 3 min at 125°C. After the vessel has cooled, decant a few milliliters of supernatant into a 10 mL disposable centrifuge tube and centrifuge at 3200 rpm for 5 min. Transfer 1 mL (1 g-equiv) liquid by pipet from the centrifuge tube to a 20 mL disposable scintillation vial, place vial on a sample concentrator, and evaporate most of the acetonitrile under a gentle stream of nitrogen with the heating block set at ca 60°C. (It is not necessary to evaporate the extract to dryness.) Add 1–2 mL water to extract and swirl to dissolve or suspend residue.

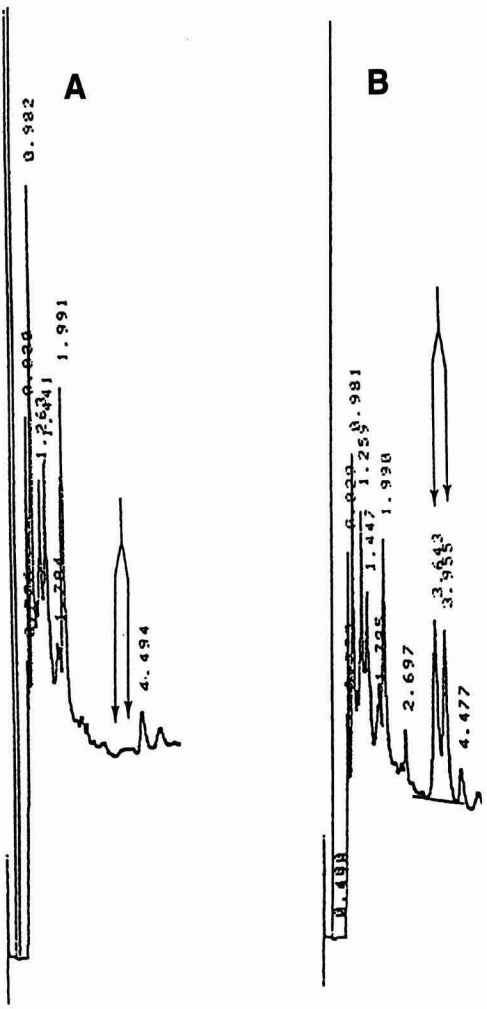

Figure 2. Chromatograms from GC–NPD of (A) control IND soil and (B) IND soil fortified with dimethomorph at 10 ppb.

Table 2. Recovery[a] of dimethomorph from soil by GC/ECNCI

Soil	Dimethomorph in control, ppb	Recovery of added dimethomorph, %		
		10 ppb	50 ppb	250 ppb
IN	1.0	107.2	100.9	94.8
AR	1.1	101.7	95.9	91.0
NJ	0.9	108.3	105.7	96.1
ND	1.2	108.1	92.9	83.4

[a] Values are averages of duplicate samples processed through the procedure.

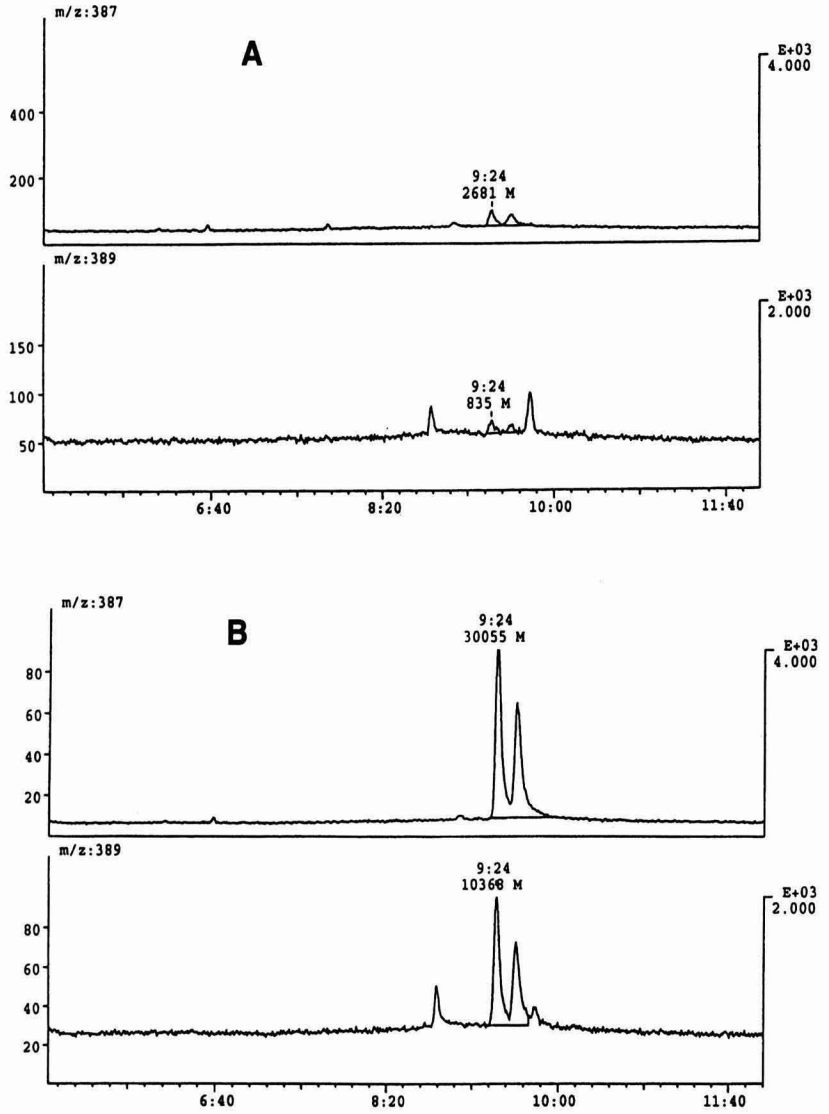

Figure 3. Chromatograms from GC/ECNCI of (A) control IND soil and (B) IND soil fortified with dimethomorph at 10 ppb.

Prepare 200 mg Bond Elut C_{18} cartridge by washing with 1 column volume (3 mL) each of hexane, CH_2Cl_2, CH_3OH, and H_2O in that order. With a Pasteur pipet, transfer extract from scintillation vial to the reservoir. Rinse vial with ca 1 mL water and transfer the rinse with the same Pasteur pipet to the reservoir. After slowly loading the cartridge with the soil extract at a rate of ca 1 drop/2–3 s, rinse cartridge with 1 column volume of H_2O followed by 1 column volume hexane. Elute analyte into a clean disposable scintillation vial with 1 column volume CH_2Cl_2. Evaporate CH_2Cl_2 under a stream of nitrogen and dissolve residue in 1.0 mL ethyl acetate.

Quantitate analyte in samples and standards by summing peak areas of the 2 peaks. Check linearity of standard curve by using the analytical standard solutions (5–50 ng/mL). The 10 ng/mL standard is used as the working standard for each set of samples analyzed. Calculate residue in parts per billion by using the equation given under *Procedure* for GC–NPD after substituting 20 for the first term (20 mL/1 mL) and 10 ng/mL for *C*(std).

Results and Discussion

Of the potential non-mass-spectrometric GC detection techniques evaluated, GC–NPD gave the best response for dimethomorph. For satisfactory performance of the GC–NPD method, the most critical factor was flow rates of eluants

Table 3. Evaluation of extractants on aged field-treated soil

Technique	Extractant	Time, min	Carbon-14 extracted, %[a]	Carbon-14 as dimethomorph, %
Shaker	10% H_2O in CH_3CN	30	52	39
Shaker	10% H_2O in CH_3CN	240	50	—[b]
Shaker	25% 0.1N HCl in CH_3CN	60	72	39
MAE	10% H_2O in CH_3CN	3	58	54
MAE	10% H_2O in CH_3CN	45	59	48
MAE	50% H_2O in CH_3OH	3	49	42
MAE	50% H_2O in CH_3OH	45	61	48
Sonication	30% 0.1N HCl in acetone	4	71	44

[a] Equals $100 \times (dpm/g_{extracted})/(dpm/g_{extracted} + dpm/g_{marc})$.
[b] Not determined.

through the SPE cartridges. If recommended flow rates were not adhered to, low recoveries would result. This effect was noted even at flow rates as low as twice the recommended values.

In selecting a MS detection technique, GC/electron ionization (EI) MS generated a molecular ion of only 10% relative intensity at *m/z* 387 and a base peak at *m/z* 301, corresponding to loss of the morpholino group from the molecular ion. GC/chemical ionization (CI) MS in positive ion and negative ion detection modes generated solely molecular species for dimethomorph, (M + H) for positive ion and M^- from electron capture detection (ECD) in the negative ion mode. However, the negative ion response was about 100-fold greater than the positive ion response. This response enhancement in GC/ECNCI was interesting, given that GC–ECD showed no such response advantage when compared with GC–NPD and was actually less sensitive than GC–NPD. Similar behavior has been observed previously in our laboratories in GC/ECNCI analyses of imidazolinone herbicides (15, 16). Again, GC/ECNCI was the superior MS detection technique while GC–ECD showed no response advantage over GC–NPD. A potential explanation for the response difference between GC–ECD and GC/ECNCI could be the range of electron energies within the detectors, with GC/ECNCI having a lower range of energies as a result of lower initial energies from the filament (70 eV). A lower range of electron energies would facilitate electron capture by compounds not possessing as high of an electron capture cross-section.

To validate the methods, control soils were spiked with the appropriate dimethomorph standard solution and carried through each procedure. For GC–NPD, recoveries were run in the fortification range of 10 to 500 ppb (Table 1). Overall, the recovery expressed as the average ± 1 standard deviation (SD) was 88 ± 11%. At the limit of quantitation (LOQ), recovery was 89 ± 12%. Control soils showed apparent dimethomorph residues of <1 ppb. The response was linear from 150 to 1200 pg injected on-column (corresponding to 5 to 40 ppb analyte in soil), and samples with higher levels were diluted to fit on the standard curve. Figure 2 shows typical GC–NPD chromatograms from a control soil and a soil fortified at 10 ppb. For GC/ECNCI, recoveries were run in the fortification range of 10 to 250 ppb (Table 2). Overall, the recovery expressed as the average ± 1 SD was 99 ± 8.8%. At the LOQ, recovery was 106 ± 6.4%. Again, control soils showed apparent dimethomorph residues of about 1 ppb. The response was linear from 5 to 50 pg injected on-column (corresponding to 5 to 50 ppb analyte in soil), and samples with higher levels were diluted to fit on the standard curve. Figure 3 shows typical chromatograms from GC/ECNCI analyses of control soil and a soil fortified at 10 ppb. The monitored ions, *m/z* 387 and 389, are ^{35}Cl and ^{37}Cl isotopes of the M^- ion.

While both methods of analysis were validated successfully and the GC–NPD approach certainly used less expensive and more widely available instrumentation, the sensitivity and specificity of GC/ECNCI offered some distinct advantages for a simplified sample cleanup. A comparison of the working standard concentrations corresponding to 10 ppb dimethomorph in soil showed a sensitivity advantage of 10 to 1 for GC/ECNCI versus GC–NPD. Because of this sensitivity enhancement, the amount of soil extract requiring processing for GC/ECNCI was reduced proportionately. The specificity of GC/ECNCI enabled a simple 1-cartridge cleanup to be developed rapidly that was patterned after one used for determination of imidazolinones in soil (15, 17). The 1-cartridge cleanup used for GC/ECNCI required processing only 1 mL crude soil extract and effected the same cleanup as the initial 2 partitioning steps used in the GC–NPD approach. The specificity of GC/ECNCI then permitted analysis of this extract without the additional 2-cartridge cleanup required for GC–NPD.

The utility of GC/ECNCI was clearly demonstrated in the rapid evaluation of extractability of dimethomorph from aged field-treated soil in a carbon-14 extractability study. This study was used to select the appropriate extractant and extraction technique for removing the parent compound from soil. The solvents and techniques evaluated and the amount of radioactivity extracted are given in Table 3, with each row representing a separate experiment. At higher levels of residual parent (>250 ppb) in the soil, the extracts were directly analyzed by GC/ECNCI after evaporation of the water in the aqueous organic extractant and dissolution of the residue in organic solvent. At lower levels or in acidic extracts, the samples were quickly processed through the 1-cartridge cleanup system prior

to analysis. (One injection of an acidic aqueous organic extract after evaporation and dissolution destroyed chromatographic peak resolution, thus, necessitating replacement of the GC column.) While column 4 of Table 3 gives the results from each experiment on the basis of the amount of carbon-14 extracted (in any form) as a percentage of total carbon-14 in the soil, column 5 lists the results on the basis of the amount of dimethomorph extracted (as determined from GC/ECNCI) as a percentage of total carbon-14 in the soil. Thus, while other approaches could extract a higher percentage of the total radioactivity in the soil (column 4), microwave-assisted extraction (MAE) with 10% water in acetonitrile most effectively extracted only the parent compound (column 5). In an additional study using MAE with a stronger extractant (25% 0.1N HCl in acetonitrile) on the marc left after MAE with 10% water in acetonitrile, only an additional 11% of radioactivity was extracted, yielding only an additional 3% of the parent compound. Thus, MAE with a stronger extractant only marginally increased the extractability of dimethomorph while releasing more soil coextractives, which would require subsequent cleanup.

In conclusion, methods of analysis using GC–NPD and GC/ECNCI have been developed for determining dimethomorph in soil at 10 ppb. MAE with 10% water in acetonitrile was determined to most efficiently extract the parent compound from soil. While GC/ECNCI was a more expensive and operationally complex technique than GC–NPD, the sensitivity and specificity of GC/ECNCI offered a simpler and faster cleanup procedure prior to final instrumental determination.

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RESIDUES AND TRACE ELEMENTS

Double Calibration (External Calibration and Stable Isotope Dilution) for Determining Selenium in Plant Tissue by Hydride Generation Inductively Coupled Plasma Mass Spectrometry

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Both external calibration (EC) and stable isotope dilution (SID) calibration are reliable methods for determining selenium by hydride generation inductively coupled plasma mass spectrometry (HG-ICP-MS), but they are rarely used simultaneously. Because these methods are independent of each other, they could be used simultaneously to test each other and to evaluate data quality directly. A double calibration approach using EC and SID simultaneously was developed to evaluate data quality directly for analysis of Se in plant tissue by HG-ICP-MS. Natural Se was used for EC. ^{77}Se-enriched Se (0.004 μg; ^{77}Se = 94.38%) was spiked to 0.5 g sample for SID (^{78}Se or ^{82}Se isotope used as reference isotope). Sample preparation and measurement was not doubled, because one spiked sample served for both EC and SID. All samples were digested with acid and heat, selenate was reduced to selenite, and selenite was measured by HG-ICP-MS. The Se determined by SID corresponded to that determined by EC with a concentration ratio of 1.02 ± 0.08 (n = 207 alfalfa samples). The measured Se in 8 reference materials also agreed well with certified values. The study showed that the double calibration approach was a direct way to evaluate data quality. This approach should be applicable to other multiisotope elements.

Selenium is one of the dual-role elements. It is essential to animals but can also be toxic at high intake levels (1). It is important to measure Se content of forage accurately and precisely because of the narrow range of animal deficiency (0.05 mg Se/kg) and toxicity (2–5 mg Se/kg) (1, 2). Determination of Se in forage remains challenging because of its low concentration and the fact that analysis requires sensitive fluorometry or neutron activation analysis for best results (3). Recently, Se has been determined in various samples by hydride generation inductively coupled plasma mass spectrometry (HG-ICP-MS; 4–7).

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Determination of any analyte requires an instrument calibration to ensure that the actual sample's analyte is quantitated. External calibration (EC) and stable isotope dilution (SID) calibration are 2 options available for ICP-MS. In the EC method, the analyte signals measured sequentially from separately prepared samples and standards are compared to quantitate the sample's analyte concentration. Good results are obtained for determining Se in plant tissue by HG-ICP-MS (8), although EC is susceptible to matrix effects (chemical, physical, specific, and nonspecific interferences during sample digestion, hydride generation, gas–liquid separation, and ICP-MS measurement), laboratory operational error (digestion and dilution error), and instrument instability during measurement.

SID is applicable only to MS (4, 5, 9–13). A standard with an enriched isotope is spiked to a sample to change the analyte's isotope abundance. The change in isotope abundance is then measured to quantitate the sample's analyte concentration. The standard and the sample are prepared and measured under identical conditions. Thus SID is robust and free of the various problems associated with EC. Good results can be obtained even if samples are not completely digested, as long as the spiked analyte is isotopically equilibrated with the sample's analyte. SID is usually the most accurate and precise among many other analytical methods, including EC.

Either EC or SID is used for determining Se in various kinds of samples by ICP-MS, but they are rarely used simultaneously (4–6, 8, 12). Data quality for any individual sample determined by either calibration method is only indirectly assured by stating the data quality of other samples such as standard reference materials.

EC and SID should serve as complementary and independent methods for each other if they could be applied simultaneously. Then, the quality of any particular sample's result could be directly evaluated. The ability to unequivocally evaluate data quality is important for precise and accurate analysis, especially for determination of trace and difficult elements such as Se. In this study, the 2 methods were used simultaneously to achieve these objectives.

Experimental

Materials

(a) *Standard reference materials (SRM).*—SRM 1515 Apple Leaves, SRM 1547 Peach Leaves, SRM 1573a Tomato Leaves,

Table 1. Sample preparation scheme

Material for analysis	^{N}BLK	^{77}BLK	^{N}CLB			$^{N+77}$CLB				^{77}SAM
Sample, g or mL	0	0	0	0	0	0	0	0	0	0.5 or 2
In-house alfalfa, g	0	0	0	0.3	0.3	0.3	0.3	0.3	0.3	0
^{77}Se-STD (0.02 mg/L), mL	0	0.2	0	0.2	0.2	0.2	0.2	0.2	0.2	0.2
^{N}Se-STD (1.00 mg/L), mL	0	0	0.4	0	0.05	0.1	0.2	0.4	0	0
Data used in SID	Yes		Yes							Yes
	^{N}CLB–^{N}BLK: For mass bias correction and mass bias drift correction ^{77}SAM–^{N}BLK: For sample calculation									
Data used in EC		Yes	Yes			Yes				Yes
	^{N}CLB: For signal drift correction $^{N+77}$CLB–^{77}BLK: For external calibration line ^{77}SAM–^{77}BLK: For sample calculation									

SRM 1575 Pine Needles, and SRM 1643d Water (National Institute of Standards and Technology [NIST], Gaithersburg, MD).

(**b**) *Reference materials (RM).*—RM 8413 Corn Kernel, RM 8418 Wheat Gluten, and RM 8433 Corn Bran (NIST).

(**c**) *Alfalfa samples.*—Two hundred and seven alfalfa samples from Wisconsin field variety demonstrations during 1992–1995 were dried at 55°C for 14 h, ground (1 mm mesh size), and stored in plastic bags at room temperature. An in-house stock of alfalfa tissue was used to make calibrators for EC.

Reagents

(**a**) *Nitric acid.*—About 16M HNO_3 (68%; trace metal grade; Fisher, Fair Lawn, NJ).

(**b**) *Hydrochloric acid.*—About 12M HCl (36%; reagent ACS grade; Fisher).

(**c**) *Perchloric acid.*—About 12M $HClO_4$ (60%; reagent ACS grade; Fisher).

(**d**) *Natural selenium standard (^{N}Se-STD).*—Elemental Se standard (Johnson Matthey Electronics, Ward Hill, MA) was dissolved in concentrated nitric acid and diluted in 1% v/v HNO_3 to make a 1 mg Se/L standard. Its natural abundance was assumed to be as follows: ^{74}Se, 0.87; ^{76}Se, 9.02; ^{77}Se, 7.58; ^{78}Se, 23.52; ^{80}Se, 49.82; and ^{82}Se, 9.17% (14).

(**e**) *^{77}Se-enriched selenium standard (^{77}Se-STD).*—Elemental ^{77}Se-enriched Se standard (10 mg; Isotec, Inc., Miamisburg, OH) was dissolved in concentrated nitric acid, prepared in 100 mL 5% v/v HNO_3, and quantitated by a reverse SID ICP-MS with ^{N}Se-STD as the primary standard. A working standard (^{77}Se-STD) containing 0.02 mg Se/L was then made from this stock solution. Isotope abundances (specified by Isotec) were: ^{74}Se, 0.06; ^{76}Se, 0.66; ^{77}Se, 94.38 ± 0.15; ^{78}Se, 3.02; ^{80}Se, 1.61; and ^{82}Se, 0.27%.

(**f**) *Sodium borohydride solution.*—1% m/m (ca 0.26M) $NaBH_4$ (powder form; Fisher) and ca 0.2% m/m (ca 0.05M) NaOH (2 pellets NaOH in 100 g solution). The solution was filtered through a glass fiber filter (nominal size, 1.0 µm; Gelman) and refrigerated in a glass bottle. This solution was stable for several weeks (8).

Procedure

The blank sample, calibrator sample, and actual sample were respectively named BLK, CLB, and SAM. Samples with natural Se isotope abundance were designated with superscript "N." Samples spiked with ^{77}Se isotope were designated with superscript "77" or "N+77."

(**a**) *Digestion.*—The blank (^{N}BLK and ^{77}BLK), calibrator (^{N}CLB and $^{N+77}$CLB), and plant tissue sample (^{77}SAM) were prepared in 50 mL Folin glass digestion tubes. ^{N}BLK was not spiked (Table 1), ^{77}BLK was spiked with 0.2 mL ^{77}Se-STD (0.004 µg ^{77}Se-enriched Se), and ^{N}CLB was spiked with 0.4 mL ^{N}Se-STD (0.4 µg natural Se). Five $^{N+77}$CLB samples were used for EC. To each of the 5 tubes were added 0.3 g in-house alfalfa tissue to compensate for the matrix effect and 0.2 mL ^{77}Se-STD. The tubes were spiked with 0, 0.05, 0.1, 0.2, and 0.4 mL ^{N}Se-STD, respectively. The ^{77}SAM samples consisted of 0.5 g tissue samples (or 2 mL NIST 1643d Water) plus 0.2 mL ^{77}Se-STD.

All samples (blank, calibrator, and actual sample) were digested with nitric acid plus perchloric acid (8). Samples were cold-soaked with 5 mL mixed acid (HNO_3–$HClO_4$, 5 + 1) overnight, covered with small glass funnels and digested at 160° ± 5°C for 3 h, uncovered and evaporated at 200° ± 5°C for 30–40 min or until ca 0.5 mL remained. After digestion, 12.5 mL 4M HCl was added to the remaining 0.5 mL digested sample. The samples were heated at ca 90°C without cover for 1 h to reduce Se(VI) to Se(IV). After cooling, the sample was diluted to a final volume of 25 mL with deionized water. *Note:* Anhydrous perchloric acid mixed with high organic content matter could be explosive at high temperature (15) and the detailed digestion (8) should be followed.

(**b**) *HG system and sample analysis.*—The isotopes ^{77}Se, ^{78}Se, ^{81}Br, ^{82}Se, and ^{83}Kr were determined with a PQ2 Turbo Plus ICP-MS (VG Plasma Quad, Fisons, Loughborough, Leicestershire, UK; 8). The mixed solution of digested sample (2M HCl at 3.3 mL/min) and sodium borohydride (1.0% w/w at 0.7 mL/min) was delivered directly into the spray chamber, which served as gas–liquid separator. The analysis sequence was 3 ^{N}BLK samples, 3 ^{77}BLK samples, 5 $^{N+77}$CLB samples, 1 ^{N}CLB sample, 15–20 ^{77}SAM samples, 1 ^{N}CLB sample, 15–20 ^{77}SAM samples, 1 ^{N}CLB sample, 15–20 ^{77}SAM samples, and 1 ^{N}CLB sample. The ^{N}CLB sample was measured to monitor the absolute signal drift, the mass bias, and the mass bias drift. These drifts were assumed to be linear with time, because the sam-

Table 2. Se in 207 alfalfa tissue samples measured by HG-ICP-MS with SID calibration (SID) and external calibration (EC)[a]

	Measured concentrations, mg/kg				Concentration ratios		
Statistic	Se78	Se82	Se78/77	Se82/77	Se78/(Se82/77)	Se82/(Se82/77)	(Se78/77)/(Se82/77)
Average	0.033	0.034	0.031	0.033	1.010	1.024	0.954
Standard deviation	0.015	0.016	0.015	0.015	0.078	0.081	0.097
Maximum	0.152	0.156	0.150	0.152	1.189	1.183	1.252
Minimum	0.015	0.016	0.017	0.017	0.760	0.788	0.667

[a] Se78, Se82, Se78/77, and Se82/77 are measured selenium concentrations based on, respectively, ^{78}Se (EC), ^{82}Se (EC), ^{78}Se/^{77}Se isotope ratio (SID), and ^{82}Se/^{77}Se ratio (SID).

ple's matrix and the calibrator's matrix were matched, and this analysis of Se in plant tissue with HG-ICP-MS was insensitive to matrix effect (8). A group of 80 samples was measured in 2 h and 20 min, giving an average rate of 1.8 min/sample.

(c) *SID calibration.*—The ^{77}Se, ^{78}Se, and ^{82}Se ion intensities of ^{N}BLK, ^{N}CLB, and ^{77}SAM were used in calibration. The signal of ^{N}BLK was subtracted from those of ^{N}CLB and ^{77}SAM. The mass bias and the mass bias drift in ^{77}SAM were corrected on the basis of data from ^{N}CLB, assuming that the blank-subtracted ion intensities of ^{N}CLB represented the true natural isotope abundance. ^{78}Se and ^{82}Se were used as reference isotopes, while ^{77}Se was used as the spike isotope. The amount of Se in ^{77}SAM samples were calculated with the following equation:

$$X = W \times \frac{M_N}{M_E} \times \frac{A_E}{A_N} \times \frac{R_X - R_E}{R_N - R_X} = \quad (1)$$

$$0.004 \times 12.77 \times \frac{R_X - 0.032}{3.10 - R_X} \text{ for } ^{78}\text{Se reference isotope}$$

$$0.004 \times 12.77 \times \frac{R_X - 0.00286}{1.21 - R_X} \text{ for } ^{82}\text{Se reference isotope}$$

where X, Se in 0.5 g actual sample (μg); W, spiked ^{77}Se-STD, 0.004 μg; M_N, atomic mass of natural Se; M_E, atomic mass of ^{77}Se-enriched Se; A_N, ^{77}Se abundance in natural Se; A_E, ^{77}Se abundance in ^{77}Se enriched Se; R_N, ratio of reference isotope to ^{77}Se in actual sample; R_E, ratio of reference isotope to ^{77}Se in ^{77}Se enriched Se; and R_X, ratio of reference isotope to ^{77}Se in the actual sample spiked with ^{77}Se enriched Se.

(d) *External calibration.*—The ^{78}Se and ^{82}Se ion intensities of ^{77}BLK, ^{N}CLB, $^{N+77}$CLB and ^{77}SAM were used in calibration. The signals of ^{77}BLK were subtracted from those of $^{N+77}$CLB and ^{77}SAM. The calibration lines were constructed on the basis of the ion intensities of ^{78}Se and ^{82}Se in $^{N+77}$CLB, and the Se concentrations in plant tissue were calculated. Results of ^{N}CLB were used to correct the time drift.

Results and Discussion

Analytical Aspects of SID Calibration

(a) *Amount of spiked ^{77}Se-STD.*—The amount of spiked ^{77}Se-STD should be selected before analysis to improve analytical precision and accuracy (9). The relative error $(\Delta X)/X$ of equation 1 can be expressed as follows:

$$\frac{(\Delta X)}{X} = \frac{(\Delta W)}{W} + \frac{R_N - R_E}{(R_N - R_X) \times (R_X - R_E)} \times (\Delta R_X) \quad (2)$$

where Δ is the absolute error of a respective term. The relative error $(\Delta W)/W$ results from spiking ^{77}Se-STD to samples. The second term on the equation's right side usually is the main source of error $(\Delta X)/X$, resulting from measuring the spiked sample's isotope ratio R_X, and depends on the spiked amount of ^{77}Se-STD. This error could be minimized if the spiked amount of ^{77}Se-STD is adjusted so that R_X is the average of R_N and R_E, or

$$\frac{X}{M_N} \times A_N = \frac{W}{M_E} \times A_E \text{ when}$$

$$R_X = \frac{R_N + R_E}{2} \text{ or } \frac{R_X - R_E}{R_N - R_X} = 1 \quad (3)$$

Therefore, whether ^{78}Se or ^{82}Se is used as the reference isotope, the spiked amount of ^{77}Se isotope is equal to the amount of ^{77}Se isotope in the original sample.

Alfalfa tissue contained 0.03–1.00 mg Se/kg on the basis of preliminary analysis. The desired amount of ^{77}Se-STD should be 0.001–0.039 μg if the highest precision is to be achieved. It is not convenient to know the amount of Se in each sample beforehand, nor is it practical to spike samples with various amounts of ^{77}Se-STD. Therefore, a single spike of 0.004 μg ^{77}Se-STD was selected to correspond to the amount of Se in 0.5 g alfalfa tissue.

The ^{82}Se isotope would be 0.00001 μg in 0.004 μg ^{77}Se-STD and 0.002 μg in 0.5 g plant tissue sample containing 0.05 mg Se/kg, assuming that an isotope's weight percentage is equal to its abundance. Therefore, the spiked ^{77}Se-STD did not significantly change the ^{82}Se isotope content in the original plant tissue sample. The ^{82}Se isotope could still be used for EC, while the ^{82}Se/^{77}Se isotope ratio was used for the SID calibration. This holds true as well when ^{78}Se was used as the reference isotope.

(b) *Isobaric interference.*—A reliable SID calibration also requires no or negligible isobaric interference. The isobaric interference is well defined when Se is measured with HG-ICP-MS: ^{77}Se by ArCl (5), ^{78}Se by Ar_2, and ^{82}Se by ^{82}Kr and BrH where Br could have originated from the plant tissue samples. As described by Zhang and Combs (7), the interference of ArCl with ^{77}Se was negligible in this study. The interference of BrH with ^{82}Se was also negligible, because Br would have volatilized during digestion (8). The measured ion intensities were

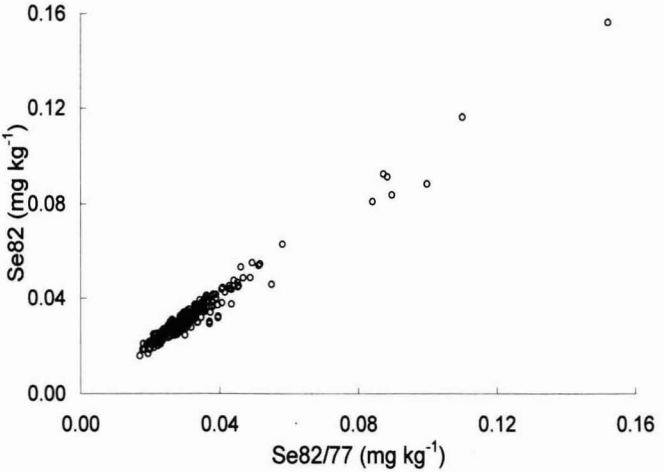

Figure 1. Relationship of measured Se in 207 alfalfa tissue by external calibration based on the ^{82}Se isotope to the stable isotope dilution calibration based on the ^{82}Se/^{77}Se ratio.

(superscript: mass), respectively, ^{77}170, ^{78}850, ^{82}120, ^{81}1600, and ^{83}65 counts/ in a ^{N}BLK sample and ^{77}4500, ^{78}4200, ^{82}1400, ^{81}1900, and ^{83}60 counts in a real ^{77}SAM sample containing 0.04 mg Se/kg, for example.

(**c**) *Isotope ratio R_N.*—The rigorous approach to SID demands that the sample's natural isotope ratio (R_N) also be measured (9). This means that samples without spiked ^{77}Se-STD are also measured. Consequently, the work load is doubled. This additional measurement does not necessarily improve data quality, because uncertainty associated with the measurement is introduced and the precision of measured isotope ratio with ICP-MS is usually 0.3 to 0.05%, which might be insufficient to differentiate Se isotope ratio variations in natural samples. The same logic was applied to ^{N}Se-STD. Therefore, the natural Se isotope ratio was assumed for unspiked samples and for ^{N}Se-STD.

(**d**) *Isotopic equilibration.*—The last important factor in obtaining a reliable SID calibration is that an isotopic equilibrium between the spiked ^{77}Se-STD and the Se in the original sample can be reached. Isotopic equilibration was assumed because the solid sample had been digested into a solution. It was known that digestion without HF may not have been absolutely complete (16). However, the isotopic equilibration between Se in solution and Se in undigested residue was expected.

Data Quality Assured by Two Independent Calibration Methods

(**a**) *Results of 207 plant tissue samples.*—The Se in 207 alfalfa samples was determined by this method. All data were included to evaluate whether the 2 calibration methods could be a tester for each other. Se concentrations designated as Se78, Se82, Se78/77, and Se82/77 were from EC based on ^{78}Se isotope, EC based on ^{82}Se isotope, SID calibration based on ^{78}Se/^{77}Se isotope ratio, and SID calibration based on ^{82}Se/^{77}Se isotope ratio, respectively. The correlations of the different concentrations are tabulated in Table 2. Considering that the averaged concentration of Se in these samples was only about 0.03 mg Se/kg, that the multistep sample preparation was used, that the results of 2 analytical methods were compared, and that all 207 data points including "poor" data points were included, it can be concluded that there was a very good correlation of these concentrations to those of Se82/77. The 2 calibration methods yielded the same results for the 7 samples having Se concentration greater than 0.08 mg/kg (Figure 1).

(**b**) *Limit of detection, dynamic range, accuracy, and precision derived from results of blank samples, NIST SRMs, and RMs.*—Twelve blank samples spiked with ^{77}Se-STD (^{77}BLK in Table 1) were analyzed with all other samples in 4 groups. The standard deviations of Se results by the SID in these 12 ^{77}BLK samples were calculated respectively for each group (i.e., 3 ^{77}BLK samples per group) and then averaged. The limit of detection for Se in plant tissue, defined as 3 times of the averaged standard deviation (SD), was 0.001 mg Se/kg (based on the ^{78}Se/^{77}Se ratio or the ^{82}Se/^{77}Se ratio). Similarly, the limit of detection for Se in plant tissue by the EC was estimated to be 0.001 mg Se/kg (based on ^{78}Se or ^{82}Se). This definition of detection limit was different from the one used by Zhang and Combs (8), which was 3 times the SD of 11 blank samples consecutively analyzed in one group. However, the estimated detection limit of 0.001 mg Se/kg agreed well with that of Zhang and Combs (8).

The Se contents of 8 NIST SRMs and RMs were also determined (Table 3). Se concentrations in final diluted solutions were 0.08 μg/L for NIST RM 8413 Corn Kernel, 0.91 μg/L for NIST 1643d Water, 51.6 μg/L for NIST 8418 Wheat Gluten, and 1–2 μg/L for other SRMs and RMs. These concentrations ranged over 4 orders of magnitude. The majority were within the ranges of the certified value ± 1 SD, and the uncertainties were smaller than or close to the certified values. The results of Wheat Gluten (RM 8418) and Tomato Leaves (SRM 1573a) by EC were slightly poorer than those by SID. Matrix effect from tomato leaves or incomplete digestion of wheat gluten may have caused the

Table 3. Se in NIST SRMs and RMs measured by HG-ICP-MS with SID calibration (SID) and external calibration (EC)[a]

		Measured, mg/kg				
		EC	EC	SID	SID	
Sample	Certified, mg/kg	Se78	Se82	Se78/77	Se82/77	*n*
Water SRM 1643[b]	11.43	10.76	10.89	13.04	12.64	
Standard deviation	0.17	1.23	1.22	0.45	0.44	3
Apple leaves SRM 1515	0.052	0.046	0.047	0.046	0.047	
Standard deviation	0.009	0.001	0.002	0.003	0.003	4
Peach leaves SRM 1547	0.120	0.119	0.122	0.117	0.121	
Standard deviation	0.009	0.004	0.006	0.011	0.011	4
Tomato leaves SRM 1573a	0.054	0.064	0.066	0.056	0.058	
Standard deviation	0.003	0.007	0.007	0.004	0.001	4
Pine needles SRM 1575	N/A	0.052	0.053	0.056	0.056	
Standard deviation		0.002	0.002	0.001	0.002	4
Corn kernel RM 8413	0.004	0.005	0.005	0.002	0.004	
Standard deviation	0.002	0.001	0.001	0.001	0.001	3
Wheat gluten RM 8418	2.58	2.22	2.28	2.38	2.52	
Standard deviation	0.19	0.05	0.05	0.42	0.39	3
Corn bran RM 8433	0.045	0.042	0.042	0.041	0.043	
Standard deviation	0.008	0.010	0.009	0.010	0.009	3

[a] Se78, Se82, Se78/77, and Se82/77 are measured selenium concentrations based on, respectively, ^{78}Se (EC), ^{82}Se (EC), $^{78}Se/^{77}Se$ isotope ratio (SID), and $^{82}Se/^{77}Se$ isotope ratio (SID).

[b] Units for water data are μg/L.

poor EC results. Such problems rarely exist with SID calibration, as long as the spiked Se reaches isotopic equilibration with the original Se in the sample.

(c) *Independence of the 2 methods.*—To use the double calibration approach to evalute data quality, the 2 methods must be independent of each other. Although the same digestion procedure was used for all samples, different sources of standards and mechanisms of calibration ensured independence. EC is a linear calibration that uses an external standard. The analyte's amount is linearly proportional to the analyte's signal. On the other hand, SID calibration is a nonlinear calibration, as implied in equation 1, and it requires an internal standard. An incorrect signal increase in either ^{78}Se or ^{82}Se generates a higher-than-true result by both EC and SID, but the result by EC would be different from that by SID. A signal variation in ^{77}Se affects SID but not EC. An overall signal increase generates higher-than-true result with EC but does not affect the result with SID. Also for data assurance by double calibration, results with one method should not be systematically higher or lower than results with the other.

Conclusion

Double calibration can be used to directly evaluate and assure the data quality of any individual sample in analysis of Se by HG-ICP-MS without increasing sample preparation and measurement or including other reference materials. The Se content is correct and accepted if the Se contents obtained by the 2 methods agree within given criteria. This approach should be applicable to other multiisotope elements, especially elements in samples that require extensive preparation before analysis.

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STATISTICAL ANALYSIS

Data Processing and Software Requirement for Analytical Method Validation

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Method documentation and validation reports are necessary steps in developing quality assurance programs in laboratories. Consequently, large amounts of data must be stored, archived, and processed in a standardized way. This paper presents these new requirements and proposes a programming approach based on Object Linking and Embedding (OLE) automation technology. Major advantages of this technique include a very flexible and modular programming technique and the possible integration of classically well known software, such as spreadsheets, into the new application. Both advantages reduce development and personnel training costs. Several examples are presented to explain some computing aspects and implementation problems.

As quality assurance systems are being developed in laboratories, the question of standardized method validation becomes more incisive. However, an agreement among agencies or authorities has not been reached, and analysts are unsure which criteria and computational techniques they must use. Several definitions and evaluation criteria are proposed for analytical method validation. But all defined procedures require traceable computation techniques and document archiving.

Only one statement is clear: a validation report must be prepared, and it must follow quality assurance guidelines; that is, it must be documented and traceable. It is then necessary to develop computerized procedures that allow flexible evolution at reduced cost. The comprehensive description of an analytical procedure, from sampling to decision-making, consists of operation-oriented documentation to describe the operating procedure and result-oriented data to illustrate its performance. Therefore, when preparing the document on method validation it is necessary to combine documentary data and experimental data. It is possible to use the recently developed Object Linking and Embedding (OLE) technology to organize all data and computations required by validation procedures within the same application. Development efficiency is very good, and the built code easily can be reused.

The proposed general approach is based on the "object-enabling software" concept, which facilitates management of these different data. This technology defines self-contained modular objects that encapsulate documentation, analytical data, and processing logic. The contents of these objects are then accessed through well-defined interfaces or programming languages. It makes possible a simple combination of different types of information: text file from word processor, raw data from database management system, and computed criteria from spreadsheet calculator.

This paper shows how this concept, when used for analytical method validation documentation, can increase modularity and hence flexibility, reduce experiment time, reduce evaluation costs, and increase the ease of use of validation figures of merit. A comprehensive approach based on Microsoft Office and Visual Basic for Applications (VBA) is proposed, and some specific aspects are described. This study also demonstrates that it is now necessary to define the rules for method validation—in some way the Good Validation Practices—which can be used to present all validation documents in a standardized manner. Data traceability and software validation must be included within these rules.

Quality Assurance in Chemical Analysis

Quality assurance is defined as "all those planned and systematic actions necessary to provide adequate confidence that the product or the service will satisfy given requirements for quality" (1). It is possible to define 4 general consumer requirements for chemical analysis:

(*1*) *Metrology compliance.*—The implied customer need is an accurate result. As the customer usually is unable to verify personally, an external control body is required. For the laboratory, accuracy compliance usually consists in taking part in proficiency tests.

(*2*) *Technical usefulness.*—An analytical measurement is performed to make a decision. The precision of the method must be consistent with the expected variability of the controlled sample. For instance, if the repeatability of the method is higher than the variability of the controlled process, the quality

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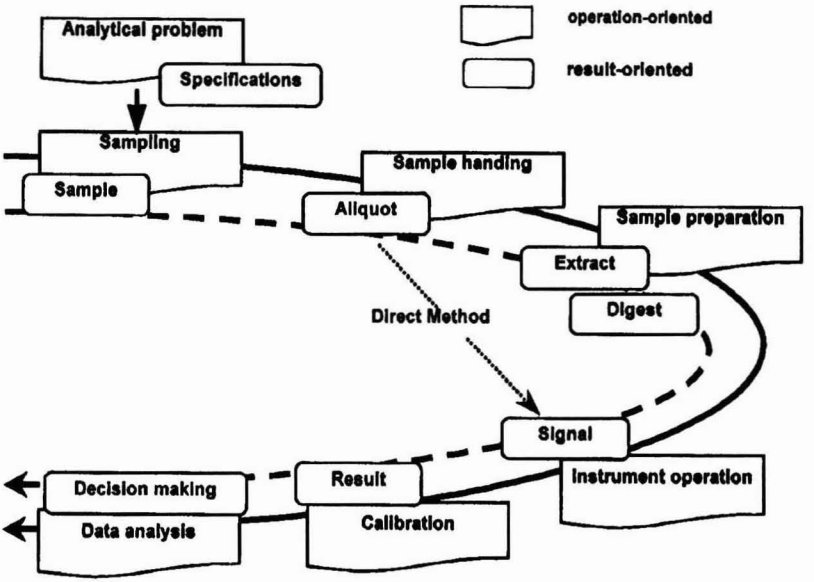

Figure 1. The analytical process from the analytical problem to the decision.

of the method is not acceptable. This requirement is sometimes defined as measurability (2).

(*3*) *Time and cost effectiveness.*—For most customers, the first requirement is a quick answer. In addition, for many applications, the analysis must be inexpensive. Both these requirements have important technical consequences. For instance, in food chemistry, most reference methods for microbiological control are being replaced by rapid methods that greatly reduce analysis time. The downside is that these methods must be calibrated with the classical reference methods.

(*4*) *Safety and cleanliness.*—The most recent requirement consists in using methods that reduce hazard for the personnel or the environment. For instance, toxic (or even forbidden) reagents, such as benzene or trichloromethane, are replaced by harmless reagents while hazardous techniques, such as radioimmunology, are being abandoned.

These requirements are interdependent, and their relative importance will depend on the selected method; for instance, the technical resources called for to reach a given precision influence the cost of a method. Three actions must be undertaken to reach quality goals as defined above. First, consumer requirements must be translated into specifications and preferably into quantitative specifications. Second, procedures must be defined to ensure that requirements are met at a given confidence level and statistical techniques are useful to reach this goal. Third, a documented organization must be set up that describes actions and allows traceability of any performance result.

Method Validation Strategy

If the definition of validation used for computer software is applied to analytical methods, validation must be broken down into separate components addressing the equipment, the analytical method run on this equipment, the analytical system, and the analytical data (3). Thus, in more general terms, method validation could be defined as the sum of sampling validation, instrument validation, and data analysis validation. But a more restrictive definition of method validation is sometimes given by standardization bodies, such as the U.S. Pharmacopoeia, which lists a set of figures of merit to be measured. Some confusion often occurs between method development and method validation. Method development consists of selecting, optimizing, and describing the analytical procedure, whereas validation consists of verifying that a procedure correctly applies.

An analytical method is a sequence of 6 operations: sampling, sample handling, sample preparation, instrument operation, calibration, and data analysis. Figure 1 presents these 6 steps as gray boxes with boldface characters, which correspond to the operation-oriented description of the method. Each operation gives an output, which is illustrated as a rounded box: sampling gives a sample, sample handling gives an aliquot, sample preparation gives an extract or a digest, instrument operation gives a signal, calibration allows transformation of the signal into a result, and data analysis leads to a decision. These outputs consist of data and represent the result-oriented aspect of the method. When considering an analytical procedure, it can be described as either an operation-oriented or a result-oriented process.

Table 1. Method validation criteria

Step	Operation-oriented strategy	Result-oriented strategy	
		Simple criteria	Global criteria
Sampling	Sampling device, sampling design, frequency and size, packaging	Representativeness	
Sample handling	Storage, transport, grinding, homogenizing and mixing operations, aliquot size, aliquot number	Homogeneity	
Sample preparation	Laboratory operations: reagents, volume, weight, temperature, glassware	Recovery yield	Accuracy Specificity
Instrument operation	Instrument operation: settings and tuning, maintenance, operation control, brand and model	Signal specificity Signal resolution	Repeatability Reproducibility Uncertainty
Calibration	Experimental design, signal processing, calculation and algorithm, quality control linear range	Sensitivity blank	Limit of detection Limit of quantitation
Data analysis	Control plan, statistical inference, risk assessment	No. of false positive and false negative	

According to this scheme, each step is performed to produce an output to be used by the next step. On the other hand, the number of steps varies according to the analytical technique or the role of the laboratory. For instance, in food chemistry, rapid methods have been developed that avoid sample preparation, such as near-infrared spectroscopy or nuclear magnetic resonance spectroscopy, which usually can be applied directly to raw food samples. Similarly, in biomedical analysis laboratories, sampling of specimens, such as blood, and sample handling can be regarded as single steps because sample collectors usually work under the direct responsibility of the laboratory.

Two strategies have been developed to validate a method: operation-oriented and result-oriented strategies. In the first case, the analytical method is described in utmost detail, and validation consists of verifying that all operations can be performed and that the required resources actually are available. This approach can be considered as an obligation of resources. The other strategy is based on the obligation of results: its actual performances are verified and validated in comparison with predefined specifications. The ultimate result-oriented method validation could consist of defining a set of performances to be reached whatever the analytical method used. The discrepancy between the 2 strategies is visible in the various implementations of the EN 45001 standard (ISO Guide 25) in Europe. In France, accreditation is often based on mastering the means necessary to apply a given standardized method (glassware, re-

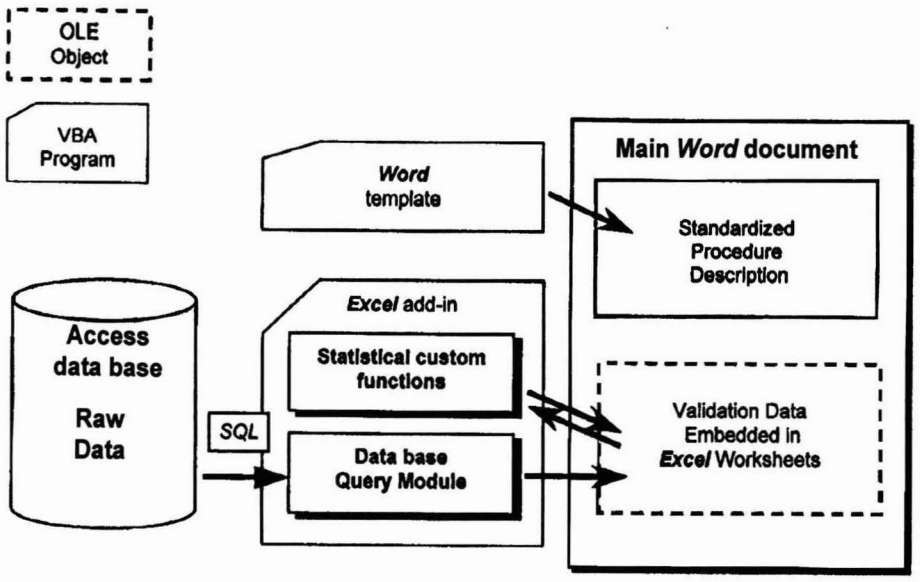

Figure 2. General organization of validation report using OLE (object linking and embedding) automation and VBA (Visual Basic for Applications) programming.

Table 2. Database structure

Field name	Type	Description
Study	String	Code that describes the study type
Date	Date	Date of collection
Sample	String	Code name of the sample used for the study
Replicate	Short integer	Number of the replicate
Result	Double precision	Observed result

agents, instruments), while in several other countries it is based more on the actual performance of the method.

Table 1 lists various criteria that were classically required to describe and validate analytical methods according to each strategy. Although result-oriented criteria usually are preferred, they are not always accessible or favorable. For instance, for sampling and sample handling, it is very difficult to quantitatively assess representativeness or homogeneity. Thus, describing these steps as operations is often preferred. On the other hand, one of the greatest advantages of the result-oriented strategy is that global criteria, listed in the last column of Table 1, can be used to qualify several steps at once. Therefore, both approaches may be used simultaneously, and method validation reports may contain a mixture of text to describe the operation-oriented part of the method along with data to illustrate its result-oriented facet. This remark is very important if one is trying to establish the best way to handle method validation documents.

A comprehensive approach based on object-oriented models, used by software designers, allows an adapted solution. Self-contained modular objects may be defined that encapsulate documentation, analytical data, and processing logic. The contents of these objects are then accessed through predefined interfaces. To easily handle the complex information collected during method validation, one can take advantage of the recently developed computerized technique known as object technology. One well-known form of object technology is object-oriented programming. An object is a self-contained software module that consists of a set of data and its associated processing information. For instance, it would be possible to describe a validation document as several object models, each consisting of defined data sets and computational techniques. A significant benefit of object technology is that an object encapsulates all of the data and processing details while hiding the inner complexity. In addition, because all implementation detail is hidden from other objects (other software modules), it is easy to modify an object's internal details without affecting other objects in the system. However, object-oriented programming technology has failed to fully address the need to simplify application development.

A new form of object technology offers more tangible benefits. Called object-enabling system software, it allows prefabricated software components to be purchased and integrated into complete solutions. If designed properly, object-enabling system software makes components usable and reusable across application boundaries by ensuring that components written by different programmers from different companies behave in a well-described and consistent manner. The object-enabling system software defines an object interface that is independent of the programming language, as well as a mechanism to ensure that connections between components are valid, even if components in a distributed system are individually upgraded or replaced.

The OLE technology is based on this new concept (4). It enables an application to expose its functionality or to control the functionality of other applications on the same computer or across networks. OLE Automation integrates 2 key elements: (*1*) Applications or software components called servers, which can be controlled because their functionality has been exposed

Table 3. Common statistics and statistical techniques involved in method validation

Criteria	Statistics	Principle	Statistical method
Accuracy	Translation bias Rotation bias	Compare measured and true values	Orthogonal regression
Calibration	Blank Sensitivity Limit of detection Limit of quantitation	Apply to replicate measurements	Classical least squares regression
Linearity	Linearity range Significance of regression Lack-of-fit test	Perform a linearity test	Linearity test
Precision	Reproducibility standard deviation Repeatability standard deviation Grand average	Based on ISO 5725 part 2	Analysis of variance
Robust precision	Reproducibility robust standard deviation Repeatability robust standard deviation Robust grand average	Based on ISO 5725 part 5	Iterative weighing algorithm
Robustness	Main factor effects Interaction effects	Perform robustness test	Robustness test
Specificity	Slope of recovery line	Based on standard addition method	Orthogonal regression

Table 4. Example of data organization for the calibration study

A	B	C	D	E	F
x_standard			y_replicate		
0.0		5.0	6.0	−4.0	2.0
2.5		122.0	117.0	109.0	113.0
5.0		224.0	229.0	225.0	230.0
7.5		350.0		347.0	
10.0		456.0	448.0	455.0	457.0

and made accessible to other applications. Examples of such servers are software such as Word, Excel, ChemDraw, Sigma Plot, or WordPerfect. (2) Other applications or development tools, called controllers, which can control servers through programming codes. Examples of controllers are Visual Basic, Visual C++, and Delphi, as well as Visual Basic for Applications (VBA), which is included in several Microsoft application programs.

OLE technology was developed by Microsoft, but it is now widely used by other software producers. Many applications today have taken advantage of OLE Automation to use code contained in other applications. OLE Automation is the part of the OLE standard that defines not only how code is shared between applications but also how public services can be shared among applications. With OLE Automation, it is possible to create applications that expose objects and their interfaces to programming tools and macro languages. OLE works independently of programming languages through a recognized standard, allowing the oldest code to be integrated as a component alongside the newest object-oriented code.

Methods

Programming Tools

To demonstrate the usefulness of this technology, consider the Microsoft Office for Windows 95 package. It includes a word processor (Word), a spreadsheet (Excel), and a database manager (Access), which are all OLE-compatible controllers and/or servers. The most interesting feature of all of these applications is that they include the unique programming language VBA, which replaces previous macro languages.

Several years ago, G. Kateman promoted spreadsheets as tools well adapted for laboratory computations. Recent papers present some computing solutions based on use of macro language or macro tools (5, 6). These solutions are interesting because it is very easy to develop a template worksheet that can be copied and used over and over again. But these templates do not take advantage of OLE Automation. Results are obtained on separate worksheets and final incorporation in any global document is not easy. Moreover, the copy process may induce many sources of inconsistency for maintaining the application while different versions of the template would coexist among different worksheets. This drawback can be avoided by taking advantage of the new add-in file. This is a program file, independent from data files, that can be used simultaneously on several worksheets.

Figure 2 illustrates the general organization designed for managing information related to method validation. The main document consists of a Word document that includes a descriptive part and an experimental part (gray box). The procedure description is standardized in accordance with the different chapters of a standard operating procedure: application field, sampling, reagents, and experimental procedure. Description consistency is preserved by means of a VBA authoring pro-

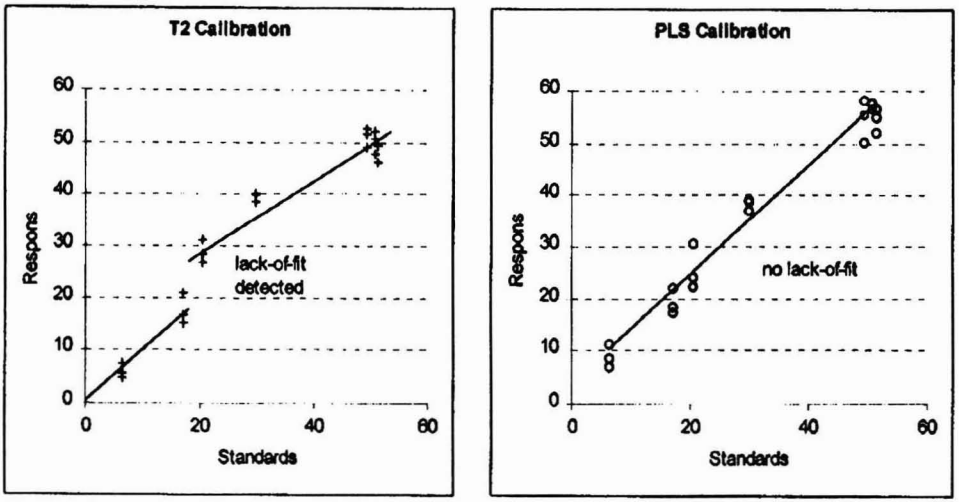

Figure 3. Comparison of calibration curves for both calibration techniques based on T_2 parameter or PLS multivariate regression.

Table 5. Linearity test table

Sources of variation	Sums of squares	Degrees of freedom	Variances	Fisher F
Regression model	SS_{reg}	1	$V_{reg} = SS_{reg}$	$F_{reg} = \frac{V_{reg}}{V_r}$
Lack-of-fit	SS_{lof}	$p-2$	$V_{lof} = \frac{SS_{lof}}{p-2}$	$F_{lof} = \frac{V_{lof}}{V_r}$
Residual	SS_r	$N-p$	$V_r = \frac{SS_r}{N-2}$	
Total	SS_{yy}	$N-1$		

gram that contains styles and functions for preparing a global framework to be used for data storage.

As shown in Figure 2, raw data are stored in a global Access database. They are extracted by using Structured Query Language (SQL) queries. SQL works on ODBC (open database connectivity) standard, and any database can be used without modification of the query statement module. These queries allow required data to be selected and copied in a specific format adapted to subsequent processing. They can be directly invoked from any embedded Excel worksheet, because VBA allows use of SQL. After the correct query is invoked, data are directly incorporated into the text document and become available for any Excel function, thanks to OLE Automation. The database structure is very simple, consisting of 5 fields (Table 2).

Classical extraction consists of selecting data from the Result field by using Study, Sample, and Date fields as keys and ordering them according to the Replicate number. However, it is useful to distribute data as a rectangular table where all rep-

Table 6. Linearity study for 2 TD-NMR techniques for water content of food

		T2 Calibration			PLS Calibration		
Standard No.	Reference	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
1	6.38	7.42	5.45	4.87	11.15	6.96	8.56
2	17.04	20.90	16.83	15.03	21.91	18.33	17.21
3	20.50	26.73	30.97	28.35	22.18	30.42	24.23
4	29.82	38.62	40.20	39.83	36.97	38.85	39.12
5	49.27	52.50	51.48	49.04	58.11	55.46	50.29
6	50.60	47.70	50.79	51.92	57.48	57.59	56.43
7	51.23	49.60	49.46	46.31	54.95	56.67	51.95

T2 Calibration

	Degrees of freedom	SS	Variance	F	Critical values
Regression	1	5251.387	5251.387	1336.184	8.862
Lack-of-fit	5	371.381	74.276	18.899	4.695
Residual	14	55.022	3.930		
Total	20	5677.789			

PLS Calibration

	Degrees of freedom	SS	Variance	F	Critical values
Regression	1	6711.912	6711.912	900.344	8.862
Lack-of-fit	5	80.770	16.154	2.167	4.695
Residual	14	104.368	7.455		
Total	20	6897.049			

Table 7. Accuracy study for cellulose determination

Feed No.	Reference	Alternative
1	2.060	2.710
2	4.935	4.220
3	5.445	4.615
4	6.505	5.800
5	7.420	6.605
6	8.260	8.375
7	8.595	8.470
8	14.085	12.525
9	15.400	15.605
10	22.935	21.810

	Accuracy	Bias
Values	1.040328255	0.124581578
dof and r^2	8	0.989069804
Student's *t*-test	1.158	
1% Critical value	3.355	

licates are on the same line to have a more explicit presentation. The best way to perform this operation in a single step is to use the TRANSFORM ... PIVOT statement. For instance, the following SQL instruction extracts all replicate measurements for a sample coded "sample 1" collected in the frame of a study called "Precision" at different dates. In this example, it is assumed that the global database is called DB1.

```
TRANSFORM Avg([DB1].Result) AS [Value]
SELECT [DB1].Study, [DB1].Sample, [DB1].Date
FROM [DB1]
WHERE ((([DB1].Study)=
"Precision" AND (([DB1].Sample)="sample 1"))
GROUP BY [DB1].Study, [DB1].Sample, [DB1].Date
PIVOT [DB1].Replicate;"
```

A code sample shown in the **Appendix** illustrates how this query can be used directly with VBA. VBA programming language replaces former macro language and adds many new features. In the most recent Office 97 version, program coding is achieved in a special editing environment, which contains many programming tools. As usual, 2 statements are available: the Sub ... End sub structure adapted to internal computations and the Function ... End function syntax essential to create custom functions, which can be used in the same way as any built-in function. The program is compiled as a complementary add-in to prevent any end-user code modification. An add-in is an external file with the .XLA extension. It can be loaded into memory and accessed by any worksheet. Compared with macros, add-ins are very convenient for controlling traceability, because they can be managed and modified independently from the worksheets that use them. They are also compiled and fully protected against unauthorized modifications. For this study, several custom functions and subroutines were coded, compiled as add-ins, and documented with an additional on-line help file.

Computational Methods

Table 3 presents the different statistical techniques used to compute validation criteria. Details on these techniques are re-

Table 8. Precision study for glucose in orange juice (20%) by LC

	Peak area			Peak height		
Laboratory No.	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
1	3.23	2.75	3.22	2.69	2.60	2.70
2	2.52	2.70		2.46	2.50	
3	2.72	2.91	2.89	2.77	2.85	2.88
4	3.36	3.10		3.49	3.39	
5a	2.77	2.93	2.81			
5b	2.78	2.73	2.77	2.88	2.89	2.88
6	2.76	2.66	2.62	2.89	2.85	2.80
7a	2.51	2.49	2.56	2.21	2.50	2.54
7b	2.54	2.38[a]	2.54	2.55	2.43	2.57
8	2.84	2.84	2.85	2.85	2.85	2.91
9	3.08	3.40[a]	3.09	3.07	3.37	3.08
		SD	RSD, %		SD	RSD, %
Classical reproducibility		0.266	9.5		0.307	11.0
Classical repeatability		0.129	4.6		0.094	3.4
Classical grand average		2.818			2.802	
Robust reproducibility		0.301	10.6		0.292	10.5
Robust repeatability		0.092	3.2		0.054	1.9
Robust grand average		2.829			2.792	

[a] Outliers.

Table 9. Custom functions used for the study

Function syntax	Description
Accuracy_TEST(reference, alternative)	Array formula that returns the accuracy and bias of an accuracy study by using orthogonal regression
Calibration_LOD(x_standard, y_replicate)	Returns the limit of detection from a calibration line based on replicates
Calibration_LOQ(x_standard, y_replicate)	Returns the limit of quantification from a calibration line based on replicates
Interlab_NBLABS(data_range)	Returns the number of laboratories having more than 2 replicates and kept for computation
Interlab_REPEAT(data_range)	Returns the repeatability standard deviation of a collaborative study
Interlab_REPROD(data_range)	Returns the reproducibility standard deviation of a collaborative study
Linearity_TEST(x_standard, y_replicate)	Array formula that returns the results of a linearity test as a table
Robust_GAVERAGE(x_data)	Returns the robust grand average of a collaborative study
Robust_REPEAT(x_data)	Returns the robust repeatability standard deviation of a collaborative study
Robust_REPROD(x_data)	Returns the robust reproducibility standard deviation of a collaborative study

ported in several textbooks (7, 8). The programming technique for custom functions is very flexible. Only some of the most common validation criteria are presented as illustrations but the method can be extended to any other figure of merit. For instance, the limit of detection x_{LOD} can be defined as the concentration corresponding to a signal of 3 blank standard deviations above the analytical blank obtained through a calibration curve:

$$x_{LOD} = \frac{a_0 + 3\, s_{a_0}}{a_1} \qquad (1)$$

where a_0 is the blank, s_{a_0} is the standard deviation, and a_1 is the sensitivity. The syntax of the corresponding custom function is as follows:

Calibration_LOD (x_standard, y_replicate)

The syntax requires 2 mandatory input arguments corresponding to 2 ranges of cells. These ranges must be organized in a specific way for the function to return a correct result. The first mandatory argument, x_standard, is a one-column range of contiguous cells containing the values of the standard solutions. The second mandatory argument, y_replicate, is a multiple column range of contiguous cells containing the instrumental responses. These columns must be joined in order, and replicates for one standard are listed in one row. This means that, when using this function, the limit of detection must computed with replicate measurements. It is not necessary for both x_standard and y_replicate zones to be contiguous, but they must have the same number of lines.

Table 4 illustrates this arrangement for 5 standard solutions of copper ranging from 0.0 to 10.0 mg/L, determined by flame atomic absorption spectrometry. Each standard, in cell range A2:A6, was measured 4 times, and replicates are stored in cell range C2:F6. To show that the program correctly handles missing data, even when they are in the interior of a cell range, 2 replicates were cancelled for the 7.5 mg/L standard solution. To obtain the limit of detection from these data, one simply inputs the following formula in any cell of the sheet that is not in the selected zones:

=Calibration_LOD(A2:A6;C2:F6)

This cell will contain the value 0.162784, which represents the limit of detection computed according to the given definition. Several controls were included in the function to prevent some misapplication: (*1*) The function returns an error if there are fewer than 3 standard solutions. (*2*) The function returns an error for non-numeric input. (*3*) If there are fewer than 2 replicates in a row, the corresponding standard solution is discarded. (*4*) If one standard solution is missing, all corresponding replicates are discarded. (*5*) If one replicate is a blank, it is considered a missing datum and not as zero.

An interesting feature of this programming technique is that all built-in Excel functions also can be used by VBA inside a custom function. For instance, the LinEst function, which computes classical least squares (CLS) statistics, can be invoked directly by the following statement:

reg_result = Application.LinEst(y_data; x_data; True; True)

where y_data and x_data are 2 cell ranges or data arrays containing responses and standard solutions. The next arguments indicate that the straight line has a constant and that statistics are required. Results are stored in the reg_result variable, which must be declared as a Variant-type variable. In fact, reg_result is an array of 10 data arranged as described in the Excel documentation. For instance, reg_result(1, 1) contains the value of the slope. Finally, writing a custom function such as Calibration_LOD simply means to correctly invoke the LinEst

Table 10. New precision criteria after deletion of 2 outlying data

Statistic	Standard deviation	RSD, %
Classical reproducibility	0.23673	8.42
Classical repeatability	0.11800	4.20
Robust reproducibility	0.28479	10.06
Robust repeatability	0.05305	1.87
Classical grand average	2.81276	
Robust grand average	2.83121	

function and combine obtained results according to equation (1), such as:

```
Calibration_LOD = (reg_result(1, 2) +
3 * reg_result(2, 2)) / reg_result(1, 1)
```

Several dozens of built-in statistical functions can be used in such a way. Among these functions, the user can find all probability and inverse probability functions, such as Tdist, which returns Student's *t*-distribution, and Tinv, which returns the inverse of the Student's *t*-distribution.

When data are stored in variables, it is preferable to declare them as double precision or variant type to attain good accuracy. We verified that the precision is around 10^{-16} when double precision is used but drops to 10^{-9} with single-precision variables. The results are even worse (around 10^{-6}) when all computations are performed by developing algorithms with For ... Next loops. These discrepancies are due only to rounding errors of the internal conversion algorithms of Windows. The accuracy of built-in Excel functions was controlled by comparing least-squares regression results on raw data with 10-digit numbers to those obtained with the S-plus software (9). As the relative difference is of about 10^{-13}, the computation results are different only when considering the 13th digit and after. These results were observed with a Pentium microprocessor. Performances can be slightly different with other microprocessors. Therefore, it is important to precisely address the computer type and the expected computation precision level when defining a software validation procedure (10). The computation precision of the computer we used was estimated to 1.192×10^{-7}.

For result traceability, spreadsheets may be deemed dangerous because automatic and transparent modifications can occur without any user notice, for instance if the "Automatic Link Update" option is set on at file opening. However, several ways to prevent data from being modified are accessible manually or through a program. Complete or partial workbooks and/or worksheets can be protected by passwords that are requested when any modification occurs. These passwords may be used to lock any cell or range of cells and still operate when the worksheets are embedded as OLE objects. Confidentiality can be obtained by hiding parts or contents of cells; it is also possible to hide a cell formula without hiding resulting values. Use of separate add-in files for developing custom functions certainly represents the safest technique, because it is completely protected by a password.

Results

According to the proposed procedure, preparation of the validation document starts by opening a Word document. Several worksheets containing raw validation data are automatically embedded. Thereafter, data are transferred from the database and specific custom functions are stored in the add-in file manually added to the embedded worksheets.

Linearity

The first step in validating an analytical method consists in defining the linear calibration range. A calibration curve is linear when the instrument response y is proportional to the analyte concentration x (11). The linear model can be expressed as:

$$y = a_0 + a_1 x + e$$

where a_0 is the analytical blank, a_1 is sensitivity, and e is instrument background noise. This model is very easy to use because model coefficients can be estimated simply by CLS regression technique when e is normally distributed. Thus, the limits of the linear domain can be defined as the experimental limits within which linearity is established with a high confidence level. The statistical test for nonlinearity is derived from a lack-of-fit test applied to univariate CLS regression (12).

Final results usually are presented as in Table 5, where N represents the total number of replicates. Fisher ratios can be used to verify 2 hypotheses. The first one is used to verify that the regression model is significant and explains the variation of the y variable. Thus, F_{reg} is compared with a critical value of the Fisher distribution law that is noted $F_{1-\alpha,1,N-p}$, where α is the accepted first type error. If F_{reg} is larger than the critical value, the hypothesis that the regression model explains the data variation can be conserved. The second hypothesis is used to verify that there is no significant deviation from the linear model: in this case it is a nonlinear. The computed ratio F_{lof} is compared with a critical value of the Fisher distribution law $F_{1-\alpha,p-2,N-p}$. If F_{lof} is above this limit, we can conclude that there is a lack-of-fit.

A linearity study was performed for a time domain nuclear magnetic resonance (TD-NMR) method used to determine the water content of food (13). Beforehand, the method was indirectly calibrated with reference food samples of known water content. Reference values were established by a classical gravimetric drying technique. In this case, 21 reference food samples of known moisture contents were used for calibration. Two calibration techniques and models can then be computed from the free induction decay signal: univariate CLS regression on the T_2 NMR parameter, or multivariate partial least squares (PLS) regression on the total signal. The first method requires the former computation of T_2, whereas the second technique is directly applicable on raw data. To check linearity, 7 new samples of known water content ranging from 6 to 52% were prepared and analyzed. For each sample, 3 replicate measurements were performed, and water contents were predicted by using both calibration models. These 2 sets of data were extracted from the database and transferred into a single embedded worksheet like in Table 6.

Linearity tests were achieved by using the Linearity_TEST custom function that is an array formula. An array formula is useful when a function returns results as a data matrix. It is entered in an array of cells and simultaneously recuperates all results. The output range of the array formula is marked in Table 6 as a black rectangle of 16 cells containing the same formula but not the same results. Two complementary formulas are added to give the critical values of the test for a 1% risk.

These values are obtained by using the built-in function Finv that returns the inverse of the Fisher F distribution. Formulas are actually written in cells to conserve the computational power of Excel. It means that, if one datum is erased because it is assumed to be an outlier, all results are immediately recalculated. The exposed results accurately reflect the true contents of the data set. Because it is possible to protect the data with passwords, any careless modification can be avoided.

These results are easy to interpret. The F_{reg} test values for the data predicted from T_2 or on the total NMR signal are highly significant and indicate that the linear regression model is acceptable to explain the global trend of the data. But in the case of T_2, the lack-of-fit test is also significant because the observed F_{lof} value 18.899 is greater than the critical value 4.695. On the contrary, for the data predicted from total signal, the lack-of-fit test is not significant.

This conclusion can be confirmed when plotting both data sets (Figure 3). The T_2 calibration line presents a rupture and a shift at about 17% of water content. It can be explained from the physical meaning of the T_2 parameter, which detects the different states of water in the food samples. At this concentration, the physical state of the water is modified. Thus, the PLS multivariate calibration technique is to be preferred because it is not influenced by this mechanism.

Accuracy

Accuracy expresses the closeness of agreement between a conventional true value of a sample and the value found by using the method. A classical way to estimate accuracy consists of evaluating a linear functional relationship between conventional true values obtained by a reference method, called x_1, and values determined on same samples with the alternative method, called x_2. Depending on the straight line computed with these experimental results, it is the possible to define 2 kinds of biases: (*1*) If the intercept is statistically different from 0.0, there is a translation bias, which modifies the results of the alternative method in a constant way, whatever the concentration level. (*2*) If the slope is statistically different from 1.0, there is rotation bias, which generates a proportional error.

The linear functional relationship can be translated into 2 mathematical models, which must be simultaneously verified:

$$x_1 = c_0 + c_1 x_2$$

$$x_2 = d_0 + d_1 x_1$$

It is then possible to demonstrate that it is not appropriate to use CLS regression technique for estimating the coefficients (14). Several other regression techniques are available that give consistent results, but the simplest is orthogonal regression, based on the least-rectangles criterion. It is related to principal component regression and consists of minimizing the sum of squared normal distances to the fitted line. It leads to a very simple solution:

$$\frac{x_1 - \bar{x}_1}{s_1} = \frac{x_2 - \bar{x}_2}{s_2} \qquad (2)$$

$$\text{If } c_0 = \frac{s_1}{s_2} \text{ and } c_1 = \bar{x}_1 - \frac{s_1}{s_2}\bar{x}_2$$

equation 2 can be rearranged as $x_1 = c_0 + c_1 x_2$.

It is also possible to test the hypothesis that estimated slope c_1 is statistically equal to 1.0 (15). The observed criterion t_{obs} follows a Student's t-distribution law, with $p - 2$ degrees of freedom. In the formula, p is the number of standard solutions and r^2 the determination coefficient:

$$t_{obs} = \frac{|c_1^2 - 1.00|}{2c_1} \frac{\sqrt{p-2}}{\sqrt{1-r^2}}$$

For the accuracy study, we used 10 different feed samples with cellulose contents ranging from 2 to 22 g/per 100 g. The cellulose content of each sample was determined by using 2 methods, the standardized official French technique and an alternative, rapid technique. The data are reported in Table 7. The main function Accuracy_TEST also consists of an array formula that returns the coefficients (slope and intercept), the number of degrees of freedom, the determination coefficient, and the Student's t-test value. This value is compared with a critical value obtained by using the Tinv custom function. Because the observed Student's t statistics, 1.158, is below the critical value at 1%, 3.355, the hypothesis that the slope is not different from 1.0 can be kept. It means that no rotational bias exists and the new method is validated for accuracy.

Precision

The statistical techniques used to compute the repeatability and reproducibility of a measurement method are those described in the ISO 5725 standard (16). The data set illustrated by Table 8 was obtained from an interlaboratory study on the determination of glucose in dilute orange juice by liquid chromatography (17). Results are expressed as grams glucose per 100 g sample and were obtained from chromatograms by using 2 techniques: one using peak area and the other using peak height for result expression and calibration.

Grand average, repeatability and reproducibility standard deviations, and relative standard deviations (RSDs) were computed according to the classical technique based on an analysis of variance and the robust technique using an iterative algorithm, as described in the recent part 5 of the ISO 5725 standard. Robust statistics are recognized as nonsensitive to outliers. Six new custom functions described in Table 9 were developed to compute these statistics. They require, as a single argument, the range of cells containing the raw data. It is interesting to note that robust repeatability is usually smaller than classical repeatability, whereas reproducibility and grand average are only slightly modified. By using Dixon's test it was possible to detect 2 outlying data for laboratories 7b and 9 (indicated in Table 8), but they do not seem to strongly influence computations. These data can be erased from the worksheet and new results are automatically recalculated without retyping formu-

lae. New data reported in Table 10 show that only some statistical criteria are lightly modified by this deletion.

Conclusions

New integration technology based on OLE automation seems to be well adapted to managing documents that contain descriptive and factual data. Moreover, this technology seems to be now rather stable as we did not encounter problems in implementing complex embedded worksheets, such as data loss or computer system crash. OLE version 2 is more reliable than version 1 and can be recommended for industrial applications. On the other hand, if we take into account the claims of several major software producers, new improvements must be expected during the coming years. Nowadays, OLE is an "informal" standard and many producers do not implement all possible extensions. For instance, the Windows 95 version of the statistical software S-PLUS from Mathsoft uses OLE simply to embed graphics but does not give access to its programming environment (9). For complex applications like this it seems advisable to use programs from a single producer, and for this reason, we did not test if it was still possible to develop this application by using Word Perfect as a word processor and Excel as a spreadsheet. Moreover, some problems would likely occur in transferring a given application to a platform other than that used to develop it.

In any case, the great advantage of this technique is that it is very convenient for rapid program development. Method validation requires complex calculation but also standardization: results are to be compared and a uniform data processing scheme must be used. As a first step, the proposed strategy consists of developing the processing model on a spreadsheet. Many laboratories are already doing this as a standard operating procedure. However, it is almost impossible to prevent inconsistencies. When using encoded, compiled, and protected custom functions it becomes a very powerful tool that can be used by any analyst without too much learning, while custom functions can be accessed simply in the classical spreadsheet environment. Later, if modifications are required, only a small part of the software has to be modified, and maintenance can be organized conforming to good automated laboratory practices requirements.

Moreover, some software producers understand that software development is a real investment. When upgraded software versions are available it is always a problem to know if older application programs will still be running. In that respect, selection of OLE technology seems to be promising, as it is a strategic choice for several software producers.

The development of automated validation procedures raises the question of what can be considered as good validation practices. The main goal of these practices could be to present, in a standardized and easy-to-understand manner, validation data and results whereas data consistency could be achieved by the classical rules of traceability.

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Appendix: Code Sample

This example shows how SQL can be used in combination with VBA to extract a complete data set from a table, called DB1, contained in a database. The name of the database is in the variable Dbname. Extracted data are copied directly into a zone starting from the "A2" cell of the active worksheet.

```
' define objects
Dim db As Database
Dim rs As Recordset
' define variables
Dim Dbname as String
Dim SQLstr as String
' intialize query
SQLstr = "TRANSFORM Avg([DB1].Result) AS [Value]"
SQLstr = SQLstr & " SELECT [DB1].Study, [DB1].Sample,
[DB1].Date"
```

```
    SQLstr = SQLstr & " FROM [DB1] WHERE
((([DB1].Study)="Precision" AND "
    SQLstr = SQLstr & " (([DB1].Sample)="sample 1"))"
    SQLstr = SQLstr & "GROUP BY [DB1].Study,
[DB1].Sample, [DB1].Date PIVOT [DB1].Replicate;"
    'open data base, its name is stored in string variable
DBname
    Set db = OpenDatabase(DBname)
    ' run query, the SQL instruction is stored in SQLstr
    Set rs = db.OpenRecordset(SQlstr, dbOpenDynaset)
    ' copy recordset in active worksheet, starting in A2 cell
    ActiveSheet.Range("A2").CopyFromRecordset rs
```

TECHNICAL COMMUNICATIONS

Expert System for Catalytic Titrimetry—Part 2: Determination of Monobasic Carboxylic Acids[1]

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An expert system (ES) to solve the problem of choosing a catalytic titrimetric procedure for determining monobasic carboxylic acids is described. Carboxylic acids were divided into 3 groups—aliphatic, aromatic, and α-aminocarboxylic acids—based on their behavior in catalytic titrations with different indicator reactions, titrant, and/or solvent and the possibility of their selective determination in the presence of other acids.

In our previous work (1), we gave a brief account on an expert system (ES) for solving the problem of choosing a catalytic titrimetric procedure (CTP) for determining organic acids. The first branching of the ES segment related to the choice of a CTP for determining organic acids is into MONOBASIC, POLYBASIC, and UNKNOWN. And then comes the branching of monobasic acids into CARBOXYLIC and NONCARBOXYLIC acids. The present paper gives a detailed description of that part of the ES concerned with the choice of a CTP for determining monobasic carboxylic acids. The detailed description will show all the elements that determine its development and will point out problems arising in catalytic titration (CT) of monobasic carboxylic acids.

Basic Principle of Catalytic Titrations

During the past 40 years, catalytic indicator reactions frequently have been used for titration end-point detection (2–9). CTs are based on titration of an inhibitor (A) with a solution of catalyst (K) to give a catalytically inactive product (AK):

$$\text{Titration reaction: } A + K \rightleftharpoons AK \qquad (1)$$

The catalyst and the inhibitor must react stoichiometrically, and the reaction rate constant should be high. The titration is performed in the presence of the components of the indicator reaction, X and Y:

$$\text{Indicator reaction: } X + (Y) \overset{K}{\rightleftharpoons} L + (O) \qquad (2)$$

The course of the titration is followed by measuring the rate of the indicator reaction, that is, by registering the changes in concentration of one of the reactants or products of the indicator reaction. Because the titrant concentration in the titrated solution before the equivalence point is very small, the rate of reaction 2 is very slow. However, after the equivalence point, the titrant reacts catalytically, and a small excess of titrant produces large changes in the rate of the indicator reaction. Concentrations of the indicator reaction components often may be much higher than the concentration of the component to be determined (the inhibitor of the process). As the catalyst is regenerated by the catalytic reaction, very small amounts of catalyst can catalyze the formation of relatively large amounts of the indicator reaction products, leading to marked changes in the chosen physiochemical property of titrated solution, which can then be measured easily. In this way, it is possible to detect the moment at which a small excess of the titrant has been added and to determine small amounts of the titrated component. These titrations have been used successfully to determine strong and weak organic acids and bases and a number of metal ions and inorganic anions (2–9).

In CT of acids, the most frequently used basic titrants are solutions of potassium hydroxide (KOH) or tetra-*n*-butylammonium hydroxide (TBAH), with either acetone dimerization and acrylonitrile polymerization as the indicator reaction (10–35). The thermometric method has been used almost exclusively to monitor the course of these titrations because of the marked heat effects of these reactions, especially of the acrylonitrile polymerization.

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[1] Part 1: *Analyst* **121**, 401(1996).

Basic Principles of Expert System Design

To attain the goals preset in ES development and to meet the requirements concerning its application (described in detail in Part 1), we divided the ES knowledge base (KB) into internal and external KBs.

In addition to the inference engine, the internal KB contains the decision-making system, that is, all rules and parameters defining the flow of consultation and the results. Parameters are defined within rules in symbolic form; for example, parameters of consultation flow and elements of the consultation results are represented as A01–A99 . . . D01–D99 (Figure 1), and results of consultation are represented as PA01–PA99 . . . PD01–PD99 (Figure 2). We also applied numerical definition of the decisions. In this way, we made design, control, and testing of the ES faster and easier, with the program listing of the decision-making system significantly reduced.

Further, we introduced graphical parameters of the flow and results of consultation. These represent the external KB, which encompasses the formalized knowledge within the KB. With graphical parameters, the whole screen can be defined as one parameter (with full freedom in choosing text, picture, table, formula, or diagram). The same parameter is also defined in the internal KB, that is, in the decision-making system, in symbolic form. This ensures that any change in the content of a graphical parameter does not affect the decision-making system, except when it is also necessary to change the logic of the system. This fact is very important in ES development, as well as in its updating, innovation, and even translation into other languages. It also permits easier formalization of knowledge for the ES and introduction of this knowledge into the ES independent of the prsonal consultant (PC) shell used in ES development (36–41). Thus, preparation, editing and drawing, altering, and updating of formalized knowledge can be done with corresponding software tools for PC, without using the development shell. This frees the domain expert from the care of the development shell during development or maintenance of the ES. The expert is free to formalize the knowledge and logic of the decision-making system in a way that is understandable to the user. It also makes it possible to reach a high level of expert reasoning, even with very complex and numerous elements of decision making.

We ensured easy portability and simple use of the developed ES from one PC to another. The user receives the ES in a runtime form, which often protects the ES from accidental damage. Thus, the developed ES can be used on any configuration starting from a minimum of PC 386. Alterations and updates in most cases consist of replacing certain graphical parameters; modifications of the decision-making system in the internal KB are very rarely needed.

We used both forward and backward chaining to develop the ES. The rule structure is generally based on IF–THEN relations (i.e., IF a certain condition is satisfied, THEN the corresponding reaction follows), as well as on backward chaining. We start from the assumption that the goal is to obtain an appropriate procedure for solving the problem (in this case, determination of a given acid by CT). It is necessary to first analyze all the elements that could influence the solution itself. During consultation, through questions posed by ES and answers given by the user (with the help of additional information and suggestions obtained from the ES), the ES defines the conditions and the environment for CT determination. Then, the ES proposes the most suitable procedure. In the course of consultation, the user obtains appropriate questions formed in a way that it is enough to type in a number from 1 to 9, depending on the question. The questions are given with plenty of additional information in the form of text, tables, schemes, formulas, and diagrams, which should enable the user to find the right answer to

```
B06 ŠKB-PARMSó
---
EXPECT:   (1 2)
GPROMPT:   "EKØ12"
TYPE:   SINGLEVALUED
GHELP:   "HEKØ12"
USED-BY:   RULEØ27 RULEØ25 RULEØ26

BØ7 ŠKB-PARMSó
---
EXPECT:   (1 2)
GPROMPT:   "EKØ15"
TYPE:   SINGLEVALUED
GHELP:   "HEKØ15"
USED-BY:   RULEØ29 RULEØ33 RULEØ3Ø RULEØ31 RULEØ32 RULEØ28

BØ8 ŠKB-PARMSó
---
EXPECT:   (1 2)
GPROMPT:   "EKØ16"
TYPE:   SINGLEVALUED
GHELP:   "HEKØ16"
USED-BY:   RULEØ29 RULEØ28
```

Figure 1. Definition of parameters B06, B07, and B08.

```
RULEØ3Ø śB-RULESć
-------

PREMISE:  ($AND
            (SAME FRAME BØØ 1)
            ($OR
              ($AND
                (SAME FRAME BØ1 2)
                (SAME FRAME BØ2 2))
              (SAME FRAME BØ1 1))
            (SAME FRAME BØ3 1)
            (SAME FRAME BØ4 2)
            (SAME FRAME BØ7 1)
            (SAME FRAME BØ9 2))
ACTION:   (DO-ALL
            (CONCLUDE FRAME POSTUPAK "PBØ6" TALLY 1ØØ)
            (PICTURE "RES1")
            (PICTURE "PØ1A")
            (PICTURE "SEMA4")
            (PICTURE "PØ3B")
            (PICTURE "SEMA8")
            (PICTURE "PØ1C")
            (PICTURE "PØ3D")
            (PICTURE "PØ1E"))
```

Figure 2. Rule for defining the consultation result (PB06).

the question. The programs in the ES ensure an automatic link between internal and external KBs, so that when a symbolic parameter appears in the internal KB, such as D05, the picture of the graphic parameter D05 is downloaded, with all the necessary details. Hence, it suffices to type the appropriate number on the keyboard as the answer. Thus, the user is not burdened either by the mode in which the ES makes decision or by the links between internal and external KBs. Furthermore, to ensure best presentation of the consultation results, to provide the user the largest amount of information for a given question, and make possible the checking of certain data, we introduced forward chaining. This permits effective solutions to tasks that would be very hard or even impossible to do with backward chaining only. Use of forward chaining ensures the most convenient flow of consultation to attain the goal, as well as convenient presentation of the results of consultation to the user.

A connection is provided between the ES and the database (DB) by automatic calling of the DB from the ES when necessary. This connection enriches the ES with additional possibilities not contained in the development PC shell. For example, the ES provides automatic call of a DB in which descriptions of all the procedures are stored, which can be followed by printing of the relevant procedure.

Structure of the Expert System

Carboxylic acids are classified into ALIPHATIC, AROMATIC, α-AMINOCARBOXYLIC, and OTHER acids on the basis of their behavior in CTs with different indicator reactions, titrant, and solvent and the possibility of selective determination of acid mixtures. The classification also simplifies use of the ES and makes updating easier. Flow charts for CT determination of monobasic aliphatic, aromatic and α-aminocarboxylic acids are presented in Figures 3–5.

We developed a completely modular ES, where each system branch represents one module. Each module can exist and function on its own, that is, independently of the rest of the ES. At the same time, each module can be relatively easily incorporated into the integral ES.

This segment contains 50 procedures from which the ES, on the basis of consultation, gives the target procedure. Furthermore, the internal KB (decision-making system) encompasses 58 rules and 78 symbolic parameters, and the external KB contains 327 graphical parameters, of which 117 are concerned with consultation flow and 210 provide descriptions of procedures.

Within the branch of monobasic carboxylic acids, we provided the special branching OTHER, which at the present encompasses no acids. This branching has been envisaged for a potential group of carboxylic compounds that would exhibit some specific properties in CT similar to those of α-aminocarboxylic acids.

The development of the ES for monobasic carboxylic acids will be illustrated with monobasic aliphatic acids.

By entering the subsystem of monobasic aliphatic carboxylic acid, the user obtains a list of acids from this group that have been titrated by CT (Figure 3, BO1; 10–12). This ensures that the user knows immediately in what cases CT can be applied successfully. However, if the acid is not in this list, then the ES checks first whether the value of acidity constant permits such a determination (Figure 3, B02). Because a value of 1×10^{-12} (in aqueous medium) was adopted as the limiting acidity constant (13), all acids from the given group can be titrated by CT, provided no other factors interfere in determination (e.g., solubility in the solvent used), of which the user is informed through HELP. (In figures, H denotes the screen supplied with HELP.)

Next, the ES determines if the analyte contains some other acid apart from the acid to be determined (Figure 3, B03). If

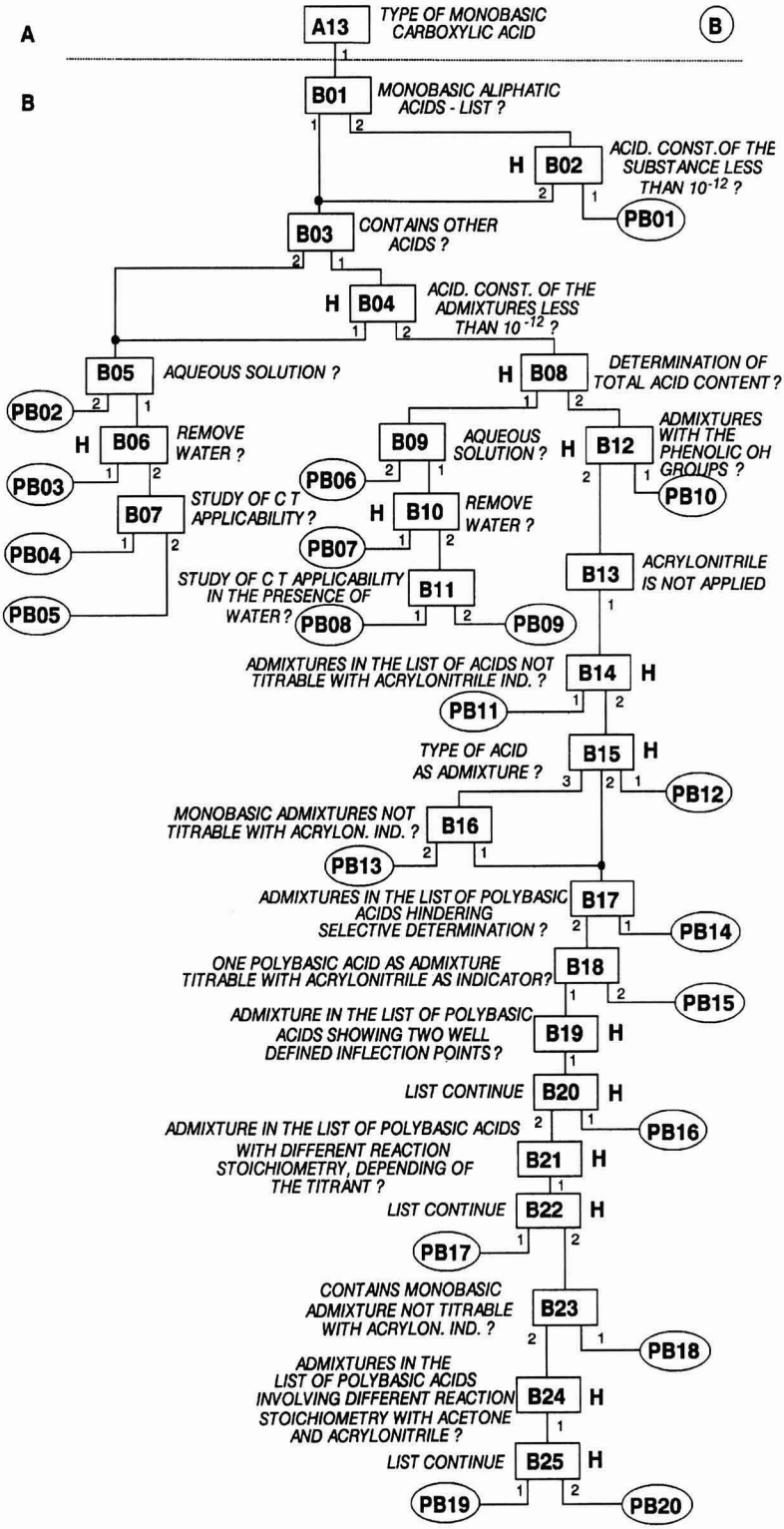


Figure 3. Flow chart for catalytic titrimetric determination of monobasic aliphatic carboxylic acids.

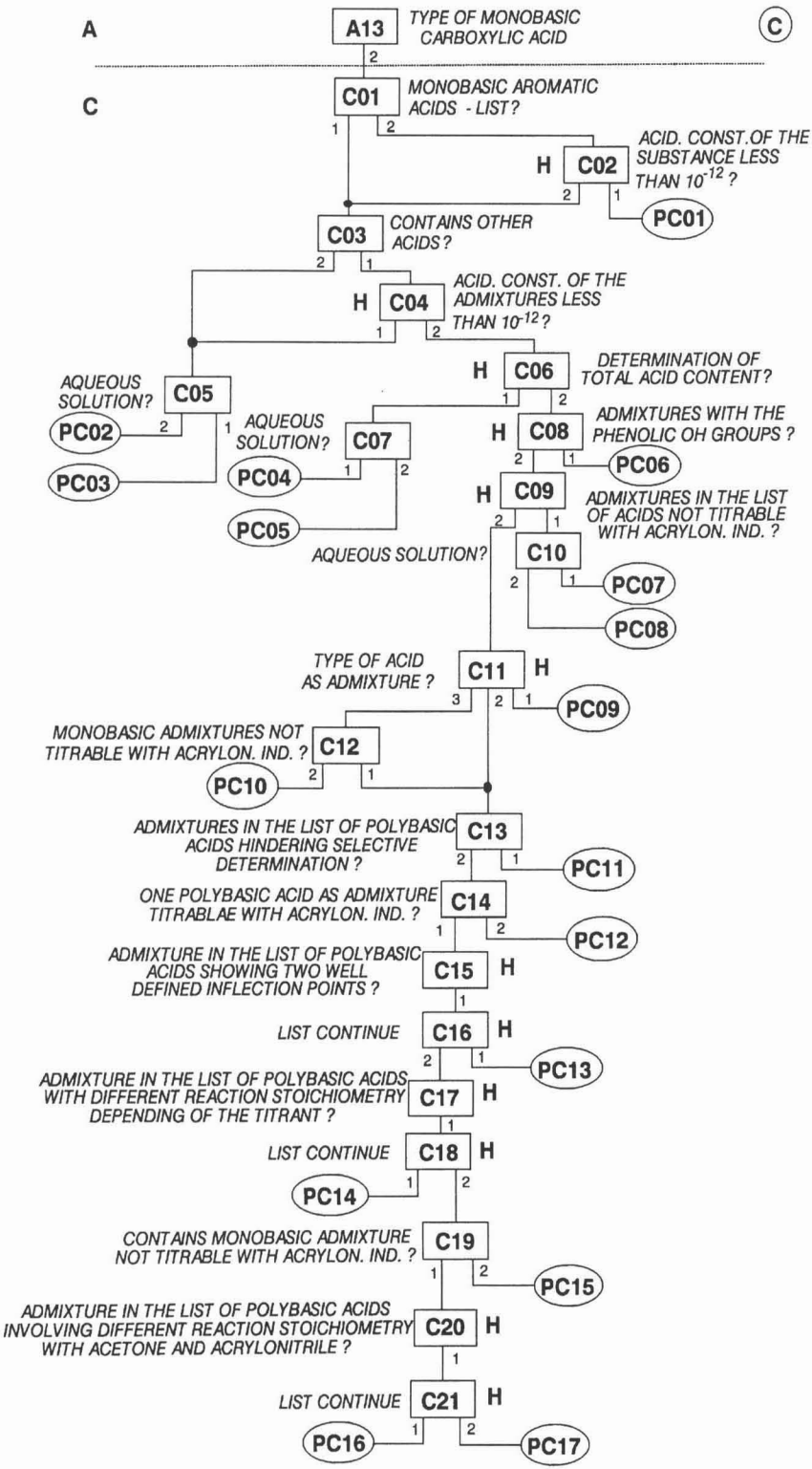


Figure 4. Flow chart for catalytic titrimetric determination of monobasic aromatic carboxylic acids.

not, the user chooses the answer 2, and then the situation is much simpler (in all cases 1 denotes YES and 2 denotes NO). Then the ES asks whether the analyte is in an aqueous solution (Figure 3, B05) because that determines the choice of the indicator reaction. If the answer is NO, the ES proposes use of acetone dimerization as indicator reaction with KOH as titrant (Figure 3, PB02). The procedure is contained in screens 7–12, including the description of the procedure and the necessary schemes, diagrams, mathematical expressions to calculate results, and a list of pertinent literature (which will be seen later on).

However, if the analyte is in an aqueous solution, the ES asks whether the user wants to eliminate water (Figure 3, B06). If the answer is YES, the ES proposes water removal by the procedure described above (Figure 3, PB03). However, if the user answers NO, the ES offers to investigate the applicability of acrylonitrile as indicator and KOH as titrant while using a mixture of dimethylsulfoxide (DMSO) and acrylonitrile as solvent (Figure 3, B07; 14). To test this possibility, the user must choose 1 to obtain corresponding procedure (Figure 3, PB04). Otherwise, the ES provides the corresponding CTP for determination with the formaldehyde indicator reaction (Figure 3, PB05).

If the answer at node B03 is YES, then the ES asks if the acidity constant of all other acids is less than 1×10^{-12} (Figure 3, B04). If YES, the ES assumes that the mixture of other acids is indifferent (of which the user is informed through HELP) and proposes the same procedure as that for analyte containing no other acids. If the acidity constants of the other acids are greater than 1×10^{-12}, the ES asks if the user wants to determine total acids (Figure 3, B08). In some cases, this determination is a satisfactory solution, such as in determination of total acids in mineral insulating oils (15, 16), aliphatic aldehydes (17), petroleum bitumen (18), coal (19), and vegetable tannins (20). HELP informs the user that CTs have been relatively seldom used for analysis of acid mixtures (21–23), so that procedures proposed in the frame of this ES are given on the basis of experience gained from CT of polybasic organic acids (10, 13, 14, 20–26), which should also be taken into account. If the answer at node B08 is YES, the branching is identical to that when other acids are absent. In certain cases, the ES proposes different procedures.

If the user does not want to determine total acids, the ES asks whether the other acids are compounds with phenolic hydroxyl groups only (Figure 3, B12), because in contrast to the carboxylic functional group, which can be titrated with acetone and propiophenone as indicators, the phenolic hydroxyl group can be titrated only with acetone (with KOH as titrant), and a selective determination is possible (Figure 3, PB10; 27).

However, if the other acids are not solely compounds with phenolic hydroxyl groups, then selective determination is possible only with acrylonitrile polymerization as an indicator reaction (Figure 3, B13). The user should investigate the possibility of applying acrylonitrile as indicator under the given experimental conditions, which, on the basis of results previously obtained, is suitable for selective determination of the analyzed mixture of acids. A screen follows, listing all possible other acids that cannot be titrated successfully with acrylonitrile as indicator and KOH as titrant (Figure 3, B14; 21). If only the acids from this list appear as other acids and if under the given conditions it is possible to titrate aliphatic carboxylic acids, the other acids will behave as inert substances, and the appropriate CTP follows (Figure 3, PB11). However, if the answer at node B14 is NO, the ES expects the user to define the type of the other acid (Figure 3, B15). That is, the user must state if the other acids are solely monobasic (answer 1), polybasic (answer 2), or a mixture of monobasic and polybasic acids (answer 3). This step is necessary because it offers a more reliable basis for proposing a procedure for selective determination.

If the other acids are monobasic only and if they do not belong to the above-mentioned groups, a selective determination is not possible, and the user is informed (Figure 3, PB12).

When the other acids are polybasic only, the ES asks if at least one of them is in a list of acids (Figure 3, B17) whose reaction stoichiometry is the same irrespective of the indicator (acetone or acrylonitrile) applied when KOH is used as titrant (10, 13, 21, 28, 29). The presence of these acids does not permit selective determination. We thought it useful to provide such a survey, so that the user, with no additional experiments, can know immediately that in the given case it is not possible to make a selective determination. If none of the other acids is in the list, then the ES asks whether the other substance is only one polybasic acid titrable with acrylonitrile as indicator (Figure 3, B18). If not, the ES informs the user that it is not possible to make a selective determination (Figure 3, PB15). If, however, the answer at node B18 is YES, then a list of polybasic acids follows (Figure 3, B19 and B20). The acids in this list exhibit 2 well-defined inflection points on the titration curves obtained with KOH as titrant and acrylonitrile as indicator (24). If the other acid is in this list, a selective determination is highly likely on the basis of the results of one titration. The screens also show the corresponding reaction stoichiometry, which is important for later calculation of results.

If the other polybasic acid is not in the list (Figure 3, B19 and B20), the user is informed through HELP, then it is possible that the concomitant acid has not been determined by CT. In that case, the user has 2 options: to investigate whether the titration curve of the other acid exhibits at least 2 well-defined inflection points under the given experimental conditions or, to consider first some other possibilities for selective determination.

Screens B21 and B22 (Figure 3) provide a list of polybasic acids with their corresponding reaction stoichiometries (23, 24). These acids are characterized by the fact that different acidic functional groups are titrated when different titrants (KOH and TBAH) are used with acrylonitrile polymerization as indicator reaction. If the other acid is in this list, the user chooses 1 to obtain the appropriate procedure (Figure 3, PB17). If, however, the other acid is not in the list, the ES recommends the procedure for the previous case, that is, when the acid is not in the list.

If this CTP also gives an unsatisfactory solution for selective determination of acids, the ES provides a third possibility for selective determination. This is the case when a different number of functional groups of the polybasic acid is titrated with different indicator reactions: acetone dimerization (titrant KOH) and acrylonitrile polymerization (titrant TBAH). The ES also ensures a survey of polybasic acids fulfilling this condition, as well as the corresponding reaction stoichiometry (Fig-

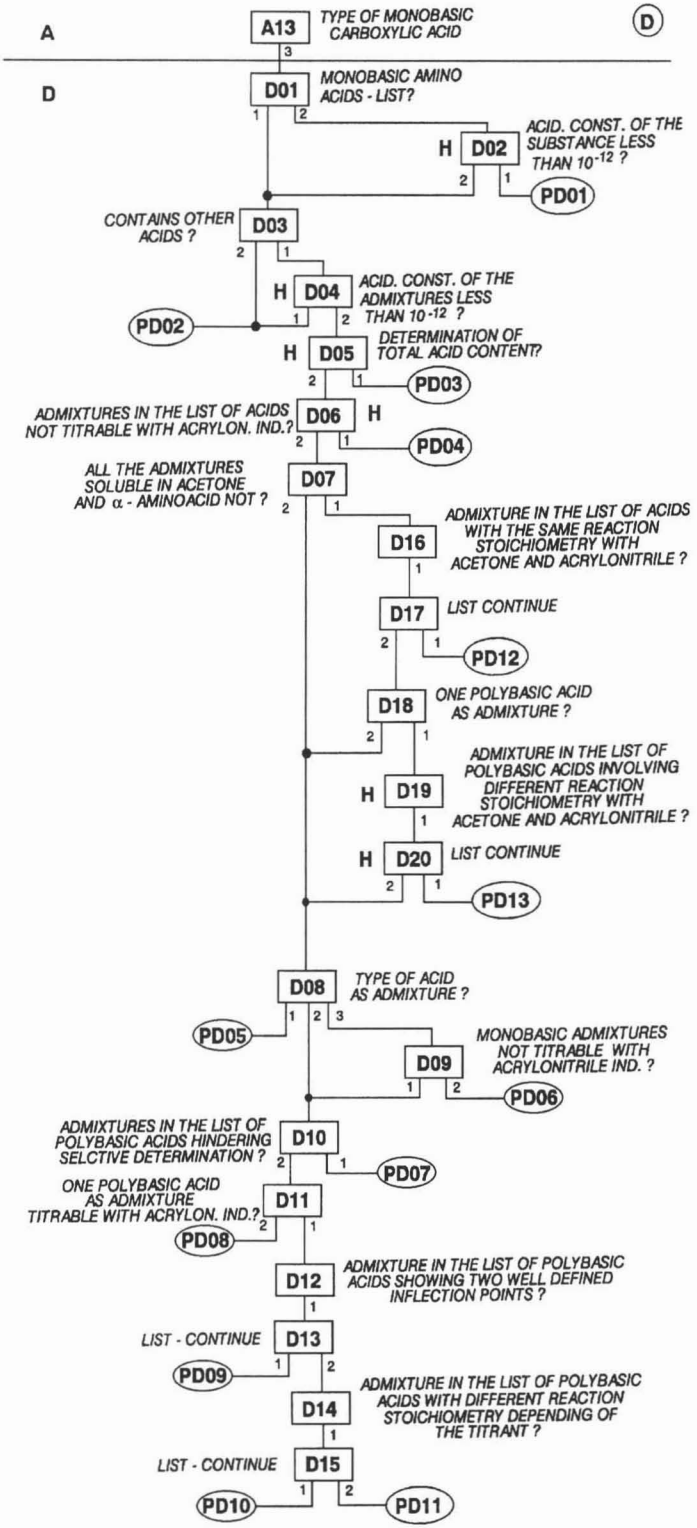


Figure 5. Flow chart for catalytic titrimetric determination of monobasic α-aminocarboxylic acids.

ure 3, B24 and B25; 10, 13, 21, 29). If the other acid is in the list, then the determination procedure follows (Figure 3, PB19). However, if the answer is NO, the ES tells the user (Figure 3, PB20) that it is not possible to do a selective determination of the monobasic aliphatic carboxylic acid.

If the other acids represent a mixture of monobasic and polybasic acids, the ES asks whether the monobasic acids are solely those that are not titrable with acrylonitrile as indicator (Figure 3, B16). If the answer is NO, the ES informs the user that selective determination of acids is not possible (Figure 3, PB13). However, if the answer is YES, then the course of consultation up to node B23 (Figure 3) is the same as when the other substances are solely polybasic acids, that is, the cases when acrylonitrile is exclusively used for indication. When acetone is used as indicator the presence of monobasic acids as other acids does not permit selective determination, the user is asked whether the analyte contains a monobasic other acid (Figure 3, B23). If the answer is YES, the ES tells the user that it is not possible to do a selective determination by CT (Figure 3, PB18).

Results and Discussion

How this part of the ES works may be illustrated with the example of glycine in a mixture with salicylic acid. The user is expected to know that glycine is an α-aminocarboxylic acid and that salicylic acid is a polybasic acid ($K_1 = 1 \times 10^{-3}$ and $K_2 = 2.5 \times 10^{-14}$). After startup of the ES, the user must indicate whether he or she wants to get basic information about CT. By choosing the appropriate numbers, he or she defines that the analyzed substance is a COMPOUND, that the compound is ORGANIC, and that the organic compound is an ACID, NOT FORMING CHELATE COMPLEXES, that the organic acid is MONOBASIC, that it is CARBOXYLIC, and that it is an α-AMINOCARBOXYLIC ACID. In this way, the user reaches the subsystem of α-aminocarboxylic acids (Figure 5). Then the user must indicate whether glycine is in the list of the α-aminocarboxylic acids that have been determined previously by CT (Figure 5, D01). Because glycine is in such a list, the user answers YES. After that, by choosing 1, the user indicates that the analyte is present with another organic acid (Figure 5, D03). By choosing 2 (Figure 5, D04) the user indicates that the acidity constant of the other acid is not less than 1×10^{-12}. For salicylic acid, the first acidity constant is greater than 1×10^{-12}, and the second is below this limit. Hence, this acid can be titrated as a monobasic acid. However, it has been reported (24) that under the given experimental conditions salicylic acid also can be titrated as a dibasic acid. It is also possible that the tabulated data for constants differ even by a factor of 20. For example, the data for salicylic acid given above (42, 43) are somewhat different from those reported by Greenhow and Spencer ($K_1 = 1 \times 10^{-3}$ and $K_2 = 4 \times 10^{-13}$; 13); that is, the second acidity constant is by about 17 times higher than the one mentioned previously. This indicates that acidity constants have to be regarded critically. Then, the user must indicate whether he or she wants to obtain total acid content (Figure 5, D05). If the answer is NO, the user is asked whether the other acid (salicylic acid) is in the list of compounds (Figure 5, D06) that appear to be nontitratable with acrylonitrile for indication. Because this is not the case with salicylic acid (it is not in the list), the answer is NO. Then, at node D07 (Figure 5), the user answers YES because salicylic acid, in contrast to glycine, is soluble in acetone. After that, the user must indicate whether salicylic acid is in the list of compounds containing all the monobasic and polybasic acids exhibiting the same reaction stoichiometry when either acetone or acrylonitrile is applied as indicator using KOH as titrant (Figure 5, D16 and D17). Because this is not the case with salicylic acid (24), the user is then asked whether the sample contains only one polybasic acid as another acid (Figure 5, D18). After the user answers YES, the next question is whether the other acid (salicylic acid) is in the list of polybasic acids exhibiting different reaction stoichiometry with acetone or acrylonitrile as indicator (Figure 5, D19 and D20). Because this is the case with salicylic acid, the answer is YES. This brings the user to the end of consultation. The ES proposes the determination procedure PD13, presented in Figure 6.

As can be seen, the ES functions on the basis of questions and answers. The presented ES was designed to pose very simple questions, requiring no specialist knowledge in chemistry from the user while providing a lot of additional information, making it seem that the user has a top expert in the field as consultant. The user is expected to know only the qualitative composition of the sample (type of the compound to be determined and the type of compound(s) appearing as admixture) and, if the analyzed acid has not been titrated previously by CT, its acidity constant and those of other acids that could be present with the analyte.

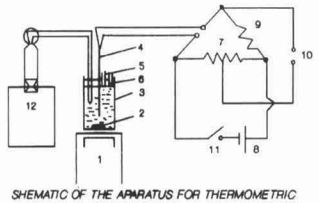
Validation of the presented ES can be viewed from 2 perspectives: whether the procedure for a CT determination of the analyzed acid has been described in the literature or not. In the former case, the ES gives the positive result with 100% confidence; that is, it offers an optimal determination procedure. If the analyzed acid has not been determined previously by CT, the ES reliability is also very high; that is, it can predict with high confidence whether the analyzed acid can be determined by CT or not, and if so, the ES proposes an optimal procedure. (Of course, a negative answer is not a consequence of a failure of the ES but of the method itself.) ES may be unreliable only when the acidity constants of the analyzed acids are close to the limiting value of 1×10^{-12}, of which the ES provides detailed information. This is related to the situation when the analyte does not contain other acids as concomitant substances. If the analyte is in a mixture with other acids, the ES offers an optimal procedure with 100% confidence, provided the user wants to determine total acid content. If, however, the user wants a selective determination, the ES gives a reliable positive answer, provided an appropriate CT procedure has been described in the literature. If this is not the case, then the ES, by analogy with similar situations, proposes a CTP. In certain circumstances, the user is expected to investigate the behavior of the analyzed/concomitant substance in the CT determination. In that case too, the ES shows a maximal reliability (except for acids with acidity constants close to 1×10^{-12}), but the specific situation will dictate whether ES can offer an appropriate determination procedure or inform the user that CT cannot be applied.

CHEMICALS AND SOLUTIONS

- All chemicals should be of reagent grade.
- Acetone, acrylonitrile, propan-2-ol and dimethylsulphoxide should be dried before use over 4A molecular sieves.
- Standard solutions of potassium hydroxide (KOH) 0.5 and 1.0mol/l in propan-2-ol. Standardize the solutions against benzoic acid by the same procedures used to titrate the sample.

TO CONTINUE PRESS ENTER

(a)



SHEMATIC OF THE APARATUS FOR THERMOMETRIC CATALYTIC TITRATIONS

1. Magnetic stirrer
2. Magnetic core
3. Dewar vessel
4. Thermistor 5 KOhm
5. Solvent inlet
6. Gas inlet
7. Potentiometer 10 KOhm
8. Ni - Cd battery 2.6 V
9. Resistor 8 KOhm
10. Chart recorder
11. Switch
12. Burette

TO CONTINUE PRESS ENTER

(b)

PROCEDURE

The procedure consists of two stages. In the first stage sample is titrated using acrylonitrile polymerization as indicator reaction and 0.5 mol/l KOH as titrant, and in the second stage acetone dimerization and 1.0 mol/l KOH as titrant. In the first stage both acids are titrated, and in the second one only the concomitant acid.

First stage

Place the measured sample in a dry titration vessel so that the acid content is about 0.1 mmol. Bubble prepurified and dried nitrogen and add to the reaction vessel 4 ml of dimethylsulphoxide, 0.5 ml of water and 2 ml of acrylonitrile. Insert the thermistor into the solution, switch on the magnetic stirrer and adjust the measuring instrument (recorder) to zero. Wait till the sample is completely dissolved and the solution thermostated.

TO CONTINUE PRESS ENTER

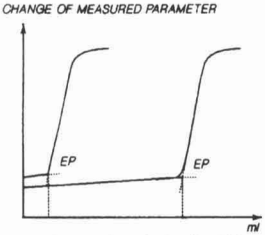
(c)

Switch on the burette and recorder simultaneously. The rate of addition of 0.5 mol/l KOH solution should be about 0.2 ml/min. The chart speed should be about 30 mm/min. After reaching the titration end-point continue on the titration for another 1 minute. Then, switch off the burette and recorder. Repeat the procedure at least three times. In the same way titrate the blank (only the mixture of solvents is titrated). Determine the end-point from the intersection of the extraplated portions of the titration curve before and after the equivalence point, as illustrated in the following figure.

TO CONTINUE PRESS ENTER

(d)

CHANGE OF MEASURED PARAMETER



Graphical determination of catalytic titration end-point

TO CONTINUE PRESS ENTER

(e)

Second stage

Place the measured sample in a dry titration vessel so that it contains about 0.03 mmol of acid. Bubble through the solution prepurified and dried nitrogen and add 5 ml of acetone. Further procedure is identical to the previous one, only the rate of titrant addition should be about 0.01 ml/min.

NOTICE

Presence of water in the sample above 0.2% hinders the determination in the second stage.

TO CONTINUE PRESS ENTER

(f)

CALCULATION OF THE RESULTS

$$\left(\frac{V1 \times c2}{a2} - \frac{V1 \times c1 \times I2}{a1 \times I1}\right) M \times 100 = \text{content of the determined substance (\%)}$$

V1 – average volume of KOH solution used for sample titration corrected by the average volume of KOH needed for the blank using acrylonitrile for indication (ml);
V2 – as for V1 but with the application of acetone (ml);
c1 and c2 – concentrations of KOH solution (mol/l);
a1 – mass of the sample in the titration using the acrylonitrile as indicator (mg);
a2 – mass of the sample in the titration using the acetone as indicator (mg);
M – molecular mass of the determined substance (g/mol);
I1 – number of acidic functional groups of the polybasic acid titrated using the acrylonitrile as indicator;
I2 – number of acidic functional groups of the polybasic acid titrated using the acetone as indicator.

TO CONTINUE PRESS ENTER

(g)

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TO CONTINUE PRESS ENTER

(h)

Figure 6. Graphic presentation of the results of consultation PD13: (a) chemicals and solutions; (b) schematic of apparatus; (c) procedure; (d) procedure (cont.); (e) end point determination; (f) procedure (cont.); (g) calculation of results; (h) literature references.

The developed ES has been in use for 2 years, and no shortcomings have been revealed. The users are satisfied with its performance.

Conclusions

The favorable characteristics of the presented ES are its full transparency, simplicity of operation, and easy updating. The independent preparation of graphic parameters significantly eases the work of both the expert of the domain and ES designer in the ES development and updating. With updating, the decision-making system very often remains unchanged, but significant gains in efficacy, speed, and reliability are obtained. The design also facilitates translation of the ES into other languages. At the same time, the ES provides a very cheap solution with a very high level of expert knowledge. Presentation of information in schemes, tables, graphs, and formulas makes use of the ES much easier.

With this ES, it is possible to determine monobasic carboxylic acids within a concentration range of 0.0001–0.5 mmol/sample. Furthermore, the ES helps solve the following analytical problems: determination of monobasic carboxylic acids in samples containing no other acids, selective determination of monobasic carboxylic acids in the presence of other acids provided the acidity constants of the admixtures are less than 10^{-12}, determination of total acid content, and selective determination of monobasic carboxylic acids in the presence of monobasic and/or polybasic acids on the basis of different behavior of the determined acid and admixture(s) in CT. The ES also provides the appropriate analytical procedures, depending on the type of solvent in which the acid to be determined is dissolved (aqueous/nonaqueous solution).

Acknowledgment

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TECHNICAL COMMUNICATIONS

Characterization of Balsamic Vinegar by Multivariate Statistical Analysis of Trace Element Content

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To characterize vinegars according to the types prescribed by Italian regulations, 8 trace elements (Cr, Mn, Co, Ni, Cu, Zn, Cd, and Pb) were determined. The data collected were successively elaborated by 3 statistical techniques: linear principal component analysis (LPCA), linear discriminant analysis (LDA), and cluster analysis (CA). LDA and LPCA best classified and discriminated the 3 types of vinegar under study, separating traditional balsamic vinegars from the other 2 types, nontraditionally aged balsamic vinegars and common vinegars. The latter 2 types were appreciably distinguished only by LDA through bidimensional analysis of discriminant scores.

Balsamic vinegar is a typical Italian product exclusively manufactured in the hinterland of Modena, whose origins date back to the 10th century (1). The term "balsamic" first appeared in 1747, but even before then, people believed that this vinegar was characterized by some particular balsamic powers (2). Nowadays, this term indicates solely its gastronomic use.

The current Italian regulation considers only 3 types of vinegar: traditional balsamic vinegar from Modena and Reggio Emilia obtained by sugar and vinegar fermentation of cooked grape juice and then aged for at least 12 years, nontraditionally aged balsamic vinegar from Modena produced through a traditional technique of vinegar and alcohol fermentation of grape juice and containing an aliquot of vinegar aged for at least 10 years, and common vinegars obtained by fermentation of red or white wines (3).

Studies have been done to identify chemical and sensory properties that could differentiate the 3 types of vinegar (4–10) and in particular to characterize the traditional balsamic vinegar from Modena by the heavy metal content (11, 12). However, the complexity of microbial and oxidative processes during vinegar maturation and the nonstandardized process for aging make it difficult to differentiate these products. Recently, Giaccio et al. (13, 14) found a compound (5-acetoxymethyl-2-furaldehyde) that is typical of the aging process and that therefore could be used to discriminate and differentiate traditional balsamic vinegars from the others.

Chemometric techniques such as linear principal component analysis (LPCA), linear discriminant analysis (LDA), and cluster analysis (CA) have been widely applied for quality control in the food industry. The aim of these techniques is to describe populations through analysis of samples. These techniques have successfully differentiated and characterized typical products according to origin (15) or extraction technologies (16).

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Experimental

Sampling

All 16 traditional balsamic vinegar samples and some nontraditionally aged balsamic samples were provided by Consorzio Produttori di Aceto Balsamico Tradizionale di Modena. Other nontraditionally aged balsamic vinegars and regular vinegars were collected from retail outlets. The total sample size was 48.

Apparatus

(**a**) *Glassware.*—Glassware was washed with nitric acid (65%, v/v) and rinsed first with cold tap water and then with deionized water.

(**b**) *Atomic absorption spectroscopy.*—Perkin-Elmer Model 4100 ZL spectrometer equipped with high-temperature graphite furnace system, autosampler, and a Zeeman baseline corrector mounted on a pyrolitic graphite tube provided with a L'Vov platform.

(**c**) *Atomic absorption spectroscopy for zinc.*—Instrumentation Laboratory Model 457 air–acetylene flame atomic absorption (FAAS) spectrophotometer.

Reagents

(**a**) *Water.*—Deionized water was obtained from a Milli-Q plus (Millipore) apparatus.

(**b**) *Triton X-100 (Fluka).*—1 mL in 1000 mL deionized water.

(**c**) *Atomic absorption reference solutions.*—Standard curves for Cr, Mn, Co, Ni, Cu, Zn, Cd, and Pb were prepared

Table 1. Analytical parameters for determination of Cr, Mn, Co, Ni, Cu, Cd, and Pb

Program	Temperature, °C	Time, s		Gas	Flow, mL/min
		Ramp	Hold		
		Chromium			
Drying	120	1	30	N_2	250
	130	10	30	N_2	250
Ashing	450	15	10	N_2	250
	1200	10	30	N_2	250
Atomization	2100	0	5	N_2	0
Clean-out	2600	1	5	N_2	250
Wavelength, nm	357.9				
Chemical modifier	15 μg $Mg(NO_3)_2$				
		Manganese			
Drying	110	1	20	N_2	250
	130	5	30	N_2	250
Ashing	1200	10	20	N_2	250
Atomization	1900	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, nm	279.5				
Chemical modifier	5 μg Pd + 3 μg $Mg(NO_3)_2$				
		Cobalt			
Drying	110	1	30	N_2	250
	130	1	20	N_2	250
Ashing	500	5	5	N_2	250
	1400	10	20	N_2	250
Atomization	2400	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, ng	242.5				
Chemical modifier	15 μg $Mg(NO_3)_2$				
		Nickel			
Drying	110	1	20	N_2	250
	130	5	30	N_2	250
Ashing	1100	10	20	N_2	250
Atomization	2300	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, nm	232.0				
Chemical modifier	—				
		Copper			
Drying	110	1	20	N_2	250
	130	5	30	N_2	250
Ashing	450	5	15	N_2	250
	1200	5	20	N_2	250
Atomization	1900	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, nm	324.8				
Chemical modifier	5 μg Pd + 3 μg $Mg(NO_3)_2$				

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

Program	Temperature, °C	Time, s		Gas	Flow, mL/min
		Ramp	Hold		
		Cadmium			
Drying	110	1	20	N_2	250
	130	5	30	N_2	250
Ashing	700	10	20	N_2	250
Atomization	1400	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, nm	228.8				
Chemical modifier	50 μg PO_4 + 3 μg Mg $(NO_3)_2$				
		Lead			
Drying	110	1	20	N_2	250
	130	5	30	N_2	250
Ashing	750	5	30	N_2	250
Atomization	1500	0	5	N_2	0
Clean-out	2400	1	2	N_2	250
Wavelength, nm	283.3				
Chemical modifier	50 μg PO_4 + 3 μg Mg $(NO_3)_2$				

by diluting 1000 mg/L stock reference solutions (20°C) for atomic absorption (Merck).

Analytical Procedure

(**a**) *Sample preparation for Cr, Mn, Co, Ni, Cu, Cd, and Pb.*—A 5 mL volume of vinegar taken from just-opened bottles was brought to volume with Triton X-100 solution in 10 mL glass volumetric flask.

(**b**) *Procedure for Cr, Mn, Co, Ni, Cu, Cd, and Pb.*—Prepared solutions were put directly in the autosampler. The automatic diluter diluted the original liquid samples from 5 to 25 times, according to the analytical sensitivity for the trace element to be determined.

To prepare standard curves for Cr, Mn, Co, Ni, Cu, Cd, and Pb, standards were made at 3 levels: 10, 20, and 40 μg/L for Cr, with a peak area of 0.005 and a concentration <0.3 μg/L for the blank; 25, 50, and 100 μg/L for Mn, Co, and Ni, with peak areas of 0.013, 0.009, and 0.004 respectively, and concentrations of <1 μg/L, <2.5 μg/L, and <1 μg/L for blanks, respectively; 50, 100, and 200 μg/L for Cu, with a peak area of 0.025 and a concentration <3 μg/L for the blank; 1.25, 2.5, and 5 μg/L for Cd, with a peak area of 0.015 and a concentration <0.2 μg/L for the blank; 50, 100, and 200 μg/L for Pb, with a peak area of 0.011 and a concentration <2 μg/L for the blank. The curves obtained for each trace element were linear, with correlation coefficients equal to 1.

Analytical parameters adopted for each element are reported in Table 1.

(**c**) *Sample preparation for Zn.*—A 2 mL volume of vinegar taken from just-opened bottles was brought to volume in a 50 mL glass volumetric flask.

(**d**) *Procedure for Zn.*—Zinc was analyzed by air–acetylene FAAS after further dilutions of samples.

To prepare a standard curve, standards were made at 3 levels: 250, 500, and 1000 μg/L. The curve obtained was linear, with a correlation coefficient equal to 1. The blank had a concentration of <50 μg/L.

Analytical parameters adopted for Zn were as follows: flame, oxidizing; wavelength, 213.9 nm; slit width, 320 μm.

(**e**) *Repeatability.*—Intralaboratory repeatabilities—expressed as means (mg/L)—and standard deviations (values in parentheses) for 10 observations were as follows: Cr, 0.105 (0.0044); Mn, 3.445 (0.110); Co, 0.0154 (0.00075); Ni, 0.113 (0.0100); Cu, 0.523 (0.0203); Zn, 7.90 (0.076); Cd, <0.005; Pb, 0.058 (0.0028).

For each type of vinegar, mean values of 3 measurements are reported in Tables 2–4.

(**f**) *Accuracy.*—Addition of well-known amounts of trace elements to samples was adopted for quality control to verify accuracy. Trace elements recovered were within acceptable ranges of concentrations.

Statistical Analysis

Computations for basic and multivariate statistics were performed with Statistica, Release 4.5.

(**a**) *Basic statistics.*—Basic statistics were obtained for each trace element in all samples, except Cd which had a concentration of <0.005 mg/L (the experimental sensitivity level). Maximum and minimum values, sample mean, median, and variance for each type of vinegar ($n_1 = n_2 = n_3 = 16$) are listed in Tables 2–4.

(**b**) *Exploratory multivariate statistics.*—LPCA is a classical method for linear transformation of original variables. This

Table 2. Data matrix and basic sample statistics for Modena and Reggio Emilia traditional balsamic vinegars (n_1 = 16)

	Concentration, mg/L						
Sample	Cr	Mn	Co	Ni	Cu	Zn	Pb
1	0.222	5.69	0.0401	0.184	0.713	12.75	0.094
2	0.215	6.509	0.0455	0.24	0.967	24.6	0.066
3	0.159	9.452	0.0355	0.189	1.656	30	0.0693
4	0.102	4.849	0.0355	0.15	0.523	17.75	0.246
5	0.223	6.607	0.0355	0.209	1.263	13.5	0.014
6	0.484	5.783	0.0247	0.332	0.412	7.37	0.036
7	0.156	6.932	0.0393	0.238	1.037	21.65	0.071
8	0.105	6.489	0.0324	0.327	0.643	27.8	0.025
9	0.108	10.4955	0.0416	0.196	1.839	29.8	0.005
10	0.154	11.388	0.0525	0.264	1.2	25.65	0.049
11	0.059	3.445	0.02	0.113	0.709	7.42	0.021
12	0.087	9.77	0.0401	0.18	0.793	14.5	0.007
13	0.144	6.139	0.0216	0.199	0.728	9.02	0.007
14	0.166	6.981	0.0385	0.255	0.419	21.15	0.01
15	0.164	6.157	0.0386	0.354	1.003	23.25	0.1
16	0.1075	4.068	0.0231	0.354	2.06	14.5	0.046
Minimum	0.059	3.445	0.02	0.113	0.412	7.37	0.005
Maximum	0.484	11.388	0.0525	0.354	2.06	30	0.246
Mean	0.165969	6.922156	0.035281	0.2365	0.997813	18.79438	0.054144
Median	0.155	6.499	0.037	0.2235	0.88	19.45	0.041
Variance	0.009532	5.082237	0.000081	0.005385	0.246105	60.00148	0.003596

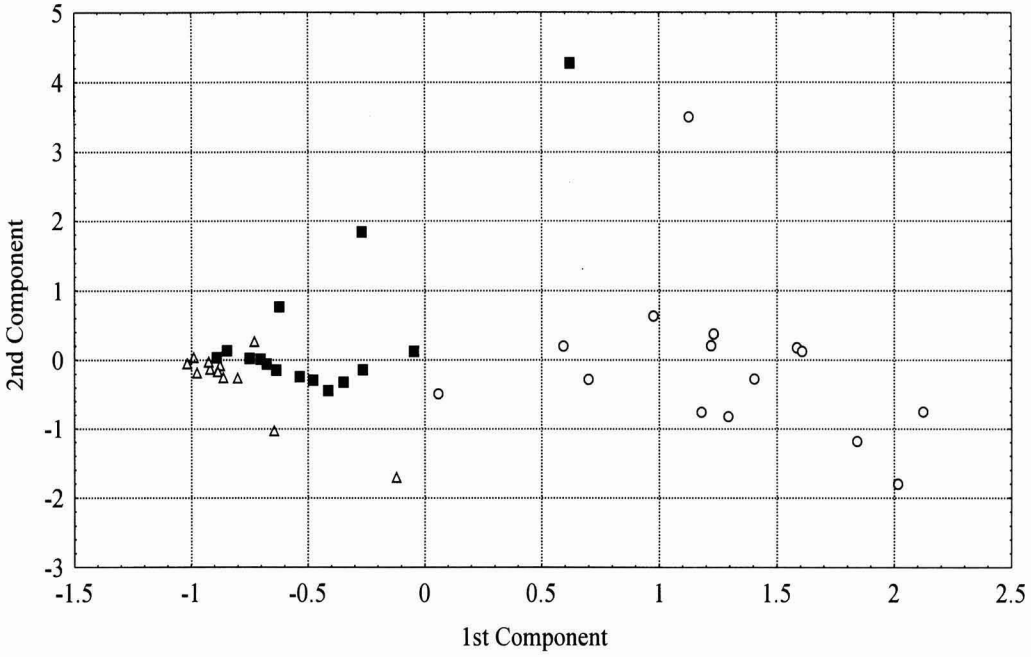

Figure 1. Projection of linear principal component scores along the first 2 eigenvector axes. ○ = G1 traditional balsamic vinegars from Modena and Reggio Emilia, ■ = G2 nontraditionally aged balsamic vinegars from Modena, △ = G3 common vinegars.

Table 3. Data matrix and basic sample statistics for Modena nontraditionally aged balsamic vinegars (n_2 = 16)

	Concentration, mg/L						
Sample	Cr	Mn	Co	Ni	Cu	Zn	Pb
1	0.141	0.832	0.0083	0.06	0.218	1.3	0.047
2	0.088	3.964	0.0133	0.153	0.468	4.05	0.012
3	0.071	0.965	0.0055	0.028	0.379	4.22	0.027
4	0.0717	1.505	0.0062	0.051	0.702	1.75	0.02
5	0.081	3.525	0.0031	0.03	0.863	1.64	0.023
6	0.0485	0.789	0.0059	0.019	0.1225	0.87	0.058
7	0.085	1.776	0.0096	0.061	0.885	2.75	0.02
8	0.058	3.907	0.0111	0.09	0.281	5.72	0.035
9	0.065	1.225	0.005	0.032	0.285	1.92	0.036
10	0.2965	1.582	0.0052	0.0985	0.472	1.57	0.016
11	0.0665	2.156	0.0062	0.056	0.664	2.5	0.006
12	0.565	2.323	0.0126	0.258	0.311	7.9	0.021
13	0.056	1.257	0.0050	0.035	0.27	1.45	0.019
14	0.06	1.335	0.0037	0.047	0.431	4.12	0.029
15	0.0762	1.046	0.004	0.039	0.453	1.6	0.078
16	0.059	1.364	0.0034	0.027	0.084	1.82	0.029
Minimum	0.0485	0.789	0.0031	0.019	0.084	0.87	0.006
Maximum	0.565	3.964	0.0133	0.258	0.885	7.9	0.078
Mean	0.118025	1.846938	0.006756	0.067781	0.430531	2.824375	0.02975
Median	0.7135	1.4345	0.0057	0.049	0.405	1.87	0.025
Variance	0.017749	1.123653	0.0000106	0.003738	0.058119	3.65672	0.000333

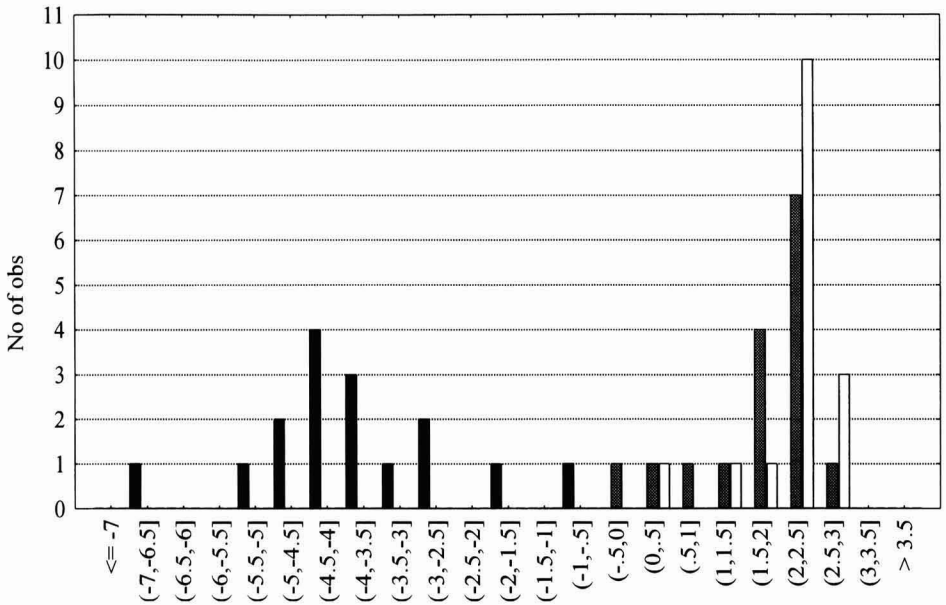

Figure 2. Distribution of data expressed as discriminant scores along the first eigenvector. ■ = G1 traditional balsamic vinegars from Modena and Reggio Emilia, ▩ = G2 nontraditionally aged balsamic vinegars from Modena, □ = G3 common vinegars.

Table 4. Data matrix and basic sample statistics for common vinegars (n_3 = 16)

	Concentration, mg/L						
Sample	Cr	Mn	Co	Ni	Cu	Zn	Pb
1	0.015	1.131	0.003	0.016	0.051	0.85	0.061
2	0.03	1.285	0.0055	0.026	0.067	1.23	0.049
3	0.041	0.59	0.003	0.012	0.049	1.07	0.068
4	0.017	1.332	0.0065	0.027	0.088	3.86	0.0703
5	0.025	1.071	0.004	0.027	0.14	1.6	0.049
6	0.022	1.112	0.0043	0.029	0.154	1.3	0.031
7	0.023	1.246	0.0043	0.022	0.195	1.6	0.043
8	0.026	0.834	0.0040	0.025	0.126	1.22	0.046
9	0.025	0.966	0.0037	0.03	0.154	1.54	0.034
10	0.027	0.565	0.003	0.012	0.031	0.86	0.095
11	0.027	0.956	0.008	0.068	0.062	1.85	0.092
12	0.027	0.95	0.0043	0.032	0.017	1.24	0.044
13	0.0084	0.946	0.0052	0.017	0.999	1.37	0.023
14	0.015	1.87	0.0055	0.055	1.822	5.6	0.377
15	0.042	2.448	0.0154	0.093	0.404	3.2	0.086
16	0.026	0.804	0.0037	0.023	0.097	1.79	0.266
Minimum	0.0084	0.565	0.003	0.012	0.017	0.85	0.023
Maximum	0.061	2.448	0.0154	0.093	1.822	5.6	0.377
Mean	0.0268	1.132875	0.005213	0.032125	0.2875	1.88625	0.089644
Median	0.0255	1.0235	0.0043	0.0265	0.1115	1.455	0.055
Variance	0.000158	0.219218	0.00000921	0.000477	0.226172	1.626905	0.009055

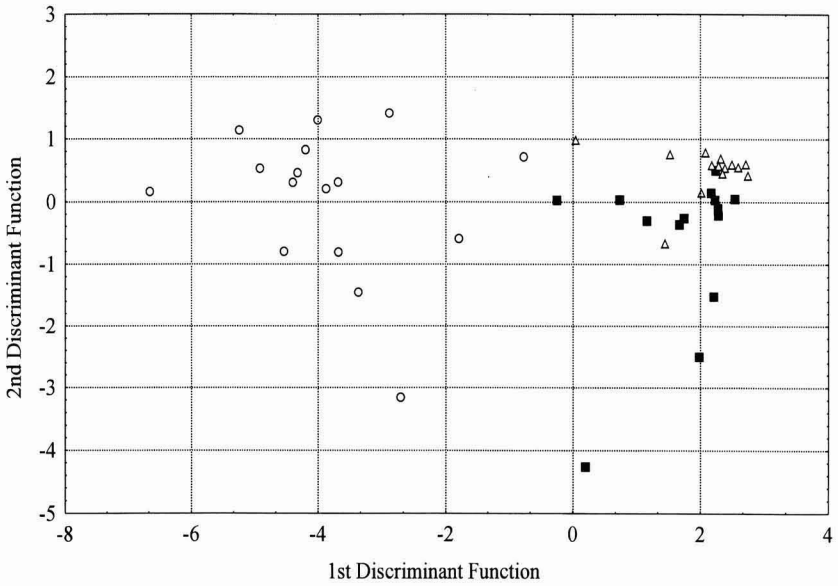

Figure 3. Distribution of data expressed as discriminant scores along 2 eigenvectors. ○ = G1 traditional balsamic vinegars from Modena and Reggio Emilia, ■ = G2 nontraditionally aged balsamic vinegars from Modena, △ = G3 common vinegars.

Table 5. Linear correlation matrix ($N = 48$)

Element	Chromium	Lead	Copper	Manganese	Nickel	Cobalt	Zinc
Chromium	1.00						
Lead	–0.17	1.00					
Copper	0.18	0.13	1.00				
Manganese	0.38[a]	–0.12	0.64[a]	1.00			
Nickel	0.65[a]	–0.10	0.56[a]	0.77[a]	1.00		
Cobalt	0.42[a]	–0.02	0.58[a]	0.93[a]	0.82[a]	1.00	
Zinc	0.34[b]	0.01	0.65[a]	0.90[a]	0.80[a]	0.92[a]	1.00

[a] A significant correlation coefficient at the 0.01 level.
[b] A significant correlation coefficient at the 0.05 level.

method gives an approximate representation of the original data matrix starting from the correlation matrix R. The overall data matrix, having $N = 48$ observations and $p = 7$ variables, was first standardized to obtained R. Details on the calculation procedures are reported elsewhere (17). The number of principal components to retain was chosen according to the total variation accounted for (17, 18).

LDA was applied to the separation of the 3 types of vinegars according to their label on the bottle. As the type of each sample was known, LDA was applied to this variable set to evaluate sample differentiation and classification of data expressed as discriminant scores. LDA has been extensively discussed by others (18–20).

CA, the classical *k*-means clustering algorithm, was adopted to explore the number of classes or natural groups (21, 22). A detailed discussion can be found in the book of Massart and Kaufman (23).

Results and Discussion

Data Matrixes and Sample Basic Statistics

For each vinegar sample group, data matrixes are given in Tables 2–4, along with corresponding basic statistics.

LPCA

Linear correlations are reported in Table 5 for a sample size of 48. Some linear correlation coefficients appeared near zero in absolute value, particularly those concerning Pb. This probably means that Pb does not contribute to group differentiation and that it can be considered as a redundant variable. Consequently, Pb was not considered further in the analysis.

Other correlations were distinctly high. The highest positive correlation was between Mn and Co (+0.93). A comparable high correlation was also observed between Zn and Co (+0.92). Further positive and significant correlations are given in Table 6.

After discarding the lead variable from the correlation matrix, a new (6×48) correlation matrix was obtained. Eigenvalues of this correlation matrix were as follows: $\lambda_1 = 4.30$, $\lambda_2 = 0.93$, $\lambda_3 = 0.47$, $\lambda_4 = 0.16$, $\lambda_5 = 0.09$, and $\lambda_6 = 0.05$.

As can be seen from Table 6, 2 principal components accounting for 87.2% of the total variation (71.7 and 15.5%, respectively) were extracted. Table 6 shows that, on the first principal component, 4 elements have a major weight—Co, Mn, Zn, and Ni—whereas for the second component, only Cr is important.

Table 6. Coefficients of correlation between the first 2 principal components and variables, associated eigenvalues, and cumulative percentages of explained variance

Variable	Principal component 1	Principal component 2
Cobalt	0.95	–0.07
Manganese	0.94	–0.16
Zinc	0.94	–0.18
Nickel	0.92	0.25
Copper	0.72	–0.40
Chromium	0.54	0.80
Eigenvalues	4.30	0.93
Cumulative, %	71.7	87.2

Data for the 3 vinegar groups were expressed graphically as a projection of linear principal component scores along the first 2 eigenvector axes (Figure 1). In this scatter plot, 3 score contours could be distinguished. A first contour given by Modena and Reggio Emilia traditional balsamic vinegars (G1) located in the first and second quadrant, a second contour represented by overlapped samples of nontraditionally aged balsamic and common vinegar (G2 and G3, respectively), and a third contour comprising only nontraditionally aged balsamic vinegars that probably differ from common vinegars in some typical elements.

The net differentiation between Modena and Reggio Emilia traditional balsamic vinegars and the other 2 groups supports similar previous results (13, 14) based on dosing with 5-acetoxymethyl-2-furaldehyde. Those studies showed that this compound is typical only of Modena and Reggio Emilia traditional balsamic vinegars and that it seemed to be formed only with the particular aging conditions of the traditional methods.

LDA

In this multivariate analysis, 2 discriminant functions were estimated, because the number of groups in this sample was 3, and 3 – 1 was the maximum number of the eigenvalues of the matrix $W^{-1}B$. The first discriminant eigenvalue (7.796) had a Wilks Λ value close to zero (0.094), whereas the second discriminant eigenvalue (0.207) had a Λ value of 0.829.

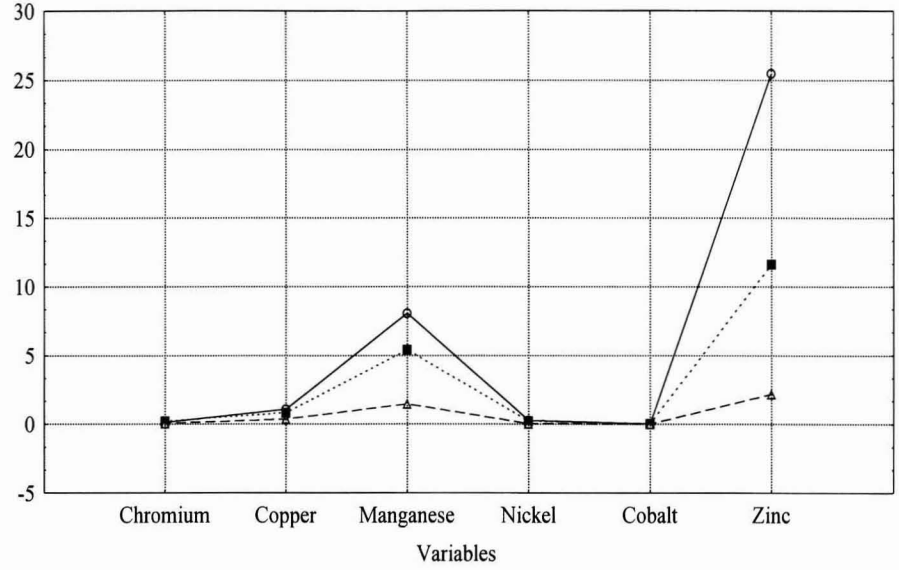

Figure 4. Mean variability for clusters: —○— = cluster 1, ...■... = cluster 2, —△— = cluster 3.

To determine the number of linear discriminant functions to retain the Bartlett's classical test, we used the following equation:

$$b = -[N - (p + g)/2 - 1] \ln \Lambda$$

where N = number of observations, p = number of variables, g = number of groups, and Λ = ratio of within-group sum of squares to total sum of squares. According to this test, at most 1 dimension would be needed to interpret group differences. However, going from 1 dimension to 2 dimensions seemed useful, particularly when projecting in the discriminant space the scores representing the vinegar samples.

The distribution of data expressed as discriminant scores along the first eigenvector is presented in Figure 2. In this one-dimensional representation of all data, the frequency histogram clearly shows that group 1, corresponding to Modena and Reggio Emilia traditional balsamic vinegars, is well separated from the other 2 groups, at least in this data set. The first group is characterized by a symmetric tendency around the mode in the score distribution, whereas the other groups appear to be mixed, at least in the discriminant score projection.

The scatter plot of the discriminant scores in Figure 3 points out the usefulness of the second dimension for the separation between nontraditionally aged balsamic vinegars (G2) and common vinegars (G3). In fact, the best separation among discriminant scores appeared to be confined to the first discriminant eigenvector. In this 2-dimensional representation, not only the centroid of the balsamic traditional vinegar (G1 centroid = –3.82, 0.04) is separated from the other 2, but even those of the other vinegars are distinct (G2 centroid = 1.70, –0.56; G3 centroid = 2.12, 0.52).

Using the values for the 2 linear discriminant functions for each sample, the group membership can be predicted through a classification rule. The result of the classification analysis can be reported in a table where actual and predicted group membership and percentage of cases assigned correctly to the group they belong to are given.

Table 7 summarizes results of the classification for the 48 vinegar samples. The diagonal shows the number of the samples classified correctly. Among the Modena and Reggio Emilia traditional balsamic vinegars (G1) and the common vinegars (G3), only 1 sample out of 16 was misclassified, for the nontraditionally aged balsamic vinegars (G2), 6 samples were assigned to the group of common vinegars.

Table 7. Classification table for 3 groups of vinegar samples: G1, Modena and Reggio Emilia traditional balsamic vinegars; G2, Modena nontraditionally aged balsamic vinegars; G3, common vinegars

	Predicted group			
Actual group	G1	G2	G3	Total
G1	15	1	0	16
G2	0	10	6	16
G3	0	1	15	16
Total	15	12	21	48
Accuracy of prediction, %	93.75	62.5	93.75	

CA

A series of 2, 3, 4, and 5 clusters were used to find a better classification of each group. From the overall analysis, better classification occurred when the total sample was subdivided

into 3 classes. In the first cluster, 8 samples of Modena and Reggio Emilia traditional balsamic vinegars were classified. In the second cluster, the remaining 8 samples of the same group were included together with 1 sample of nontraditionally aged balsamic vinegar. In the third cluster, the remaining 31 vinegars were included: all common vinegars, 10 Modena nontraditional balsamic vinegars, and 5 Modena nontraditionally aged balsamic vinegars. All products appeared to be mixed.

In Figure 4, mean variabilities for the clusters are shown for each trace element under study. Maximum variability is clearly associated with Mn and Zn.

Conclusions

LPCA and LDA were applied successfully to differentiation of 3 types vinegars using trace element concentration as variables. Both differentiated Modena and Reggio Emilia traditional balsamic vinegars from others. In particular, when LDA was applied, no discriminant score overlapping between traditional balsamic vinegars and the other 2 types was observed. To differentiate between the 2 remaining groups, 2 dimensions were needed. This result could be also deduced from centroid coordinates.

On the other hand, CA did not discriminate the various types of vinegars.

This research represents a first basic study for creation of a database for vinegar production.

Acknowledgment

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TECHNICAL COMMUNICATIONS

Integration and Calculation Improvements in Monensin Liquid Chromatographic Method

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The liquid chromatographic method for monensin in premix and animal feeds was approved by the AOAC INTERNATIONAL Methods Committee on Feeds, Fertilizer, and Related Agricultural Topics and adopted by the Official Methods Board in January 1997. Improvements have been made in the integration and calculation to include additional monensin factors. The original method accounted for monensin factors A and B only. The improved method also includes monensin factors C and D, Monensin factors C and D account for approximately 2.4% of the total biopotency of monensin in both the premix and feed.

Monensin (Figure 1) is a polyether monocarboxylic acid ionophore marketed as a feed additive for poultry (Coban) for prevention of coccidiosis and for beef cattle (Rumensin) for improved feed efficiency (1, 2). Until recently, the approved AOAC methods for determining monensin in feeds were microbiological (3). In January 1997, AOAC INTERNATIONAL adopted a liquid chromatographic (LC) method for determining monensin in premix and animal feeds, AOAC Method **997.04** (4–6). This LC method has been modified to account for the bioactivity contributions of monensin factors C and D.

METHOD

Principle

Monensin is extracted from premixes and feeds with methanol–water (90 + 10, v/v). The extracts are analyzed by LC with postcolumn derivatization. A schematic of the LC system is shown in Figure 2. More specific information regarding the LC system, other apparatus, reagents, sample preparation, and system suitability can be found in AOAC Method **997.04** (6). With AOAC Method **997.04**, 4 monensin factors can be detected—monensin A, monensin B, monensin C, and monensin D. Monensin factors C and D coelute in this system. Because the bioactivities of monensin factors C and D are the same (4), coelution of these 2 factors is allowed to reduce analysis time.

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LC Determination

Before analyzing test samples, ensure that the LC system meets system suitability parameters. The most critical parameters in the HPLC measurement system are water content, reactor temperature, acid/vanillin concentrations, and flow rates. Measure peak area response (PR) at the retention volume of monensin factor A, factor B, and factor C/D. Approximate retention times for the monensin factors are monensin A, 640 s; monensin B, 560 s; and monensin factors C/D, 730 s. Figures 3 and 4 show chromatograms of a Rumensin Type A medicated article and a Monensin Type C medicated poultry feed.

Calculations

Using the measured responses of monensin A from the working standards, construct a linear regression plot of monensin A. Determine the slope and y intercept of the calibration line. When constructing the linear regression plot, ensure that the potencies for the working standards have been corrected for standard purity. (*Note*: Refer to the Reference Standard Profile, available from Eli Lilly and Company, for the monensin factor A content of the current reference standard.)

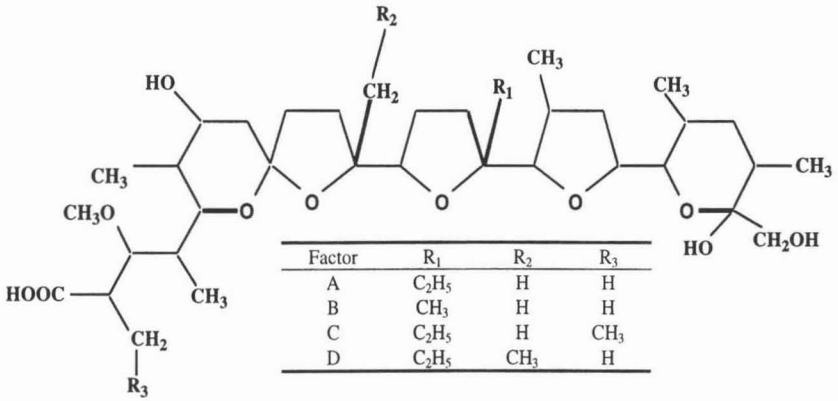
Determine the concentration of the individual monensin factors A, B, and C/D in each sample as follows:

$$\text{Biopotency} = \frac{y - b}{m} \times \frac{V}{W_s} \times f \times \text{BCF}$$

where y = peak area of monensin in the sample; b = y intercept of standard area curve; m = slope of standard curve; V = extraction volume (mL); W_s = weight of sample (g); f = dilution factor; BCF = biopotency conversion factor (factor A; 1.00, factor B, 0.28; and factor C/D, 1.5). Add the biopotency values for monensin factor A, monensin factor B, and monensin factor C/D to obtain the total biopotency for monensin.

Results and Discussion

The original method accounted for monensin biopotency from monensin factors A and B only. However, levels of monensin factors C/D can be detected not only in bulk and in Type A medicated article products but also in final finished feeds. A comparison of assay results based monensin factors A and B versus results based on monensin factors A, B, and C/D for 110 production lots of monensin granulations were evaluated over a 3-year period. In addition, a comparison of assay



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

Figure 1. Structure of monensin factors.

$$S1 \rightarrow P1 \rightarrow A \rightarrow C \rightarrow T \leftarrow P2 \leftarrow S2$$
$$\downarrow$$
$$CO \rightarrow D \rightarrow R$$

Figure 2. Diagram of LC postcolumn derivatization system. S1 = HPLC mobile phase, methanol–water–glacial acetic acid (94 + 6 + 0.1, v/v/v); degassed. P1 = pulse-dampened HPLC pump operating at 0.7 mL/min. A = autosampler. C = 4.6 mm × 25 cm Whatman 5 ODS-3 HPLC column, or equivalent. T = 90 degrees Tee (SSI01-0165 or equivalent) plumbed such that the inlet flows directly oppose one another. P2 = reagent pump operating at 0.7 mL/min. S2 = color reagent consisting of methanol–sulfuric acid–vanillin (95 + 2 + 3, v/v/w). CO = 0.020″ × 20′ stainless steel coil enclosed in a 98.0°C ± 0.5°C heater. (*Warning*: Do not use Teflon for the 0.020″ × 20′ coil. Teflon has been shown to cause problems with the monensin assay.) D = variable wavelength absorbance detector operating at 520 nm with a rise time of 1.0 and a range of 0.2 (or as needed). R = recorder, integrator or computer measurement device.

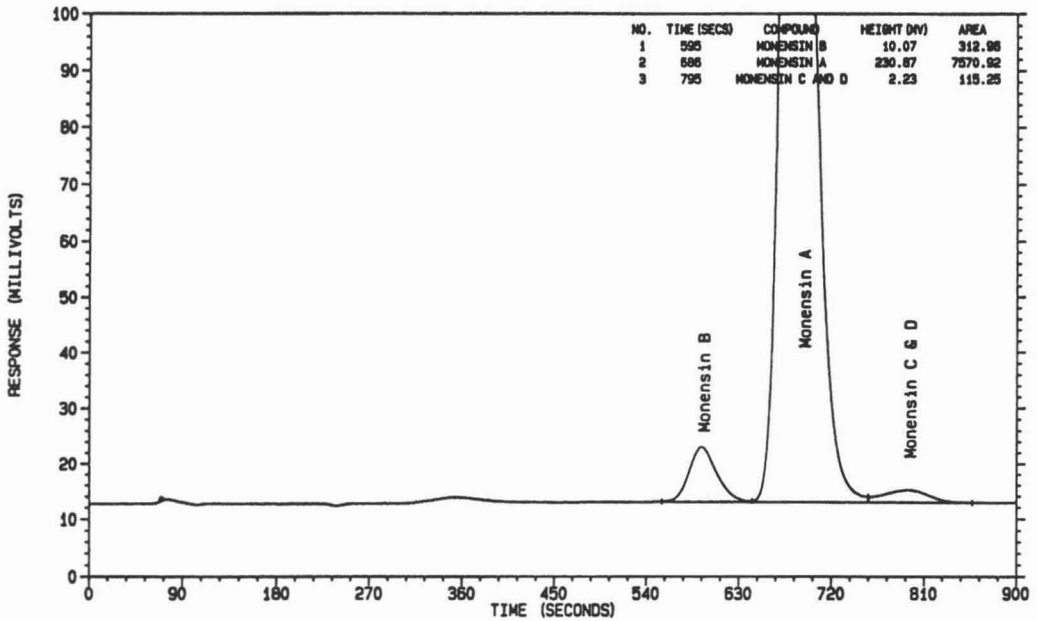


Figure 3. Chromatogram of Rumensin 80 Type A medicated article.

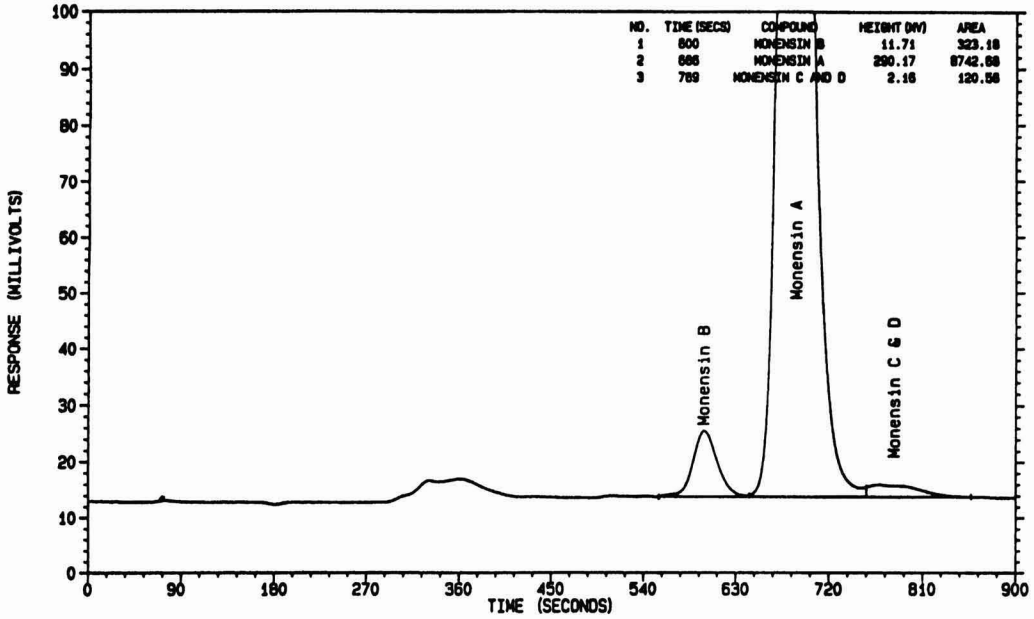


Figure 4. Chromatogram of monensin Type C medicated poultry feed.

Table 1. Percentage of monensin factor C/D in monensin granulations, premix, and feed

Monensin product	No. of lots	Range of monensin factor C/D in product, %	Mean, %
Monensin granulated	110	2.0–3.6	2.8
Coban 60 Type A Medicated Article	20	2.1–2.6	2.4
Rumensin 80 Type A Medicated Article	20	1.9–2.7	2.3
Type C medicated feed	60	1.3–4.8	2.3
Overall	210	1.3–4.8	2.4

results was conducted on 20 production lots of Coban and 20 lots of Rumensin Type A medicated articles. Sixty lots of Type C medicated feeds from sites across the United States and Canada also were evaluated. Results are given in Table 1. On average, addition of monensin factors C/D accounts for approximately 2.0 g/ton (about 2.3%) of the overall monensin biopotency in the final medicated feeds.

Conclusions and Recommendation

The integration and inclusion of monensin factors C and D into the calculation of monensin biopotency allows for a more accurate determination of total monensin bioactivity, making LC results more closely in agreement with previously reported microbiological results. It is recommended that AOAC Method **997.04** be modified to include monensin factors C/D in the calculation.

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TECHNICAL COMMUNICATIONS

Determination of Ammonia and Aliphatic Amines in Food by Ion Chromatography with Double-Cell Bulk Acoustic Wave Detection

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An ion chromatographic (IC) method with double-cell bulk acoustic wave (DCBAW) detection is described for determination of ammonia and low-molecular-mass aliphatic amines in food. A 9 MHz AT-cut quartz crystal is used as the resonator. This detection technique provides high sensitivity, good reproducibility, and wide work region. Its sensitivity is independent of background conductivity of the mobile phase in the range 10–2700 μS. Detection limits (peak = 3δ) for NH_4^+, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$, and $(CH_3)_3NH^+$ were 0.02, 0.05, 0.13, and 0.6 μg/mL, respectively. Relative standard deviations for NH_4^+, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$, and $(CH_3)_3NH^+$ were 0.7, 1.0, 0.8, and 1.2%, respectively. For IC analysis, the analytical column is a Shim-pack IC-C1 column and the mobile phase is 2.0 mmol/L nitric acid solution at a flow rate of 1.5 mL/min. Detection with DCBAW was compared with series bulk acoustic wave and conventional conductivity. The method was applied to analyses of egg, fish, and cured meat.

N-Nitroso compounds are carcinogens that can be formed by the reaction of nitrite with secondary and tertiary amines. They are formed in vivo in the stomach and small intestine of experimental animals after concurrent ingestion of nitrite and precursor amines (1). There is concern that consumption of amines in human diets may play an important role in the development of certain cancers. Methylamine, dimethylamine, and trimethylamine are precursors of *N*-nitrosamines (2). Ammonia and trimethylamine are of particular interest because they are used as indicators of food spoilage.

Gas chromatography is widely used for determining low-molecular-mass aliphatic amines (3, 4) in air. Conventional liquid chromatography is not suitable for aliphatic amines because aliphatic compounds lack natural chromophores or fluorophores required for photometric or fluorometric detection and must first be derivatized to generate photometrical and fluorometrical active adducts (5–9). However, derivatization is tedious. Hence, whenever available with sufficient sensitivity, direct detection of aliphatic amines separated by ion chromatography (IC) is preferable (10–13).

Because of its simplicity and reliability for inorganic and organic ions, conventional conductivity detection continues to be the most common detection technique for IC. However, other detection techniques have been used (5, 14). In this work, a double-cell bulk acoustic wave (DCBAW) detector is used to detect ammonia and aliphatic amines in food.

The response of bulk acoustic wave sensing devices in liquid is affected by liquid properties such as density, viscosity, specific conductivity, and permittivity (15, 16). Series bulk acoustic wave (SBAW) detection has been used successfully (14, 17–19). Compared with conventional conductivity, SBAW has the advantages of high sensitivity, low cost, and simplicity of construction. Nevertheless, its sensitivity is also affected by the background conductivity of the mobile phase.

A DCBAW sensor was designed and constructed in our laboratory (20). This detector further improves the sensitivity and reduces the effect of background conductivity.

The aim of this work is to develop a method for ammonia and low-molecular-mass primary, secondary, and tertiary amines in food using IC with DCBAw detection.

Experimental

DCBAW Detection Apparatus

The DCBAW detector was made in our laboratory (20). A 9 MHz AT-cut quartz crystal was mounted on top of a Teflon column with one side facing liquid. The 2 opposite electrodes were separated by 2 flow-through conductivity cells: an adjustment cell, and a sample cell. The adjustment cell was used to adjust the frequency responses of the quartz crystal to the sample cell, in which the liquid was in contact with the quartz crystal. Its cell constant can be changed by adjusting the position of the Teflon column holding the crystal. The optimal cell constant of the sample cell was 0.83 cm. Frequency signal from the detector was sent either to a digital counter to give a direct frequency recording or to the frequency-to-voltage conversion circuit connected to the chromatography workstation to get voltage information. A frequency-to-voltage converter made in this laboratory was

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Table 1. Sensitivity of DCBAW detector at different conductivities in the adjustment cell and the sample cell[a]

	Sensitivity (kHz/μS)						
G_2, μS	300	400	500	600	700	900	1200
10	4.3	5.0	6.1	7.2	7.7	5.6	3.8
50	4.4	4.9	6.0	7.3	7.7	5.5	3.8
100	4.9	5.0	6.0	7.2	7.6	5.7	4.0
500	3.6	5.1	6.1	7.2	7.8	5.8	4.1
1000	3.0	4.3	6.2	7.3	6.5	5.3	2.8
2000	1.1	2.7	5.1	7.1	5.7	4.6	2.3
2700	0.7	1.0	3.4	6.9	4.4	3.8	1.4

[a] G_1, conductivity in adjustment cell; G_2, conductivity in sample cell.

used to transform the frequency signal of the DCBAW detector to a CR-4A Chromatopac data processor (Shimadzu Co., Kyoto, Japan).

Chromatographic Apparatus

The chromatographic apparatus consisted of a Shimadzu LP-6A liquid delivery pump, a SLC-6B system controller, a SIL-6B autoinjector, and a CTO-6AS column oven. The analytical column was a Shim-pack IC-C1 column (50 mm id × 15 m, stainless) filled with 10 μm particle size polystyrene–divinylbenzene resin incorporating a sulfonic acid base as a functional group. A Shim-pack IC-GC1 guard column (4.0 id × 10 mm) preceded the analytical column.

Reagents

Trimethylamine hydrochloride, dimethylamine hydrochloride, and methylamine hydrochloride were purchased from Sigma (St. Louis, MO). Other chemicals were from Beijing Reagents Co. (Beijing, China). All reagents were analytical-reagent grade and were used as received. Deionized water was distilled and passed through a Millipore (Bedford, MA) Milli Q purification apparatus. Milli-Q water was used to prepare the mobile phase and aqueous solutions. Stock solutions (0.1M) of ammonia and amines were prepared in aqueous hydrochloric acid and diluted as required.

Procedures

The column was conditioned with the mobile phase for 30 min prior to injection of 10 μL sample solutions. Column temperature was maintained at 30°C. To obtain optimum response of the DCBAW detector and lowest noise level, the temperature of the DCBAW detector was kept the same as that of the column, 30°C. The mobile phase was 2.0 mmol/L nitric acid at a flow rate of 1.5 mL/min.

Samples

(**a**) *Egg.*—Eggs were deproteinized according to the method of Scheller et al. (21). Homogenized liquid egg sample (2–3 g) was mixed with 50 mL of a 0.2M HCl–methanol (1 + 1) mixture. Separation of the precipitate by centrifugation gave the deproteinized sample solution. This was passed through a Sep-Pak C_{18} cartridge. The first 2 mL eluate was discarded and the remainder was filtered through a 0.45 μm filter for analysis.

(**b**) *Fish or meat.*—Homogenized fish sample (4 g) or cured meat sample (5 g) was treated in the same way as egg. The sample solution was diluted as necessary and applied to the IC column.

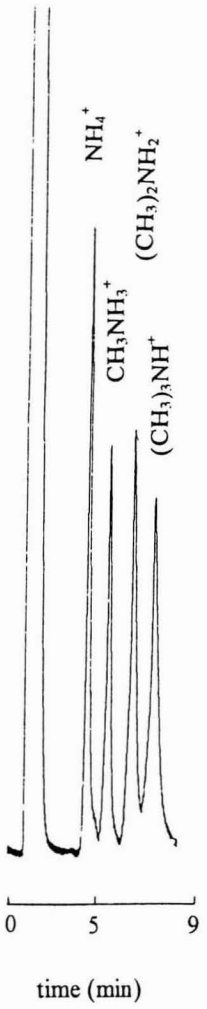

Figure 1. Chromatogram of a mixed standard solution.

Results and Discussion

Selection of DCBAW Working Conditions

For the DCBAW detector, the frequency shift (Δf) between the sample and the reference solution versus their difference in conductivity (ΔG) is defined by

$$\Delta f = \lambda \Delta G$$

where λ incorporates all design parameters of the detector, including the cell constants of the adjustment cell and the sample cell. When all design parameters are kept unchanged, the Δf versus ΔG relationship is linear. Optimal cell constants of the sample cell and the adjustment cell were 0.83 and 1.0 cm, respectively.

For nonsuppressing IC analysis of cation, the conductivity of the mobile phase decreases when the tested ions elute, giving rise to an increase in the frequency of the detector. With the conductivity changing in the adjustment cell, the phase angle of the crystal shifts. The effect of the conductivity in the adjustment cell on the frequency characteristics and work region of the DCBAW detector was investigated. Table 1 shows that the response sensitivity of the DCBAW detector was much less dependent on the background conductivity of solution in the sample cell and satisfactory when the conductivity of the solution in the adjustment cell was about 600 μS.

Response stability and sensitivity of the DCBAW detector depend on temperature. Hence, detector temperature must be maintained constant. In our experiments, the DCBAW detector was kept at the same temperature as the column, 30°C.

Chromatographic Separation

The DCBAW detector was used with the working conditions described above. The concentration of the mobile phase greatly affects retention time and resolution of the investigated ions. High concentrations reduce retention times and result in bad separation of ions, whereas low concentrations make analysis time intolerably long. Also, when the conductivity in the sample cell is outside the range 10–2700 μS, the sensitivity of the detector decreases. Figure 1 shows good resolution of a mixed standard solution containing 6.0 ppm ammonium chloride, 3.5 ppm methylamine hydrochloride, 5.0 ppm dimethylamine hydrochloride, and 10 ppm trimethylamine hydrochloride. The cations NH_4^+, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$, and $(CH_3)_3NH^+$ eluted at 4.62, 5.77, 7.11, and 8.28 min, respectively.

Quantitative Characteristics

Quantitative characteristics such as linear dynamic ranges, correlation coefficients, and detection limits are given in Table 2.

Analytical accuracy was evaluated through recovery studies using egg, fish, and cured meat samples spiked with mixed standard solution containing ammonium chloride, methylamine hydrochloride, dimethylamine hydrochloride, and trimethylamine hydrochloride. Recoveries varied from 92 to 108% (n = 11), with means of 103% for ammonium chloride, 97% for methylamine hydrochloride, 102% for dimethylamine hydrochloride, and 98% for trimethylamine hydrochloride.

Instrument reproducibility was tested in 11 consecutive chromatograms of a mixed standard solution. Relative standard deviations were 0.7, 1.0, 0.8, and 1.2% for NH_4^+, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$, and $(CH_3)_3NH^+$, respectively.

Table 2. Quantitative characteristics

Ion	Linear range, μg/mL	Correlation coefficient	Detection limit, μg/mL (signal/noise = 3)
NH_4^+	0.1–400	0.9997	0.02
$CH_3NH_3^+$	0.2–250	0.9990	0.05
$(CH_3)_2NH_2^+$	0.7–800	0.9986	0.13
$(CH_3)_3NH^+$	1.5–1200	0.9981	0.60

Sample Analysis

Figure 2 shows the chromatogram of an egg sample that had been stored for 20 days at 25°C. As can be seen from Table 3, the concentrations of ammonia and aliphatic amines in fresh eggs were below the method's detection limit. After storage for 20 days at 25°C, 5.42 μg NH_4^+/mL and 2.74 μg $(CH_3)_3NH^+$/mL were found in the egg sample solution.

Fresh fish (sardines) samples were compared with those stored for 3 and 5 days at 25°C (Table 3). In fresh fish samples, levels of NH_4^+ and $(CH_3)_3NH^+$ were 4.96 and 3.61 μg/mL, respectively. $CH_3NH_3^+$ and $(CH_3)_3NH_2^+$ were not detected. After storage, the concentrations of ammonia and aliphatic amines in samples increased greatly. After 5 days at 25°C, the levels of NH_4^+ and $(CH_3)_3NH^+$ were up to 588 and 712 μg/mL, respectively. Method precision was not influenced by the fish matrix.

Meat samples that had been salted and stored for 1 day at 25°C were compared with those that had been salted and stored for 30 days at 25°C. A representative chromatogram of meat sample stored for 30 days at 25°C is shown in Figure 3. On average, 938 μg NH_4^+/mL, 126 μg $(CH_3)_2NH_2^+$/mL and

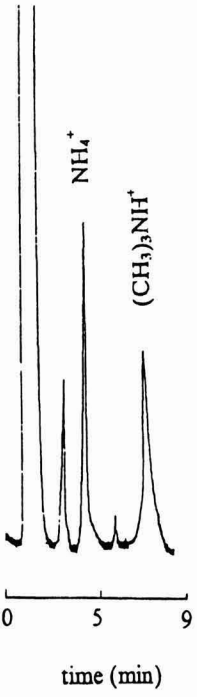

Figure 2. Chromatogram of an egg sample.

Table 3. Analysis of samples

Sample	Ion	Mean, μg/mL	RSD, % (n = 7)
Egg, fresh	NH_4^+	ND[a]	
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	ND	
	$(CH_3)_3NH^+$	ND	
Egg, stored at 25°C for 20 days	NH_4^+	5.42	2.1
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	ND	
	$(CH_3)_3NH^+$	2.74	3.3
Fish, fresh	NH_4^+	4.96	2.8
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	ND	
	$(CH_3)_3NH^+$	3.61	2.6
Fish, stored at 25°C for 3 days	NH_4^+	383	1.9
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	51.6	1.8
	$(CH_3)_3NH^+$	279	2.0
Fish, stored at 25°C for 5 days	NH_4^+	588	1.7
	$CH_3NH_3^+$	12.1	3.1
	$(CH_3)_2NH_2^+$	106.3	2.4
	$(CH_3)_3NH^+$	712	1.5
Cured meat, stored at 25°C for 1 day	NH_4^+	10.4	3.2
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	ND	
	$(CH_3)_3NH^+$	4.12	3.0
Cured meat, stored at 25°C for 30 days	NH_4^+	938	1.6
	$CH_3NH_3^+$	ND	
	$(CH_3)_2NH_2^+$	126	2.3
	$(CH_3)_3NH^+$	502	2.1

502 μg $(CH_3)_3NH^+$/mL were found in meat samples stored for 30 days at 25°C.

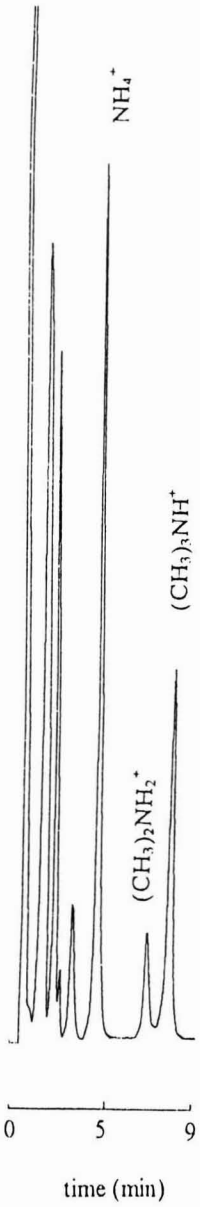

Figure 3. Chromatogram of a cured meat sample stored for 30 days at 25°C.

Comparison of DCBAW, SBAW, and Conventional Conductivity Detection

The electrical circuits of the DCBAW (18) and the SBAW (20) detectors are shown in Figure 4, where C_1 and C_2 are solution capacitances in the adjustment and the sample, respectively; R_1 and R_2 are the solution resistances in the adjustment and the sample; C_0 is shunt capacitance, C_m, L_m, and R_m are motional capacitance, motional inductance, and motional resistance, respectively.

By adjusting the conductivity in the adjustment cell, the phase angle of the crystal can be shifted. According to Table 1, when conductivity in the adjustment cell is about 600 μS, the sensitivity of the DCBAW detector is independent of background in the mobile conductivity range of 10–2700 μS, whereas the sensitivity of the SBAW detector is slightly affected by background only in the mobile conductivity range of 150–1200 μS. When conductivity is 3000 μS, the SBAW detector does not work satisfactorily (14). As such, the working region of the DCBAW detector is much wider than that of the SBAW detector.

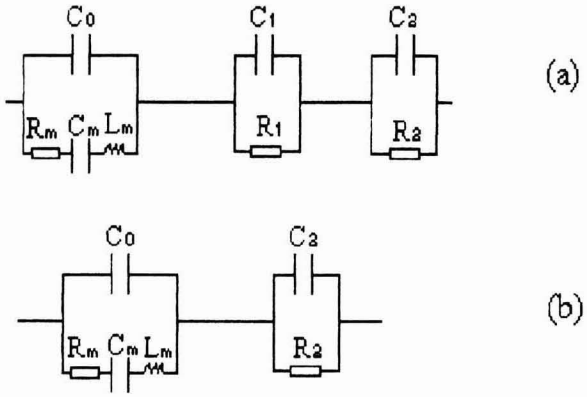

Figure 4. Electrical circuit for (a) DCBAW and (b) SBAW detectors.

Moreover, the crystal in the DCBAW detector does not contact the liquid directly, so the stability of the crystal oscillation is not affected by the solution flowing through the sample cell. Hence, the DCBAW detector is also advantageous over the SBAW detector in terms of the stability of crystal oscillation.

The sensitivity of the conventional conductivity detector is greatly affected by the background conductivity of the mobile phase. The conductivity of the mobile phase in single column IC is generally 100–1500 μS (22).

Compared with a conventional conductivity detector, DCBAW and SBAW detectors also manifest themselves in high sensitivity, low cost, and simple construction (interference of the double layer capacitance on the detector response is eliminated without the complicated construction).

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